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Semicarbazide-Sensitive Amine Oxidase
&
5-HT in Dental Pulp

by

Michael I. O Sullivan

A dissertation submitted to the University of Dublin, Trinity College in candidature for the degree of Doctor of Philosophy

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Table of Contents

Declaration

I certify that none of the work presented in this thesis has been submitted for any degree at this, or any other University, and that the work described in this thesis is entirely my own.

Michael I. O'Sullivan

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Summary

This thesis investigated the behavior of semicarbazide sensitive amine oxidase (SSAO) and the unique activity that it possesses towards 5-hydroxytryptamine (5-HT) in various dental pulp tissues. This summary presents the findings of each chapter of this thesis.

The substrate specificity of SSAO in human dental pulp homogenates was investigated. Both benzylamine and 5-HT were found to be substrates for SSAO in that tissue.

The substrate specificity of SSAO in porcine dental pulp was investigated. Microsomal fractions were investigated and found to contain two forms of SSAO. 5-HT and the majority of benzylamine deamination were found catalysed by separate entities, whereas 5-HT and β-phenylethylamine (PEA) compete for the one active site.

Thermostability of the 2 forms of SSAO in porcine dental pulp microsomes differed substantially. When the microsomal enzyme was heated for 15 min and assayed at 70 °C the benzylamine deaminating enzyme lost 90% of activity, whereas the 5-HT deaminating SSAO remained at about 120 % of control activity (37 °C) for 60 min. Temperature activation of microsomal SSAO at 37 °C was found that raised the activity to 550 % (of control) for 5-HT and 250 % for benzylamine after 3 hours and this persisted for up to 30 hours. However, this activation was not always reproducible, suggesting that there is another factor other than temperature responsible for activation. The SSAO activities in the microsomal fraction could be solubilised in 1 % Triton X-100.

3-methylhistamine was found to be a substrate for porcine dental pulp microsomes but any activity towards histamine was too low to be detected. SSAO may be the main regulator of histamine metabolism in that tissue as both metabolic pathways are dependent on SSAO, as no MAO activity could be detected.

Partial purification of porcine dental pulp microsomal SSAO was accomplished using a Superdex 200 column. This resolved in two peaks of activity at 110 and 440 kDa for both benzylamine and 5-HT deaminating activity. The two peaks of activity towards benzylamine were found to have similar kinetic constants. When the 440 kDa fraction was re-run in the Superdex column peak activity was found at 220 kDa. This suggests that the enzyme can exist in monomer, dimer and tetramer form. Western blot analysis of the 110 and 440 kDa peaks resulted in bands in the 110 kDa region confirming the presence of SSAO. When urea was used as a dissociation agent, distinct areas of SSAO activity were not resolved when the samples were gel-filtered through a Superdex 200 column.

A method was developed for isolating histologically undisturbed human dental pulp tissue from third molar teeth. Immunohistochemical labelling of specimens of human dental pulp with monoclonal anti-tryptophan hydroxylase antibodies and avidin-biotin complex immunolabelling demonstrated the presence of tryptophan hydroxylase in the odontoblastic layer and in nerve tissue. 5-HT reactive nerves have not been previously described in dental pulp. Immunohistochemical labelling of specimens of human dental pulp with polyclonal anti-SSAO antibodies (raised in rabbit to bovine lung microsomal SSAO) and avidin-biotin complex immunolabelling demonstrated the presence of SSAO in the odontoblastic layer, blood vessels and in nerve tissue. The presence of SSAO in nerve tissue had not been previously described.

Cultured murine odontoblasts (MO6G3) were used as a model for oxidative stress in dental pulp tissue. A single application of hydrogen peroxide tested 10, 23 and 36 hours later was found to have little effect on murine odontoblasts at concentrations up to 100 μM. A single application of 5-HT was found to have little effect on cell viability up to concentrations of 1 mM. However a decrease in viable cells was observed at 10 and 23 hours with respect to controls but this returned to the level of controls at 36 hours at 33 °C in 5 % CO_2 and air.

Cloning of SSAO from a murine dental pulp cDNA library was attempted. However PCR produced multiple products when oligonucleotide primers were used directed against both ends of the coding sequence or the conserved site containing the tyrosine

residue that is converted to the organic cofactor. The presence of SSAO in neo-natal murine dental pulp could not be confirmed.

Abbreviations

All abbreviations listed here are defined in the text as they appear.

ABC	Avidin-biotin complex
Ach	Acetylcholine
β-APN	β-aminopropionitrile monofumarate salt
ATP	Adenosine triphosphate
B24	3,5-Ethoxy-4-aminomethyl pyridine
BAT	Brown adipose tissue
°C	Degrees Celsius
$CaCl_2$	Calcium chloride
CAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene-related peptide
Ci	Curie (2.22×10^{12} dpm)
CNS	Central nervous system
DAO	Diamine oxidase
DMEM	Dulbecco's modification of Eagle's medium
DNA	Deoxyribonucleic acid
DNTB	5,5'-dithiobis(2-nitrobenzoic acid)
DOPA	3,4-Dihydroxyphenylalanine
DPM	disintegrations per minute
DPPE	N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine-HCl
DSA	Diazotised sulphanilic acid (4-diazobenzene sulphonic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide, reduced form
FCS	Foetal calf serum
FDH	Formaldehyde dehydrogenase
G	Gram
G	Acceleration due to gravity
GABA	γ-Aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
GSH	Reduced glutathione
GSSG	Glutathione
H	Hour
HBS	Hanks buffer solution
HDC	Histidine decarboxylase
HETEs	Hydroxyeicosatetraenoic acids
HEV	High endothelial venules
HIV	Human immunodeficiency virus
HMT	Histamine-*N*-methyltransferase
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HSF	Histamine supressor factor
HVAP-1	Human vascular adhesion protein-1
5-HT	5-Hydroxytryptamine (Serotonin)
IC_{50}	Inhibitor concentration giving 50% reduction of enzyme activity

IFN-γ	Interferon-γ
IL-1	Interleukin 1
IP3	Inositol-1,4,5-trisphosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
KCN	Potassium cyanide
KDa	KiloDalton
K_i	Inhibitor constant
K_m	Michaelis constant
L	Litre
LDH	Lactate dehydrogenase (EC 1.1.1.27)
M	Metre
M	Moles per litre
MAb 1B2	Monoclonal antibody 1B2
MAO	Monoamine oxidase (EC 1.4.3.4)
M_{ao}	Standard medium without amine oxidase activity
M_{cat}	Standard medium with 10mM 3-amino-1,2,4-triazole
MDL 72145	(E)-2-(3,4-dimethoxyphenyl)-3-fluoroallylamine
MDL 72161	(E)-β-phenyl-3-fluoroallylamine
MDL 72274	(E)-β-phenyl-3-chloroallylamine
MDL 72974A	(E)-2-(4-fluorophenethyl)-3-fluoroallylamine
Min	Minute
MO6G3	Murine odontoblast cell lines
Mol	Mole
M_r	Molecular mass
M_s	Standard medium
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
NAD	β-Nicotinamide adenine dinucleotide
NADH	β-Nicotinamide adenine dinucleotide, reduced form
NADP	β-Nicotinamide adenine dinucleotide phosphate
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
NCBI	National centre for biotechnological information
NKC	Natural killer cells
NO	Nitric oxide
P_c	Hydrostatic pressure of blood
P_i	Tissue pressure
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEA	β-phenylethylamine
pfu	Plaque-forming units
PI	Phosphatidylinositol
PKC	Protein kinase C
PLN	Peripheral lymph node
PLP	Pyridoxal phosphate
PPO	2,5- diphenyloxazole
PQQ	Pyrroloquinoline quinone
QIP	Quench indication parameter
ROS	Reactive oxygen species
Rpm	Revolutions per minute
S	Second

S.A.	Specific activity
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide-gel electrophoresis
SDW	Sterile distilled water
SMB	Salt-magnesium buffer
SNAP	*S*-nitroso-*N*-acetyl-DL-penicillamine
SNOG	*S*-nitrosoglutathione
sp. Act.	specific activity
SSAO	Semicarbazide-sensitive amine oxidase (EC 1.4.3.6)
TCDO	Tetrachlorodecaoxygen
TNF-α	Tumour necrosis factor α
TOPA	3,4,6-Trihydroxyphenylalanine
TRIS	Tris (hydroxymethyl) aminomethane
TTBS	Tween-Tris buffered saline
TXB2	Thromboxane B2
UV	Ultra violet
V_e	Elution volume
V_{st}	Volume of solvent
V_t	Total bed volume
V_x	Column volume
VAP-1	Vascular adhesion protein-1
VIP	Vasoactive intestinal polypeptide
V_{max}	Maximum enzyme velocity
VSMC	Vascular smooth muscle cell
v/v	volume by volume
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

DETAILED LIST OF CONTENTS

CHAPTER ONE
General Introduction

CHAPTER TWO
Kinetic Characteristics of SSAO in Human and Porcine Dental Pulp

CHAPTER THREE
Partial Purification of Porcine Membrane-Bound SSAO

CHAPTER FOUR
Histology and Immunocytochemistry of Human Dental Pulp

CHAPTER FIVE
The Response of Murine Odontoblasts to Oxidative Stress

CHAPTER ONE

General Introduction

General Introduction

The work described in this thesis concerns the amine oxidase activities, particularly the semicarbazide-sensitive amine oxidases (SSAO) in dental pulp and their possible roles in that tissue.

1.1 Biogenic amines

These amines first came into prominence when adrenaline was extracted from adrenal glands and it was demonstrated that the pressor response to adrenaline was similar to that seen when sympathetic nerves were stimulated (Langley, 1901). This response was evident even if the nerve had degenerated. Elliot (1904) proposed that adrenaline was the chemical stimulant released by the nerve into contact with the muscle.

Several other amines are now thought to act as neurotransmitters or neuromodulators, both centrally and peripherally. Such amines include 5-HT, noradrenaline, dopamine, tyramine, histamine, phenylethylamine, octopamine, spermine and spermidine.

1.1.1 Amine oxidases

In 1928, an enzyme called "tyramine oxidase" was discovered that was capable of oxidative deamination of tyramine (O' Hare, 1928). The following reaction was proposed to explain the reaction as it was demonstrated that one molecule of oxygen was absorbed for every molecule of tyramine, with one molecule of ammonia being released:

$$R\text{-}CH_2\text{-}NH_2 + H_2O + O_2 = R\text{-}CHO + H_2O_2 + NH_3$$

(O' Hare, 1928; Bernheim, 1931).

In 1929, another amine oxidase, which was capable of deaminating histamine, was discovered (Best, 1929). This "histaminase" was isolated from the lungs of ox and horse and was shown to reduce the vasodilator properties of histamine in a time-dependent manner. In dogs, the kidney and intestines contained substantial amounts of this enzyme (Best & McHenry, 1930). This reaction was oxygen-dependent and

was inhibited by the presence of cyanide. Ammonia was a product of this reaction. The ammonia nitrogen was derived from the side-chain amino group of histamine (McHenry & Gavin, 1935).

Tyramine oxidase and histaminase differed from each other in terms of their location and also susceptibility to cyanide. Zeller (1938) discovered that histaminase was also capable of deaminating the short chain diamines, putrescine and cadaverine. He suggested a new terminology for these enzymes. Tyramine oxidase, as described by O' Hare (1928) and Blaschko (1937), was renamed *monamine oxidase (MAO)*, and the histaminase of Best (1929) became *diamine oxidase.*

Shortcomings of this classification came to light when it was reported that monoamine oxidase was capable of metabolising long chain aliphatic diamines, but diamine oxidase was not (Blaschko & Duthie, 1945). Further differences necessitated the introduction of new terminology. Blaschko (1959) devised two classes of enzyme based on their sensitivity to inhibition by carbonyl reagents. The classes are:

(i) Amine oxidases that are not affected by carbonyl reagents. Included in this group are the monoamine oxidases described by Zeller and a number of FAD-dependent enzymes including mouse liver histaminase and the enzyme isolated from the cytosolic portion of rat liver which deaminates the secondary amine groups of the polyamines, spermine and spermidine.

(ii) Amine oxidases that are susceptible to carbonyl reagents, for instance semicarbazide and hydroxylamine. This group includes the classical histaminase and amine oxidases derived from plants, bacteria, plasma and rabbit liver. Also included are ruminant plasma-borne polyamine oxidases that deaminate the polyamines spermine and spermidine at the primary amine group (Morgan, 1985) and benzylamine oxidase (Blaschko *et al.*, 1959).

The second group has been renamed the semicarbazide-sensitive amine oxidases [amine: oxygen oxidoreductase (deaminating) (copper-containing); EC 1.4.3.6] (Callingham and Barrand, 1987). This is because semicarbazide was only effective

against the second group but some other carbonyl reagents were also able to inhibit from the first group.

This semicarbazide-sensitive amine oxidase group (SSAO) has expanded to incorporate lysyl oxidase. This enzyme is concerned with the biosynthesis of collagen (Siegel & Fu, 1976) and cross-linkage formation (Kagan, 1986). Also included are other tissue-bound amine oxidases of unknown function (Callingham & Barrand, 1987).

1.1.2 Location of SSAO

SSAO is widely distributed throughout the animal world, and is also found in plants and micro-organisms. In humans it is most commonly found in association with blood vessels and smooth muscle (Lewinsohn, 1984). SSAO has also been demonstrated in white and brown rat adipocytes (Raimondi *et al.*, 1991) as well as rat articular cartilage (Lyles & Bertie, 1987). Bovine lung (Lizcano *et al.*, 1990) and optic nerve (Fernandez de Arriba, 1990), rat aorta (Cao Danh *et al.*, 1985) and porcine dental pulp (Norquist & Oreland, 1989) also contain activity.

Subcellularly, the enzyme is associated with the plasmalemma and microsomal fractions (Barrand & Callingham, 1982; 1984). The enzyme is a glycoprotein (Yasunobu, 1976) and its position in the membrane of rat vascular smooth muscle suggests that it may be an ectoenzyme. Holt and Callingham (1993) discovered that, in rat, it was possible to inactivate almost half the enzyme activity by prior perfusion with the non-permeating agent, diazotised sulphanilic acid (DSA). The enzyme's location in other tissues has not been determined. In blood, SSAO is soluble in plasma. Two SSAO enzymes are active in sheep plasma (Elliot *et al.*, 1992), one of which is similar to that contained in the arterial wall.

1.1.3 Organic cofactors and reaction

The organic cofactor associated with SSAO has not been fully elucidated. A carbonyl group must be involved as the enzyme is sensitive to semicarbazide and other carbonyl reagents. Pyridoxal phosphate (PLP) was suggested and this group was reported to be present in preparations of the enzyme (Davison, 1956). Spectral data

also demonstrated that PLP may be present (Yamanda & Yasunobu, 1963). Acid hydrolysis of the pig plasma benzylamine oxidase enzyme in argon in the presence of phenylhydrazine allowed the isolation of an adduct which was purified by high performance liquid chromatography (HPLC) and identified as pyridoxal phosphate by spectrophotometry, spectrofluorimetry and mass spectrometry (Buffoni, 1990).

Pyrroloquinoline quinone (PQQ) became a possibility when it was found to be involved in bacterial redox systems (Duine & Frank, 1981). Resonance Raman spectroscopy also favoured PQQ, but this was undermined by the discovery of PQQ contamination of the pronase used to hydrolyse the proteins for this technique (Buffoni, 1988).

Janes *et al.* (1990), upon examining the peptide sequence of bovine serum amine oxidase, found 6-hydroxy DOPA (also known as TOPA) to be a possible cofactor. This cofactor is formed by the hydroxylation of a specific tyrosine residue in the polypeptide. SSAO catalyses a double displacement reaction of the amino-transferase type (Oi *et al.*, 1970):

$$E\text{-}CHO + R\text{-}CH_2\text{-}NH_2 = E\text{-}CH_2\text{-}NH_2 + R\text{-}CHO$$
$$E\text{-}CH_2\text{-}NH_2 + O_2 + H_2O = E\text{-}CHO + NH_3 + H_2O_2$$

This reaction is dependent on the presence of oxygen. Since SSAO from several sources has been shown to contain copper a mechanism for this oxidation by bovine serum amine oxidase which is consistent with the presence of TOPA and Cu^{2+} has been proposed (Hartmann & Klinman, 1991). The mechanism involves the formation of an oxidised TOPA-substrate imine complex that forms an aldehyde-TOPA complex by proton transfer. The latter complex is formed by way of a carbanion intermediate. The aldehyde is released from the TOPA by hydrolysis. TOPA is reduced with the help of the Cu^{2+} with hydrogen peroxide and ammonia being formed. Bellelli and co-workers (1991) postulated that the rate-limiting step in the whole sequence was the proton transfer step. However, not all SSAO enzymes have been demonstrated to contain copper (Blaschko, 1974; Barrand & Callingham, 1984).

In those enzymes that have been shown to contain copper there appears to be one copper atom per subunit. Most SSAO enzymes have two subunits (Knowles & Yadav, 1984) of approximately 90 kDa (Buffoni & Blaschko, 1964). Both subunits possess active sites (Janes & Klinman, 1991), but it may be that the dimeric enzyme exhibits half-site reactivity (Collison, 1989).

1.1.4 Substrate Specificity

There appear to be quite large variations between species and tissues in the specificities of SSAO, and to a lesser extent MAO. Table 1.1 summarises the generally accepted specificities of SSAO and the monoamineoxidase. The possible significance of these activities will be discussed in following sections, as will some reported species and tissue differences.

SUBSTRATE	SSAO	MAO-A	MAO-B
Benzylamine	+++	–	+++
Dopamine	+	++	++
Methylamine	+++	–	–
PEA	++	(+)	+++
Tyramine	(+)	+++	+++
5-HT	+*	+++	–
Aminoacetone	++	–	–
Noradrenaline	–	++	++

*In pig dental pulp only

Table 1.1. Substrate specificity for SSAO and MAO. Data for SSAO from Buffoni, (1993). Data for MAO from Tipton *et al.*,(1986).

1.1.5 Inhibitors

One of the criteria for demonstrating the presence of SSAO in tissues is to detect activity towards benzylamine that can be inhibited by 0.1 - 1 mM semicarbazide (Lyles 1994). Inhibitors of MAO, such as clorgyline, pargyline and selegiline do not affect this activity. Activity may also be inhibited by derivatives of hydrazine and

allylamine (Lyles, 1984). Some cloroallylamines, such as MDL 72274A, are potent inhibitors of SSAO (Lyles *et al.*, 1987). B24 (3,5-diethoxy-4-aminomethylpyridine) has been reported to be a selective inhibitor of SSAO (Bertini *et al.*, 1985).

Procarbazine and methylhydrazine are also dose-dependent inhibitors of SSAO activity in rat aorta, brown adipose tissue (BAT), brain, heart, liver and lung, the latter being the more potent (Holt & Callingham, 1993). Little effect of these compounds on MAO activity was observed except in the liver, where hepatic MAO-B activity was potentiated by methylhydrazine and in BAT where both methylhydrazine and procarbazine caused a dose-dependent increase in MAO-A activity (Holt & Callingham, 1993).

1.1.6 Physiology

The physiological function of SSAO has not been elucidated to date, but four possible roles have been proposed. The first is in the scavenging of locally-released or circulating amines. The location of the enzyme in vascular smooth muscle means that the enzyme, as it is membrane-bound and thought to be at least in part externally facing, will be in contact with the circulating blood. Thus the enzyme may be important in metabolising circulating amines such as methylamine, tryptamine and tyramine in several species and, in ruminants, dopamine. Methylamine is a substrate for SSAO (Precious & Lyles, 1988) but not for MAO (Precious *et al.*, 1988; Yu 1989), therefore SSAO may be involved in its endogenous turnover. Methylamine, which is produced as a breakdown product of several biochemical pathways, is metabolised by SSAO as effectively as benzylamine in some tissues (Lyles *et al.*, 1990) and thus the enzyme may act to reduce its levels *in vivo*. Methylamine is a cytotoxic endogenous amine with the ability to inhibit insulin release from pancreatic cells and to interfere with intracellular processing of certain plasma membrane receptors (Precious *et al.*, 1988). SSAO in conjunction with MAO is involved in the removal of trace amines, such as tyramine in isolated perfused mesenteric arterial bed of the rat (Elliot *et al.*, 1989) and the contractile response of rat aorta to tryptamine is potentiated by inhibitors of SSAO (Lyles & Taneja, 1987). Dopamine originates in mast cells (Falck *et al.*, 1964) and is rapidly broken down by SSAO (Sharman, 1987). The same may be true with amine substrates such as spermine and spermidine

produced by microbial fermentation in the rumen of ruminants and some drugs such as mescaline and primaquine (Blaschko *et al.*, 1959), although not all forms of SSAO deaminate these amines. This was thought to be protective, but no evidence exists that the polyamines are absorbed from the rumen and the products of the reaction are more toxic than the initial compounds (Byrd *et al.*, 1977).

The exogenous substance benzylamine is a preferential substrate of both soluble and membrane-bound SSAO (Lewinsohn *et al.*, 1978). However benzylamine can also be metabolised by MAO.

Aminoacetone is rapidly oxidised by SSAO. It is formed by the breakdown of two amino acid, glycine and *L*-threonine (Ray & Ray, 1983) and oxidative deamination produces methylglyoxal as an aldehyde product. SSAO activity towards aminoacetone has also been reported in rat aorta (Lyles & Chalmers, 1992), bovine lung (Lizcano *et al.*, 1994) and human umbilical artery (Lyles & Chalmers, 1992). Methylglyoxal and other aldehyde products are themselves toxic (see Section 1.1.7).

The second function may be to generate hydrogen peroxide for local "second messenger" use. H_2O_2 has suggested functions in transmembrane signalling (Mukherjee & Mukherjee, 1982) and may have some role in the control of vasomotor tone (Callingham & Barrand, 1987). The hydrogen peroxide formed by the deamination of the amines by SSAO has also been shown to augment insulin action (Enrique-Tarancon *et al.*, 1998). Thus any protective role of SSAO in removing trace amines must be compared to the relative toxicity of the metabolic products, as the latter may be far more damaging.

The presence of SSAO in brown adipose tissue suggests a different role for the enzyme. The production of H_2O_2 may again act as a signal within the tissue, but neither the signal nor the responses to the signal are known (see Section 1.4).

The third function involves the binding of lymphocytes to lymph nodes. Vascular adhesion protein-1 (VAP-1) is a sialoglycoprotein endothelial cell adhesion molecule that mediates the initial L-selectin-independent interaction between lymphocytes and

endothelial cell under non-static conditions (Salmi & Jalkanen, 1992). VAP-1 mediates adhesion in high endothelial venules (HEV) of CD8-positive and CD16-positive lymphocytes. VAP-1 is thought to contain both *N*- and *O*-linked sugars with abundant sialic acid residues. The latter are necessary for the adhesive properties of hVAP-1 (human vascular adhesion protein-1: Salmi & Jalkanen, 1996). HVAP-1 consists of a 170- to 180-kDa dimer composed of two 90 kDa subunits (Salmi & Jalkanen, 1996). Expression is principally in the HEVs of peripheral lymph node (PLN) type lymphatic tissues (Salmi & Jalkanen, 1992). In the HEVs, lymphocyte surface receptors bind to specific ligands and a cascade of events (Springer, 1994; Butcher & Picker, 1966) cause the lymphocytes to leave the blood and extravasate between the endothelial cells into the surrounding lymphatic tissue (Girard & Springer, 1995). Upregulation of hVAP-1 expression occurs after prolonged chronic inflammation in different tissues, such as gut, tonsil, skin and synovium (Salmi *et al.*, 1992; Arvilommi *et al.*, 1996). The presence of VAP-1 is defined by a mAb 1B2 (monoclonal antibody 1B2) that immunoprecipitates 90- and 170-kDa glycoproteins (Streeter *et al.*, 1988).

Human VAP-1 cDNA sequence has been identified (Smith *et al.*, 1998). It encodes a type II transmembrane protein of 84.6 kDa with six potential *N*-glycosylation sites and three potential *O*-glycosylation sites in the long extracellular domain. HVAP-1 is a unique adhesion molecule that has significant homology to the SSAO family of enzymes and activity towards certain monoamines. Mouse VAP-1 cDNA has also been identified and cloned to produce an 84.5 kDa molecule (Bono *et al.*, 1998). Antibodies specific for mVAP-1 recognised a 110 /220-kDa antigen, suggesting that mVAP-1 exists as a dimer like hVAP-1. It possesses 83% homology with hVAP-1 and also possesses activity towards monoamines. The cDNA sequences of hVAP-1, mVAP-1 and human umbilical cord are compared in Table 1.4.

The fourth function may be the effect of SSAO on glucose transport and GLUT4 recruitment to the cell surface in adipose cells (Enrique-Taracon *et al.*, 1998). GLUT4 is an isoform of glucose transporter found in adipose tissue, cardiac and skeletal muscle. It is localised as an intracellular storage pool that is recruited as vesicles to the cell surface in the presence of insulin. An other isoform, GLUT1 is present on the

plasma membrane but GLUT-4, once recruited, is present in greater quantities and accounts for most of the insulin-stimulated glucose transport in adipose and muscle cells (Gould & Holman, 1993; Mueckler, 1994).

Vesicle immunolocalisation analysis indicated that GLUT4-containing vesicles from rat adipocytes contained substantial levels of SSAO activity (Enrique-Taracon *et al.*, 1998). Antibodies specific for SSAO protein were localised to the same site. Immunotitration of intracellular GLUT4 vesicles indicated that SSAO and GLUT4 co-localise in an endosome compartment in rat adipocytes, 3T3-L1 adipocytes and rat skeletal muscle. The presence of benzylamine and low concentrations of vandate in isolated rat adipocytes stimulated glucose transport, but this stimulation was inhibited by the presence of semicarbazide. Benzylamine and vandate initiated a recruitment of GLUT4 to the plasma membrane of adipose cells. Stimulation of glucose transport was also inhibited by catalase, suggesting that hydrogen peroxide may be involved in the regulation of this process.

1.1.7 Pathology

1.1.7.1 Plasma-bound SSAO

There is a decrease in plasma SSAO activity in patients who have received severe burns (Lewinsohn, 1984). Given that there is evidence than the products of metabolism of biogenic amines by SSAO result in the formation of more toxic compounds, such as hydrogen peroxide and ammonia (Byrd *et al.*, 1977), it may be a protective mechanism in humans to decrease activity that leads to the formation of toxic products.

In patients with hard tissue tumours there is a decrease in SSAO activity (Lewinsohn, 1984). This may be as a result of an as yet unknown direct influence of the tumour on the enzyme activity, or else competition between the two for essential elements, for example copper (Elliot *et al.*, 1991). Elliot (1991) has also suggested that the influence may be hormonal in nature, as a result of the decrease in activity of plasma SSAO in diabetic ewes in the latter stages of pregnancy.

1.1.7.2 Membrane-bound SSAO

The two most investigated sources of membrane-bound SSAO activity are the smooth muscle cells of blood vessels and brown adipose tissue (BAT).

Methylamine is a good substrate for SSAO in rat aorta and human umbilical artery and is a useful substrate to observe as MAO shows no activity towards it (Lyles *et al.*, 1990). Unfortunately, the assays that have been used for methylamine deamination are difficult and time-consuming and rather few studies have involved this substrate. Methylamine does not demonstrate cytotoxicity towards cultured vascular smooth muscle cells that contain SSAO (Blicharski, 1992), but is known to be able to disrupt intracellular processing and recycling of certain plasma membrane receptors (Precious *et al.*, 1988). This may be due to the production of formaldehyde during the deaminating process. This may be of importance in states where the methylamine concentration is raised, such as in certain physiological (pregnancy and exercise) and pathological (diabetes and uraemia) conditions (Kapeller-Adler, 1970). Formaldehyde may react with H_2O_2 and also interact with the ε-amino group of the basic amino acid lysine (Trezl *et al.*, 1992). SSAO must thus act to decrease the concentration of methylamine, even though the products of the reaction may themselves be harmful.

Acrolein is an α,β-unsaturated aldehyde found in car exhaust fumes, cigarette smoke, cyclophosphamide. It is also a reaction product of allylamine metabolism by cardiovascular tissue homogenates (Nelson & Boor, 1982) and porcine SSAO (Boor *et al.*, 1990). This may be prevented by prior inhibition of the enzyme by selective SSAO inhibitors.

The alkylamine allylamine (3-aminopropene) displays cardiac toxicity in experimental animals (see Boor and Ferrans, 1985). The main effects are seen in terms of myocardial necrosis and intimal proliferation of the smooth muscle of the aorta and coronary arteries. This results in transmural scarring with ventricular aneurysm formation and metaplasia of the endocardial cartilage (Boor & Ferrans, 1985; Boor & Hysmith, 1987). The damage is due to the toxic products of the alkylamine as the inhibition of the SSAO prevents this both *in vitro* (Boor *et al.*, 1990) and *in vivo* (Boor & Nelson, 1980). Acrolein plays a major role in the toxicity

but Ramos and co-workers (1988) demonstrated that the addition of catalase to the cultures also reduced the damage. This suggests that H_2O_2, which is produced during deamination, contributes to the cytotoxicity.

Aminoacetone is an aliphatic amine that is formed by the mitochondrial metabolic product of glycine and threonine (see Section 1.1.6). The product of this metabolism is the highly toxic substance, methylglyoxal, which is able to produce damaging effects on the neuromuscular, respiratory and cardiac tissue (Conroy, 1979). Circulating semicarbazide-sensitive amine oxidase is raised both in type I (insulin-dependent), in type II (non-insulin-dependent) diabetes mellitus and even in childhood type I diabetes at first clinical diagnosis (Boomsma, 1999). Cultured rat vascular smooth muscle cells are not damaged by aminoacetone metabolism if they are able to synthesise SSAO in the presence of catalase (Blicharski, 1992).

1.1.8 DNA Sequences of SSAO

The DNA sequences for SSAO isolated from different tissues have been described. These include SSAO from human placenta (Zhang & McIntire, 1996), human retina, mouse 3T3 adipocytes (Moldes *et al.*, 1999), rat adipocyte plasma membrane (Morris *et al.*, 1997), bovine serum (Mu *et al.*, 1994) and bovine membrane (Hogdall *et al.*, 1998). The use of The National Centre for Biotechnological Information (NCBI) browser ENTREZ PubMed (http://www.ncbi.nlm.nih.gov/PubMed/) to determine DNA sequences and BLAST facility (http://ncbi.nlm.nih.gov/BLAST/) to compare sequences has been pivotal in determining the possible functions of SSAO. Near complete homology exists between human placental SSAO and human vascular adhesion protein-1 (Smith *et al.*, 1998) and mouse SSAO and mouse vascular adhesion protein-1 (Bono *et al.*, 1998). An example of a BLAST search to compare two DNA sequences is provided in Table 1.2. The relative homology between SSAO and VAP-1 sequences identified is presented in Tables 1.3 & 1.4. The FASTA sequences were compared using the CLUSTWAL 1.7 software (multiple sequence alignment at http://dot.imgen.bcm.edu:9331/multi-align/multi-align.html) and then converted using BOXSHADE 3.21 for printing and shading of multiple alignment files (http://www.ch.embnet.org/software/BOX_form.html).

Human retinal SSAO has been excluded as it demonstrates poor homology with other SSAO and VAP-1 sequences described.

Comparison of the nucleotide sequences for SSAO and diamine oxidases has revealed a highly conserved region at the active site near the pre-TOPA tyrosine coding sequence. This feature has been used to clone further SSAO forms (Zhang & McIntire, 1996).

Table 1.2

```
MSSAO:      2    actgcagccagctcagacccgcctcccaaccctagctccggcttccctgctccttcccac 61
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     186  actgcagccagctcagacccgcctcccaaccctagctccggcttccctgctccttcccac 245

MSSAO:      62   cctggcaaaagcagactacaaagaagctctgctattgcctagcgcctgggagaccccagc 121
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     246  cctggcaaaagcagactacaaagaagctctgctattgcctagcgcctgggagaccccagc 305

MSSAO:      122  caaaaggagtccatttctggtagaagcctgtccatcaagaagcaatgacccagaagacca 181
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     306  caaaaggagtccatttctggtagaagcctgtccatcaagaagcaatgacccagaagacca 365
                                                      M  T  Q  K  T

MSSAO:      182  ccctagtgctcctggctctggctgtcatcaccatctttgctttggtttgtgtcttgctag 241
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     366  ccctagtgctcctggctctggctgtcatcaccatctttgctttggtttgtgtcttgctag 425
                 T  L  V  L  L  A  L  A  V  I  T  I  F  A  L  V  C  V  L  L

MSSAO:      242  ctggcaggagcggagatggggtggactgagccaacctcttcattgcccctctgttcttc 301
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     426  ctggcaggagcggagatggggtggactgagccaacctcttcattgcccctctgttcttc 485
                 A  G  R  S  G  D  G  G  G  L  S  Q  P  L  H  C  P  S  V  L

MSSAO:      302  ctagtgtccagccccggacacaccctagccagagccagccgtttgcagacctgagcccag 361
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     486  ctagtgtccagccccggacacaccctagccagagccagccgtttgcagacctgagcccag 545
                 P  S  V  Q  P  R  T  H  P  S  Q  S  Q  P  F  A  D  L  S  P

MSSAO:      362  aggagctgacagctgtgatgagcttcctgaccaagcacctggggccagggctggtggatg 421
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     546  aggagctgacagctgtgatgagcttcctgaccaagcacctggggccagggctggtggatg 605
                 E  E  L  T  A  V  M  S  F  L  T  K  H  L  G  P  G  L  V  D
```

Table 1.2 (continued)

```
MSSAO:      422  cagcccaggctcgaccctcggacaactgtgtcttctcagtagagttgcagctgcctgcca 481
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     606  cagcccaggctcgaccctcggacaactgtgtcttctcagtagagttgcagctgcctgcca 665
                 A  A  Q  A  R  P  S  D  N  C  V  P  S  V  E  L  Q  L  P  A

MSSAO:      482  aggctgcagccctggcccacttggacagaggggggccccccacctgtgagagaggcattgg 541
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     666  aggctgcagccctggcccacttggacagaggggggccccccacctgtgagagaggcattgg 725
                 K  A  A  A  L  A  H  L  D  R  G  G  P  P  P  V  R  E  A  L

MSSAO:      542  ccatcatcttctttggtggacaacccaagcccaacgtgagtgagctggtggtggggcccc 601
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     726  ccatcatcttctttggtggacaacccaagcccaacgtgagtgagctggtggtggggcccc 785
                 A  I  I  F  F  G  G  Q  P  K  P  N  V  S  E  L  V  V  G  P

MSSAO:      602  tgcctcaccctcctacatgcgggatgtgactgtggagcgccacggcggccccctgccct 661
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     786  tgcctcaccctcctacatgcgggatgtgactgtggagcgccacggcggccccctgccct 845
                 L  P  H  P  S  Y  M  R  D  V  T  V  E  R  H  G  G  P  L  P

MSSAO:      662  attaccggcgtcctgtgttggacagagagtatcaggatatcgaggagatgatcttccaca 721
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     846  attaccggcgtcctgtgttggacagagagtatcaggatatcgaggagatgatcttccaca 905
                 Y  Y  R  R  P  V  L  D  R  E  Y  Q  D  I  E  E  M  I  F  H

MSSAO:      722  gagagctgccccaagcttctggactcctccatcactgttgcttctacaaacaccagggac 781
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     906  gagagctgccccaagcttctggactcctccatcactgttgcttctacaaacaccagggac 965
                 R  E  L  P  Q  A  S  G  L  L  H  H  C  C  F  Y  K  H  Q  G

MSSAO:      782  agaacctgctaacaatgactacagccccccgtggtttgcaatcaggggaccgggccacct 841
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:     966  agaacctgctaacaatgactacagccccccgtggtttgcaatcaggggaccgggccacct 1025
                 Q  N  L  L  T  M  T  T  A  P  P  G  L  Q  S  G  D  R  A  T
```

Table 1.2 (continued)

```
MSSAO:      842  ggtttggcttgtattacaatctctcagggctgggttttaccctcaccccattggcttgg 901
MVAP-1:    1026  ggtttggcttgtattacaatctctcagggctgggttttaccctcaccccattggcttgg 1085
                 W  F  G  L  Y  Y  N  L  S  G  A  G  F  Y  P  H  P  I  G  L

MSSAO:      902  agcttctgatagatcataaggccctggatcctgccctgtggaccatccagaaggtattct 961
MVAP-1:    1086  agcttctgatagatcataaggccctggatcctgccctgtggaccatccagaaggtattct 1145
                 E  L  L  I  D  H  K  A  L  D  P  A  L  W  T  I  Q  K  V  F

MSSAO:      962  atcaaggccgttactatgagagtctcactcagctggaggaccagtttgaggctggcctgg 1021
MVAP-1:    1146  atcaaggccgttactatgagagtctcactcagctggaggaccagtttgaggctggcctgg 1205
                 Y  Q  G  R  Y  Y  E  S  L  I  Q  L  E  D  Q  F  E  A  G  L

MSSAO:     1022  tgaatgtggtattggtcccaaacaatggtacaggtgggtcctggtctctaaagtcctcag 1081
MVAP-1:    1206  tgaatgtggtattggtcccaaacaatggtacaggtgggtcctggtctctaaagtcctcag 1265
                 V  N  V  V  L  V  P  N  N  G  T  G  G  S  W  S  L  K  S  S

MSSAO:     1082  tgccaccgggcccagctccccctctgcagttccatccccagggaccccggttcagtgtcc 1141
MVAP-1:    1266  tgccaccgggcccagctccccctctgcagttccatccccagggaccccggttcagtgtcc 1325
                 V  P  P  G  P  A  P  P  L  Q  F  H  P  Q  G  P  R  F  S  V

MSSAO:     1142  aggggagccaagtgtcttcttccttgtgggcttttttcttttggccttggagctttcagtg 1201
MVAP-1:    1326  aggggagccaagtgtcttcttccttgtgggcttttttcttttggccttggagctttcagtg 1385
                 Q  G  S  Q  V  S  S  S  L  W  A  F  S  F  G  L  G  A  F  S

MSSAO:     1202  gcccaaggatctttgatatccgctttcaaggggagagggtggcctatgaaatcagtgtcc 1261
MVAP-1:    1386  gcccaaggatctttgatatccgctttcaaggggagagggtggcctatgaaatcagtgtcc 1445
                 G  P  R  I  F  D  I  R  F  Q  G  E  R  V  A  Y  E  I  S  V
```

Table 1.2 (continued)

```
MSSAO:    1262 aggaggccatagccctctacggtggaaattccccagcatcaatgtcgacctgctacgtgg 1321
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:   1446 aggaggccatagccctctacggtggaaattccccagcatcaatgtcgacctgctacgtgg 1505
               Q  E  A  I  A  L  Y  G  G  N  S  P  A  S  M  S  I  C  Y  V

MSSAO:    1322 acggtagctttggcattggcaaatactctacccccctgatccgaggggtagactgtcctt 1381
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:   1506 acggtagctttggcattggcaaatactctacccccctgatccgaggggtagactgtcctt 1565
               D  G  S  F  G  I  G  K  Y  S  T  P  L  I  R  G  V  D  C  P

MSSAO:    1382 acctcgccacctatgtggactggcacttccttctggaatctcaggccccaagacactac 1441
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:   1566 acctcgccacctatgtggactggcacttccttctggaatctcaggccccaagacactac 1625
               Y  L  A  T  Y  V  D  W  H  F  L  L  E  S  Q  A  P  K  T  L

MSSAO:    1442 gtgatgcattttgtgtgtttgaacagaaccagggcctcccactccggcggcaccactcag 1501
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:   1626 gtgatgcattttgtgtgtttgaacagaaccagggcctcccactccggcggcaccactcag 1685
               R  D  A  F  C  V  F  E  Q  N  Q  G  L  P  L  R  R  H  H  S

MSSAO:    1502 atttctactcccattattttgggggtgttgtggggacggtgcttgtggtcagatccgtgt 1561
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:   1686 atttctactcccattattttgggggtgttgtggggacggtgcttgtggtcagatccgtgt 1745
               D  F  Y  S  H  Y  F  G  G  V  V  G  T  V  L  V  V  R  S  V

MSSAO:    1562 ctaccttgctcaattacgactacatatgggacatggtcttccaccccaatggggccatag 1621
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:   1746 ctaccttgctcaattacgactacatatgggacatggtcttccaccccaatggggccatag 1805
               S  T  L  L  N  Y  D  Y  I  W  D  M  V  F  H  P  N  G  A  I

MSSAO:    1622 aagtcaaattccacgccacaggctatatcagctcagctttcttcttcggtgctggtgaga 1681
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:   1806 aagtcaaattccacgccacaggctatatcagctcagctttcttcttcggtgctggtgaga 1865
               E  V  K  F  H  A  T  G  Y  I  S  S  A  F  F  F  G  A  G  E
```

Table 1.2 (continued)

```
MSSAO:          1682 agtttgggaaccgagttggggcgcacacgctgggcacggtacacacccacagcgctcact 1741
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:         1866 agtttgggaaccgagttggggcgcacacgctgggcacggtacacacccacagcgctcact 1925
                     K  F  G  N  R  V  G  A  H  T  L  G  T  V  H  T  H  S  A  H

MSSAO:          1742 tcaaagtggatctggatgtggcagggctgaagaactgggcctgggcagaggatatggctt 1801
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:         1926 tcaaagtggatctggatgtggcagggctgaagaactgggcctgggcagaggatatggctt 1985
                     P  K  V  D  L  D  V  A  G  L  K  N  W  A  W  A  E  D  M  A

MSSAO:          1802 ttgtccccacgattgtaccttggcaaccggagtaccagatgcagaggctgcaggtgactc 1861
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:         1986 ttgtccccacgattgtaccttggcaaccggagtaccagatgcagaggctgcaggtgactc 2045
                     F  V  P  T  I  V  P  W  Q  P  E  Y  Q  M  Q  R  L  Q  V  T

MSSAO:          1862 ggaagctgctggagacagaggaggaggctgccttcccactgggggcgccaccccacgct 1921
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:         2046 ggaagctgctggagacagaggaggaggctgccttcccactgggggcgccaccccacgct 2105
                     R  K  L  L  E  T  E  E  E  A  A  F  P  L  G  G  A  T  P  R

MSSAO:          1922 acctgtacctggccagtaaccacagcaacaagtggggtcataggcggggctaccgcatcc 1981
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:         2106 acctgtacctggccagtaaccacagcaacaagtggggtcataggcggggctaccgcatcc 2165
                     Y  L  Y  L  A  S  N  H  S  N  K  W  G  H  R  F  G  Y  R  I

MSSAO:          1982 agatactcagctttgctggaaagcccttgccccaggaaagtcccatagagaaagccttca 2041
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MVAP-1:         2166 agatactcagctttgctggaaagcccttgccccaggaaagtcccatagagaaagccttca 2225
                     Q  I  L  S  F  A  G  K  P  L  P  Q  E  S  P  I  E  K  A  F
```

Table 1.2 (continued)

```
MSSAO:      2042 cctgggggaggtatcacttggctgtgacccagaggaaggaggaggagcctagcagctcta 2101
MVAP-1:     2226 cctgggggaggtatcacttggctgtgacccagaggaaggaggaggagcctagcagctcta 2285
                 T  W  G  R  Y  H  L  A  V  T  Q  R  K  E  E  E  P  S  S  S

MSSAO:      2102 gcatcttcaaccagaacgacccgtggacccccactgtggacttcaccgacttcatcagca 2161
MVAP-1:     2286 gcatcttcaaccagaacgacccgtggacccccactgtgaacttcaccgacttcatcagca 2345
                 S  I  F  N  Q  N  D  P  W  T  P  T  V  N  F  T  D  F  I  S

MSSAO:      2162 atgagaccattgctggagaggacttggtagcctgggtgacggctggctttttacacatcc 2221
MVAP-1:     2346 atgagaccattgctggagaggacttggtagcctgggtgacggctggctttttgcacatcc 2405
                 N  E  T  I  A  G  E  D  L  V  A  W  V  T  A  G  F  L  H  I

MSSAO:      2222 ctcatgcagaagatatccccaacacggtgactgcggggaactcagtgggcttcttcctcc 2281
MVAP-1:     2406 ctcatgcagaagatatccccaacacggtgactgcggggaactcagtgggcttcttcctcc 2465
                 P  H  A  E  D  I  P  N  T  V  T  A  G  N  S  V  G  F  F  L

MSSAO:      2282 ggccgtataacttctttgacgaggacccctccttccactctgctgactccatctatttcc 2341
MVAP-1:     2466 ggccgtataacttctttgacgaggacccctccttccattctgctgactccatctatttcc 2525
                 R  P  Y  N  F  F  D  E  D  P  S  F  H  S  A  D  S  I  Y  F

MSSAO:      2342 gggagggccaggatgccacggcctgtgaggttaaccccttggcttgcctgtcccagactg 2401
MVAP-1:     2526 gggagggccaggatgccacggcctgtgaggttaaccccttggcttgcctgtcccagactg 2585
                 R  E  G  Q  D  A  T  A  C  E  V  N  P  L  A  C  L  S  Q  T

MSSAO:      2402 ccacctgtgccccgaaattcctgccttctcccatgggggctttgcttacagagacaatt 2461
MVAP-1:     2586 ccacctgtgccccgaaattcctgccttctcccatgggggctttgcttacagagacaatt 2645
                 A  T  C  A  P  E  I  P  A  F  S  H  G  G  F  A  Y  R  D  N
```

Table 1.2 (continued)

```
MSSAO:      2462 gaactgtttctaagtatccctccctcgctcctgctcagaccatgtgctcacttccccatg 2521
MVAP-1:     2646 gaactgtttctaagtatccctccctcgctcctgctcagaccatgtgctcacttccccacg 2705

MSSAO:      2522 ccattaagtgtccccaagatggacaatctagccaagagctgggaagtagcgcaacagccg 2581
MVAP-1:     2706 ccattaagtgtccccaagatggacaatctagctaagagctgggaagtagcgcaacagccg 2765

MSSAO:      2582 ggcagtacacagagcaattcgattgaagatctggttccttctgtccccacatctttgatg 2641
MVAP-1:     2766 ggcagtacacagagcaattcgattgaagatctggttccttctgtccccacatctttgatg 2825

MSSAO:      2642 tcccctctctcttctgctgccctccttgtctctccctctctgcttggagcatcctgagcc 2701
MVAP-1:     2826 tcccctctctcttctgctgccctccttgtctctccctctctgcttggagcatcctgagcc 2885

MSSAO:      2702 catggaaacctgatgcacagggacaatgaactttgttggttgtgcctgtactgagttcct 2761
MVAP-1:     2886 catggaaacctgatgcacagggacactgaactttgttggttgtgcctgtactgagttcct 2945

MSSAO:      2762 gccttgggagaatagccttgttggagcctggagtaatggctatgttttgttttgttttga 2821
MVAP-1:     2946 gccttgggagaatagccttgttggagcctggagtaatggctatgttttgttttgctttga 3005

MSSAO:      2822 ataaggctccttttccccatccccaccgcaccccctatttggctttcatttaaaagctta 2881
MVAP-1:     3006 atatggctccttttccccaccccccaccgcaccccctatttggctttcatttaaaagctta 3065

MSSAO:      2882 tgatagctttgaggactctgcaatgaggataactctctagagacccccaaagtagggtct 2941
MVAP-1:     3066 tgatagctttgaggactctgcaatgaggataactctctagagacccccaaagtagggtct 3125
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MSSAO:      2942 ctctgagtctctcacatcaacagattcttcatctacatccctttcctacttaagcctctt 3001
                 ||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
MVAP-1:     3126 ctctgagtctctcacatccacagattcttcatctacatccctttcctacttaagcctctt 3185

MSSAO:      3002 tcatttcca 3010
                 |||||||||
MVAP-1:     3186 tcatttcca 3194
```

Table 1.2. Result of a BLAST comparison of the nucleotide sequences of *Mus musculus* semicarbazide-sensitive amine oxidase mRNA (MSSAO) and *Mus musculus* vascular adhesion protein-1 (MVAP-1)

AF115411: *Mus musculus* semicarbazide-sensitive amine oxidase mRNA, complete cds

AF054831: *Mus musculus* vascular adhesion protein mRNA, complete cds

HSU39447: Human placenta copper monamine oxidase mRNA, complete cds

AF067406: Human vascular adhesion protein-1 (VAP1) mRNA, complete cds

RNU72632: *Rattus norvegicus* membrane amine oxidase mRNA, partial cds

BOVFRA: *Bos taurus* serum amine oxidase mRNA, complete cds

BTY15774: *Bos taurus* mRNA for copper amine oxidase

	AF115411	AF054831	HSU39447	AF067406	RNU72632	BOVFRA	BTY15774
AF115411	*	99	84	84	92	81	82
AF054831	99	*	84	84	92	81	82
HSU39447	84	84	*	100	82	85	86
AF067406	84	84	100	*	82	85	86
RNU72632	92	92	82	82	*	81	83
BOVFRA	81	81	85	85	81	*	94
BTY15774	82	82	86	86	83	94	*

Table 1.3. BLAST results of homology between published semicarbazide-sensitive amine oxidases and vascular adhesion protein-1 sequences expressed as a percentage value. One point is awarded for a matched basepair sequence and two points deducted for a mismatch. The open gap penalty was 5 points and gap extension 2 points. The total points are presented as a percentage of the total number of basepairs.

AF115411: *Mus musculus* semicarbazide-sensitive amine oxidase mRNA, complete cds

AF054831: *Mus musculus* vascular adhesion protein mRNA, complete cds

HSU39447: Human placenta copper monamine oxidase mRNA, complete cds

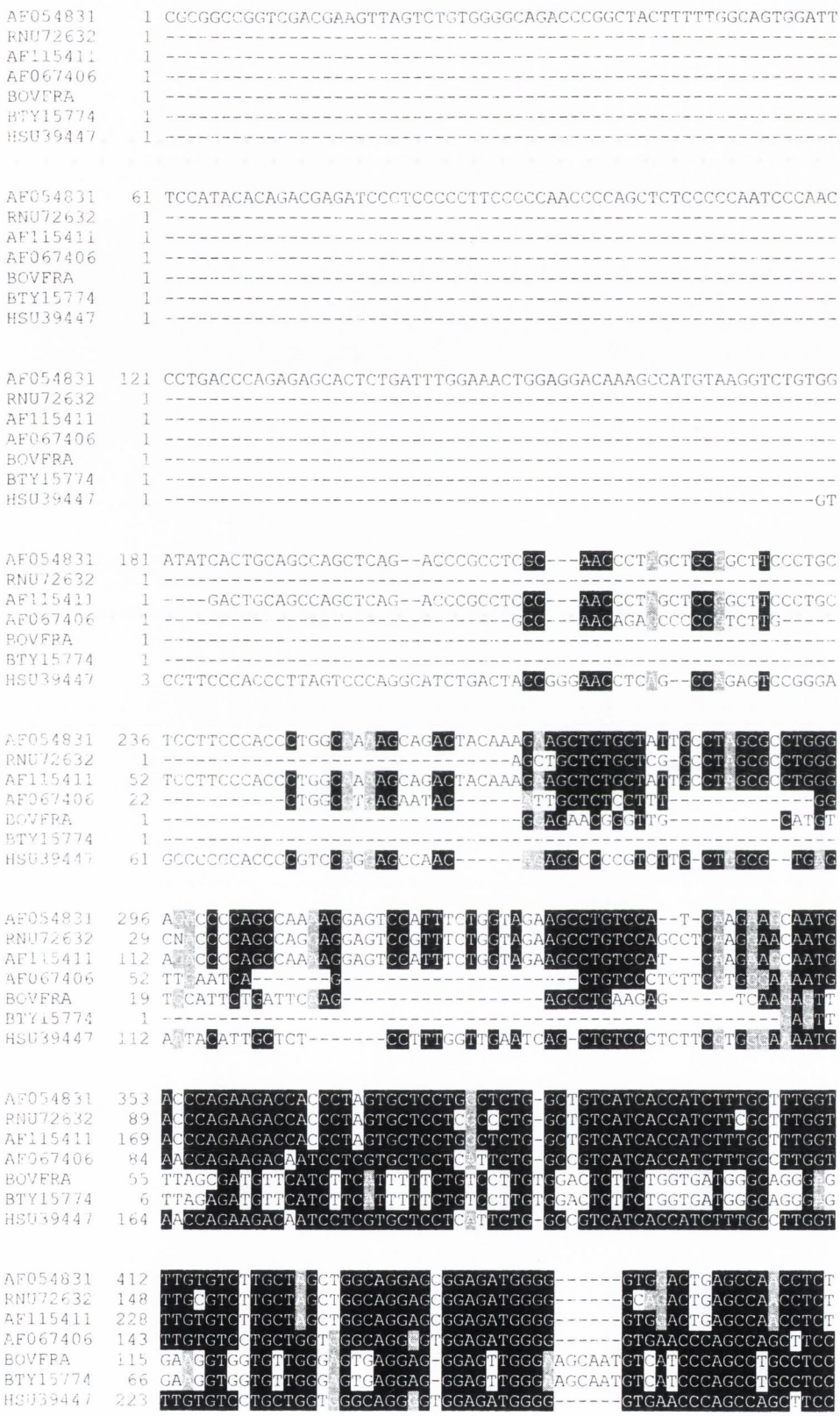
AF067406: Human vascular adhesion protein-1 (VAP1) mRNA, complete cds

RNU72632: *Rattus norvegicus* membrane amine oxidase mRNA, partial cds

BOVFRA: *Bos taurus* serum amine oxidase mRNA, complete cds

BTY15774: *Bos taurus* mRNA for copper amine oxidase

Table 1.4



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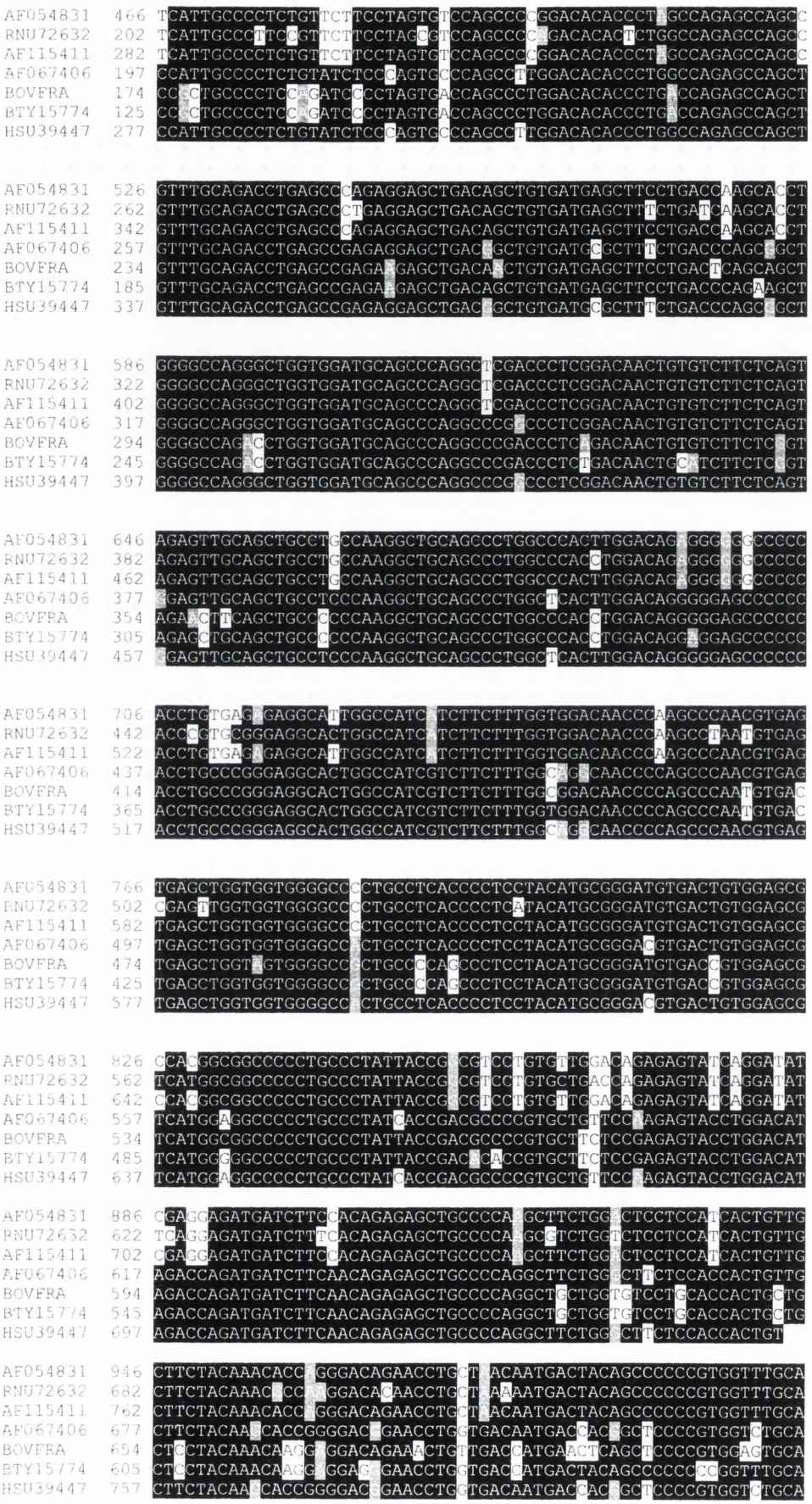

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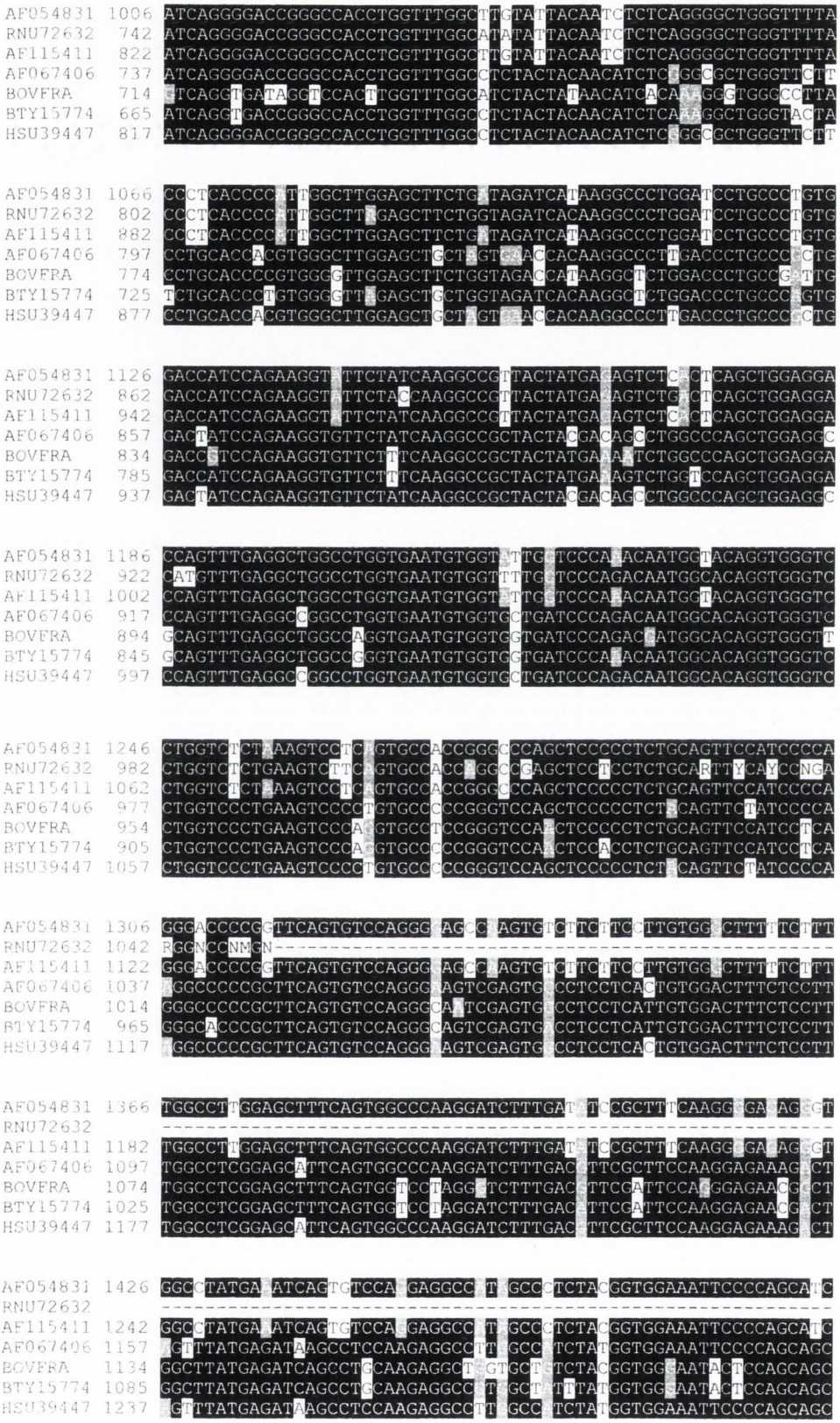

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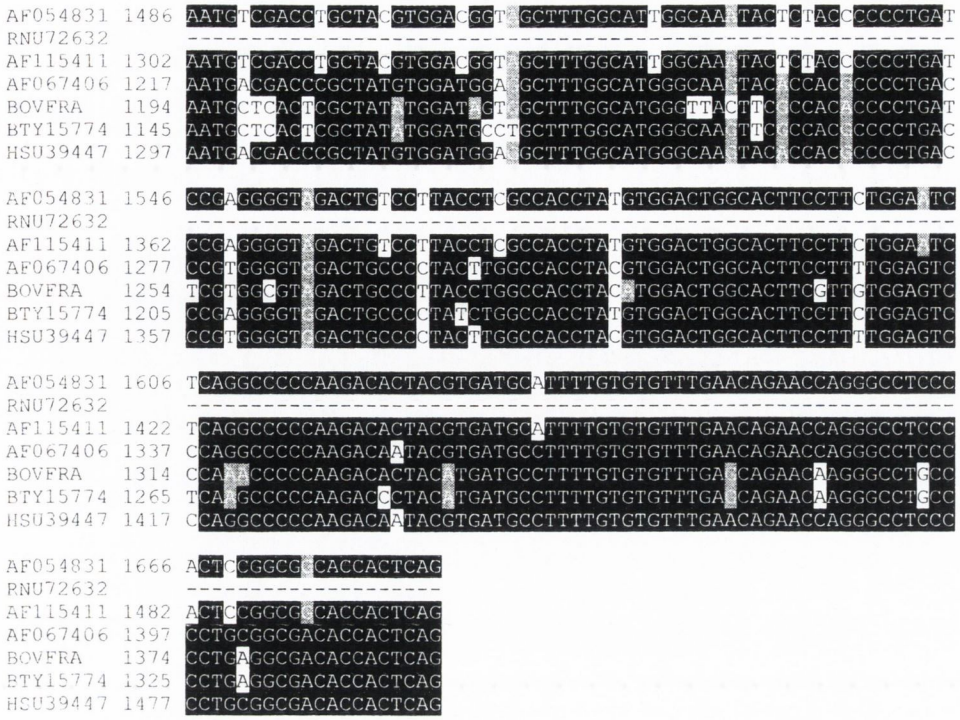


Table 1.4. A multiple alignment of SSAO /VAP-1 sequences. The DNA sequences ere compared using the CLUSTWAL 1.7 software programme (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) and then converted using BOXSHADE 3.21 for printing and shading of multiple alignment files (http://www.ch.embnet.org/software/BOX_form.html) as described in Section 1.1.8.

AF115411: *Mus musculus* semicarbazide-sensitive amine oxidase mRNA.

AF054831: *Mus musculus* vascular adhesion protein mRNA.

HSU39447: Human placenta copper monamine oxidase mRNA.

AF067406: Human vascular adhesion protein-1 (VAP1) mRNA.

RNU72632: *Rattus norvegicus* membrane amine oxidase mRNA, partial cds.

BOVFRA: *Bos taurus* serum amine oxidase mRNA.

BTY15774: *Bos taurus* mRNA for copper amine oxidase.

1.2 Serotonin

In the last century a potent vasoconstrictor was found to be contained in coagulated blood. In 1948 this substance was isolated by Rapport *et al.* and identified as 5-hydroxytryptamine (5-HT). Although 5-HT (also known as serotonin) is located in nearly all parts of the body, its functions have not been fully elucidated. Of dental relevance is the fact that 5-HT is a substrate for porcine dental pulp SSAO (Norqvist *et al.*, 1982), raising the possibility that SSAO in that tissue may be involved in the inflammatory process.

There are three main sites where it is located (Hindle 1994):

(a) Intestine. 90 % in the enterochromaffin cells and 10% in the myenteric plexus of the intestine.

(b) Blood. 5-HT is absorbed from the plasma and stored in platelets. It is released when the platelet aggregates at a damaged site.

(c) CNS. 5-HT is primarily located in the synaptic vesicles in the central nervous system areas, specifically the raphe nuclei and adjacent nuclear groups.

1.2.1 Serotonin Synthesis

5-HT is synthesised from approximately 1% of the total dietary intake of the amino acid tryptophan. The amino acid is converted to 5-hydoxytryptophan by tryptophan hydroxylase. This rate-limiting step takes place in the enterochromaffin cells of the small intestine. 5-hydroxytryptophan is then converted to 5-HT by aromatic L-amino acid decarboxylase (see Hindle 1994).

1.2.2 Metabolism and storage

Metabolism of 5-HT is similar to that of other biogenic amines principally involving the mitochondrial enzyme monoamine oxidase, although in some instances a semicarbazide sensitive amine oxidase (SSAO) may also contribute. This is detailed in Figure 1.1. 5-HT may enter the bloodstream after being released from chromaffin cells or nerve endings. The bulk of this is removed by the liver or endothelial cells (principally pulmonary) and deaminated (Vanhoutte and Cohen, 1983). The remaining

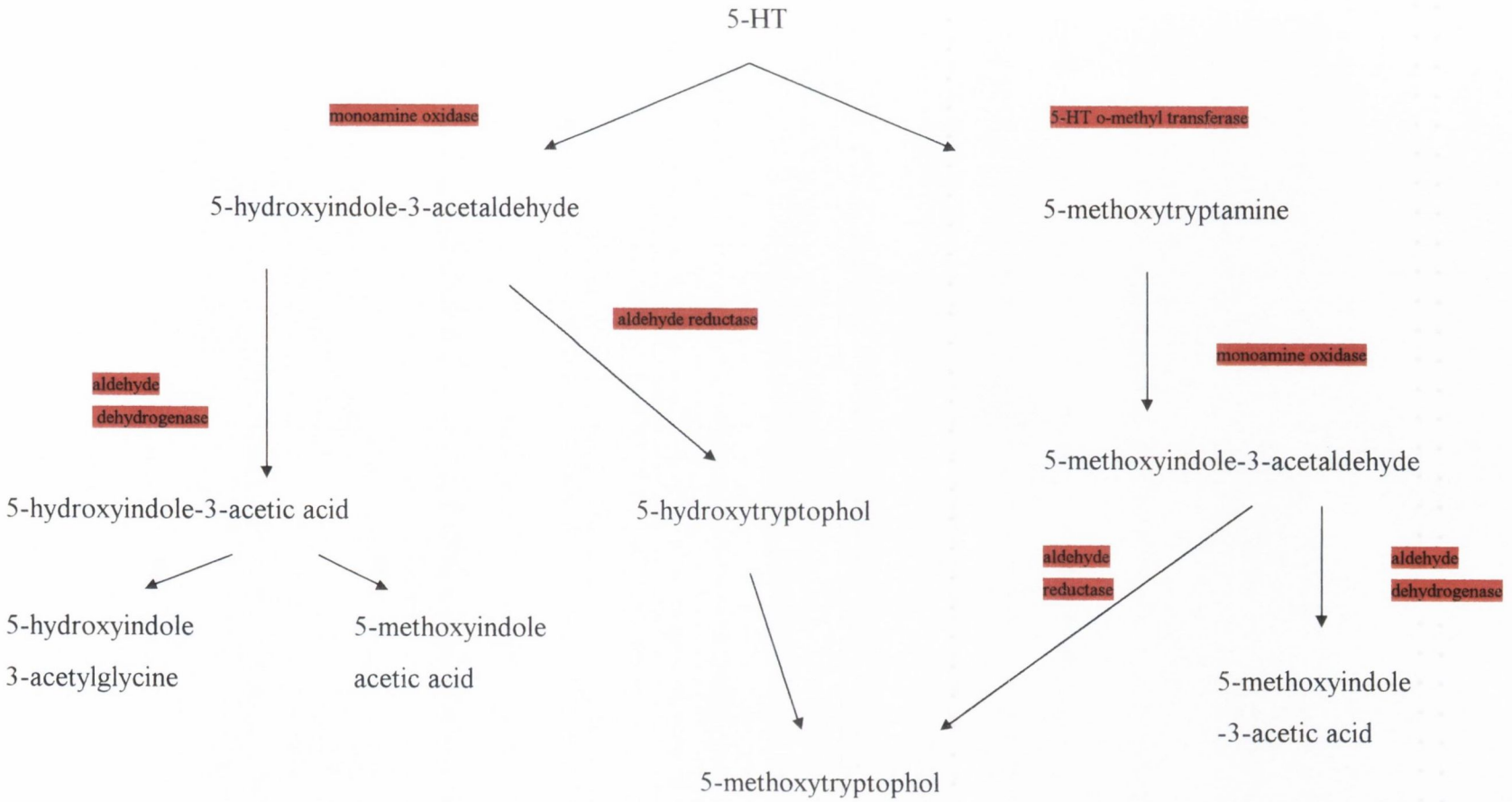


Figure 1.1. Metabolic pathway of 5-HT.

5-HT is stored in dense granules in platelets. 5-HT is also located in mast cells in rodents, but not in humans (Parratt & West, 1957).

1.2.3 Release of Serotonin

In rodents 5-HT is released together with histamine when the mast cell degranulates. 5-HT is a mediator of acute inflammation and modulation of its activity has helped delineate a model for acute inflammation in these species (Spector & Willoughby 1958, Gershon & Ross 1961, Bromley et al. 1984). When applied to the plantar surface of rat paw it causes marked albumin rich oedema, but it does not increase local blood flow (Owen 1977).

In humans when the platelets aggregate during tissue damage the stored 5-HT is released and it amplifies the local effect of other mediators to promote further aggregation (De Clerck & David 1981, De Clerck & Herman 1983). It also promotes release of further inflammatory mediators and sensitises or directly activates nociceptors.

The inflammatory mediators released in response to 5-HT in isolated rat dental pulp include prostacyclins, but neither thromboxane A_2 (Hirafuji & Ogura, 1987) nor prostaglandin E_2 (Hirafuji *et al.*, 1982) are released. 5-HT stimulates the release of arachidonic acid metabolites from tissues of various species e.g. rat dental pulp tissue (Hirafuji *et al.*, 1982), rat adipose tissue (Shaw & Ramwell, 1968), isolated perfused lungs of guinea pig (Alabaster & Bakhle, 1970) and perfused frog spinal cord (Ramwell *et al.*, 1966). However 5-HT does not release PGE_2 from some tissues, including rat cerebral cortex (Wolfe *et al.*, 1975) and rabbit reno-medullary interstitial cells in culture (Zusman & Keiser, 1977).

Two theories for this release have been proposed. First, 5-HT has been proposed as a phenolic co-factor for the cyclo-oxygenation of arachidonic acid (Sih *et al.*, 1971; Egan *et al.*, 1978; Baumann *et al.*, 1979), or secondly it may increase the availability of free arachidonic acid by facilitating its release from cellular phospholipid stores (Hirafuji *et al.*, 1982; Levine & Moskowitz, 1979).

5-HT causes nociceptor activation and increased vascular permeability in mice and rats when applied to the bases of blisters raised on skin (see Goodman & Gilman, 1975). It also induces firing of dental pulp nerves in cats when applied to the bases of deep prepared tooth cavities (Olgart, 1974). In the dorsal vein of the human hand 5-HT potentiates the pain produced by bradykinin (Sicuteri, 1968).

In the dental pulps of beagle dogs the application of 5-HT (1 mg/ml) to the dentine surface caused both sensitisation and increased responsiveness to stimuli including probing, air blasts, cold (ethyl chloride) and application of hypertonic solutions of glucose (saturated) and $CaCl_2$ (3.5 M, 4.9 M and saturated) (Ngassapa *et al.*, 1992). In that study the sensitising effect of 5-HT was far more potent than that of calcitonin gene-related peptide (rat CRGP 2 μg/μl). The effects of 5-HT application over the 15 experimental animals tested (2 teeth per animal) were significant for all the parameters tested, except for cold (Table 1.5). The responses for probing and air-blasting appeared to be more easily provoked and be of longer duration. CGRP application for the 11 experimental animals tested failed to alter the response to probing and air blasting. One fibre began continuous firing and one additional fibre responded to osmotic stimulation after CGRP application.

	Continuous Firing Without Stimulation	**Drilling**	**Probing**	**Air Blast**	**Osmotic**	**Cold**
Before 5-HT	0	30	15	14	13	0
After 5-HT	11	30	30	30	30	3

Table 1.5. The application of 5-HT to the dental pulp of beagle dogs and the effect on nerve firing when the pulps were challenged with different stimuli. The numbers represent the number of dental pulps (out of 30) that fired when stimulated. From Ngassapa *et al.*(1992).

The mechanism by which 5-HT exerts its sensitising effect has not been fully elucidated. In man the sensitising effect is inhibited by the 5-HT_3 receptor antagonist ICS 205.930 (Richardson *et al.* 1985), but this compound does not affect the oedema in carageenan-induced inflammation. However, the specificity of the inhibitor ICS 205.930 is not fully established and it is possible that other receptors are also inhibited by this compound (Boeckaert *et al.*, 1990).

5-HT may, in addition, modulate the responses of other inflammatory mediators including neuropeptides like CGRP and substance P. 5-HT, as well as other mediators released during inflammation produces a dose-related stimulation of CGRP release from bovine pulpal nerve terminals (Jackson et al., 1992). CGRP involvement in neurogenic inflammation is well established. It is suggested that CGRP is a transmitter in the central and peripheral nervous systems and could be involved in nociceptive processing (Kruger *et al.*, 1985). It is released from perivascular nerves (Zaidi *et al.*, 1985), causes vasodilatation (Brain *et al*, 1985) and extends the response time of substance P (Le Greves *et al.*, 1985).

