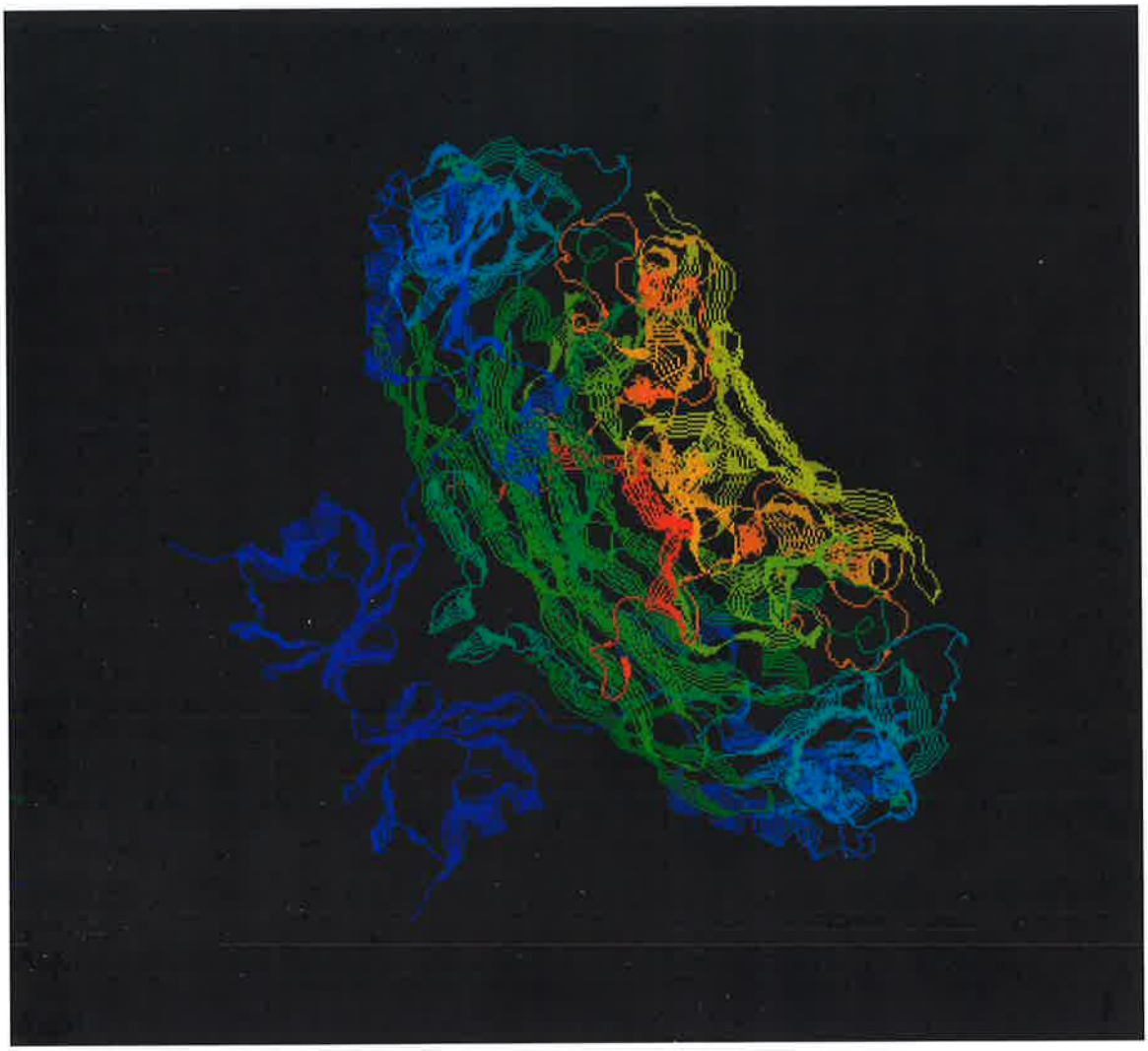
Human Retroplacental Serum Polyamine Oxidase

VOLUME I



Three-Dimensional Structure of an *E. coli* Amine Oxidase.
E. coli amine oxidase viewed perpendicular to the molecular dyad axis pointing at 45° from the horizontal. The amine oxidase chains are shown as 'strands' coloured according to a scheme which codes residues by their position in a macromolecular chain. Each chain is drawn as a smooth spectrum from blue at the *N*-terminus through cyan to green, yellow and orange to red at the *C*-terminus. β-sheets are represented as flat stranded ribbons and α-helices by coils (both 1.52 Å), turns and other residues are represented by a narrower ribbon (both 0.4 Å). Each subunit of the mushroom-shaped homodimer comprises four domains. The stalk domains (residues 1-85) can be clearly seen at the *N*-terminus. The active sites, which each include a copper atom and TOPA quinone cofactor, are located within the large β-sandwich domain which makes up the bulk of the molecule. This is the first amine oxidase crystal structure to be reported [529]. The figure was generated using RasMol version 2.6 for Microsoft Windows, © 1996 R. Sayle, Biomolecular Structures Group, Glaxo Wellcome Research and Development, Stevenage, Hertfordshire, UK obtained by anonymous FTP from ftp://ftp.dcs.ed.ac.uk/pub/rasmol/v2.6beta/. Coordinates (entry 1oac, version of February 1996) used for the *E. coli* amine oxidase structure were obtained from the Protein Data Bank [1141,1142] at the Brookhaven National Laboratory, NY by gopher://pdb.pdb.bnl.gov:70/00/FTP/fullrelease/uncompressed_files/oa/pdb1oac.ent. The secondary structure was assigned from the PDB file.

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Human Retroplacental Serum Polyamine Oxidase

Purification and Characterization

A thesis submitted to
The University of Adelaide
for the degree of
Doctor of Philosophy
in the Faculty of Medicine

by

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The epigraph is taken from *Why Purify Enzymes?* [1820], which borrows extensively from *For the Love of Enzymes: The Odyssey of a Biochemist*, Harvard University Press 1989 [1821].

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To my parents and mentors

[Enzyme purification] may seem like the ascent of an unchartered mountain: the logistics like those of supplying successively higher base camps. Protein fatalities and confusing contaminants may resemble the adventure of unexpected storms and hardships. Gratifying views along the way feed the anticipation of what will be seen from the top. The ultimate reward of a pure enzyme is tantamount to the unobstructed and commanding view from the summit. Beyond the grand vista and the thrill of being there first, there is no need for descent, but rather the prospect of even more inviting mountains, each with the promise of even grander views.