Substance P induces many of the events associated with neurogenic inflammation. It alters vascular permeability and calibre and releases a relaxant factor from vascular endothelium. It also stimulates the release of interleukins, tumour necrosis factor and arachidonic acid (Lotz *et al.*, 1988).

Substance P induces plasma extravasation and vasodilatation when perfused over a blister base in rat hind foot pad (Andrews & Helme, 1989; Andrews *et al.*, 1989; Khalil & Helme, 1989). The former response is maintained throughout the 30 minute stimulation period (Khalil & Helme, 1989), but the latter undergoes rapid tachyphylaxis (Moskowitz et al., 1987; Khalil & Helme, 1990). Substance P is most likely released from unmyelinated primary afferent nerve fibres with polymodal nociceptors (Hokfelt *et al.*, 1975) that cause neurogenic inflammation (Lembeck & Holzer, 1979). Perfusion with 5-HT in the rat hind paw blister model of inflammation before perfusion with substance P altered the resultant response. The vasodilatation did not undergo the normal rapid tachyphylaxis but was maintained throughout the period of stimulation and the resultant vasodilatation was significantly greater than

that observed with substance P alone. The plasma extravasion response was an initial inhibition during the stimulation period with substance P. That was followed by a post-stimulation period of enhancement (Khalil & Helme, 1990). Substance P alone produces only a single enhancing effect. It is most likely that capsaicin-sensitive nerves are involved in the modulatory effects as they are responsible for the vasodilatation and plasma extravasation seen with 5-HT (Khalil & Helme, 1989) and the vasodilatation response to substance P (Andrews & Helme, 1989). Pretreating the rats with capsaicin removed the modulatory effect of prior perfusion with 5-HT on the substance P response. The continual presence of 5-HT is not necessary for this effect (Khalil & Helme, 1990).

1.2.4 Serotonin Receptors

In 1957 it was recognised that more than one 5-HT receptor existed, so two subtypes were distinguished (Gaddum & Picarelli). These were called D- and M- serotonergic receptors depending on whether or not 5-HT action was inhibited by dibenamine or morphine.

It is now accepted that there are many types of receptor and that such a simple classification is not possible. A recent review (Hindle 1994) listed all the known receptor types. This is based on the work of Hoyer and Schoeffer (1991), Peroutka (1990, 1993) and Zita & Fillion, (1992). The present situation, with the addition of the work of Kim *et al.* (1992), Mawe *et al.* (1986), Branchek *et al.* (1988) and Gershon *et al* (1990) is summarised in Table 1.6.

5-HT Receptor	Agonist Ligands	Antagonist Ligands	Location	Tissue Activity
5-HT$_1$	Flesinoxan	Cyanopindol	1. Cerebral cortex	Anxiolytic
5-HT$_{1A}$	Urapidil	Spiperone		Hypotension
	5-CT	Metitpin	2. Raphe nucleus	
	Spiroxatrine	Pindolol	3. Hippocampus	
	Buspirone	Methiothepin	4. Arterioles	
	8-OH			
	DPAT			
5-HT$_{1B}$	5-CT	Propanolol		Autoreceptor
	Metergoline	Metitpin		(↓ Ach & Noradrenaline release)
5-HT$_{1C}$	α-Methyl 5-HT	Ritanserin	Choroid plexus	Vasodilatation
	1-Methyl 5-HT	Mesulergine		
		Ketanserin		
		Mianserin		
5-HT$_{1D}$	Sumatriptan	Metitpin	1. Substantia nigra	A. Migraine
	Metergoline		2. Caudate	B. Cerebral blood flow
	Methysergide		3. Globus Pallidus	
			4. Intracranial vessels	

Table 1.6. Classification of 5-HT receptors (adapted by Hindle, 1994 from the work of Bobker & Williams, 1990).

5-HT Receptor	Agonist Ligands	Antagonist Ligands	Location	Tissue Activity
5-HT$_{1P}$	5-hydroxyindalpine	BRL24924 N-acetly-5-hydroxytryptophal -5-hydroxytryptophan amide	1. Enteric nervous system 2. Skin 3. Heart 4. Dental pulp	A. Neurotransmitter
5-HT$_2$	α-Methyl 5-HT	Ketanserin Ritanserin Mianserin Spiperone Cyproheptadine Metitpin	1. Cerebral cortex 2. Spinal cord 3. Veins 4. Arteries 5. T 6. Platelets 7. Rat SA node	1. Anxiety 2. Depression 3. Pain signals 4. Preganglionic sympathetic excitation 5.Contractile activity: arteries, veins, T, bronchi 6. Platelet aggregation 7. Heart rate

Table 1.6. Classification of 5-HT receptors (continued)

5-HT Receptor	Agonist Ligands	Antagonist Ligands	Location	Tissue Activity
5-HT$_3$	2-Methyl-5-HT Phenylbiguanide	Odansetron Granisetron Zacopride MDL7222 ICS205930	1. Cerebral cortex 2. Nucleus tractus solitarus 3. Hippocampus 4. Enteric neurones 5. Nerve endings 6. Primary afferent nerve fibre	1. Anxiety 2. Cognition 3. Nausea 4. Pain signals 5. Neural transmission
5-HT$_4$	Metoclopramide Cisapride Zacopride Renzapride BRL24924	ICS205930 SDZ205557	1. Superior colliculi 2. Cerebral cortex 3. Guinea pig ileum 4. Human: Right atrium	1. EEG activity, gastric motility, cardiac inotropy

Table 1.6. Classification of 5-HT receptors (continued)

Receptor Subtype	Second Messenger	Effect	Ion Channels	Conductances
5-HT_{1A}	cAMP	Decrease	Hyperpolarisation	$\downarrow K^+$
5-HT_{1B}	cAMP	Decrease	Hyperpolarisation	?
5-HT_{1C}	PI	Increase	Hyperpolarisation	$\downarrow Cl^-$
5-HT_{1D}	cAMP	Decrease	?	?
5-HT_2	PI	Increase	Depolarisation	$\downarrow Cl^-$
5-HT_3	Unknown		Depolarisation	$\uparrow Na^+ K^+$
5-HT_4	cAMP	Increase	Depolarisation	$\uparrow Na^+ K^+$

Table 1.7. The coupling of 5-HT to specific receptors (adapted from Bobker & Williams, 1990). PI = phosphatidylinositol. cAMP = cyclic adenosine monophosphate.

5-HT receptors may be directly coupled to specific membrane channels (receptor gated ion channels), or may act via intracellular secondary messengers (Table 1.7). Some activate membrane phospholipase C, e.g. 5-HT_{1C} and 5-HT_2, whereas other receptors are linked to adenylate cyclase. 5-HT_3 receptors are directly linked to ion channels (Peters & Lambert, 1989). The receptors on sensory neurones may be involved in the direct pain-producing effect of 5-HT as they are linked to ligand-gated sodium channels (Rang *et al.*, 1991). Other effects of 5-HT include modulation of voltage-gated calcium channels (Dunlap & Fischbach, 1981) and inhibition of a slow hyperpolarising after-potential (Christian, Taylor & Weinreich, 1989).

There is some evidence that 5-HT itself may have a direct effect on the endothelium when it is released by activated platelets (Kishi & Numano, 1989). Using cultured fetal bovine aorta as a source of endothelial cells, thromboxane A_2 and serotonin caused cell damage in a dose- and time-dependent manner. Methysergide only partly

blocked the damage, while both the prostacyclin analogue, ZK 36374 and the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, prevented injury. It was postulated that substances that increased cyclic AMP levels may have a protective effect against damage caused by materials released from platelets (Kishi & Numano, 1989).

Removal of serotonin from the damaged site has been shown to produce accelerated wound-healing (Wolin *et al.*, 1994). In that study tetrachlorodecaoxygen (TCDO), a wound healing agent, was shown to form a complex with haemoglobin which was capable of removing serotonin. This may be the mechanism by which wound-healing may be facilitated by that TCDO.

1.2.5 Serotonin and Blood Flow

The name 'amphibaric hormone' was given to 5-HT due to its many actions on cardiovascular tissue (Page, 1954). In patients with carcinoid syndrome both vasodilatation and vasoconstriction may be observed and there is no simple explanation for the different responses observed with smooth muscles from various sites (Vanhoutte and Cohen, 1983). Serotonin may also produce 'indirect' effects as it may act both as a β-adrenergic or an α-adrenergic agonist (Vanhoutte *et al*, 1981; Clement *et al*, 1969; Curro *et al.*, 1978).

5-HT released from aggregated platelets causes vasodilatation at the level of the arteriole, increased capillary and venular permeability and venoconstriction. This is modulated through the 5-HT_2 receptor. It also potentiates the responses to other neurohumoral mediators. 5-HT increases the vasoconstrictor responses to noradrenaline and angiotensin II (Van Nueten *et al.*, 1981; de le Lande *et al.*, 1967; Van Nueten *et al.*, 1982; Rapoport & Bevan, 1982). This will promote local stasis and oedema.

Sensitivity of vascular smooth muscle can be modulated both acutely and chronically (Vanhoutte & Cohen, 1983). The constriction responses to serotonin are exaggerated in the short term in cutaneous veins by cooling and, over a longer period of time, in

the coronary arteries and by hypoxia (Vanhoutte & Shepherd, 1970; Van Nueten & Vanhoutte, 1980; Van Nueten, Van Beek & Vanhoutte, 1980).

High concentrations of serotonin will displace noradrenaline from adrenergic nerves (Vanhoutte, *et al.*, 1981; Mc Grath, 1978) causing α-adrenergically-mediated vasoconstriction in most blood vessels, but β-adrenergic relaxation in the facial and coronary veins (Pegram, Bevan & Bevan, 1976; Cohen, Shepherd & Vanhoutte, 1983). However, low doses of serotonin inhibit the release of noradrenaline (Mc Grath, 1978).

Endothelial cells reduce the constriction produced as a result of serotonin release by aggregated platelets (Cohen, *et al.*, 1982 & 1983) probably by causing enzymatic breakdown of the 5-HT, but also by the endothelium causing vascular relaxation when exposed to various agents (Furchgott *et al.*, 1981; Vanhoutte & De May, 1983; Vanhoutte & Rimele, 1983). Concentrations of 5-HT that altered local arterial pressure in the vessels supplying the dental tissues (but not systemic arterial pressure measured in a limb to a significant effect) decreased the dental pulp blood flow significantly ($p < 0.05$, $30.7 \pm 15.2\%$), as measured by laser Doppler flow. This is in agreement with other research using radiolabelled microspheres (Kim *et al.*, 1986, 1992). Local application of 5-HT caused an insignificant ($p > 0.05$) decrease in pulpal blood flow ($9.3 \pm 5.2\%$), as measured by laser Doppler flow (Liu *et al.*, 1990). In that case the effect of the 5-HT may have been offset by the excitation of local pulpal sensory nerves leading to the release of vasodilatory neuropeptides such as substance P (Gazelius & Olgart, 1980).

1.3 HISTAMINE

1.3.1 Introduction

Histamine was first isolated in 1911 (Barger & Dale). Its functions in allergy and anaphylaxis have been described and most of the early research was dedicated to examining the pathological actions of histamine. More recently the role of histamine in physiological processes have been studied even though the functions that it regulates are not fully elucidated (Hough & Green, 1984).

1.3.2 Synthesis and Deamination

Histamine is derived from the decarboxylation of histidine by a pyridoxal phosphate-dependent enzyme, histidine decarboxylase (HDC). This enzyme is not a single entity as HDC isoenzymes with differing isoelectric points have been isolated (Savany & Cronenberger, 1982: Watanabe & Wada, 1983). Two separate HDC cDNAs have been demonstrated with 86.1% homology in their derived amino acid sequences (Joseph *et al.*, 1990; Yamamoto *et al.*, 1990).

Storage of histamine occurs in the cytoplasmic granules of mast cells (Riley & West, 1966) and basophils (Merget *et al.*, 1990), where it is bound to the anionic side-chains of proteoglycans in the granule matrix. It is released in response to IgE or non-IgE specific mechanisms. The inhibitory effect of dexamethasone on mitogen-activated HDC may explain its effect in allergic inflammation.

Oxidative deamination is carried out by either diamine oxidase (DAO), which is a semicarbazide-sensitive amine oxidase enzyme, or histamine-*N*-methyltransferase (HMT) followed by MAO-B or DAO (Green *et al.*, 1987) (Figure 1.2). The route of breakdown is specific to the species and organ. In human brain HMT is the main pathway, but in invertebrates DAO is the major route.

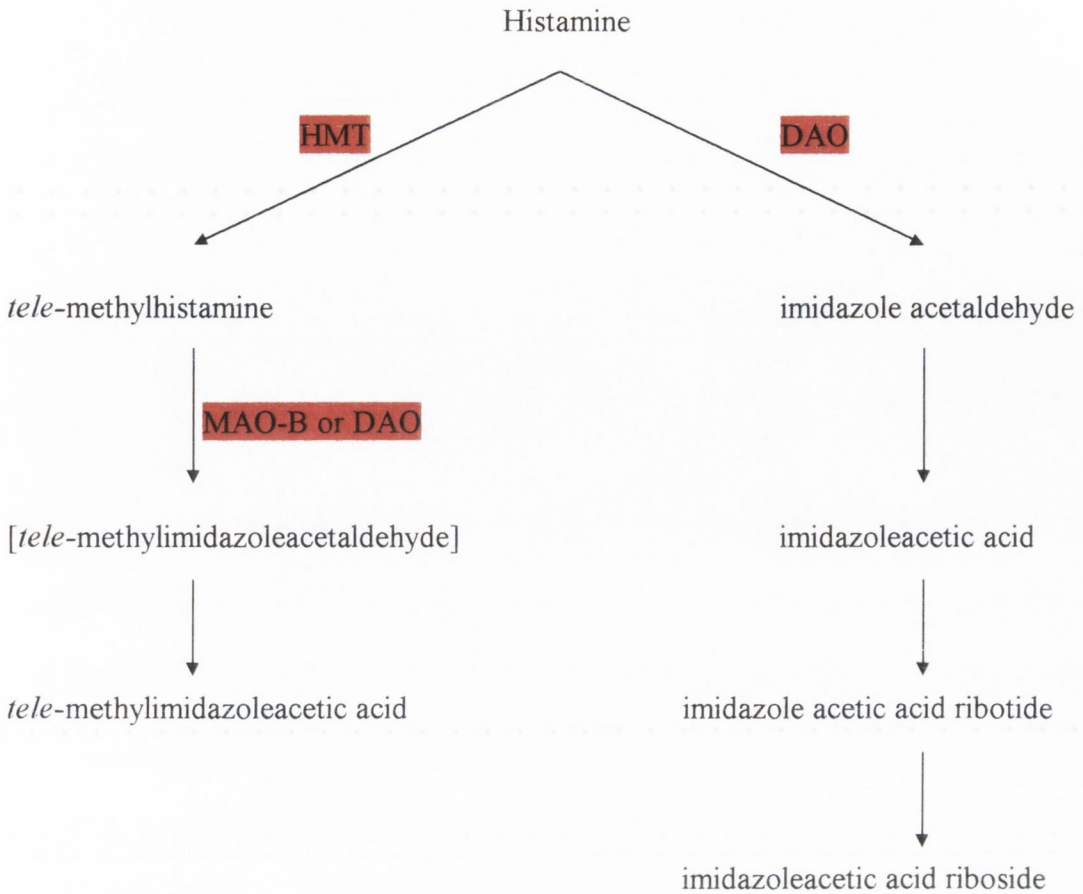


Figure 1.2. The two major metabolic pathways of histamine. HMT = histamine methyl transferase; DAO = diamine oxidase (semicarbazide-sensitive amine oxidase); MAO-B = monoamine oxidase B. From Green *et al.* (1987).

1.3.3 Histamine receptors

There are three receptor subtypes for histamine, designated H1, H2 and H3 (see Table 1.8).

The H1 receptor was first demonstrated by the inhibition of contractile response of isolated guinea pig ileum (Hill *et al*, 1977) by the specific antagonist pyrilamine and subsequently by binding studies with the [^{3}H] labelled compound. It was proposed that the antagonist binding site was the H1 receptor as its dissociation constant (K_d) correlated with the amount required to inhibit half of the contractile response of the guinea pig ileum to histamine (EC_{50}). Similar numbers of binding sites for the antagonist were observed on rat, guinea pig and rabbit ileal membranes . However the contractile effects of histamine varied among species (Chang *et al*, 1979).

The activated H1 receptor increases vascular permeability and releases catecholamines from chromaffin tissue. Antagonism of the H1 receptor in the brain produces marked sedative effects.

Studies involving the inhibitor [^{3}H]-pyrilamine indicate that extracellular concentrations of Na^+, Mg^{2+}, Mn^{2+} and guanine nucleotides may alter the agonist affinity in guinea pig brain membranes and that dithiothreitol also increased agonist affinity in guinea pig ileum (Chang & Snyder, 1980: Donaldson & Hill, 1987). Mitsuhashi and Payan (1989) suggested that the microenvironment may explain the apparent diversity of the H1 receptors and that the receptor may also be influenced by different G-proteins, as shown with other G-protein-coupled membrane receptors (Koo *et al*, 1983).

The high-affinity H1 receptor on BC3H1 smooth muscle cells was changed to a low-affinity receptor by incubation with glycosylation inhibitors (tunicamycin or swainsonine) without decreasing the number of receptors (Mitsuhashi & Payan, 1989). Substances that inhibit carbohydrate binding also inhibited [^{3}H]-pyrilamine binding to differentiated smooth muscle cell membranes, but not to undifferentiated cell membranes. The relative molecular mass (M_r) of the H_1 receptor, as determined by sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE), decreased from 68,000 (the M_r in differentiated cells) to 40,000 (the M_r in undifferentiated cells) after treatment with N-glycanase (Mitsuhashi & Payan, 1989). This suggests that N-glycosylation may also contribute to the variation seen with the H1 receptor.

The H1 receptor may also have one or more disulphide bridges. SDS-PAGE analysis indicated a M_r in the range 350,00 to 400,000 in the absence of 2-mercaptoethanol, as detected by [^{125}I]-iodoazidophenpyramine incorporation into the H1 receptor as a result of irreversible photoaffinity labelling. In the presence of 2-mercaptoethanol the M_r decreased to 56,000 and 47,000. (Ruat *et al*, 1988). Following treatment with a protease the M_r of the 56,000 Da peptide decreased to 47,000, suggesting that the latter may be a cleavage product of the larger molecule.

The H1 receptor is coupled to phosphatidylinositol hydrolysis pathways. Stimulation induces the hydrolysis of phosphatidylinositol to form inositol-1,4,5-trisphosphate (IP3) and diacylglycerol. This results in intracellular Ca^{2+} mobilisation and activation of protein kinase C (PKC) (Hill, 1990). The action of PKC in cells may vary since subtypes of the enzyme exist, as determined by cloning studies (Boyer *et al*, 1989).

The H2 receptor is coupled to the adenylate cyclase and its stimulation increases the level of intracellular cyclic adenosine monophosphate (cAMP) (Black *et al.*, 1972). It may also increase the levels of Ca^{2+} in parietal cells (Chew, 1986). The same occurs in human HL-60 granulocytic cells in a dose dependent manner. The Ca^{2+} has been shown to originate from intracellular stores and histamine stimulation also results in increased levels of IP3 (Mitsuhashi *et al*, 1989).

The chemokinetic response of neutrophils is markedly increased upon H2 receptor stimulation and decreased by antagonists (Anderson *et al*, 1977: Seligman *et al*, 1983). The inhibition occurs by the mechanism involving elevation of cAMP, but the mechanisms involved in stimulation are not known. It appears that there may be alternative paths that result in stimulation of neutrophils.

Combination of histamine with albumin increases the level of intracellular calcium in HL-60 cells and this complex has been used as an active ligand to identify the H2 receptor bearing leukocytes (Melmon *et al*, 1972).

The H2 receptor cDNA from parietal cells has been cloned (Gantz *et al*, 1991). The deduced amino acid sequence displays similar structural moieties to many other G-protein coupled receptors in which the peptide spans the membrane several times with an extracellular $-NH_2$ terminal and an intracellular -COOH terminal. The first extracellular domain exhibits a possible N-glycosylation site. Cysteine residues are located on the second and third extracellular sites (Gantz *et al*, 1991).

Histamine-induced gastric acid secretion is competitively inhibited by H2 receptor antagonists such as cimetidine and ranitidine and is an established treatment for gastric ulcers.

The H3 receptor is pre-synaptically located on histaminergic nerve endings and modulates histamine synthesis and release in brain (Arrang *et al.*, 1983). Exogenous histamine decreases the release and formation of endogenous histamine and this autoregulation was found to occur in the parts of the brain where histamine nerve endings were located. This suggests that these nerve endings contain H3 receptors. Arrang (1992) demonstrated this type of regulation at the level of the posterior hypothalamus, suggesting that autoreceptors may exist at the level of the perikarya or dendrites. The H2 receptor also inhibits the release of monoamines in the brain and peripheral tissues and neuropeptides from unmyelinated C-fibres (Arrang *et al.*, 1992: Schwartz *et al.*, 1991).

Through the use of specific agonists for each receptor i.e. (*R*)α-methylhistamine (H1, Arrang *et al.*, 1987), (*R*)α, (S)β-dimethyl-histamine (H2, Lipp *et al*, 1992) and imetit (H3, Garbarg *et al.*, 1992) the functions of histamine in the brain has been partly elucidated. The H3 receptor ligand plays a part in cortical activation and arousal (Schwartz *et al.*, 1991). Agonists increase slow-wave sleep and decrease wakefulness and antagonists increase arousal (Lin *et al.*, 1990: Schwartz *et al.*, 1991).
H3 receptors in the airways are located pre-synaptically on both pre- and post-ganglionic cholinergic fibres and thus modulate the release of acetylcholine in an inhibitory manner (Barnes, 1992). The receptors also inhibit pre-synaptically the release of tachykinins from unmyelinated C-fibres (Barnes, 1992).

	H1	H2	H3
Number of amino acids	491 a.a. (bovine 488 a.a. (guinea pig) 486 a.a. (rat)	358 a.a. (rat) 359 a.a. (dog, human)	?
Chromosome localisation	Chromosome 3	?	?
Highest brain densities	Thalamus Cerebellum Hippocampus	Striatum Cerebral cortex Amydgala	Striatum Frontal cortex Substantia nigra
Autoreceptor	No	No	Yes
Affinity for histamine	Micromolar	Micromolar	Nanomolar
Characteristic agonists	2(methylchlorophenyl) -histamine	Impromidine, Sopromidine	(R)α-methylhistamine, Imetit
Characteristic antagonists	Mepyramine	Cimetidine	Thioperamide
Radioligands	[^{3}H]-Mepyramine [^{125}I]-Iodo-bolpyramine	[^{3}H]-Tiotidine [^{125}I]-Iodo-aminopotentidine	[^{3}H]-(R)α-methyl-histamine, [^{125}I]-Iodo-phenpropit
Second messengers	Inositol phosphates Arachidonic acid cAMP	CAMP Arachidonic acid Ca^{2+}	Inositol phosphates

Table 1.8. Properties of the three histamine receptors subtypes (adapted from Arrang, 1994).

The structure of the H3 receptor has not yet been fully elucidated. It appears that it belongs to the same family of G-protein coupled receptors as the other histamine receptors (Arrang *et al.*, 1990). The H3 receptors in parietal cells are negatively coupled to phospholipase C (Cherifi *et al.*, 1992).

1.3.4 Similarities between serotonin and histamine receptors

There appears to be some degree of a cross-over of the functional activities of serotonin and histamine. Many cell surfaces express both histamine and serotonin receptors, so determining whether each amine can act as a receptor agonist of the other is difficult. Three serotonin receptors, 5HT1a, 5HT1c and 5HT2b, have been cloned and expressed in the Xenopus oocyte membrane (Julius *et al.*, 1988) by using low-stringency cross-hybridisation techniques (Pritchett *et al.*, 1988: Fargin *et al.*, 1988).

The mRNA for the 5HT1c receptor was transcribed *in vitro* from the cloned 5HT1c receptor cDNA and inserted into Xenopus oocytes (Shichijo *et al.*, 1991). Histamine and agonists of the H1 receptor induced $^{45}Ca^{2+}$ efflux in 5HT1c-receptor-RNA-injected oocytes, but not in uninjected or water-injected oocytes. However, the mechanism of action appears different from the standard pharmacological behaviour of histamine as antagonists to the H1, H2 and H3 receptors failed to inhibit this efflux, even at high concentrations (10^{-6} M) (Shichijo *et al.*, 1991).

1.3.5 Biological effects of histamine

Local allergic reactions are usually confined to the target organ, but may be more widespread in anaphylactic reactions. These may result in response to stimuli such as insect stings, drugs or anaesthetic agents. A large rise in plasma histamine is observed in such cases and the larger the rise the greater the effect (Ennis & Lorenz, 1984). At levels <1 ng/ml symptoms may include a mild cutaneous reaction, metallic taste, nasal congestion and nausea. Increased levels lead to skin reactions, cardiac arrhythmias, tachycardia, hypotension and gastrointestinal disturbances. Plasma histamine concentrations >12 ng/ml may be life-threatening with pronounced

hypotension, ventricular fibrillation, bronchospasm, cardiac and respiratory arrest (Pearce, 1991).

Histamine regulates the release of acid by gastric parietal cells (see Black & Shankley, 1987) by interacting with acetylcholine and gastrin. Two theories have been proposed to explain the method of modulation (see Black & Shankley, 1987). The *permission* theory proposes that these three agonists act on the receptors on the parietal cells but the effects of the acetylcholine and gastrin are only observed if the receptor also has histamine present. The *transmission* hypothesis states that both acetylcholine and gastrin act on the mast cells. The mast cell, in return, release the final common stimulant for regulating the parietal cells, histamine.

The effect of histamine on muscle is species- and site-dependent. In the cardiovascular system the effect is mainly vasodilatation. This produces the characteristic drop in blood pressure and flushing due to a decrease in total peripheral resistance (Owen, Harvey & Boyce, 1982). A positive inotropic and chronotropic cardiac effect is also observed (Levi *et al.*, 1982). In other muscle groups the main reaction to histamine is contraction. This is seen to an exaggerated extent with asthmatics, as marked bronchoconstriction that may compromise the airway.

Histamine functions as a neuroregulator and neurotransmitter in the brain (Prell & Green, 1986). Sources are the mast cells and histaminergic neurones (Schwartz *et al.*, 1990). The former are found in association to blood vessels. The latter are relatively few in number, at least in the mammalian CNS, but the axons divide into fibres that inervate much of the brain (Schwartz *et al.*, 1990). Possible roles for these nerves include vasopressin release, ACTH and prolactin secretion, increased blood pressure and heart rate, thermoregulation, vasodilatation and modulation of the arousal mechanisms (see Schwartz *et al.*, 1990).

Histamine has also been implicated in immune surveillance and carcinogenesis (Batholeyns & Bouclier, 1984). Through the actions of H2 receptors, histamine suppresses lymphocyte proliferation, T-cell mediated cytotoxicity of allogenic target cells, lymphokine production, natural killer cell cytotoxicity and antibody production

by B-lymphocytes (Bach *et al.*, 1985). Histamine may also activate suppressor T-lymphocytes. This occurs via lymphokine histamine suppressor factor (HSF) produced by certain T-cell H2 receptors. HSF blocks the proliferation of lymphocytes by increasing the production of monocytes, and it may also activate suppressor T-cells (Pearce, 1991).

Acting on the H2 receptor, histamine decreases the production of complement component C2 in human monocytes (Lappin & Whaley, 1980) and also C2, C5 and factor B production in mouse peritoneal macrophages (Ooi, 1982: Falus & Meretey, 1989). It decreases the production of C3, but the biosynthesis of this component is increased through the H1 receptor.

Histamine also modifies cytokine-induced complement and fibrinogen synthesis, enhancing interleukin-6-stimulated C3 and fibrinogen expression (Rokita *et al.*, 1992), but it suppresses interferon-γ (IFN-γ) induced C3 secretion in mouse primary hepatocytes.

Cytokines are influenced by histamine that acts both as a target of cytokines and a regulator of cytokine-receptor interactions. Histamine (via the H2 receptor) decreases endotoxin induced Interleukin 1 (IL-1) production (Dohlsten *et al.*, 1988) and tumour necrosis factor α (TNF-α) production in human monocytes, but it has no effect on IL-6 production (Vannier *et al.*, 1991). In contrast, in the mouse peritoneal, macrophage histamine has a slight enhancing effect on IL-1 production (Okamoto & Nakano, 1990).

Histamine also inhibits IL-2 production and decreases the production of IFN-γ, although the latter may be indirectly caused by the inhibition of the former (Carlsson *et al.*, 1985). It also affects the production of some neurotransmitters and pituitary hormones that modulate the cytokine network. These include adrenocorticotropic hormone, β-endorphin, α-melanocyte-stimulating hormone and prolactin (all of which are enhanced) and thyroid stimulating hormone (which is suppressed) (Knigge & Warberg, 1991).

The cytokines in return exert control over the production and release of histamine. IL-1 may enhance histamine production, stimulated by bacterial endotoxin, in mouse macrophages. If histamine release is stimulated by IL-1 this may lead to increased local production of IL-6 by cells with active histamine receptors. IL-1 potentiates the effect of histamine on the release of prostaglandin and monohydroxyeicosatetraenoic acids (HETEs) by endothelial cells and hematopoietic precursor cells (Revtyak *et al.*, 1988). IL-3 induces histamine as well as IL-4 and IL-6, but not IL-2 or IFN-γ production. IL-8 has a positive priming effect on IL-3-enhanced histamine release (Bischoff *et al.*, 1991). IL-5 also primes human basophils towards the histamine-induced release by C5a, anti-IgE and fMLP (Bischoff *et al.*, 1990).

Increased levels of histamine and histidine decarboxylase (HDC) and decreased concentrations of amine oxidases are associated with tumour growth in both experimental animals and humans (Bartholeyns & Fozard, 1985). Tumour growth may be inhibited by H2 receptor antagonists and H1 receptor agonists. H1 antagonists may promote growth (Bartholeyns & Fozard, 1985: Burtin *et al.*, 1982).

Histamine has also been described as an intracellular messenger in human platelets (Saxena *et al.*, 1989: Gerrard *et al.*, 1993). Intracellular concentrations of histamine and HDC activity have been correlated with cell growth (Kahlson & Rosengren, 1971). Inhibitors of HDC decreased phorbol ester or collagen-induced platelet aggregation. This was reversed by N,N-diethyl-2-[4-(phenylmethyl)phenoxy}-ethanamine-HC1 (DPPE), a non-specific histamine antagonist. In saponin-permeabilized platelets histamine reversed the effect of inhibitors of DPPE or other HDC inhibitors on platelet aggregation.

1.3.6 Histamine and SSAO

The metabolism of histamine has been dealt with in Section 1.3.2. The breakdown of histamine in dental pulp, if catalysed by oxidative deamination (MAO or DAO), may have implications for inflammation in dental pulp. The biological effects of histamine have been discussed in Section 1.3.5 in terms of its role in inflammation, blood flow, cytokine stimulation /inhibition and as an intra-cellular messenger. Modulation of the histamine present in dental pulp by modulating its rate of metabolism may open new

means by which inflammation may be controlled. If oxidative deamination occurs then metabolite production may also be important in terms of hydrogen peroxide release as will be discussed in Section 1.4.

1.4 Hydrogen peroxide, biogenic amines and cell death

In the developing blastocyst extracellular hydrogen peroxide is believed to cause apoptosis of the inner cell mass destined to develop into trophectoderm. This hydrogen peroxide is thought to be produced by the metabolism of polyamines by amine oxidases (Parchment 1993).

Hydrogen peroxide may be the most important reactive oxygen species (ROS) that impact on human spermatozoa. The effect seen depends on the concentration of hydrogen peroxide. High concentrations cause lipid peroxidation and cell death. Low concentrations cause sperm immobility, mostly by a mechanism involving depletion of intracellular ATP and the resulting decrease in the phosphorylation of axonemal proteins (de Lamirande and Gagnon, 1995).

The generation of hydrogen peroxide at low levels may act as an intra- or inter-cellular messenger capable of promoting growth. The exact mechanisms of this action are unknown, but they are thought to involve direct interaction with specific receptors or oxidation of growth signal transduction molecules, such as protein kinases, protein phosphatases, transcription factors or transcription inhibition factors (Burdon, 1995). It may also alter the activity of signal transduction proteins through the route of altering the levels of reduced glutathione (GSH) and oxidised glutathione (GSSG) in the cell. It is suggested that there exists a balance between the level of hydrogen peroxide that causes cell proliferation and that that causes lipid peroxidation and cell death (Burdon, 1995). Increased levels cause an initial proliferation but if prolonged may result in death of the cell.

Reduced glutathione may have a part to play in preventing cell death by buffering oxidative stress. In human U937 monocytic cells apoptosis was found to occur simultaneously with depletion of GSH that was extruded from the cell. However the

modulation of intracellular GSH did not influence the overall level of apoptosis, so GSH depletion alone does not cause cell death (Ghibelli *et al.*, 1995).

It has also been proposed that hydrogen peroxide is involved in cell death observed with certain pharmacological agents such as sodium nitroprusside, a vasodilator and nitric oxide donor, when used against murine neuroblastoma N1E-115 cells (Yamada *et al.*, 1996).

Perfusion of rabbit lung with 3 X 10^{-5} M hydrogen peroxide raised the overflow of thromboxane B2 (TXB2) and perfusion pressure. It caused oedema and epithelial distress (evidenced by increased 6-oxo-PGF1α). However cell death did not result, as determined by lactate dehydrogenase release (Corten *et al.*, 1991).

Apoptosis of human natural killer cells (NKC) was prevented by catalase and levels of hydrogen peroxide greater than 1 μM induced apoptosis in both NKC and human T cells, the latter being two to five times more susceptible. The apoptosis observed with hydrogen peroxide was independent of the synthesis of protein or mRNA. However it was blocked by the endonuclease inhibitor aurin tricarboxylic acid. It was also inhibited by herbimycin A, an inhibitor of tyrosine kinase, suggesting that the cell death was dependent upon protein kinases (Hansson *et al.*, 1996).

Myoblasts are also susceptible to apoptosis in response to increased concentrations of hydrogen peroxide as well as to other reactive oxygen intermediates and nitric oxide (Stangel *et al.*, 1996). This may partly explain the reason why in certain degenerative and metabolic muscle diseases the cells die without any notable inflammatory response. Cultured rat cortical neurones are also sensitive to low concentrations of hydrogen peroxide, which results in cell death (Whittemore *et al.*, 1994).

Hydrogen peroxide is released following phagocytosis of mycobacteria and the physiological result of this may be to kill the cell wherein the bacteria are contained. This is the proposed mechanism for the hydrogen peroxide-mediated apoptotic death of *M. avian* mycoplasma intracellular-infested monocytes. This may be why the parasite rarely causes disease in healthy individuals, but does so in human

immunodeficiency virus (HIV) type I where the monocyte population can be defective or absent can infect almost every area (Laochumroonvorapong *et al.*, 1996).

H_2O_2 is relatively non-toxic, however the presence of transition metals it can be converted to the highly toxic $^{\bullet}OH^{-}$ radical. The hydroxyl radical has been proposed as the major damaging species formed under Fenton systems under biologically-relevant conditions (Halliwell & Gutteridge, 1992). Similar metal-catalysing Fenton reactions resulting in hydroxyl radical release have been reported with thiol oxidation in cultured V79 cells using dithiothreitol (DTT, concentration range 0.2-1.0 mM)(Held *et al.*, 1996).

1.5 Human dental pulp

Dental pulp is composed of loose connective tissue surrounded by a rigid casing of dentine and enamel. It has three principal functions: (1) it maintains the vitality of the cellular components by providing oxygen and nutrients, (2) it elaborates and repairs the calcified tissue in which it is contained and (3) it responds to irritation by producing patterns of neuronal activity that evoke painful stimuli (Van Hassel, 1971). Dental pulp is derived embryologically from the cephalic neural crest. Because of this surrounding enamel and dentine, the monitoring and regulation of blood flow is critical. Its low compliance environment prevents the pulp volume from increasing during periods of high vascular permeability and vasodilatation.

1.5.1 Anatomical zones and cells of the pulp

The pulp consists of four distinct areas (Figure 1.3) (see Trowbridge and Kim, 1993). The outermost zone is labelled the *odontoblast layer*. This is composed of the cell bodies of the odontoblasts, the cells responsible for the formation of dentine (dentinogenesis) during tooth formation (developmental or primary dentine), physiologically after tooth formation (secondary dentine), or in response to injurious agents (tertiary dentine). Odontoblasts produce a matrix of collagen fibres and proteoglycans that may undergo mineralisation. During maturation the cells undergo a change in shape from cuboidal to tall columnar. Extensions of these cells reach into the dentine and are called odontoblastic processes. This layer also contains some nerves and blood vessels.

The second area is called the *cell poor zone*. This layer is beneath the odontoblast layer and is in the region of 40 μm wide. It is traversed by nerves, blood vessels and cytoplasmic processes of fibroblasts, but contains few cells.

The *cell rich zone* contains a large number of fibroblasts. Odontoblasts that become damaged are replaced by specific cells that originate from this layer (Fitzgearld *et al.*, 1990). Immature fibroblasts are not well developed, polygonal in shape and are dispersed throughout the pulp (Avery, 1971). As the cell matures the cell changes to a stellate shape with many cell-to-cell contacting processes. Organelles appear within the cell, *e.g.* Golgi complexes and rough endoplasmic reticulum. Secretory vesicles are also present and collagen fibrils appear along the outer surface of the cell body. The number of cells decreases when the pulp matures (Avery, 1971). Lymphocytes and macrophages may also be found in this area. Lymphocytes of both the B and T variety have been identified in the pulp, T8 being the most common T cell found (Hahn *et al.*, 1989). Macrophages are involved in phagocytosis, endocytosis and antigen processing for T lymphocytes (see Trowbridge, 1990). Macrophages also secrete factors such as interleukin-1 and various cytokines.

The innermost layer is the *pulp proper*. This consists of nerves, blood vessels and cells embedded in a ground substance. The ground substance consists mainly of proteoglycans and glycoproteins. The principal proteoglycans include hyaluronic acid, dermatan sulphate, heparin sulphate and chondroitin sulphate (Mangkornkarn & Steiner, 1992). Principal glycoproteins present in this layer include fibronectin, laminin and tenascin.

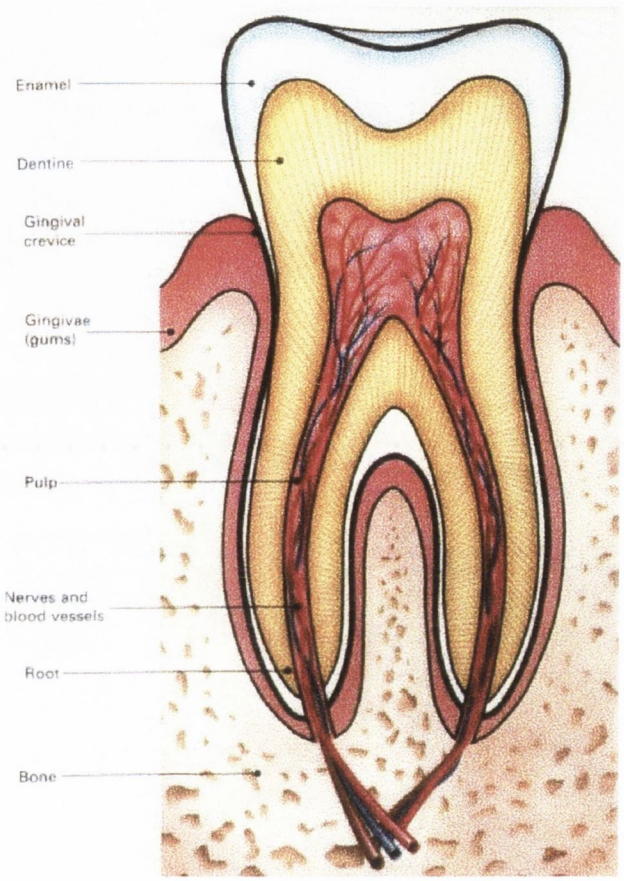


Figure 1.3. The structure of dental pulp.

1.5.2 Blood supply

Blood supply to the pulp enters mainly through the apical foramina (Figure 1.4), but is also supplied via lateral canals. Main vessels in the pulp consist of arterioles of below 100 μm and venules of less than 200 μm diameter. From the arterioles numerous capillaries branch off at right angles and form the subodontoblastic capillary plexus. In the root canal region the capillaries are arranged in a fine cross-fenced terminal network, but in the pulp horn region there is a dense pin-loop network (Takahashi *et al.*, 1982). The greatest volume of blood flow is in the region of the pulp horn (Meyer & Path, 1979). The venous system occupies the greater portion of the central area of the pulp, with smaller venules coalescing to form venules of greater diameter as they progress from the tooth. The walls of the venules are thin with a patchy muscular coat. Three features of the vasculature of the pulp are unusual: arteriovenous anastamoses, venous-venous anastamoses and U-turn loop arteries (Takahashi, 1982). The precise function of these peculiarities is not known, but they may be involved in regulation of blood flow (Kim, 1985). The blood flow to dental pulp in dogs has been demonstrated to be relatively high when compared to the resting blood flow in more cellular tissues (Tonder, 1980) suggesting that blood flow may be in excess of metabolic requirements.

The sympathetic system is believed to control the blood flow (Kim *et al.*, 1983). Electrical stimulation of the sympathetic nerve fibres or infusion with noradrenaline decreases the blood flow (Edwall, 1971; Tonder & Naess, 1978) and tissue pressure (Kroeger, 1968). However antagonists of α-adrenoceptors do not completely inhibit the response to stimulation (Edwall et al, 1985), suggesting other factors may be involved. Neuropeptide Y has been isolated in feline dental pulp (Edwall et al, 1985). It is primarily located in the principal cells of the superior cervical ganglion and transferred to the dental pulp along the post-ganglionic sympathetic axons of the inferior alveolar artery and the lingual nerve. The peptide causes vasoconstriction of blood vessels that is resistant to α-adrenoceptor blockade. Noradrenaline and Neuropeptide Y may act in tandem to regulate pulpal blood flow, the latter being more sustained in its effect lasting for several minutes (Olgart *et al.*, 1987).

Sympathetic vasomotor control in dental pulp is susceptible to inhibition in the form of mechanical insult and rapid changes in pulpal temperature (Ahlberg & Edwall, 1977). This suggests that part of the sympathetic control is located within the pulp and that endogenous substances may suppress vasomotor tone (Olgart *et al.*, 1989).

The presence of parasympathetic system vasomotor control in dental pulp is controversial. Histochemical studies have demonstrated the presence of acetylcholine esterase (Avery & Chiego, 1990), which is responsible for degradation of acetylcholine (Ach). Vasoactive intestinal polypeptide (VIP)-like immunoreactivity has also been described in mammalian dental pulp (Uddman *et al.*, 1980) and as this is often co-localised with Ach containing nerves, suggesting that vasodilator nerves may be present. VIP has been demonstrated to cause vasodilation in dental pulp (Olgart, 1989) and inhibit sympathetic vasoconstrictor tone.

The pulp is encased in a rigid dentine structure and thus is unable to increase in volume. In acute inflammation the sudden increase in blood flow is restricted by the dentine and so pressure in the pulp increases. Vasodilators actually reduce blood flow in the pulp (Tonder, 1976; Kim *et al.*, 1982). This was originally thought to occur as a result of the elevated tissue pressure actually exceeding that of the blood vessels and therefore compressing the vessels to reduce the amount of blood flow, the "Self-Strangulation Theory". The mechanism of strangulation is based on Starlings Law (1896) governing transcapillary water flow. On the arterial side of the capillary, the hydrostatic pressure of the blood (P_c) and the colloid-osmotic pressure of the interstitial fluid (π_i) will tend to move fluid out of the vessel. On the venous side, where the blood pressure (P_c) is lower, the plasma colloid osmotic pressure (π_p) will move the fluid towards the vessel. The tissue pressure (P_i) normally approximates atmospheric pressure and has little influence. However, if the P_i drops it will draw fluid from the vessels and if it is raised the opposite will occur. The Self-Strangulation Theory is shown in Figure 1.5 in which the main feature is the "vicious circle" established that leads to an increase in tissue pressure until the venous system is occluded and pulpal death occurs.

However, more recent studies have demonstrated that self-strangulation rarely occurs. The inflammation in dental pulp has been demonstrated to occur more locally without spreading throughout the pulp and that the pressure increase observed rarely spreads to the apical pulp (Van Hassel, 1971; Stenvik *et al.,* 1972). Heyeraas and Kvinnsland (1992) found that increased intra-pulpal pressure promoted fluid absorption back into the blood in cat dental pulp. This was recorded using a micropuncture technique and laser Doppler flowmetry to simultaneously measure the tissue pressure and blood flow during neurogenic inflammation. This increased blood flow was evident for greater than 8 hours, leading the authors to propose that plasma proteins and other macromolecules must have been removed by lymphatic drainage.

There are three main mechanisms in dental pulp that can break the "vicious cycle". The first is the lymphatic system, with an increased net absorption in adjacent healthy tissue that prevents the increase in P_i from spreading; the second is the absorption of fluid into the capillaries in the adjacent non-inflamed area and the third localised increased hydrostatic tissue pressure which prevents accumulation of interstitial fluid in the dental pulp (Tonder, 1983) (Figure 1.6).

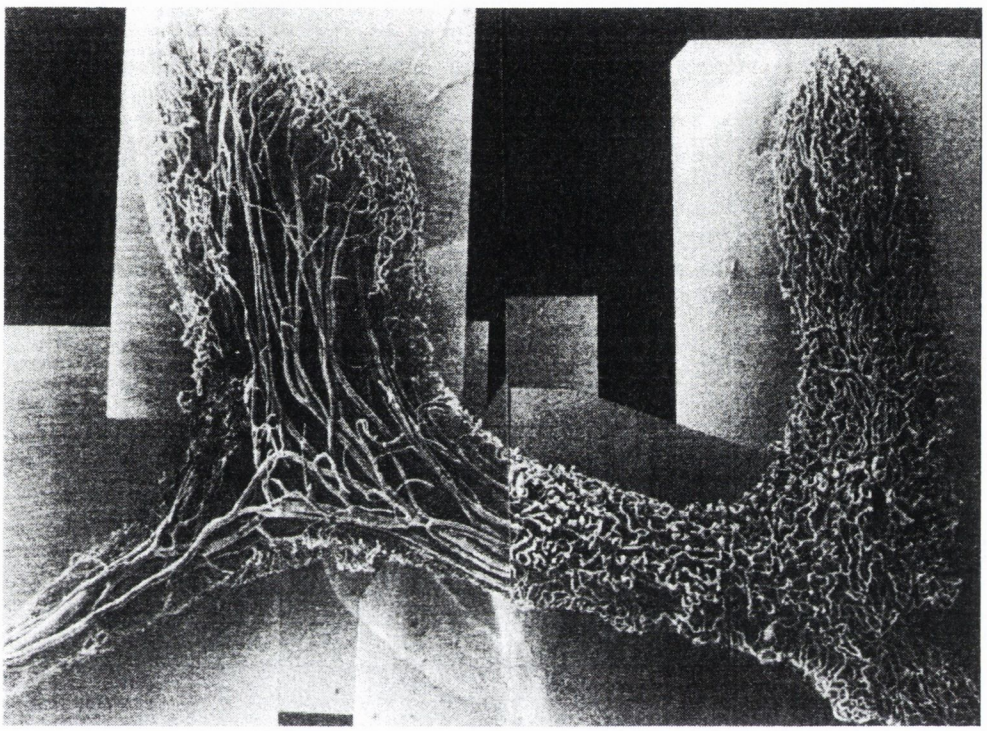


Figure 1.4. Resin cast of the vasculature of a canine molar. On the right side the peripheral vasculature can be seen. On the left side the peripheral vasculature has been removed to show the central pulp vessels. From Ten Cate, A.R. *Oral Histology*, Mosby, St Louis, USA.

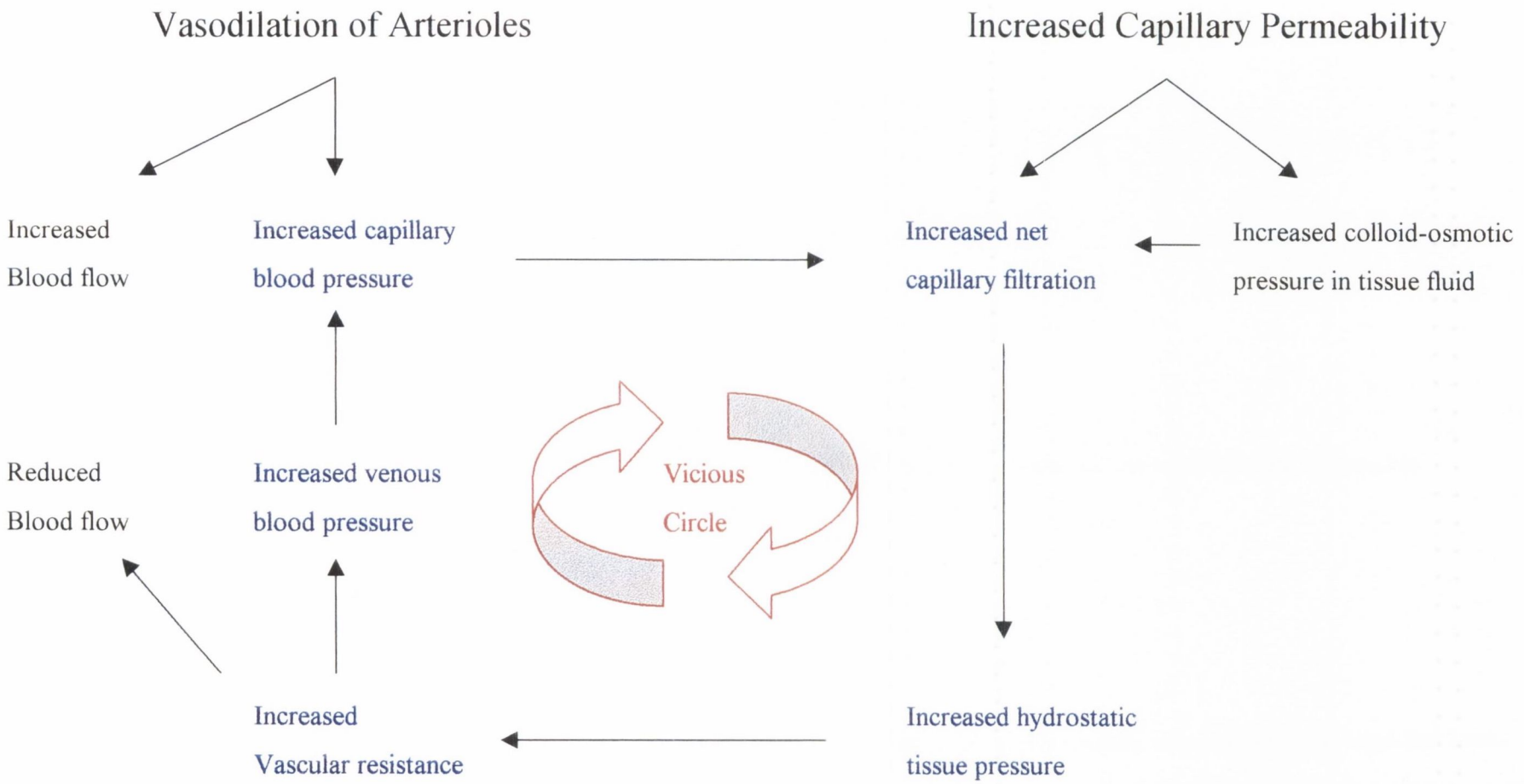


Figure 1.5. The "Self Strangulation Theory" model of pulpal inflammation leading to a "vicious circle" of inflammation. Adapted from Tonder (1983).

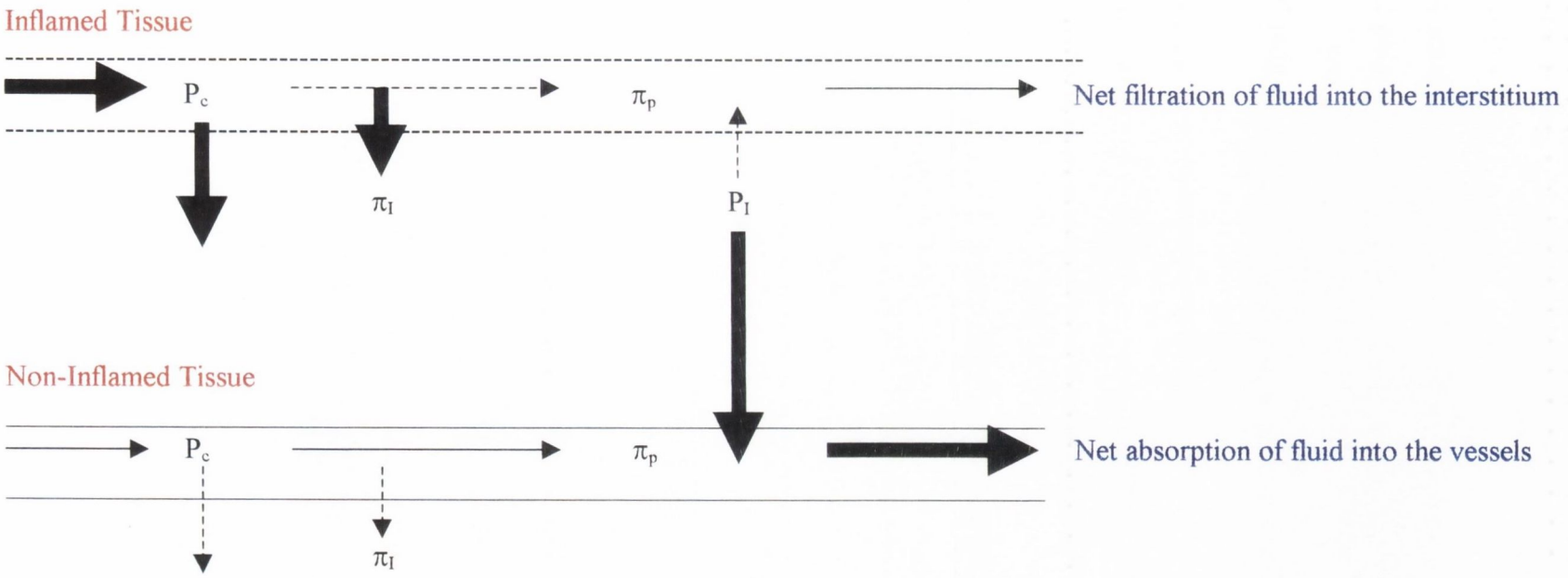


Figure 1.6. Diagrammatic representation of a capillary in inflamed tissue (broken lines) and in nearby non-inflamed tissue (solid lines). Arrows indicate the relative magnitude and direction of flow. P_c = capillary hydrostatic pressure; P_I = interstitial fluid hydrostatic pressure; π_I = interstitial fluid colloid osmotic pressure; π_p = plasma colloid osmotic pressure. Adapted from Tonder (1983).

1.5.3 Nerve supply

Dental pulp is unique in that the sensation experienced in response to various stimuli is one of pain. The sensory fibres are mainly of the myelinated A-δ (sharp, piercing pain) and unmyelinated C (dull aching, burning pain) varieties. The A fibres have a diameter of 1 to 4 μm and conduct with a mean velocity of 13 m / sec. The C fibres are smaller being less than 1 μm in diameter and conducting in the region of 1 m / sec. A small number of very fast conducting fibres called Aγ has also been reported in dental pulp (Seesle, 1979). The conduction velocity of these fibres is in the region of 30 m / sec and they are usually associated with proprioception. Their function in dental pulp has not been elucidated to date.

The fibres originate from the trigeminal nerve and enter the pulp with the blood vessels and proceed through the radicular pulp to the cell rich zone in the coronal pulp. Here, they branch into a plexus of single nerve axons called the plexus of Raschkow. In this plexus the A-δ fibres emerge from their myelin sheath and branch out to form the subodontoblastic plexus. They then leave the Schwann cells to terminate as a free nerve ending between the odontoblasts, many terminating in the extracellular spaces of the cell rich zone or the odontoblast layer (Gunji, 1982). Some fibres pass into the predentine, some extending on to the dentine to a maximum of 100 - 150 μm (Gunji, 1982: Byers & Kish, 1976: Lilja, 1979).

Sympathetic nerves from the superior cervical ganglion form plexuses around the mature arterioles (Anneroth & Nordenberg, 1968) and when activated cause constriction of the vessel. Fibres of both cholinergic and adrenergic origin have been identified in close proximity to odontoblasts (Avery, *et al.*, 1980). They may have a role in dentine formation (dentinogenesis) (Avery, 1981).

1.6 Dental aspects of SSAO

1.6.1 5-HT in dental pulp

5-HT is released and it amplifies the local effect of other mediators to promote further aggregation (De Clerck & David 1981, De Clerck & Herman 1983). It also promotes release of further inflammatory mediators and sensitises or directly activates nociceptors (as discussed in Section 1.2.3).

In the dental pulps of beagle dogs the application of 5-HT (1 mg/mL) to the dentine surface caused both sensitisation and increased responsiveness to a range of stimuli (Ngassapa *et al.*, 1992 as discussed in Section 1.2.3). 5-HT may, in addition, modulate the responses of other inflammatory mediators including neuropeptides like CGRP and substance P (as discussed in Section 1.2.3).

Through the actions of H2 receptors, histamine suppresses lymphocyte proliferation, T-cell mediated cytotoxicity of allogenic target cells, lymphokine production, natural killer cell cytotoxicity and antibody preparation by B-lymphocytes (Bach *et al.*, 1985). Histamine may also activate suppressor T-lymphocytes. This occurs via lymphokine histamine suppressor factor (HSF) produced by certain T-cell H2 receptors. HSF blocks the proliferation of lymphocytes by increasing the production of monocytes, or it may activate suppressor T-cells (Pearce, 1991). Histamine has also been implicated in immune surveillance and carcinogenesis (Batholeyns & Bouclier, 1984).

1.6.2 Histamine in dental pulp

Acting on the H2 receptor, histamine decreases the production of complement component C2 in human monocytes (Lappin & Whaley, 1980) and also C2, C5 and factor B production in mouse peritoneal macrophages (Ooi, 1982: Falus & Meretey, 1987). It decreases the production of C3, but biosynthesis is increased through the H1 receptor.

Histamine also modifies cytokine induced complement and fibrinogen synthesis, enhancing interleukin 6 stimulated C3 and fibrinogen expression (Rokita *et al.*, 1992), but suppresses IFN-γ-induced C3 secretion in mouse primary hepatocytes.

Cytokines are influenced by histamine that acts both as a target of cytokines and a regulator of cytokine-receptor interactions. Histamine (via the H2 receptor) decreases endotoxin induced Interleukin 1 (IL-1) production (Dohlsten *et al.*, 1988) and tumour necrosis factor α (TNF-α) production in human monocytes, but has no effect on IL-6 production (Vannier *et al.*, 1991). However, in the mouse peritoneal macrophage histamine has a slight enhancing effect on IL-1 production (Okamoto & Nakano, 1990).

Histamine also inhibits IL-2 production and decreases the production of IFN-γ, although the last of these may be indirectly caused by the inhibition of IL-2 (Carlsson *et al.*, 1985). It also effects the production of some neurotransmitters and pituitary hormones that modulate the cytokine network. These include adrenocorticotropic hormone, β-endorphin, α-melanocyte-stimulating hormone and prolactin (enhanced) and thyroid stimulating hormone (suppressed) (Knigge & Warberg, 1991).

The cytokines in return exert control over the production and release of histamine. IL-1 may enhance production by bacterial endotoxin in mouse macrophages. If histamine is stimulated by IL-1 this may lead to increased local production of IL-6 by cells with active histamine receptors. IL-1 potentiates the effect of histamine on the release of prostaglandin and monohydroxyeicosatetraenoic acids (HETEs) by endothelial cells and hematopoietic precursor cells (Revtyak *et al.*, 1988). IL-3 induces histamine as well as IL-4 and IL-6, but not IL-2 or IFN-γ production. IL-8 has a positive priming effect on IL-3-enhanced histamine release (Bischoff *et al.*, 1991). IL-5 also primes human basophils for the histamine induced release by C5a, anti-IgE and fMLP (Bischoff *et al.*, 1990).

Increased levels of histamine and histidine decarboxylase and decreased concentrations of amine oxidases are associated with tumour growth in both experimental animals and humans (Bartholeyns & Fozard, 1985). Tumour growth may be inhibited by H2 receptor antagonists and H1 receptor agonists. H1 antagonists may promote growth (Bartholeyns & Fozard, 1985: Burtin *et al.*, 1982).

Histamine has also been described as an intracellular messenger in human platelets (Saxena *et al.*, 1989: Gerrard *et al.*, 1993). Intracellular concentrations of histamine and HDC activity have been correlated with cell growth (Kahlson & Resengren, 1971). Inhibitors of HDC decreased phorbol ester or collagen-induced platelet aggregation. This was reversed by N,N-diethyl-2-[4-(phenylmethyl)phenoxy}-ethanamine-HC1 (DPPE), a histamine non receptor antagonist. In saponin-permeabilized platelets histamine reversed the effect of inhibitors of DPPE or other HDC inhibitors on platelet aggregation.

1.6.3 Amine oxidases in dental pulp

1.6.3.1 Semicarbazide-sensitive amine oxidase

The products of SSAO metabolism of any primary monoamine are an aldehyde, ammonia and hydrogen peroxide (H_2O_2) (Barrand and Callingham, 1984). As a result of this the role of SSAO in removing trace amines must be compared to the relative toxicity of the metabolic products, as the latter may be far more damaging. H_2O_2 also has suggested functions in transmembrane signalling (Mukherjee and Mukherjee, 1982), so may have some role in the control of vasomotor tone (Callingham and Barrand, 1987). This correlates with the idea put forward by Pierce *et al.* (1990) that the toxic products may be involved in tissue renewal (as discussed in Section 1.4).

Lysyl oxidase initiates the biosynthesis of cross-links in elastin and collagen (Siegel, 1979) by catalysing the deamination of the ε-amino groups of lysyl and hydroxylysyl residues in those molecules. The activity of lysyl oxidase in the bovine incisor odontoblast/predentine layer was found to be in the region of 4 to 11 times greater than in the sub-odontoblastic layer or in the central pulp tissue (Numata & Hayakawa, 1986, see Table 1.9). The incisors tested were from bovine second incisor teeth of various stages of development.

Region	Stage of development	Counts/hour per mg protein	Number of samples examined
Odontoblast/ predentine layer	Crown formed Crown almost formed	504.6 ± 42.5 431.7 ± 85.1	5 6
Sub-odontoblast layer	Crown formed Crown almost formed	92.5 ± 25.3 120.6 ± 64.6	5 6
Central pulp	Crown formed crown almost formed	47.5 ± 26.8 36.1 ± 12.2	6 5

Table 1.9. The regional activity of lysyl oxidase in the bovine incisor (reproduced from Numata & Hayakawa, 1986).

Porcine dental pulp is capable of deaminating 5-HT (Norqvist *et al.*, 1981,1982). This property is unique in that no other SSAO enzyme has demonstrated 5-HT as a substrate. This suggests a specific role for pulpal SSAO not observed elsewhere. The distribution of SSAO activity is variable (Table 1.10) with the highest concentrations present in the peripheral pulp tissue (Norqvist *et al.*, 1988). The authors of the latter paper stated that lysyl oxidase and SSAO were probably the same enzyme.

Dental pulp	Enzyme activity (pmol/mg/protein min)
Peripheral pulp tissue	19.8 ± 12.5
Central pulp tissue	5.0 ± 2.6
Odontoblast residues	4.5 ± 2.9

Table 1.10. Distribution of SSAO activity versus 5-HT in pig dental pulp. From Norqvist *et al.*, (1988).

The porcine samples were from unerupted teeth that demonstrated complete crown formation but no root formation. The porcine enzyme is located in the plasma membrane, as the SSAO activity towards 5-HT moves to a higher equilibrium density on centrifugation when treated with digitonin. This is in agreement with the work of

Wibo *et al.* With rat aorta (1980) and Barrand and Callingham with rat brown adipose cells (1982, 1984).

In developing porcine dental pulp two types of amine oxidation were observed. The first was a 'soluble' form that may have been a result of blood contamination of the sample as the developing pulp has a large blood supply. This enzyme was similar to plasma benzylamine oxidase in its behaviour. The second 'membrane bound' variety was also capable of being inhibited by semicarbazide, but resistant to clorgyline (Norqvist, Fowler and Oreland, 1981). It was not possible to 'solubilise' the membrane-bound form by treatment with Triton X-100 (0.1% v/v) or up to 4M urea. In that study both the 'soluble' and 'membrane-bound' enzymes demonstrated activity towards benzylamine, tryptamine, tyramine, 2-phenethylamine and 5-HT. The last two substrates are deaminated primarily by the membrane-bound enzyme.

The SSAO from porcine dental pulp also was inhibited by aminoguanidine (IC_{50} 60 μM), hydroxylamine (IC_{50} 5 μM) and phenylhydrazine (IC_{50} 10 μM), but was resistant to heating to 55 °C for 100 minutes, unlike MAO (Norqvist, Oreland and Fowler, 1982). The membrane bound form of SSAO demonstrated no activity towards cardaverine or putrescine.

1.6.3.2 Monoamine oxidase in dental pulp

Monoamine oxidase activity is mainly located in the mitochondria (Schnaitman & Greenwalt, 1968) with about a quarter of the activity being found in the microsomal fraction (Hawkins, 1952). Monoamine oxidase A preferentially deaminates 5-HT, whereas the MAO-B form deaminates benzylamine and β-phenylethylamine. Other amines, for instance tyramine, noradrenaline and dopamine, can be metabolised by both forms. Most porcine tissues have been found to only contain MAO-B activity (Lyles & Greenwalt, 1978). If this is the case for porcine dental pulp then metabolism of 5-HT may not be mediated by MAO, which would be unique. Previous work has found benzylamine, tyramine, tryptamine and β-phenylethylamine to be substrates for MAO-B in porcine dental pulp (Norqvist *et al.*, 1981).

1.7 Aims of Thesis:

1. To demonstrate the presence of SSAO in human dental pulp.
2. To localise the SSAO in human dental pulp.
3. To determine the kinetic behaviour of SSAO in human dental pulp.
4. To complete further kinetics studies in porcine dental pulp.
5. To determine if one or more forms of SSAO exist in porcine dental pulp.
6. To identify and clone murine SSAO.
7. To determine the responses of murine odontoblasts to oxidative stress.

CHAPTER TWO

Kinetic Characteristics of SSAO in Human and Porcine Dental Pulp

2.1 Materials

2.1.1 Sources of Reagents

Reagents are listed in alphabetical order. The source of named reagents is indicated by the suppliers' name.

PRODUCT	SUPPLIER
Inhibitors	
Clorgyline hydrochloride	Sigma Chemicals
Deprenyl hydrochloride	Sigma Chemicals
Phenelzine sulphate	Aldrich Chemicals
Semicarbazide hydrochloride	Sigma Chemicals
Radiochemicals	
[7-^{14}C] Benzylamine hydrochloride (Specific activity 59 mCi/mmol)	Amersham P.L.C.
5-Hydroxy [side-chain 2-^{14}C]tryptamine creatine sulphate (Specific activity 55 mCi/mmol)	Amersham P.L.C.
[^{14}C]Methylamine hydrochloride (Specific activity 50 mCi/mmol)	Amersham P.L.C.
2-Phenylethylamine (ethyl-1-^{14}C] hydrochloride (Specific activity 56 mCi/mmol)	Amersham P.L.C.
Substrates	Sigma Chemicals
Ascorbic acid	Sigma Chemicals
Benzylamine hydrochloride	Sigma Chemicals
3,4-Dihydroxyphenylethylamine (dopamine) hydrochloride	Sigma Chemicals
Histamine dihydrochloride	Sigma Chemicals
5-Hydroxytryptamine creatine sulphate	Sigma Chemicals
Kynuramine	Sigma Chemicals

Octopamine	Sigma Chemicals
β-Phenylethylamine hydrochloride	Sigma Chemicals

Miscellaneous Reagents

Bovine serum albumin (BSA fraction V)	Sigma Chemicals
Citric acid	BDH Chemicals
Ethyl acetate	
Folins & Ciocalteau's phenol reagent	BDH Chemicals
2,5-Diphenyloxazole (PPO)	Sigma Chemicals
Quenched ^{14}C standards	Packard Instruments
S-Nitroso-*N*-acetyl-DL-penicillamine	Sigma Chemicals
S-Nitrosoglutathione	Sigma Chemicals
Toluene	BDH Chemicals

All other general laboratory reagents were purchased from BDH Chemicals or Lennox Laboratories

2.1.2 Addresses of Suppliers

The full names and addresses of suppliers are listed below in alphabetical order:

Amersham	Amersham P.L.C. *through* P.J. Brennan & Co. Ltd., Stillorgan Industrial Estate, Stillorgan, Co. Dublin.
BDH	BDH Chemicals Ltd., Poole, Dorset, England.

Lennox	Lennox Laboratories Ltd., J.F.K. Drive, Dublin 12.
Packard	Packard Instrument Co. Inc., 2200 Warrenville Road, Downers Grove, Illinois, U.S.A.
Vector	Vector Laboratories, 16 Wulfric Square, Bretton, Peterborough, PE3 8RF, Scotland.

2.2 Methods

2.2.1 Preparation of Solutions

All materials were weighed using a Mettler Model K7T top-loading balance (1g-800 g) or a Mettler College 150 analytical balance (2 mg-1 g).

Volumes from 1 µl to 5 ml were dispensed using a range of Gilson Pipetman automatic pipettes. All pipettes were checked at regular intervals for accuracy according to the manufacturer's instructions.

The pH of solutions was determined using a Corning pH meter, model 240 equipped with a Corning general-purpose combination electrode. The pH meter was calibrated at the start of each day using BDH standard buffer solutions of pH 4.0, 7.0 and 10.0.

All solutions, unless stated otherwise, were prepared in distilled water, which had been de-ionised by passage through a Millipore Milli-U10 model water purification system.

2.2.2 Preparative Centrifugation

Centrifugations were performed using a Sorvall RC-5B with a GSA rotor (6 X 250 ml, *g* maximum 27,600 *g*.

Ultracentrifugations were performed using a Sorvall ultracentrifuge with a AH-629 rotor (6 X 36 ml, *g* maximum 151,200 *g*) or for smaller volumes a Beckman TL100 Ultracentrifuge (6 X 6 ml, *g* maximum 541,000 *g*).

All centrifugations were performed at temperatures of 0-4°C. Rotors were pre-cooled before use. The *g* force quoted refers to the relative centrifugation at the bottom of the centrifugation tube.

2.2.3 Preparation of pulpal homogenates

Porcine

Pig heads were obtained from 22-24 week-old piglets after veterinary inspection. Samples were obtained within 6-7 hours after slaughter, during which period they were refrigerated. The mandible was removed using a band-saw and a horizontal section was made along the body of the mandible half way between the upper and lower borders. This exposed the developing permanent teeth that could be easily extracted and the pulps removed. Root formation had not begun so the pulpal tissue could be removed with a small periosteal elevator (Ash Dental Instruments Ltd., UK). In the region of 800-900 mandibles were used and the yield of dental pulp per jaw was in the region of 3 g.

The pulps were pooled and homogenised 1:4 (w/v) in 100 mM sodium /potassium phosphate buffer with 0.025 M sucrose, pH 7.2 in an Ultra-Turrax homogeniser at 0°C. The homogenates were centrifuged at 13,000 *g* for 15 minutes. The resultant supernatant was then further centrifuged at 100,000 *g* for 1 hour. The pellet was

resuspended 1:2.5 (w/v) in 100 mM sodium /potassium phosphate buffer, pH 7.2 and this formed the microsomal fraction (Figure 2.1).

Human

Human third molar teeth were used as described in detail in Section 4.3. This involved freezing the teeth upon extraction in dry ice. The teeth were rapidly defrosted and the pulps extracted after rupturing the surrounding calcified tissue with a vice. The pulps were then stored at -80 °C until required. About 550 human teeth were used for this purpose. The weight of pulp retrieved per tooth was in the region of 10-12 mg.

The pulps were homogenised 1:4 (w/v) in 100 mM sodium phosphate buffer, pH 7.2 in an Ultra-Turrax homogeniser at 0 °C. Crude homogenates were used for experiments.

2.2.4 Protein assay

Protein was determined using the Markwell *et al.* (1978) method.

Reagent A	2 % (w/v) sodium carbonate
	0.4 % (w/v) sodium hydroxide
	0.16 % (w/v) sodium potassium tartrate
	1 % (w/v) SDS
Reagent B	6 % (w/v) copper sulphate ($CuSO_4.5H_2O$)
Reagent C	100 volumes Reagent A
	1 volume Reagent B
Reagent D	Folin and Ciocalteau's reagent 1:1 (v/v)
	with de-ionised H_2O

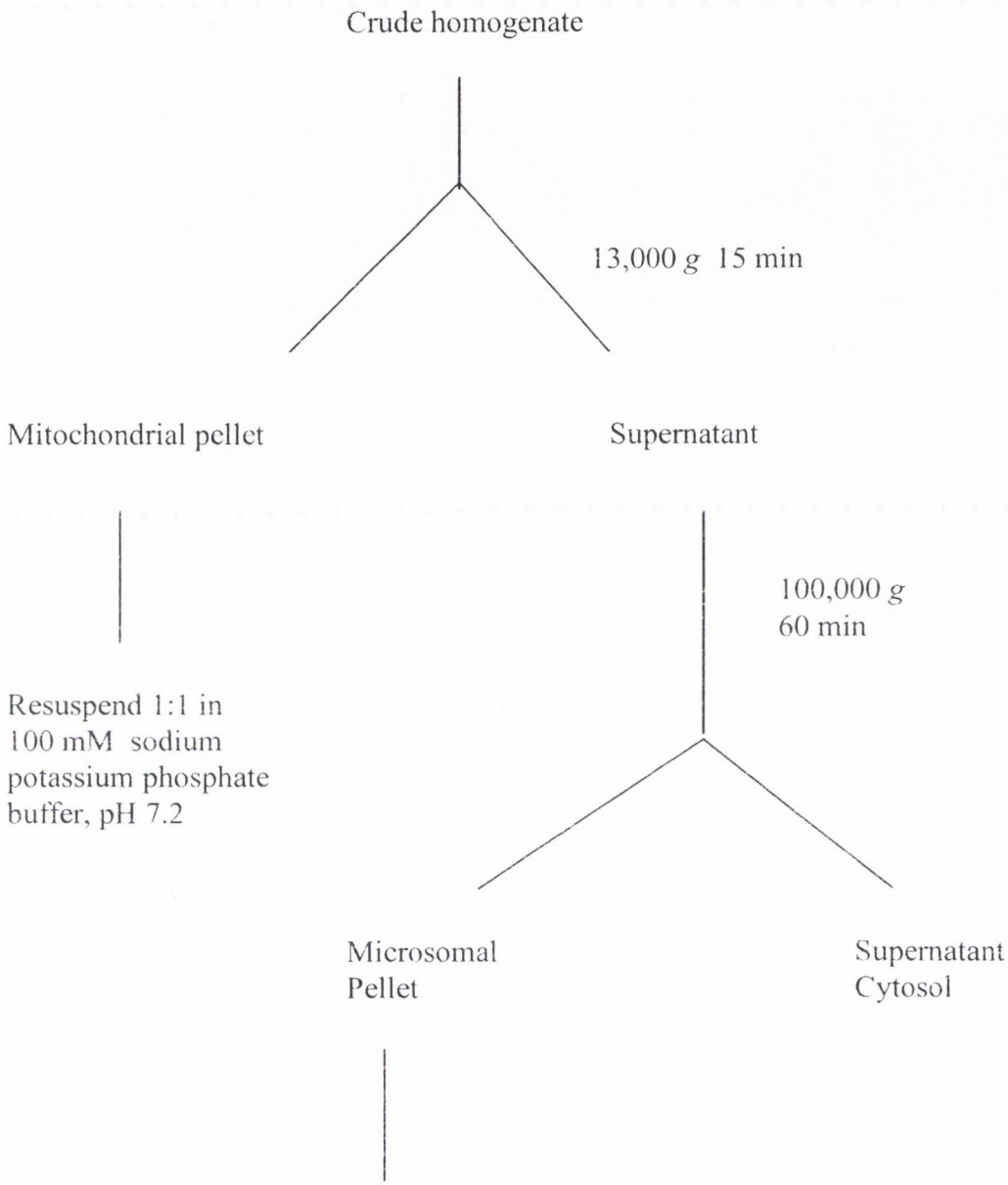


Figure 2.1. The centrifugation protocol for porcine dental pulp

Samples of various dilutions were made up to 1ml with de-ionised H_2O. Three ml of Reagent C was added to each sample, vortex mixed and left to stand at room temperature for 15 minutes. Thereafter 0.3ml of Reagent D was added and vortex mixed. After a further 45 minutes at room temperature the absorbance was determined at 660 nm. Standards were processed in a similar manner with bovine serum albumin protein concentrations from 0 - 100 μg. A representative standard curve is shown in Figure 2.2.

2.2.5 Radiochemical assay and kinetic studies

The activities of SSAO and MAO were determined radiochemically using a modification of the method of Otsuka and Kobayashi (1964). The aldehyde product was extracted in mixture containing toluene-ethyl acetate (1:1, v/v) and 0.6 % 2,5-diphenyloxazole (PPO). All incubation times were chosen so that the deamination of the biogenic amines and enzyme concentration were linear with respect to time.

Assays were performed in scintillation vials in a shaking waterbath at 37°C. All solutions were incubated at 37 °C for 5 min prior to any reaction.

The assays contained 100 μl of enzyme solution, 25 μl of radioactively-labelled substrate (X 9 final desired concentration), inhibitors as required and the volume was brought up to 225 μl with 100 mM sodium /potassium phosphate buffer, pH 7.2. For the time courses the reaction was started by the addition of the substrate, but for the kinetic experiments the reaction was stared by the addition of the enzyme (including any inhibitors). All inhibitors were pre-incubated with the enzyme for 30 minutes before the reaction was started. The reactions were halted by the addition of 100 μl of 2 M citric acid. Blanks included all the materials for the assay but the citric acid was added before the substrate. For the kinetic experiments blanks were recorded at each substrate concentration.

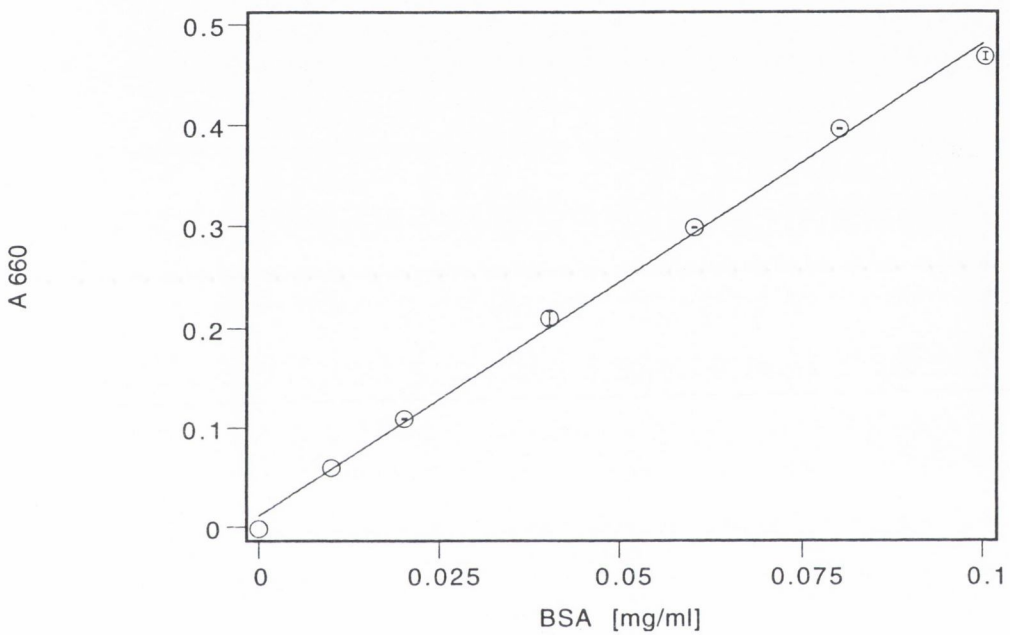


Figure 2.2. Sample standard curve for a Markwell *et al.* (1978) protein assay. The absorbance was determined spectrophotometrically at 660 nm as described in Section 2.2.4. The points shown are mean values ± S.E.M. of three separate experiments.

After termination of the reaction 4 ml of scintillant (toluene-ethyl acetate (1:1, v/v) containing 0.6 % PPO) was added. The tube were capped and mixed thoroughly using a vortex mixer. The tube was then placed in a -20 °C freezer for at least 4 hours or at -70°C for 30 minutes. This caused the aqueous layer to freeze whereas the organic layer, containing the reaction product, remained liquid. The organic phase was poured off into scintillation vials and counted by liquid scintillation spectroscopy in a Packard TriCarb 1500 or a TriCarb 2100 counter.

2.2.6 Calculation of radiochemical concentrations

The following formula was used to calculate the substrate and radioactive concentrations for the radiochemical assays described in Section 2.2.5. These were based on experiments where 25 µl of radioactive substrate was to be added to a total volume of 225 µl.

$$\frac{9 \times [\text{final substrate}]}{\text{litre}} * \frac{1}{1000\ \text{ml}} * \text{total volume} = \text{total µmol substrate}$$

$$\text{total µmol of substrate} * \text{final µCi µmol}^{-1} = \text{µCi required}$$

$$\text{µCi required} * \frac{\text{µl}}{\text{µCi of radioactive solution}} = \text{Volume of radioactive solution required}$$

$$\text{µCi} * \frac{\text{µmol}}{\text{µCi of radioactive solution}} = \text{Radioactive µmol}$$

$$\text{Total µmol - radioactive µmol} = \text{unlabelled substrate µmol}$$

$$\frac{\text{Unlabelled substrate µmol}}{\text{total volume - radioactive volume}} * \frac{\text{mol}}{10^6\ \text{µmol}} * \frac{10^3\ \text{ml}}{\text{litre}} * \frac{M_r\ \text{substrate}}{\text{mol}}$$

$$= \text{unlabelled solution in g/l}$$

2.2.7 Correction for Counting Efficiency

Conversion from c.p.m. to d.p.m was necessary when the TriCarb 1500 liquid scintillation spectrometer was used. Quenched ^{14}C standards were obtained from Packard Instrument Co. Ltd. A correlation between counting efficiency and a quench indication parameter (QIP) was established. The QIP employed was based on an external standard measurement.

The counting efficiency was obtained by calculating the ratio of the observed counts to the total d.p.m.

$$\text{Efficiency (\%)} = (\text{c.p.m.}/\ \text{d.p.m.}) * 100$$

The percentage efficiencies were plotted against the corresponding QIP values, as shown in Figure 2.3. This permitted the radioactivity of any samples to be expressed

in d.p.m. values with correction for counting efficiency. Quench correlation curves were determined regularly as the counting efficiency of the liquid scintillation counter was variable.

The TriCarb 2100 scintillation spectrometer automatically calculated the conversion from c.p.m. to d.p.m.

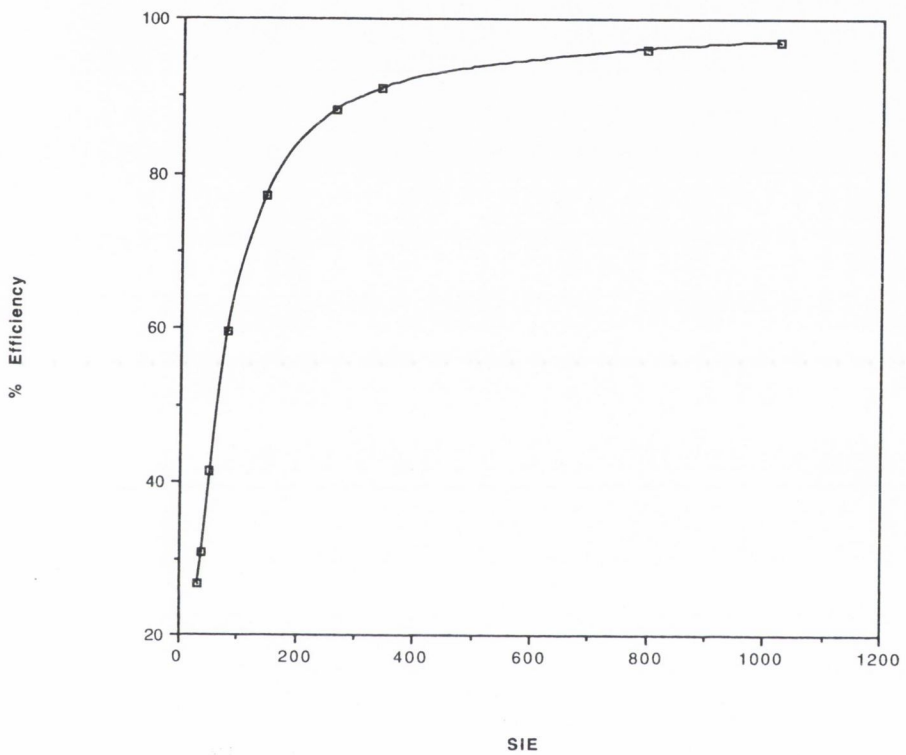


Figure 2.3. The correction for counting efficiency for radiochemical assays to be quantified by liquid scintillation counting. A correlation between counting efficiency and quench indication parameter (QIP) was plotted using quenched ^{14}C standards as described in Section 2.2.7 to permit the radioactivity of any sample to be expressed in d.p.m. values.

2.2.8 Estimation of Specific Activities of SSAO from d.p.m. Measurement

The specific activity ($nmol.min^{-1}.mg^{-1}$ protein) of each substrate was experimentally determined by measuring the d.p.m. value. The rate of product formation (specific activity, S.A.) could be calculated using the following formula which allows for extraction efficiencies of the products formed (see Table 2.1), the duration of the reaction and the protein concentration of the sample:

S.A. = d.p.m * 100/X * Y * 1/t * 1/μl protein per reaction * 1000/(mg/ml protein)

where X is the extraction coefficient for the amine product, t the duration of the reaction, Y the conversion factor from d.p.m. to pmoles and is calculated in the following equation:

$$Y = mmol/mCi * mCi/2.22 \times 10^{9} \text{ d.p.m.} * 10^{9} pmol/nmol$$

Substrate	Mean Extraction Efficiency (%)
Benzylamine	86.8
5-HT	74.4
PEA	92.5

Table 2.1. Extraction Efficiency of radiolabelled amine metabolites in toluene-ethylacetate-0.6% PPO scintillant. From Fowler & Oreland (1980).

2.2.9 Curve Fitting and Statistical Analysis

K_m and V_{max} values were determined using the commercial software programme MacCurveFit Version 1.2. (Kevin Raner Software, Australia).The programme fits the recorder experimental data to the Michaelis-Menten equation which describes the behaviour of enzymes in the presence of increasing concentrations of substrate (Michaelis & Menten, 1913). The formula is :

$$v = V_{max}[S] / (K_m + [S])$$

where v is the observed velocity of the reaction, [S] the substrate concentration, V_{max} the maximum enzyme velocity and K_m is the Michaelis constant.

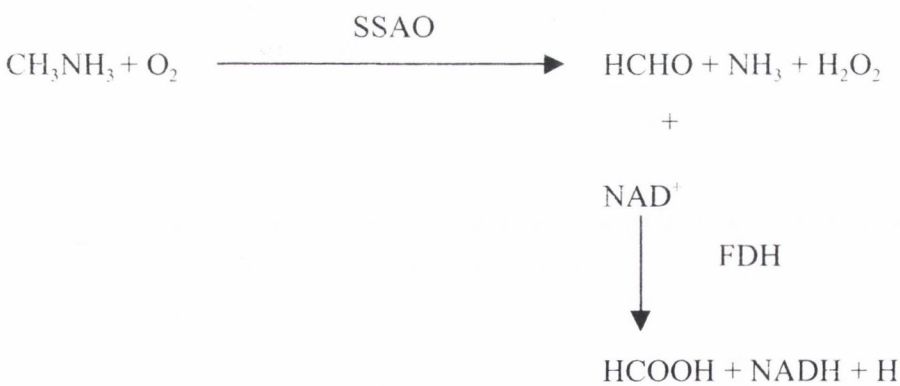
IC_{50} values (the inhibitor concentration that gives 50% inhibition of the reaction) were determined using MacCurveFit using the equation:

$$F(x) = a*((\exp(b*(x+c))-1)/(\exp(b*(x+c))+1))+d$$

The software programme Cricketgraph III Version1.5 (Computer Associates International Inc., New York, USA) was used to fit data to a straight line or interpolate values. This programme was also used to plot saturation curves according to Lineweaver & Burk (1934).

2.2.10 Spectrophotometric assay for methylamine deamination.

This reaction is based on the principal that one of the products of the reaction (HCHO) can react with NAD^+ in the presence of formaldehyde dehydrogenase (FDH) to form NADH. The formation of NADH can be followed at 340 nm.



The reaction mixture contained 300 µl of sample, 30 µl of FDH (0.1 units, one unit of FDH will oxidise 1 µmol of formaldehyde to formic acid per min at pH 7.5 at 37 °C), 102 µl NAD^+ (200 µM final concentration) and substrate. The total reaction volume was brought to 3 ml by adding 50 mM sodium /potassium phosphate buffer, pH 7.2.

Blanks contained no substrate. After pre-incubating at 37 °C for 5 min the reaction was started by the addition of substrate to the sample cuvette and the reaction was followed spectrophotometrically at 340 nm. Blank rates were recorded with buffer in place of substrate.

2.2.11 Radiochemical methylamine assay

This method involves the removal of the unreacted substrate by adsorption to a 3cm amberlite C6-50 column and the determination of the reaction products by liquid scintillation spectroscopy (Tipton & Youdim,1976).

Amberlite columns were prepared in syringes (3cm X 1cm). A separate column was used for each reaction mixture. The reaction volume used was 100 µl, comprising 50 µl of 2 mM (1.0 µCi/µmol) methylamine and 50 µl sample. The reaction was started by adding the latter solution and proceeded for up to 90 min. The reaction was stopped by placing the tubes in ice. Blanks were kept on ice for the 90 min.

One ml of distilled H_2O was passed through the columns, followed by the reaction mixture. The column was then washed with a further 1 ml of H_2O. The eluate was collected and combined with 6 ml of Bray's scintillation cocktail (Table 2.2) and counted by liquid scintillation spectroscopy.

A positive control was provided by adding 50µl of substrate to the Bray's scintillation cocktail (Bray, 1960).

PPO	4 g
POPOP	0.2 g
Ethyleneglycol	20 ml
Naphthaylene	60 g
Ethanol	100 ml

Table 2.2. The composition of Brays reagent. The solution was made to 1 litre with *p*-dioxane.

2.2.12 Detection of hydrogen peroxide

Colourimetric detection of Hydrogen peroxide.

During the peroxidase catalysed conversion of H_2O_2 to water 4-aminophenazone can act as a proton and electron donor (Yamada *et al.*, 1979). Oxidised 4-aminophenazone then condenses with 2,4-dichlorophenol to form the red coloured dye quinoneimine that can be detected at 500 nm.

Assays were performed on 96 well microtitre plates in a total volume of 225 µl and the reaction mixture included 140 µl of the colourimetric solution. 10 µl of enzyme and 50 µl of substrate dissolved in 1 mM sodium /potassium phosphate buffer, pH 7.8. Inhibitors were added (X 8 the final concentration) dissolved in 1 mM sodium /potassium phosphate buffer, pH 7.8 containing 20 µg/ml sodium azide.

The colourimetric mixture consisted the materials listed in Table 2.3 dissolved in 1 mM sodium /potassium phosphate buffer, pH 7.8.

horseradish peroxidase (Type II)	200 µg/ml
2,4-dichlorophenol	0.2 mg/ml
sodium azide	0.2 mg/ml
4-aminophenazone	0.2 mg/ml

Table 2.3. The composition of the colourimetric solution. The solution was prepared to a volume of 10 ml.

Reactions were carried out at 37 °C and stopped by placing the plate on ice for 5 min and adding 50µl of phenelzine (500 µM). The samples were then allowed to warm to room temperature and the absorbance was then recorded at 505nm. After the blank values were subtracted the reading was compared to a standard curve of H_2O_2 (0-25 µl of a solution of 1 nmol/µl).

Fluorimetric Determination of Hydrogen peroxide.

In this reaction adrenaline was used as the reducing agent to give rise to adrenolutine, which is highly fluorescent. This occurs by the "trihydroxyindole" reaction (Lund, 1950; Callingham & Cass, 1963). In the presence of hydrogen peroxide and horseradish peroxidase, adrenaline forms adrenaline-quinone, which cyclises to form adrenochrome (brownish-pink in colour). Under alkaline conditions in the presence of ascorbate this is converted to adrenolutine.

Assays were carried out in scintillation vials in a total volume of 200 µl. This consisted of 10 µl of enzyme that had been mixed with MnO_2 (20 mg/ml) and briefly centrifuged for 1 min in a benchtop centrifuge to sediment the MnO_2, 50 µl of amine substrate (X 4 final concentration ± inhibitors) and 140 µl of 200 µM sodium phosphate buffer, pH 7.2 containing 0.2 mg/ml of sodium azide. The blanks contained no substrate. The reaction was completed at 37°C in a shaking waterbath after vortex mixing and was stopped by placing the tubes in ice for 5 min. When the mixture had returned to room temperature mixes were performed as shown in Table 2.4.

1.	50 µl of 9 mg/ml adrenaline bitartrate and 2 mg/ml Type II horseradish peroxidase in distilled water	
2.	3 mins later	100 µl ascorbate solution*
3.	3 mins later	2 ml distilled H_2O

Table 2.4. The compounds added to the reaction mixture.

* 1:1 (v/v) solution of 20% (w/v) NaOH and 1 % (w/v) ascorbate.

The fluorescent intensity was recorded in a luminesence spectrofluorimeter (Perkin Elmer) (activation filters 405 nm, emission filters above 500 nm). The values were compared to a standard curve of H_2O_2 (0-25 µl of a solution of 1 nmol/µl).

2.2.13 Inhibitor potencies of semicarbazide and phenelzine

The inhibitor potencies of semicarbazide and phenelzine were measured by radiochemical assay, as described in Section 2.2.5, with benzylamine or 5-HT as substrates. 100 µl of enzyme sample was incubated with 10µl of clorgyline (10^{-2} M) for 30 min. These were then mixed with 70 µl of 100 mM sodium /potassium phosphate buffer, pH 7.2, and 20 µl of semicarbazide (10^{-10} - 10^{-1} M) or phenelzine (10^{-10} - 10^{-2} M) for a further 30 min. Then 25 µl of radioactive substrate was added, either 25 µmol benzylamine (1.0 Ci/mol) or 40 µmol 5-HT (3.0 Ci/mol). Control reactions were completed where no inhibitor was included and blanks contained the same materials but 2M citric acid was added before the substrate. The reaction was allowed to proceed for 60 min and then stopped by the addition of 100 µl of 2 M citric acid. The radioactive product was extracted and counted as described in Section 2.2.5.

The rate of inhibition of porcine microsomal SSAO by semicarbazide and phenelzine was determined by spectrophotometric coupled assay based on the method of Houslay and Tipton (1973). Benzylamine (final 50 µM) or 5-HT (final 100 µM) were used as substrates in a reaction mixture containing 660 µM NAD^+, 0.015 units ALDH (one unit of ALDH activity is the amount that catalyses the production of 1 µmol NADH per minute at 37 °C, pH 8.8 in the presence of 3 mM acetaldehyde), inhibitor (semicarbazide or phenelzine) at a final concentration of 10^{-2} M and the volume made to 300 µl with 100 mM phosphate buffer, pH 7.2. 700 µl of solubilised microsomal SSAO (see Section 2.2.19) that had been incubated with clorgyline (final 10^{-3} M) at 37 °C for 30 min were added to start the reaction. The formation of NADH was followed spectrophotometrically at 340 nm. Blank reaction and controls without inhibitor were also performed.

2.2.14 Localisation of SSAO activity

Samples of porcine dental pulp were assayed with radiolabelled 50 µmol benzylamine (1.0 Ci/mol) or 5-HT (3.0 Ci/mol) for 60 min as described in Section 2.2.5, for SSAO activity in all the fractions of the centrifugation process described in Figure 2.1. The total SSAO activity for each fraction was then determined.

2.2.15 Contributions of SSAO and MAO to the deamination of benzylamine, 5-HT and PEA

The reaction mixture consisted of 100 µl of enzyme sample and 20 µl of each inhibitor used (X 10 final concentration) and the volume was made to 200 µl by the addition of 100 µM sodium /potassium phosphate buffer, pH 7.2. Final concentrations of inhibitors were semicarbazide 10^{-3} M, deprenyl 10^{-6} M, clorgyline 10^{-3} / or 10^{-6} M, β-APN (β-aminopropionitrile monofumarate salt) 10^{-3} M, KCN (potassium cyanide) 10^{-3} M and a combination of semicarbazide and clorgyline each at 10^{-3} M. Pre-incubation with the enzyme lasted 30 mins before the addition of 50 µmol benzylamine (1.0 Ci/mol), 500 µmol 5-HT (1.0 Ci/mol) or 20 µmol PEA (2-phenethylamine) (2.5 Ci/mol). The reaction duration was 45 min for the benzylamine assays, 60 min for the PEA assays and 90 min for the 5-HT assays. The reaction was stopped by the addition of 100 µl of 2 M citric acid. Blanks contained the same materials but the citric acid was added before the substrate. The times for the reactions had been determined by time courses without any inhibitors. The radioactive product was extracted and counted as in Section 2.2.5.

2.2.16 Determination of IC_{50} / K_i values from substrate competition experiments

100µl of enzyme sample and 20µl clorgyline (10^{-3}M) were incubated for 30 min at 37 °C before being added to 25 µl of radioactive substrate, either 25 µmol benzylamine (1.0µCi/µmol) or 40 µmol 5-HT (3.0 µCi/µmol) and 22.5 µl of unlabelled substrate (X10 final desired concentration, see Table 2.5 for concentration ranges). 60 µl of 100 mM phosphate buffer, pH 7.2 was added to bring the volume to 225 µl. The reactions were stopped after 60 min by the addition of 100 µl of 2 M citric acid. Blanks were included for all assays as were controls without cold substrate. The radioactive product was extracted and counted as in Section 2.2.5.

Assuming competitive inhibition the K_i values were calculated from the IC_{50} values using the following formula:

$$K_i = IC_{50} / (1 + [S] / K_m)$$

where the K_m is the Michaelis constant and [S] the substrate concentration used in the assay.

Substrate	Concentration Range
Kynuramine	10^{-1} - 10^{-6} M
Octopamine	10^{-1} - 10^{-6} M
Histamine	10^{0} - 10^{-6} M
Dopamine*	10^{-1} - 10^{-6} M

Table 2.5. The various concentration ranges of unlabelled substrate employed in the determination of IC_{50} / K_i values from substrate competition experiments
* 0.64 mM ascorbic acid included to prevent auto-oxidation and did not effect the rate of the reaction.

2.2.17 Spectrophotometric assay for kynuramine oxidation

A spectrophotometric assay for MAO activity based on the disappearance of kynuramine was developed in 1960 (Weissbach *et al.*). The assay is based on the fact that kynuramine is oxidised to an aldehyde that could either condense to 4-hydroxyquinoline or undergo further oxidation to the acid or 2,4-dihydroxyquinoline. The reaction is followed by either observing the disappearance of substrate at 358-360 nm or the appearance of quinoline product at 315 and 329 nm.

The reaction consisted of 250 µl of enzyme sample that had been pre-incubated at 37 °C for 30 min with 25 µl clorgyline (10^{-2} M initial concentration), kynuramine at a final concentration of 0-250 µmol. The volume was brought to 3 ml through addition of 100 mM sodium phosphate buffer, pH 7.2. The reactions were started by the addition of enzyme and followed spectrophotometrically at either 360 or 315 nm. Blank rates were recorded in the absence of substrate.

2.2.18 Activation experiments and the effect of thiol groups on SSAO activity

The time-dependent activation of SSAO activity towards benzylamine at 37 °C has been demonstrated (Lizcano, 1994). Up to a 20-fold increase in activity has been demonstrated with the activation commencing at about 60-90 min and continuing for up to 60 hours.

100 µl of enzyme sample and 80 µl of 20 mM phosphate buffer, pH 9.5 were placed in a shaking water bath at 37 °C and incubated for different times. 30 min before the addition of radiochemical substrate 20 µl of clorgyline (10^{-2}M initial concentration) was added. When the results were plotted, pre-incubation time included the 30 min where the clorgyline was added. The substrates (25 µl) used were either 100 µmol benzylamine (1.0 µCi/µmol) for 30 min or 100 µmol 5-HT (3.0 µCi/µmol) for 90 min. Blanks contained the same materials but had the citric acid added before the substrate. The radioactive product was extracted and counted as described in Section 2.1.3. Pre-incubation times of up to 210 minutes were used.

A second experiment was conducted over a longer time course. To limit evaporation and ensure homogeneity of the enzyme sample 500 µl of sample was placed in a closed Eppendorph tube in a shaking waterbath at 37 °C. At different times 100 µl samples were withdrawn after the tube had been inverted and the contents sonicated for 5 seconds, then 20 µl of clorgyline (10^{-2}M) added with 80 µl of 20 mM phosphate buffer, pH 9.5. After a further 30 min substrates (25 µl of 100 µmol benzylamine (1.0 µCi/µmol) 100 µmol 5-HT (3.0 µCi/µmol) were added and the reactions allowed to proceed for 30 min (benzylamine) or 60 min (5-HT). Blanks contained the same materials but had the citric acid added before the substrate. The radioactive product was extracted and counted as described in Section 2.2.5.

The volume of remaining enzyme sample in the Eppendorph was measured and this was compared between samples. Incubation times ranged up to 32 hours.

The effect of thiol oxidation was evaluated in porcine dental pulp microsomal SSAO. The effect of *S*-nitrosoglutathione (SNOG) (final 1 mM) over 2 hours and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) (final 0.1 mM) with /without DTT (final 0.3 mM) over a 5 hour period. The oxidative deamination of 50 μM benzylamine (1 Ci/mol) and 100 μM 5-HT (3 Ci/mol) was measured radiochemically as described in Section 2.2.5. The MAO activity was inhibited by pre-incubation with clorgyline (final 10^{-3}M). Control reactions without NO donors were run each time interval. Blank reactions where 2 M citric acid was added before the substrate were measured.

2.2.19 Solubilisation of porcine membrane-bound SSAO

Solubilisation of the membrane bound SSAO by detergent treatment has been demonstrated previously for brown adipose tissue in the rat (Barrand & Callingham, 1984) but not for porcine SSAO (Norqvist, Fowler & Oreland, 1981).

One volume of porcine microsomes were mixed with one volume of 100 μM sodium phosphate buffer, pH 7.2 containing 1 %, 2 % or 3 % Triton X-100 and mixed on an orbital shaker for 2 hours at 4 °C. A control without Triton X-100 was also used. The mixture was then centrifuged at 100,000 *g* for 60 min. The pellet was resuspended in 0.5 ml of 100 μM sodium /potassium phosphate buffer, pH 7.2. A portion of both pellet and supernatant were taken for protein assays at dilutions of 1:50 and 1:100. SSAO assays were carried out on both fractions. The assays contained 100 μl of enzyme sample, 80 μl of 100 mM sodium phosphate buffer, pH 7.2 and 20 μl of 10^{-3} M clorgyline. This was incubated at 37 °C in a shaking waterbath for 30 min before the addition of 25 μl of radioactive substrate, either a final concentration of 100 μmol benzylamine (1.0 μCi/μmol) or 100 μmol 5-HT (3.0 μCi/μmol). Blanks contained the same materials but with the citric acid added before the substrate and positive controls involved the use of 100 μl of porcine microsomes. The reaction was stopped after 90 min by the addition of 100μl 2M citric acid. The radioactive product was extracted and counted as described in Section 2.2.5.

The total protein and activity in each phase were calculated as a percentage of the total.

2.2.20 Mixed substrate analysis

The [^{14}C] metabolite formation from incubating porcine microsomes with 18.6 μmol benzylamine (1.0 μCi/μmol), 38.6 μmol 5-HT (3.0 μCi/μmol) and 44 μmol PEA (2.5 μCi/μmol) was recorded, both individually and also as mixtures. The substrate concentrations were set at their K_m values (see Section 2.2.5). If a single active site metabolises two different substrates then:

$$v_T = \frac{v_A (1 + [A] / K_A) + v_B (1 + [B] / K_B)}{1 + [A] / K_A + [B] / K_B}$$

v_T = product formation from a combination of substrates A and B

v_A, v_B = product formation from substrates A and B alone

[A], [B] = assay concentrations of A and B

K_A, K_B = Michaelis constants for A and B

(Segel, 1975).

If the actual K_m concentrations of A and B are used then the equation becomes:

$$v_T = [v_A(1+1) + v_B(1+1)] / 1 + 1 + 1$$

$$\therefore \quad v_T = (v_A(2) + v_B(2)) / 3$$

$$\therefore \quad v_T = (v_A + v_B) / 1.5$$

$$\therefore \quad 0.67 = v_T / (v_A + v_B)$$

The assays contained 25 μl of the substrate(s) and the volume was brought to 115 μl with 100 mM sodium /potassium phosphate buffer, pH 7.2. The enzyme sample

(100 µl) was mixed with 10 µl of clorgyline (10^{-2} M) and incubated for 30 min and the reaction was then started by addition of the enzyme to the substrate(s). The reaction was run for 60 min and stopped by adding 100 µl of 2 M citric acid. Blanks had the same contents but the citric acid was added to the enzyme before the substrate. The radioactive product was extracted and counted as described in Section 2.2.5.

2.2.21 The inhibition of [^{14}C] metabolism with a different unlabelled substrate

If a single active site is saturated with an unlabelled substrate at the [V_{max}] for that substrate, and then a second [^{14}C] labelled substrate added then there will be competitive inhibition between the substrates and a reduced amount of radioactive product will be formed than if there was no unlabelled substrate. However, if 2 different active sites/enzymes are involved the unlabelled substrate should not interfere with the formation of product from the radioactive substrate.

Samples of porcine microsomes (100 µl) were incubated at 37 °C with 10 µl of clorgyline (10^{-2} M) for 30 min. 22.5 µl of 5-HT (final concentration 4 mM) and 67.5 µl of 100 mM sodium /potassium phosphate buffer, pH 7.2, were then added together with either 18.6 µmol benzylamine (1.0 µCi/µmol) or 44 µmol PEA (2.5 µCi/µmol) were added and the reaction was allowed to proceed for a further 60 min. Blanks had the same contents but the citric acid was added to the enzyme before the substrate. Positive controls were matched to each reaction but without the 5-HT. The radioactive product was extracted and counted as described in Section 2.2.5.

2.2.22 Inhibition of porcine microsomal SSAO deamination by competition

The inhibition of SSAO deamination of 5-HT by benzylamine and PEA, of benzylamine deamination by 5-HT and PEA and of deamination of PEA by benzylamine and 5-HT were determined. Optimum concentrations of inhibitors were determined by inhibiting deamination of 100 µmol 5-HT (3.0 Ci/µmol) with benzylamine or PEA in the concentration range of 10^{-5}-10^{-2} M, or 100 µmol PEA (1 Ci/µmol) with benzylamine in the concentration range of 10^{-5}-10^{-2} M. Porcine microsomal SSAO was incubated with clorgyline (final concentration 10^{-3} M) for 30

min at 37 °C before 110 µl of this mixture was added to 22.5 µl of inhibitor (X 10 final concentration), 25 µl radiolabelled solution (X 9 final concentration) and 67.5 µl of 100 mM sodium /potassium phosphate buffer, pH 7.2, to a total volume of 225 µl. The reactions were halted after 60min by the addition of 100 µl of 2 M citric acid. Blanks were included where the citric acid was added at the start of the reaction.

IC_{50} values were determined for each combination and these used to determine the concentration range employed in the inhibition of the deamination of radiolabelled substrate by unlabelled inhibitor.

Deamination of radiolabelled benzylamine (25-500 µmol, 1.0 Ci/mol) was inhibited by 5-HT in the concentration range 0.1-10 mM. Deamination of radiolabelled PEA (25-500 µmol, 1.0 Ci/mol) was inhibited by 5-HT in the concentration range 10-500 µmol, or by benzylamine in the range 0.1-10 mM and deamination of radiolabelled 5-HT (25-500 µmol, 3.0 Ci/mol) was inhibited by benzylamine in the concentration range 0.1-10 mM. 110 µl of porcine microsomal SSAO (pre-incubated with clorgyline for 30 min at 37 °C) was added to 22.5 µl of inhibitor (X 10 final concentration) and 25 µl of radiolabelled substrate (X 9 final concentration) and 67.5 µl of 100 µmol phosphate buffer, pH 7.2 in a total volume of 225 µl. The reaction was halted after 75 min by the addition of 100 µl of 2 M citric acid.

2.2.23 Loss of porcine microsomal SSAO activity at 60 and 70 °C

A method of examining the involvement of one enzyme or two different enzymes is to record the alteration in activity at increased temperatures. A difference may be due to changes in the active site that occur at increased temperature or different rates of denaturation of the proteins.

Porcine microsomal protein was incubated with clorgyline at a final concentration of 10^{-3} M for 30 min at 37 °C. 90 µl of 100 mM sodium /potassium phosphate buffer, pH 7.2, per reaction was placed in 2 shaking waterbaths, one at 60 °C and the other at 70 °C and incubated for 5 min before 110 µl of the protein /clorgyline mixture was

added. Reactions at 60 °C and 70 °C were started at different time intervals by the addition of 25 µl of either 50 µ mol benzylamine (1.0 µCi/µmol) or 100 µmol 5-HT (3.0 µCi/µmol). After 30 min each reaction was halted by the addition of 100 µl of 2 M citric acid. Blanks contained the same materials but the citric acid was added before the radioactive substrate.

2.2.24 Inhibition of porcine microsomal SSAO deamination of 5-HT and benzylamine after heat treatment

Porcine microsomal SSAO was incubated with clorgyline at a final concentration of 10^{-3} M for 30 min. The SSAO was the heated to 70°C for 15 min. 110 µl of this mixture was then added to (a) 25 µl 5-HT (100 µmol, 3.0 Ci/mol) and 22.5 µl of benzylamine (0.1-10 mM) or (b) 25 µl benzylamine (50 µmol, 1.0 Ci/mol) and 22.5 µl of 5-HT (0.1-10 mM) and 67.5 µl of 100 µM phosphate buffer, pH 7.2, in a total volume of 225 µl, at 70 °C. The reactions were stopped after 75 min by adding 100 µl of 2 M citric acid.

2.2.25 Inhibition of porcine microsomal SSAO deamination of benzylamine by 5-HT after heat treatment

Porcine microsomal SSAO was incubated with clorgyline at a final concentration of 10^{-3} M for 30 min. The SSAO was the heated to 70°C for 15 min. 110 µl of this mixture was then added to 25 µl 5-HT (25-500 µmol, 3.0 Ci/mol) and 22.5 µl of benzylamine (0.1-10 mM) and 67.5 µl of 100 µM phosphate buffer, pH 7.2, in a total volume of 225 µl. The reactions were stopped after 75 min by adding 100 µl of 2 M citric acid.

2.2.26 Michaelis-Menten kinetics for heat-treated porcine microsomal SSAO

Porcine microsomal SSAO was incubated with clorgyline at a final concentration of 10^{-3} M for 30 min. The SSAO was the heated to 70°C for 15 min. 110 µl of this mixture was then added to 25 µl 5-HT (25-500 µmol, 3.0 Ci/mol) 90 µl of 100 µM sodium /potassium phosphate buffer, pH 7.2, in a total volume of 225 µl, assayed at 37 °C. The reactions were stopped after 60 min by adding 100 µl of 2 M citric acid.

2.2.27 Oxidative deamination of 3-methylhistamine by porcine dental pulp microsomal SSAO

Deamination of 3-methylhistamine by solubilised porcine dental pulp microsomes was measured spectrophotometrically based on the method of Holt and co-workers (1997). 4-aminoantipyrine acts as a proton donor in the peroxidase reaction with hydrogen peroxide produced by the oxidative deamination of an amine to form oxidised 4-aminoantipyrine that condenses with vanillic acid to produce a quinoneimine dye that absorbs maximally at 498 nm. 700 µl of solubilised microsomes that had been pre-incubated with clorgyline (final concentration 10^{-3}M) for 30 min were added to 100 µl of 3-methylhistamine (X10 final concentration) and 200 µl of the colourimetric solution (4-aminoantipyrine 500 µM, vanillic acid 1mM, peroxidase 4 U/ml in 0.1 M sodium /potassium phosphate buffer, pH 7.2). The absorbance change was measured at 498 nm. Kinetic constants were determined using 3-methylhistamine in the concentration range 0.025-10 mM. The percentage of the total 3-methylhistamine deamination as a result of SSAO or MAO activity was determined by pre-incubating solubilised microsomal samples for 30 min in the presence of (a) no inhibitor (total activity), (b) clorgyline (10^{-3}M final concentration) (SSAO activity), (c) semicarbazide (10^{-3}M) and deprenyl (10^{-6}M) (MAO-A activity), (d) semicarbazide (10^{-3}M) and clorgyline (10^{-6}M) (MAO-B activity) and (e) semicarbazide (10^{-3}M) and clorgyline (10^{-3}M) (negative control) before starting the reaction by the addition of 5 mM 3-methylhistamine.

2.3 Results

2.3.1 Radiochemical assay

In all assays the product formation was linear with time for the periods used.

Human K_m Values for Crude Homogenate

The kinetic constants are given in Table 2.6 and graphs of the kinetic behaviour in Figures 2.4 & 2.5.

Substrate	K_m (μM)	V_{max} (nmol/hour/mg protein)
Benzylamine	254 ± 55	370 ± 44
5-HT	318 ± 54	52 ± 5

Table 2.6. The K_m and V_{max} values for human dental pulp crude homogenate SSAO. Values are the mean ± S.E.M. of experiments completed in triplicate on three separate homogenate preparations.

Porcine K_m Values for microsomal and cytosolic fractions

The kinetic constants are given in Table 2.7 and graphs of the kinetic behaviour in Figures 2.6 to 2.11.

Substrate	Fraction Tested	K_m (μM)	V_{max} (nmol/min/mg protein)
Benzylamine	Microsomal	18.6 ± 1.2	72.23 ± 4.17
Benzylamine	Cytosolic	35 ± 1.7	53.36 ± 3.82
5-HT	Microsomal	38.6 ± 2.0	33.47 ± 0.42
5-HT	Cytosolic	911 ± 262	12.01 ± 2.65
PEA	Microsomal	44.1 ± 6	26.19 ± 1.40
PEA	Cytosolic	257.6 ± 45	4.41 ± 0.43

Table 2.7. The K_m and V_{max} values for the oxidative deamination of amine substrates by porcine dental pulp SSAO microsomal and cytosolic fractions as determined by radiochemical assay as described in Section 2.2.5. Values are the mean ± S.E.M. of experiments completed in triplicate.

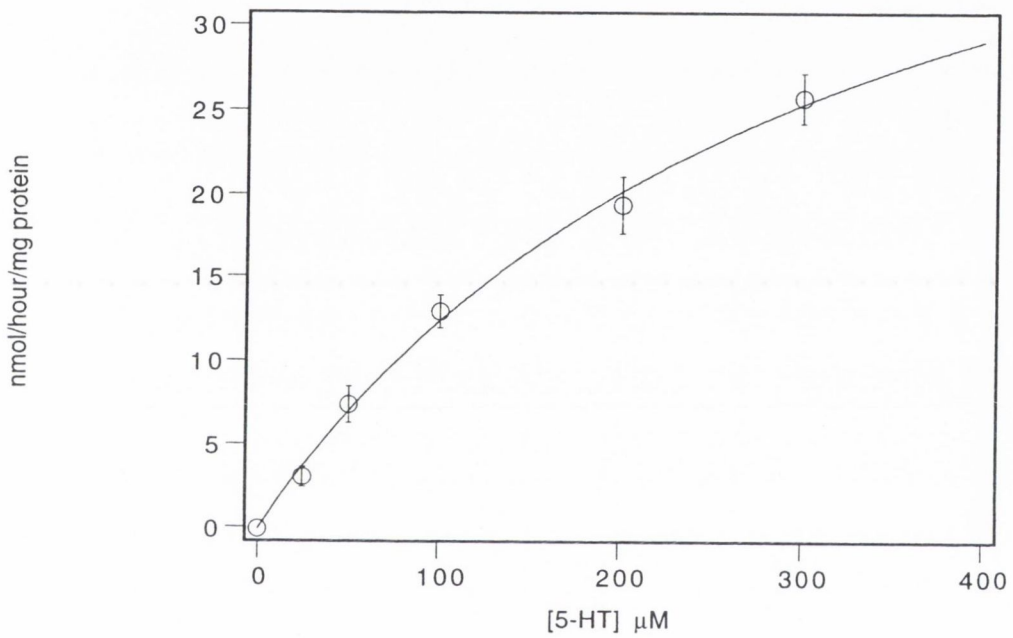


Figure 2.4. Michaelis-Menten plot for the oxidative deamination of 5-HT by SSAO from human dental pulp homogenates.

The oxidative deamination of 5-HT by human dental pulp SSAO was determined by radiochemical assay as described in Section 2.2.5 over the range of substrate concentrations indicated. The points shown are mean values ± S.E.M. of two separate experiments.

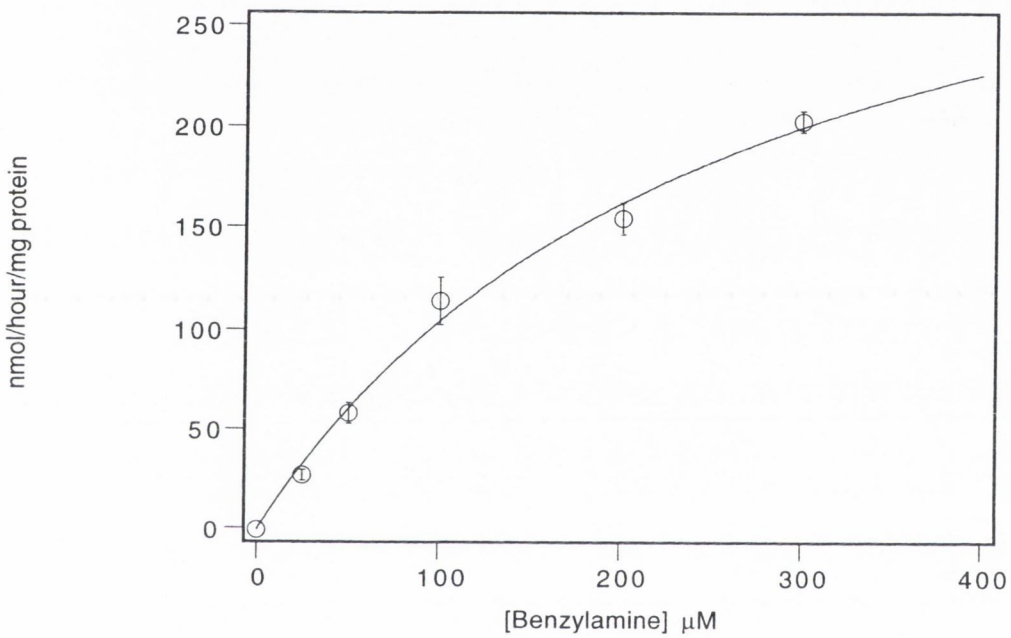


Figure 2.5. Michaelis-Menten plot for the oxidative deamination of benzylamine by SSAO from human dental pulp homogenates.

The oxidative deamination of benzylamine by human dental pulp SSAO was determined by radiochemical assay as described in Section 2.2.5 over the range of substrate concentrations indicated. The points shown are mean values ± S.E.M. of three separate experiments.

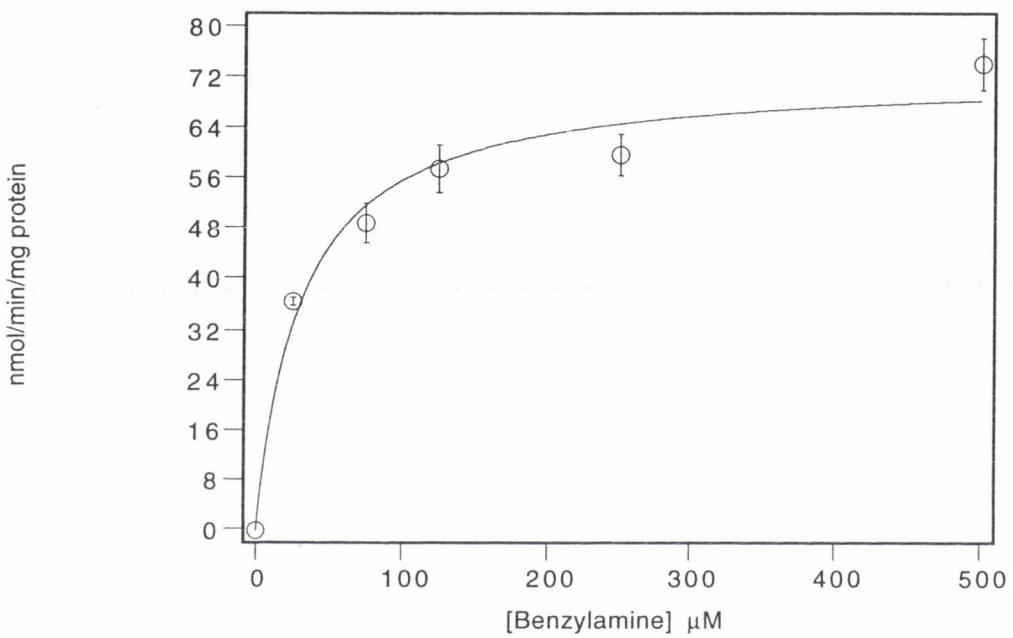


Figure 2.6. Michaelis-Menten plot for the oxidative deamination of benzylamine by SSAO from porcine dental pulp microsomes.

The oxidative deamination of benzylamine by porcine dental pulp microsomal SSAO was determined by radiochemical assay as described in Section 2.2.5 over the range of substrate concentrations indicated. The points shown are mean values ± S.E.M. of three separate experiments.

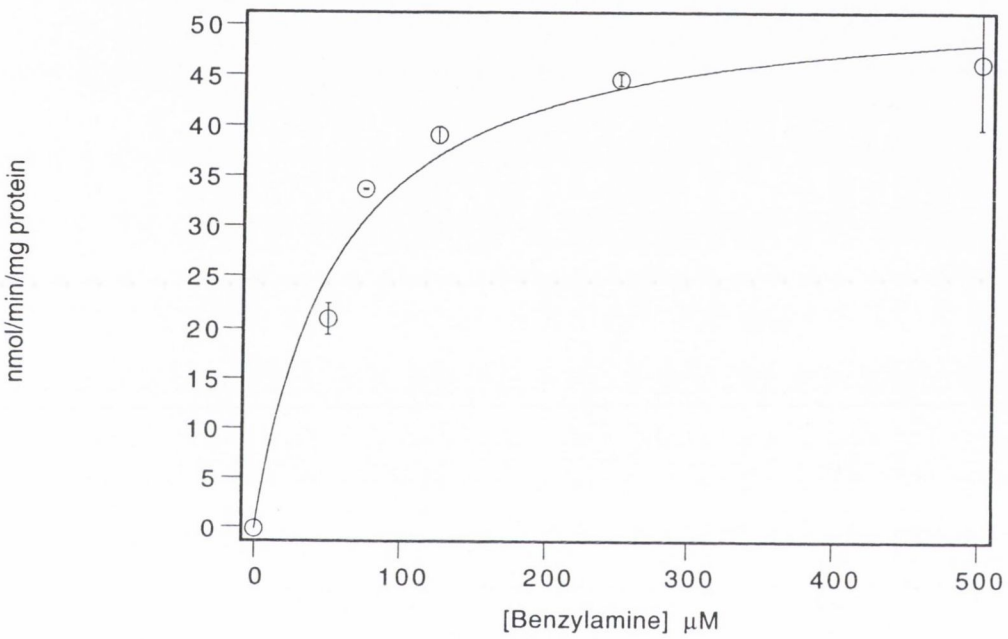


Figure 2.7. Michaelis-Menten plot for the oxidative deamination of benzylamine by SSAO from porcine dental pulp cytosol.

The oxidative deamination of benzylamine by porcine dental pulp microsomal SSAO was determined by radiochemical assay as described in Section 2.2.5 over the range of substrate concentrations indicated. The points shown are mean values ± S.E.M. of three separate experiments.

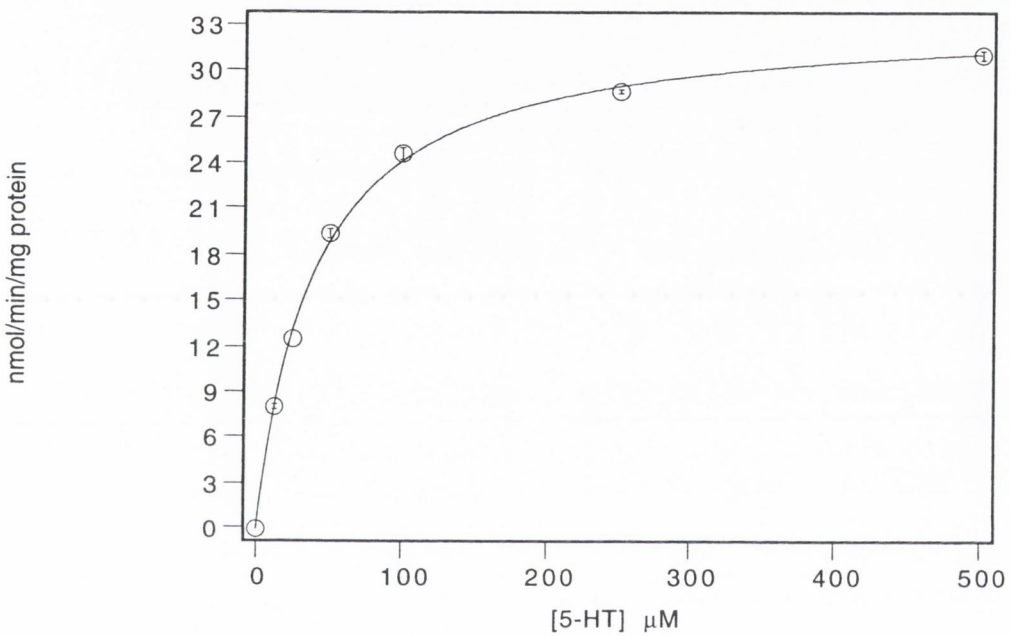


Figure 2.8. Michaelis-Menten plot for the oxidative deamination of 5-HT by SSAO from porcine dental pulp microsomes.

The oxidative deamination of 5-HT by porcine dental pulp microsomal SSAO was determined by radiochemical assay as described in Section 2.2.5 over the range of substrate concentrations indicated. The points shown are mean values ± S.E.M. of three separate experiments.

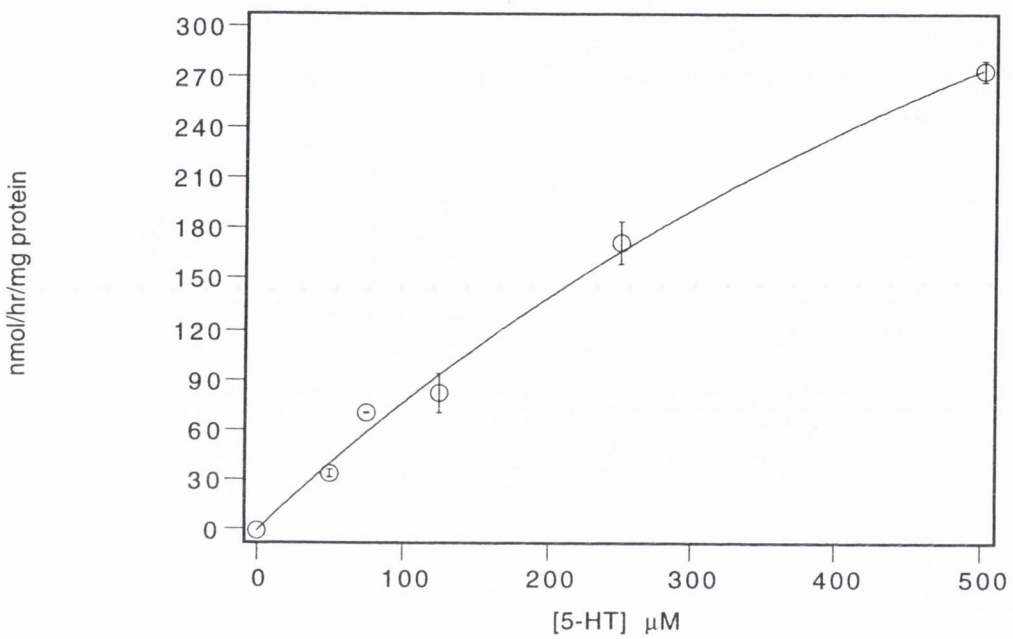


Figure 2.9. Michaelis-Menten plot for the oxidative deamination of 5-HT by SSAO from porcine dental pulp cytosol.

The oxidative deamination of 5-HT by porcine dental pulp cytosolic SSAO was determined by radiochemical assay as described in Section 2.2.5 over the range of substrate concentrations indicated. The points shown are mean values ± S.E.M. of three separate experiments.

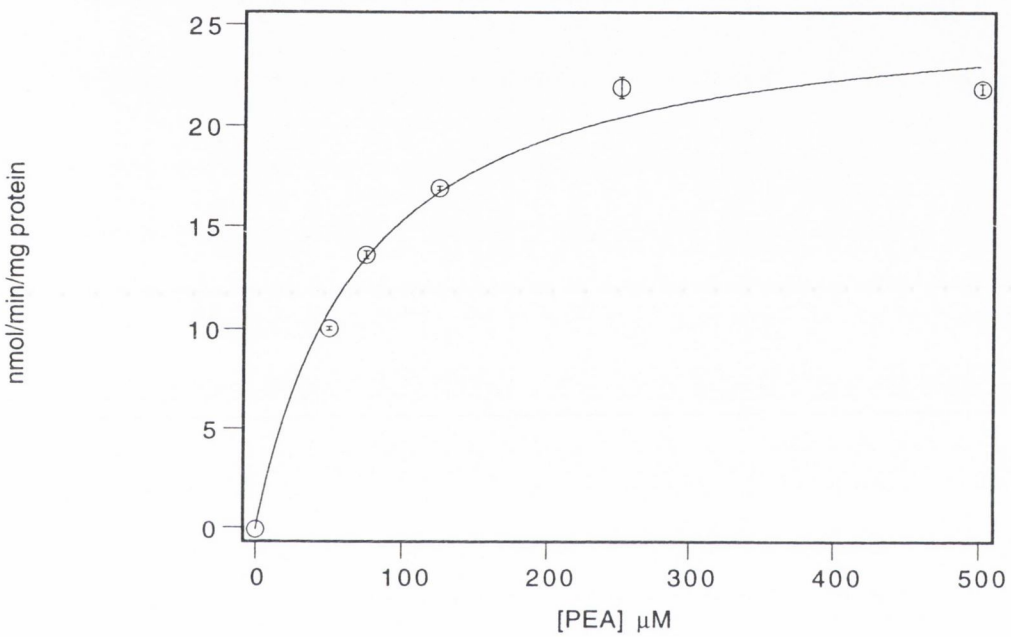


Figure 2.10. Michaelis-Menten plot for the oxidative deamination of PEA by SSAO from porcine dental pulp microsomes.

The oxidative deamination of PEA by porcine dental pulp microsomal SSAO was determined by radiochemical assay as described in Section 2.2.5 over the range of substrate concentrations indicated. The points shown are mean values ± S.E.M. of three separate experiments.

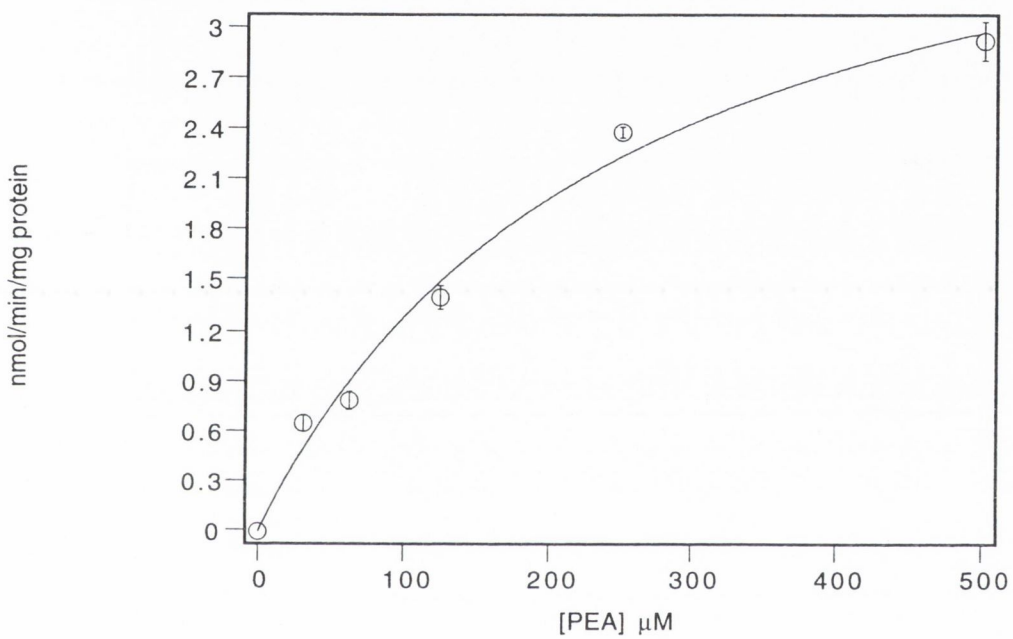


Figure 2.11. Michaelis-Menten plot for the oxidative deamination of PEA by SSAO from porcine dental pulp cytosol.

The oxidative deamination of PEA by porcine dental pulp cytosolic SSAO was determined by radiochemical assay as described in Section 2.2.5 over the range of substrate concentrations indicated. The points shown are mean values ± S.E.M. of three separate experiments.

2.3.2 Spectrophotometric assay for methylamine deamination.

This coupled assay could not reproducibly detect NADH formation with the dental pulp preparations, although control experiments with pure bovine lung SSAO were successful. This may have been as a result of contaminating NADH oxidase activities in the pulp preparations or to the low activity demonstrated by the SSAO in other assays. The addition of rotenone, a NADH oxidase inhibitor, to a final concentration of 2.5 μmol did not produce any significant difference.

2.3.3 Radiochemical assay for methylamine deamination

The time course run with porcine samples over 90 minutes failed to produce any significant product. This result in combination with those from Section 2.3.2 suggests that methylamine is not a substrate for porcine dental pulp SSAO.

2.3.4 Detection of hydrogen peroxide assays

The experiments described in Sections 2.2.12 failed to produce measurable amounts of product when either benzylamine or 5-HT were used as substrates. Doubling the amount of enzyme sample did not produce a measurable rate. This, as for Section 2.3.2, may have been due to impurities in the membrane, in this case endogenous reducing agent or to the low activity of the SSAO.

2.3.5 Inhibitor potency of semicarbazide and phenelzine

Both semicarbazide and phenelzine were good inhibitors capable of producing complete inhibition of SSAO activity in porcine microsomal fractions at concentrations of 10^{-3} M (Figures 2.12 & 2.13). However, phenelzine appears to be a much more potent inhibitor with IC_{50} values against 5-HT and benzylamine in the 10^{-8} M range, whereas the IC_{50} values for semicarbazide against the same substrates was in the 10^{-5} M range. Maximum inhibition was observed after 10 min of the reaction with both substrates for both inhibitors (Figures 2.14 & 2.15).

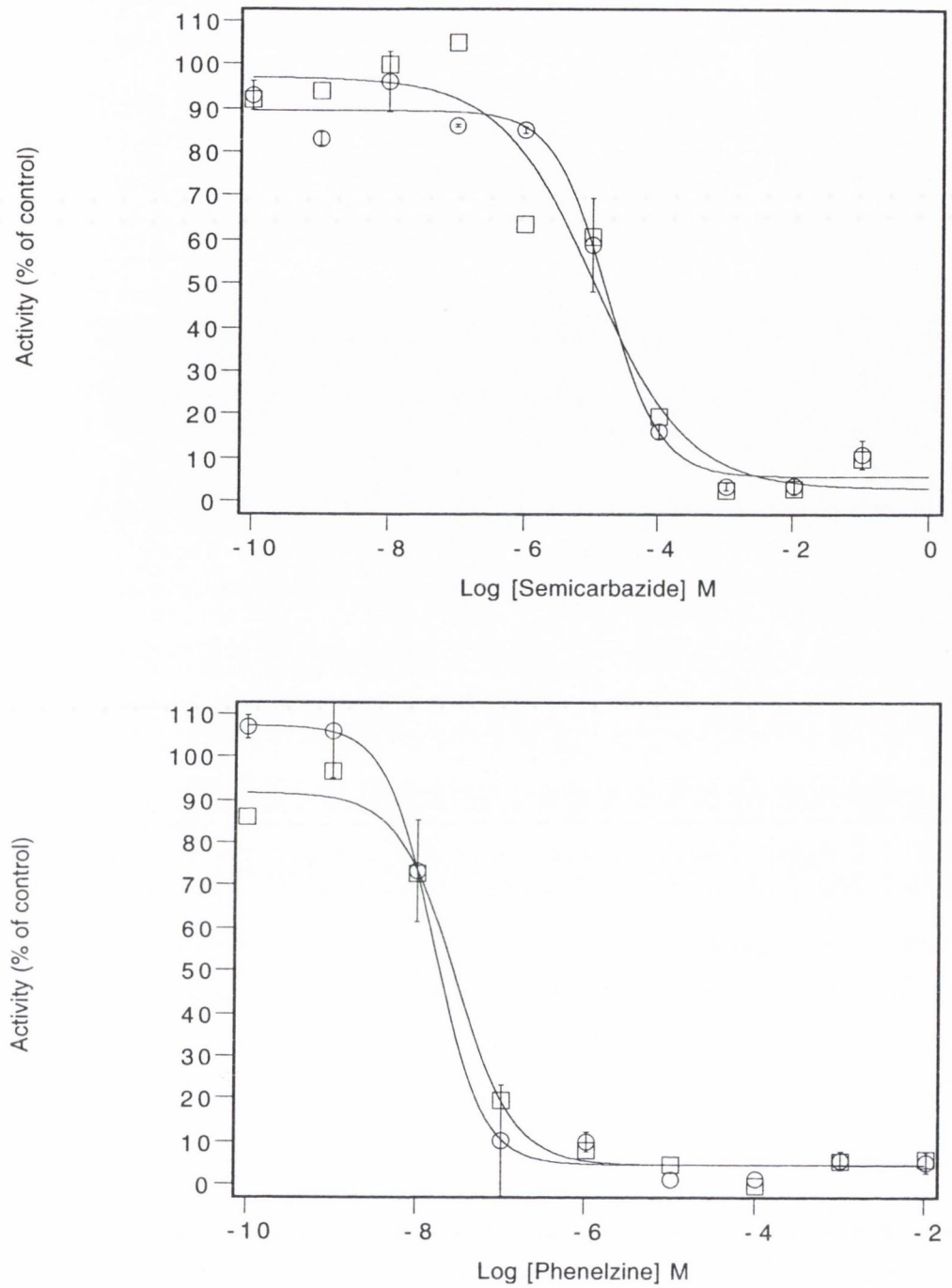


Figure 2.12. The inhibition of oxidative deamination of 25 μM benzylamine by porcine dental pulp microsomal SSAO in the presence of various concentrations of (a) semicarbazide and (b) phenelzine determined radiochemically as described in Section 2.2.13. Each curve represents separate inhibition of different microsomal preparations. Points shown are the mean values ± S.E.M. of three separate experiments. The curves were fitted using MacCurveFit Version 1.2.

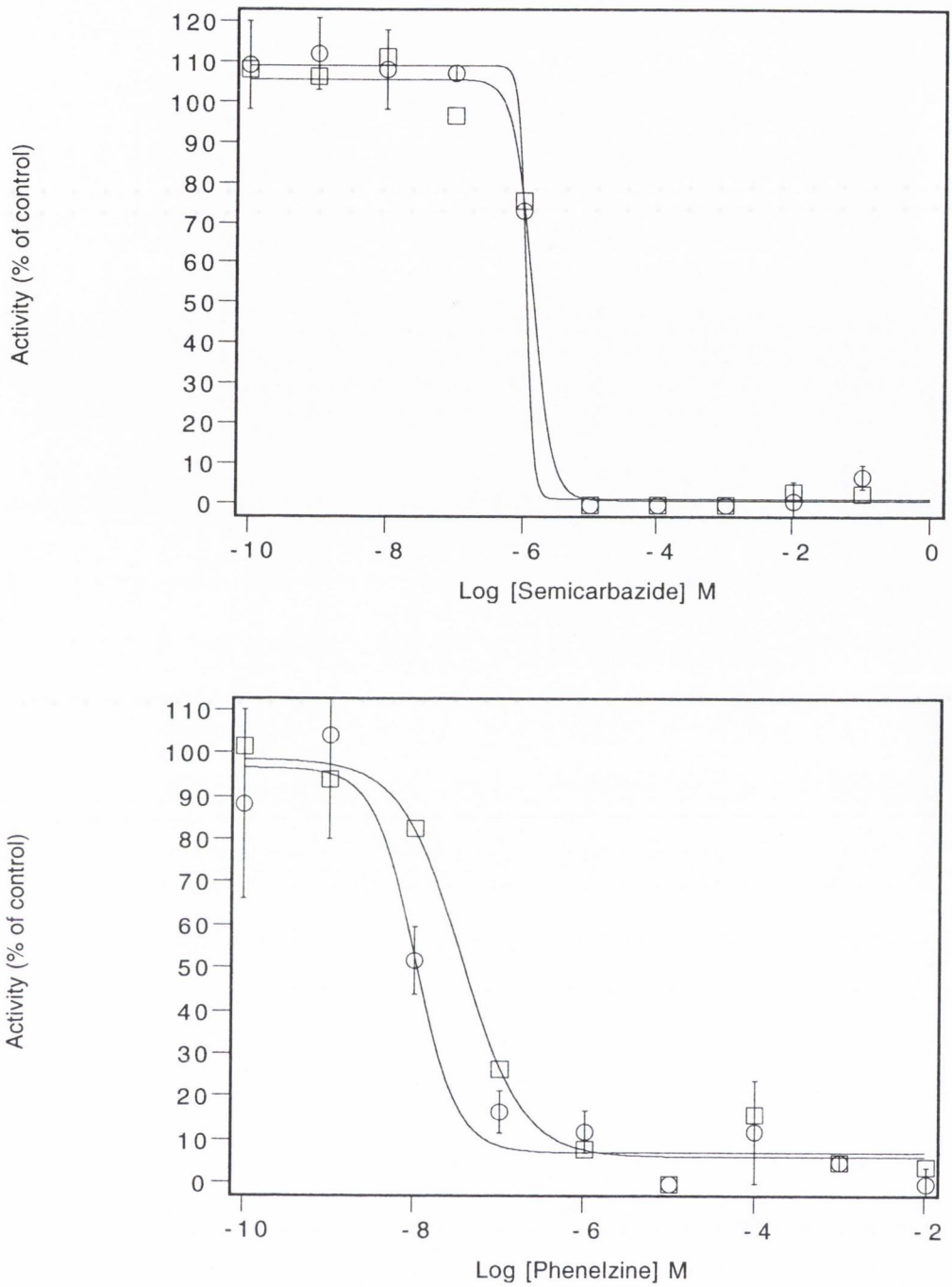


Figure 2.13. The inhibition of oxidative deamination of 40 μM 5-HT by porcine dental pulp microsomal SSAO in the presence of various concentrations of (a) semicarbazide and (b) phenelzine determined radiochemically as described in Section 2.2.13. Each curve represents separate inhibition of different microsomal preparations. Points shown are the mean values ± S.E.M. of three separate experiments. The curves were fitted using MacCurveFit Version 1.2.

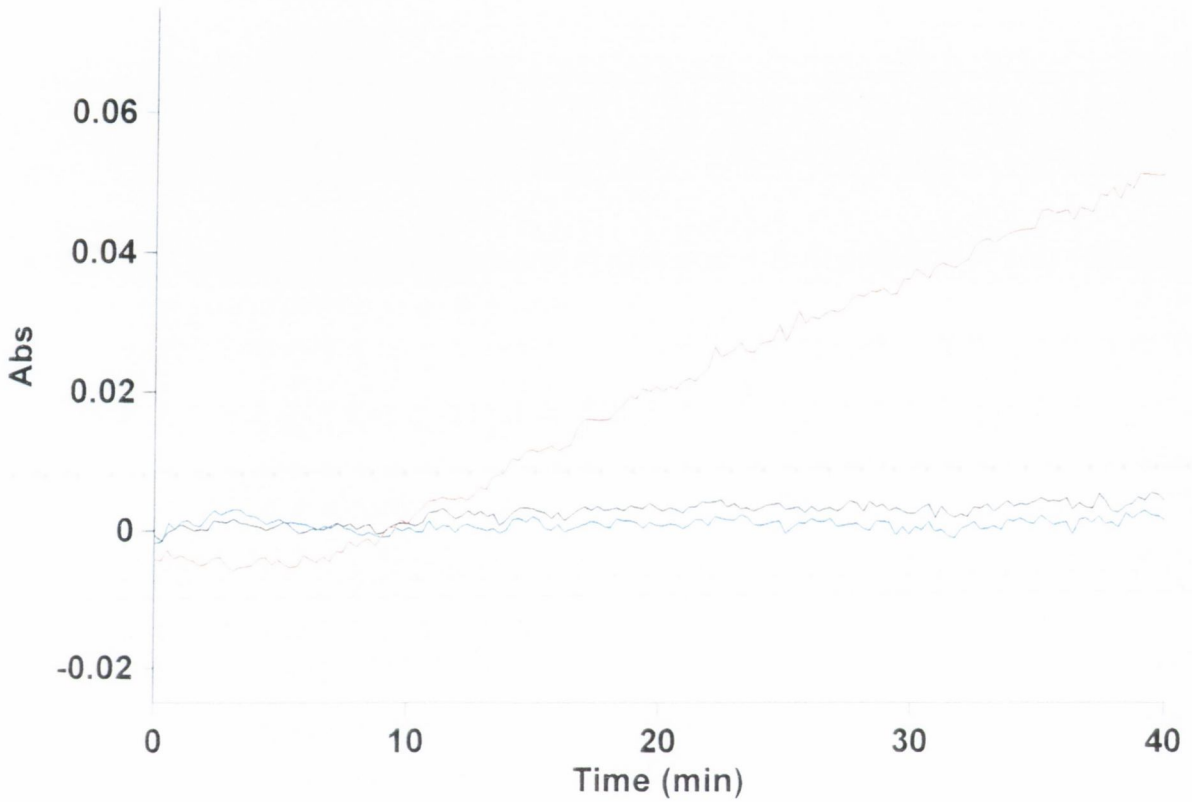


Figure 2.14. The inhibition of oxidative deamination of 50 μM benzylamine by Triton X-100-solubilised porcine dental pulp microsomal SSAO in the presence of 10^{-3} M semicarbazide or 10^{-3} M phenelzine determined by coupled spectrophotometrical assay as described in Section 2.2.13 based on the method of Houslay and Tipton (1973). Each experiment was completed in triplicate. The red plot is control activity, the black plot represents semicarbazide (final 10^{-3}M) inhibition of microsomes pre-treated with clorgyline (final 10^{-3}M) and the blue plot represents phenelzine (final 10^{-3}M) inhibition of microsomes pre-treated with clorgyline (final 10^{-3}M).