—Arthur Kornberg

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ABSTRACT

Polyamine oxidases catalyse the oxidation of polyamines to aminoaldehydes that have been shown to have antimicrobial, anti-inflammatory, antiproliferative and immunomodulatory effects *in vitro*. The enzymes have been localized to placenta, liver, kidney, small intestine, macrophages and neutrophils. Plasma polyamine oxidase activity increases during human pregnancy and is especially high in retroplacental blood. Polyamine oxidases have a role in a broad range of physiological and pathophysiological functions. Therefore, an understanding of their role would have wide importance. Identification and characterization of isolated polyamine oxidases would facilitate an understanding of their biological role.

Limitations to our understanding of the polyamine oxidases have included a lack of specific reagents and definitive characterization of the oxidases. Neither monoclonal antibodies nor a sequence for the human retroplacental serum polyamine oxidase have previously been available. Therefore, the human retroplacental enzyme was purified using adaptations of conventional biochemical and novel affinity methods. Monoclonal antibodies were produced to the purified enzyme and used for its further, large scale, purification with immunoaffinity techniques, providing enzyme for detailed structural and functional analyses. The enzyme was subsequently identified as an amiloride-sensitive copper-containing amine oxidase by *N*-terminal sequence analysis of the immunoaffinity purified enzyme subunit. Substrate selectivity of the enzyme, based on relative catalytic efficiency, k_{eff}, was:
histamine > putrescine > N^1-acetylspermine > spermidine > spermine > N^1-acetylspermidine.

The enzyme was sensitive to carbonyl group reagents but not monoamine oxidase inhibitors. Metabolic studies indicated that the enzyme acts by cleaving its polyamine substrates at secondary amino groups in an EC 1.5.3 manner, suggesting a broader classification for the enzyme than the current EC 1.4.3.6 classification of amiloride-sensitive copper-containing amine oxidase.

The enzyme was found to exist as at least two multiple forms that are composed of homodimers of M_r 108,000 glycoprotein subunits. Deglycosylation of the enzyme reduced the apparent M_r of the enzyme subunit to 86,000, consistent with the calculated molecular mass of the mature enzyme subunit (83,415.6 Da). Atomic absorption spectroscopy indicated one copper atom per subunit. Sequence similarities with other amine oxidases suggested that the copper atom is at the active site of the enzyme along with 2,4,5-trihydroxyphenylalanine quinone.

PUBLICATIONS

Publications arising from these studies:

Storer, R.J., Ferrante, A. and Morgan, D.M.L. (1998) Human Retroplacental Serum Polyamine Oxidase: Purification and Characterization. *Biochemical Journal,***000**, 000–000. (*in preparation*)

Storer, R.J. and Ferrante, A. (1998) Radiochemical assay of diamine oxidase. **In:** *Methods in molecular biology. Vol. 79*: *Polyamine protocols,* (Morgan, D.M.L. *ed.*), Chapter 10, pp. 91–95, Humana Press, Totowa, NJ.

Storer, R.J. and Ferrante, A. (1998) Hydrogen peroxide assay for amine oxidase activity. **In:** *Methods in molecular biology. Vol. 79*: *Polyamine protocols,* (Morgan, D.M.L. *ed.*), Chapter 9, pp. 81–90, Humana Press, Totowa, NJ.

Storer, R.J., Ferrante, A. and Morgan, D.M.L. (1994) Human retroplacental polyamine oxidases. **In:** *Proceedings of the 6th amine oxidase workshop and 5th trace amine conference. A joint international IUPHAR satellite meeting,* (Yu, P.H., Tipton, T.F.and Boulton, A.A. *eds.*) *C.06* [abstract], *Saskatoon, Canada.*

Storer, R.J. and Ferrante, A. (1990) Purification of retroplacental polyamine oxidizing enzymes. **In:** *Kokusai taiban godokaigi,* (Taki, I. and Soma, H. *eds.*) *E9* [abstract], The Japan Placental Group, The European Placenta Group and The Rochester Trophoblast Society. Proceedings of the International Conference on Placenta, *Tokyo, Japan.*

Ferrante, A., Storer, R.J. and Cleland,L.J. (1990) Polyamine oxidase activity in rheumatoid arthritis synovial fluid. *Clinical and Experimental Immunolology* **80**, 373–375.

Storer, R.J., Ferrante, A., Bates, D.J., Zola, H. and Morgan, D.M.L. (1988) Retroplacental polyamine oxidase: antiinflamatory, antitumour and immunosuppressive properties; further characterization, **In:** *Placental and endometrial proteins: basic and clinical aspects,* (Tomada, Y., Mizutani, S. Narita, and Klopper, A. *eds.*) pp. 349–352, VSP BV, Utrecht, The Netherlands. Proceedings of the 6th International Congress on Placental and Endometrial Proteins, *Nagoya, Japan.*

DECLARATION

All of the results described in this dissertation are from experiments conducted in the University of Adelaide Department of Paediatrics, and Department of Immunopathology at the Women's and Children's Hospital. I hereby declare that this submission is my own work and that it contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and that, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due acknowledgement is made in the text.

Procedures with animals were carried out in accordance with the 'Code of Practice for the Care and Use of Animals for Scientific Purposes', NH&MRC/CSIRO/AAC guidelines and the Prevention of Cruelty to Animals Act 1985 with procedures recommended by, and with approval of, the Ethics Committees of the Women's Children's Hospital and The University of Adelaide.

Retroplacental blood and synovial fluid collections were carried out in accordance with the National Health & Medical Research Council statement on human experimentation, the guidelines recommended by, and with the approval of the Ethics Committees of the Queen Victoria Hospital, the Women's Children's Hospital (formerly the Adelaide Children's Hospital) and The University of Adelaide.

Subject to the provisions of the Copyright Act, 1968, I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

R. James Storer
London,
February 1998

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While the assistance of those mentioned above is acknowledged, I must accept the ultimate responsibility for the intellectual content and any errors herein.