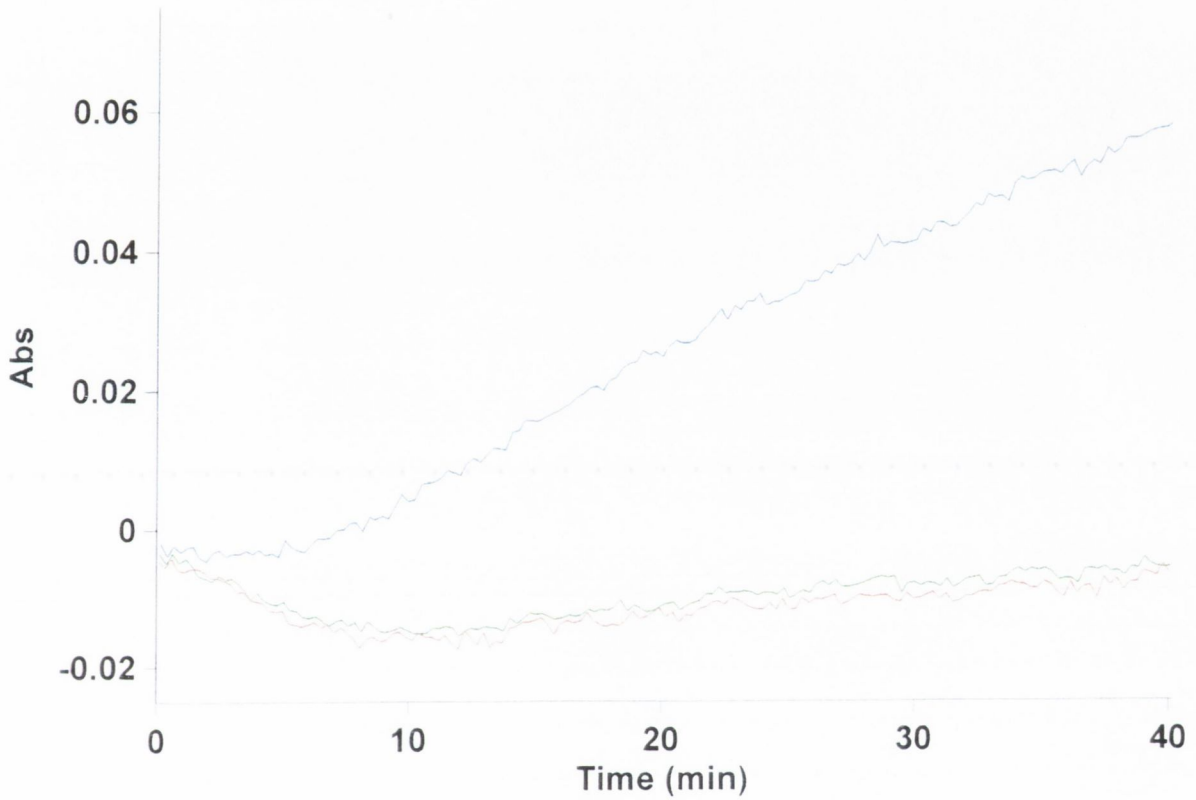


Figure 2.15. The inhibition of oxidative deamination of 100 μM 5-HT by Triton X-100 solubilised porcine dental pulp microsomal SSAO in the presence of 10^{-3} M semicarbazide or 10^{-3} M phenelzine determined by coupled spectrophotometrical assay as described in Section 2.2.13 based on the method of Houslay and Tipton (1973). Each experiment was completed twice in triplicate (only one plot is shown). The blue plot is control activity, the green plot represents semicarbazide (final 10^{-3} M) inhibition of microsomes pre-treated with clorgyline (final 10^{-3} M) and the red plot represents phenelzine (final 10^{-3} M) inhibition of microsomes pre-treated with clorgyline (final 10^{-3} M).

2.3.6 Localisation of SSAO activity

The location of SSAO activity in porcine dental pulp is demonstrated in Table 2.8 (a) & (b).

(a)

Location	Specific SSAO activity towards benzylamine (nmol/min/mg protein)	Total SSAO activity towards benzylamine (nmol/min)
Homogenate	14.2 ± 1.8	2125 ± 270
Mitochondria	17.8 ± 0.5	2228 ± 62
Microsome	35.7 ± 1.3	1000 ± 34
Cytosol	19.0 ± 0.9	3137 ± 148

(b)

Location	Specific SSAO activity towards 5-HT (nmol/min/mg protein)	Total SSAO activity towards 5-HT (nmol/min)
Homogenate	0.3 + 0.01	45.3 + 1.2
Mitochondria	0.46 + 0.1	57.6 + 12.5
Microsome	8.18 + 0.2	29.3 + 5.3
Cytosol	0.33 + 0.004	56.0 + 0.7

Table 2.8 (a) & (b). The location of SSAO activity in porcine dental pulp as determined radiochemically through the oxidative deamination of (a) 50 μM benzylamine (1 Ci/mol) and (b) 100 μM 5-HT (3 Ci/mol) as described in Section 2.2.14. Values shown are mean values ± S.E.M. of three separate experiments.

The highest concentration of SSAO activity per mg of protein was in the microsomal fraction when 5-HT and benzylamine were used as substrates. The SSAO activity towards 5-HT is four to five times less than that for benzylamine. The mitochondrial fraction contains significant SSAO activity for both substrates tested probably as a

result of the collagenous nature of the pulp that hinders rupturing of the tissue during homogenisation.

2.3.7 Contributions of SSAO and MAO to the deamination of benzylamine, 5-HT and PEA in porcine and human tissue

Porcine pulpal microsomes & human crude homogenates

The results are displayed in Table 2.9.

2.3.8 Substrate competition experiments using porcine dental pulp microsomal SSAO

The K_i values are given in Table 2.10 and graphically in Figures 2.16 to 2.28. All curves were fitted using MacCurveFit Version 1.2.

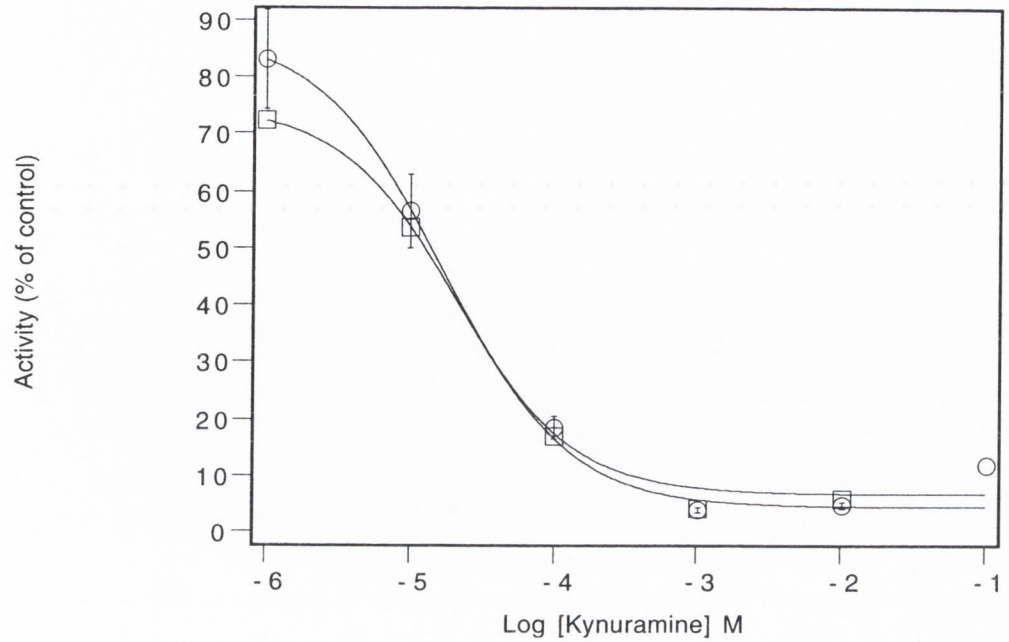
Unlabelled substrate	Radiolabelled substrate	K_i (μM)
Kynuramine	Benzylamine	18 ± 10
Kynuramine	5-HT	5.25 ± 0.2
Histamine	Benzylamine	1054 ± 30
Histamine	5-HT	2950 ± 400
Octopamine	Benzylamine	1429 ± 521
Octopamine	5-HT	5805 ± 878
Dopamine	Benzylamine	328 ± 15
Dopamine	5-HT	391.5 ± 67

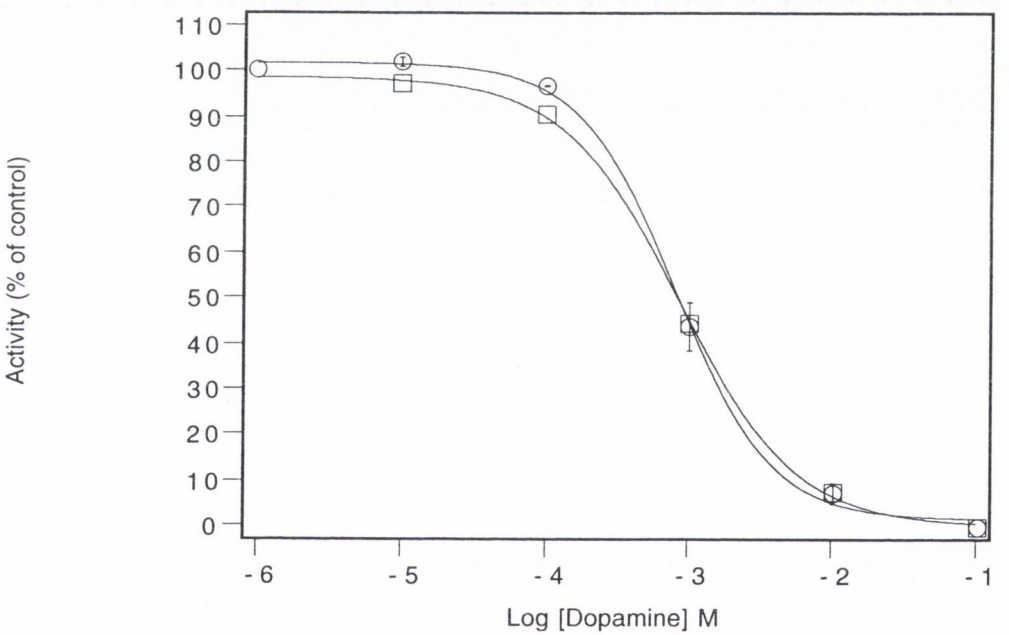
Table 2.10. K_i values from substrate competition experiments using porcine dental pulp microsomal SSAO. Inhibition of oxidative deamination of benzylamine or 5-HT by SSAO was determined radiochemically as described in Section 2.2.16. Values shown are the mean ± S.E.M. from 3 experiments

Inhibitor	Concentration	Percentage of activity remaining in porcine microsomes		
		Benzylamine	PEA	5-HT
semicarbazide	10^{-3} M	4.1 ± 1.7	35 ± 1.8	57.8 ± 3
deprenyl	10^{-6} M	93.6 ± 2.9	69 ± 3.2	91.4 ± 7.9
clorgyline	10^{-3} M	89.1 ± 2	63.4 ± 3.7	33.8 ± 3.5
clorgyline	10^{-6} M	102.2 ± 7.2	91.5 ± 4.3	46.1 ± 4
Semicarbazide + clorgyline	10^{-3} M + 10^{-3} M	3.6 ± 2.5	3.8 ± 1.8	0
KCN	10^{-3} M	94.1 ± 0.6	91.5 ± 12.7	93.9 ± 1.3
β-APN	10^{-3} M	111.9 ± 0.5	36 ± 7.1	63.4 ± 4.6

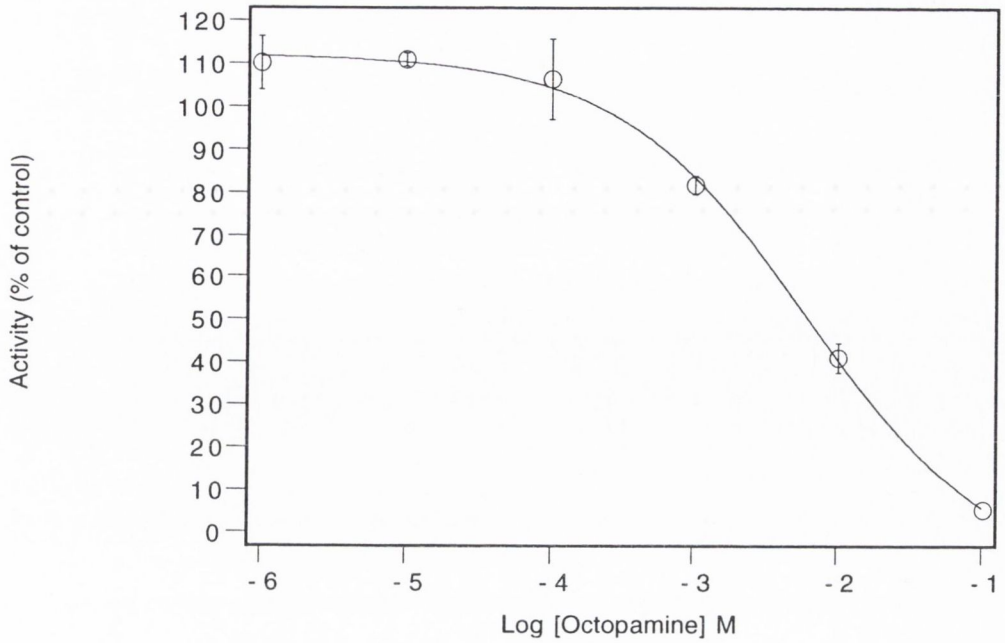
Inhibitor	Concentration	Percentage of activity remaining in crude human homogenate	
		Benzylamine	5-HT
clorgyline	10^{-3} M	66 ± 0.5	87.7 ± 12
Semicarbazide + deprenyl	10^{-3} M + 10^{-6} M	3.3 ± 0.5	9.1 ± 0.6
Semicarbazide + clorgyline	10^{-3} M + 10^{-6} M	28 ± 1.5	0
Semicarbazide + clorgyline	10^{-3} M + 10^{-3} M	0	0

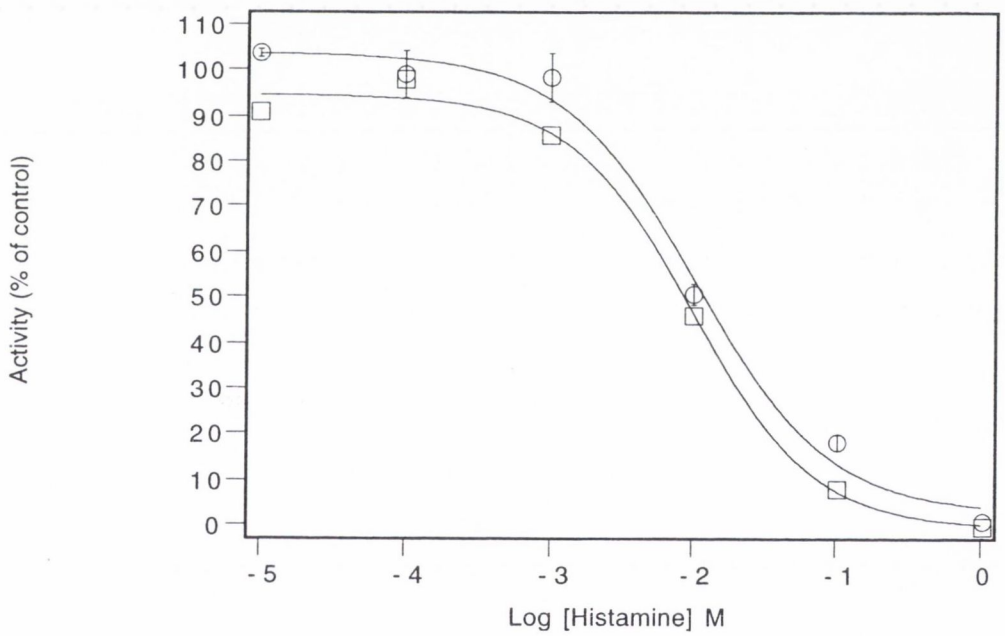
Table 2.9. Contributions of SSAO and MAO to the oxidative deamination of benzylamine, 5-HT and PEA in porcine dental pulp microsomes and benzylamine and 5-HT in crude human homogenates as determined radiochemically as described in Section 2.2.15. Values are the mean ± S.E.M. of two experiments each completed in triplicate.



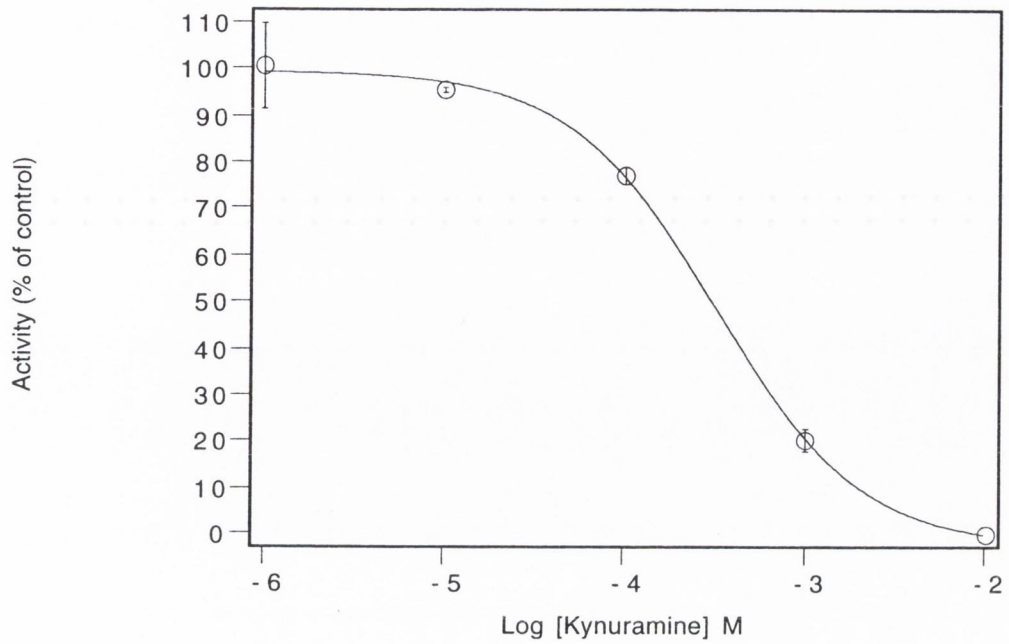


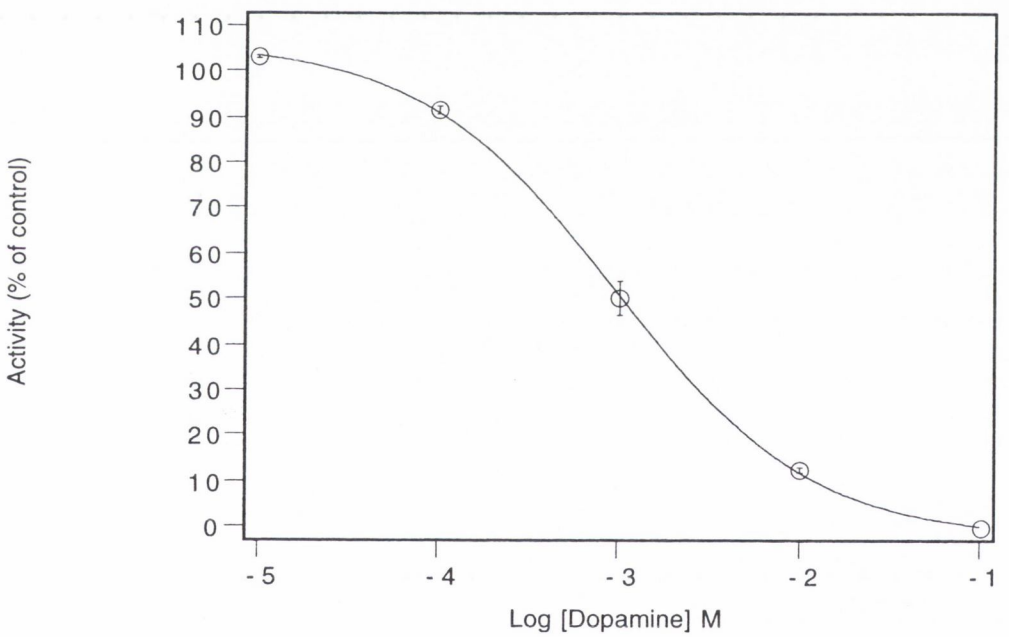
Figures 2.16 (top) & 2.17 (bottom). Inhibition of oxidative deamination of benzylamine by porcine dental pulp microsomal SSAO by kynuramine (Figure 2.16) and dopamine (Figure 2.17) as determined by the radiochemical assay described in Section 2.2.16. Each point is the mean ± range of experiments completed in duplicate. Each line denotes an experiment completed on different porcine dental pulp preparations. All curves were fitted using MacCurveFit Version 1.2.



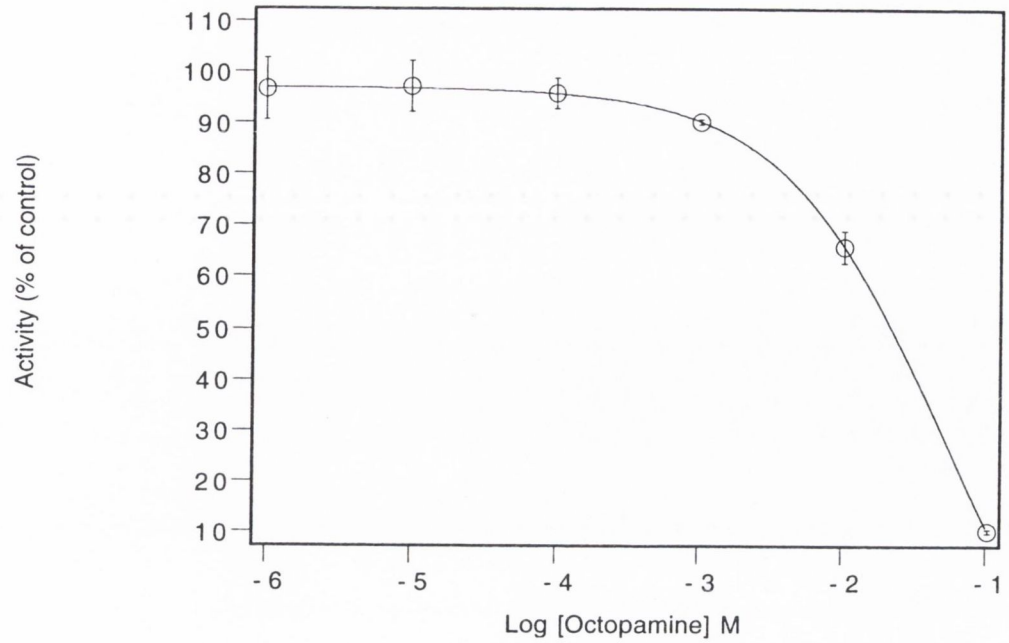


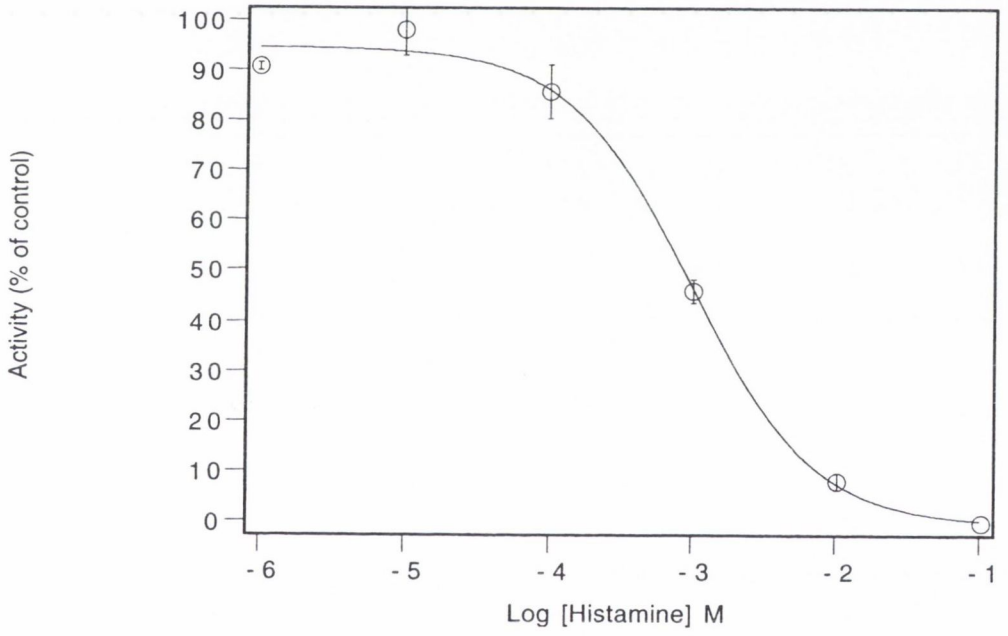
Figures 2.18 (top) & 2.19 (bottom). Inhibition of oxidative deamination of benzylamine by porcine dental pulp microsomal SSAO by octopamine (Figure 2.18) and histamine (Figure 2.19) as determined by the radiochemical assay described in Section 2.2.16. Each point is the mean ± range of experiments completed in duplicate. Each line denotes an experiment completed on different porcine dental pulp preparations. All curves were fitted using MacCurveFit Version 1.2.



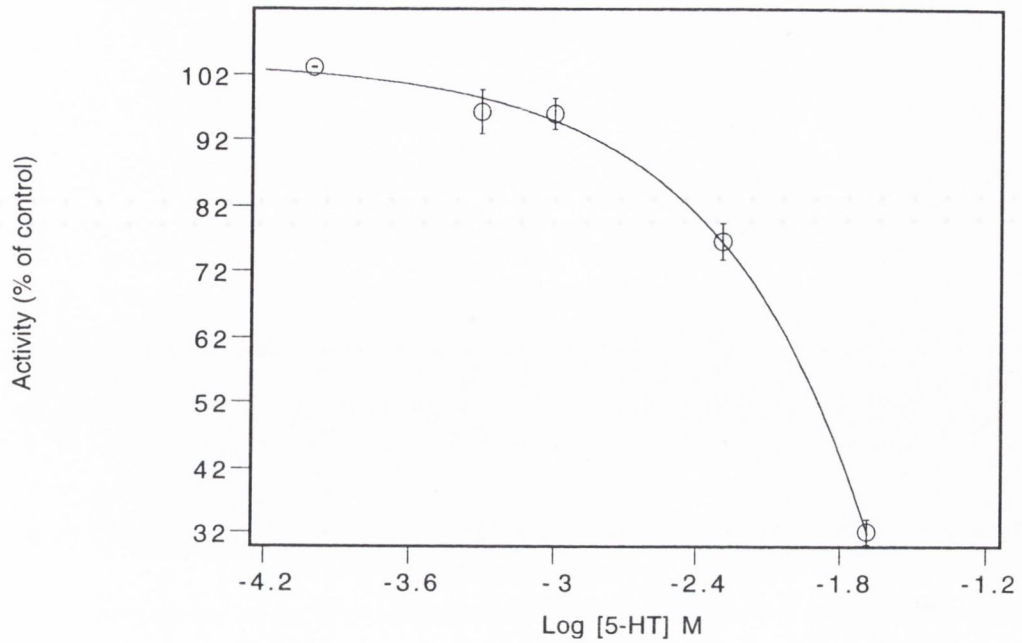


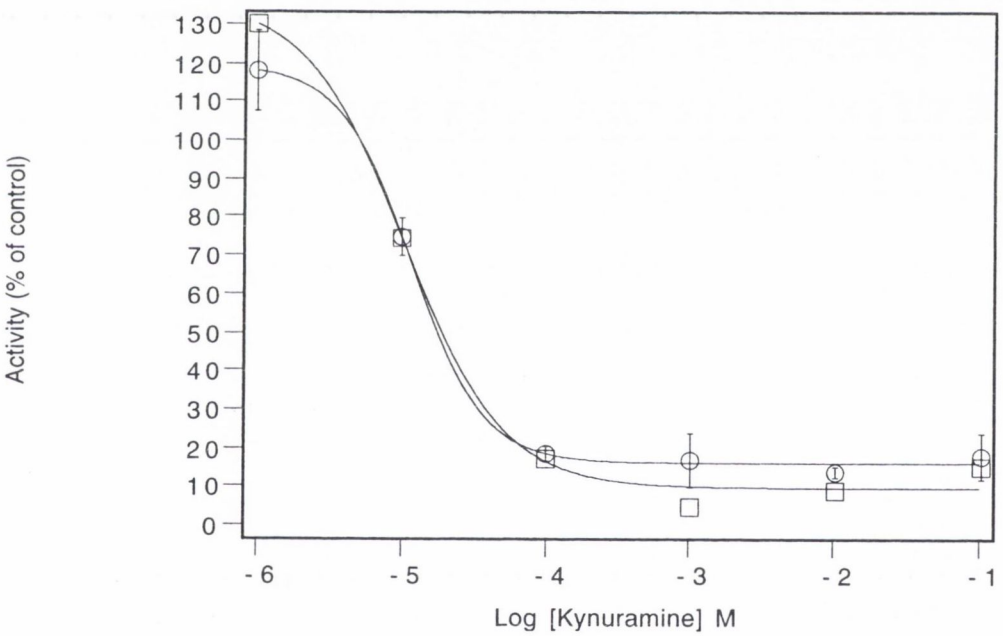
Figures 2.20 (top) & 2.21 (bottom). Inhibition of oxidative deamination of benzylamine by porcine dental pulp cytosolic SSAO by kynuramine (Figure 2.20) and dopamine (Figure 2.21) as determined by the radiochemical assay described in Section 2.2.16. Each point is the mean ± range of experiments completed in duplicate. Each line denotes an experiment completed on different porcine dental pulp preparations. All curves were fitted using MacCurveFit Version 1.2.



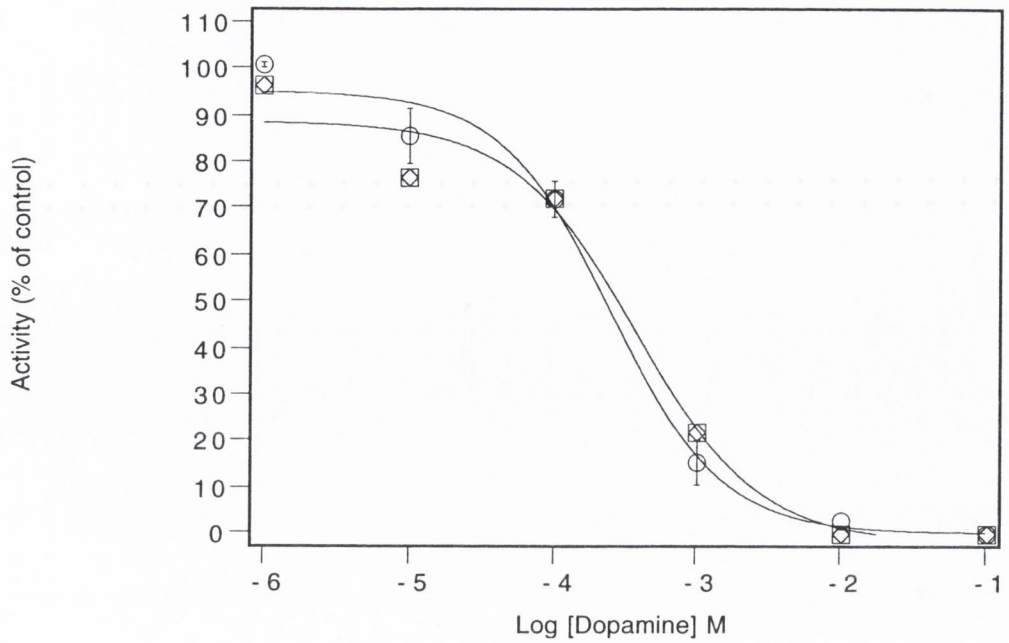


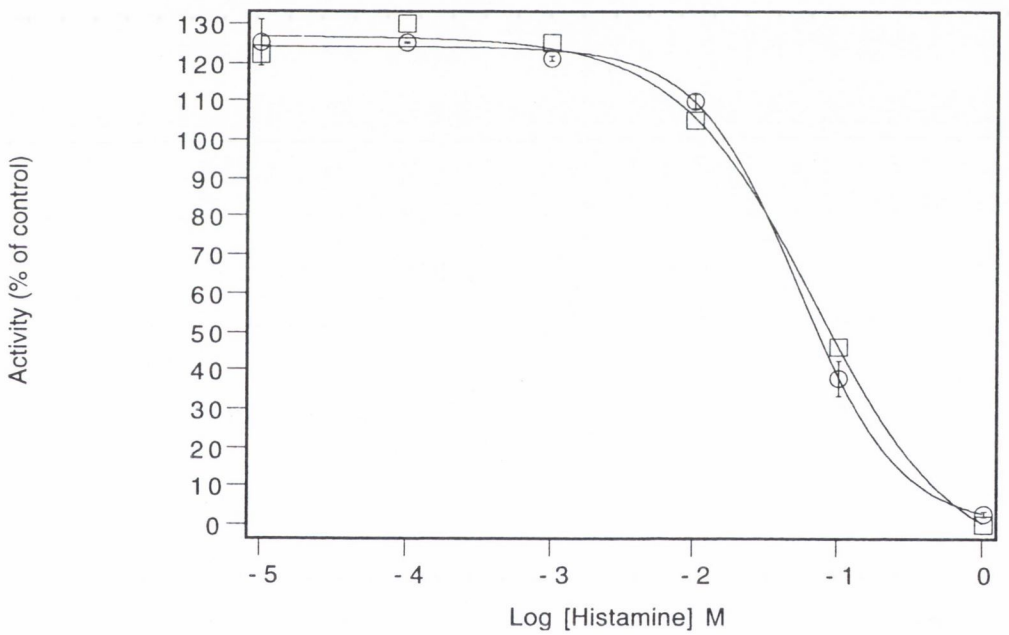
Figures 2.22 (top) & 2.23 (bottom). Inhibition of oxidative deamination of benzylamine by porcine dental pulp cytosolic SSAO by octopamine (Figure 2.22) and histamine (Figure 2.23) as determined by the radiochemical assay described in Section 2.2.16. Each point is the mean ± range of experiments completed in duplicate. Each line denotes an experiment completed on different porcine dental pulp preparations. All curves were fitted using MacCurveFit Version 1.2.





Figures 2.24 (top) & 2.25 (bottom). Inhibition of oxidative deamination of benzylamine by porcine dental pulp cytosolic SSAO by 5-HT (Figure 2.24) and of the inhibition of oxidative deamination of 5-HT by porcine microsomal SSAO by kynuramine (Figure 2.25) as determined by the radiochemical assay described in Section 2.2.16. Each point is the mean ± range of experiments completed in duplicate. Each line denotes an experiment completed on different porcine dental pulp preparations. All curves were fitted using MacCurveFit Version 1.2.





Figures 2.26 (top) & 2.27 (bottom). Inhibition of oxidative deamination of 5-HT by porcine dental pulp microsomal SSAO by dopamine (Figure 2.26) and histamine (Figure 2.27) as determined by the radiochemical assay described in Section 2.2.16. Each point is the mean ± range of experiments completed in duplicate. Each line denotes an experiment completed on different porcine dental pulp preparations. All curves were fitted using MacCurveFit Version 1.2.

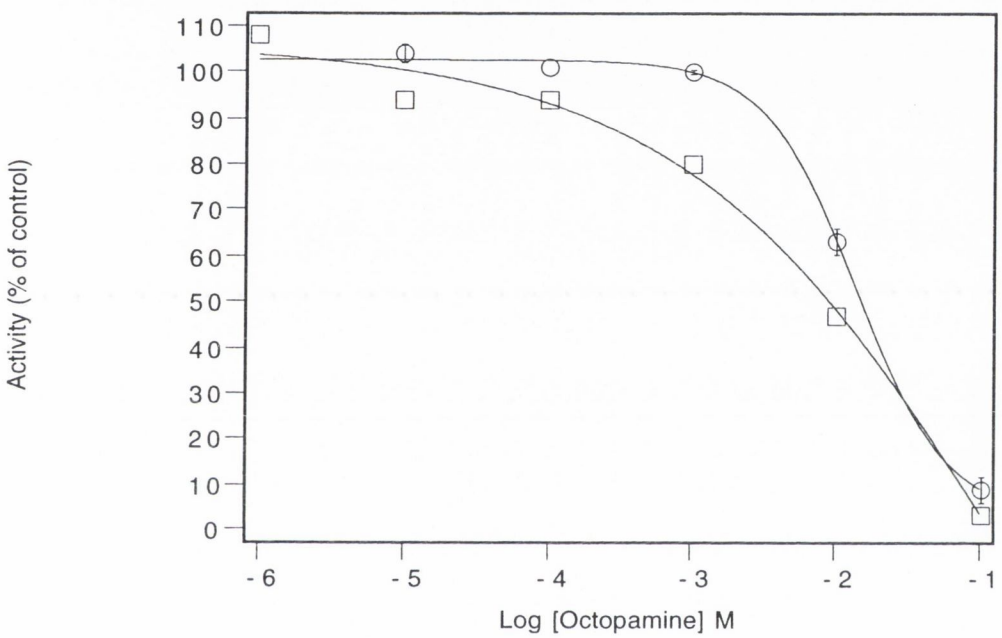


Figure 2.28. Inhibition of oxidative deamination of 5-HT by porcine dental pulp microsomal SSAO by octopamine as determined by the radiochemical assay described in Section 2.2.16. Each point is the mean ± range of experiments completed in duplicate. All curves were fitted using MacCurveFit Version 1.2.

2.3.9 Spectrophotometric assay for kynuramine deamination

This assay failed to produce a repeatable rate for either the disappearance of kynuramine or the appearance of aldehyde. The enzyme was also solubilised with 1 % Triton, as described in Section 2.2.19, but the resultant reaction did not produce a rate. This may be because the rate of reaction was below the sensitivity of the spectrophotometrical assay.

2.3.10 Activation experiments and the effect of thiol groups on SSAO activity

Incubation of the enzyme sample at 37 °C resulted in activation after 90 min when benzylamine was used as a substrate and the activation increased to 350 % at 210 min. For 5-HT, the activation was detected after 90 min and the activity increased to 250 % of the controls (Figure 2.29). Evaporation may have contributed to the apparently increased rates and therefore the second, longer duration experiments were performed with the sample in sealed Eppendorph tubes to restrict material loss (Figure 2.30).

The results from these experiments confirmed that activation had occurred with both substrates with benzylamine reaching 260 % and 5-HT 550 % of the controls (see Figures 2.29 & 2.30). At the end of 32 hours the 5-HT activity returned to its control activity, but the benzylamine remained at nearly double the control.

However, the reproducibility of the activation is variable. The experiment was performed repeatedly under identical conditions and did not demonstrate activation on all occasions.

The effect of *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and *S*-nitrosoglutathione (SNOG) are detailed in Figure 2.31. SNOG produced a decrease in activity over the period tested. Benzylamine oxidative deamination by porcine dental pulp microsomes was inhibited to a greater extent than that for 5-HT, measuring 50 $\pm$ 3 % at 1 hour and 49 $\pm$ 5 % at two hours compared to 10 $\pm$ 1 % and 26 $\pm$ 1 % respectively.

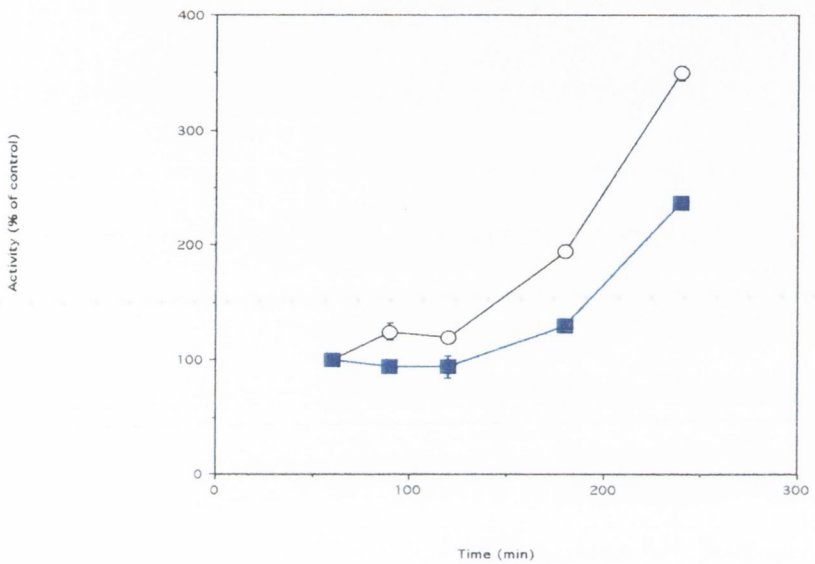
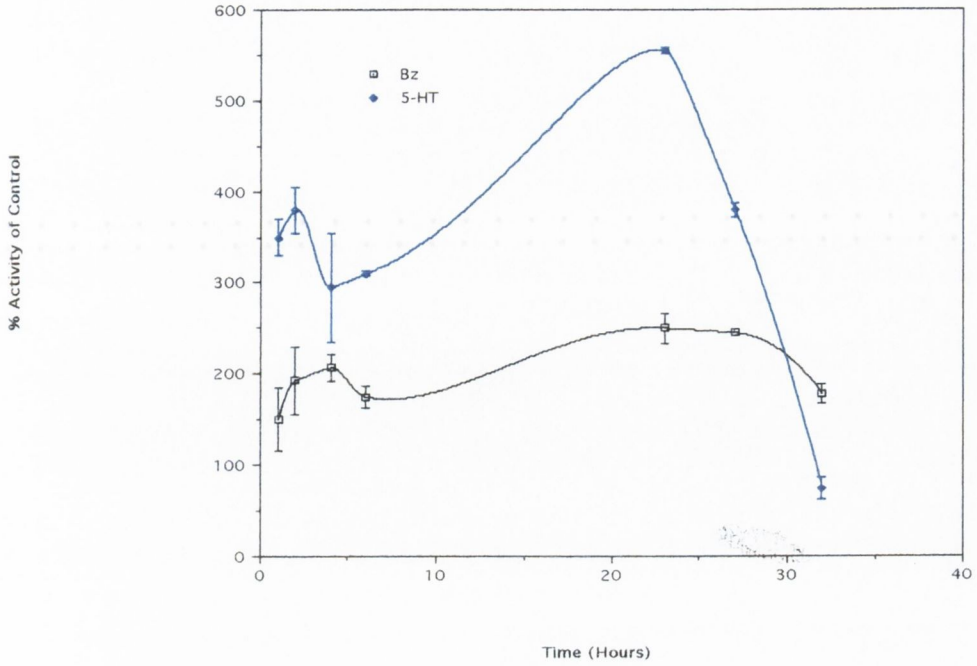


Figure 2.29. The activation at 37 °C of SSAO activity over 240 min towards benzylamine (O) and 5-HT (■) measured radiochemically as described in Section 2.2.18. The points shown are mean values ± S.E.M. of three separate experiments.



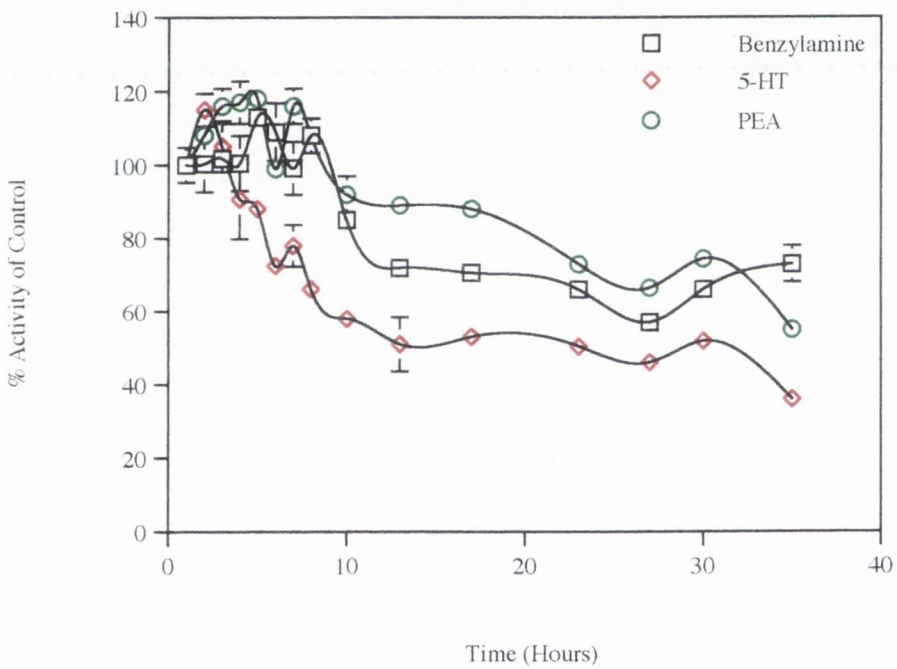


Figure 2.30 (a) & (b). The activation at 37 °C of SSAO activity over 1920 min towards benzylamine, PEA and 5-HT as determined radiochemically as described in Section 2.2.18. The points shown are mean values ± S.E.M. of three separate experiments. Figure 2.30 (a) demonstrates activation, whereas Figure 2.30 (b) does not.

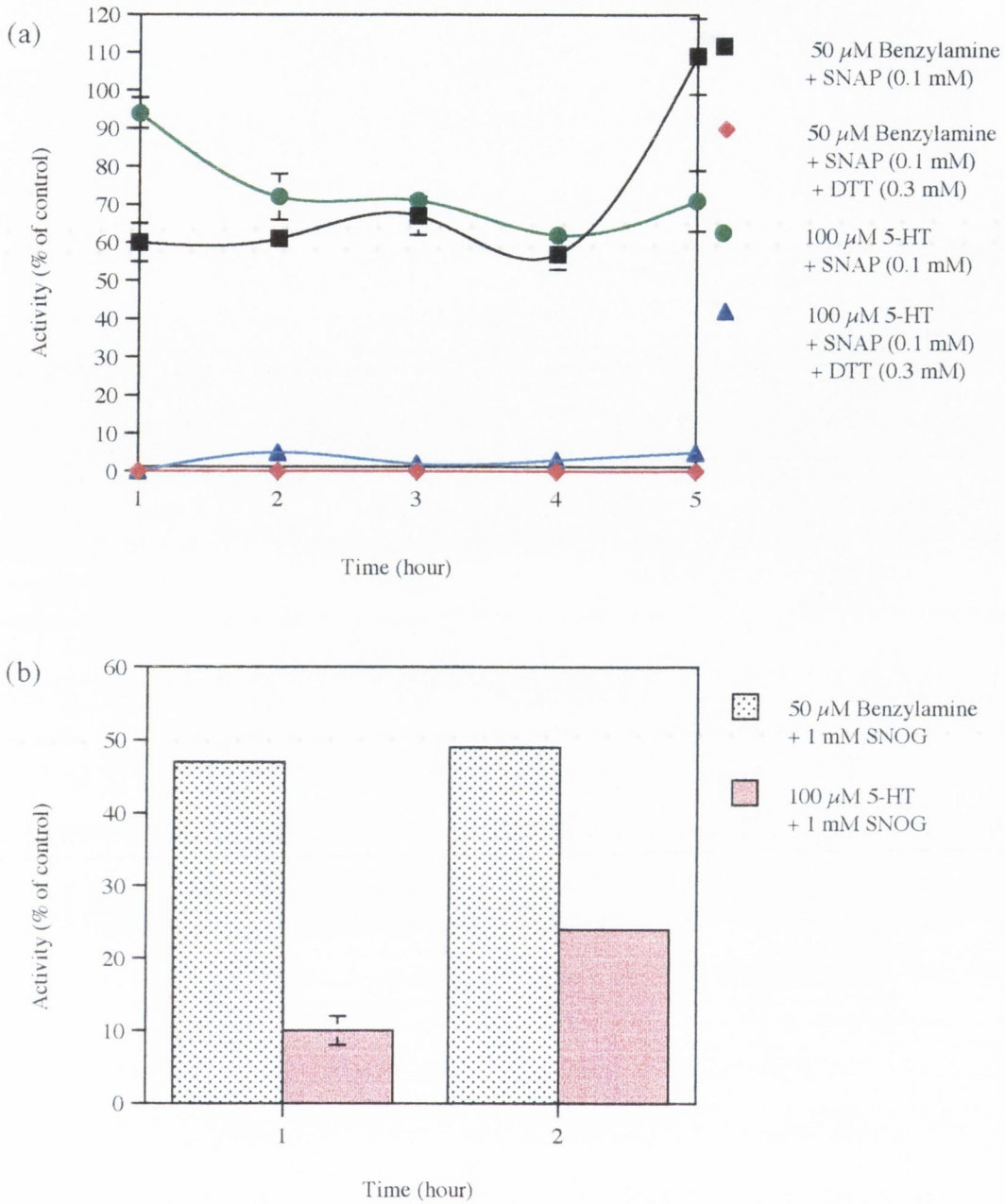


Figure 2.31 (a) & (b). The effect of thiol oxidation in porcine dental pulp microsomal SSAO. (a) *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP, final 0.1 mM) with /without dithiothreitol (DTT)(final 0.3 mM) was incubated at 37 °C for 1-5 hours with sample tissue. (b) *S*-nitrosoglutathione (SNOG, 1 mM) was incubated at 37 °C for 1-2 hours with sample tissue. The oxidative deamination of 50 μM benzylamine (1 Ci/mol) and 100 μM 5-HT (3 Ci/mol) was then measured radiochemically as described in Section 2.2.5. The MAO activity was inhibited by pre-incubation with clorgyline (final 10^{-3}M). Control reactions without NO donors were run each time interval. Blank reactions where 2 M citric acid was added before the substrate, were measured.

2.3.11 Solubilisation of membrane-bound SSAO

The protein concentration in the supernatant doubled to about 50 % of the total protein at Triton concentrations of 0.5 % and higher (see Figure 2.32). The SSAO activity towards benzylamine and 5-HT was substantially solubilised at 0.5 % Triton X-100, but concentrations of 1 % or higher were required to achieve the maximum solubility (see Figures 2.33 & 2.34).

A concentration of 1 % Triton X-100 was used in all further solubilisation experiments.

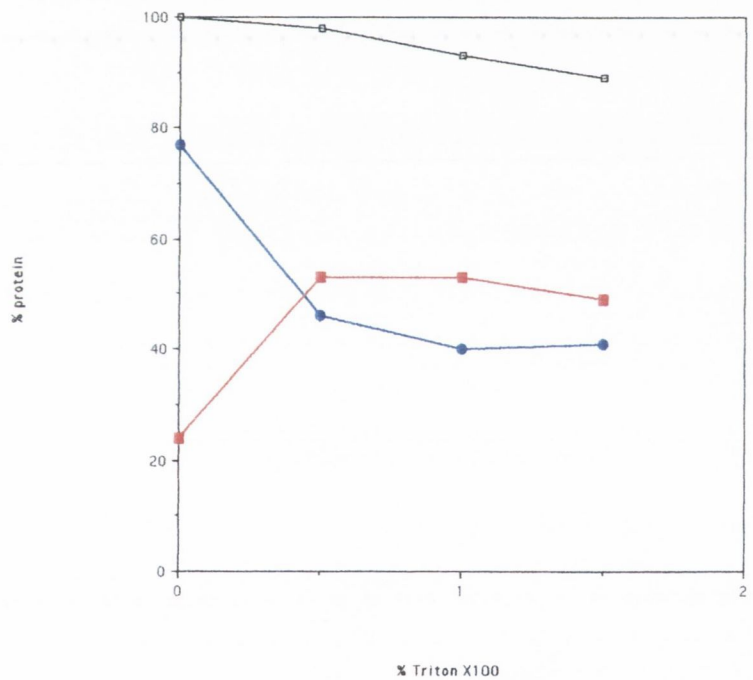
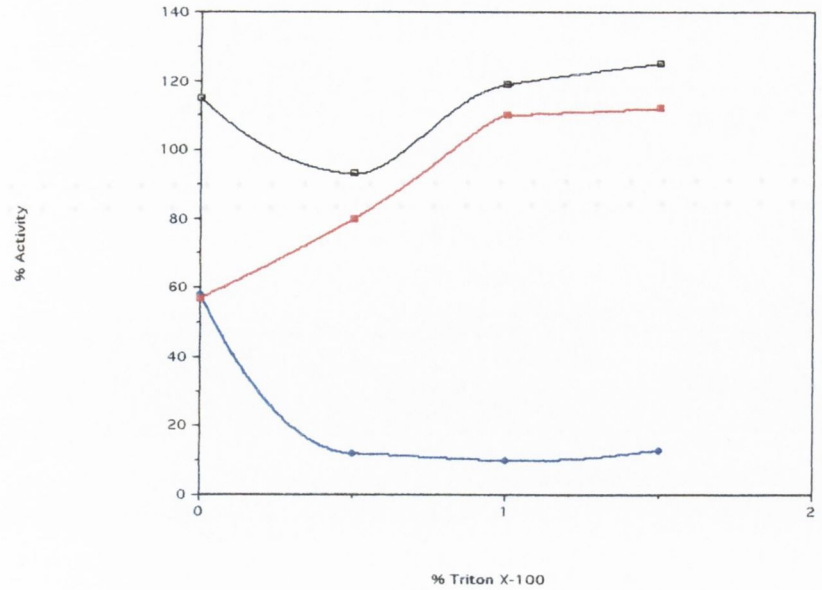


Figure 2.32. The effect of increasing Triton X-100 concentration on protein concentration in the cytosol and pellet as described in Section 2.2.19. Values shown are the mean ± S.E.M. from 2 experiments performed in triplicate. The black curve represents the total protein concentration, the blue curve the percentage microsomal protein and the red curve the percentage cytosolic protein at different Triton X-100 concentrations.



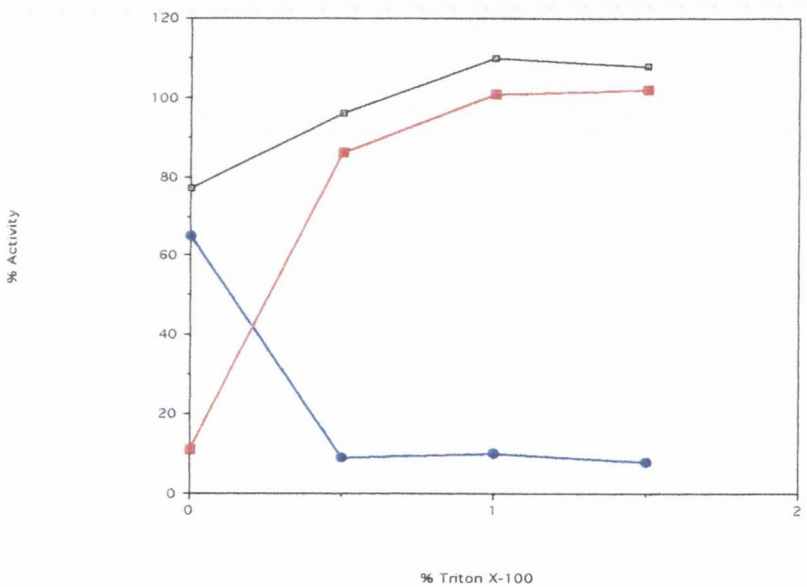


Figure 2.33 (top) & 2.34 (bottom). The effect of increasing Triton X-100 concentrations on the location of porcine dental pulp SSAO activity as measured radiochemically through the oxidative deamination of by the deamination of benzylamine (Figure 2.31) and 5-HT (Figure 2.32) as described in Section 2.2.19. Values shown are the mean ± S.E.M. from 2 experiments performed in triplicate. The black curve represents the total activity of the sample, the blue curve the percentage microsomal activity and the red curve the percentage cytosolic activity at different Triton X-100 concentrations.

2.3.12 Mixed substrate analysis

Substrates	Ratio of $v_T / (v_A + v_B)$
Benzylamine + 5-HT	1.00 ± 0.06
Benzylamine + PEA	0.8 ± 0.0005
PEA + 5-HT	0.681 ± 0.004

Table 2.11. Mixed substrate analysis results. The oxidative deamination of the substrates benzylamine, 5-HT and PEA (at the respective K_m concentrations), alone and in combination in porcine dental pulp microsomal SSAO was determined radiochemically as described in Section 2.2.20

v_I = product formation from a combination of substrates A and B

v_A, v_B = product formation from substrates A and B alone

The results are the means $\pm$ S.E.M. of 2 experiments, each completed in triplicate.

The results indicate that the same enzyme or active site is involved in PEA and 5-HT metabolism and that the two substrates compete for the same active site. However, there are different enzymes or active sits involved in benzylamine and 5-HT oxidation. The relationship between benzylamine and PEA is not simple. It may be that PEA is a substrate for both enzymes /active sites and that it competes with benzylamine for one site and that PEA alone is deaminated at the second site that does not metabolise benzylamine.

2.3.13 Substrate competition experiments

Substrates	% of Control Activity Remaining
5-HT + PEA*	18 % ± 1.5
5-HT + benzylamine*	33 % ± 2.7

Table 2.12. Substrate competition of the oxidative deamination of radiolabelled PEA or benzylamine in porcine dental pulp microsomes determined radiochemically as described in Section 2.2.21 in the presence of unlabelled substrate at the V_{max} concentration. * denotes radioactive substrate.
The values shown are means ± S.E.M. of an experiment performed in triplicate.

The results are not conclusive in demonstrating the relationship between the oxidative deamination of the three substrates tested. There was however a marked decrease in PEA deamination when inhibited by 5-HT at the V_{max} concentration, than that seen with benzylamine. This may suggest that there is more of a competitive relationship between PEA and 5-HT than between benzylamine and 5-HT. However, the V_{max} amine concentration used were of a magnitude that substrate inhibition may have impeded binding of the radiolabelled amine.

2.3.14 Loss of porcine microsomal SSAO activity at 60 and 70 °C

The results demonstrate that oxidative deamination of benzylamine and 5-HT by porcine dental pulp microsomal SSAO respond in a different manner at increased temperatures (Figure 2.35). At 60 °C the SSAO activity towards benzylamine is slightly raised but is almost completely eliminated at 70 °C when assayed at that temperature. In contrast, the SSAO activity towards 5-HT increases markedly at 60 °C and remained constant over the period observed. The SSAO activity towards 5-HT at 70 °C remained constant up to 45 min before the activity decreases markedly at that time when assayed at that temperature.

This difference in temperature stability may suggest that there are two forms of SSAO present in the porcine dental pulp microsomes. One activity, towards benzylamine is denatured rapidly at 70 °C, where the activity towards 5-HT is more resistant.

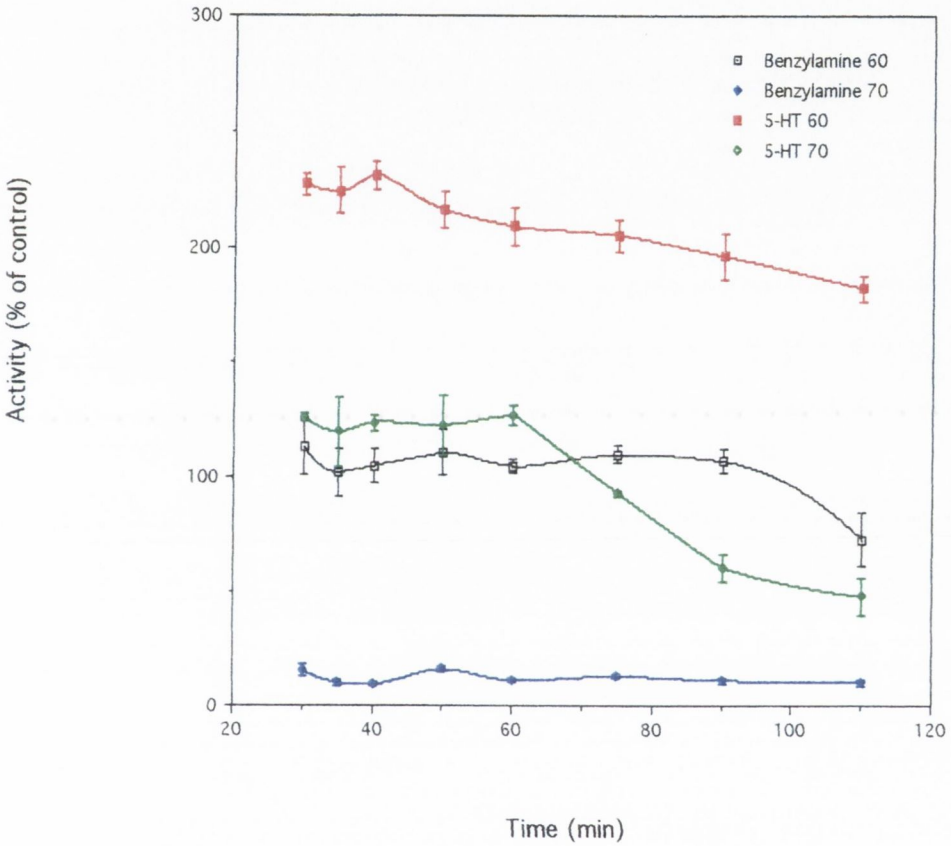


Figure 2.35. The percentage change in porcine dental pulp microsomal SSAO at 60 °C and 70 °C as determined radiochemically by the oxidative deamination of benzylamine and 5-HT and assayed at 60 °C and 70 °C respectively as described in Section 2.2.23. The values shown are means ± S.E.M. of an experiment performed in triplicate.

2.3.15 Inhibition of porcine dental pulp microsomal SSAO deamination of radiolabelled substrates by competition

The inhibition of oxidative deamination of radiolabelled substrates is presented in Figures 2.36 & 2.37. From these curves the K_i values were determined as described in Section 2.2.20. and appropriate inhibitor concentrations determined for inhibiting the oxidative deamination activity of the substrates (Endrenyi & Kwong, 1972).

The inhibition of oxidative deamination of PEA by 5-HT demonstrates competition for the one active site (Figure 2.38). The inhibition of oxidative deamination of PEA by benzylamine is also competitive (Figure 2.39), but the interaction between benzylamine and 5-HT is complex (Figures 2.40 & 2.41).

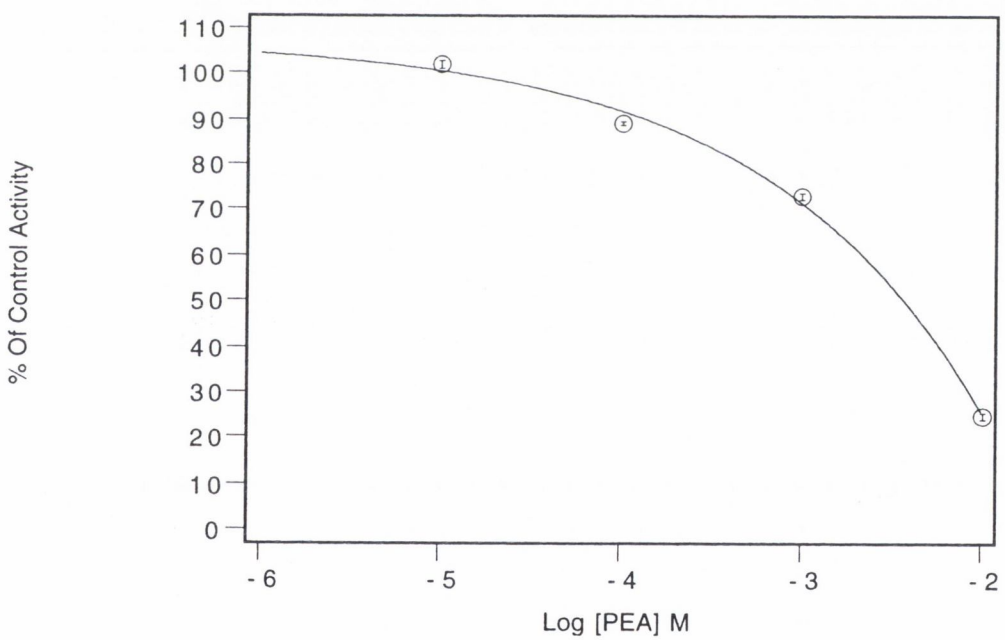


Figure 2.36. Inhibition of oxidative deamination of 5-HT by porcine dental pulp microsomal SSAO by PEA as determined by the radiochemical assay described in Section 2.2.22. Each point is the mean ± range of experiments completed in duplicate.

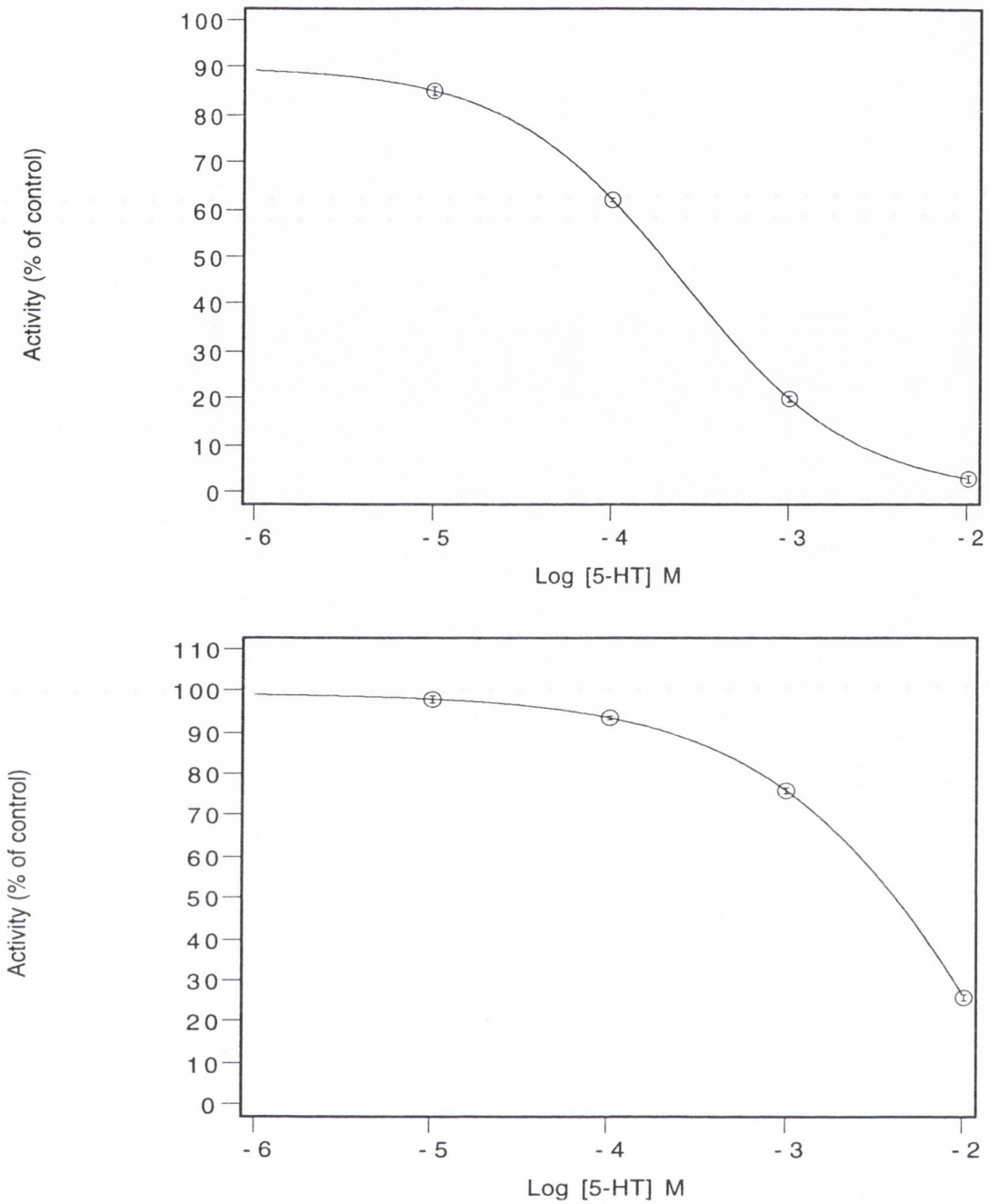


Figure 2.37. Inhibition of oxidative deamination of (a) benzylamine and (b) PEA by porcine dental pulp microsomal SSAO by 5-HT as determined by the radiochemical assay described in Section 2.2.22. Each point is the mean ± range of experiments completed in triplicate.

(a)

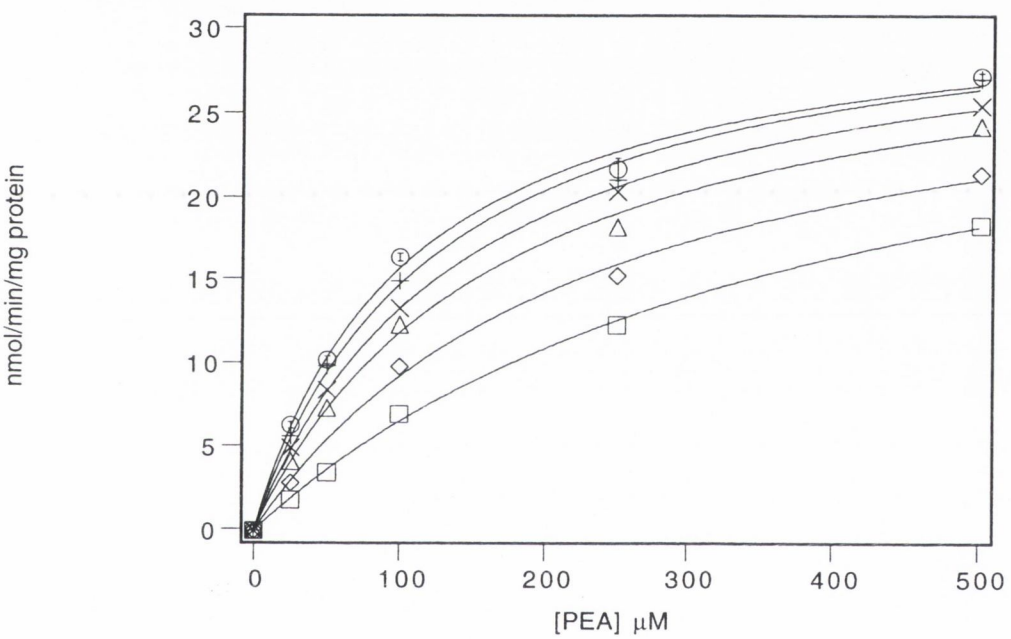
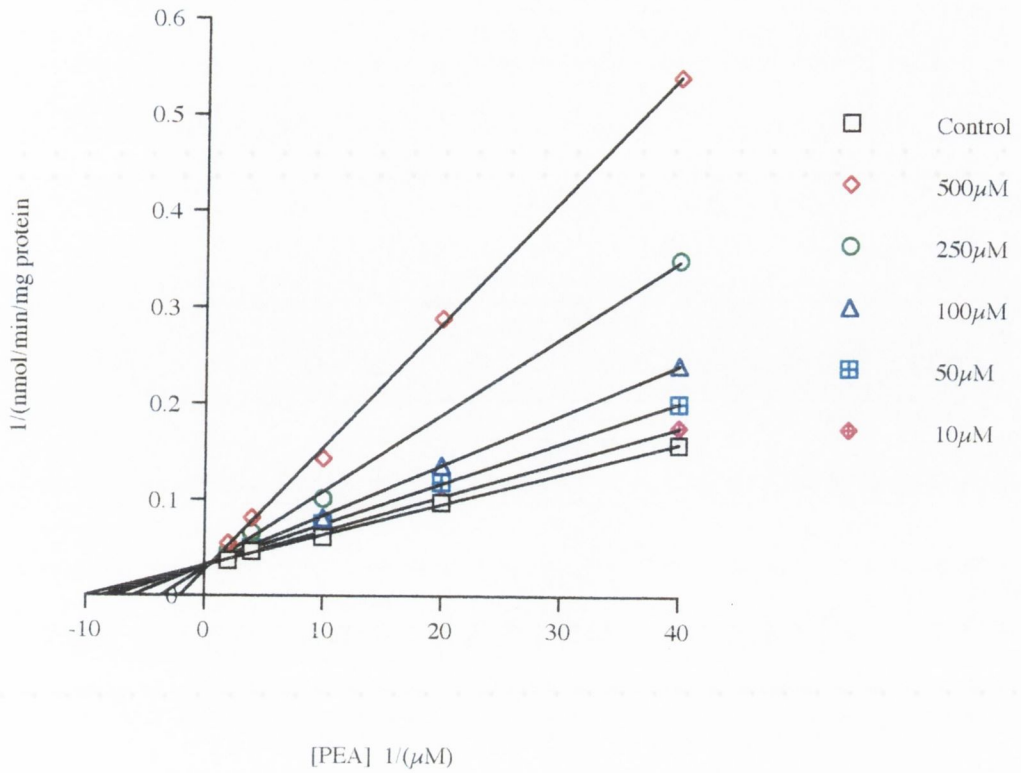


Figure 2.38. Inhibition of porcine dental pulp SSAO oxidative deamination of PEA by 5-HT, as determined radiochemically, as described in Section 2.2.22. The data is presented as (a) Michaelis-Menten plots in the presence of (O) 0 μM, (+) 10 μM, (X) 50 μM, (△) 100 μM, (◊) 250 μM and (□) 500 μM 5-HT, (b) Lineweaver-Burk plots to determine the apparent values of K_m/V_{max} and (c) as plots of K_m/V_{max} against concentration of 5-HT.

(b)



(c)

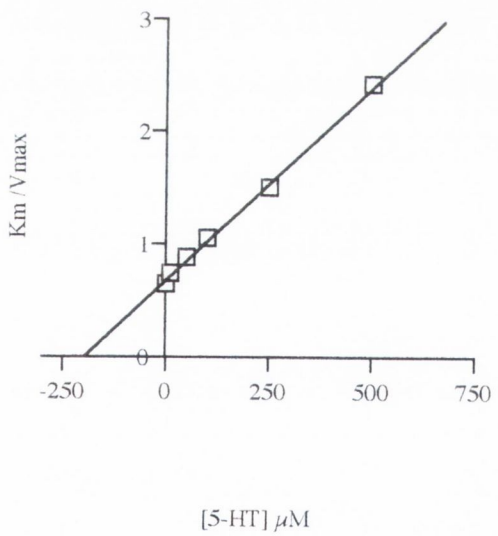


Figure 2.38 (Continued). Inhibition of porcine dental pulp SSAO oxidative deamination of PEA by 5-HT, determined radiochemically, as described in Section 2.2.22. Error bars are not included for clarity in plots (b) & (c).

(a)

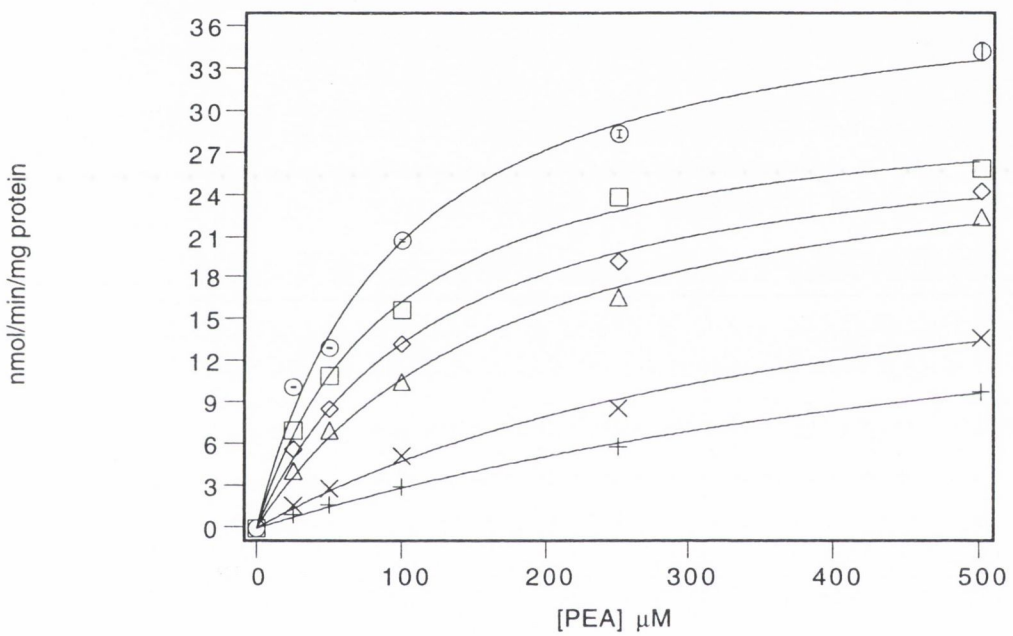
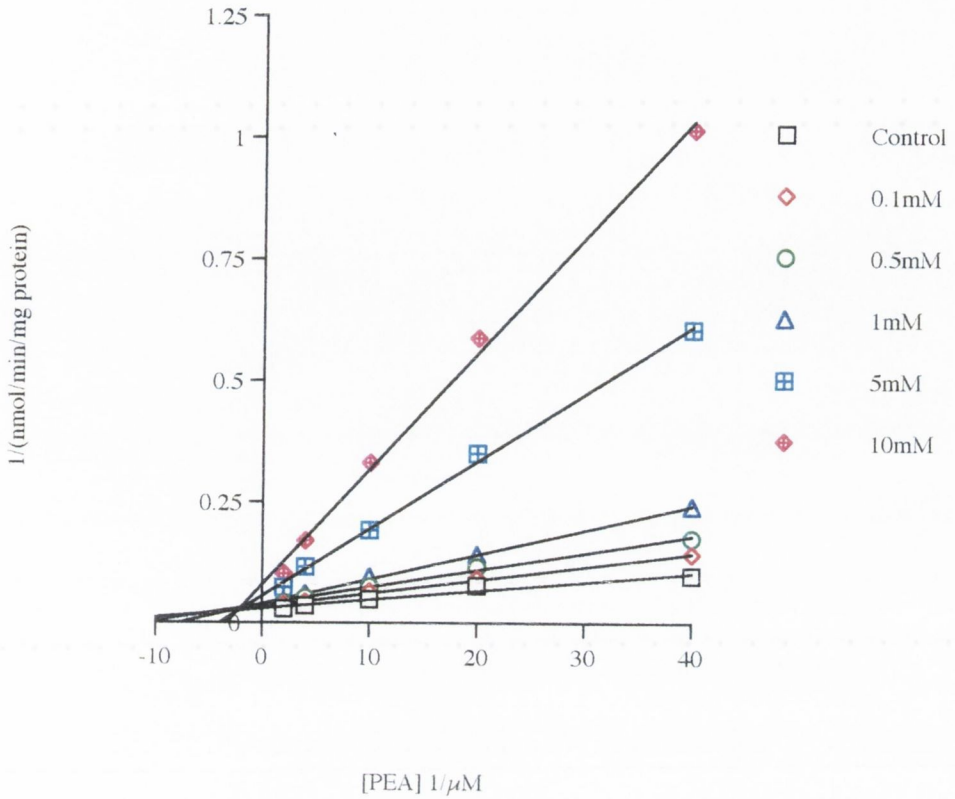
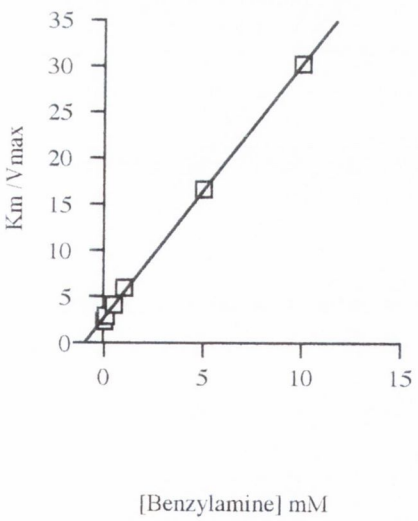


Figure 2.39. Inhibition of porcine dental pulp SSAO oxidative deamination of PEA by benzylamine, as determined radiochemically, as described in Section 2.2.22. The data is presented as (a) Michaelis-Menten plots in the presence of (O) 0 μM, (□) 0.1 mM, (◊) 0.5 mM, (△) 1 mM, (X) 5 mM and (+) 10 mM benzylamine (b) Lineweaver-Burk plots to determine the apparent values of K_m/V_{max} and (c) as plots of K_m/V_{max} against concentration of benzylamine. Error bars are not included for clarity in plots (b) & (c).

(b)



(c)



(d)

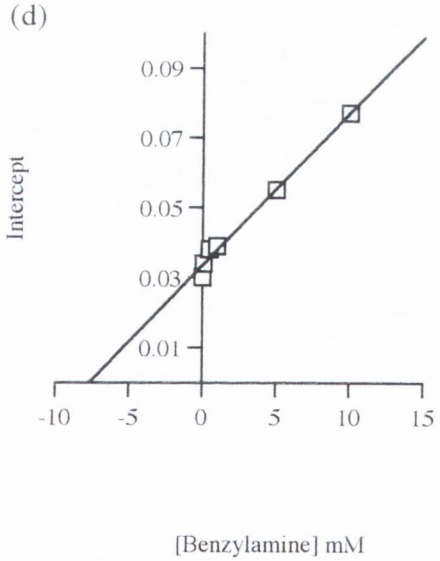


Figure 2.39 (Continued). Inhibition of porcine dental pulp SSAO oxidative deamination of PEA by benzylamine, determined radiochemically, as described in Section 2.2.22.

(a)

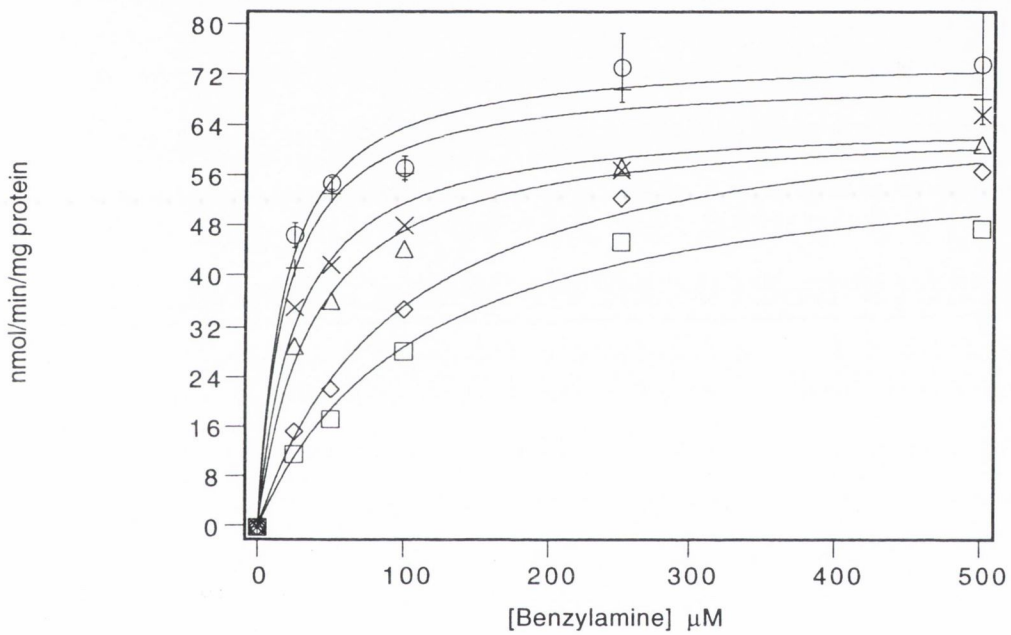
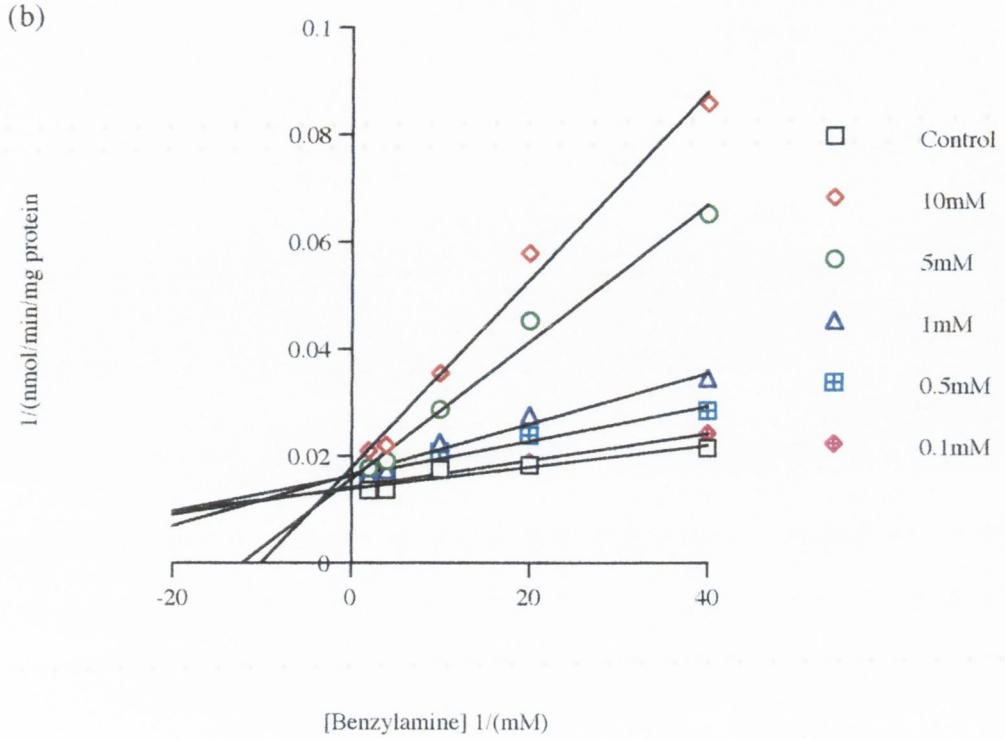


Figure 2.40. Inhibition of porcine dental pulp SSAO oxidative deamination of benzylamine by 5-HT as determined radiochemically as described in Section 2.2.22. The data is presented as (a) Michaelis-Menten plots in the presence of (O) 0 µM, (+) 0.1 mM, (X) 0.5 mM, (△) 1 mM, (◊) 5 mM and (□) 10 mM 5-HT, (b) Lineweaver-Burk plots to determine the apparent values of K_m/V_{max} and (c) as plots of K_m/V_{max} against concentration of 5-HT. Error bars are not included for clarity in plots (b) & (c).

(b)



(c)

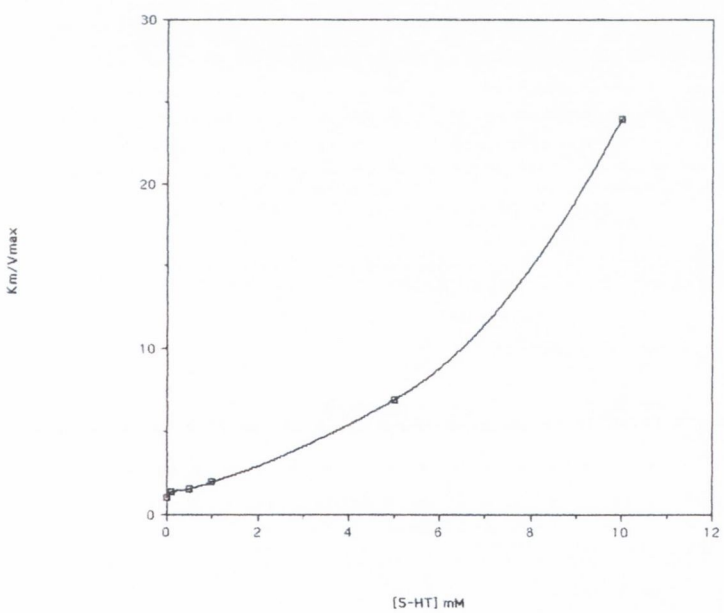


Figure 2.40 (Continued). Inhibition of porcine dental pulp SSAO oxidative deamination of benzylamine by 5-HT, determined radiochemically, as described in Section 2.2.22.

(a)

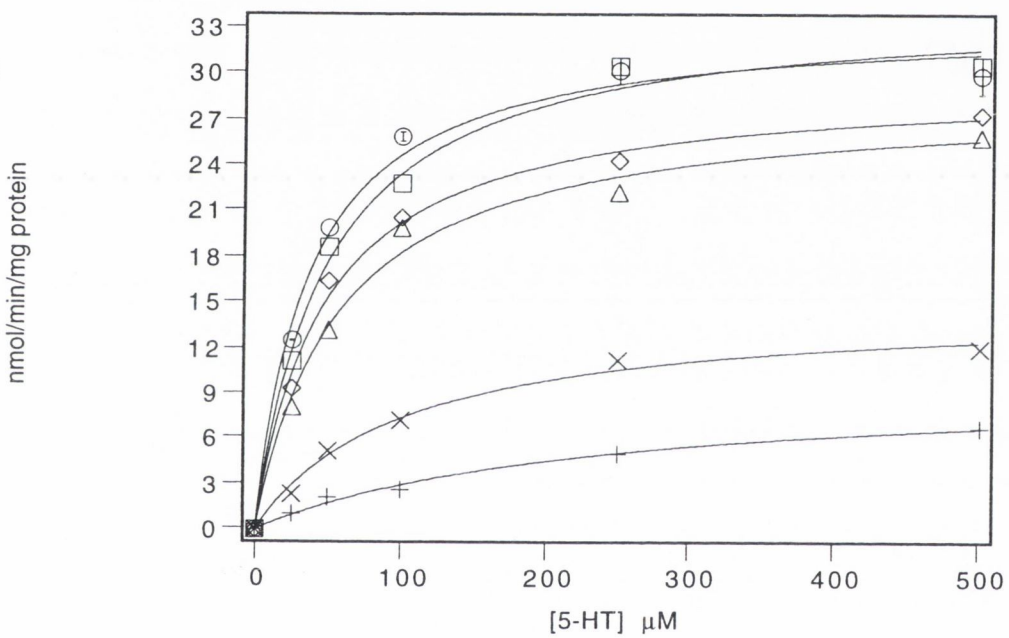


Figure 2.41. Inhibition of porcine dental pulp SSAO oxidative deamination of 5-HT by benzylamine, as determined radiochemically, as described in Section 2.2.22. The data is presented as (a) Michaelis-Menten plots in the presence of (O) 0 μM, (+) 0.1 mM, (X) 0.5 mM, (△) 1 mM, (◊) 5 mM and (□) 10 mM 5-HT, (b) Lineweaver-Burk plots to determine the apparent values of K_m/V_{max} and (c) as plots of K_m/V_{max} against concentration of 5-HT. Error bars are not included for clarity in plots (b) & (c).

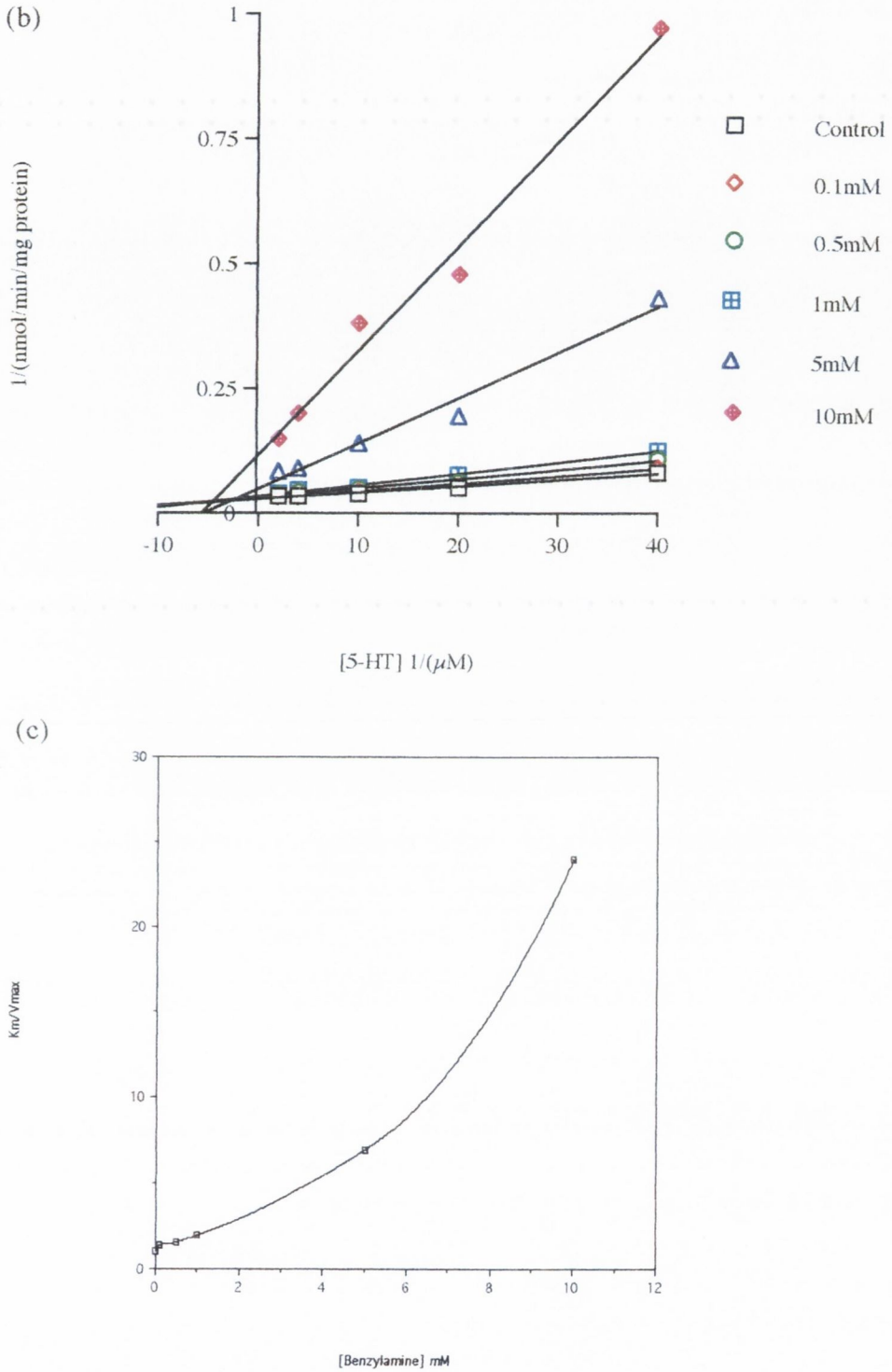


Figure 2.41 (Continued). Inhibition of porcine dental pulp SSAO oxidative deamination of 5-HT by benzylamine, determined radiochemically, as described in Section 2.2.22.

Assay substrate	Competing substrate	Inhibition	K_m/V_{max} versus [I]	K_i^{slope} (mM)	$K_i^{ntercept}$ (mM)
PEA	5-HT	Competitive	Linear	0.2	n.d.
PEA	Benzylamine	Mixed	Linear	1.0	7.5
Benzylamine	5-HT	Mixed	Parabolic	n.d.	n.d.
5-HT	Benzalymine	Mixed	Parabolic	n.d.	n.d.
*5-HT	Benzalymine	Mixed	Parabolic	n.d.	n.d.

*Heat-treated enzyme and assayed at 37 °C

n.d. = not determined

Table 2.13 Inhibition of the porcine-SSAO catalysed oxidation of radioactively labelled substrates by unlabelled substrates.

2.3.16 Inhibition of porcine microsomal SSAO deamination of 5-HT by benzylamine after heat treatment

Heat treatment at 70 °C for 15 min selectively removed the majority of benzylamine deaminating activity (See Section 2.2.23) in porcine dental pulp microsomes. Inhibition of 5-HT deamination by benzylamine demonstrated the same complex interaction as observed prior to heat treatment. This suggested that the enzyme that deaminates 5-HT was not the same as that responsible for the majority of benzylamine deamination (Figure 2.44). Inhibition of 5-HT deamination by benzylamine after heat treatment (Figure 2.42) showed a marked difference to the inhibition seen with the native SSAO (Figure 2.37). Benzylamine inhibited the 5-HT deamination to a far lesser extent after heat treatment. The inhibition of benzylamine metabolism by 5-HT after heat treatment showed a K_i of 6.8 ± 0.7 mM (Figure (2.42).

2.3.17 Michaelis-Menten kinetics for heat-treated porcine microsomal SSAO

The kinetic constants for benzylamine and 5-HT after heat treatment at 70 °C for 15 min and assayed at 37 °C are presented in Table 2.14. Comparison between the heat-treated and native SSAO activity when benzylamine was used as a substrate showed a similar K_m but a 23 % decrease in V_{max}. On the other hand the activity when 5-HT was used as a substrate showed almost no difference in K_m or V_{max}.

The SSAO catalyzed deamination of benzylamine, after heat treatment at 70 °C for 15 min and assayed at 37 °C, contrasted with the results shown for activity when assayed at 70 °C (Figure 2.35) where in the region of 90 % of all deaminating activity was removed for this substrate. This result suggested that a large percentage of SSAO activity towards benzylamine was recovered when the microsomes were cooled from 70 °C to the assay temperature at 37 °C.

Substrate	**5-HT**	**Benzylamine**
K_m (μM)	38.6 ± 2.0	18.6 ± 1.2
V_{max} (nmol/min/mg protein)	33.5 ± 0.4	72.2 ± 4.2
K_m Heat Treated (μM) assayed at 37 °C	35.6 + 5	40.5 + 9
V_{max} Heat Treated (nmol/min/mg protein) assayed at 37 °C	33.4 + 1.2	55.8 + 3.0

Table 2.14. The kinetic constants for porcine dental pulp microsomes both in the native form and after heat treatment at 70 °C for 15 min and assayed at 37 °C (see Figure 2.43).

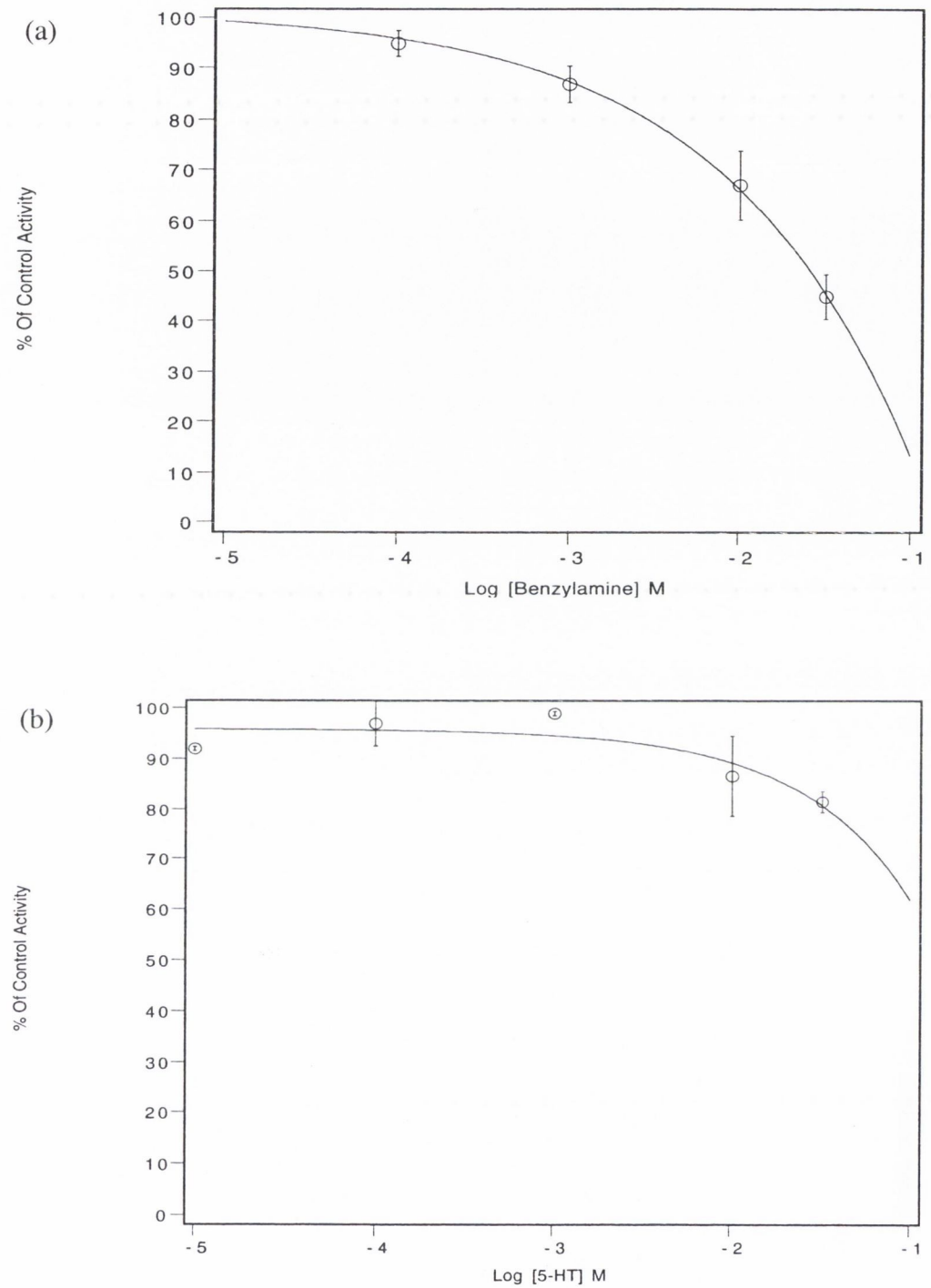


Figure 2.42. Inhibition of oxidative deamination of (a) benzylamine by porcine dental pulp microsomal SSAO by 5-HT assayed at 70 °C (b) 5-HT by porcine dental pulp microsomal SSAO heat-treated at 70 °C for 15 min by benzylamine assayed at 70 °C as determined by the radiochemical assay described in Sections 2.2.24. Each point is the mean ± S.E.M. of experiments completed in triplicate.

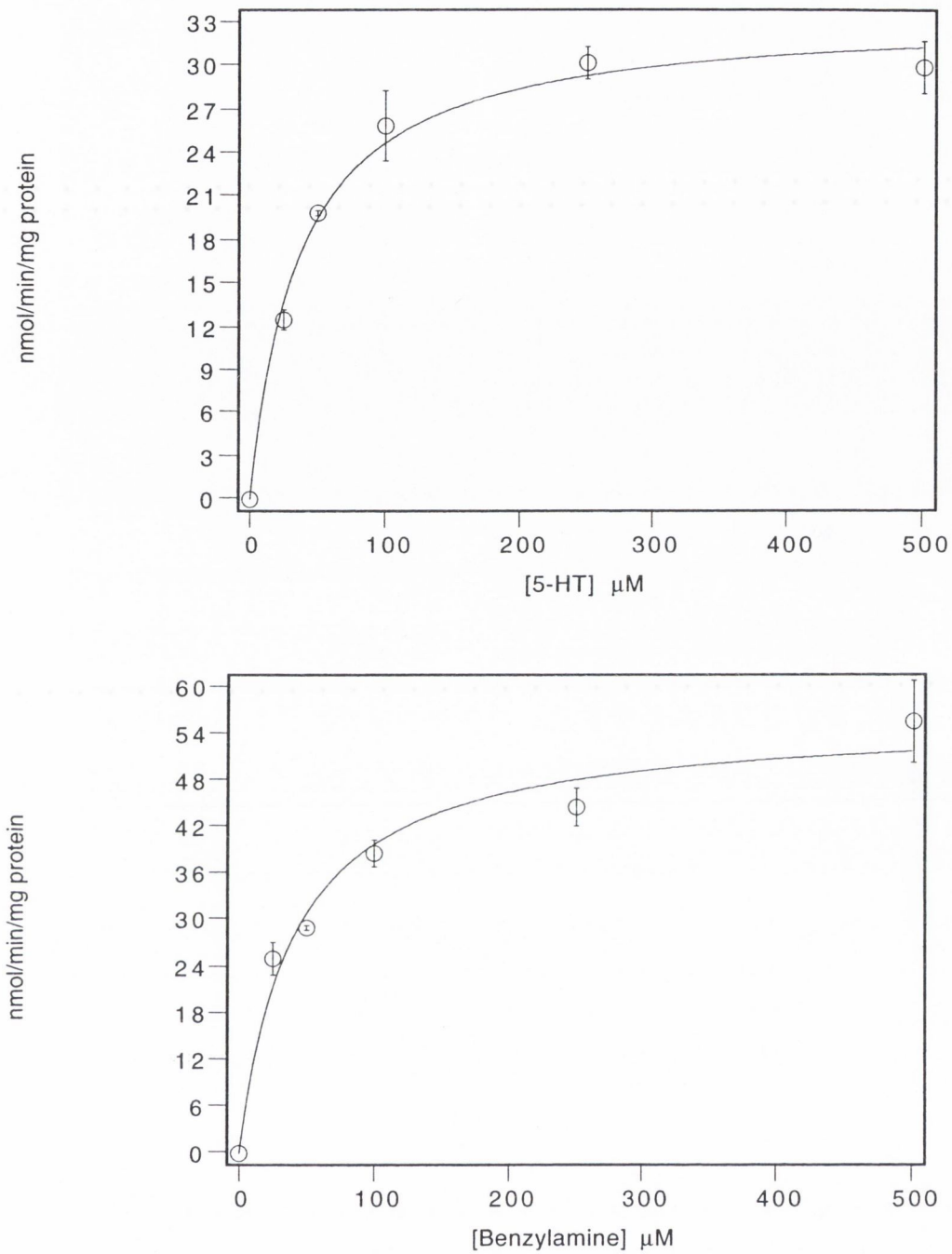


Figure 2.43. Michaelis-Menten plot for the oxidative deamination of (a) 5-HT and (b) benzylamine by porcine dental pulp microsomal SSAO heat-treated at 70 °C for 15 min. The oxidative deamination was determined by radiochemical assay at 37 °C as described in Section 2.2.26 over the range of substrate concentrations indicated. The points shown are mean values ± S.E.M. of three separate experiments.

(a)

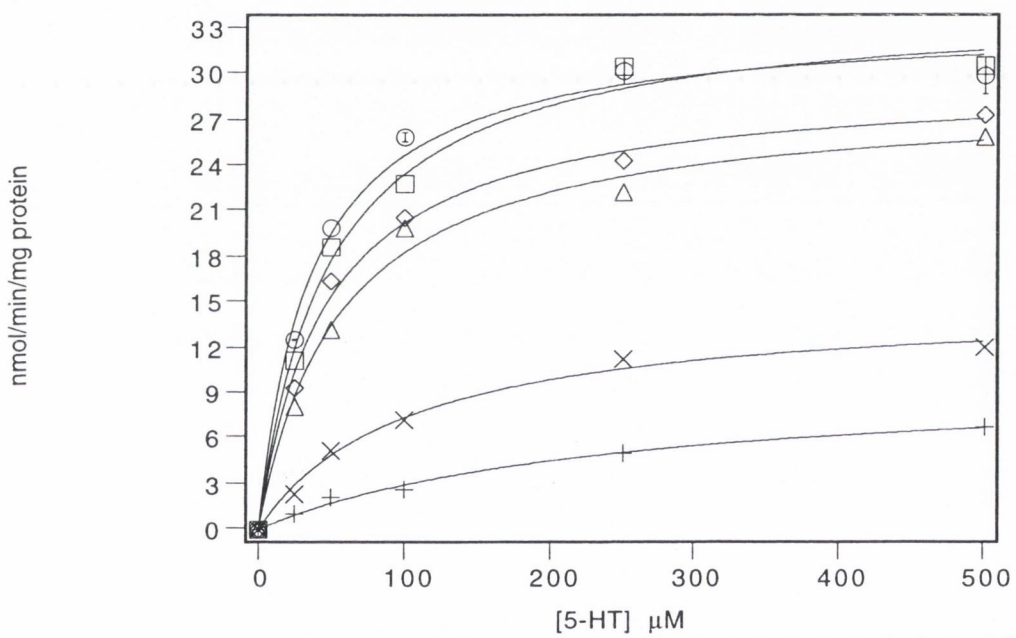


Figure 2.44. Inhibition of heat-treated (70 °C for 15 min) porcine dental pulp SSAO oxidative deamination of 5-HT by benzylamine as determined radiochemically at 37 °C as described in Section 2.2.25. The data is presented as (a) Michaelis-Menten plots in the presence of (O) 0 μM, (+) 10 μM, (X) 50 μM, (△) 100 μM, (◊) 250 μM and (□) 500 μM 5-HT, (b) Lineweaver-Burk plots to determine the apparent values of K_m/V_{max} and (c) as plots of K_m/V_{max} against concentration of 5-HT. Error bars are not included for clarity in plots (b) & (c).

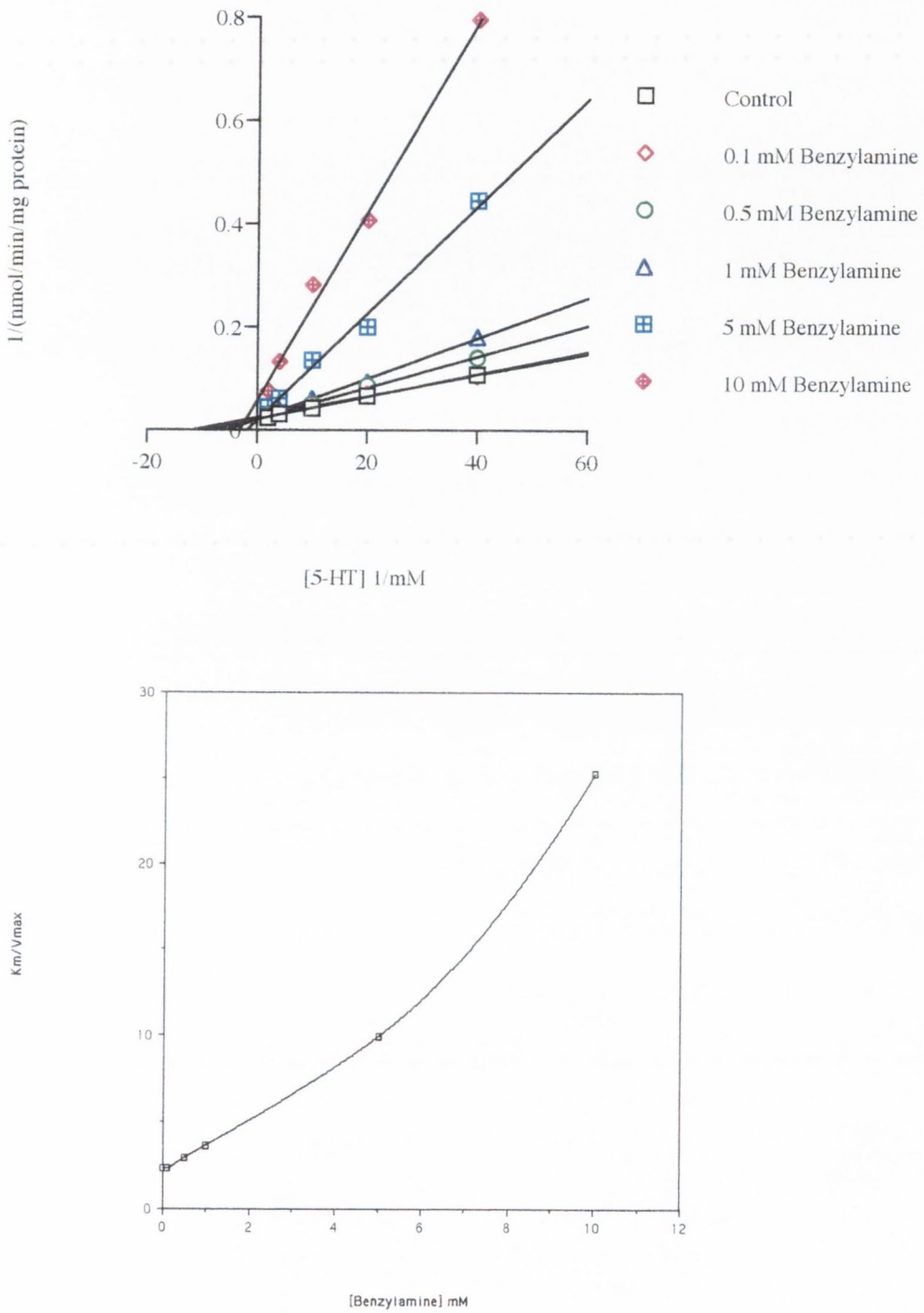


Figure 2.44 (Continued). Inhibition of heat-treated porcine dental pulp SSAO oxidative deamination of 5-HT by benzylamine as determined radiochemically at 37 °C as described in Section 2.2.25.

2.3.18 Oxidative deamination of 3-methylhistamine by porcine dental pulp microsomal SSAO

The activity observed was low, but SSAO was found to be responsible for all metabolism of 3-methylhistamine (Figure 2.45). The K_m value for SSAO metabolism was 6.43 ± 1.2 mM and the V_{max} 119 ± 10 nmol/hour/mg protein (Figure 2.46). There is a time lag (τ) evident in the SSAO activity before activity is observed. This may suggest that clorgyline (10^{-3}M) may be acting as a competitive inhibitor of peroxidase activity, as described previously for clorgyline and beef plasma amine oxidase (Houslay & Tipton, 1975).

SSAO activity may therefore be responsible for all histamine deamination in porcine dental pulp microsomes, deaminating histamine to imidazole acetaldehyde (K_i of 1054 + 30 μM in substrate competition with 50 μM benzylamine and a K_c of 2950 ± 400 μM in substrate competition with 100 μM 5-HT) in one pathway and *tele*-methylhistamine to *tele*-methylimidazoleacetaldehyde in the other.

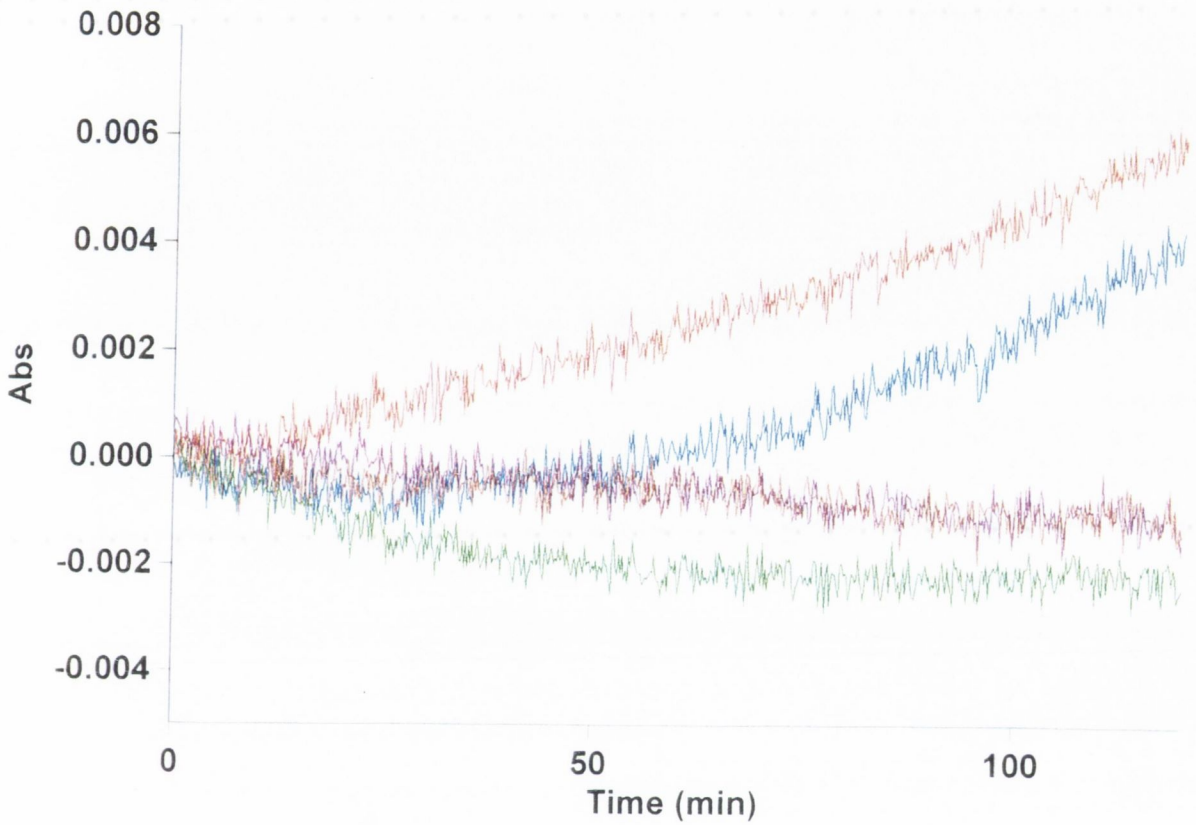


Figure 2.45. The kinetic plot for 3-methylhistamine (5 mM final concentration) metabolism by porcine dental pulp amine oxidases. The activity was determined by coupled spectrophotometric assay using 4-aminoantipyrine as a proton donor in the peroxidase reaction with hydrogen peroxide produced as a product of the deamination of 3-methylhistamine. The oxidised 4-aminoantipyrine condensed with vanillic acid to produce a quinoneimine dye that was be followed at 498 nm as described in Section 2.2.27 (Holt *et al.*, 1997). The rates are: control activity (red), SSAO activity (blue), MAO-B (wine), MAO-B (purple) and a negative control (green).

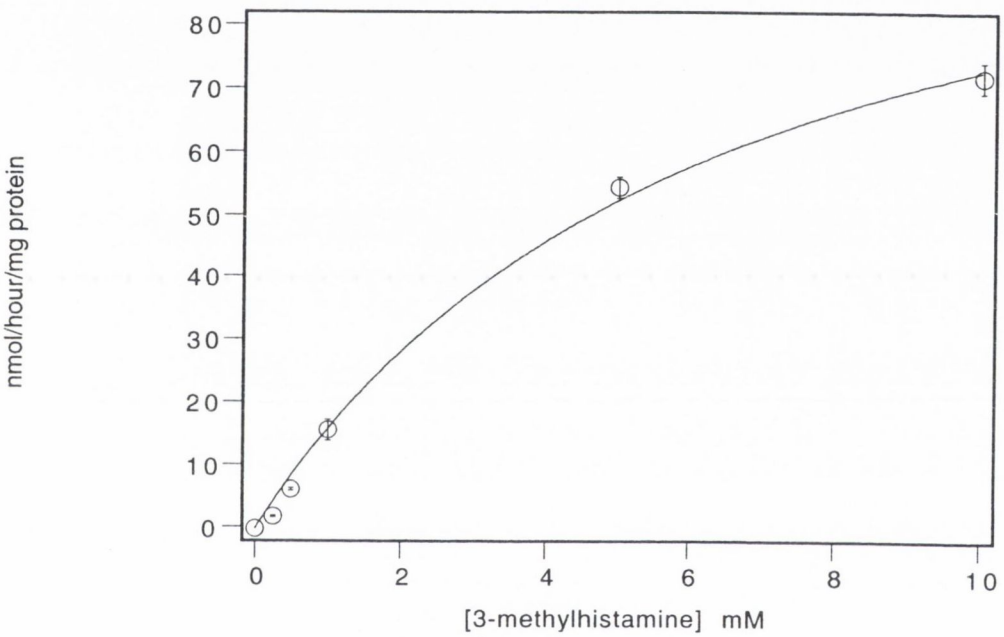


Figure 2.46. Michaelis-Menten plot for 3-methylhistamine oxidative deamination by porcine dental pulp microsomal SSAO. The activity was determined by coupled spectrophotometric assay, over the range of substrate concentrations indicated, using 4-aminoantipyrine as a proton donor in the peroxidase reaction with hydrogen peroxide produced as a product of the deamination of 3-methylhistamine. The oxidised 4-aminoantipyrine condensed with vanillic acid to produce a quinoneimine dye that was be followed at 498 nm as described in Section 2.2.27 (Holt *et al.*, 1997). The points shown are mean values $\pm$ S.E.M. of three separate experiments.

2.4 Discussion

Porcine dental pulp homogenate contains both forms of MAO and also SSAO. Benzylamine is primarily deaminated by SSAO, whereas almost 40-45 % of 5-HT and 65 % of PEA are deaminated by SSAO (when assayed radiochemically with 50 μM benzylamine or 100 μM 5-HT). PEA deamination by SSAO is higher than found in other tissues (Lizcano, 1990). This is in agreement with earlier work with dental pulp (Norqvist, Fowler & Oreland, 1981). The remainder of the 5-HT is principally deaminated by MAO-A (<55 %) and MAO-B (10 %) and PEA by MAO-A (<10 %) and MAO-B (30 %).

In crude human dental pulp homogenates SSAO deaminated 66 % of the benzylamine (when assayed radiochemically with 100 μM benzylamine) with 28 % being metabolised by MAO-B. SSAO was found to metabolise 88 % of the 5-HT (when assayed radiochemically with 100 μM 5-HT), the remainder (10 %) being metabolised by MAO-A.

Benzylamine is a preferred substrate for SSAO as expected (Blaschko, 1974). Only in the porcine dental pulp microsomes had deamination of 5-HT been previously demonstrated (Norqvist, Fowler & Oreland, 1981). This has now been confirmed for human dental pulp. It opens the possibility that SSAO may have a role to play in the control of the inflammatory process. Inhibitor studies with microsomes have also demonstrated that both kynuramine and dopamine may be metabolised by SSAO, neither of which had been reported before. This is based on the inhibition of a radiolabelled substrate by unlabelled kynuramine and dopamine. Kynuramine, like 5-HT and tryptamine, is also derived from tryptophan (Makino & Arai, 1955). Methylamine does not appear to be a substrate.

The spectrophotometric assay for kynuramine deamination was not sensitive enough to detect the reaction. This was also a problem with the hydrogen peroxide detecting assays. Impurities in the microsomal fraction may be responsible for this by interfering with the products of the reaction. However, solubilisation of the microsomal SSAO still did not result in a detectable rate with benzylamine as a

substrate using these procedures. The other obstacle to the reactions was the low activity of the enzyme, in particular with 5-HT where highly radioactive solutions, in the order of 3 Ci/mol, were required to allow detection of measurable amounts of product.

Both semicarbazide and phenelzine have been shown to be potent inhibitors of SSAO activity (Andree & Clarke, 1982) and this is also true for the enzyme from porcine dental pulp microsomes (Norqvist *et al.*, 1982). Phenelzine is a superior inhibitor than semicarbazide with an IC_{50}, being an order of magnitude lower, under the conditions used here. However, phenelzine has been reported to inhibit MAO activity in some tissues (Houslay & Tipton, 1975), so the true SSAO inhibition may not be evident.

Attempted solubilisation of porcine dental pulp microsomal SSAO by sonication, urea and Triton X-100 treatment was previously reported not to be successful (Norqvist, Fowler & Oreland, 1982). The concentration ranges used were 0.1%. 1% Triton X-100 was found to be capable of releasing most of the SSAO activity into the supernatant but further increasing the Triton X-100 concentration did not release further SSAO.

The activation of SSAO deamination of benzylamine has been reported for bovine lung microsomal SSAO (Lizcano, 1994). Uncertainty exists as to the physiological significance for this as benzylamine is not a physiological substrate. Activation in bovine lung SSAO has been demonstrated with both the microsomal fraction and the purified enzyme (Lizcano, 1994). The same researcher found the activation described to be reversible by denaturation in urea followed by dialysis. Other factors may also affect activation such as endogenous inhibitors present in the tissue that may be more thermolabile than the stable SSAO, but this is not the case for the bovine lung enzyme as the pure form also demonstrates activation. Porcine dental pulp microsomes demonstrated a profound activation towards both 5-HT and benzylamine. 5-HT is the first physiological substrate for which activation has been demonstrated. Such a substrate may be deaminated at a much increased rate *in vivo,* maintained at a constant 37 °C, than *in vitro* and therefore SSAO may play a much more important role in the

deamination of 5-HT than MAO. This might suggest that the SSAO becomes "de-activated" during the collection and treatment of the porcine dental pulp. The activation, when observed, persisted for 32 hours at 37 °C for benzylamine deamination, but activity towards 5-HT was progressively lost after about 20 hours. The mechanism and significance of this activation of porcine dental pulp SSAO requires further research. It appears that some other factors may be involved in the "activation" reported here, since the reults were variable. The experiment was repeated on 5 separate occasions, each with a different SSAO preparation (with similar specific activities and protein concentrations), and activation was only observed twice.

A possible kinetic mechanism that might account for substrate-dependant activation is:

$$E + S_1 \underset{k_{1r}}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_2} ES_1^* \xrightarrow{k_3} E + P_1$$

$$E + S_2 \underset{k_{1r}*}{\overset{k_1*}{\rightleftharpoons}} ES_2 \xrightarrow{k_2*} ES_2^* \xrightarrow{k_3*} E + P_2$$

where for S_1 k_2 is the rate limiting step and for S_2 k_3* is the rate limiting step. A constant temperature of 37 °C may cause conformational change that increases k_2 and k_2* without affecting k_3 or k_3*. This activation would be seen with S1 but not S_2. This may explain why in bovine lung SSAO the activation is only seen for benzylamine. In porcine dental pulp both enzymes appeared to demonstrate activation, at least on some occasions. Given the failure of porcine dental pulp SSAO to activate on all runs of the experiment, it is unlikely that temperature alone causes activation in dental pulp. Another, as yet unidentified, factor may alter the kinetic constant k_2 in conjunction with the constant temperature. Assuming the activation is similar to that observed for bovine lung SSAO then conformational change at the active site is responsible for the

changes observed. Mild thiol group oxidation by nitric oxide has been shown to affect the activity of GAPDH (Albina *et al.*, 1999; Ishii *et al.*, 1999). As hydrogen peroxide is a product of the SSAO oxidative deamination of amines it may be capable of oxidising the active site and hence altering enzyme activity. Amine oxidase activity from rat liver has been shown to be altered by sodium nitroprusside and 5,5'-dithiobis(2-nitrobenzoic acid) (Symes & Sourkes, 1975).

Experiments with the NO-donor *S*-nitrosoglutathione (SNOG) showed that SSAO activity towards both benzylamine and 5-HT as substrates decreased. The benzylamine and 5-HT deaminating activity decreased in the region of 50 % and 90 %, respectively, after 1 hour pre-incubation and 50 % and 75 % after 2 hours pre-incubation. The effect of *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) showed that 5-HT oxidising SSAO activity decreased from almost 100% of control activity at 1 hour to a constant 70 % over the next 4 hours, but that benzylamine deaminating activity was constant at 60 % until the 5th hour, when activity increased to 110 % of control activity. These experiment demonstrate that thiol oxidation may not be responsible for SSAO activation, but that the enzymes that deaminate 5-HT and benzylamine show different sensitivities to nitric oxide donors.

Heat treatment of porcine dental pulp SSAO revealed a marked difference between the deamination of 5-HT and benzylamine at 60 °C and 70 °C. At 60 °C, 5-HT deaminating activity was greater than double the control activity at 37 °C. This increase in activity was still evident after 110 minutes. At 70 °C, 5-HT deaminating activity was in the region of 30 % higher than the control activity at 37 °C, but after 60 minutes the activity decreased progressively fall to 50 % of the control activity after 110 minutes. Benzylamine deamination at 60 °C was marginally greater than the control, remaining constant until the activity fell to below that of the control at 110 minutes. However, at 70 °C the benzylamine deaminating activity was less than 15 % of the control for all time points observed. The difference in effects seen with benzylamine and 5-HT may thus indicate two separate enzymes, one active towards 5-HT and relatively thermostable with perhaps a low activity towards benzylamine, and the other more thermolabile and active towards benzylamine. It is noteworthy that

there was a small residual towards benzylamine (about 15 % of the origional) after extended incubation at 70°C. This suggests that the enzyme that is active towards 5-HT also has a relatively low activity towards benzylamine. The possibility that the two activities result from two active sites on the same enzyme with different stabilities cannot be excluded. However, no SSAO enzyme has previously been shown to contain two distinct active sites.

When the porcine dental pulp microsomes were heated to 70 °C for 15 min and SSAO activity towards benzylamine measured at 37 °C, activity was found to return to 77 % of the native enzyme assayed at 37 °C. This contrasted with a 90 % decrease in activity when activity was measured at 70 °C, implying that SSAO activity returned on reduction of temperature. Heat denaturation of most enzymes result in irreversible changes, although a number of heat-stable proteins, including growth hormone and *Taq* polymerase, are known. The present results suggest that reversible denaturation may be involved with SSAO. Alternatively, heat-treatment of porcine dental pulp microsomes induced changes in the membrane that blocked SSAO activity towards benzylamine without denaturing the enzyme. Therefore the loss in activity seen may have been as a result of membrane changes and not SSAO activity alterations. In contrast the 5-HT deaminating activity of SSAO did not appear to be altered by the heat-treatment.

The results of the mixed-substrate analysis experiments also suggest the involvement of more than one enzyme, as was first suggested by Norqvist and co-workers (1982). One appears to be capable of deaminating benzylamine and another 5-HT and PEA. The relationship between 5-HT and PEA is competitive. There was no significant competition between benzylamine and 5-HT agreeing with the idea that there are two potential sites for deamination. The relationship between PEA and benzylamine is not as clear. This may result from PEA being a substrate for both the enzymes that deaminates benzylamine and that oxidising 5-HT.

The inhibition of the oxidative deamination of [^{14}C] labelled substrates by unlabelled substrates at the V_{max} concentration was not conclusive. This may have been due to the

fact that the V_{max} value for 5-HT was two orders of magnitude greater than the radiolabelled substrate and could have blocked the movement of radiolabelled PEA and benzylamine. A similar trend to the mixed substrate competition experiments was evident in that the amount of radiolabelled PEA product was in the region of 2 / 3 of that produced by benzylamine, suggesting that PEA may be competing with 5-HT, whereas benzylamine was not.

The inhibition of the oxidative deamination of [^{14}C] labelled substrates by unlabelled substrates indicated competition between PEA and benzylamine for oxidation at the one active site. However, the inhibition of benzylamine oxidation by 5-HT and the inhibition of 5-HT oxidation by benzylamine were complex and not as simple as suggested by mixed-substrate analysis. Inhibition at 37 °C did not appear simply competitive and plots of the apparent K_m/V_{max} against the concentrations of unlabelled inhibiting substrates were positive parabolic. Parabolic inhibition can arise from there being two binding sites on the same enzyme form for an inhibitor. The activity remaining towards benzylamine following heat treatment was too low to permit similar studies. However 5-HT deamination was inhibited in a mixed type fashion by benzylamine and again the dependence on the apparent K_m/V_{max} values on the benzylamine was complex, suggesting more than one binding site for the inhibitory substrate. Inhibition of the heat-treated enzyme by benzylamine might be expected in view of the residual activity towards benzylamine in that preparation. However, the inhibitory results indicate a hitherto unknown, complexity in the kinetic behaviour of the activities towards both benzylamine and 5-HT that require further study. PEA and 5-HT also demonstrate competition for a single active site in agreement with the mixed substrate experiments.

Histamine, when measured indirectly by substrate competition with 5-HT and benzylamine has a K_m value in the region of 1-2 mM. The other catabolic pathway of histamine breakdown involves histamine-N-methyltransferase (HMT) followed by further breakdown by either MAO-B or SSAO (Green *et al.*, 1987). 3-Methylhistamine, the product of HMT breakdown is almost exclusively deaminated by SSAO in porcine dental pulp microsome. The time-lag evident in the presence of

clorgyline (10^{-3} M) may suggest that clorgyline inhibits peroxidase activity. The mechanism may be the following:

$$S \xrightarrow{E_1} P_1 \xrightarrow{E_2} P_2$$

where the time taken for P_1 to reach steady-state can be represented by τ since an increase in the K_m for that enzyme would increase τ as $\tau \propto V_{max}/ K_m$ for enzyme 2 (Tipton, 1992). Thus if clorgyline competitively inhibits peroxidase activity or decreases the V_{max} of that enzyme it will increase the time lag (τ). In the present case the inhibition appears to be competitive as the V_{max} was the same as V_{max} of the control experiment.

2.5 Conclusions

From these experiments the following conclusions may be drawn:

1. Benzylamine, 5-HT, PEA are substrates for porcine dental pulp microsomal SSAO.
2. Kynuramine and dopamine may be substrates for porcine dental pulp microsomal SSAO as demonstrated by the mixed substrate experiments, although no activity could be directed towards them.
3. Benzylamine and PEA are substrates for porcine dental pulp cytosolic SSAO.
4. Benzylamine and 5-HT are substrates for human dental pulp SSAO.
5. Semicarbazide and phenelzine are effective inhibitors of porcine dental pulp microsomal SSAO, the latter being more efficient.
6. Porcine dental pulp microsomal SSAO exhibits activation of deamination of both benzylamine and 5-HT when pre-incubated at 37 °C for greater than 90 min and that this activation continues for up to 32 hours. This activation is not predictable and may involve other factors not yet identified.
7. Porcine dental pulp microsomal SSAO deamination of 5-HT and benzylamine at 60 and 70 °C demonstrates marked differences between their thermostability. The majority of the benzylamine deaminating activity is selectively lost by incubating

the enzyme at 70 °C for 15 minutes, although it is regained on returning to a temperature of 37 °C.

8. There appears to be two separate enzyme activities that are sensitive to semicarbazide and phenelzine in porcine dental pulp microsomes. It may also be the case that one SSAO enzyme in porcine dental pulp possesses two active sites, although this has not been demonstrated for any other SSAO enzyme.
9. 5-HT and the majority of benzylamine oxidative deamination occur independently, whereas 5-HT and PEA compete for the one active site.
10. Solubilisation of porcine dental pulp microsomal SSAO activity is possible with 1 % Triton X-100 for both 5-HT and benzylamine.
11. SSAO is the major enzyme involved in the breakdown of 3-methylhistamine in porcine dental pulp microsomes.

CHAPTER THREE

Partial Purification of Porcine Membrane-Bound SSAO

3.1 Introduction

The mixed substrate experiments described in Chapter Two indicated that there were two distinct membrane-bound enzyme activities in porcine dental pulp. One of these is capable of metabolising benzylamine and the other 5-HT and PEA. This infers either that there are two distinct semicarbazide-sensitive amine oxidases in the membrane or that the enzyme contains two active sites. Partial purification of the SSAO activity by utilising both gel permeation chromatography and ion exchange separation was completed in order to investigate this further. The former separates out molecules of different sizes. It is based on the principal that the molecules are partitioned between the carrying solvent and a stationary porous matrix of defined porosity. Molecules larger than this pore size cannot enter the matrix and so pass through the column rapidly. Smaller molecules pass into the matrix and hence take longer to pass through the column. Therefore all molecules are eluted in order of their decreasing size.

Ion exchange separation is based on the principal that proteins carry both positively and negatively charged groups. When proteins are passed through a charged column some will be adsorbed on the basis of their charge and will be retained in the column allowing later elution with buffer of different pH or higher ionic strength.

3.2 Methods

3.2.1 Preparation of samples

Ten ml of porcine dental pulp microsomes was isolated, as discussed in Section 2.2.3. The enzyme was then solubilised in a final concentration of 1% Triton X-100, as described in Section 2.2.19. Five ml were used for the ion exchange and the remainder of the sample was then concentrated back to 1 ml volume by centrifugal ultrafiltration, using an Amicon semi-permeable membrane with a nominal molecular weight cut-off of 10,000 kDa (Amicon, Stonehouse, Gloucester, UK), at 5000 *g* for 50 min. It was then used in the gel permeation chromatography.

3.2.2 Superdex 200 gel permeation chromatography

3.2.2.1 Gel-filtration under non-denaturing conditions

The Superdex 200 column was cleaned with 0.2 M sodium hydroxide and flushed with 20 mM sodium phosphate buffer, pH 7.2 containing 1 % Triton X-100, prior to use. The FPLC system employed was the Pharmacia-LKB FPLC (Pharmacia House, Milton Keynes, UK) which was run at 0.3 ml/min. The eluent was 20 mM sodium phosphate buffer, pH 7.2 containing 1% Triton X-100, and 1 ml of sample (10 mg/ml) was loaded by way of a three way valve.

Samples from different runs were collected as 1.0, 0.5 and 0.3 ml fractions and assayed using the radiochemical method for activity towards both benzylamine and 5-HT. K_m values were determined and in areas of high activity.

Western Blot analysis was performed in the areas of high enzyme activity, as described in Section 4.3.5.2. The high molecular weight areas that demonstrated activity were re-run through the column to determine if they could be resolved into more than one enzyme, or if the enzyme existed in different forms, *e.g.* as a dimer.

3.2.2.2 Gel-filtration in the presence of urea

Urea is a denaturing agent capable of minimising the effect of tertiary and quaternary protein interactions by transforming proteins to random coil configurations. This allows separation from other interacting molecules and a more accurate determination of molecular weight of all the proteins, even those that are not globular in shape.

The column was prepared with 20 mM sodium phosphate buffer, pH 7.2 containing 1 % Triton X-100 and 2 M urea. The protein sample (10 mg) was mixed with urea (final concentration 8 M). This was in order to allow full denaturation of the protein in the sample. The column was set at a lower urea concentration to allow the protein structures to reform. 1 ml of sample (10 mg/ml) was loaded by way of a three way valve. Fractions of 0.3 ml were recovered and assayed using the radiochemical method for activity towards benzylamine. Fractions were also assayed using ELISA in 96 well plates, as described in Section 4.3.5.1, using 200 µl of eluent as sample. They

were labelled with 1:100 dilution of anti-SSAO antibodies (anti-bovine lung SSAO, raised in rabbit) and 1:1000 dilution of anti-rabbit IgG before developing colour in 3 mg / 10 ml 2,2' azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid in 0.1 M citrate buffer, pH 4.3. After 30 min the absorbance of each well was read at 405 nm in an optical plate reader.

3.2.3 Determination of molecular weights

The volume of solvent between the injection point and the peak maximum of solute is called the elution volume (V_e). If a molecule is larger than the pore size of the stationary phase it will not enter that phase and the elution volume is the exclusion volume of the column (V_x). The elution volume of any particle will depend on how much of its time in the column that it spends in and outside the stationary phase. This should be constant and is called K_d.

Therefore:

$$V_e = V_x + K_d (V_{st})$$

where V_{st} is the volume of solvent that is contained in the stationary phase. V_{st} is not easily determined and so $V_t - V_x$ is used where V_T is the total bed volume. This is determined by measuring the elution volume of a molecule of smaller dimension than the pore size of the stationary phase. If K_{av} is representative of the fraction of the stationary phase available for diffusion for a given molecule then:

$$K_{av} = (V_e - V_x) / (V_T - V_x)$$

and $K_{av} = K_d$.

The elution volumes of proteins are determined by their hydrodynamic radius. This is directly related to the molecular weight for globular proteins, so the elution volume is approximately a linear function of the logarithm of the molecular weight (see Preneta, 1989).

A calibration graph was constructed for the Superdex 200 column using Sigma (Sigma Laboratories Ltd, UK) standard proteins of known molecular weight (see Table 3.1). The elution volume of each standard protein was determined from the chromatogram. The elution volume was plotted against the logarithm of the molecular

weight. From this graph the molecular weights of unknown samples can be determined from respective elution volumes.

Molecular Weight Markers (Sigma)	
Marker	Molecular Weight
Thyroglobulin (bovine)	669 kDa
Apoferritin (horse spleen)	443 kDa
Alcohol dehydrogenase (yeast)	150 kDa
Albumin (bovine serum)	66 kDa
Ovoalbumin	45 kDa
Carbonic anhydrase	29 kDa

Table 3.1. The standard proteins used in the calibration graph to determine the molecular weight of unknown samples.

3.2.4 MonoQ ion exchange chromatography

The Mono Q ion exchange utilises a charged polyether matrix to adsorb and retain proteins. The FPLC system employed was the Pharmacia-LKB FPLC run at 1.0 ml/min. It was flushed with 20 mM sodium phosphate buffer, pH 7.2 containing 1 % Triton X-100 prior to use. The solubilised sample was loaded (5 ml at 2 mg/ml) and was eluted with increasing stepwise concentrations of NaCl up to 2 M.

One ml fractions were collected and assayed for activity against both benzylamine and 5-HT.

3.2.5 Radiochemical assay

The radiochemical assay involved the same principal at that described in Section 2.2.5. The assays contained 100 µl of enzyme solution, 20 µl of clorgyline (10^{-2} M) and 80 µl of 100 mM phosphate buffer, pH 9.5, were incubated in a shaking waterbath at 37 °C for 30 min before addition of 25 µl of radioactively labelled substrate (9 times final desired concentration). Reactions were stopped by addition of 100 µl of

2 M citric acid and the labelled product was extracted and determined as described in Section 2.2.5. Blanks were made containing the same materials but with the citric acid added before the substrate. The radioactively labelled substrate used were 100 μM benzylamine (1.0 Ci/mol) or 100 μM 5-HT (3.0 Ci/mol). Reaction times varied from 60-180 min. Results were expressed as dpm or percentage of total radiolabelled substrate.

Samples were taken from areas that demonstrated peak activity and K_m values were determined in an attempt to distinguish if two separate enzymes existed. All assays were performed in duplicate.

3.3 Results

3.3.1 Superdex 200 gel permeation chromatography

The data are shown in Figure 3.1. Two peaks of activity are evident for activities towards both benzylamine and 5-HT. For 0.3 ml fractions the peaks were in the fraction 30 -35 (peak at elution volume 19.9 ml) and 44 - 53 (peak at elution volume 14.1 ml). There was an area of lower activity in the region of elution volume 11.1-12.6 ml. Samples from both of these peaks were assayed against benzylamine (concentration range 10 - 100 μM) as the 5-HT activity was too low to measure.

The K_m value for the activity in the fraction at elution volume 9.9 ml was 51 $\pm$ 8 μM and that for the fraction at elution volume 14.1 ml was 73 $\pm$ 17 μM (see Figure 3.2a & b). The V_{max} value for the activity in the fraction at elution volume 9.9 ml was 5014 $\pm$ 365 nmol/min/mg protein and that for the fraction at elution volume 14.1 ml was 4967 $\pm$ 610 nmol/min/mg protein (Figure 3.2a & b). Western blot analysis showed a band at 110 kDa for both peaks (Figure 3.3).

The fraction at elution volume 9.9 ml (the higher molecular weight peak) was further run through the column and assayed for activity towards benzylamine. No activity was detectable. In order to concentrate activity, 2 further columns of Triton X-100 solubilised porcine dental pulp microsomal SSAO were run in the Superdex 200 column. Fractions at elution volume 9.6-10.2 ml from each run were pooled and

concentrated to 0.5 ml in an Amicon semi-permeable membrane with a nominal molecular weight cut-off of 10,000 kDa. This concentrated sample was run through the column. The resultant profile demonstrated one peak of activity at an elution volume of 12.3 ml (Figure 3.4).

The use of urea is a denaturing agent did not reveal distinct zones of SSAO when enzyme activity in the fractions was measured using ELISA (Figure 3.5). The recovery of SSAO activity towards benzylamine was very low and it was undetectable when 5-HT was used as a substrate.

3.3.2 Determination of molecular weights

The elution volumes of the molecular weight markers are shown in Figure 3.6. From this graph the areas of high SSAO activity can be compared in terms of molecular weight with known markers. The activity peak at fraction 33 (elution volume 9.9 ml) corresponds to a molecular weight of 440 kDa and the peak at fraction 47 (elution volume 14.1 ml) of 110 kDa.

When the 440 kDa peaks were pooled and concentrated the resultant peak after running the sample through the Superdex column was a single peak in the 220 kDa range.

3.3.3 MonoQ ion exchange chromatography

This column when run with porcine pulpal microsomes that had been solubilised with 1 % Triton X-100 failed to produce any evidence of resolution of the SSAO activity into separate fractions (Figure 3.7). The total recovery from the column was very low, in the region of 10 % of the total applied activity. There was essentially a complete loss of 5-HT activity whilst some activity towards benzylamine remained. The use of urea in the column failed to produce distinct areas of activity (Figure 3.8).

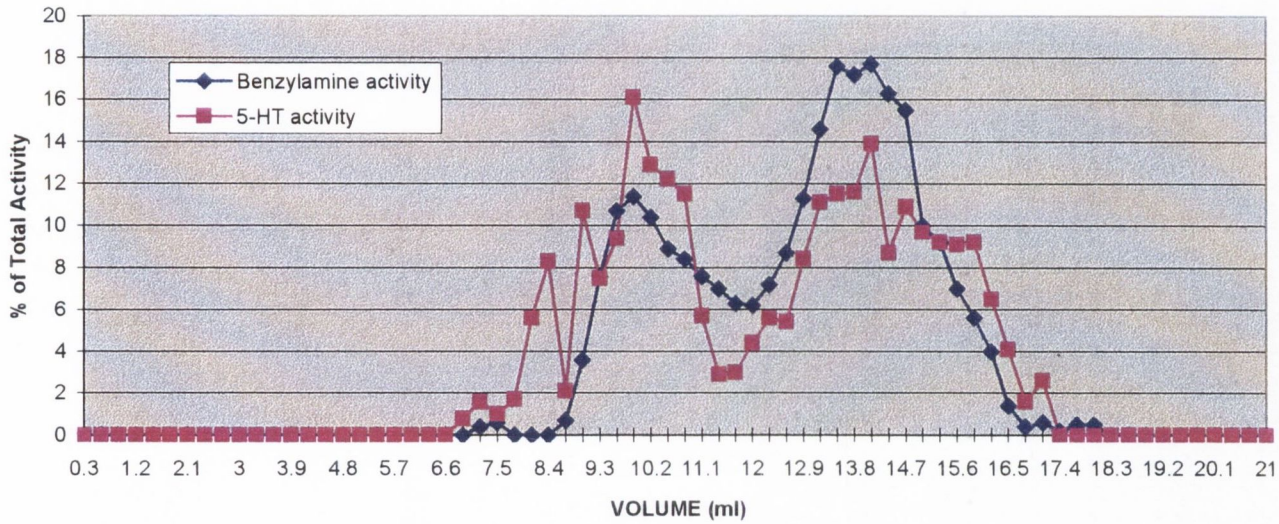
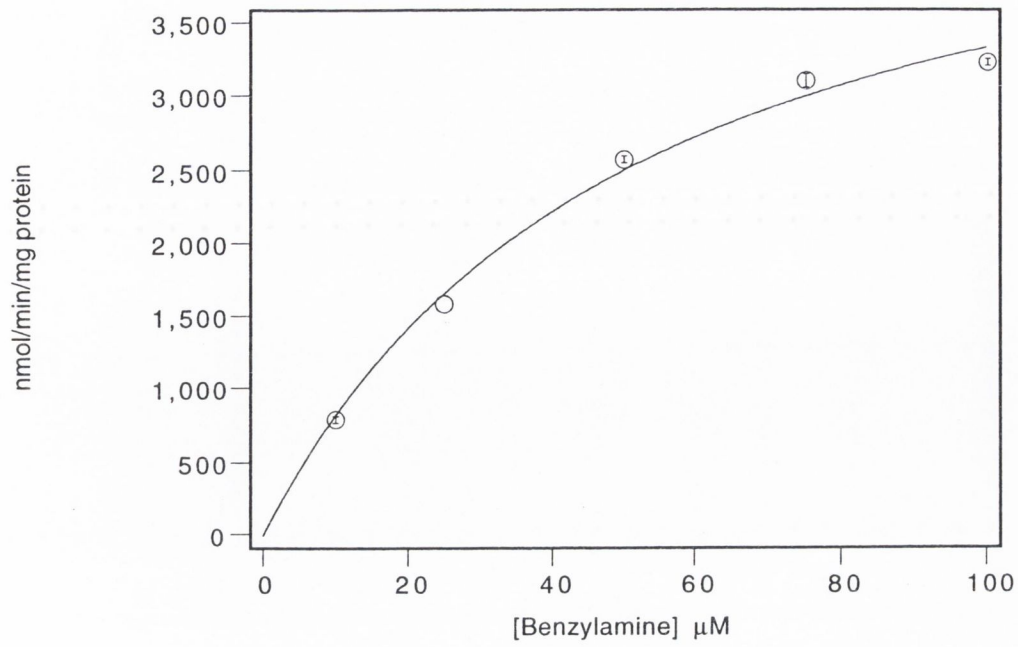
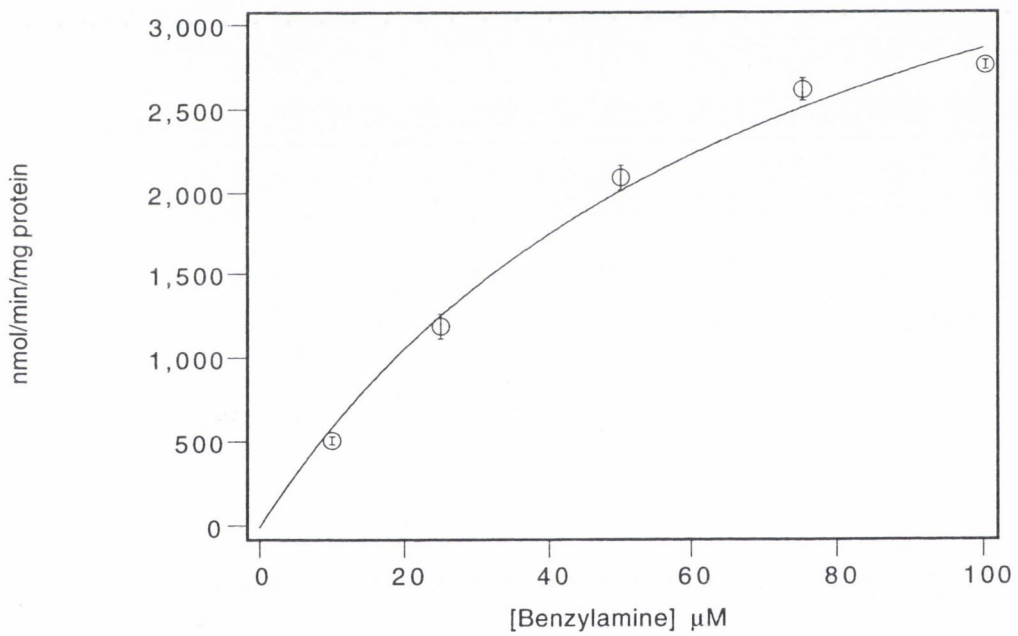


Figure 3.1. Gel filtration of Triton X-100 solubilised porcine dental pulp microsomal SSAO on a Superdex 200 column. SSAO activity towards 100 μM benzylamine (1 Ci/mol) and 100 μM 5-HT (3 Ci/mol) in the different fractions eluting from a Superdex 200 column was determined radiochemically as described in Section 2.2.5. The 5-HT activity in the figure has been arbitrarily magnified 10-fold for comparative purposes.