PREFACE

The amine oxidases have attracted a wide range of interest because of their ubiquity and their role in a broad spectrum of physiological and pathophysiological functions including non-specific immunity (antimicrobial activity), antitumour, anti-inflammatory and immuno-suppressive activity as described in the Introduction, which also discusses various physical and catalytic properties of these enzymes and their substrates. This thesis describes the purification and characterization of human polyamine oxidases found in retroplacental serum, the most active source of pregnancy-associated polyamine oxidases, as recognised by Morgan. The general methods used are described in Chapter 2. Following Morgan's work it was found that the polyamine oxidase activity in retroplacental serum could be resolved into two forms as described in Chapter 3, but is was not clear whether the two forms were identical or represented distinct enzymes as suggested by Crabbe and others. The purified retroplacental serum polyamine oxidase was found to have the antimicrobial, antiproliferative, immuno-suppressive and anti-inflammatory biological activity associated with the bovine serum enzyme and unpurified retroplacental and pregnancy serum. Attempts to identify and characterize the retroplacental serum polyamine oxidases using novel affinity methods is also described in Chapter 3. Following the preparation of purified enzymes they were used to generate monoclonal antibodies as described in Chapter 4. The monoclonal antibodies were produced in large quantities and used for the immunoaffinity purification of the human retroplacental serum polyamine oxidases, resulting in significant improvement of enzyme yield and purity as described in Chapter 5 along with further characterization of the relative molecular mass of the enzymes. Furthermore, these monoclonal antibodies are unique biochemical reagents and provide a basis for the development of sensitive and specific immunochemical detection systems for the enzymes. Detailed characterization studies including the protein sequencing and steady-state kinetics of the enzymes allowing the identification of the enzymes are described in Chapter 6. The results of the various purification and characterization studies are discussed in Chapter 7 where the results are discussed in the context of other studies and attempts are made to rationalise the similarities and differences. Finally, conclusions about the nature of the enzymes are summarized.

To assist the reader, this dissertation is divided into two volumes. Volume I contains the preliminaries, literature review, methods, descriptions of the experimental work and the results, discussions and conclusions. Volume II contains the appendices, publications arising from this study and references.

ABBREVIATIONS

Ø (column) diameter
ΔAB$^+$ human AB$^+$ serum from healthy nonpregnant donors, heated to 56 °C for 30 min
ΔFBS fetal bovine serum, heated to 56 °C for 30 min to inactivate complement proteins
2D-PAGE two-dimensional polyacrylamide gel electrophoresis
aa amino acid
AA arachadonic acid
Ab antibody
ABP amiloride binding protein
ABP_HUKI.s human kidney ABP/DAO sequence [M55602]
ABP_HUMN.s human diamine oxidase sequence [EMBL 78212]
ABP_HUMAN human amiloride-binding protein precursor
ABP_RATC.s rat colon/lung ABP/DAO sequence [X73911]
ABTS 2,2[1]-azino-bis-(3-ethylbenzthiazoline-6-sulphonate)
ALL acute lymphoblastic leukemia
AML acute myoblastic leukemia
AMO_ECOL.s *E. coli* amine oxidase (maoA) sequence [L47571]
AMO_HANS.s *Hansenula polymorpha* AO sequence [X15111]
anh. anhydrous
AO amine oxidase (EC 1.4.3 or 1.5.3)
AO_ANTHR.s *Anthrobactr* methylamine oxidase (maoxII) sequence [L12990]
AO_BOVIN.s bovine serum/liver copper amine oxidase sequence [S69583]
AO_KAERO.s *K. aerogenes* AO sequence [D10208]
AO_LENSC.s lentil seedling AO sequence [X64201]
AO_PISUM.s pea seedling AO sequence [L39931]
APS ammonium persulphate
BAO benzylamine oxidase
BCA bicinchoninic acid (4,4′-dicarboxy-2,2′-biquinoline)
BHK baby hamster kidney
BIS *N,N′*-bis-methylene acrylamide
BPB bromophenol blue
BSA bovine serum albumin
CAPS 3-[cyclohexylamino]-1-propanesulphonic acid
CBB Coomassie Brilliant Blue
CF cystic fibrosis
CHO chinese hamster ovary
CI Colour Index
CK-2 casein kinase II
con A concanavalin A
CTC copper-tartrate-carbonate
DAO diamine oxidase = histaminase (EC 1.4.3.6 although may have EC 1.5.3 activity)
DEAE diethylaminoethyl
DFMO α-difluoromethylornithine
DMPTU dimethylphenylthiourea
DMSO dimethylsulphoxide
DNS dansyl
DOC deoxycholate, sodium
DPM disintergrations per minute
DPTU diphenylthiourea

DPU diphenylurea
DTT dithiothreitol
EBI European Bioinformatics Institute
EC Enzyme Commission (nomenclature recommended by NC-IUBMB *q.v.*)
EIA enzyme immunoassay
ELISA enzyme linked immunoassay
EMBL European Molecular Biology Laboratory
EPR electron paramagnetic (or spin) resonance
FAD flavin–adenine dinucleotide
FBS fetal bovine serum
fMLP *N*-formyl-methionyl-leucylphenylalanine (peptide)
ftp file transfer protocol
GABA γ-aminobutyric acid (γ-aminobutyrate)
G6PD glucose-6-phosphate dehydrogenase
GSH reduced glutathione
HAT hypoxanthine/aminopterin/thymidine
HECS human endothelial cell supernatant
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HES hybridoma enhancing supplement
HGPRT hypoxanthine guanine phosphoribosyl transferase
HIC hydrophobic interaction chromatography
HMP hexose monophosphate
HMT histamine methyltransferase (EC 2.1.1.8)
HPLC high-pressure (-performance) liquid chromatography
HRPO horseradish peroxidase
HT hypoxanthine/thymidine
HVA homovanillic acid
IBM-PC/DOS IBM personal computer/disk operating system
IEF isoelectric focusing
Ig immunoglobulin
IL-2 interleukin-2
IUdR iododeoxyuridine
LPS lipopolysaccharide
LT leukotriene
mAb monoclonal antibody
MAO monoamine oxidase (EC 1.4.3.4 although may have a 1.4.3.6 phenotype)
MAOx methylamine oxidase (EC 1.4.3)
MGBG methylglyoxyl-bis(guanylhydrazone)
MLR mixed lymphocyte (culture) reaction
MS-Windows Microsoft® Windows
NC-IUBNB Nomenclature Committee of the International Union of Biochemistry
NMDA *N*-methyl-D-aspartate
ODC ornithine decarboxylase
ONPG *o*-nitrophenol-β-D-galactopyranoside
OPA *o*-phthalaldehyde
PA polyacrylamide (gel)
PAGE polyacrylamide-gel electrophoresis
PAO polyamine oxidase (EC 1.5.3.11, EC 1.4.3.6, or EC 1.4.3.4)
PBS Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+}
PBS-T PBS containing 0.05% Tween 20