Figures 3.2a (top) & 3.2b (bottom). Kinetic behaviour for the peak areas of activity against benzylamine (1.0 Ci/mol) after gel-filtration of Triton X-100 solubilised porcine dental pulp microsomes through a Superdex 200 column as determined radiochemically as described in Section 2.2.5. Figure 3.2a corresponds to the peak at fraction 33 (elution volume 9.9 ml) and Figure 3.2b to the peak at fraction 47 (elution volume 14.1 ml). The values are means ± S.E.M. of the experiment completed in triplicate.

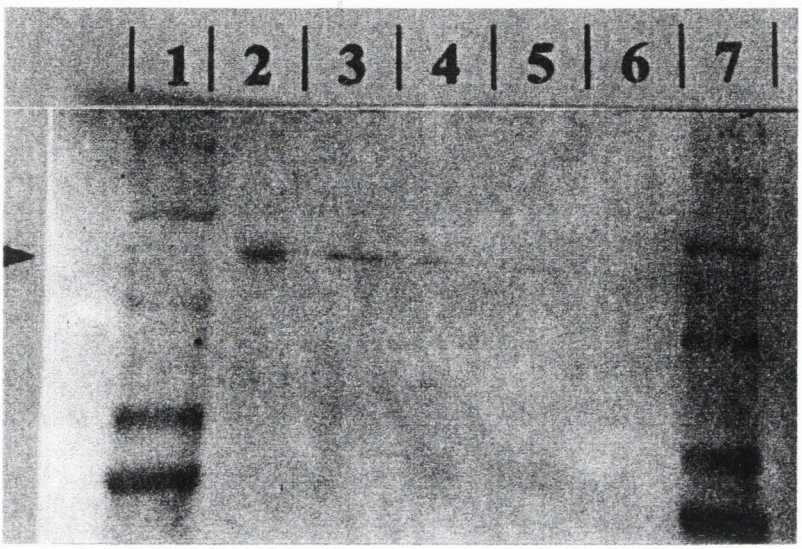


Figure 3.3. Western blot of SSAO from Triton X-100 solubilised porcine dental pulp SSAO fractions after partial purification by running in a Superdex 200 column. The fractions associated with peak activity towards benzylamine and 5-HT, as assayed radiochemically by the procedure described in Section 2.2.5, were tested. These were elution volumes 9.9 ml (lane 2), 10.2 ml (lane 3), 13.8 ml (lane 4), 14.1 ml (lane 5), 14.4 ml (lane 6). Molecular weight markers are in lanes 1 & 7. The arrowed band is 110 kDa.

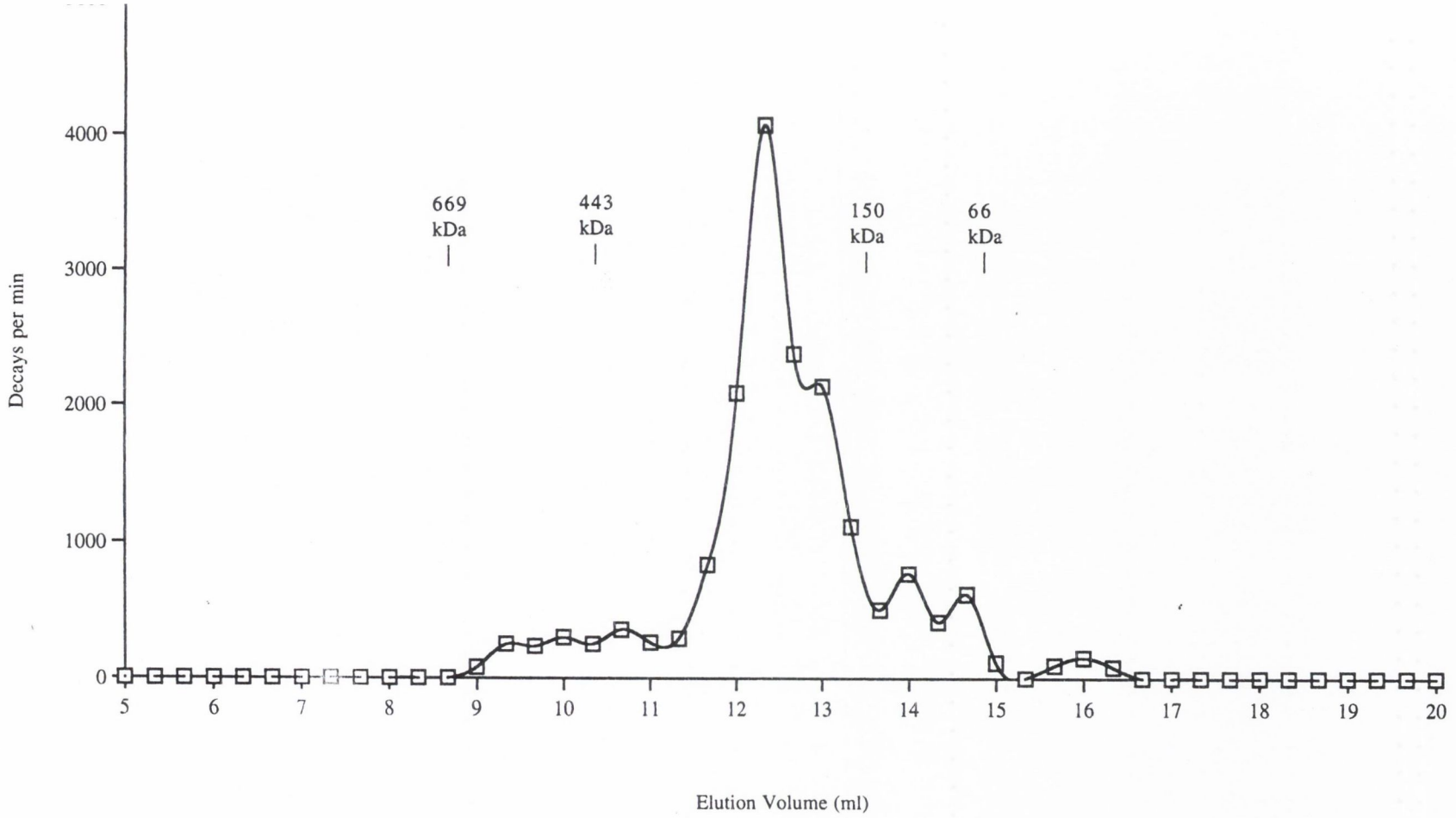


Figure 3.4. Gel filtration of Triton X-100 solubilised fractions 32, 33 and 34 (pooled and concentrated) on a Superdex 200 column. SSAO activity towards 100 μM benzylamine (1 Ci/mol) in the different fractions was determined radiochemically as described in Section 2.2.5.

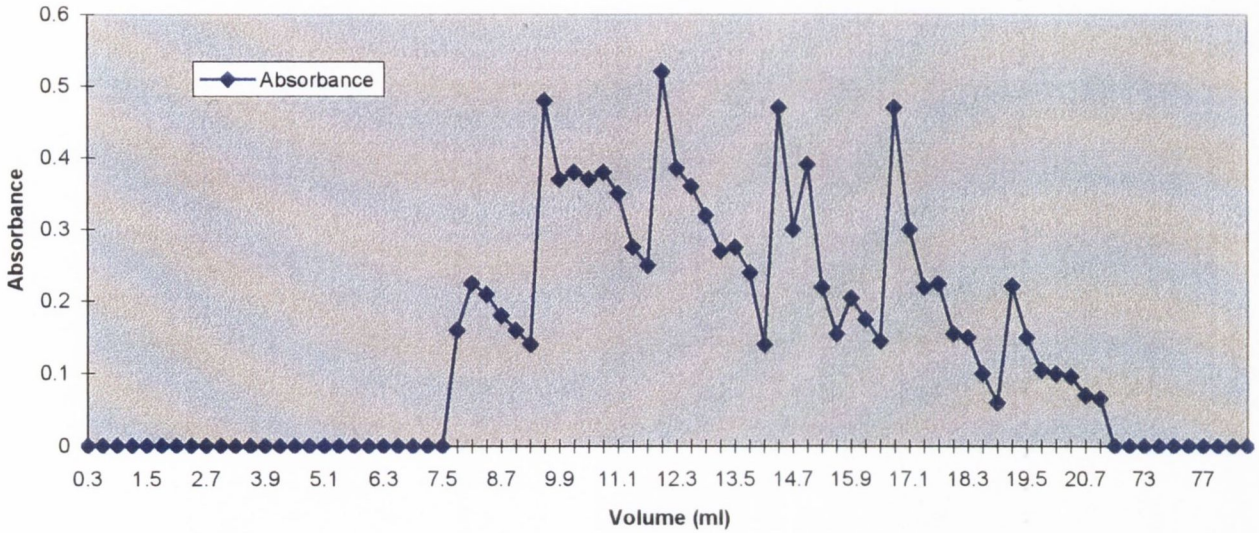


Figure 3.5. ELISA absorbance values for fractions of urea-treated Triton X-100 solubilised porcine dental pulp in a Superdex 200 column as described in Section 3.2.2.2. The protein sample (10 mg) was mixed with urea (final concentration 8 M). Fractions were assayed using ELISA in 96 well plates as described in Section 4.2.5.1 using 200 µl of eluent as sample, labelling with 1:100 dilution of anti-SSAO antibodies (anti-bovine lung SSAO, raised in rabbit) and 1:1000 dilution of anti-rabbit IgG before developing colour in 3 mg / 10 ml 2,2' azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid in 0.1 M citrate buffer, pH 4.3. After 30 min the plates were read at 405 nm in an optical plate reader.

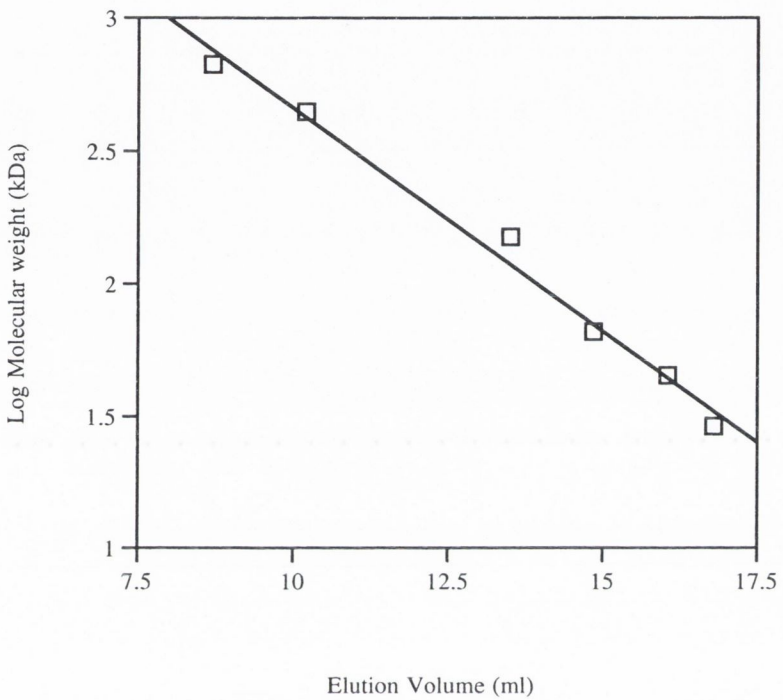


Figure 3.6. Calibration graph for the Superdex 200 column using Sigma (Sigma Laboratories Ltd, UK) standard proteins of known molecular weight (see Table 3.1). The elution volume of each standard protein was determined from the chromatogram. The elution volume was plotted against the logarithm of the molecular weight. From this graph the molecular weights of unknown samples can be determined from respective elution volume.

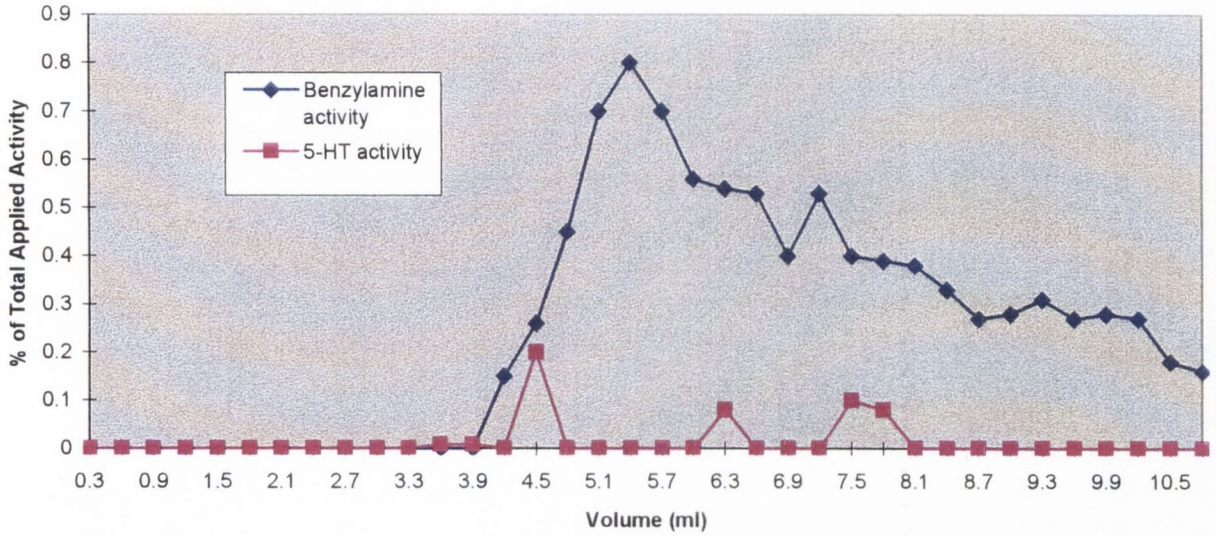


Figure 3.7. Percentage applied activity in each fraction of Triton X-100 solubilised porcine dental pulp microsomes eluted from a MonoQ column. The activities in each fraction were determined radiochemically as described in Section 2.2.5 with 100 μM benzylamine (1 Ci/mol) and 100 μM 5-HT (3 Ci/mol).

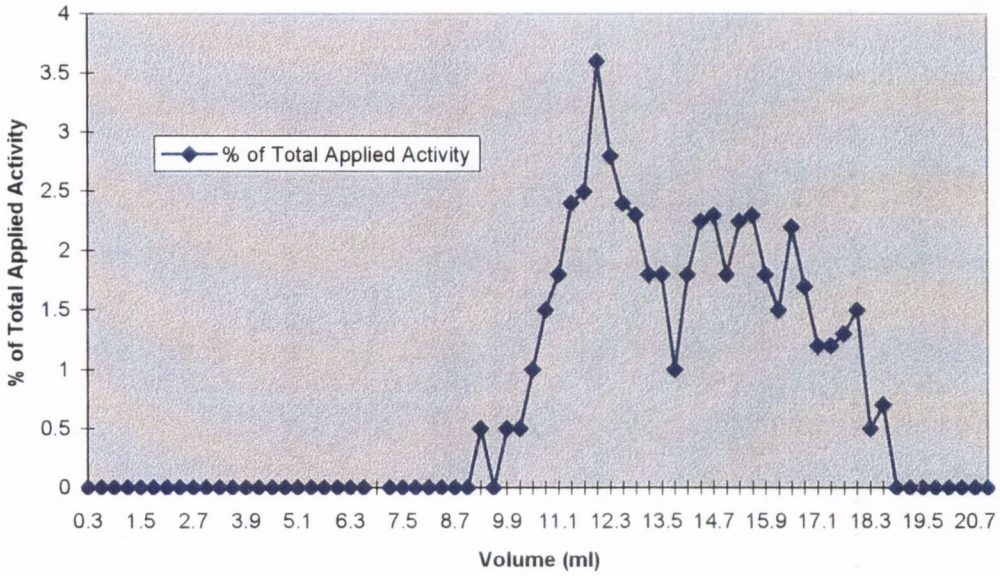


Figure 3.8. Percentage applied activity in each fraction of Triton X-100 solubilised porcine dental pulp microsomes eluting from a MonoQ column after urea treatment. Activity was determined radiochemically as described in Section 2.2.5 with 100 μM benzylamine (1 Ci/mol) as a substrate.

3.4 Discussion

3.4.1 Superdex 200 Gel Permeation Chromatography

This form of chromatography separated out the SSAO activity towards benzylamine and 5-HT into two distinct areas with apparent molecular weights of 110 kDa and 440 kDa. This might represent either two separate enzymes or a monomeric and tetrameric form of the enzyme. The kinetic constants were determined with benzylamine alone, as the activity with the 5-HT was too low to assay. Both the K_m and V_{max} values suggest that the 110 kDa and 440 kDa sites are the same and that one is a tetramer of the other. When the areas of peak activity were run in a Western Blot using anti-bovine lung microsomal SSAO bands corresponding to 110 kDa molecular weight were observed for all peaks of activity. This suggests that the 440 kDa form is a tetramer that is denatured by the SDS in the running gel.

Re-running combined and concentrated 440 kDa fractions through the Superdex 200 column produced a single peak of activity with benzylamine as a substrate at 220 kDa. SSAO existing as a dimer has been described for bovine lung SSAO (Lizcano, 1994). Benzylamine oxidising activity is therefore present in the 440, 220 and 110 kDa molecular weight forms of SSAO in porcine dental pulp.

When freshly recrystallised urea was used as a denaturing agent for the protein before being gel-filtered through the column it failed to produce distinct peaks of SSAO activity when assayed with either benzylamine or 5-HT as substrates. This may reflect the inability of the denatured enzyme to refold under the conditions used here. Alternatively inactivation by carbamoylation might have occurred during the urea treatment.

3.4.2 MonoQ ion exchange chromatography

When the fractions of the porcine pig pulpal SSAO were assayed radiochemically with benzylamine and 5-HT as substrates no resolved peaks were produced. The activity when 5-HT was used as a substrate was very low and the percentage activity recovered was much less than that recovered when benzylamine was used as a

substrate. Selective loss of 5-HT activity may be as a result of the two enzymes being two distinct entities.

The primary aim of this work was an attempt to resolve the two SSAO enzymes that has been inferred from the work reported in chapter 2. However, the procedures used did not succeed in this respect. The relatively small amount of starting material available precluded a more elaborate procedure. From the work presented here it would seem that the two SSAO enzymes in porcine dental pulp are rather similar in their gross molecular properties and that studies at the DNA level might be the most appropriate procedure for investigating differences at a more precise level.

3.5 Conclusions

1. Semicarbazide-sensitive amine oxidase catalysed deamination of benzylamine or 5-HT at approximate molecular weights 110 kDa and 440 kDa could be detected when solubilised porcine dental pulp microsomes were gel-filtered through a Superdex 200 column.
2. Western Blot analysis of each peak of activity from the Superdex column resulted in bands in the region of 110 kDa suggesting that the 440 kDa enzyme was a tetramer of the 110 kDa enzyme.
3. The K_m values for the two areas of peak activity with benzylamine as a substrate were 51 $\pm$ 8 (110 kDa material) and 73 $\pm$ 17 (440 kDa material) μM and the V_{max} values 5014 $\pm$ 365 and 4967 $\pm$ 610 nmol/min/mg protein respectively, suggesting that the active sites may be the same.
4. The 440 kDa peak, on re-running through the Superdex column, produced a peak of activity at 220 kDa.
5. The use of urea as a dissociation agent did not resolve distinct areas of activity towards benzylamine when the samples were gel-filtered through a Superdex 200 column.
6. The small amounts of material available and the low activity precluded further studies. In order to accumulate sufficient material for further research cloning and expression of SSAO is required.

CHAPTER FOUR

Histology & Immunocytochemistry of Human Dental Pulp

Histology & Immunocytochemistry of Human Dental Pulp

4.1 Introduction

Obtaining specimens of dental pulp using current techniques is both time-consuming and expensive. There is a risk that both histological and biochemical information may be lost during the processing. The length of time involved may permit cell disruption and breakdown of the less robust cell lines.

The original methods involved decalcification and fine sectioning (Coleman & Desalva, 1966; Brain, 1967). As well as being time-consuming, these procedures require special equipment and specific training. They are also not efficient in that decalcification requires at least 48 hours before the pulp can be accessed (Balogh, 1963). Furthermore, fine sectioning involves cutting the surrounding calcified structures and the pulp tissue simultaneously which is likely to damage the fragile soft tissue.

Other methods that have been used involve applying force to crack the enamel and dentine. This can be done by direct force *e.g.* a hammer and chisel or after scoring the root with a bur. The predictability of these methods is unclear as the fracture may not be controlled and they will not work for multirooted teeth. Excessive cutting with a rotary instrument may cause aspiration of the odontoblasts (Shovelton, 1968) and disrupt the histological detail.

4.2 Materials

4.2.1 Sources of Reagents

Reagents are listed in alphabetical order. The source of named reagents is indicated by the suppliers name.

PRODUCT	SUPPLIER
Inhibitors	
Clorgyline hydrochloride	Sigma Chemicals
Semicarbazide hydrochloride	Sigma Chemicals
Substrates	
Ascorbic acid	Sigma Chemicals
Benzylamine hydrochloride	Sigma Chemicals
5-Hydroxytryptamine creatine sulphate	Sigma Chemicals
Antibodies	
Anti-SSAO (from bovine lung). Polyclonal antibody raised in rabbit.	A kind gift from Drs Mercedes Unzeta & Jose-Miguel Lizcano de Vega, Universitat Autonoma de Barcelona, Spain
Anti-tryptophan hydroxylase (from mouse)	Sigma Chemicals
Miscellaneous Reagents	
Bovine serum albumin (BSA fraction V)	Sigma Chemicals
Citric acid	BDH Chemicals
Vectastain® ABC	Vector Laboratories

All other general laboratory reagents were purchased from BDH Chemicals or Lennox Laboratories.

4.2.2 Addresses of Suppliers

The full names and addresses of suppliers are listed below in alphabetical order:

BDH	BDH Chemicals Ltd., Poole, Dorset, England.
Lennox	Lennox Laboratories Ltd., J.F.K. Drive, Dublin 12.
Sigma	Sigma Aldrich Ltd., Poole, Dorset, England.
Vector	Vector Laboratories, 16 Wulfric Square, Bretton, Peterborough, PE3 8RF, Scotland.

4.3 Methods

4.3.1 Isolation of human dental pulp

The extracted tooth was rinsed in cold saline and placed on a bed of dry ice (solid carbon dioxide) in a polystyrene container. The entire tooth was rapidly frozen by this procedure and its temperature within the container was maintained at approximately – 30 °C. This temperature will remain constant for a period that depends on the insulating capacity of the container, the ambient temperature and the size of the dry ice block used. A closed polystyrene box (24 cm X 24 cm X 24 cm, wall thickness 4.5 cm) initially containing a cylindrical block of dry ice (diameter 8.5 cm, height 11.5 cm) has been found to be satisfactory for storage periods of eight to ten hours under operating theatre conditions (temperature range 17 – 21 °C).

When the pulp was required, the tooth was taken from the container with a tweezers and held under cold (10^{0} – 20 °C) running tap water for 5 seconds. This partially defrosts the pulpal tissue and facilitates its separation from the dentine. Failure to do this results in tearing the pulp in an uncontrolled fashion and its separation from the dentine is more difficult.

Access to the pulp tissue was achieved by breaking the brittle enamel and dentine using manually controlled force in a mechanical vice (Record 1 TON-E-B, Record Tools Ltd, Sheffield, England). The tooth was placed in the vice with the root apices upwards (Figure 4.1). The jaws were closed on the facial-lingual height of contour points of the crown. Crushing force was applied gradually until the enamel and dentine of the crown fracture. Each individual root was then placed between the jaws of the vice and broken open in a similar fashion.

The comminuted tooth was placed on a suitable impermeable work surface under good lighting. Using College Tweezers (Ash Dental Instruments, England) the hard tissue may be carefully dissected away from the pulp (Figure 4.2). The time taken to remove the pulpal tissue in this way was, at most, 2 minutes.

The pulp may then be used immediately, refrozen for later use, or embedded and sectioned for histological examination. Alternatively, if histological examination is the main focus, root apices are cut off the extracted tooth which is placed in formol-saline for twenty-four hours before freezing and processing (Masterson, 1966). This is because the fixed pulp is more robust and the odontoblast fibrils are better preserved.

4.3.2 Other calcified tissues

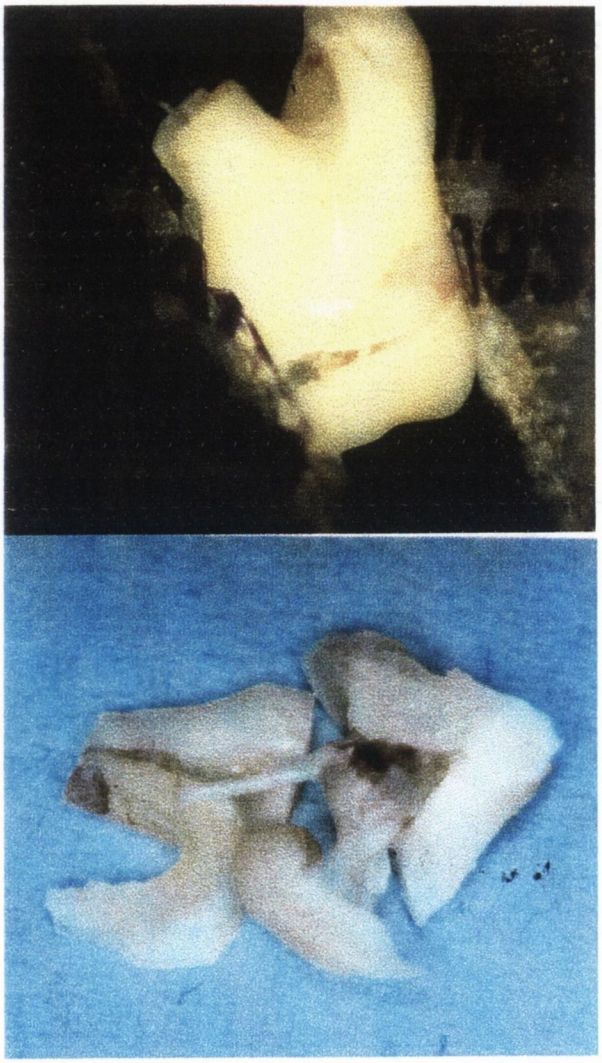
This technique may also be applied to the study of bones. To test this fresh rat femurs were obtained, frozen in dry ice and fractured open with a crushing force as described in Section 4.3.1. The marrow was then removed intact and processed for histological examination (see Section 4.3.4).

4.3.3 Comparison of pulps retrieved at different time intervals (after extraction)

To examine the effect produced by various time intervals between tooth extraction and freezing the tooth, cell membrane integrity was investigated by enzymatic means in a small sample of teeth. The enzyme studied was lactate dehydrogenase (LDH). This stable enzyme is contained in most cells and leaks from them if the membrane integrity is disrupted. It has been previously used as a marker of membrane damage in many cell types, including hepatocytes (Cherney, 1987), fibroblasts (Duffy & Flint, 1987), thyroid cells (Wroblewski & Gregory, 1961) and cardiac cells (Mosinger, Stejskal & Mosinger, 1977).

Four intact, non-carious, third molars, being extracted for orthodontic reasons, were obtained from each of two patients. One sample from each patient was frozen immediately and the others at one, three and seven days. The samples not frozen at once were stored in sterile normal saline at 4 °C in a refrigerator. Frozen samples were stored at -80 °C until required.

The dental pulps were separated from the surrounding calcified tissue as described. The intact pulps were then weighed and placed in four centrifugation tubes containing 3.0 ml of 0.025 M sucrose / 0.05 M sodium phosphate buffer (pH 7.2) containing 0.1 % w/v collagenase (Sigma Ltd. Mo. USA).



Figures 4.1 (top) & 4.2 (bottom). The tooth is placed in a manual vice (root apices upwards) and crushed (Figure 4.1). The dental pulp is then accessible and can be carefully separated from the surrounding hard tissue (Figure 4.2).

The samples were then centrifuged at 100,000 *g* for 60 minutes in a Beckman TL-100 Ultracentrifuge (Beckman Inst. Ltd. USA) and the resulting supernatants were assayed for LDH by a method developed from that of Kornberg (1955) to determine LDH leakage from sample cells. The pellets were resuspended in buffer, homogenised in an Ultra-Turrax T25 (Janke and Kunkel, GMBH & Co., Germany) and centrifuged as before. This supernatant represented the total released tissue LDH and data was presented as LDH released in each sample as a percentage of total LDH content of the sample. Final assays contained 100 mM potassium phosphate buffer (pH 7.0), 0.2 mM reduced form of nicotinamide adenine dinucleotide (NADH), 1 mM sodium pyruvate and 500 µl of the supernatant in a total volume of 3 ml. The oxidation of NADH was monitored spectrophotometrically (HP8452A Diode Array Spectrophotometer, Hewlett Packard, USA) at 340 nm.

4.3.4 Histological verification of intact specimens

Samples of human dental pulp were obtained as described as above and wax embedded overnight. Ten µm vertical sections were cut and the sections were placed on glass slides and left to dry. The samples were then stained with haemotoxylin and eosin using the following technique:

1. The sections were hydrated through xylene for 20 min followed by a graded alcohol series (100 %, 96 %, 70 %) for 5 min each to water.
2. The sections were incubated with Mayer's haemalum for 15 min before being washed in water for 10 min.
3. Excess haemalum was removed with dilute hydrochloric acid and the samples were placed in eosin solution for 1 min, washed and dehydrated through alcohol to xylene.
4. DPX mountant was placed on coverslips and the samples were covered and left to dry at room temperature.

4.3.5 Antibodies against SSAO

Polyclonal antibodies against bovine lung SSAO have been demonstrated to react with SSAO from other species (Lizcano, 1994). ELISA and Western blot analysis were used to confirm cross-reactivity with human SSAO.

4.3.5.1 ELISA

Ten human third molars were homogenised in 50 mM sodium /potassium phosphate buffer, pH 7.2, in a ratio of 1:4 w/v. The homogenate were diluted at various concentrations in 0.1 M H_2CO_3 / Na_2CO_3 pH 9.6 and a total volume of 200 µl per well left overnight at 4 °C in a 96-well plate. The sample wells were washed twice with 200 µl Tween-Tris buffered saline (TTBS) for 10 minutes each and then incubated for 90 min with 200 µl of 3 % Marvel in TTBS. The wells were washed in 200 µl TTBS three times for 5 min each before addition of 100 µl of the primary anti-SSAO antibody (dilution range 1:10 to1:100) in blocking buffer for 90 min. The sample wells were washed three times for 5 min, in 200 µl TTBS before addition of 100 µl of peroxidase-linked anti-rabbit IgG (1:1000 dilution) for one hour. The wells were rinsed with three times by 5 min washings with 200 µl TTBS and colour was developed in 3 mg/ 10 ml of 2.2'azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid in 0.1 M citrate buffer, pH 4.3. Absorbance was recorded at 30 min at 405 nm. Blanks were used with either no dental pulp homogenate or no primary antibody, but otherwise treated as before.

4.3.5.2 Western blot analysis

The slab gel plates for the SDS-PAGE (Laemmli, 1970) were cleaned with ethanol and wiped with a clean tissue prior to swabbing with acetone and a final rinse of ethanol. The plates were left to air-dry. The glass plate sandwich was assembled and clamped after insertion of a 1.5 mm spacer using the "ATTO" mini-gel electrophoresis unit.

SDS (20 %)	0.1 ml
H_2O	8.04 ml
30 % Acrylamide-bisacrylamide (30:0.8)	6.66 ml
4X Resolving buffer	5 ml

Table 4.1. 10% separating gel constituents. The amount of acrylamide-bisacrylamide was altered to produce gels of different percentages.

The running gel was prepared as shown in Table 4.1. After degassing for 5 min at room temperature 0.2 ml of 10% ammonium persulphate was added, mixed and 20 µl of TEMED added. The resolving gel mixture was added carefully to avoid bubbles in the gel to 1 cm from the bottom of the bottom of the sample wells. This gel was then covered with distilled water and allowed to set. When it had set the water was drained off and the stacking gel (see Table 4.2) layered on top.

SDS (20 %)	25 µl
4X Stacking gel buffer	1.25 ml
30 % acrylamide-bisacrylamide (30:0.8)	0.666 ml
H_2O	3.07 ml

Table 4.2. Stacking gel constituents.

After degassing the stacking gel mix for 5 min at room temperature 50 µl of 10 % ammonium persulphate was added, followed by 10 µl of TEMED. The mixture was then added onto the separating gel, in sufficient quantity to fill the remaining space between the plates, and the comb for the wells was inserted into the stacking gel, carefully avoiding trapping air bubbles beneath it. The apparatus was left to set undisturbed.

After polymerisation the comb was removed to expose the wells and the wells were filled with reservoir buffer. The gel was then used immediately. The gel was placed in

the electrode assembly which was filled with 1X SDS electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3).

Ten human third molars were homogenised in 50 mM phosphate buffer, pH 7.2, at a ratio of 1:4 w/v. Forty µl of sample was mixed with 20 µl buffer (25 µl mercaptoethanol and 500 µl sample buffer (see Table 4.3) and boiled for 3 min to denature the proteins. Forty µl of this mixture was placed in the sample wells for the SDS-PAGE and 6 µl of molecular mass marker (27,000-180,000 M_r, Sigma) was placed in each of the marker lanes. Blank wells were filled with 40 µl of blank sample buffer.

Distilled water	8 ml
Tris HCL 0.5 M pH 6.8	2ml
Glycerol	1.6 ml
10 % (w/v) SDS	3.2 ml
0.1 % (w/v) bromophenol blue	0.4 ml

Table 4.3 Sample buffer

The electrophoresis assembly was attached to the power pack and the voltage set at 150V for 2.5 hours or until the tracker dye reached the bottom of the gel. Following electrophoresis the gel was equilibrated in cold transfer buffer for 15 min.

Glycine	0.192 M
Tris, pH 8.3	25 mM
SDS	1.3 mM
Methanol	15 % (v/v)

Table 4.4. Transfer buffer constituents.

The nitrocellulose and blotting paper (Whatman 3 mm filter paper) were also placed in transfer buffer for 15 min. The order of the components in the semi-dry blotter was from the top: 3 sheets of blotting paper, the gel, nitrocellulose (Bio-Blot-NC) and 3 sheets of blotting paper. This was run at 110mA for 1 hour.

After transfer the blot was briefly washed in TTBS and blocked by incubating in 3 % Marvel in TTBS overnight at 4 °C. The nitrocellulose was rinsed in TTBS and then washed 3 times in TTBS for 15 min each, prior to incubation with the primary antibody (1:100 dilution of anti-SSAO in 3 % Marvel / TTBS) for 1 hour on an orbital shaker at room temperature. The nitrocellulose was then rinsed in TTBS and then washed 3 times in TTBS for 15 min each before incubation for 1 hour with the biotinylated secondary antibody (anti-rabbit IgG, Vectastain®, Vector Laboratories, Scotland).

The nitrocellulose was rinsed in TTBS and then washed 3 times in TTBS for 15 min each before incubation in the ABC reagent (see Section 4.3.5.3) for 1 hour. The nitrocellulose was then rinsed in TTBS and then washed three 15 min periods in TTBS.

The blot was developed in one of the two chromogenic solutions shown in Table 4.5. Colour development was halted by rinsing with tap water.

1.

60 mg 4-chloronaphthol in 20 ml methanol
80 ml of 20 Mm Tris-HCL, pH 7.2
60 µl of 30 % H_2O_2

2.

8 mg diaminobenzidine in 20 ml TTBS
20 µl of 30 % H_2O_2

Table 4.5. The two chromogenic solutions used to develop colour with Western Blotting. The former method is more specific but less sensitive.

4.3.5.3 Immunohistochemical labelling

The Vectastain® ABC is a commercially available kit used for immunohistochemical staining. It is based on the principal that avidin is a basic glycoprotein which has a high affinity for the water-soluble vitamin biotin. Biotin may be linked to a variety of molecules, including antibodies and more than one molecule of biotin may be added to each protein. The basic technique is that a primary antibody which is attached to a particular antigen is linked to a secondary antibody that is biotinylated. The biotin in turn attaches to a preformed avidin and biotinylated horseradish peroxidase macromolecular complex (Hsu, Raine & Fanger, 1981).

The laboratory steps are:

1. The embedded paraffin sections were rehydrated by placement in zylene for 20 min, followed by a graded ethanol series (100 %, 96 %, 70 %, 0 % for 5 min each).
2. The endogenous peroxidase activity was quenched by 0.5 % w/v H_2O_2 in phosphate buffered saline, pH 7.2 (PBS) for 30 minutes.
3. The samples were washed in PBS for 20 min.
4. The samples were blocked with normal rabbit serum for 20 min (3 drops to 10 ml in PBS).
5. As per Step 3.
6. The samples were blotted dry.
7. The samples were covered with primary anti-SSAO antibody (1:200 in PBS) for 30 min (blanks were incubated with rabbit normal serum for the same length of time).
8. As per Step 3.
9. The samples were covered with biotinylated secondary antibody (anti-rabbit IgG, 1 drop to 10 ml with PBS) for 30 min.
10. As per Step 3.
11. The samples were covered with the avidin-biotin complex (ABC) mixture (prepared 60 min beforehand) for 60 min.
12. As per Step 3.

13. The samples were covered with diaminobenzidine / H_2O_2 for 5 min or until colour developed.
14. The samples were washed for 5 min in H_2O.
15. The samples were dehydrated through a graded ethanol series (70 %, 96 %, 100 % for 5 min each) to zylene for 20 min.
16. The samples were covered with coverslips with DPX mountant and dried at room temperature before viewing.

Blanks were run with each staining with (i) no primary antibody, (ii) no secondary antibody, (iii) pre-immune serum and (iv) primary antibody that had been combined 30 min previously with bovine lung microsomes with the latter at a 20 times higher protein concentration than that of the anti-SSAO antibody. Commonly 5-10 times the protein concentration is used but as the bovine lung microsomes were not pure 20 times concentration was used to ensure excess bovine lung SSAO.

4.3.6 Primary antibodies

Two primary antibodies were used for immunochemical investigation. The first was the anti-SSAO (bovine lung) raised in rabbits as described in Section 4.2.1. It was used in dilutions of 1:40, 1:100, 1:200, 1:500 and 1:100 in PBS.

The second antibody was monoclonal anti-tryptophan hydroxylase (Sigma Bio-sciences, UK) derived from the WH-3 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from BALB/c mice. This was obtained as ascites fluid with 0.1 % sodium azide as a preservative. It was used at dilutions of 1:200, 1:500 and 1:1000 in PBS.

4.3.7 Frozen sections of human dental pulp

Ten human third molar teeth were frozen immediately after extraction, crushed in a mechanical vice and then refrozen as described in Section 4.3. The frozen pulps were cut into 50 μm sections and placed on glass slides while still frozen and refrigerated at -70 ^{0}C until required.

4.3.8 Formazan identification of enzyme activity

Histological demonstration of oxidative enzyme systems has been in use since 1946 (Oster & Schlossman). To demonstrate the presence of MAO activity these workers incubated tissue specimens with tyramine and demonstrated the aldehyde product using Schiff's reagent. However the product is soluble and this procedure has been criticised for poor localisation of the product (Gomori, 1950). The use of tetrazolium salts to localise the MAO activity was first proposed by Glenner *et al.*, (1957). The mechanism of action is that nitro-blue tetrazolium acts as an electron acceptor in the oxidative deamination of substrates such as tryptamine catalysed by MAO. This yields a formazan which is insoluble and so precipitates onto the sample localising the enzyme activity.

Component	**Quantity**
Benzylamine / serotonin	To final [1mM]
Sodium sulphate	4 mg
Nitro-blue tetrazolium	5 mg
0.1 M phosphate buffer, pH 7.6	5 ml
H_2O	15 ml

Table 4.6. The incubation solution used in the formazan identification of enzyme sites.

The samples and glass slides were placed in the incubation solution (as described in Table 4.6) and maintained at 37 °C for 45 and 90 min. Blank samples were run without the addition of substrate. The samples were then counterstained with methylene green and washed in running water and fixed in buffered 4 % formaldehyde for 24 hours, then dehydrated in graded ethanol solutions (70 %, 96 % and 100 %, each for 5 min) and then placed in zylene for 20 min. The samples were then covered by coverslips coated in DPX mountant.

4.4 Results

4.4.1 Isolation of dental pulp

More than two hundred and fifty pulps have been removed from extracted teeth. Both human and animal incisors and molars were sampled. Removal of intact pulps from teeth with more than two roots, with roots fused together and in which more than one canal was present in a single root caused the greatest difficulty. When more than two roots are present each root should be split individually. This may require the removal of the hard tissue of the crown and one root before adequate access is obtained to the other root(s). Where roots are fused or there is more than one root canal per root, the number of fracture sites on the root must be increased to create smaller dentine fragments that can be carefully teased from the pulpal tissue.

4.4.2 Comparison of pulps retrieved at different time intervals (after extraction)

Delay in freezing samples led to a relative rise in supernatant LDH concentration (Figure 4.3). There was an increased amount of damage to the cell membranes even if the sample tissue was refrigerated at 4 °C. For a delay of one day or more the relative LDH concentration present in the supernatant doubled. The immediately frozen sample demonstrated some LDH leakage, possibly due to membrane rupture during extraction and removal of the surrounding calcified tissue. Therefore, tissue for use both in histological and kinetic experiments should be frozen without delay upon removal from the mouth.

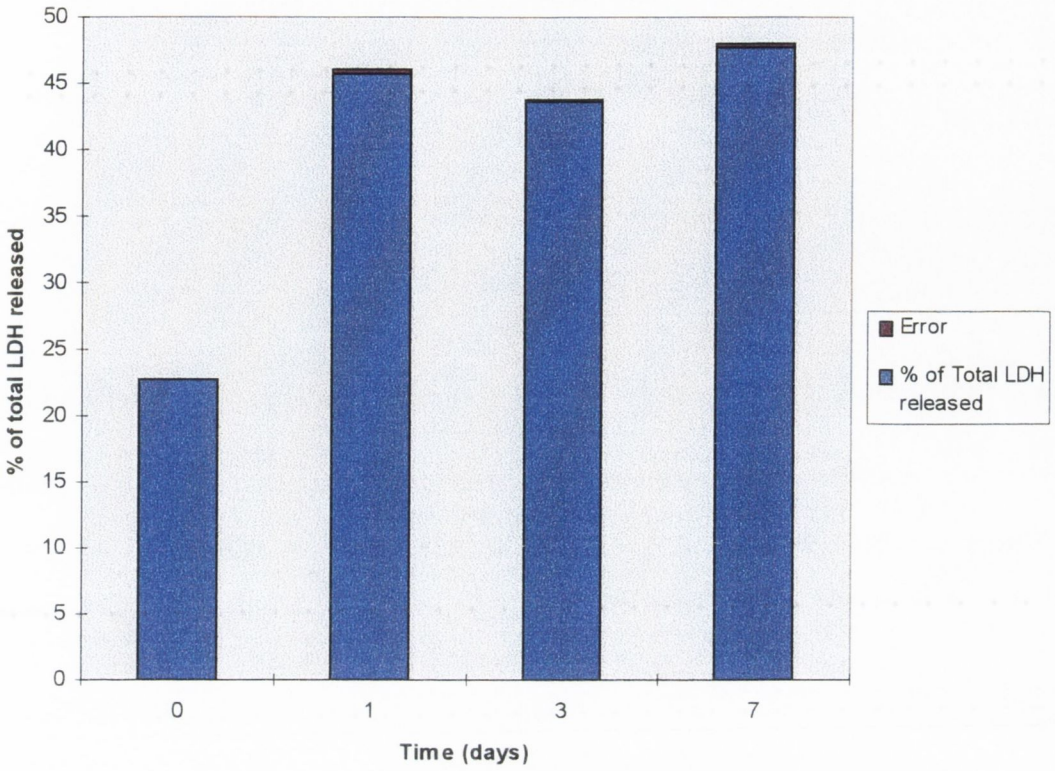


Figure 4.3. Teeth were extracted and assayed for released LDH immediately or stored at 4 °C for 1, 3 or 7 days before being assayed as described in Section 4.3.3. This was in order to observe the effect of delay in use of dental pulp. The LDH is expressed as a percentage of the total sample LDH.

4.4.3 Histological verification of intact specimen

Histological sections of dental pulp fixed immediately after extraction and frozen at 24 hours is shown in Figures 4.4, 4.5 & 4.6. The sections demonstrate that the normal histological features have been well preserved. An intact odontoblastic layer is visible complete with the odontoblastic processes. Beneath the odontoblastic layer the cell free zone (of Weil) and cell rich zone are evident. There were no signs of inflammation.

A histological section of the rat femur (X400) is seen in Figure 4.7. It demonstrates normal cell series with myeloid, erythroblastic and megakaryocyte cells in evidence. There were signs of inflammation.

4.4.4 Antibodies against SSAO

4.4.4.1 ELISA

The relationship of absorbance to concentration of primary anti-SSAO antibody is presented in Figure 4.8. It demonstrates that the antibody recognises antigens in the human dental pulp and that by increasing the concentration of antibody the absorbance increased, initially linearly but then tending towards a plateau at higher concentrations.

4.4.4.2 Western blot analysis

The blot is shown in Figure 4.9. It demonstrates that human dental pulp SSAO when blotted with anti-bovine lung SSAO produced two distinct bands. A similar pattern has been described for bovine lung (Lizcano, 1994).

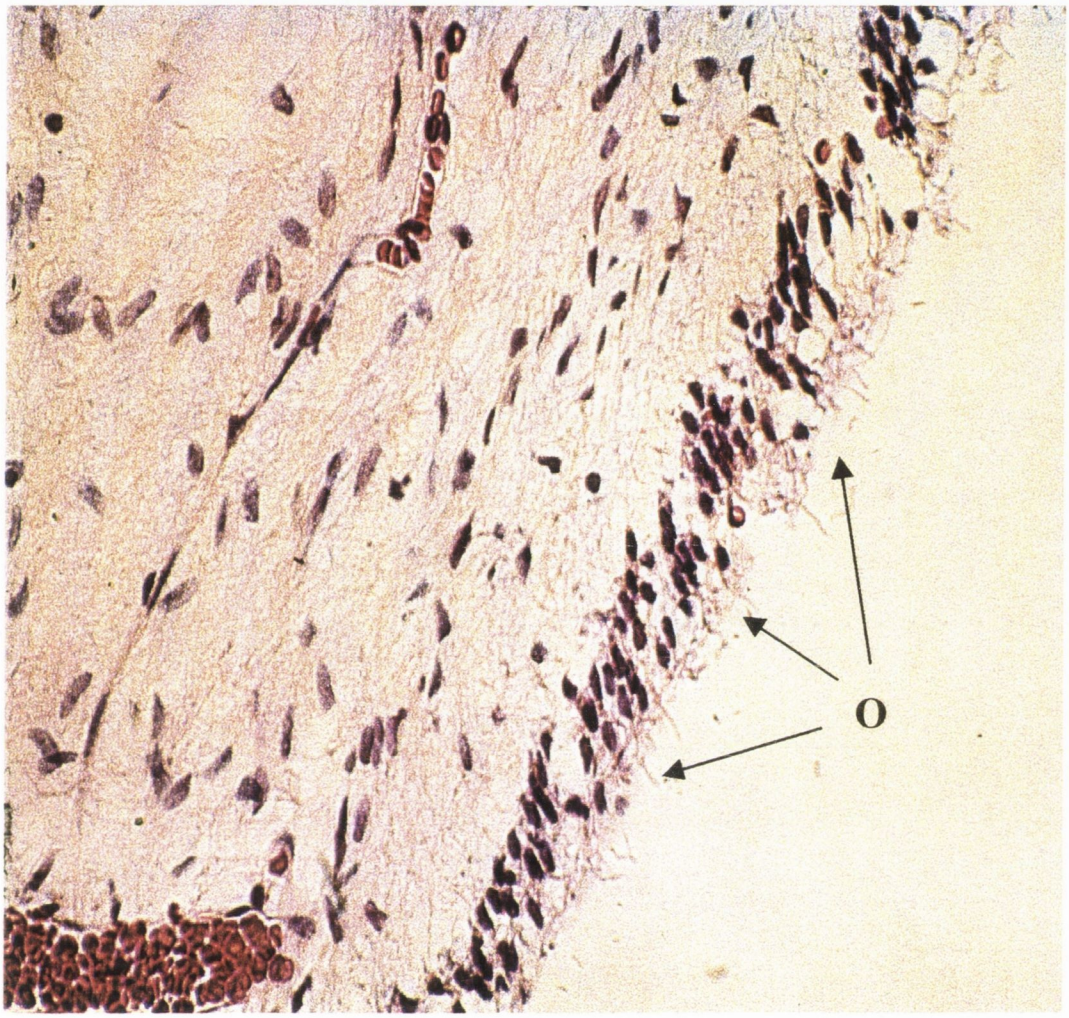


Figure 4.4. A histological section (Haemotoxylin & Eosin stained) of human dental pulp (X400). The samples were prepared as described in Section 4.3.1 and stained as described in Section 4.3.4 prior to microscopic evaluation. The section demonstrates normal histological features of dental pulp, including an intact odontoblast layer (O).

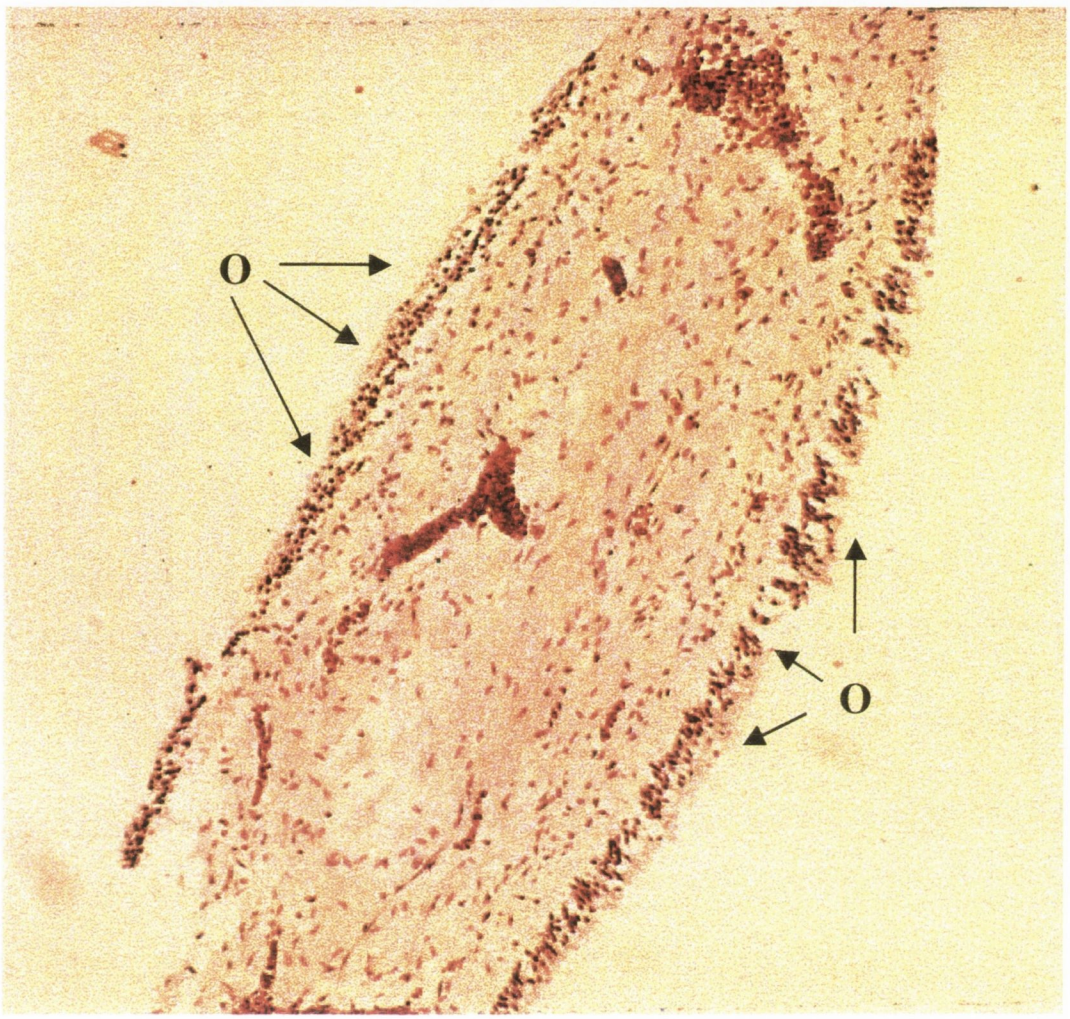


Figure 4.5. A histological section (Haemotoxylin & Eosin stained) of human dental pulp (X80). The samples were prepared as described in Section 4.3.1 and stained as described in Section 4.3.4 prior to microscopic evaluation. The section demonstrates a normal histological appearance of coronal dental pulp without evidence of inflammation. The odontoblast layer is marked (O).

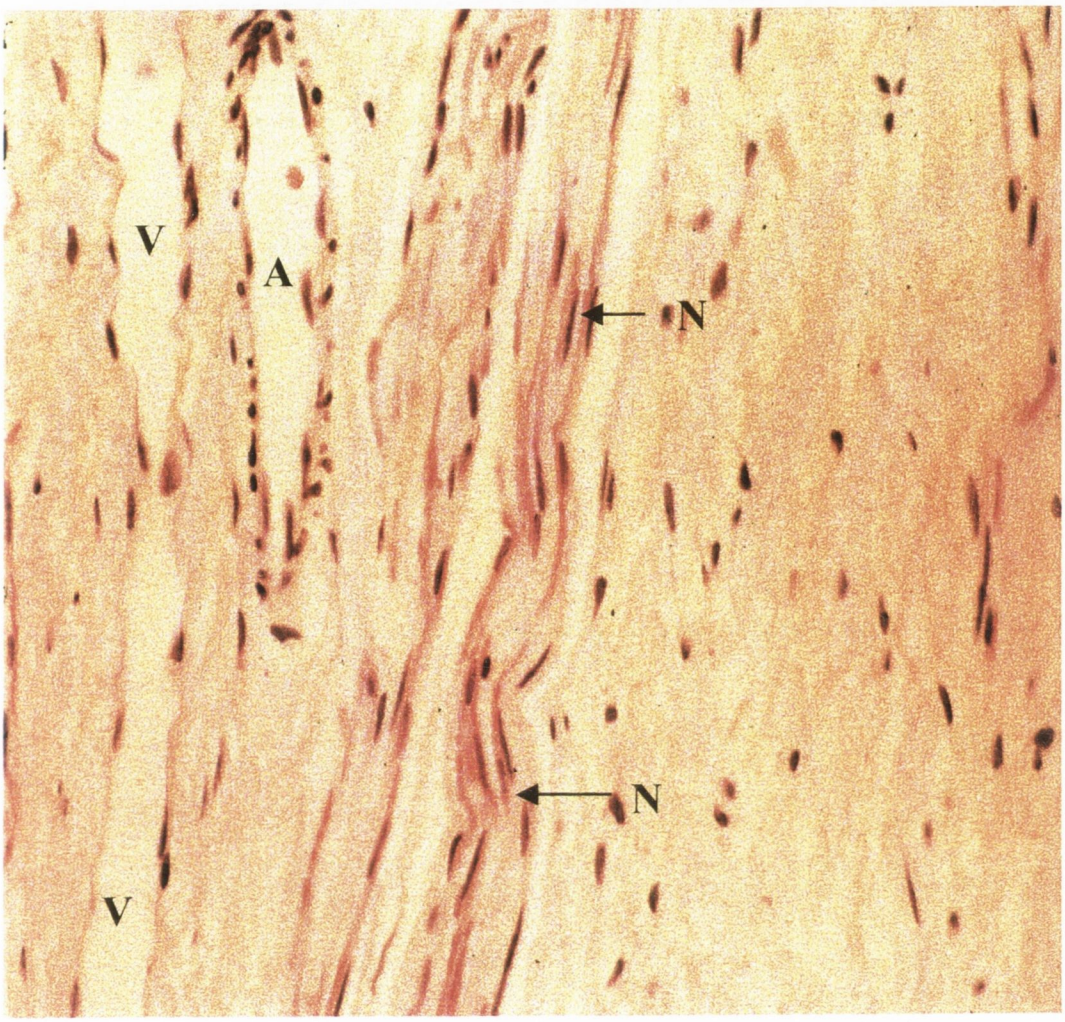


Figure 4.6. A histological section (Haemotoxylin & Eosin stained) of human dental pulp (X500). The samples were prepared as described in Section 4.3.1 and stained as described in Section 4.3.4 prior to microscopic evaluation. The section shows a longtitudinal view of small venule (V), a transverse view of an arteriole (A) and nerve tissue (N) in a normal histological arrangement.

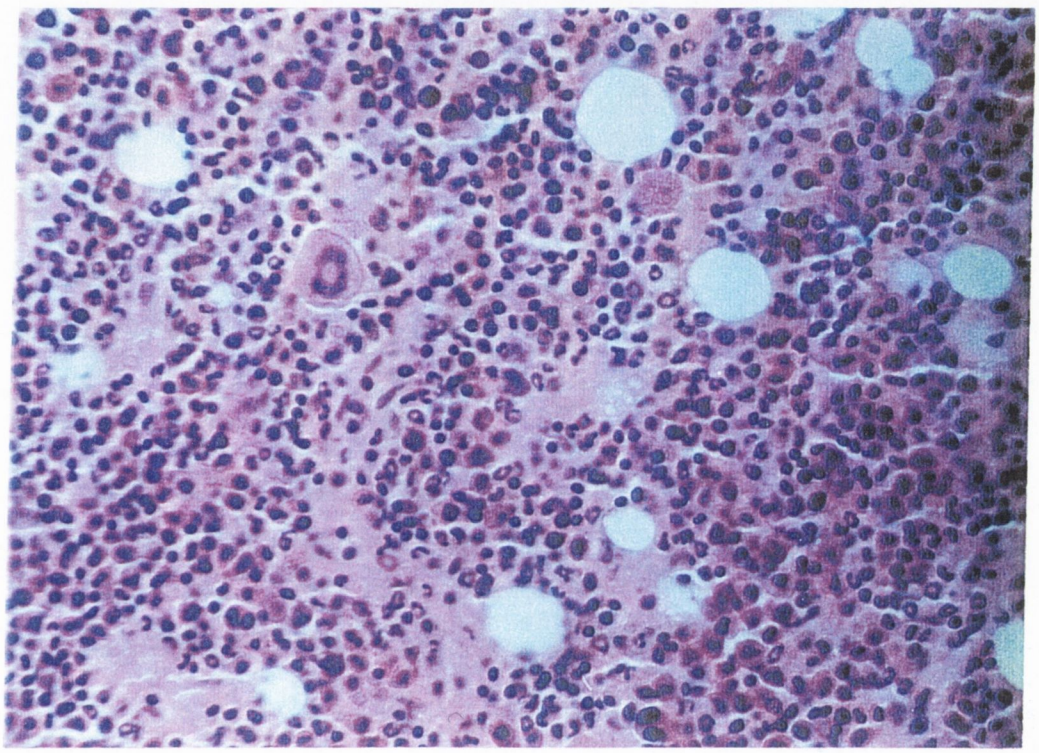


Figure 4.7. A histological section (Haemotoxylin & Eosin stained) of rat femur marrow (X200). The samples were prepared as described in Section 4.3.1 and stained as described in Section 4.3.4 prior to microscopic evaluation. The section shows a normal histological appearance.

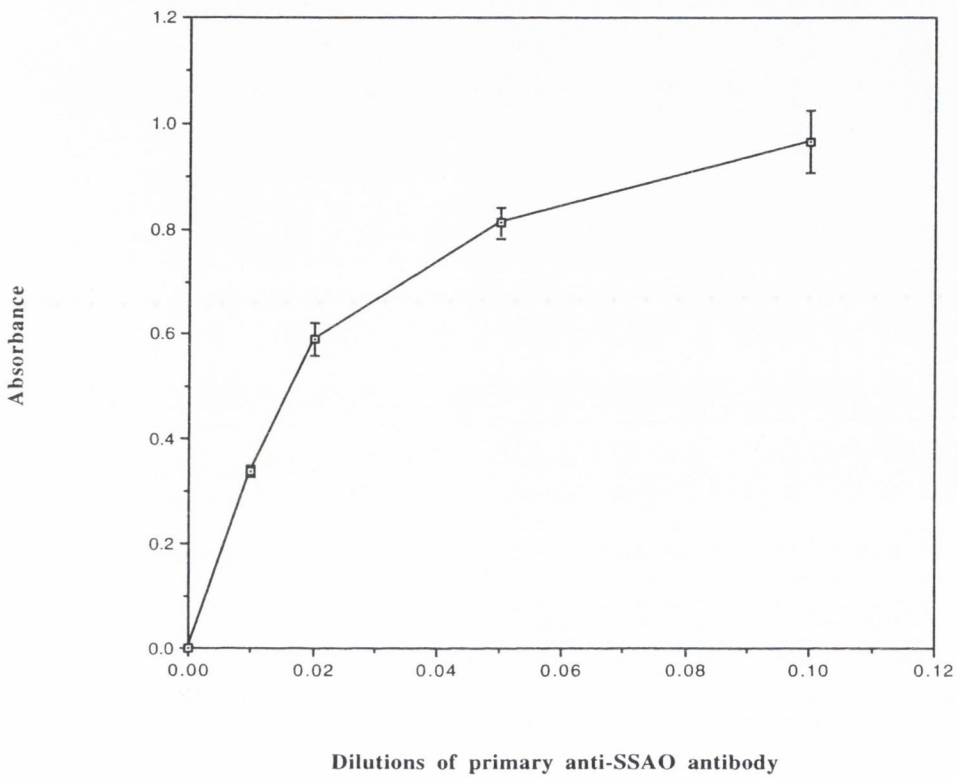


Figure 4.8 ELISA plot demonstrated changes in absorbance with various dilutions of primary anti-SSAO antibodies. Values shown are means ± S.E.M. of the triplicate determinations.

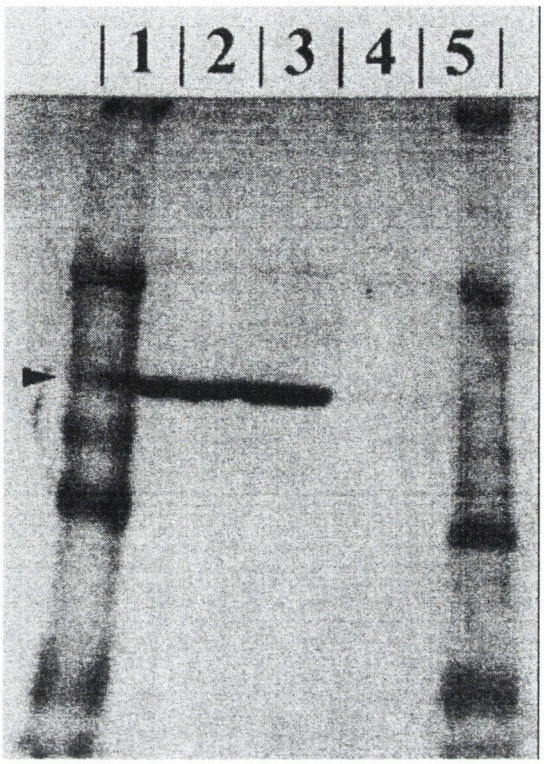


Figure 4.9 Western blot analysis using anti-SSAO antibodies against human dental pulp homogenate. Lanes 1 & 5 are Sigma markers and lanes 2 and 3 contain human dental pulp sample. The arrow corresponds to 110 kDa.

4.4.5 Immunohistochemical Labelling

(i) Anti-SSAO antibodies

None of the blank samples gave significant staining. The 1:200 primary antibody dilution gave the best staining of the tissue preparation. Below this concentration there was increased background staining and above this amount of staining was too faint.

The staining was demonstrated in three areas (Figures 4.10, 4.11 & 4.12). The first was in the odontoblastic layer and this appeared to be more evident in the coronal than the root portion. The second was the lining of the blood vessels, both thin-walled

venules and arterial vessels. The third was in nerve tissue and this was demonstrated in both in transverse and longitudinal sections.

(ii) Anti-tryptophan hydroxylase antibodies

None of the blank samples gave significant staining. The 1:1000 primary antibody dilution gave the best staining of the tissue preparation. All higher concentrations resulted in increased background staining.

Staining was demonstrated in two areas (Figure 4.13, 4.14 & 4.15). The first was in the odontoblastic layer and the second in nerve tissue and this was evident both in longitudinal and transverse sections.

4.4.6 Formazan identification of enzyme site

Formazan crystal formation to identify SSAO activity was noted with the samples incubated with 1 mM benzylamine and 5-HT (in the presence of 10^{-3}M clorgyline). However the staining was very faint and was difficult to distinguish due to the lack of orientation of the sample. Counterstaining with methylene green improved the orientation but obscured some of the staining (Figures 4.16, 4.17 & 4.18) Counterstaining with other dyes (methylene blue, haemotoxylin & eosin) resulted in greater difficulty in localising the formazan crystals. The area that stained most clearly was in the odontoblastic layer. However, the blank samples also demonstrated some staining in that area, but this was to a lesser extent. SSAO activity was clearer when benzylamine was used as a substrate than 5-HT. This is in agreement with the kinetic studies on these substrates in Chapter 2.

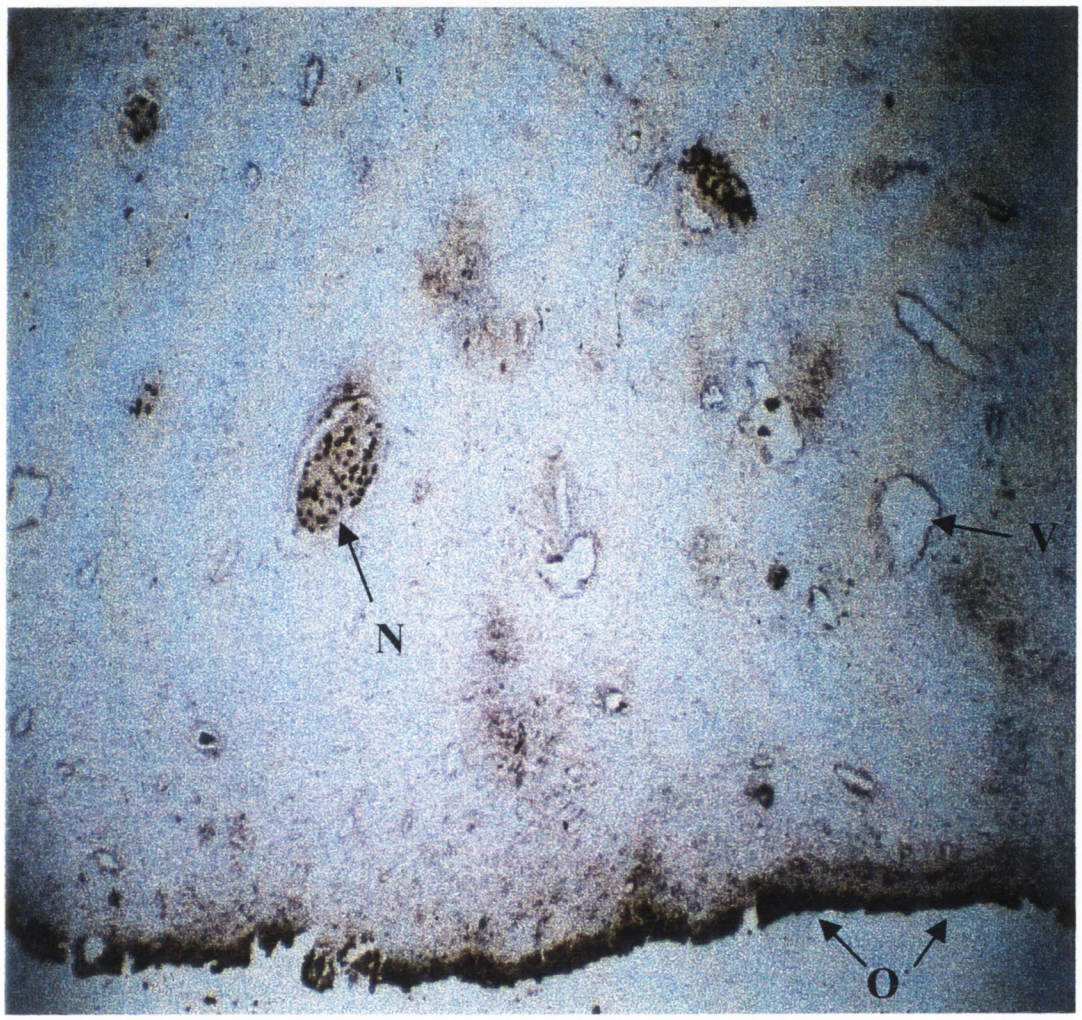


Figure 4.10. A histological section of human dental pulp immuno-stained for SSAO (X200). The samples were prepared as described in Section 4.3.1 and treated with a 1:200 dilution of the primary anti-SSAO antibody and stained using the Vectastain® ABC kit, as described in Section 4.3.5.3 prior to microscopic examination. The view shows the presence of staining in the odontoblast layer (O), venules (V) and neural tissue (N).

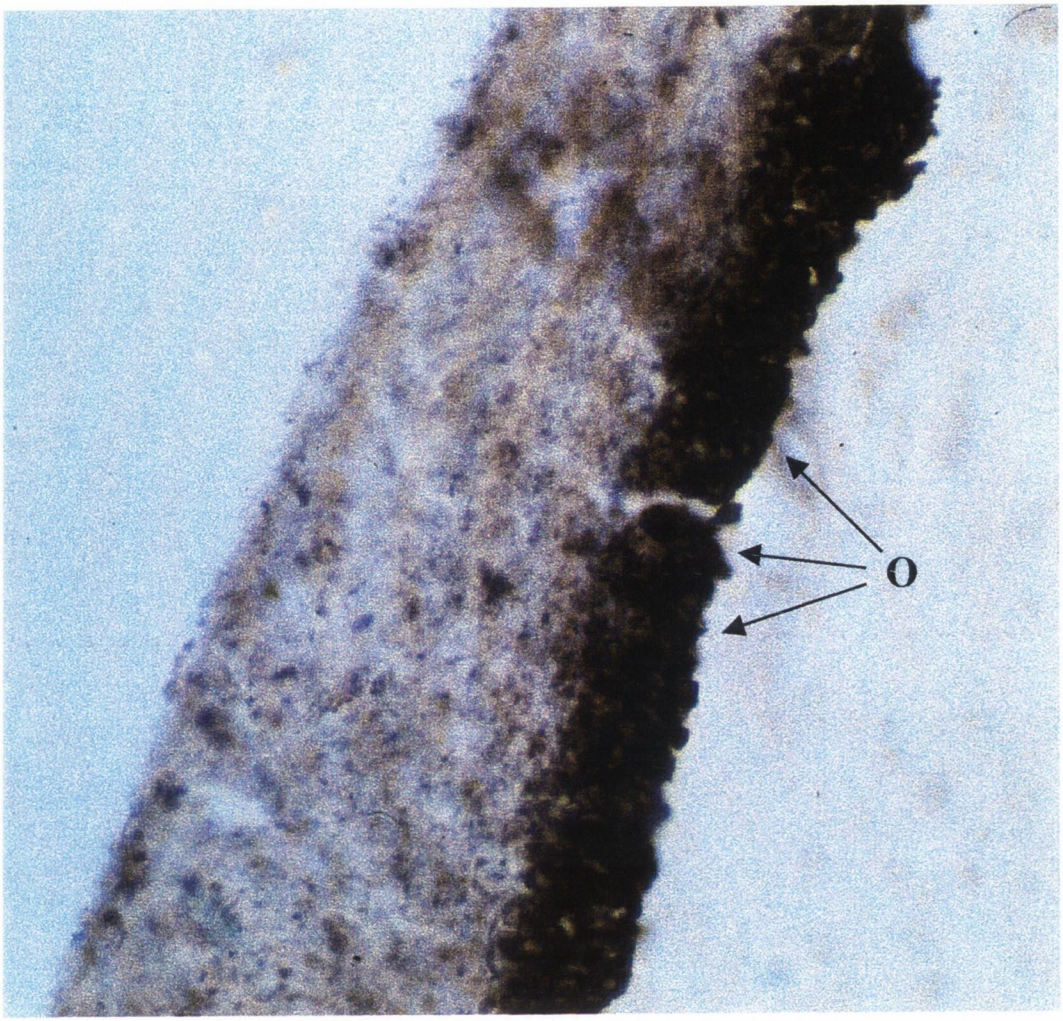


Figure 4.11. A histological section of human dental pulp immuno-stained for SSAO (X400). The samples were prepared as described in Section 4.3.1 and treated with a 1:200 dilution of the primary anti-SSAO antibody and stained using the Vectastain® ABC kit, as described in Section 4.3.5.3 prior to microscopic examination. The section shows the intense staining of the odontoblast layer (O).