PCR polymerase chain reaction
PCZ procarbazine (*N*-isopropyl-α-(2-methyl hydrazino)-*p*-toluamide hydrochloride)
PDB (Brookhaven) Protein Data Base
PEAO 2-phenylethylamine oxidase (EC 1.4.3.-)
PEG polyethylene glycol
Pg prostaglandin
PHA phytohaemagglutinin
PHYLIP phylogeny inference package
PITC 1,4-phenylenediisothiothiocyanate
Plasma AO plasma amine oxidase = serum amine oxidase = diamine oxidase
PLA_2 phospholipase A_2
PMA phorbol 12-myristate 13-acetate
PMN polymorphonuclear nucleocytes (neutrophils)
PNGase F peptide:N-glycosidase F (EC 3.5.1.52)
POPOP 1,4-di(2-(5-phenoxazolyl))-benzene
PPO 2,5-diphenoxazole
PQQ pyrroloquinoline quinone
PSG penicillin, streptomycin and glutamine medium supplement (*q.v.* §2.8.1.1)
PTH phenylthiohydantoin
PVA polyvinyl alcohol
PVDF polyvinylidene fluoride
RA PBMNC rheumatoid arthritis peripheral blood mononuclear cells
RA SFMNC rheumatoid arthritis synovial fluid mononuclear cells
RPS retroplacental serum
RID radial immunodiffusion
RPMI 1640 Roswell Park Memorial Institute 1640 medium (*q.v.* §2.8.1.1)
SD standard deviation
SDS sodium dodecyl sulphate
SE-HPLC size-exclusion high pressure liquid chromatography
SOD superoxide dismutase
SSAO semicarbazide-sensitive amine oxidase; EC 1.4.3.6?
SWR standard working reagent
TAO tyramine oxidase (EC 1.4.3.4)
[^{3}H]TdR [^{3}H]thymidine deoxyribose
TCA trichloroacetic acid
TEMED *N,N,N′,N′*-tetramethylethylenediamine
t.l.c. thin layer chromatography
TNF tumour necrosis factor
TOPA 2,4,5-trihydroxyphenylalanine
TPQ 2,4,5-trihydroxyphenylalanine quinone
Tris tris(hydroxymethyl)aminomethane
TST Tris-saline-Tween 20
Tx thromboxane

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Chapter 1

INTRODUCTION: POLYAMINES AND THEIR OXIDASES

1.1 Introduction

This chapter provides a comprehensive survey of polyamines, their oxidases and the consequences of their interactions. It is not intended to be exhaustive review of all aspects of the literature, but to focus instead on areas that are of interest, with the general aim of placing these areas within the context of other areas in the field and setting the research described in this dissertation in a broader context. This review discusses polyamines, their physiology and their pathophysiology; amine oxidases (especially the human pregnancy-associated enzymes), their classification, distribution, structure, substrate and inhibitor specificity, physiology and pathophysiology; the biological effects of oxidized polyamines and consequences of polyamine oxidation; the pregnancy-associated amine oxidases and their purification.

1.2 Polyamines

The polyamines are nonprotein, aliphatic amines of low molecular mass (*vide* §1.2.2). They are usually known by their trivial names: putrescine, cadaverine, spermine and spermidine. Putrescine and cadaverine are the simple diamines, 1,4-diaminobutane and 1,5-diaminopentane respectively; and spermine and spermidine are aminopropyl derivatives of putrescine, *N,N′*-bis(3-aminopropyl)-1,4-diaminobutane and *N*-(3-aminopropyl)-1,4-diaminobutane. Throughout this dissertation, the term 'polyamines' includes in its meaning 'diamines'[1.] [1,2]. Polyamines are found ubiquitously in nature [3-15]. Spermidine and putrescine are present in virtually all living organisms and spermine is present in virtually all cells of higher eukaryotes [6]. The ubiquity of the polyamines suggests their appearance at an early stage of evolution and their persistence during evolutionary development.

1.2.1 Historical Introduction to the Polyamines

In a letter written in November 1677 to Viscount Brouncker, the then President of the Royal Society, the Dutch microscopist Antoni van Leeuwenhoek described crystals he observed in a

[1.] 'When I use a word,' Humpty Dumpty said, in rather a scornful tone, 'it means just what I choose it to mean—neither more nor less.' [16,17].

sample of human semen. His famous letter, in which he also announces his discovery of spermatozoa, was published in *Philosophical Transactions* for 1678 [18] and includes a marginal engraving of the crystals that he saw with his primitive microscope (Figure 1.1). Leeuwenhoek's original sketch was probably distorted by the engraver, but most certainly what he saw were crystals of spermine phosphate (Figure 1.2). It is now known that the slow deposition of spermine as its insoluble phosphate salt is a result of the liberation of inorganic phosphate by the enzymatic hydrolysis of phosphorylcholine present in seminal fluid [19-21].

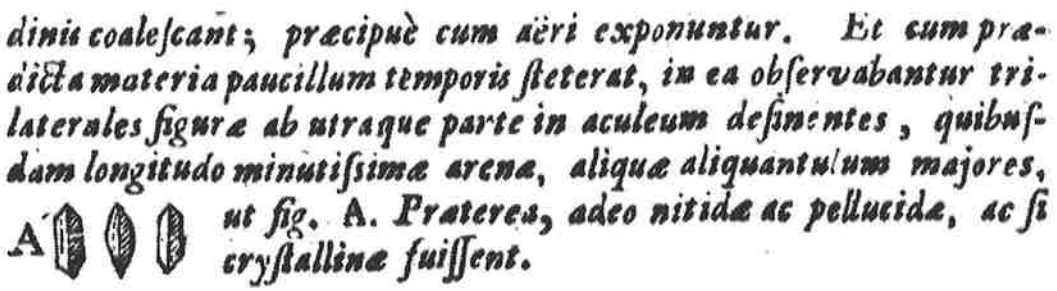

dinis coaleſcant; præcipuè cum aëri exponuntur. Et cum prædicta materia paucillum temporis ſteterat, in ea obſervabantur trilaterales figuræ ab utraque parte in aculeum deſinentes, quibuſdam longitudo minutiſsimæ arenæ, aliquæ aliquantulum majores, ut fig. A. Prateres, adeo nitidæ ac pellucidæ, ac ſi cryſtallinæ fuiſſent.

FIGURE 1.1. Extract of Leeuwenhoek's Letter to Viscount Brouncker, from Phil. Trans. Vol. XII, 1678. No. 142, p. 1042. It shows a Latin translation of Leeuwenhoek's [Dutch] description of crystals [of spermine phosphate] in human semen and includes the engraving (Fig. A.) from a drawing sent by Leeuwenhoek. The Latin text has been translated as: 'When this matter had stood a little while, some three-sided bodies were seen in it, terminating at either end in a point; some were the length of the smallest grain of sand, and some were a little bigger, as in Fig. A. They were further as bright and clear as if they had been crystals.' [22,23].