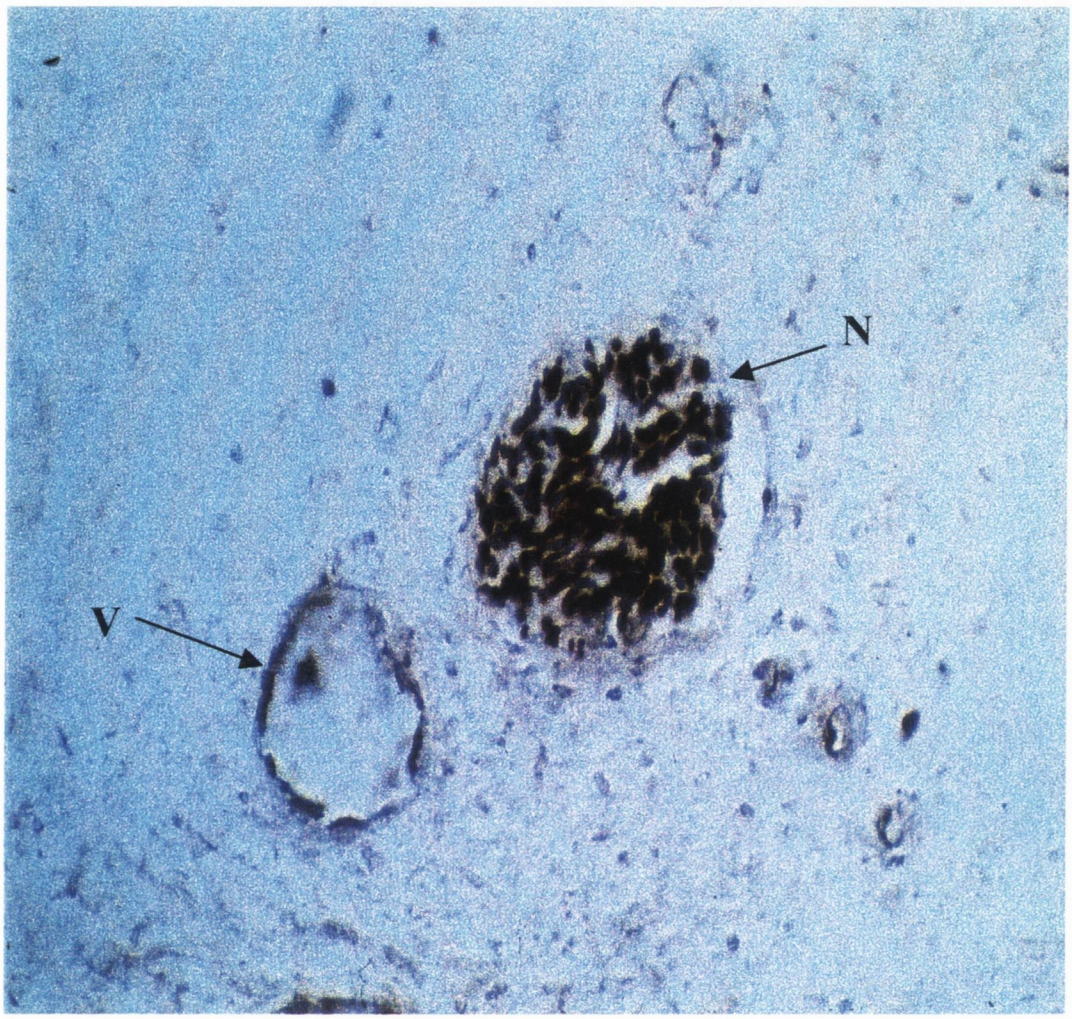


Figure 4.12. A histological section of human dental pulp immuno-stained for SSAO (X500). The samples were prepared as described in Section 4.3.1 and treated with a 1:200 dilution of the primary anti-SSAO antibody and stained using the Vectastain® ABC kit, as described in Section 4.3.5.3 prior to microscopic examination. The section shows staining in a thin-walled blood vessel (V) and nerve tissue (N).

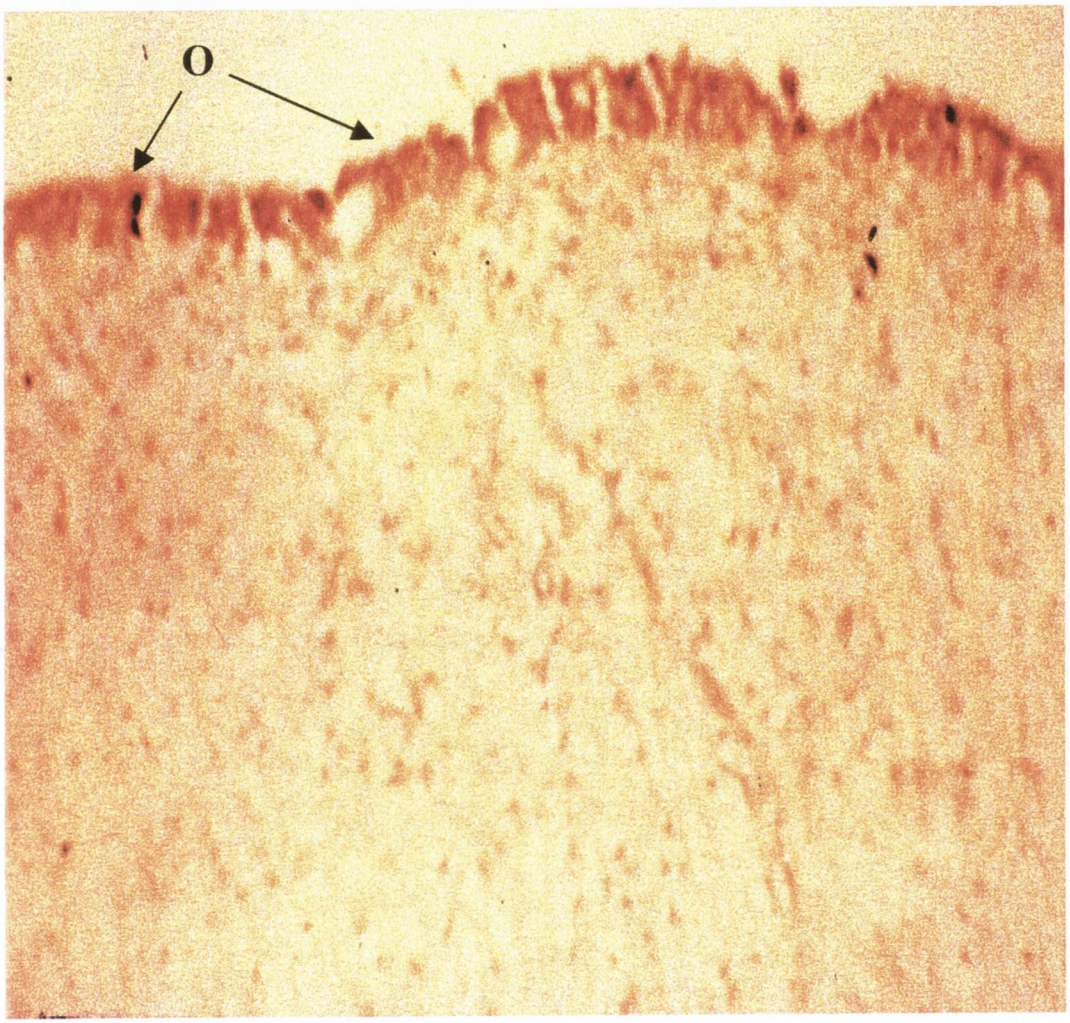


Figure 4.13. A histological section of human dental pulp immuno-stained for tryptophan hydroxylase (X400). The samples were prepared as described in Section 4.3.1 and treated with a 1:200 dilution of the primary anti-tryptophan hydroxylase antibody and stained using the Vectastain® ABC kit, as described in Section 4.3.5.3 prior to microscopic examination. The section shows the staining evident in the odontoblast layer (O).

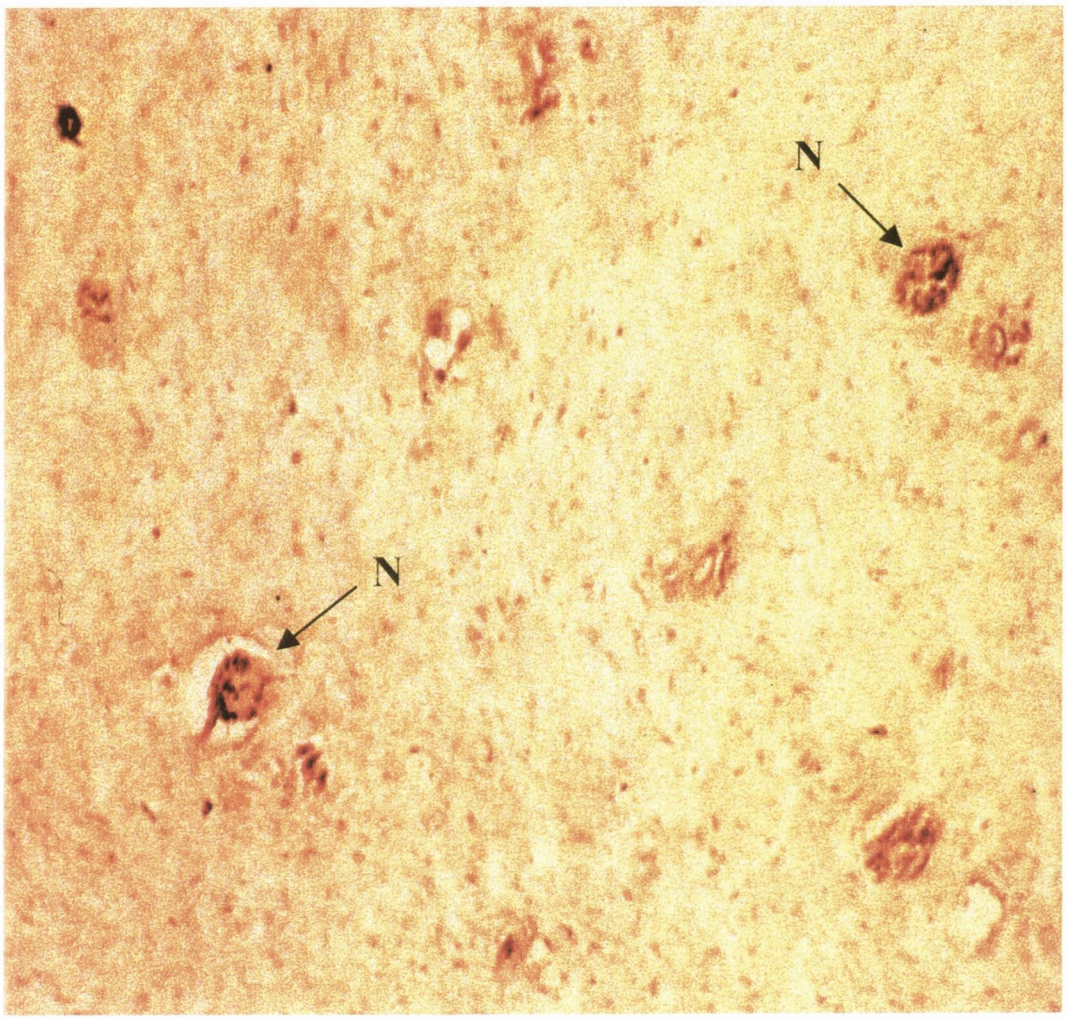


Figure 4.14. A histological section of human dental pulp immuno-stained for tryptophan hydroxylase (X200). The samples were prepared as described in Section 4.3.1 and treated with a 1:200 dilution of the primary anti-tryptophan hydroxylase antibody and stained using the Vectastain® ABC kit, as described in Section 4.3.5.3 prior to microscopic examination. The section shows staining in neural tissue (N).

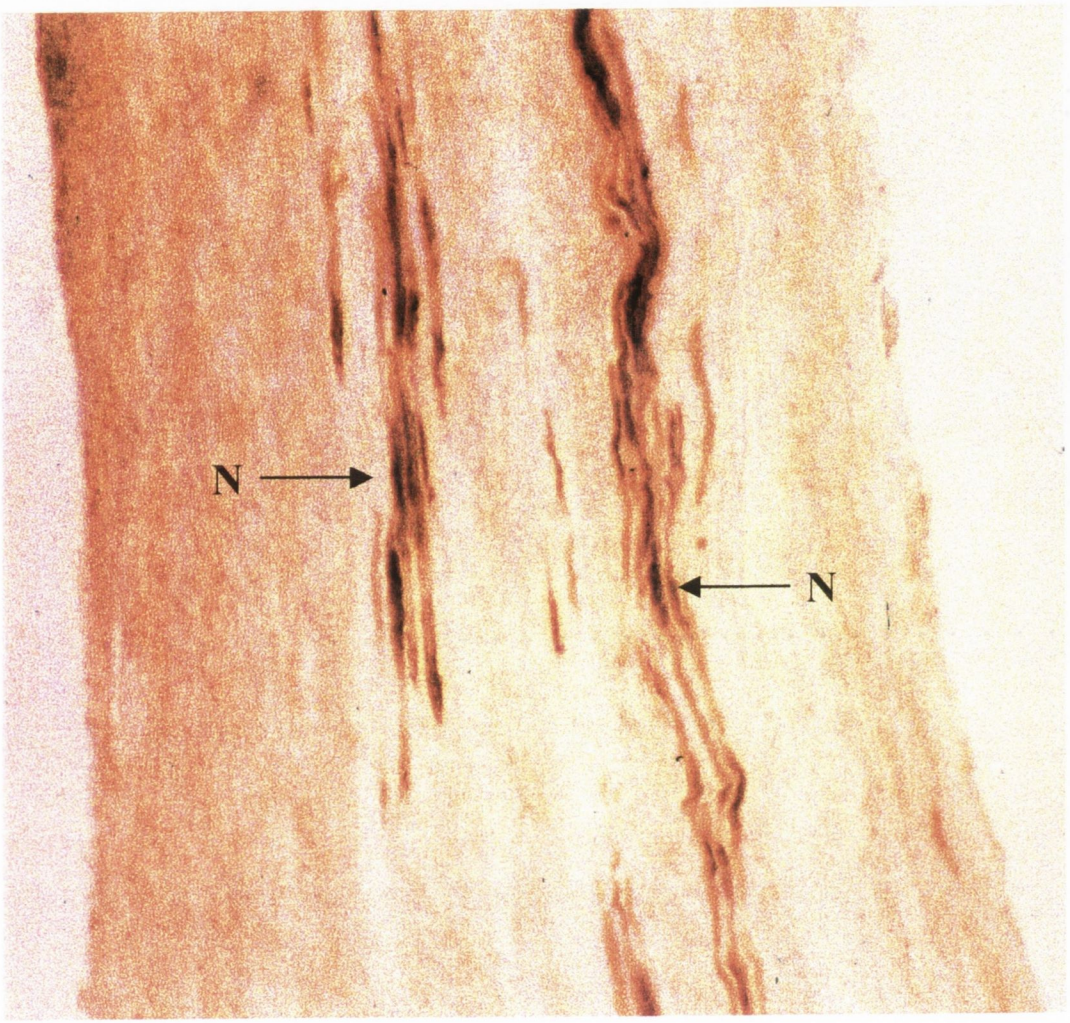


Figure 4.15. A histological section of human dental pulp immuno-stained for tryptophan hydroxylase (X400). The samples were prepared as described in Section 4.3.1 and treated with a 1:200 dilution of the primary anti-tryptophan hydroxylase antibody and stained using the Vectastain® ABC kit, as described in Section 4.3.5.3 prior to microscopic examination. The section shows staining of nerve tissue in longtitudinal section (N).

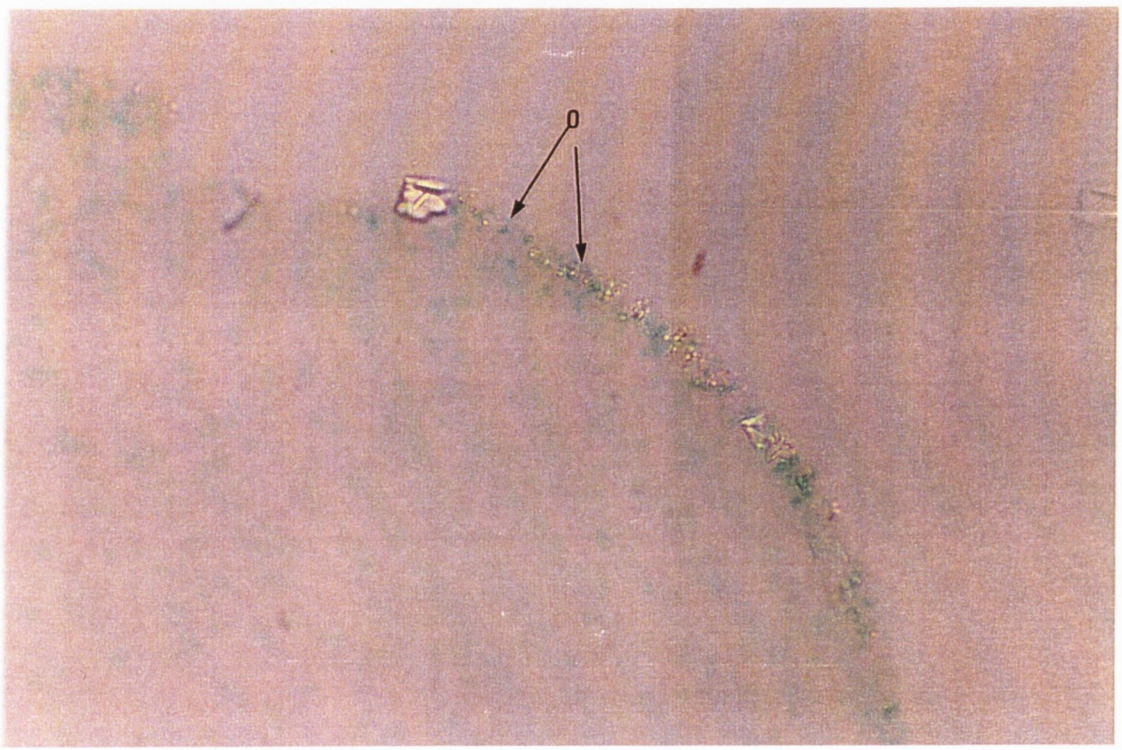


Figure 4.16. A histological section of human dental pulp stained for total amine oxidase activity (X200). Samples were prepared as described in Section 4.3.7 and then incubated in the solution described in Section 4.3.8 in order to identify active amine oxidases through formazan localisation using benzylamine as a substrate. Counterstaining was performed with methylene green before microscopic examination. The odontoblast layer is arrowed (o).

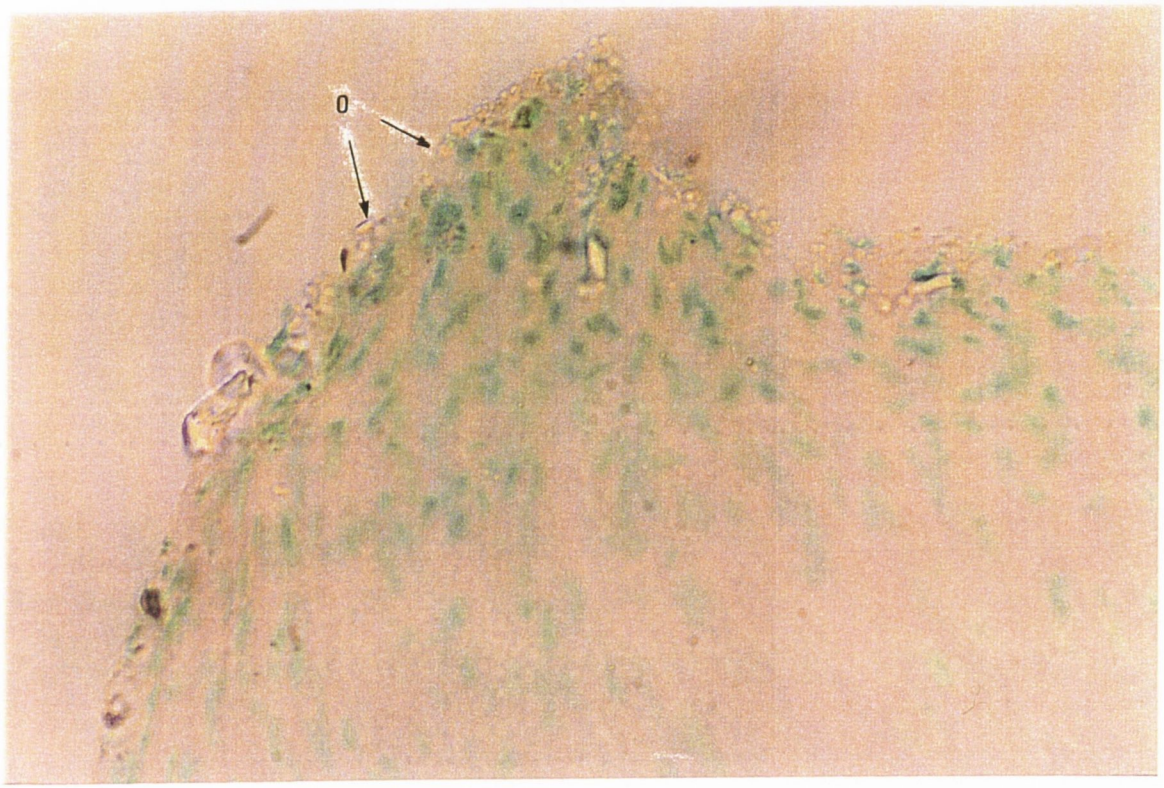


Figure 4.17. A histological section of human dental pulp stained for clorgyline-resistant amine oxidase activity (X400). Samples were prepared as described in Section 4.3.7 and then incubated in the solution described in Section 4.3.8 in order to identify active enzyme sites through formazan localisation using benzylamine as a substrate. Counterstaining was performed with methylene green before microscopic examination. The odontoblast layer is arrowed (o).

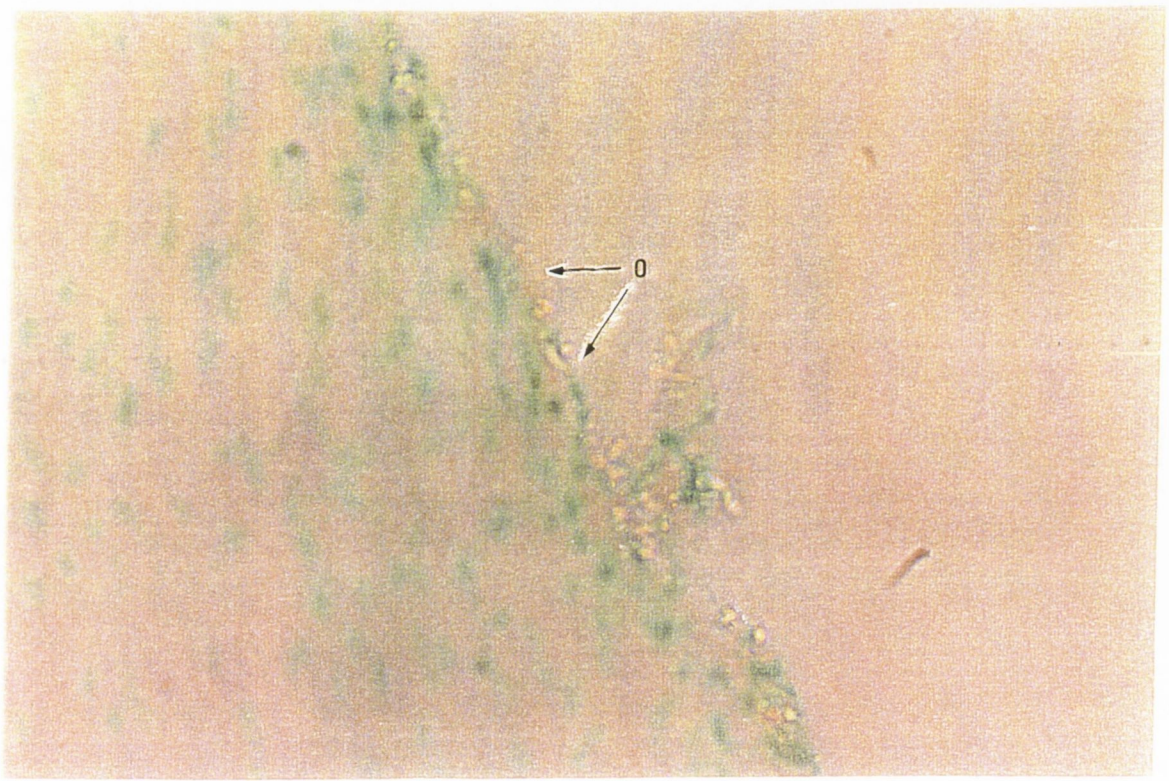


Figure 4.18. A histological section of human dental pulp stained for amine oxidase activity (X400). Samples were prepared as described in Section 4.3.7 and then incubated in the solution described in Section 4.3.8 in order to identify active enzyme sites through formazan localisation using 5-HT as a substrate. Counterstaining was performed with methylene green before microscopic examination. The odontoblast layer is arrowed (o).

4.5 Discussion

This method of obtaining dental pulp tissue for biological manipulation offers useful advantages over those previously used. It is simple and quick to perform. It requires minimal expertise and low-cost and uncomplicated equipment. Polystyrene containers are readily available in most laboratories and can be easily purchased. Carbon dioxide costs in the region of £3 per block. It may be purchased or made more economically using compressed gas and a dry ice machine ('Icemaker', RB Radley, Cambridge, England). A single block may be used for multiple specimens. Samples collected over the course of a session are placed on the ice as they are extracted and the pulps are removed at an appropriate time subsequently.

When the tooth is frozen immediately upon removal, the potential for alteration of physical or biological features of its pulp is considerably reduced in comparison with currently-used techniques. Thus, cell necrosis, membrane breakdown and enzymatic autodigestion in the pulpal tissues should be considerably reduced by the rapid cooling and maintenance at low temperature. LDH assays used as indicators of cell damage demonstrate breakdown of cell integrity if the sample is not frozen immediately and that such changes are minimised by rapid freezing.

The use of dry ice and rapid freezing of the specimens improves the possibility of examining the physiological and biochemical processes occurring in the dental pulp. To date enzyme studies of dental pulp tissues have had to use immature teeth before any root formation has commenced to achieve access for sampling (Numata and Hayakawa 1986). When there is no root the process of pulp removal is facilitated as direct access to the pulp chamber is possible. Little research has been reported on the biological processes in more mature, fully-formed, human teeth. The method of access to the dental pulp described here should permit investigation of aspects of pulp biology that may change rapidly after tooth extraction.

The presence of odontoblastic fibrils in the histological specimens is also unusual. In routine sectioning these processes are normally lost by aspiration (Shovelton, 1968) or ruptured. Reasons for their presence using this technique may include the rapid defrosting of the frozen specimens and the resultant shrinking of the pulp tissue away from the dentine pulling the fibrils from the dentinal tubules. This may explain why the odontoblastic processes were retained.

The presence of SSAO in human blood vessels has been reported previously (Coquil *et al.*, 1973; Lewinsohn, 1981, 1984). It has been postulated that this functions to scavenge circulating amines (Lyles *et al.*, 1990). However, the products of this reaction can in some instances be more toxic than the amine. Physiological examples include the conversion of methylamine to formaldehyde by SSAO in homogenates of rat aorta, human umbilical cord artery and the human plasma enzyme (Precious *et al.*, 1988; Lyles *et al.*, 1990) and metabolism of aminoacetone to form methylglyoxal in human umbilical cord artery (Lyles & Chalmers, 1992) and bovine lung (Lizcano *et al.*, 1994). A non-physiological example is allylamine (3-aminopropene), an industrial alkylamine used in the pharmaceutical industry. It is highly toxic when administered by a variety of routes (Boor & Hysmith, 1987), but this toxicity can be prevented through the use of SSAO inhibitors (Boor *et al.*, 1990) leading to the conclusion that the product acrolein is the damaging element. The production of H_2O_2 and its effects have previously been discussed in Section 1.4.

The presence of SSAO has not been reported previously in human dental pulp neural tissue, nor any other neural tissue. This finding is unique as it suggests function for dental pulp SSAO not observed with other SSAO enzymes. It may be functioning as a enzyme responsible for the breakdown of neurotransmitters, most notably 5-HT. It opens the possibility of neurogenic inflammation being effected directly from 5-HT releasing nerves and its modulation by SSAO.

The SSAO in the odontoblast layer could be from one of three sites. It could be from the dense capillary bed that is located just beneath the odontoblast layer from which capillaries extend to the odontoblastic layer, from the odontoblasts themselves or from

the neural tissue that lies beneath the odontoblasts. From the plexus of Raschkow A-δ fibres emerge from their myelin sheath and branch out to form the subodontoblastic plexus. They then leave the Schwann cells to terminate as a free nerve ending between the odontoblasts, many terminating in the extracellular spaces of the cell rich zone or the odontoblast layer (Gunji, 1982).

In order to locate the presence of a source of 5-HT in human dental pulp anti-tryptophan hydroxylase was employed. It is the specific step in the production of 5-HT and the distribution of tryptophan hydroxylase coincides with the distribution of 5-HT in other tissues and therefore measurement of the site of tryptophan hydroxylase should provide an indirect measure of the location of 5-HT. Thus antibodies raised against tryptophan hydroxylase should detail the location of 5-HT in the tissue (Cash *et al.*, 1985). The 5-HT pathway has been discussed in Section 1.2.2.

Tryptophan hydroxylase was stained in preference to the detection of 5-HT in the pulpal tissue since, if this were labelled there could be many false positive results from 5-HT present in blood vessels and in plasma that had leaked from capillaries. Thus this would not have reflected the indigenous 5-HT. Similarly, localisation of the breakdown products of 5-HT would have presented the same difficulties.

Tryptophan hydroxylase was demonstrated in two areas one of which was in neural tissue. The presence of 5-HT reactive nervous tissue has not been previously described for human dental pulp. Circulating levels of 5-HT have been used in studies of inflammation, but it was assumed that the 5-HT was extra-neuronal in origin (Kerezoudis *et al.*, 1994). Serotoninergic neurones are principally located in the central nervous system with cell bodies in the raphe area and with terminals distributed throughout the CNS. They have also been identified in the peripheral nervous system, principally in the gastro-intestinal tract.

The release of 5-HT by nervous tissue could contribute to the inflammatory process itself by causing nociceptor activation (see Goodman & Gilman, 1975) and potentiation of the pain producing activity of bradykinin (Sicuteri, 1968). It may also

amplify the local effect of other mediators to promote aggregation of platelets (De Clerck & David 1981, De Clerck & Herman 1983).

The other site where tryptophan hydroxylase was demonstrated was in the odontoblastic layer. Here, as for the SSAO, it may be present either in association with actual odontoblast cells or with the nerves terminals that are intimately associated with them. Release of 5-HT in this area may have a direct role in inflammation, but its metabolism may have a second messenger function via the release of H_2O_2. Burdon (1995) suggested that there exists a balance between the level of hydrogen peroxide that causes cell proliferation and that that causes lipid peroxidation and cell death. Increased levels cause an initial proliferation but if these are prolonged may result in death of the cell.

In response to the invasion of bacteria and their products one of two histological events are observed in human teeth *in vivo*. The first is when there is a low-grade inflammation in the dental pulp. The reaction involves the deposition of reactionary dentine on the surface of the pulp chamber beneath the advancing dental caries. This dentine is in general, imperfectly formed and the degree of irregularity depends on the degree of irritation of the odontoblasts (for review see Soames & Southam, 1989). The function of the reactionary dentine is to increase the depth of tissue between the pulp and the advancing bacteria. The second observed pattern is where the bacterial load and rate of demineralisation of enamel and dentine is so great that the odontoblasts do not produce dentine but undergo rapid necrosis, resulting in more rapid bacterial infection of the pulp chamber.

It could be postulated that the 5-HT in the area will be increased in the presence of inflammation and that this will lead to an increase in H_2O_2 produced from the breakdown of the 5-HT by SSAO. The H_2O_2 in turn may have an effect on the odontoblast function and its ability to lay down protective reactionary dentine. It may be similar to the circumstances described by de Lamirande and Gagnon (1995) for human spermatozoa, where a low level of H_2O_2 may stimulate, while a high level may cause cell death.

4.6 Conclusions

The following conclusions were drawn from the experiments detailed:

1. Human dental pulp could be isolated in a histologically undisturbed manner from human third molar teeth.
2. This method of freezing/crushing may be applied to other calcified tissues, *e.g.* rat femur.
3. To retain the integrity of the pulpal samples they should be frozen as soon as possible upon extraction.
4. The antibodies raised in rabbit against bovine lung SSAO demonstrate cross-reactivity with human dental pulp SSAO.
5. Immunohistochemical labelling of specimens of human dental pulp with bovine anti-SSAO and avidin-biotin complex immunolabelling demonstrated the presence of SSAO in the odontoblastic layer, the lining of the blood vessels and in nerve tissue.
6. Immunohistochemical labelling of specimens of human dental pulp with monoclonal anti-tryptophan hydroxylase antibodies and avidin-biotin complex immunolabelling demonstrated the presence of 5-HT in the odontoblastic layer and nerve tissue.
7. Histological demonstration of SSAO in frozen sections of human dental pulp by localising the oxidative process with formazan dye demonstrated the presence of the enzyme in the odontoblastic layer, but staining was faint.

CHAPTER FIVE

The Response of Cultured Murine Odontoblasts to Oxidative Stress

5.1 Introduction

Oxidative deamination of biogenic amines by amine oxidases releases hydrogen peroxide as a product of the reaction. Dental interest to date in hydrogen peroxide release has been on its effect on blood flow, as blood flow is decreased in dog dental pulp in response to free radicals (Okabe, 1994). The exact mechanism by which free radicals induce changes in vascular conductance is still unclear, they may act directly on vascular smooth muscle or modify vascular tone by causing the production of, or altering the activity of, endogenous vasoactive mediators (Okabe, 1994). The effects of oxidative stresses on odontoblasts and its sequelae have not been reported. The biological effects of hydrogen peroxide on various cultured cells have been discussed in Section 1.4.

The culturing of human dental pulp has proven difficult to date. In a study of donor variability in the proliferation of human dental pulp fibroblasts Moule *et al.* (1995) found that "in addition to the difficulties and variability in establishing cell lines, the human pulp fibroblasts also showed great variability in proliferative activity which could not be accounted for by donor age, source or passage number". Of the 49 explants cultured only 3 survived long-term passage and freezing and the time to first passage varied from 8-43 days (mean 24 $\pm$ 11).

In the dental literature most of the cell culture work is carried out *in vivo* on explants from a single tooth with the experiment running over 1-3 days. This leads to problems with the interpretation of the findings of these studies. Ideally, an established cell line is preferable to ensure that the results will be reproducible and therefore to allow more definite conclusions to be drawn.

Murine odontoblast cell lines (MO6G3) have been successfully immortalised (MacDougall *et al.*, 1995). Essentially, molars from E-18 Swiss Webster neo-natal mice were dissected, the dental papilla mesenchyme isolated, and pulp cells dissociated. Pulp cells (5×10^5 /well) were plated as monolayers and grown in alpha-MEM supplemented with 10 % FCS, 100 units/ml penicillin and streptomycin, 50 µg/ml ascorbic acid. Cultures were maintained for 6 days at 37 °C in a humidified

atmosphere of 95 % air and 5 % CO_2, with media changes every two days. Immortalisation was performed using a recombinant defective retrovirus containing the temperature-sensitive SV-40 large T antigen cDNA and the neomycin (G418) resistance gene recovered from CRE packaging cells. Cultures were infected for 24 hours with CRE conditioned medium containing 8 μg/ml of polybrene, the media was replaced with selective media containing 300 μg/ml of G418, and the cultures incubated at 33 °C for one month with media changes every 3-5 days. Neomycin resistant cells were cloned by serial dilution to single cells in 96-well culture plates and grown in selection medium at 33 °C.

5.2 Materials

5.2.1 Sources of Reagents

Reagents are listed in alphabetical order. The source of named reagents is indicated by the suppliers' name.

PRODUCT	SUPPLIER
Inhibitors	
Clorgyline hydrochloride	Sigma Chemicals
Semicarbazide hydrochloride	Sigma Chemicals
3-amino-1,2,4-triazole	Sigma Chemicals
Substrates	
Ascorbic acid	Sigma Chemicals
Foetal calf serum	Gibco Ltd.
Penicillin /streptomycin	Sigma Chemicals
Glutamine	Sigma Chemicals
Trypsin	Sigma Chemicals
5-HT creatine sulphate	Sigma Chemicals
Hydrogen peroxide	Sigma Chemicals
α-MEM medium	Sigma Chemicals
Miscellaneous Reagents	
RAPI-DIFF II stain pack	

MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide)	Sigma Chemicals
Poly-prep glass slides	Sigma Chemicals
Shandon filter cards	Life Sciences
Cytotoxicity Kit	Boehringer Mannheim
DPX mountant	Sigma Chemicals

All other general laboratory reagents were purchased from Sigma Chemicals, BDH Chemicals or Lennox Laboratories

5.2.2 Addresses of Suppliers

The full names and addresses of suppliers are listed below in alphabetical order:

Boehringer Mannheim	Boehringer Mannheim, Beverly, Massachusetts, USA.
Gibco	Gibco BRL Life Technologies, Gaithersburg, Maryland, USA.
Life Sciences	Life Sciences Ltd.. Shandon Corporation, Pittsburgh, USA.

5.3 Methods

5.3.1 Media

MO6G3 cells were seeded at a density of 1X 10^5 cells /ml for all experiments. The standard medium (M_S) used contained 20 % foetal calf serum (FCS), 100 units /ml penicillin and streptomycin, units /ml and 50μg /ml of ascorbic acid (MacDougall *et al.*, 1995). Two other similar media were used. The first contained 10 mM of the

catalase inhibitor, 3-amino-1,2,4-triazole, (Ziemann *et al.*, 1999) in addition to the standard medium (M_{cat}) and in the second the FCS had been pre-incubated at 37 °C for 30 min with clorgyline and semicarbazide (Final concentrations 10^{-3}) to remove all amine oxidase activity (M_{ao}).

5.3.2 MTT Assay

The MTT assay is a quantitative assay of cell viability based on the principal that live cells can turn a tetrazolium salt, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide), into a dark formazan product (for review see Mossmann, 1983). The reaction takes place in the mitochondria of the cells. The advantages of the assay are that it is rapid and does not require the use of a radioisotope.

The following protocol was used:

1. The MO6G3 cells from the 3rd or 4th passage and were seeded 24 hours beforehand in 96 well plates in standard medium at 33 °C in a 5 % CO_2 /air mixture. 1 hour before the addition of the test material the media were changed to one of the 3 media (M_s, M_{cat} and M_{ao}).
2. Hydrogen peroxide was added to cells in either M_s or M_{cat} at a final concentration of 10^{-10} – 10^{-2} M. 5-HT was added to second series of cells in either M_s or M_{ao} at a final concentration of 10^{-9} – 10^{-2} M. Assays of cell viability were performed at 10, 23 and 36 hours.
3. A stock of MTT was made by dissolving 5 mg/ml of MTT in HBS and frozen in aliquots of 200 µl. Before use the aliquots were thawed and diluted to 5 ml in HBS.
4. 50 µl of stock solution was added to the 200 µl of cell containing medium in each well. Blanks were included where medium alone was used. Cultures were incubated at 37 °C for 30 min in the dark.
5. The medium was then aspirated off and 25 µl of Sorenson's buffer added to each well (Sorenson's buffer: 0.1 M glycine, 0.1 M NaCl, adjusted to pH 10.5 with 0.1 M NaOH).
6. 200 µl of DMSO was added to each well and incubated for 10 minutes.
7. The absorbance was recorded at 570 nm in a microtitre-plate reader ($Thermo_{max}$ Microplate Reader).

5.3.3 Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme released upon cell lysis and can be assayed in the supernatant. The proportion of LDH can then be calculated for a given supernatant sample. An increase in the amount of dead or plasma membrane-damaged cells will result in an increase in LDH activity in the culture supernatant (Korzeniewski *et al.*, 1983). A colourimetric assay has been developed that measures the amount of lactate through a coupled assay that results in the reduction of a tetrazolium salt INT to a formazan dye. This dye has a broad absorption maximum at 500 nm.

$$NAD^+ + \text{lactate} \xrightarrow{\text{LDH}} \text{pyruvate} + NADH$$

$$NADH + \text{tetrazolium salt INT} \xrightarrow{\text{diaphorase}} NAD^+ + \text{formazan}$$

The following protocol was used:

1. The MO6G3 cells from the 3rd or 4th passage and were seeded 24 hours beforehand in 96 well plates in standard medium at 33 °C in a 5 % CO_2 /air mixture. 1 hour before the addition of the test material the media were changed to one of the 3 media (M_s, M_{cat} and M_{ao}).
2. Hydrogen peroxide was added to cells in either M_s or M_{cat} at a final concentration of 10^{-10} — 10^{-3} M. 5-HT was added to second series of cells in either M_s or M_{ao} at a final concentration of 10^{-9} — 10^{-3} M. Assays of cell viability were performed at 10, 23 and 36 hours.
3. 100 µl of supernatant was recovered from 96-well plates containing MO6G3 cells.
4. This was mixed with 100 l of reaction mixture (Cytotoxicity Kit, Boehringer).
5. Negative controls used 100 µl of medium in the place of supernatant and positive controls consisted of MO6G3 cells that were incubated in Triton X-100 (final 1 % v/v) for 1 hour prior to addition of 100 l of reaction mixture.
6. The plates were incubated at for 30 min.
7. The absorbance (Abs) was read on a plate reader at 500 nm.

8. The percentage LDH released was calculated using the formula:

$$\% \text{ LDH release} = \frac{[(\text{A sample}) - (\text{A negative control})] * 100}{(\text{A positive control}) - (\text{A negative control})}$$

5.3.4 Morphological staining of odontoblast cells

Morphdogical staining differentially stains nuclear material and cytoplasm. This enablesthe characteristic signs of necrosis and apoptosis to be highlighted. Signs of necrosis include nuclear swelling, chromatin flocculation and cell membrane lysis, whereas signs of apoptosis include cell membrane blebbing, nuclear shrinkage and chromain condensation, cytoplasmic constriction and the formation of apoptotic bodies. Gorman, 1994).

1. Murine odontoblast cells were seeded at 1 X 10^5 cells /ml were seeded onto 24-wellplates and incubated overnight at 33 $^{\circ}C$ in a 5 % CO_2 /air mixture.
2. 1 hour before the addition of the test material the media were changed to one of the 2 media (M_{cat} or M_{ao}).
3. Hydrogen peroxide was added to cells in M_{cat} at a final concentration of 0-750 µM.5-HT was added to second series of cells in M_{ao} at a final concentration of 0-750 µM.
4. Morphological staining was carried out 10 hours after the addition of hydrogen peroxide /5-HT.
5. Cells were gently scraped and resuspended in standard medium. 100 µl of cell suspension was loaded into a cytospin chamber and spun for 2 min at 200 rpm.
6. Slides were then air-dried for 15 min.
7. Stairing was initiated by placing the slide in fixative for 10 s (100 % methanol).
8. The slides were drained and placed in acid dye for 10 s (eosin Y (0.1 %, w/v), formaldehyde (0.1 %, w/v), sodium phosphate dibasic (0.4 %, w/v), potassium phosphate monobasic (0.4 %, w/v)).

9. The slides were drained and placed in basic dye for 10 s (methylene blue-polychrom (0.4 %, w/v), azure A (0.4 %, w/v), sodium phosphate dibasic (0.4 %, w/v), potassium phosphate monobasic (0.4 %, w/v)).
10. The slides were rinsed in water and air-dried. Samples were then mounted in DPX mountant.
11. The cells were viewed by light microscopy at X 40 magnification. 100 cells in three fields were counted and the number of necrotic, apoptotic and normal cells counted.

5.4 Results

5.4.1 MTT assay

The addition of hydrogen peroxide to MO6G3 cells in standard medium had little effect on cell viability in the concentration range 10^{-8} – 10^{-4} M. However, at higher concentrations a marked decrease in cell viability was observed, to less than 20 % of controls at concentrations of 10^{-3} and 10^{-2} M. At 10^{-9} M a small increase in cell viability was observed (Figure 5.1a).

Addition of 10 mM 3-amino-1,2,4-triazole to the standard medium in order to inhibit endogenous catalase activity present in the medium produced a similar pattern when the cells were exposed to hydrogen peroxide (Figure 5.1b).

The addition of 5-HT to MO6G3 cells in standard medium demonstrated a different pattern to that observed for hydrogen peroxide. Addition of 5-HT decreased cellular viability over 10 and 23 hours in a dose-dependent manner. This effect was transient, with the activity returning to that of controls at 36 hours (Figure 5.2a).

Inhibiting the amine oxidase activity of the standard medium did not alter the cellular response seen with standard medium alone (Figure 5.2b).

The effect of 5-HT did not result in as much loss of cell viability at higher concentrations as hydrogen peroxide. The highest concentrations tested (10mM) resulted in 30-40 % loss of viability. The effect of hydrogen peroxide persisted, whereas the cell viability returned to that of the controls when challenged with 5-HT.

The addition of 1 mM of both semicarbazide and clorgyline to the medium decreased cell viability, as measured by assaying MTT. This decrease was most pronounced (76 % of control activity) at 10 hours, less so at 23 hours (82 % of control) and returned to near control levels at 36 hours (3 % of control). The addition of 10 mM of 3-amino-1,2,4-triazole caused an increase in cell growth at 10 hours (120 % of control) returning to near control levels at 23 and 36 hours (106 and 110 % of control, respectively) (Figure 5.3).

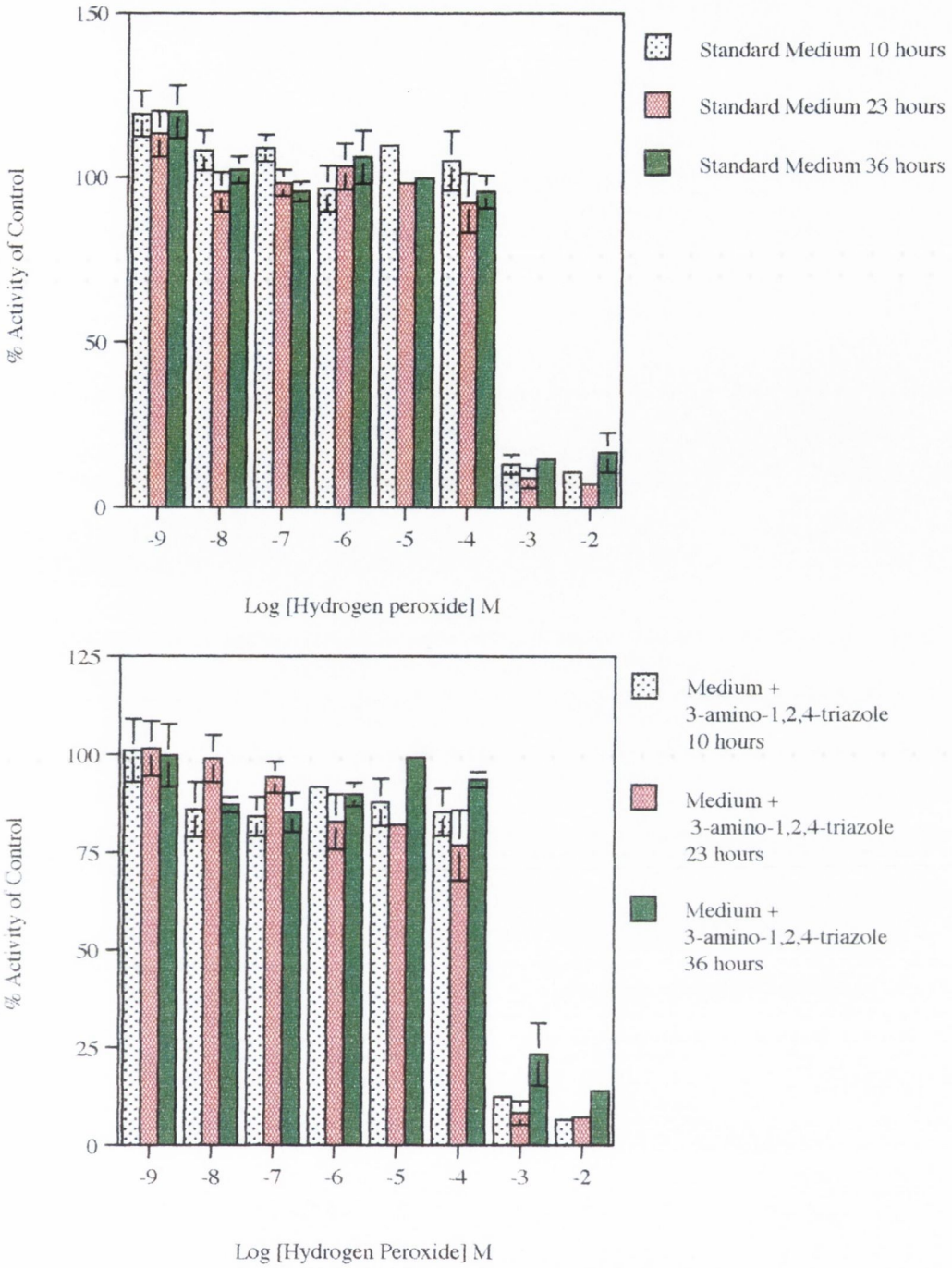


Figure 5.1a & b. The effect of hydrogen peroxide on odontoblast cell viability. MO6G3 cells from the 3rd or 4th passage and were seeded 24 hours beforehand in 96 well plates in standard medium at 33 °C in a 5 % CO_2 /air mixture. 1 hour before the addition of the test material the media were changed to one of the 2 media. Hydrogen peroxide was added to cells in either standard medium (a) or medium containing a catalase inhibitor (b) (10 mM of 3-amino-1,2,4-triazole) at a final concentration of 10^{-10}–10^{-2} M. MTT assays were performed at 10, 23 and 36 hours as described in Section 5.3.2 and compared to controls in the respective media. Each value is the mean ± SEM of experiments completed in triplicate.

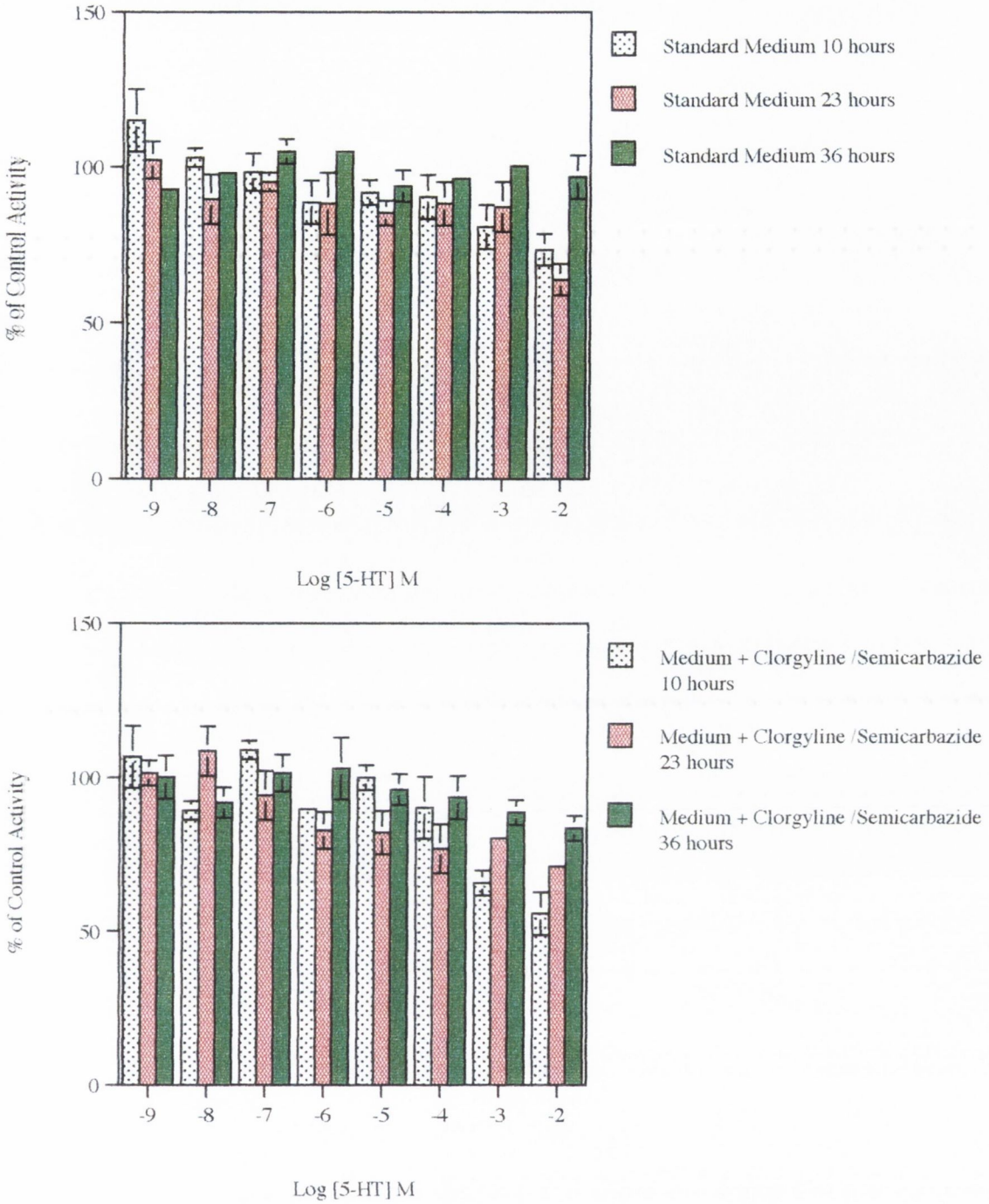


Figure 5.2a & b. The effect of 5-HT on odontoblast cell viability. MO6G3 cells from the 3rd or 4th passage and were seeded 24 hours beforehand in 96 well plates in standard medium at 33 °C in a 5 % CO_2 /air mixture. 1 hour before the addition of the test material the media were changed to one of the 2 media. 5-HT was added to cells in either standard medium (a) or medium containing amine oxidase inhibitors (b) (1 mM of both semicarbazide and clorgyline) at a final concentration of 10^{-10} – 10^{-2} M. MTT assays were performed at 10, 23 and 36 hours as described in Section 5.3.2 and compared to controls in the respective media. Each value is the mean ± SEM of experiments completed in triplicate.

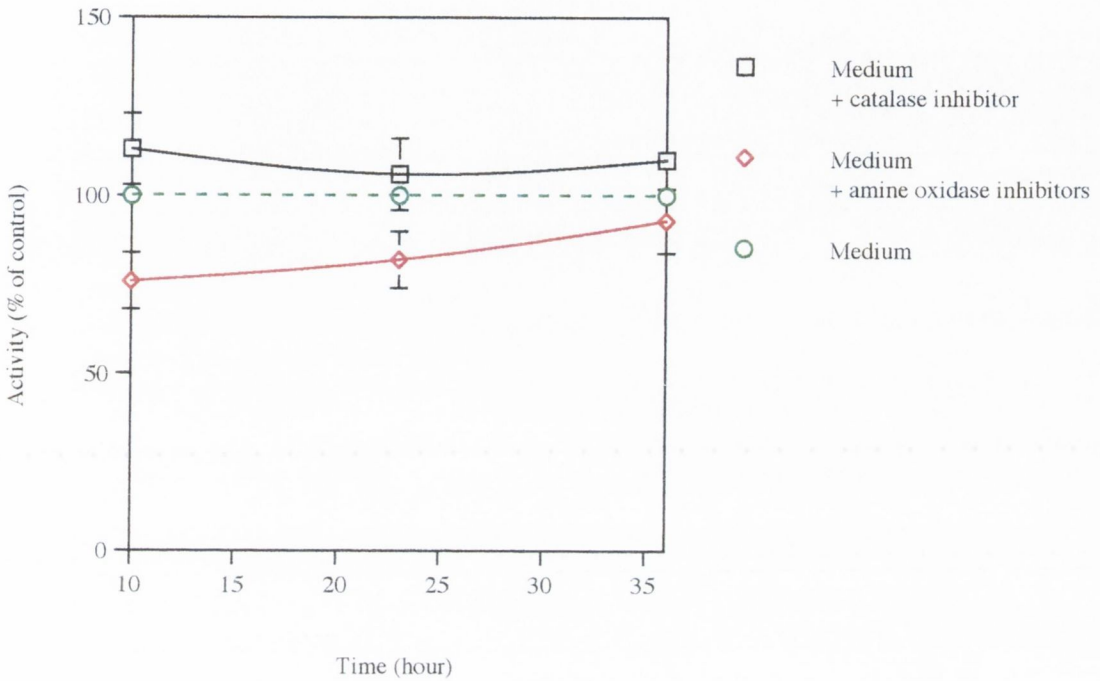


Figure 5.3 The effect on murine odontoblast viability of addition of 1 mM of both semicarbazide and clorgyline or of 10 mM 3-amino-1,2,4-triazole to the standard medium, compared to the standard medium alone. MO6G3 cells from the 3rd or 4th passage and were seeded 24 hours beforehand in 96 well plates in standard medium at 33 °C in a 5 % CO_2 /air mixture. The media were changed to one of the 2 media 10, 23 and 36 hours before MTT assays were performed as described in Section 5.3.2. Each value is the mean ± SEM of experiments completed in triplicate.

5.4.2 LDH Assay

The addition of hydrogen peroxide to MO6G3 cells in standard medium showed little LDH release in the concentration range 10^{-8}–10^{-4} M. However, at higher concentrations a marked increase in cytosolic LDH was observed at concentrations of 10^{-3} and 10^{-2} M, where essentially all the cells appeared to rupture (Figure 5.4a).

Addition of 10 mM 3-amino-1,2,4-triazole to the standard medium in order to inhibit endogenous catalase activity present in the medium produced a similar pattern when the cells were exposed to hydrogen peroxide with total release of LDH at a H_2O_2 concentration of 10^{-2} M (Figure 5.4b). However at 10^{-3} M the LDH release was slightly lower than when compared to the standard medium.

The addition of 5-HT to MO6G3 cells in standard medium demonstrated a different pattern to that observed for hydrogen peroxide. Addition of 5-HT to cells in standard medium caused little release of LDH over the first 2 days (<10 %), but this increased to 20-25 % when measured at 36 hours (Figure 5.5a).

Inhibiting the amine oxidase activity of the standard medium produced a similar trend, but there was a marked increase in LDH release at 23 hours. This release was similar to that at 36 hours at the same concentration of 5-HT (Figure 5.4b).

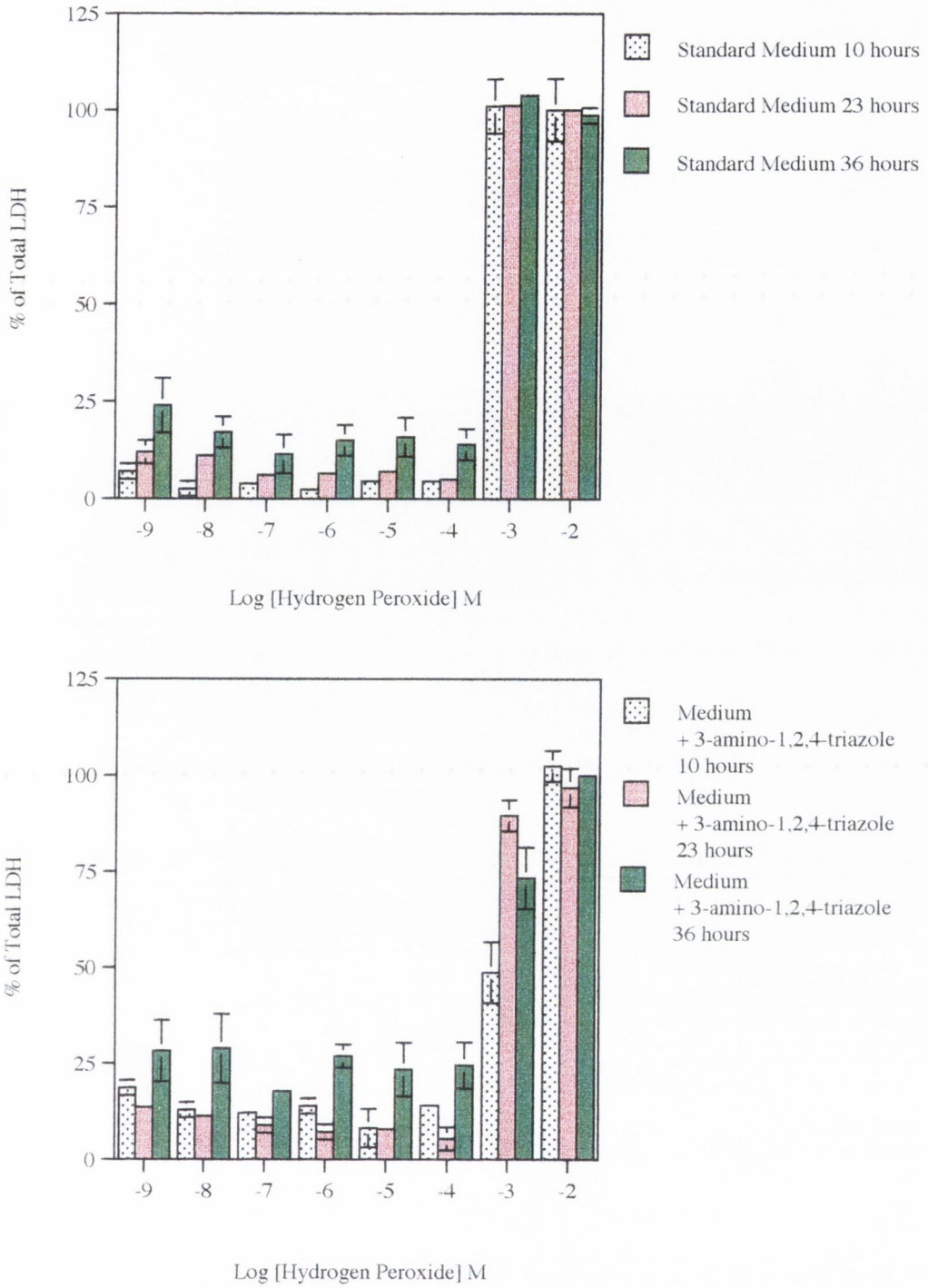


Figure 5.4a & b. The effect of hydrogen peroxide on odontoblast cell viability. MO6G3 cells from the 3rd or 4th passage and were seeded 24 hours beforehand in 96 well plates in standard medium at 33 °C in a 5 % CO_2 /air mixture. 1 hour before the addition of the test material the media were changed to one of the 2 media. Hydrogen peroxide was added to cells in either standard medium (a) or medium containing a catalase inhibitor (b) (10 mM of 3-amino-1,2,4-triazole) at a final concentration of 10^{-10} – 10^{-2} M. LDH assays were performed at 10, 23 and 36 hours as described in Section 5.2.3. Each value is the mean ± SEM of experiments completed in triplicate.

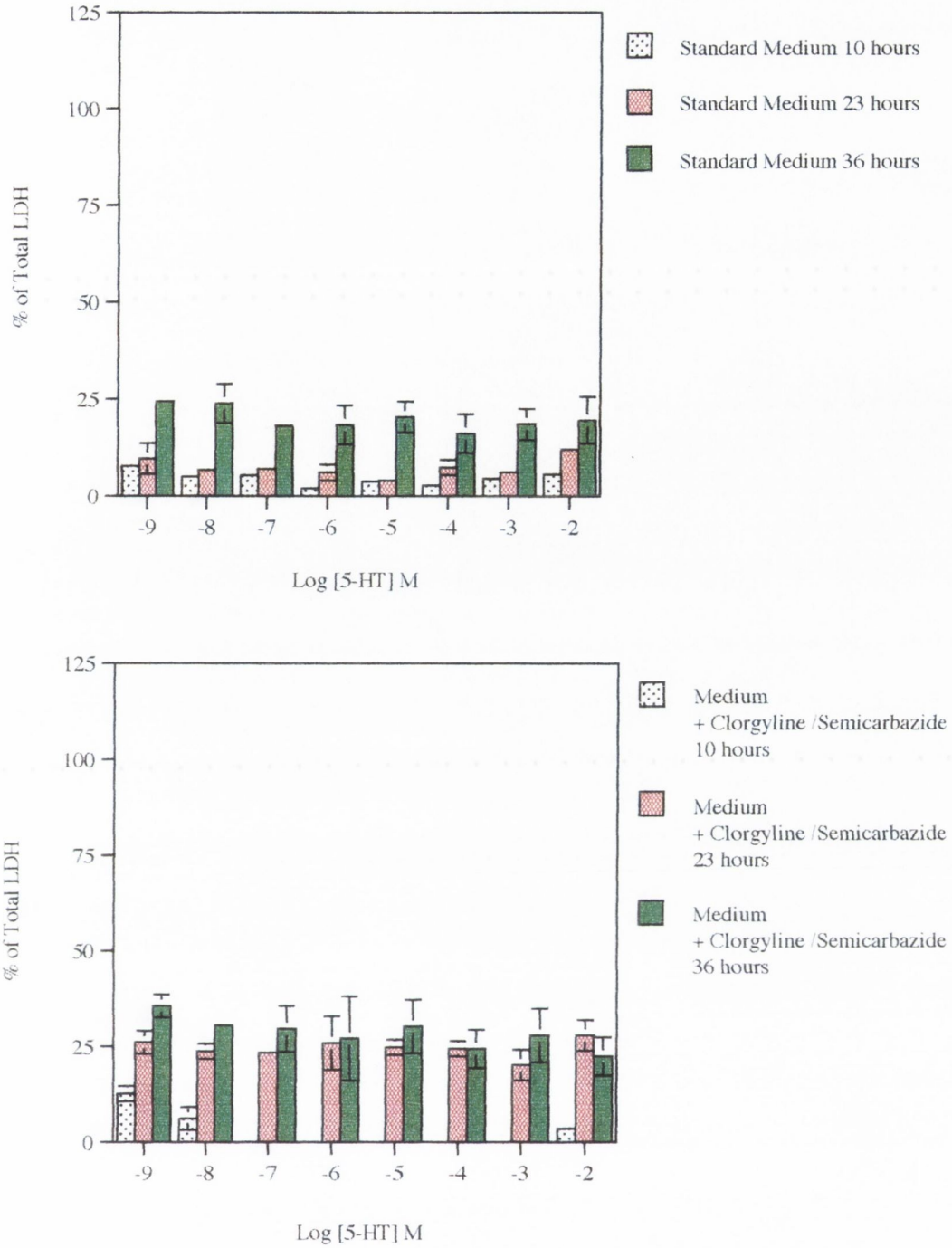


Figure 5.5a & b. The effect of 5-HT on odontoblast cell viability. MO6G3 cells from the 3rd or 4th passage and were seeded 24 hours beforehand in 96 well plates in standard medium at 33 °C in a 5 % CO_2 /air mixture. 1 hour before the addition of the test material the media were changed to one of the 2 media. 5-HT was added to cells in either standard medium (a) or medium containing amine oxidase inhibitors (b) (1 mM of both semicarbazide and clorgyline) at a final concentration of 10^{-10} – 10^{-2} M. LDH assays were performed at 10, 23 and 36 hours as described in Section 5.2.3. Each value is the mean ± SEM of experiments completed in triplicate.

5.2.1 Morphological staining of cells

The addition of hydrogen peroxide to MO6G3 cells demonstrated an increase in cell apoptosis and necrosis when the concentration was increased from 0.25 mM to 750 mM, with a subsequent loss of normal cells (Figure 5.7). At a concentration of 0.75 mM H_2O_2 75-80 % of the cells had undergone apoptosis or necrosis.

The addition of 5-HT to MO6G3 cells did not result in as much cell death as the addition of similar concentrations of H_2O_2. At a concentration of 0.75 mM H_2O_2 approximately 50 % of the cells showed normal features. The mode of cell death was predominantly apoptotic in nature (Figure 5.8)

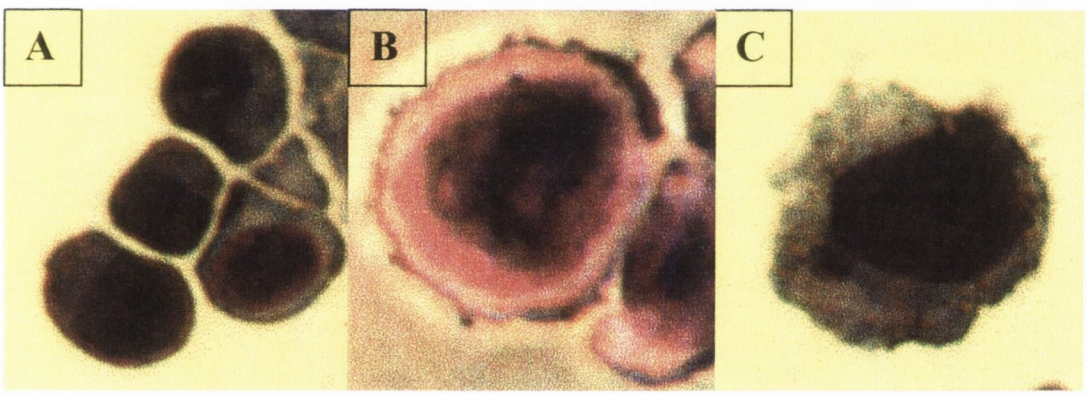


Figure 5.6 RAPI DIFF II staining of murine MO6G3 cells. Normal cells are shown in (a). Signs of necrosis (b) include nuclear swelling, chromatin flocculation and cell membrane lysis, whereas signs of apoptosis (c) include cell membrane blebbing, nuclear shrinkage and chromatin condensation, cytoplasmic constriction and the formation of apoptotic bodies.

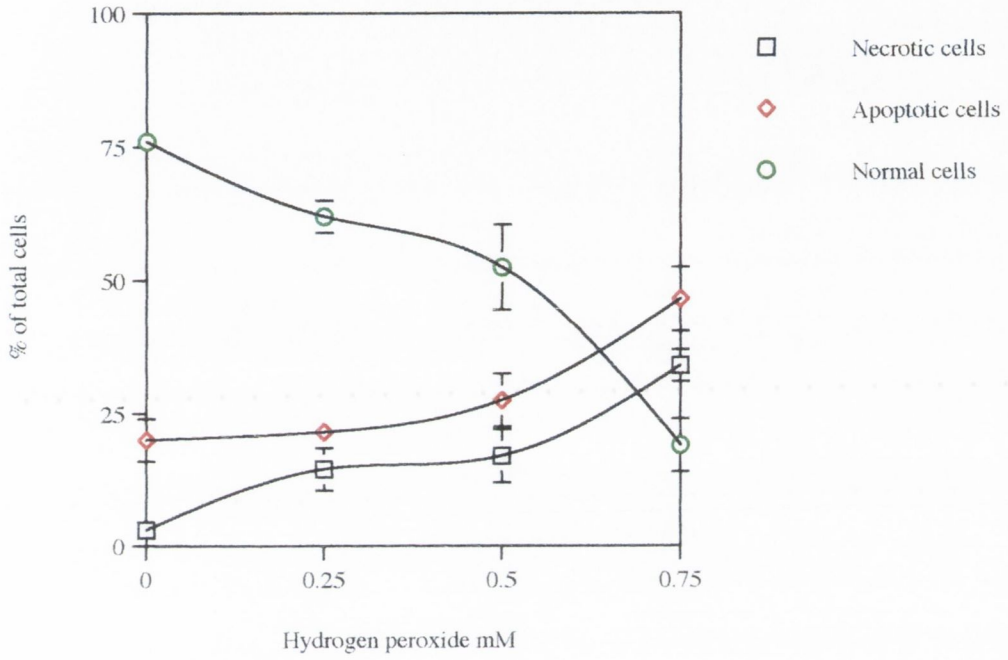


Figure 5.7. The effect of hydrogen peroxide on odontoblast cell viability. MO6G3 cells from the 3rd or 4th passage and were seeded 24 hours beforehand in 96 well plates in standard medium at 33 °C in a 5 % CO_2 /air mixture. 1 hour before the addition of the test material the media were changed from standard medium to medium containing a catalase inhibitor (10 mM of 3-amino-1,2,4-triazole). Hydrogen peroxide was added to cells at a final concentration of 0-0.75 mM. Cells were then observed by morphological staining as described in Section 5.3.4 and categorised as necrotic, apoptotic or of normal appearance. Each value is the mean ± SEM of experiments completed in triplicate.

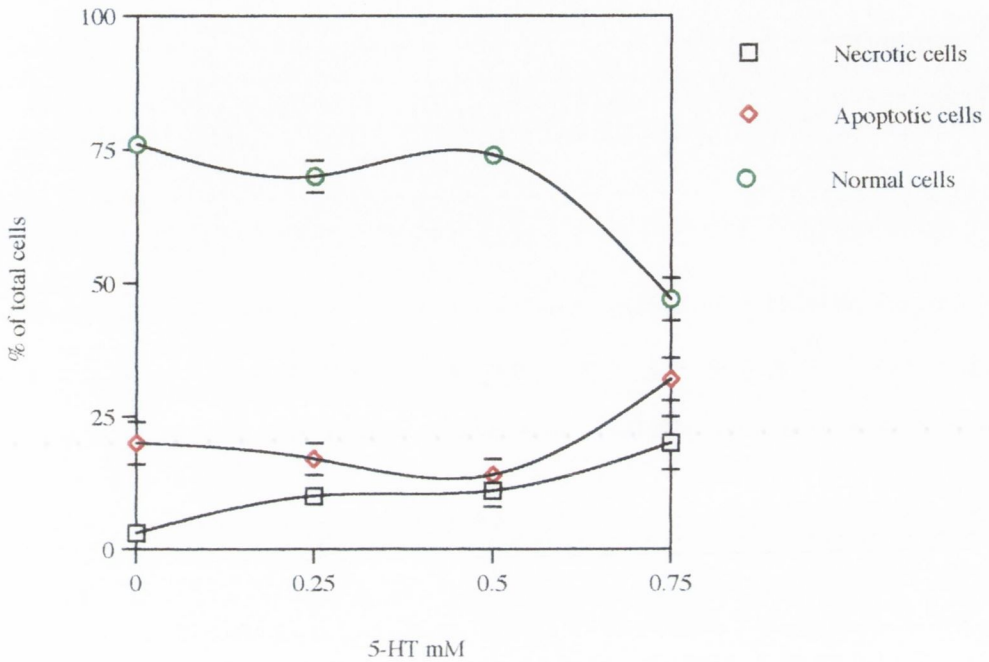


Figure 5.8. The effect of 5-HT on odontoblast cell viability. MO6G3 cells from the 3rd or 4th passage and were seeded 24 hours beforehand in 96 well plates in standard medium at 33 °C in a 5 % CO_2 /air mixture. 1 hour before the addition of the test material the media was changed from standard medium to medium containing amine oxidase inhibitors (1 mM of both semicarbazide and clorgyline). 5-HT was added to cells at a final concentration of 0-0.75 mM. Cells were then observed by morphological staining as described in Section 5.3.4 and categorised as necrotic, apoptotic or of normal appearance. Each value is the mean ± SEM of experiments completed in triplicate.

5.5 Discussion

Immortalised murine odontoblasts offer a stable cells line with which to observe cell behaviour. The problems of donor and proliferation variability experienced with human primary cultures (Moule *et al.*, 1995) are not seen, permitting meaningful analysis of experimental results. The aim of these initial experiments was to determine the effect of hydrogen peroxide on cell viability and its concentration dependence. Different effects of hydrogen peroxide are seen over a range of concentrations in other tissues, *e.g.*, human spermatozoa and the developing blastocyst (see Section 1.4). High concentrations of hydrogen peroxide in spermatozoa cause lipid peroxidation and cell death. Low concentrations produce different responses causing sperm immobility, mostly by a mechanism involving depletion of intracellular ATP and the resulting decrease in the phosphorylation of axonemal proteins (de Lamirande and Gagnon, 1995). It is suggested that there exists a balance between the level of hydrogen peroxide that causes cell proliferation and that that causes lipid peroxidation and cell death (Burdon, 1995). Increased levels cause an initial proliferation but if prolonged may result in death of the cell. Such toxic effects may have relevance to the clinical and histological situation observed with dental caries. Caries is classified into three grades by rate of attack (see Soames & Southam, 1989, pp20-23). The first is rampant or acute caries. This is rapidly progressing caries involving many teeth and on surfaces of teeth normally immune to decay. The rapid destruction of the enamel and dentine leads to early pulpal involvement with little or no formation of secondary dentine. Secondary dentine is mineralised tissue normally laid down by odontoblasts in response to toxic stimuli, but with rampant caries the odontoblasts are overwhelmed and die. The second grade is slowly progressive or chronic caries. It progresses much more slowly and involves the pulp at a much later stage. Its rate is such that it allows the odontoblasts to lay down secondary dentine and form an effective barrier between the pulp and the advancing caries. The third grade is arrested caries, where the rate of caries attack becomes so slow that the secondary dentine walls off the caries so effectively it does not progress.

In the developing blastocyst extracellular hydrogen peroxide is believed to cause apoptosis of the inner cell mass destined to develop into trophectoderm. This

hydrogen peroxide is thought to be produced by the metabolism of polyamines by amine oxidases (Parchment, 1993). This raises the possibility that hydrogen peroxide and amine oxidases may be responsible for cell turnover and tissue renewal.

Amine oxidases are not the only source of hydrogen peroxide in dental pulp as hydrogen peroxide is released in the area of inflammation, possibly from white blood cells (Okabe, 1994). In addition to affecting odontoblast function, reactive oxygen species (ROS) can influence pulpal haemodynamics. The sacroplasmic reticulum of vascular smooth muscle is thought to be the target for ROS action. Early exposure of ring preparations of rabbit lingual arteries to the ROS generated from dihydroxyfumarate plus Fe^{3+}-ADP or from H_2O_2 plus $FeSO_4$ depresses the phasic response of noradrenaline-induced contraction and potentiates the tonic response (Okabe *et al.*, 1991). This is thought to be due to decreased Ca^{2+} release from the sacroplasmic reticulum and may produce an increased, voltage-dependent influx of Ca^{2+}, which is reflected by the enhanced tonic response of the noradrenaline contraction.

Semicarbazide sensitive amine oxidase (SSAO) may also influence inflammation in human and porcine dental pulp by metabolism of the inflammatory mediator 5-HT (see Chapter 2). 5-HT is an important modulator of neurogenic inflammation both directly and by potentiation of the release of other inflammatory mediators (see Chapter 1, Section 1.2.3 for review). The fact that both hydrogen peroxide and 5-HT are intrinsically linked to SSAO suggests that modulation of the enzyme activity may have direct effects on the inflammatory process.

The direct effects of both hydrogen peroxide and 5-HT were examined. Standard medium containing foetal calf serum will contain endogenous amine oxidase activity (Buffoni, 1993) and catalase activity (Chance *et al.*, 1979) that may mask the effects seen on addition of hydrogen peroxide or 5-HT. Amine oxidase activity was removed by incubating the calf serum with both clorgyline and semicarbazide at a concentration of 10^{-3} M for 30 min at 37 °C, prior to addition to the medium. The inhibitors were used to ensure the changes observed were a as result of direct 5-HT actions and not a product of amine oxidase metabolism activity endogenous in calf

serum The catalase inhibitor, 3-amino-1,2,4-triazole, was pre-incubated for 60 min at 37 °C at a concentration of 10 mM. This concentration was found to remove endogenous catalase activity in standard medium (Ziemann *et al.*, 1999) and so the effects observed would reflect the concentration of hydrogen peroxide added.

The addition of amine oxidase inhibitors to the medium decreased the cell viability when measured by MTT assay at 10 and 23 hours, but this returned to near control levels at 36 hours. This was not reflected in the LDH assays implying that cell fuction may have been depressed over the first two periods of observation, but cell rupture did not occur. The addition of a catalase inhibitor to the medium increased cell viability, in particular after 10 hours, reducing to near control levels therafter.

The 5-HT and hydrogen peroxide were applied to the cells as one acute dose. Murine odontoblasts were found to be resistant to both hydrogen peroxide and 5-HT. Cell death was observed at millimolar concentrations similar to results with cultured glial cells (Marangolo, 1999), but up to this concentration little difference was observed with respect to the controls. Cell death in response to 5-HT application caused little deviation from the controls. However, at higher concentrations tested in standard medium there was a drop in cell viability at 10 and 23 hours, but this had returned to control levels at 36 hours. The decrease in cell viability was not reflected in LDH release suggesting that cellular activity may have been temporarily reduced, but not resulting in cell death. In the medium without amine oxidase activity a progressive decrease in cell viability was observed upon increasing 5-HT concentrations. This effect was greatest at 10 hours but less pronounced at 23 hours, and by 36 hours the viability had almost reached control levels. Possible explanations for this may be that all the 5-HT had been metabolised, or that the initial burst of hydrogen peroxide released as a result of 5-HT deamination caused cell death. However, the fact that MAO and SSAO activities were inhibited makes this unlikely. A more probable cause is that a portion of the cells died at the initial stages of the experiment and that the unaffected cells continued to proliferate.

The node of cell death at 10 hours with high concentrations of both 5-HT and hydrogen peroxide (0.25-0.75 mM) was a combination of apoptosis and necrosis. Both forms of cell death increased as the concentration of test material increased.

This introductory examination of the murine odontoblast response to 5-HT and hydrogen peroxide has shown that the cells are quite resistant to high concentrations of both materials. Further work is required to examine other pathways towards cell death, including caspase activation and NF-Kappa-B releases and the role they play, if any, in the response to 5-HT and hydrogen peroxide. The effect of 5-HT and hydrogen peroxide should also be examined at repeated low doses to examine any changes in response to chronic stimulation of the murine odontoblasts.

5.6 Conclusions

1. The addition of hydrogen peroxide to murine odontoblasts produced little difference to cell viability over controls when tested in the concentration range 10^{-9}-10^{-4} M. However, at concentrations of 1 and 10 mM almost complete cell death was observed when measured using MTT assays and measuring LDH release.
2. The effect of adding 10 mM of a catalase inhibitor, 3-amino-1,2,4-triazole, to the medium produced the same effect on the cells tested over the period of observation. The addition of the catalase inhibitor did not affect control cell growth over the period of observation.
3. The addition of 5-HT to murine odontoblasts produced little difference from controls when tested over the concentration range 10^{-9}-10^{-3} M. However, at concentrations of 1 and 10 mM cell death was observed in up to 40 % of samples when measured using MTT assays and measuring LDH release. As the concentration of 5-HT increased, a decrease in activity with respect to control activity was observed over 10 and 23 hours. This however returned to near control levels at 36 hours. The decrease observed at 10 and 23 hours increased as the 5-HT concentration increased.
4. The effect of adding amine oxidase inhibitors to the medium resulted in no change in the sensitivities of the cells to 5-HT over the period of observation to standard

medium. The decrease in cell viability over the first two observation times was more marked in the presence of amine oxidase inhibitors, but the viability at 36 hours had returned to near control levels. The addition of the amine oxidase inhibitors did not affect control cell growth over the period observed.

5. Morphological staining of murine odontoblasts 10 hours after addition of 0.25-0.75 mM hydrogen peroxide or 5-HT indicated cell death at higher concentrations of both. The 5-HT produced less cell death than hydrogen peroxide of equivalent concentration. The mode of cell death was a combination of apoptosis and necrosis. Apoptosis was marginally more prevalent at lower concentrations tested, but at increased concentrations the levels of necrosis increased.

CHAPTER SIX

Molecular Biology of SSAO

6.1 Introduction

The main problem encountered in the study of dental pulp SSAO is accumulation of sufficient material with which to complete experiments. Cloning and expression of SSAO has the advantage that it provides a readily available source of the enzyme. Purification and determination of the nucleotide sequence may provide insight into the structure and function of the enzyme in dental pulp. Analysis may also provide information on the different substrate specificity for the dental pulp enzyme compared to other known SSAO sequences. Comparison of nucleotide sequences may also provide more information on possible functions of the enzyme as happened for VAP-1 (Smith *et al.*, 1998).

6.2 Materials

6.2.1 Sources of Reagents

Reagents are listed in alphabetical order. The source of named reagents is indicated by the suppliers name.

PRODUCT	SUPPLIER
Expand™ High Fidelity PCR System	Boehringer Mannheim
Murine odontoblast cDNA library	A kind gift from Dr. Mary MacDougall, University of Texas Health Sciences Centre, San Antonio, Texas, USA.
Wizard™ Lambda Preps DNA Purification System	Promega
Wizard™ PCR Preps DNA Purification System	Promega
Taq polymerase	Promega
DNA ligase	Promega
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Boehringer Mannheim
5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal)	Boehringer Mannheim

Molecular weight markers	Gibco BRL
dNTPs	Boehringer Mannheim
PBluescript II KS (-)	Stratagene
Quantum Prep® Plasmid Miniprep Kit	Bio-Rad
Oligonucleotide primers	MWG AG
DNA sequencing kit	Applied Biosystems

All other general laboratory reagents were of analytical- or molecular biology-grade and purchased from Sigma Chemicals, BDH or Boehringer Mannheim.

6.2.2 Suppliers

The full names and addresses of suppliers are listed below in alphabetical order:

Applied Biosystems	Applied Biosystems, Perkin-Elmer Ltd., Warrington, UK.
BDH	BDH Chemicals Ltd., Poole, Dorset, England.
Bio-Rad	Bio-Rad Laboratories, Massachusetts, USA.
Boehringer Mannheim	Boehringer Mannheim, Beverly, Massachusetts, USA.
Gibco	Gibco BRL Life Technologies,

	Gaithersburg, Maryland, USA.
MWG	MWG AG Biotech, Ebersberg, Germant.
Promega	Promega Corporation, Madison, Wisconsin, USA.
Sigma Chemicals	Sigma-Aldrich Chemical Co., Poole, Dorset, England.
Strategene	Stratagene, La Jolla, California, USA.

6.3 Methods

6.3.1 Quantification and amplification of Murine cDNA library

An overnight culture of *Escherichia coli* strain LE392 was prepared in Luria-Bertani broth (LB, 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl in 1 litre of H_2O, pH 7.4, sterile) (Lennox, 1955) at 37 °C in an orbital incubator (Model G25, Gallenkamp, USA). The murine odontoblast cDNA library was sequentially diluted in the range 10^{-2}-10^{-7} in salt-magnesium buffer (SMB, 5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 ml 1M Tris-HCl, pH 7.5, 5 ml 2 % (w/v) gelatin, made to 1 litre with H_2O and sterilised by autoclaving). 100 µl of each dilution of library was added to 100 µl of the overnight culture and incubated for 30 min at 37 °C in a waterbath. Then 3 ml of 1 % LB-top agarose (pre-heated to 50 °C) was added to each reaction and plated onto Luria-Bertani agar (LB, 0.2 % (w/v) maltose, 10 mM $MgSO_4$ and 6 g agar in LB, pH 7.4, sterile) (Lennox, 1955) onto 90 mm plates and incubated overnight at 37 °C. The

number of plaques was used to calculate the plaque-forming units /ml of the murine odontoblast library.

Dilutions of murine odontoblast library, in sufficient quantities to produce confluent bacterial lysis, were incubated as before with overnight cultures of *E coli* strain LE392 as described above, mixed with 1 % top agarose and plated onto LA plates and incubated overnight at 37 °C. 2 ml of SMB was added onto each plate and incubated at 37 °C for 30 min. The plates were then scraped to remove all the top agarose and 330 µl of chloroform added for each plate. This was incubated at 37 °C for 30 min in a waterbath and then centrifuged at 8,000 *g* for 10 min at 4 °C and the supernatant removed. This represented the lysate that was stored at 4 °C.

6.3.2 Lambda DNA purification

Lambda DNA purification was completed with the Wizard™ kit. To remove the lambda coat the lysate was further centrifuged at 10,000 *g* for 10 min at 4 °C and the supernatant discarded. The pellet was resuspended in 500 µl of Phage buffer and incubated with 12 µl of proteinase K (20 mg/ml) at 37 °C for 30 min. The resuspended phage was centrifuged at 10,000 *g* (Centrifuge 5417C, Eppendorf) and the supernatant recovered. The lambda DNA purification was completed without a vacuum manifold as directed by the manufacturer (Promega, TB#142) and the resultant DNA stored at 4 °C.

6.3.3 Polymerase chain reaction (PCR)

Polymerase chain reactions were performed with the following primers synthesised by MWG Biotech AG. The primers were stored at stock concentrations of 100 pmol in sterile water at –20 °C:

The primers used included:

Forward primer 1: 5'-GGAGTCCATTTCTGGTAGAAGC-3'

Reverse primer 1: 5'-GCTCTTGGCTAGATTGTCCATC-3'

Forward primer 2: 5'-TGAACAGAACCAGGGCCTCC-3'

Reverse primer 2: 5'-CTGGCCAGGTACAGGTAGCG-3'

The primers were selected on the basis of homology with mouse VAP-1 and rat SSAO basepair alignment. Primers #1 were directed against either end of entire coding sequence, while primers #2 were directed against the conserved region encoding the tyrosine residue specific for the TOPA cofactor present in SSAO (see Section 1.1.1.3).

Amplification reaction were completed in 0.5 ml microfuge tubes (Eppendorf) in a Perkin Elmer Cetus DNA thermal cycler in 50 µl volumes containing 1X Expand™ HF reaction buffer, 0-3.0 mM $MgCl_2$, 200 µM (each) dATP, dCTP, dGTP and dTTP, 5 pM (each) forward and reverse primer, 1 µl DNA and 2.6 U Expand™. The reaction was overlaid with 40 µl sterile mineral oil. The initial cycle profile is given in Table 6.1.

1X	denature template 2 min at 94°C
30X	denaturation at 94 °C for 15 s annealing at for 30 s elongation at 72 °C for 3 min
1X	elongation for 7 min at 72 °C
1X	holding at 4 °C

Table 6.1. The initial cycle parameters for PCR reactions in a Perkin Elmer Cetus DNA thermal cycler using Expand™.

Initial reactions were completed to determine the optimum $MgCl_2$ and DNA concentrations. The optimum $MgCl_2$ concentration was then used for all further PCR reactions involving Expand™ and primers #1..

A portion of the PCR products was diluted with 1X running buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) and run on a 0.8 % TBE-agarose (108 g Tris-base, 55 g boric acid, 9.3 g EDTA, 0.8 g agarose, made to 1 litre, pH 8.2)-

ethidium bromide (4 μg /100 ml TBE gel) gel run at 70 mA. The gels were then viewed under ultra-violet (UV) light transilluminator (345 nm).

PCR reaction products of interest were pooled and run in combined wells. A 0.8 % TBE-agarose-ethidium bromide gel was run at 70 mA. The bands of interest were excised using a sterile scalpel.

6.3.4 PCR Product DNA purification

The bands excised from the 0.8 % TBE-agarose gel were purified using the Wizard™ PCR Preps DNA Purification System according to the manufacturer's instructions (Promega, TB#118) apart from the final elution from the minicolumn where the DNA was eluted with 25 μl of sterile distilled water (SDW) instead of 100 μl in order to concentrate the DNA.

Verification of the purified DNA was completed by running 1 μl from each colony on a 0.8 % TBE-agarose gel as described in Section 6.3.3.

6.3.5 Ligation of DNA fragments

The purified DNA products from the PCR reactions was ligated to pBluescript II KS(-) phagemid (Figure 6.1) digested with appropriate digestion enzymes. Ligation was performed by the addition of adenosine residues to the 3' ends of the PCR products. This was mediated by the terminal transferase activity of *Taq* polymerase (Marchuk *et al.,* 1991). The resultant adenosine overhangs were ligated to a pBluescript overhang vector. T-overhang vectors were created by incubating pBluescript II KS(-) DNA that had been cleaved by *Eco*RV (to generate "blunt ends") in a PCR reaction containing 1X *Taq* reaction buffer, 2 mM $MgCl_2$, 250 mM dTTP and 5 U *Taq* polymerase. The reaction was run for 2 hours at 70 °C. In this reaction *Taq* polymerase adds a single thymidine to the 3' end of the vector, thus allowing ligation to PCR products with adenosine overhangs.

Ligation reactions were completed with a 3:1 ratio of insert to vector DNA in 1X ligase buffer, with 1 U of T4 DNA ligase in a total volume of 10 µl. Reactions were run overnight at 14 °C.

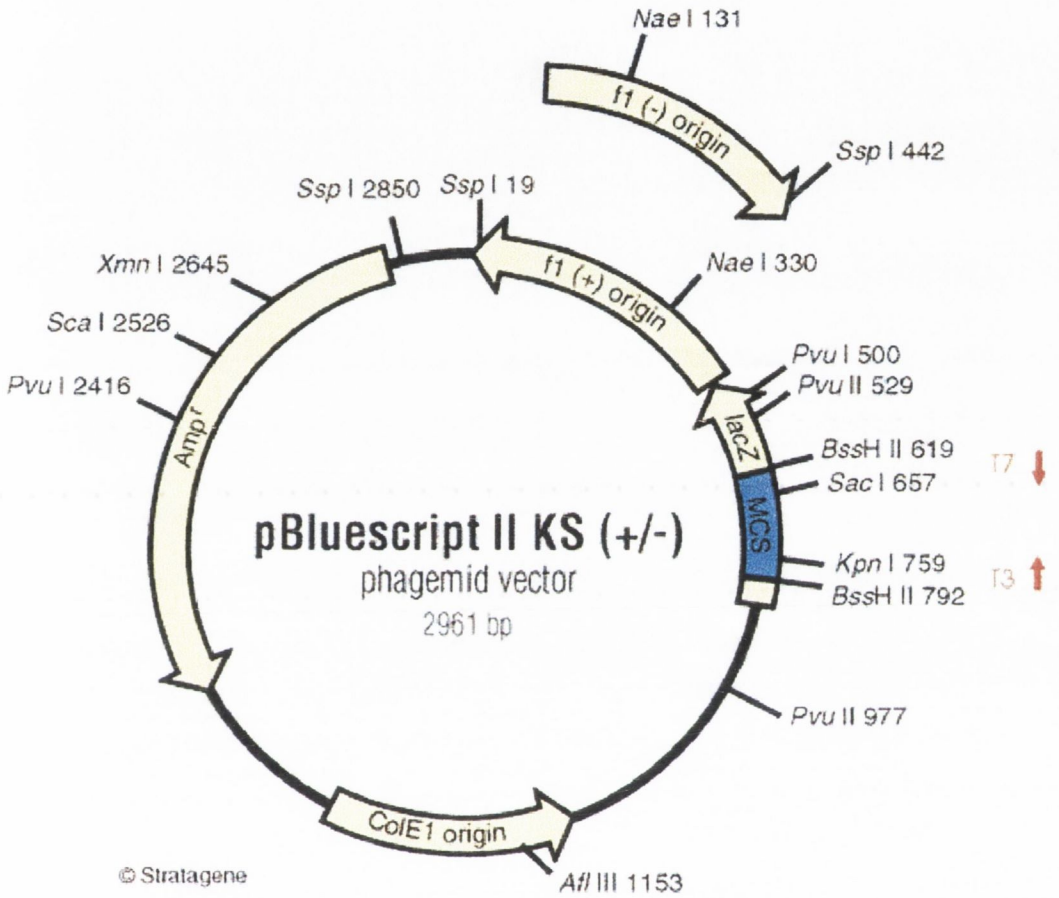


Figure 6.1. Restriction map of pBluescript II KS(-) phagemid. MCS represents the multiple cloning site which is flanked by T3 and T7 promoters and contains 20 restriction sites (Genebank #X52329).

6.3.6 Transformation of competent *E coli* prepared using Calcium Chloride

Transformation of *E coli* with calcium chloride ($CaCl_2$) was completed by the protocol of Sambrook *et al.* (1989a). A single colony of *E. coli* XL1 Blue was transferred to 100 ml of LB broth and incubated for 3 hours in an orbital incubator at 37 °C. The cells were transferred to sterile Falcon 2070 tubes and kept on ice for 10 min. They were then centrifuged at 5,000 *g* in a Sorvall SS34 rotor (Dupont) for 10

min at 4 °C. The supernatant was drawn off and the pellets resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and further centrifuged at 5,000 *g* for 10 min at 4 °C. The supernatant was drawn off and the pellet resuspended in 4 ml of ice-cold 0.1 M $CaCl_2$. 200 µl aliquots of this suspension containing the competent cells were transferred to a sterile microcentrifuge tubes and DNA added. The contents were gently mixed and stored on ice for 30 min. The tubes were placed in a water-bath at 42 °C for 90 sec and then rapidly transferred to an ice-bath for 2 min. 800 µl of LB broth was added to each tube and incubated at 37 °C for 45 min. Controls were run with either no DNA or containing undigested vector.

100 µl from each tube was added to separate 90 mm plates onto LA plates containing 100 µg ampicillin/ml, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 100 µg 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) and incubated for 20 hours at 37 °C. Recombinants were identified using blue-white selection (Sambrook *et al.*, 1989b).

6.3.7 Colony screening

This procedure was based on the method of Gouill and Dery (1991). With the aid of a Gilson P20 pipette tip a colony was picked up from the selective plate described in Section 6.3.6. This is lightly touched onto the surface of a fresh plate to preserve the clone. The remainder of the colony was transferred to a 1.5 ml Eppendorf tube containing 8 µl of lysis buffer (9 volumes 0.25 % bromophenol blue, 0.25 % xylene cyanol, 25 % Ficoll type 400, 11 volumes of SDW and 40 volumes of 0.2 N NaOH, 1 % SDS). The mix was resuspended 4-5 times with a pipette.

3 µl of buffer 2 (3 M potassium acetate, 1.8 M formic acid) was added onto the side wall of the Eppendorf and centrifuged at 10,000 *g* for 4 min. The supernatant was loaded onto a 0.8 % agarose gel and run as described in Section 6.3.3.

6.3.8 Restriction enzymes

Colonies with inserts were further purified using the Bio-Rad Quantum Prep® Plasmid Miniprep Kit according to the manufacturer's instructions. Restriction enzymes were selected using the mouse VAP-1 restriction map (DNA Strider™ 1.2, Christian Marck, Dept. de Biologie Cellulaire et Moleculaire Direction des Sciences de la Vie, CEA, France). *Bam*H I and *Hind* III were used for restriction in reactions containing the appropriate 1X reaction buffer (Section 6.3.3), 1 µl of restriction enzyme, 5 µl of plasmid and the reaction volume brought to 20 µl with sterile distilled water (SDW). The reactions were performed overnight at 37 °C in a water bath.

6.3.9 Sequencing reactions

The first 200 basepairs in the forward and reverse direction were sequenced using the DNA Sequencing Kit (Applied Biosystems) using *AmpliTaq* polymerase. The reverse primer was specific for upstream of the T3 promoter region and the forward primer was specific for downstream of the T7 promoter region in pBluescript II KS(-). The forward primer was 3'-TGACCGGCAGCAAAATG-5' and the reverse primer 5'-GGAAACAGGTATGACCATG-3'. The reactions contained 4 µl of reaction mixture, 4 µl of colony DNA, 1 µl of either M13F or M13R and 1 µl of SDW. A positive control was performed with pGEM instead of the colony DNA. The mixture was covered with 20 µl of mineral oil and the reaction proceeded as in Table 6.2.

25X	denaturation at 96 °C for 10 s annealing at 50 °C for 30 s elongation at 60 °C for 4 min
1X	holding at 4 °C

Table 6.2. The cycle parameters for DNA sequencing using M13F and M13R primers and the DNA Sequencing Kit.

10 µl of each reaction mixture was added to 50 µl of ethanol and placed at –20 °C for 10 min. The mixtures were then centrifuged at 5,000 *g* for 15 min. The ethanol was

decanted off and the product sent for sequencing to Dr David Noone, Department of Genetics, Trinity College, Dublin.

6.3.10 Isolation of Mouse genomic DNA

Mouse genomic DNA was isolated from mouse blood by the method of Sambrook *et al.*, (1989c). 6 ml of fresh blood was collected in a tube containing 1 ml of acid citrate dextrose solution (0.48 g citric acid, 1.32 g sodium citrate, 1.47 g glucose made to 100 ml with sterile distilled water). This mixture was centrifuged at 1300 *g* for 15 min and the buffy coat removed using a pasteur pipette. The buffy coat was then recentrifuged at 1300 *g* for 15 min and the pellet resuspended in 5 ml of sterile extraction buffer (10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 20 μg /ml RNAse and 0.5 % SDS). The solution was incubated for 1 hour at 37 °C. Proteinase K was added to a final concentration of 100 μg /ml and the solution incubated for 3 hours at 50 °C in a water-bath.

The solution was cooled to room temperature and extracted with an equal volume of phenol equilibrated with 0.5 mM Tris-HCl. The aqueous phase was then pipetted off and re-extracted in phenol equilibrated with 0.5 mM Tris-HCl.

0.2 volume of 3 M ammonium acetate was added to the aqueous phase and then 2 volumes of ethanol. The solution was mixed gently and centrifuged at 5000 *g* for 5 min at room temperature. The DNA precipitate was washed twice in 100 % ethanol and the pellet of DNA resuspended in 1 ml of sterile distilled water and stored at 4 °C.

6.4 Results

6.4.1 Quantitation of cDNA library

The murine odontoblast cDNA library was found to contain 1.12 X 10^8 plaque-forming units (pfu) /ml. Dilution factors of greater than 10^{-4} resulted in confluent lysis and were used in amplification of the library.

6.4.2 PCR products using primers #1

The anticipated SSAO band, if present, was estimated to be in the region of 2,400 basepairs when primers #1 were used. A product was present in that region (Figure 6.2) when the $MgCl_2$ concentration was 2.0 mM. Sequential dilutions of DNA indicated that when 1-4 µl of DNA was added the product was visible, with the optimum being 1 µl in a 50 µl reaction (Figure 6.3). Alteration of conditions (temperature, number of cycles, cycle parameters and primer concentration) failed to remove smaller molecular-weight bands.

6.4.3 DNA purification of PCR product

Two large wells were combined, each containing 60 µl of PCR reaction product from the 2.0 & 2.5 mM $MgCl_2$ reactions from Figure 6.2 and run on a 0.8 % agarose gel (Figure 6.4) as described in Section 6.3.3. The two bands in the 2.4 kb area were further purified and the purified DNA tested on a 0.8 % agarose gel (Figure 6.5). A clearly defined band was produced in the anticipated area.

6.4.4 Digestion of pBluescript(-)

Digestion of pBluescript II KS(-) was completed after 12 hours at 37 °C. 6 hours was insufficient time for the enzyme to completely digest the DNA present (Figure 6.6). 12 hours was used in all further digestions.

6.4.5 Colony screening

Twenty-one white colonies (white) were tested for the presence of inserts. Thirteen were found to have inserts (Figure 6.7). The inserts were detected by an increase in number of basepairs (in the region of 2.4 kb) over the controls without inserts (blue colonies).

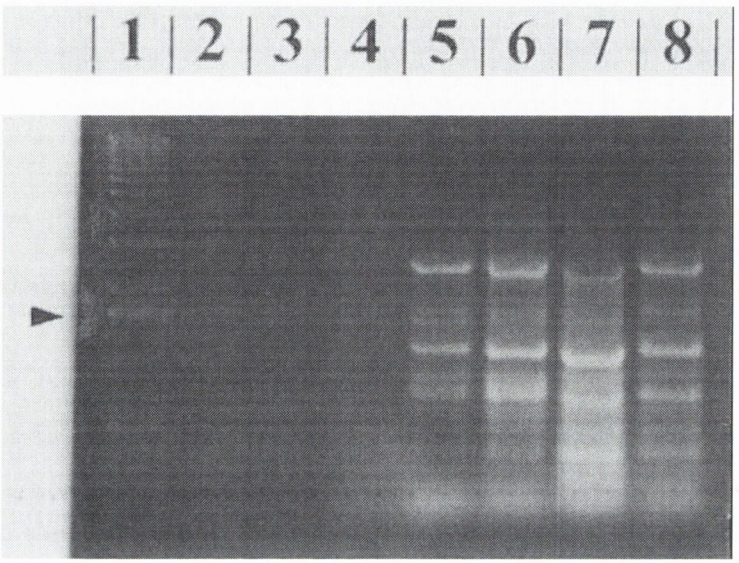


Figure 6.2 The effect of varying the $MgCl_2$ concentrations on PCR products from DNA purified from a murine dental pulp odontoblast library. A 0.8 % agarose gel containing DNA amplified from a cDNA library from murine odontoblasts amplified with Expand™ polymerase, forward oligonucleotide primer 5'-GGA GTC CAT TTC TGG TAG AAG C-3'and reverse primer 5'-GCT CTT GGC TAG ATT GTC CAT C-3'as described in Section 6.3.3. One thousand-base-pair DNA ladder size standards are shown Lane 1 and the 1.5 kb standard is arrowed. Lanes: 2, control reaction without DNA; 3, control reaction without primers; 4, 1.5 mM $MgCl_2$; 5, 2.0 mM $MgCl_2$; 6, 2.5 mM $MgCl_2$; 7, 3.0 mM $MgCl_2$; 8, 3.5 mM $MgCl_2$. Products at 2.4 kb are visible in lanes 5-8, but with most product in lanes 5 & 6.

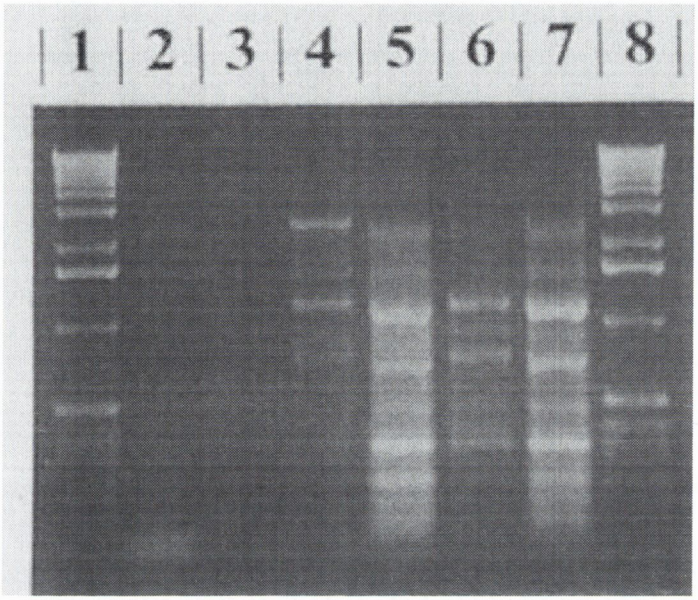


Figure 6.3 The effect of varying the DNA concentration on PCR products from DNA purified from a murine dental pulp odontoblast library. A 0.8 % agarose gel containing DNA amplified from a cDNA library from murine odontoblasts amplified with Expand™ polymerase, forward oligonucleotide primer 5'-GGA GTC CAT TTC TGG TAG AAG C-3'and reverse primer 5'-GCT CTT GGC TAG ATT GTC CAT C-3'as described in Section 6.3.3 at 2.0 mM $MgCl_2$. One thousand-base-pair DNA ladder size standards are in lane 1 & 8 and the 1.5 kb marker is arrowed. Lanes: 2, control reaction without DNA; 3, control reaction without primers; 4, 1.0 μl DNA; 5, 2.0 μl DNA; 6, 3.0 μl DNA; 7, 4.0 μl DNA.

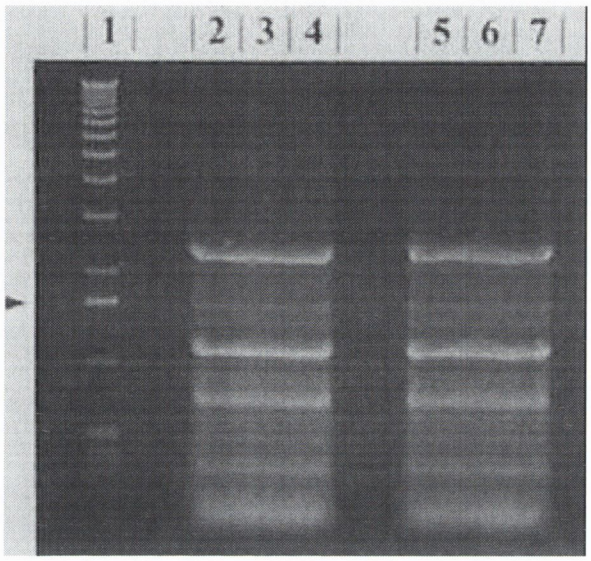


Figure 6.4 Pooling of PCR products from murine dental pulp odontoblast. A 0.8 % agarose gel containing DNA amplified from a cDNA library from murine odontoblasts amplified with Expand™ polymerase, forward oligonucleotide primer 5'-GGA GTC CAT TTC TGG TAG AAG C-3'and reverse primer 5'-GCT CTT GGC TAG ATT GTC CAT C-3'as described in Section 6.3.3. One thousand-base-pair DNA ladder size standards are shown in lane 1 and the 1.5 kb marker is arrowed. Lanes: 2-4, 60 µl of PCR product; 5-7, 60 µl of PCR product.

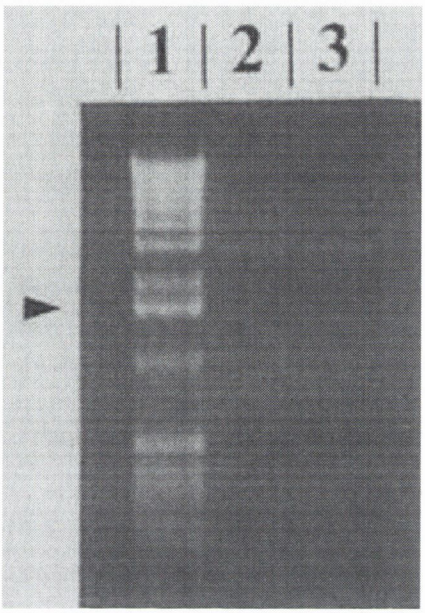


Figure 6.5 A 0.8% agarose gel containing DNA purified from a cDNA library from murine odontoblasts amplified with Expand™ polymerase, forward oligonucleotide primer 5'-GGA GTC CAT TTC TGG TAG AAG C-3'and reverse primer 5'-GCT CTT GGC TAG ATT GTC CAT C-3'as described in Section 6.3.3 using the Wizard™ PCR Preps DNA purification kit. One thousand-base-pair DNA ladder size standards are shown in lane 1 and the 1.5 kb marker is arrowed. Lanes: 2, 1 μl of purified DNA from lanes 2-4 in Figure 6.4; 2, 1 μl of purified DNA from lanes 5-7 in Figure 6.4.

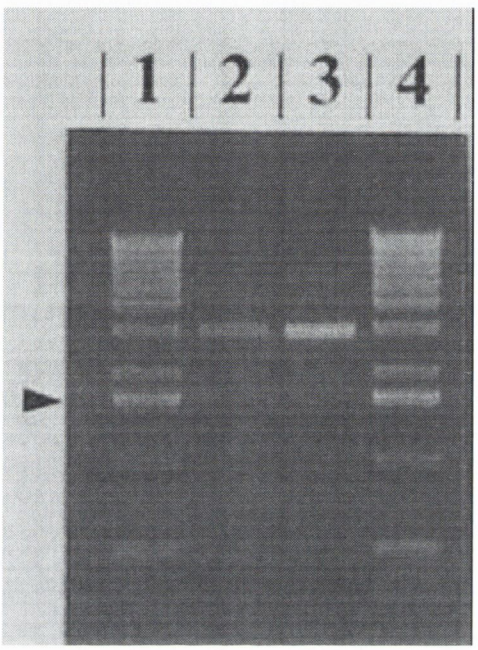


Figure 6.6 A 0.8% agarose gel showing the digestion of pBluescript II KS(-) using *Eco*RI after 2 time periods. One thousand-base-pair DNA ladder size standards are shown in lane 1 and the 1.5 kb marker is arrowed. Lanes: 2, 1 μl of digested pBluescript II(-) DNA after 12 hours digestion by *Eco*RI; 3, 1 μl of digested pBluescript II(-) DNA after 6 hours digestion by *Eco*RI. After 6 hours of digestion supercoiled DNA is still evident as the larger product band.

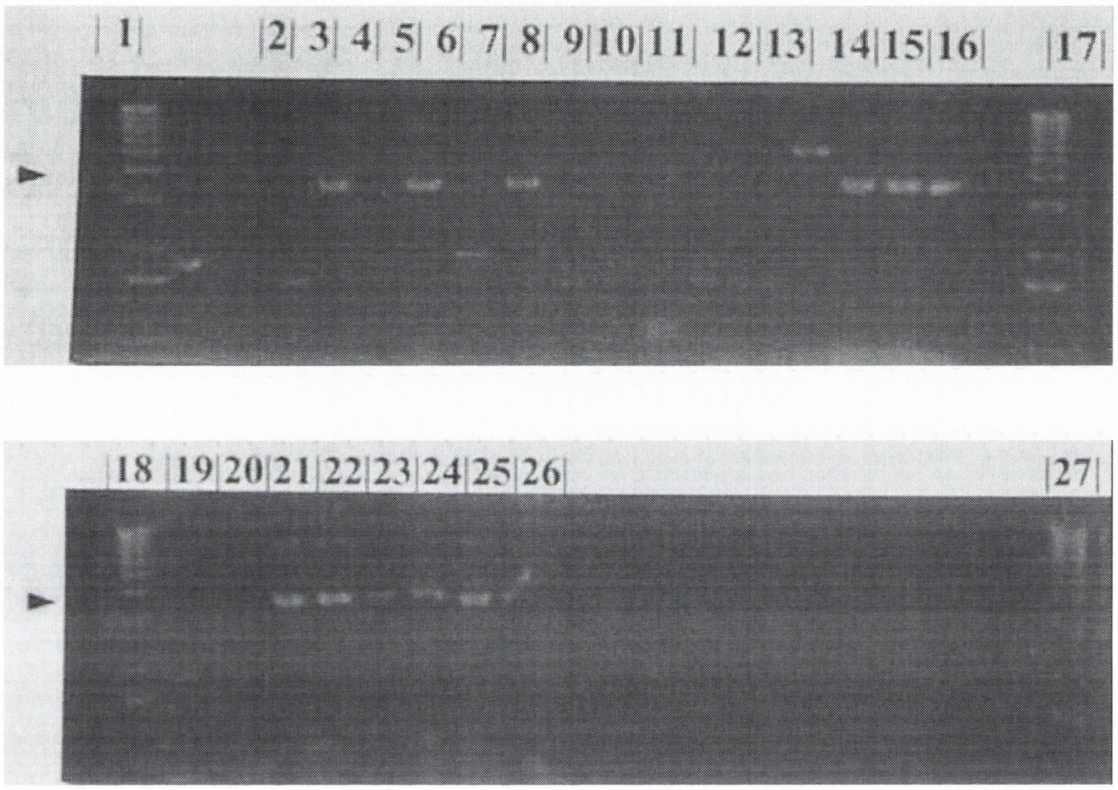


Figure 6.7. A 0.8 % agarose gel of positive (white) colonies. One thousand-base-pair DNA ladder size standards are shown in lanes 1, 17, 18 & 27 and the positive colonies are arrowed. Lanes: 2-16 & 21-26 positive (white colonies) of which the following lanes demonstrate inserted DNA- 3, 5, 7, 13, 14, 15, 16, 21, 22, 23, 24, 25, & 26. Lane 20 is blank and lane 19 a positive control (blue colony without inserted DNA).

6.4.6 Restriction enzymes

The restriction enzymes used were consistent with mouse VAP-1. *Bam*H I alone digested the vector into 2 fragments of approximately 2.7 & 3.0 kb. *Hind* III digested the vector into two fragments of approximately 0.7 & 5 kb. Combination of both restriction enzymes produced 3 fragments approximately 0.7, 2.0 & 3.0 kb. This suggested a vector basepair alignment as shown in Figure 6.8.

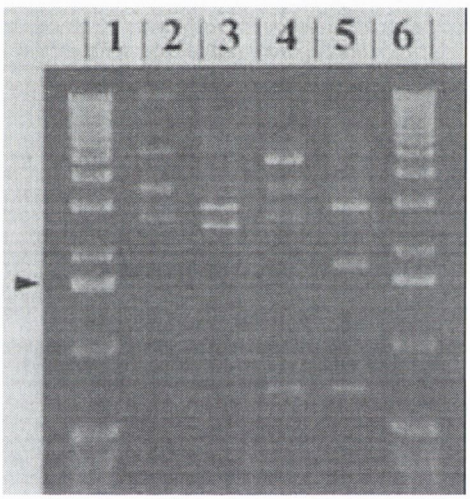


Figure 6.8. A sample 0.8 % agarose gel of digestion of the DNA from colony numbers 15 (see Figure 6.7). One thousand-base-pair DNA ladder size standards are shown in lanes 1 & 6 and the 1.5 kb marker is arrowed. Lanes: 2, uncut vector; 3, digestion with *Bam*H I; 4, digestion with *Hind* III; 5, digestion with *Bam*H1 & *Hind* III.

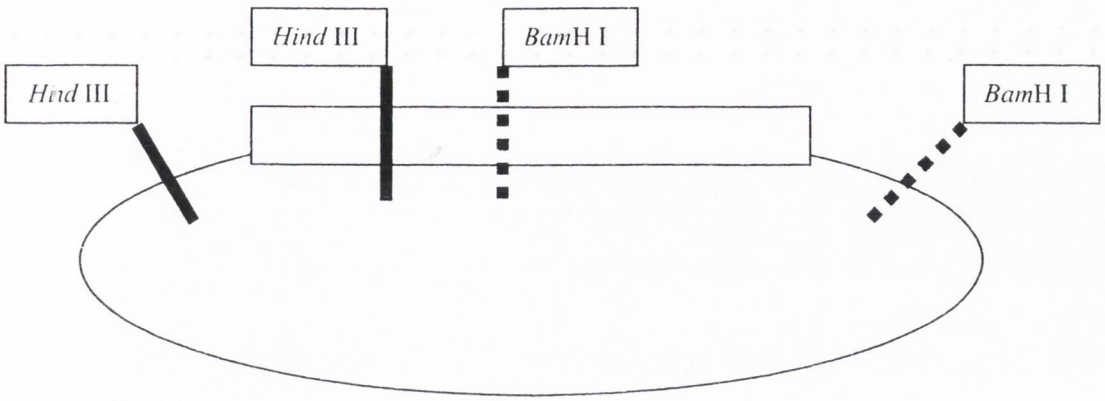


Figure 6.9. Proposed vector /inserted DNA relationship based on digestion by *Bam*H I & *Hind* III. The vector is represented by the circle and the inserted DNA by the rectangle. Dotted lines indicate the proposed restriction sites.

6.4.7 Sequencing

The sequences determined through the use of the M13F and M13R primers were as follows:

Forward primer:
TCCCCCCCCGGGTGGTTTNNNNNATNTTNCTTNAATTACANNAGNCCCGGCCNNTTGNAA
ATCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGAT
TGGATCCGTGAAGTATTCGAGGCCACCGCAGCAGGTCTGGCGGCATATTTCGGCACGCTG
GAAGCGCTGGAAGCCGCTTCGATTGAAGAGCTGCAAAAGGTGCCTGATNTTGGCATTGTC
GTTGCATCCCACGTTCACAACTTCTTTGCCGAAGAAAGCAACCGCAATGTCATCAGCGAG
CTGTTGGCGGAAGGTGTTCACTGGCCTGCGCCGATCGTTATCAACGCGGAANANATTGAC
AGCCCGTTTGCTGGTAAAACCGTGGTGCTTACGGGCCAGCTTAAGCCAGATGTTCGCGTT
GAATGACNCCTAAAGCTCCACTGGTCNAACTGGGCGCGAAATTCNCNGGCAGCGTTTTCA
AAAAAAACCGATCTGGTGATNNCGGGTTGAANCTGCAGATTCTAAACTGGCGAAAGGGG
CCAGAAACTGGGCATTGAATTCNTCCACAAACGGAAATGCTGCGTTTGCTGGTTACTAAN
NTGAAAAAA ACACTGNTTGAANT

Reverse primer
TGGGGGGGGGNNNNCCNCTTTTTTTNGANTTCANNAAANTAGGGCCCCAGGAAATNAAA
GCTGGGTACCGGGCCCCCCCTCGCNNGTCGANNGTATCGATAAGCTTGATTGGGAATCCA
GTGTGTGAAGAATACTGATGCCGATGCCTGCGGCTACCATCGGGAACAGCGTCGCCGGAT
GTCCAATCTCCTGCACAATATTCGCCTGAATACCATTGCGCGCCNTCGCTGCGTCAATCAG
CGGTCGGCTGCCTGACGCGTAATCCTGCAACACCAATTTCGCTCCCTGTAATGCCTGCCAG
GGGTACATAATCTTCCACGGCCAAAGCGCTATCGCGATGGCAAAGCAGAAAGAAAGGCT
CGGAAAGAATCGCTTCACATTGCAGATCGCCCACTGGGCCTGGATTCGATGACGATGCCC
AAAATCGACATCTCCCTGGGCGAATACTTTCCCATTACCCACTGCTGCNGAAGAATCGTG
CANGANAAACTGAATATCTGGATAGCGGNAATGNTTTTCAGCAATGCATTTGGCNGAATA
AGATGCNCCNGAAATTGTCTNGCTNGCANCGAACCCAAACTTTTCCCCTCCATTTGTTTGC
CCCATTC CCCCGGTTTCCCNCANCNTNCTN

BLAST comparisons of these sequences revealed that the insert in contained within the pBluescript KS(-) was genomic *E. coli* DNA and not compatible with any known SSAO sequence. The sequence detedted has 98 % homology with both *E coli* genomic DNA, Kohara clone #417 and *E. coli* K-12 MG1655.

6.4.8 PCR with primers #3 and nested primers

PCR reactions, using either Expand™ or *Taq* polymerases, with primers #3 directed towards the conserved area coding the tyrosine associated with the cofactor TOPA with DNA purified from the cDNA library of murine odontoblasts and murine genomic DNA (isolated from mouse white blood cells) failed to produce products of the anticipated size when run out in agarose gels (Figure 6.10).

Nested primers were then used combining primers #1 and #2. Nested primers were used as the first set of primers (#1) directed against a target nucleotide sequence produced multiple products that could not be resolved to a single product by altering the PCR conditions. A second set of primers (#2), designed to recognise an area within the nucleotide sequence amplified by the first set of primers was used against the products of the first PCR reaction in an effort to increase sensitivity and specificity of DNA amplification (Jackson *et al.,* 1992). Primers #1 were used in PCR reactions with (i) genomic DNA, (ii) DNA isolated from murine odontoblast cDNA library, as described in Section 6.3.3 using Expand as a polymerase, at an annealing temperature of 55 °C (Figure 6.11).

Primers #2 were then used in a PCR reaction with *Taq* polymerase and 1 μl of DNA product from each reaction with primers #1 at a $MgCl_2$ concentration of 1.5 mM.

The genomic DNA failed to produce a product and the library DNA produced multiple products, none of which were of the predicted 460 basepair size (Figure 6.12). Both reactions were then repeated at 58 °C and the results were similar (Figure 6.13).

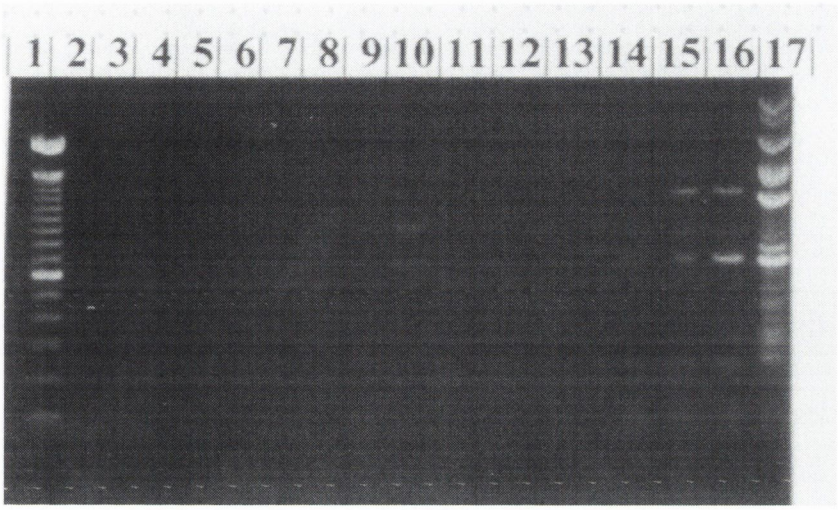


Figure 6.10. A 1 % agarose gel containing mouse genomic DNA and DNA amplified from a cDNA library from murine odontoblasts amplified with the forward oligonucleotide primer 5'-TGAACAGAACCAGGGCCTCC-3' and the reverse primer 5'-CTGGCCAGGTACAGGTAGCG-3' using Expand™ polymerase at an annealing temperature of 55 °C as described in Section 6.3.3. One hundred-base-pair DNA ladder size standards are shown Lanes 1 & 17 and the 0.6 kb standard is arrowed. Lanes: 2, reaction with no DNA; 3, reaction with no primers; 4, reaction with no forward primer; 5, reaction with no reverse primer; 6, 0.5 mM $MgCl_2$ using mouse genomic DNA; 7, 1.0 mM $MgCl_2$ using mouse genomic DNA; 8, 1.5 mM $MgCl_2$ using mouse genomic DNA; 9, 2.0 mM $MgCl_2$ using mouse genomic DNA; 10, 2.5 mM $MgCl_2$ using mouse genomic DNA; 11, reaction with no DNA amplified from a cDNA library from murine odontoblasts; 12, reaction with no primers; 13, 1.0 mM $MgCl_2$ using DNA amplified from a cDNA library from murine odontoblasts; 14, 1.5 mM $MgCl_2$ using DNA amplified from a cDNA library from murine odontoblasts; 15, 2.0 mM $MgCl_2$ using DNA amplified from a cDNA library from murine odontoblasts; 16, 2.5 mM $MgCl_2$ using DNA amplified from a cDNA library from murine odontoblasts.

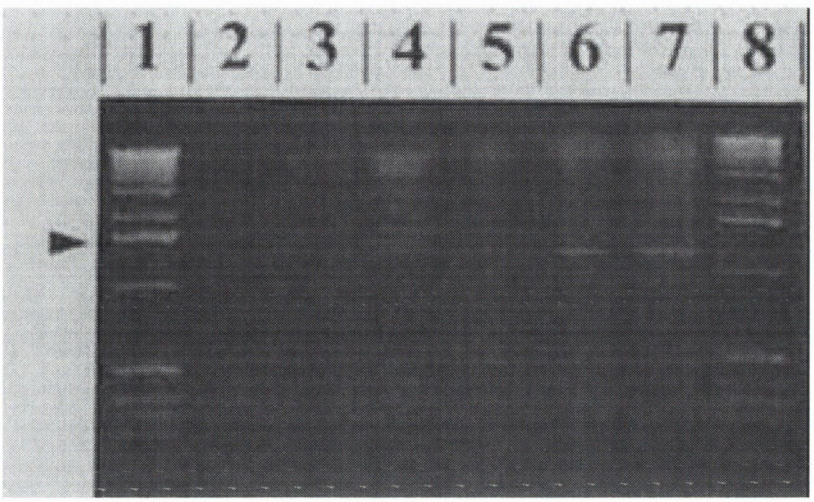


Figure 6.11. A 0.8 % agarose gel containing genomic DNA and DNA amplified from a cDNA library from murine odontoblasts amplified with the forward oligonucleotide primer 5'-GGA GTC CAT TTC TGG TAG AAG C-3'and the reverse primer 5'-GCT CTT GGC TAG ATT GTC CAT C-3' using Expand™ polymerase at an annealing temperature of 55 °C as described in Section 6.3.3. One hundred-base-pair DNA ladder size standards are shown Lanes 1 & 8 and the 0.6 kb standard is arrowed. Lanes: 2, 1.0 mM $MgCl_2$ with mouse genomic DNA; 3, 1.5 mM $MgCl_2$ with mouse genomic DNA; 4, 2.0 mM $MgCl_2$ with mouse genomic DNA; 5, 1.0 mM $MgCl_2$ with DNA isolated from mouse odontoblast cDNA library; 6, 1.5 mM $MgCl_2$ with DNA isolated from mouse odontoblast cDNA library; 7, 2.0 mM $MgCl_2$ with DNA isolated from mouse odontoblast cDNA library.

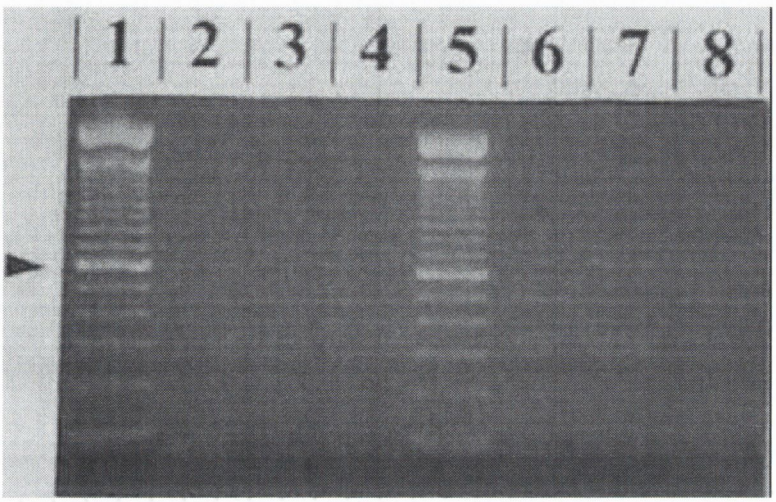


Figure 6.12. Nested primers. A 1 % agarose gel containing 1 μl of product from Lane 3 in Figure 6.11 (Mouse genomic DNA amplified with Expand™ polymerase at a $MgCl_2$ concentration of 2.0 mM) or 1 μl of product from lane 5 in Figure 6.11 (DNA purified from a cDNA library of murine odontoblasts amplified with Expand™ polymerase at a $MgCl_2$ concentration of 1.5 mM) amplified with forward oligonucleotide primer 5'-TGAACAGAACCAGGGCCTCC-3' and reverse primer 5'-CTGGCCAGGTACAGGTAGCG-3'using *Taq* polymerase at an annealing temperature of 55 °C as described in Section 6.3.3. One hundred-base-pair DNA ladder size standards are shown Lanes 1 & 5 and the 0.6 kb standard is arrowed. Lanes: 2, 1.5 mM $MgCl_2$ with mouse genomic DNA; 3, 2.0 mM $MgCl_2$ with mouse genomic DNA; 4, 2.5 mM $MgCl_2$ with mouse genomic DNA; 5, 1.5 mM $MgCl_2$ with DNA isolated from mouse odontoblast cDNA library; 6, 2.0 mM $MgCl_2$ with DNA isolated from mouse odontoblast cDNA library; 7, 2.5 mM $MgCl_2$ with DNA isolated from mouse odontoblast cDNA library.

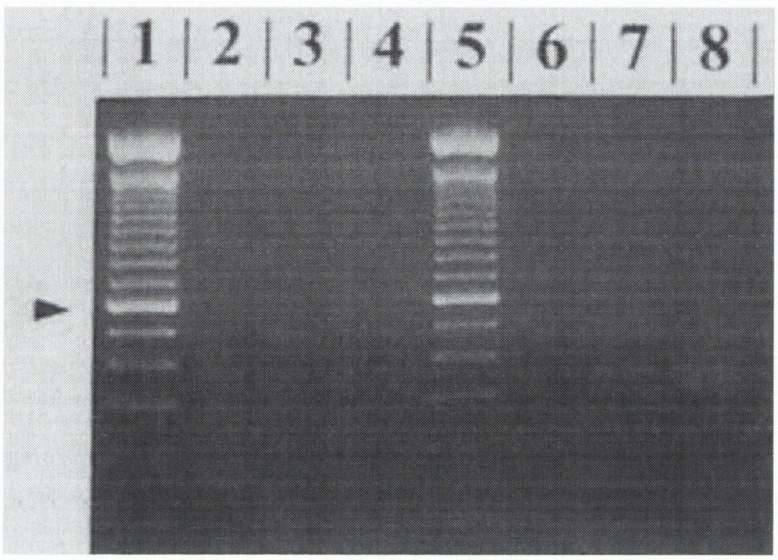


Figure 6.13. Nested primers. A 1 % agarose gel containing 1 μl of product from Lane 3 in Figure 6.11 (Mouse genomic DNA amplified with Expand™ polymerase at a $MgCl_2$ concentration of 2.0 mM) or 1 μl of product from lane 5 in Figure 6.11 (DNA purified from a cDNA library of murine odontoblasts amplified with Expand™ polymerase at a $MgCl_2$ concentration of 1.5 mM) amplified with forward oligonucleotide primer 5'-TGAACAGAACCAGGGCCTCC-3' and reverse primer 5'-CTGGCCAGGTACAGGTAGCG-3'using *Taq* polymerase at an annealing temperature of 58 °C as described in Section 6.3.3. One hundred-base-pair DNA ladder size standards are shown Lanes 1 & 5 and the 0.6 kb standard is arrowed. Lanes: 2, 1.5 mM $MgCl_2$ with mouse genomic DNA; 3, 2.0 mM $MgCl_2$ with mouse genomic DNA; 4, 2.5 mM $MgCl_2$ with mouse genomic DNA; 5, 1.5 mM $MgCl_2$ with DNA isolated from mouse odontoblast cDNA library; 6, 2.0 mM $MgCl_2$ with DNA isolated from mouse odontoblast cDNA library; 7, 2.5 mM $MgCl_2$ with DNA isolated from mouse odontoblast cDNA library.

6.5 Discussion

Two sets of primers were designed to clone the DNA responsible for the synthesis of SSAO in murine genomic DNA and DNA purified from a cDNA library of murine odontoblasts. The first set was designed to extract the complete coding sequence, in the region of 2,400 basepairs. Expand™ polymerase was the polymerase of choice as it demonstrates a 3-fold increased fidelity over *Taq* polymerase (Barnes, 1994). Expand™ polymerase has an error rate of 8.5 X 10^{-5} and is a combination of *Taq* and Pwo polymerase. It is capable of amplifying DNA fragments up to 5 kb from human genomic DNA. Pwo polymerase possesses inherent 3'-5' exonuclease proof-reading activity (Barnes, 1994) to reduce errors.

The second set of primers was designed to be specific for the coding region for the TOPA co-factor. A similar approach was successful when cloning SSAO from human placenta (Xiaoping & McIntire, 1996). The second set of primers were amplified with both Expand™ and *Taq* polymerases as a product was anticipated in the 460 basepair region and so the proof-reading of both enzymes should have been adequate.

When amplified with the primers directed against the whole coding region DNA purified from the cDNA library of murine odontoblasts produced multiple products. Altering the conditions failed to reduce the number of products amplified. There was however a product at 2.4 kb that was in the correct range for SSAO and appeared as a single band. This band was purified and expressed using the pBluescript II KS(-) phagemid. However, the product proved to be an *E. coli* sequence and did not correspond to any known SSAO sequence.

Mouse genomic DNA was then used as a template DNA in the place of the DNA purified from the cDNA library. This was to avoid any *E. coli* that may contaminate the PCR reactions when using cDNA amplified from a library. When tested with primers directed against the complete coding region smearing of products was observed. Smearing is commonly caused by high template DNA concentrations or high $MgCl_2$ concentrations. However, decreasing the amount of DNA 10-fold failed to remove the smearing and the $MgCl_2$ concentrations used were as low as 0.5 mM.

The smearing was present when the DNA was amplified by either Expand™ or *Taq* polymerases.

The use of primers directed against the TOPA site failed to produce a single product in the anticipated 460 base-pair range. It would be anticipated that all SSAO enzymes and possibly others that possess TOPA as a co-factor would be in evidence as the area is so conserved. A possible explanation is that the DNA isolated from the mouse blood was contained in a section of genomic DNA of very high molecular weight, in excess of 200 kb, and thus not have been extracted (Sambrook *et al.*, 1989c). Also *Taq* polymerase may not be as efficient amplifying DNA from genomic DNA due to the large number of basepairs (P. Bono, MIT University, Boston, USA, personal comm.). Expand™ should have been more efficient in reading genomic DNA.

Nested PCR was used in an effort to extract a product from the smeared products produced when mouse genomic DNA was amplified with primers directed against the complete coding sequence for SSAO. The products of this reaction were amplified with a second set of primers specific for the smaller TOPA co-factor portion of the coding sequence. Nested PCR improves the sensitivity and specificity of DNA amplification (Jackson *et al.*, 1992). However, multiple products were obtained upon amplification with both Expand™ and *Taq* polymerases, even when the annealing temperature was increased.

There is no evidence that murine foetal dental pulp contains SSAO and therefore the mRNA for it. If the enzyme was present, it might be sufficiently different from that in the sources so far studied to not anneal tightly to the primers used. However, current evidence suggests strong similarities between the TOPA sequences of SSAOs from different sources and therefore one might expect this to be so for the mouse. The question also exists as to whether or not the foetal murine teeth actually contain SSAO. The mRNA for blood vessels was likely to be present in the preparation from which the cDNA library was produced (MacDougall, University of Texas Health Science Centre, USA, personal comm.) and therefore one would expect the cDNA for murine VAP-1 to be present, if it exists in foetal murine teeth. There are no data

available as to the presence of VAP-1 in foetal tissue as the sequences so far identified were from adult mouse kidney. The amplification of a genomic *E. coli* contaminant indicates that the experimental procedure is working. Thus this appears to cast doubt on the applicability of the cDNA library obtained, although other genes, including dentine sialoprotein and dentine phosphoprotein, have been successfully isolated from the library (MacDougall *et al.*, 1997)

Future attempts to clone murine dental pulp SSAO should focus on using the available clones from sources previously identified to contain SSAO /VAP-1 enzymes. The use of a similar clone to screen the plated murine odontoblast cDNA library under conditions of low stringency may yield positive results. The use of lower stringency may help identify cDNA sequences that are similar to SSAO, but may contain important differences that may explain the differences observed with other dental pulp SSAO enzymes.

6.6 Conclusions

The cloning of SSAO from mouse genomic DNA and DNA purified from a murine odontoblast cDNA library was not successful. Oligonucleotide primers directed against the coding region for the tyrosine residue that links the organic co-factor failed to produce a product in the range anticipated from comparison with known SSAO sequences. Other oligonucleotide primers directed against the complete coding sequence amplified multiple products, but the product at the correct size proved not to correlate with any known SSAO sequence.

The use of nested primers combining the two sets of oligonucleotide primers, used in an effort to increase the sensitivity and specificity of the PCR reaction produced multiple products, but none in the size range anticipated.

CHAPTER SEVEN

General Discussion

SSAO in dental pulp may be linked to inflammation in three ways. The first is through the metabolism of 5-HT in porcine (Norqvist *et al,* 1982 and this thesis) and human dental pulps (this thesis). 5-HT is an important mediator in neurogenic inflammation and has many modes of action. It acts directly by stimulating release of prostacyclins (Hirafuji & Ogura, 1987) and arachidonic acid metabolites from rat dental pulp and indirectly by modulating the responses of other inflammatory mediators including CGRP in bovine pulpal nerve terminals (Jackson *et al.,* 1992) and substance P in the rat hind paw blister model (Andrews & Helme, 1989; Khalil & Helme, 1989). Ngassapa and co-workers (1992) demonstrated in dental pulps of beagle dogs that application of 5-HT (1mg /ml) increased both sensitisation and increased responsiveness to a range of stimuli. In fact spontaneous firing of unstimulated nerves was reported in 11 out of 30 teeth tested and the magnitude of the response observed with all stimuli exceeded that seen with CGRP (2 μg /μl). Sites of 5-HT formation in human dental pulp were localised using anti-tryptophan hydroxylase antibodies and found to occur in the odontoblast layer and nerve tissue. The presence of 5-HT in pulpal nerve tissue has not been previously described and suggests that it may also be acting as a neurotransmitter in this tissue. Release of 5-HT from nerve tissues may be important in the modulation of neurogenic inflammation.

The second is the two metabolic pathways for breakdown of histamine. In porcine dental pulp SSAO may be responsible for the breakdown of histamine to imidazole acetaldehyde as a result of competition experiments (K_i 1054 $\pm$ 30 μM in competition with benzylamine and 2950 $\pm$ 400 μM in competition with 5-HT). However, direct SSAO activity with histamine as a substrate was too low to be measured. SSAO catalysed the breakdown of *tele*-methylhistamine to *tele*-methylimidazole-acetaldehyde (K_m 6.4 $\pm$ mM, V_{max} 119 $\pm$ 10 nmol/hour/mg protein). The latter pathway may also be catalysed by MAO-B (Green *et al.,* 1987) but in porcine dental pulp this did not appear to be the case. This means that SSAO maybe responsible for both breakdown pathways of histamine in porcine dental pulp. Histamine has many actions in inflammation (see Chapter One, Section 1.3.5) thus increasing the importance of SSAO in dental tissues.

A third way in which SSAO may be linked to inflammation and cell turnover is through the release of hydrogen peroxide as a product of the oxidative deamination of biogenic amines. Hydrogen peroxide has been linked to differentiation of developing tissues (Parchment, 1993), and responses seen to hydrogen peroxide depend on the dosage. High doses cause lipid peroxidation and cell death (de Lamirande & Gagnon, 1995), whereas low doses may act as inter- and intra-cellular messengers capable of promoting growth (Burdon, 1995). A single application of hydrogen peroxide to cultured murine odontoblasts did not result in cell death at concentrations up to 1 mM concentration. Further work is required to examine the effects of lower doses of hydrogen peroxide over extended periods to observe changes in cell growth and proliferation. Hydrogen peroxide generated by amine oxidase metabolism may combine with that released as a defense against bacteria and other noxious agents by white blood cells (Laochumroonvorapong *et al.*, 1996) to affect both cells and alter local vasomotor tone. This occurs by depression of the the phasic responses to noradrenaline-induced contraction and potentiating the tonic response (Okabe, 1994). The possibility also exists that in the presence of transition metals hydrogen peroxide will convert to the toxic hydroxyl radical in a Fenton reaction.

Metabolism of biogenic amines by SSAO may also produce toxic aldehydes, such as the formaldehyde production upon deamination of methylamine. However oxidative deamination of methylamine could not be detected in porcine dental pulp. Other amines like aminoacetone and allylamine produce toxic aldehyde products and require testing as substrates for SSAO in dental pulp.

SSAO in human dental pulp has been localised immunocytochemically using anti-SSAO antibodies. It exists in the odontoblast layer, blood vessels and nerve tissue. SSAO has not been described in nerve tissue prior to this thesis. It raises the possibility that it acts as a neurotransmitter metabolising enzyme in this tissue. This reinforces the unique nature of this enzyme in dental pulp. The presence of SSAO in the odontoblasts layer links the enzyme to odontoblasts function and the possibility exists that the enzyme may modulate activity of these cells, but this requires further research.

New functions of SSAO in general have come to light in the lasts few years. The cloning and sequencing of mouse VAP-1 (Smith *et al.,* 1998) and the discovery that its sequence is identical to SSAO in that tissue suggests a role for SSAO in the binding of lymphocytes to blood vessel walls. VAP-1 mediates the initial L-selectin-independent interaction between lymphocytes and endothelial cells under non-static conditions (Salmi & Jalkanen, 1992). Expression of VAP-1 is principally in the peripheral lymph nodes (Salmi & Jalkanen, 1992). Interestingly, VAP-1 expression is upregulated in the presence of chronic inflammation in tissues such as gut, tonsil, skin and synovium (Salmi *et al.,* 1992). If the same process occurs in dental pulp with SSAO then its influence in inflammation may be far greater than thought to date.

Vesicle immunolocalisation analysis indicates that GLUT4-containing vesicles from rat adipocytes contain substantial levels of SSAO activity (Enrique-Tarancon *et al.,* 1998). Immunotitration of GLUT4 vesicles indicate that SSAO and GLUT4 co-localise in an endosomal compartment in rat adipocytes, 3T3-L1 adipocytes and rat skeletal muscle. Benzylamine was found to initiate recruitment of GLUT4 to the plasma membrane of adipose cells. Stimulation of glucose transport was found to be hydrogen peroxide dependent as catalase inhibited any stimulation (Enrique-Tarancon *et al.,* 1998). This opens the possibility that substrates for SSAO may be involved in glucose transport. Thus if the expression of SSAO was to be upregulated like VAP-1 in inflammation, SSAO may be important in energy metabolism in cells like odontoblasts under inflammatory conditions.

The accumulation of sufficient quantities of dental pulp to perform kinetic and other experiments is extremely difficult. Human dental pulp weighs approximately 25 mg and the availability of sample teeth is low. It was only possible to carry out limited kinetic investigations on crude homogenates in human dental pulp due this problem. Both 5-HT (K_m 318 $\pm$ 54 μM, V_{max} 52 $\pm$ 5 nmol/hour/mg protein) and benzylamine (K_m 254 $\pm$ 55 μM, V_{max} 370 $\pm$ 44 nmol/hour/mg protein) were found to be substrates for SSAO in human dental pulp. SSAO was found to be responsible for 88 % of the 5-HT metabolism in crude homogenates when tested with 100 μM 5-HT as a substrate.

The growth of odontoblasts from primary cultures is difficult with limited success having been reported due to donor and proliferation variability between samples (Moule *et al.*, 1995). Established cell lines of transformed murine odontoblasts were acquired to perform initial experiments on the effect of hydrogen peroxide and 5-HT on these cells. The cells were resistant to hydrogen peroxide up to 100 μM concentration when tested at 10, 23 and 36 hours. There was also resistance to 5-HT, with limited cell death up to a concentration of 1 mM concentration. Further work is necessary in this area to observe the effects of chronic application of both hydrogen peroxide and 5-HT in these cells. Cell lines offer the opportunity to demonstrate changes in cells without the problems of variability seen with primary cultures. However, some transormed cell lines may be particularly resistant to apoptosis.

Much of the kinetic work in this thesis focused on porcine dental pulp microsomes after the observation that 5-HT was a substrate for SSAO in this tissue (Norqvist *et al.*, 1982). Furthermore the tissue was more readily accessible and the yield per jaw was in the region of 3 g. The most noteworthy features were that two SSAO enzymes were found to exist in these microsomes, one that carries out the 5-HT, PEA and some benzylamine oxidation and the other enzyme the majority of the benzylamine oxidation. The two forms demonstrated different thermostability at 70 °C with the 5-HT-metabolising enzyme remaining stable when assayed at 70 °C for 60 min and the benzylamine-metabolising enzyme losing activity after 15 min. This may in future work, be used to separate the two forms of the enzyme. This combined with the successful solubilisation of the enzymes in 1 % Triton X-100 may provide a basis for future purification of the 5-HT-metabolising form of SSAO. Surprisingly, when the benzylamine-deaminating form of SSAO in porcine dental pulp homogenates was heated to 70 °C and assayed at 37 °C the activity returned to near normal values. This is unusual in that thermal denaturation at such high temperatures is usually irreversible.

Activation of microsomal SSAO at 37 °C was first reported with benzylamine as a substrate in bovine lung (Lizcano, 1994). Activation was found to occur in porcine dental pulp at 37 °C when both 5-HT and benzylamine were used as substrates. This is

the first time that activation has been demonstrated for a physiological substrate with SSAO. However, the activation was not always seen (only in 2 out of 5 experiments) suggesting that another, as yet unidentified, factor must be involved in the activation of dental pulp SSAO. The influence of thiol group oxidation was examined and found not to be responsible for activation, but a difference in thiol function was observed with both SNAP and SNOG for the 5-HT- and benzylamine-metabolising forms of the enzyme, suggesting that their active site configurations differs in the arrangement or involvement of thiol groups.

Partial purification of the porcine dental pulp microsomal SSAO by gel-filtration through a Superdex column identified two peaks of activity of the enzyme when both 5-HT and benzylamine were used as substrates. One peak was at 110 kDa and the other at 440 kDa. Re-running the second 440 kDa peak through the same column and testing the activity with benzylamine produced a single peak at 220 kDa. This suggests that the enzyme can exist in three active forms, a monomer, dimer and tetramer. The failure to separate the two enzyme activities by this procedure or by ion-exchange chromatography suggests that they are quite similar in gross molecular properties, although the possability that the two enzyme activities reside on the same protein cannot be excluded.

Cloning of murine dental pulp SSAO from a neo-natal murine dental pulp cDNA library using oligonucleotide primers based on known SSAO and VAP-1 sequences was not successful. The presence of VAP-1 and SSAO in neo-natal primary teeth could not be confirmed and the possibility exists that these enzymes are not expressed in these tissues. The way to avoid the problem of limited dental material in the future is through cloning and expression of dental pulp SSAO. Purification and sequencing of further SSAO enzymes should give further information regarding their function. In particular the expression and purification of human dental pulp SSAO would be of interest to determine the structural features of the active site that facilitate the metabolism of 5-HT in this tissue.

Although the function of SSAO in porcine and human dental pulp has not been fully elucidated some progress has been made in understanding its properties and

behaviour. SSAO would appear to be intimately linked with the inflammatory process in these tissues and the exciting future potential exists for modulation of the inflammatory process through SSAO. This, however may be a complex task as the inflammatory process is in part controlled by substances that are both substrates and products of the oxidative deamination deamination of 5-HT and histamine.

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