Vauquelin described the same crystals in a paper published in 1791 [24]. He was the first to attempt their chemical investigation, but erroneously concluded that they were calcium phosphate. Charcot published a paper on the crystallization of spermine phosphate in 1853 [25]. In 1865 the crystals were rediscovered by Boettcher [26] and they became known as 'Boettcher's crystals'. In 1878 Schreiner showed that the crystals were a phosphate salt of an organic base [27] known as 'spermatin' [28], a name probably derived from 'spermatine' that was previously used for a specific protein of semen by Berzelius in 1833 [29]. The modern trivial name 'spermin(e)' was first used by Landenburg and Abel in 1888 [30]. The correct chemical structure of the crystals was elucidated between 1924 and 1926 by Rosenheim *et al.* in England, who confirmed the structure by synthesis [31-36]; and by Werde's group in Germany [37-39].

The Russian physiologist Poehl, who isolated spermine phosphate from testis and other organs in 1891 [40,41], reported that spermine had valuable therapeutic properties. He advocated the use of spermine as a therapeutic agent in his '*Sperminetheorie*', which was described in his monographs *Die physiologisch-chemischen Grundlagen der Sperminetheorie* and *Rational Organotherapy* [42,43]. Poehl's books describe treatment of numerous diseases with spermine. Although his chemical invesitgation of spermine correctly identifies its chloroplatinate as $C_{10}H_{26}N_4.4HCl.2PtCl_2$ [40], his pharmacological and clinical work has been discredited [19,31]

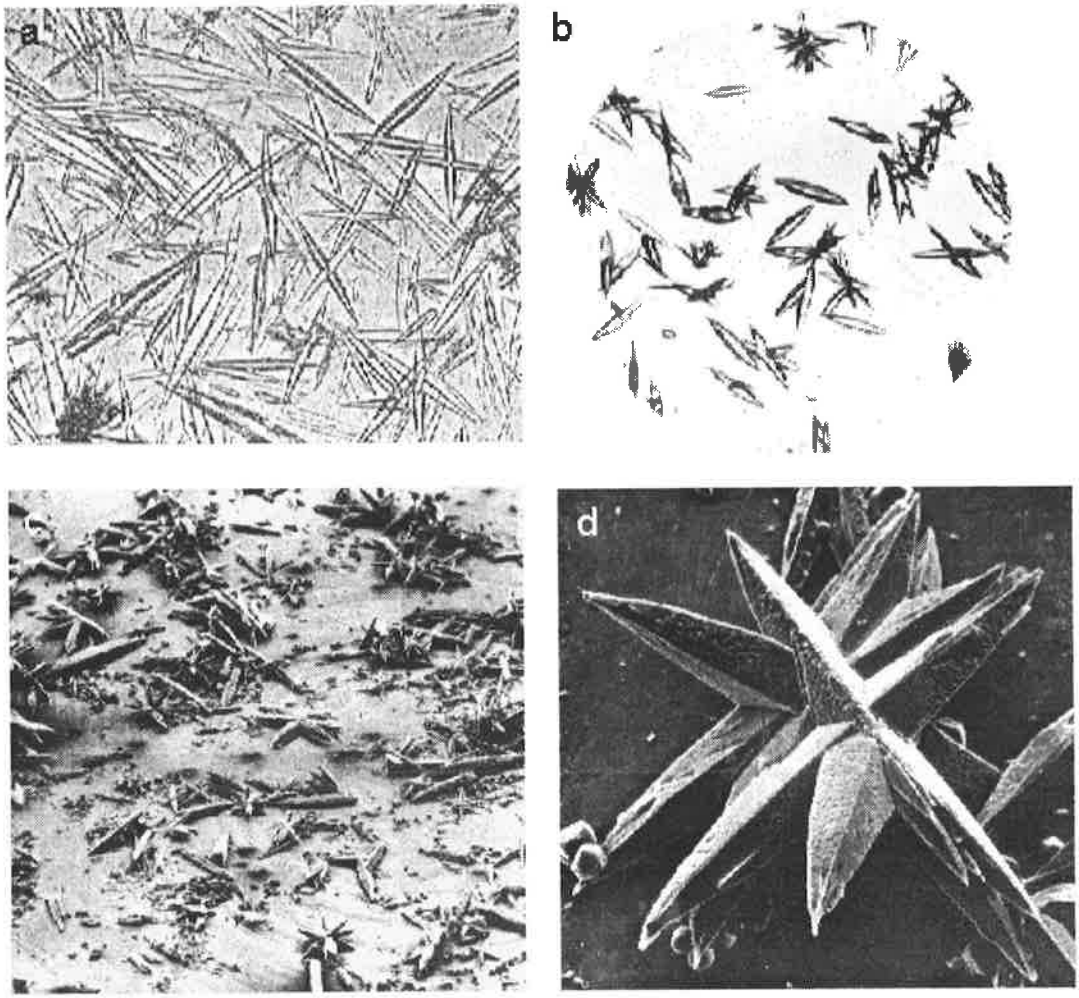


FIGURE 1.2. Crystals of Spermine Phosphate in Human Semen. Photomicrographs after (a) Poehl [41], (b) Rosenheim [31]. Scanning electron micrographs from a Cambridge Stereoscan Mark IIa SEM (c) ×50, (d) ×350 (After Smith [50]).

because of his commercial exploitation of the assumed therapeutic properties of spermine. Indeed, administered parenterally, spermine is known to be nephrotoxic [44,45]. Other toxicities have also been reported [46]. The nephrotoxicity may be due to the presence of amine oxidases in kidney, in particular those associated with the renal epithelium [47,48]. It has been suggested that some of the toxic effects of spermine might be related to products of its oxidation [44]: the highly reactive amino aldehydes [49] (*vide* §1.2.6.7, 1.4.4, 1.5 *et seq.*). Fortunately for recipients of his therapy, Poehl's commercial material, '*Sperminum Poehl*', contained only traces of spermine [31].

In 1887 Brieger isolated three bases from cultures of *V. cholerae*, putrescine (1,4-diaminobutane), cadaverine (1,5-diaminopentane), and 1,3-diaminopropane [51]. In 1927 Rosenheim *et al.* identified another biochemical base, spermidine [52].

A vast polyamine literature now exists, with many reviews of the physiological roles of polyamines [8-14,50,53-95]. The roles of polyamines in the biochemistry of particular organs or

metabolic pathways are included in a number of monographs or reviews [6,7,15,85,95-125]. Comprehensive books have been written [3,4,87,126-137]. A four volume series called *Advances in Polyamine Research* [138-141] and proceedings of various symposia have been published [142-154].

1.2.2 Chemistry of the Common Polyamines

The polyamines are aliphatic nitrogenous bases (Figure 1.3).

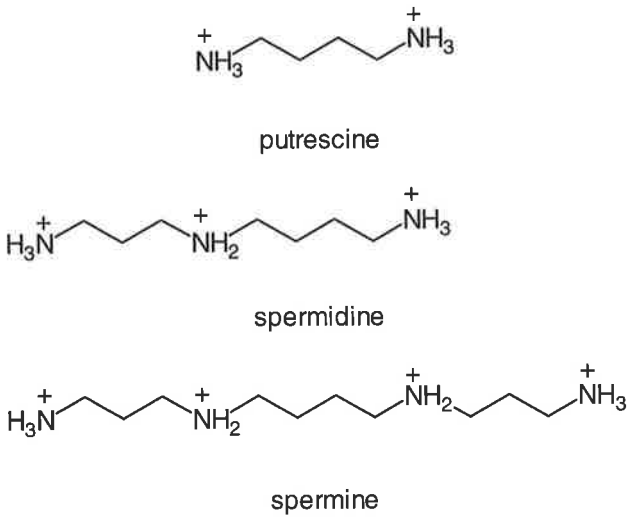


FIGURE 1.3. Structural Formulae of the Natural Polyamines

At physiological pH, the primary and secondary amino groups of putrescine, spermidine and spermine are protonated so that they exist as stable polycations with two, three and four basic centres respectively. They are very soluble in water and have weak chelating capacity [93]. Some of their properties are listed in Table 1.1.

TABLE 1.1. Physicochemical Properties of Spermine, Spermidine and Putrescine

Name	*Chemical Name*	*Molecular formula*	M_r	pK_a
Putrescine	1,4-diaminobutane	$C_4H_{12}N_2$	88.12	8.71
Spermidine	*N*-(3-aminopropyl)-1,4-diaminobutane	$C_7H_{19}N_3$	145.25	10.96 9.91 8.51
Spermine	*N,N'*-bis(3-aminopropyl)-1,4-diaminobutane	$C_{10}H_{26}N_4$	202.34	10.86 10.05 8.82

(After Morgan [6])

Polyamines can be regarded functionally as organic polycations. Interchangeability between polyamines and Mg^{2+} and other inorganic polycations has been described [93]. The polyamines are flexible molecules with positive charges distributed along the aliphatic carbon chain. The

charges on the polyamines are separated in space by specific interatomic distances dictated by the aliphatic carbon chain. Inorganic polycations, such as Mg^{2+} and Ca^{2+}, on the other hand represent point-like charges. Their positive charge enable the polyamines to form ion-pairs with negatively charged molecules. The separation of charge by specific distances give the polyamines a greater specificity of interaction than the inorganic polycations. Polyamine binding energy increases with the number of charges. Electrostatic interaction with DNA, RNA, proteins and sugars may occur (*q.v.* §1.2.6.3).

1.2.3 Polyamine Biosynthesis and Interconversion

1.2.3.1 Ornithine Decarboxylase

Ornithine decarboxylase (ODC; EC 4.1.1.17) catalyses the first and key step of polyamine biosynthesis, the decarboxylation of ornithine to form putrescine (Figure 1.4). Ornithine decarboxylase is present in very small amounts in quiescent cells, and its activity can be increased as much as 20- to 30-fold within a few hours of exposure to trophic stimuli [155].

1.2.3.2 Spermidine and Spermine Synthases

The aminopropyltransferase, spermidine synthase (EC 2.5.1.16), catalyses the addition of an aminopropyl group to putrescine forming spermidine. The aminopropyl group is derived from decarboxylated S-adenosylmethionine. Addition of another aminopropyl group converts

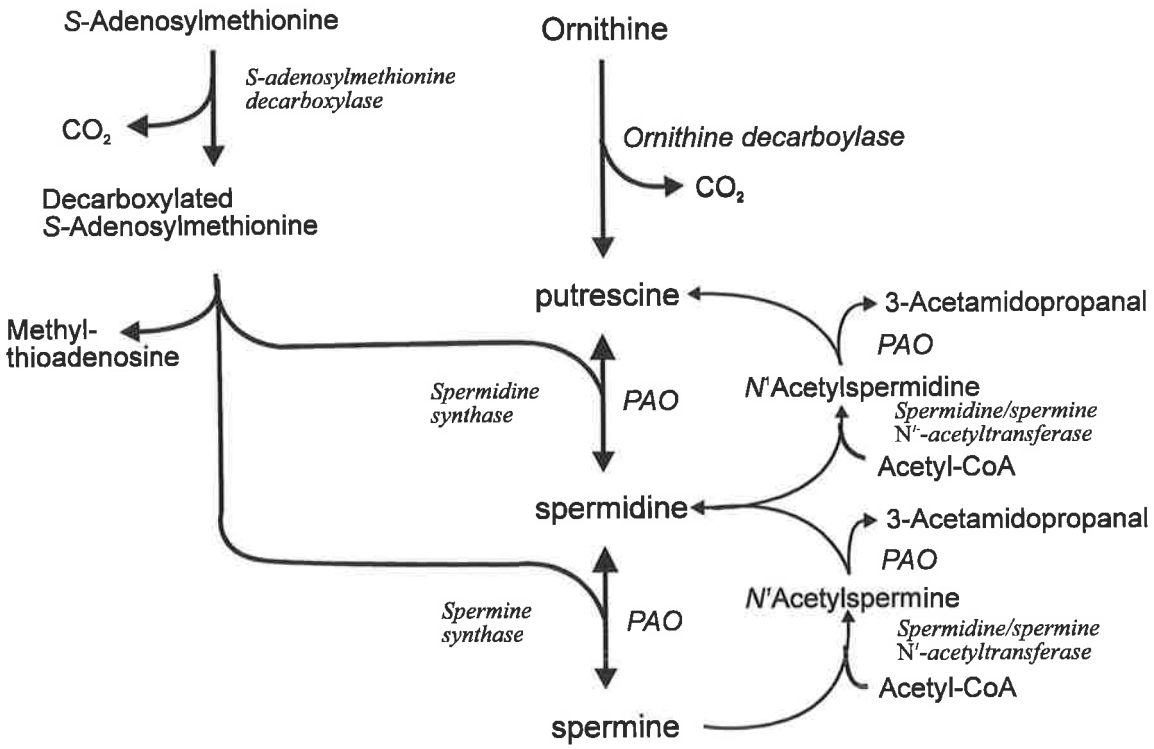


FIGURE 1.4. Polyamine Synthesis and Interconversion in Mammalian Cells.
PAO = polyamine oxidase/diamine oxidase.

spermidine to spermine, and this aminopropyl group also comes from decarboxylated S-adenosylmethionine in a reaction catalysed by a second aminotransferse, spermine synthase (EC 2.5.1.22) [156]. The other product of the aminopropyltransferase reactions is 5'-methylthioadenosine, which is in turn metabolised to methionine and adenosine, which are salvaged in mammalian cells [7].

1.2.3.3 Regulation of Polyamine Biosynthesis

The three enzymes that regulate the synthetic pathway shown in Figure 1.4 are ornithine decarboxylase, *S*-adenosylmethionine decarboxylase and spermidine/spermine *N*'-acetyltransferase. The biochemistry, biology and molecular biology of these three key regulatory enzymes have been extensively studied and are reviewed elsewhere [3,4,6,7,9,15,104,107].

Polyamine synthesis increases when cells are stimulated to increase their rate of growth. Both ornithine decarboxylase and S-adenosylmethionine decarboxylase (EC 4.1.1.50) activities can undergo enormous changes with rapid response times to diverse stimuli [7,104,157]. These enzymes are extraordinary labile. They are among the most rapidly degraded [or perhaps inactivated] proteins in mammalian cells [158].

1.2.3.4 Interconversion

Conversion of spermine to spermidine and of spermidine to putrescine *in vivo* has been known since the work of Siimes [112]. The aminopropyltransferase reactions that form spermidine and spermine are effectively irreversible. However, spermidine and spermine can be converted back into putrescine as shown in Figure 1.4. This pathway of polyamine catabolism is called the interconversion pathway [112,159-161] and is catalysed by polyamine oxidases (diamine oxidase (DAO) or polyamine oxidase (PAO), EC 1.4.3.6 and 1.5.3.11 respectively). PAOs may act directly on polyamines themselves, catalysing direct interconversion, or they may act on acetyl derivatives. Acetylation of an aminopropyl group of spermine using acetyl CoA is catalysed by spermidine/spermine N^1-acetyltransferase, to give N^1-acetylspermine. This acetylated polyamine is then oxidized by PAOs to form spermidine and 3-acetamidopropanal. Similarly, spermidine is also acetylated and the N^1-acetylspermidine thus formed may be cleaved at the internal nitrogen by PAO yielding putrescine and 3-acetamidopropanal. Putrescine can then be recycled or further metabolized to γ-aminobutyric acid. β-Alanine (3-aminopropionic acid) may be derived from the aldehyde [56].

The acetyltransferase activity is normally low or absent in cells, but is highly inducible, notably by chemicals which are toxic for cells [162] or by thermal stress [163].

The 'tissue type' polyamine oxidase (PAO, EC 1.5.3.11) involved in intracellular polyamine

interconversion in rat liver has been characterized by Hölttä [164]. It has been suggested that monoacetyl derivatives of the polyamines, N^1-acetylspermidine and N^1-acetylspermine, are better substrates of PAO than the polyamines themselves [165,166]. A reaction scheme has been proposed which includes as its first step N^1-acetylation, and as the second step the oxidative cleavage of the acetyl derivatives [159,165]. This reaction scheme has been widely advocated by Seiler and his colleagues [159,161]. However, the finding that acetylated polyamines are higher affinity substrates for the enzyme may be as a consequence of the high pH and extended reaction times used in the assay, favouring the oxidation of the acetylated polyamines [165,167]. 'Serum' amine oxidases (DAO, EC 1.4.3.6) are also capable of catalysing a reaction that is in essence equivalent to the polyamine interconversion reaction: aldehydes are formed by the oxidative deamination of the aminopropyl moieties of spermidine and spermine so that spermidine may be formed from spermine, and putrescine from spermidine [168,169]. The division between 'tissue type' and 'serum' amine oxidases represents just one of the many classification schemes attempted for the amine oxidases as discussed in following sections.

The interconversion pathway according to the scheme of Seiler *et al.* produces spermidine and spermine from putrescine and degrades the polyamines by N^1-acetylation and oxidative splitting of the acetyl derivatives (*q.v.* Figure 1.4). The putrescine moiety is conserved, but the aminopropyl moieties of spermidine and spermine are irreversibly eliminated. In a steady-state situation ornithine decarboxylation has the function of producing putrescine to make up irreversible losses of the putrescine moiety. Irreversible losses may occur through: (1) terminal polyamine metabolism (oxidation at primary amino groups) and (2) transport [101]. All intermediates of the interconversion pathway are substrates of EC 1.4.3.6 amine oxidases involved in terminal polyamine catabolism [102,159,170-172] and compounds formed as a result of this sort of metabolism have been identified in urine [167,173,174].

1.2.4 Acetylated Polyamines

Acetylated polyamine derivatives exist *in vivo.* Curiously, cellular acetylpolyamine concentrations are usually very low, even under conditions of enhanced polyamine metabolism [56,175-178], or inactivation of PAO [172,179], and no biochemical or pharmacological effects have been observed through elevated tissue acetylpolyamine levels [159]. Thus, the products of polyamine acetylation appear to be without specific significance, though they may have metabolic or regulatory functions as described below.

Acetylation reduces the positive charge of the polyamines. Thus, the directional flow of polyamines through lipophilic membranes from one hydrophilic region (intracellular compartment) to another (extracellular space) may be facilitated through a process of acetylation

and deacetylation [78]. The finding that acetylated polyamines are an excretory form, both of mammalian cells [180] and vertebrates [181], suggests that acetylation may be an important step in polyamine transport and elimination. This is supported by the occurrence of acetylated polyamines in normal urine [8,181,182]. However, acetylation does not appear to be an absolute requirement for their elimination. The finding that acetylation of the primary amino groups prevented the uptake of the polyamines by cultured L1210 leukaemic cells [183,184] also supports the suggestion that acetylation is an important step in polyamine elimination, but appears to contradict its involvement in transport.

The biological activity of polyamines and products of their oxidation may be modified by acetylation. Acetylation may be a means of terminating interactions of polyamines with polyanionic binding sites inactivating their biological activity [159,171]. Acetylation of aminopropyl groups reduces the cytotoxic effect of aldehydes produced by enzymatic polyamine oxidation [2] (*q.v.* §1.5 *et seq.*).

1.2.5 Polyamine Catabolism

In contrast to the extensive studies of polyamine biosynthesis, polyamine catabolism has received relatively less attention. As mentioned, interconversion catabolism involves polyamine oxidation at secondary amino groups and terminal catabolism involves oxidative deamination of primary amino groups yielding compounds that cannot be directly reconverted into polyamines.

The catabolic pathways of putrescine include those shown in Figure 1.5. Putrescine has diverse fates. Instead of conversion into spermidine, putrescine may be oxidatively deaminated to γ-aminobutyraldehyde by mammalian DAOs. One molecule each of ammonia and hydrogen peroxide are also formed during the reaction (Scheme 1.1). γ-Aminobutyraldehyde can be further oxidized to γ-aminobutyrate (GABA), or Δ^1-pyrroline [185,186] (an internal aldimine ring, the spontaneously cyclized form of γ-aminobutyraldehyde), which may be converted into 2-pyrrolidone [186-188] and 5-hydroxy-2-pyrrolidone [189]. GABA, a neurotransmitter and inhibitor of protein synthesis [190], is formed by the action of aldehyde dehydrogenase (EC 1.2.1.3) [191,192]. γ-Aminobutyraldehyde may be reduced by an aldehyde reductase or an alcohol dehydrogenase to yield 4-amino-1-butanol [193]. The molybdenum-hydroxylases, aldehyde oxidase (EC 1.2.3.1) and xanthine oxidase (EC 1.2.3.2) may also catalyse aminoaldehyde oxidation [194-197].

It has been established, that in eukaryotes GABA is synthesized from glutamic acid by glutamic acid decarboxylase, EC 4.1.1.15. However, there is evidence that putrescine is also converted to GABA *in vitro* and *in vivo* [160,198-208] via a direct pathway involving DAO [191,204,209]. The relationship between polyamines and GABA has been reviewed [210,211].

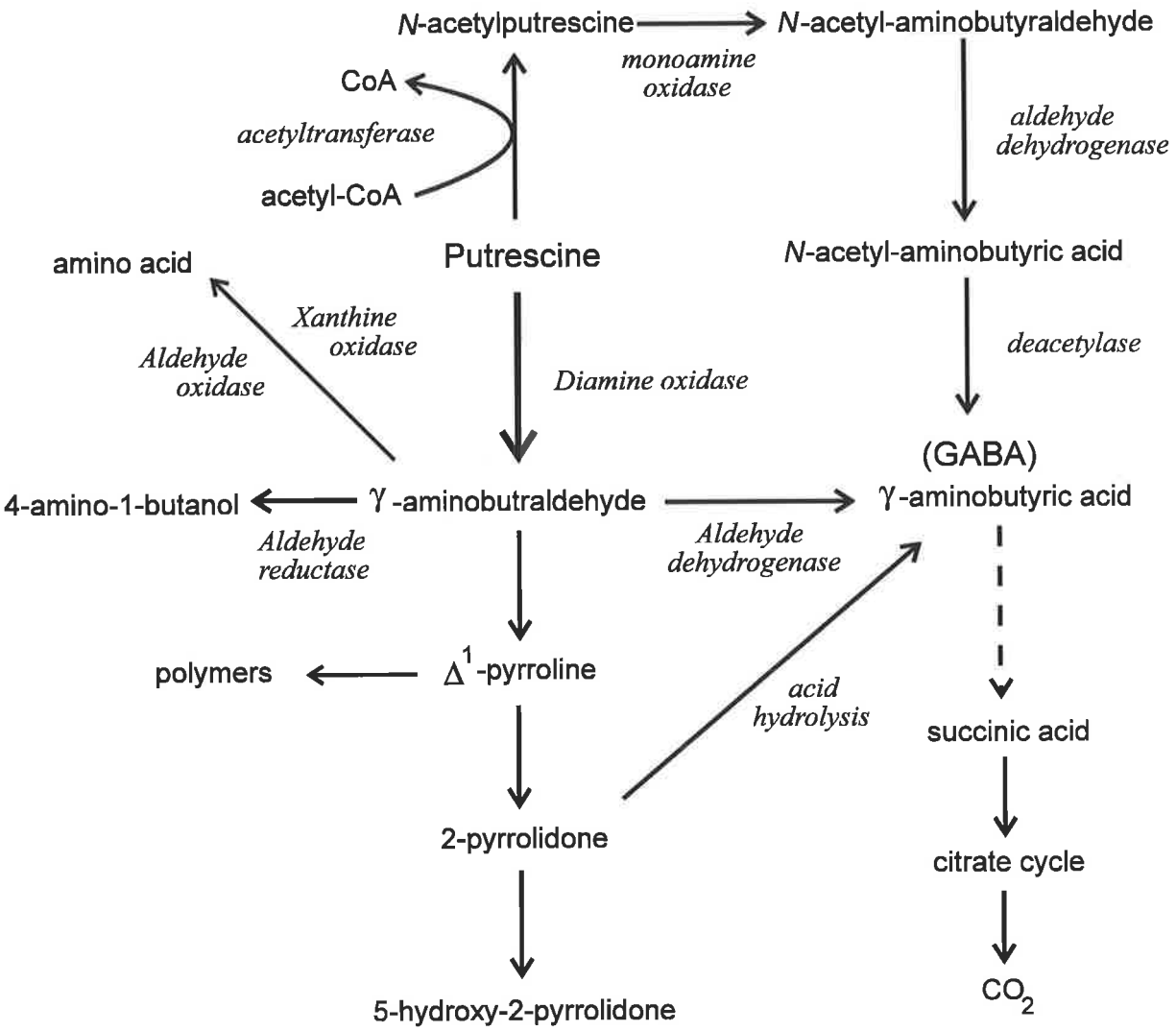


FIGURE 1.5. Putrescine Catabolism in Mammalian Tissues

Putrescine may be acetylated by the acetyl CoA dependent microsomal enzyme, *N*-acetyltransferase, and the monoacetylated putrescine oxidized by monoamine oxidase. This pathway can also give rise to GABA and is more important in GABA formation from putrescine in those tissues like brain which have little DAO [191,201,212].

The enzymatic oxidative catabolism of polyamines results in the formation of hydrogen peroxide and aminoaldehydes according to Scheme 1.1.

$$R'CH_2NHR'' + H_2O \rightarrow R'CHO + H_2NR'' + H_2O_2 \qquad \textit{Scheme 1.1}$$

Oxidation of polyamines may take place at either the primary or secondary amino groups. Where oxidation occurs at primary amino groups, the enzyme acts to produce aldehyde derivatives. The dialdehyde 4,9-diazadodecanedial (*N,N'*-bis(3-propanal)-1,4-diaminobutane) is formed from spermine and the aminomonoaldehyde *N*-(4-aminobutyl)-3-aminopropanal from spermidine,

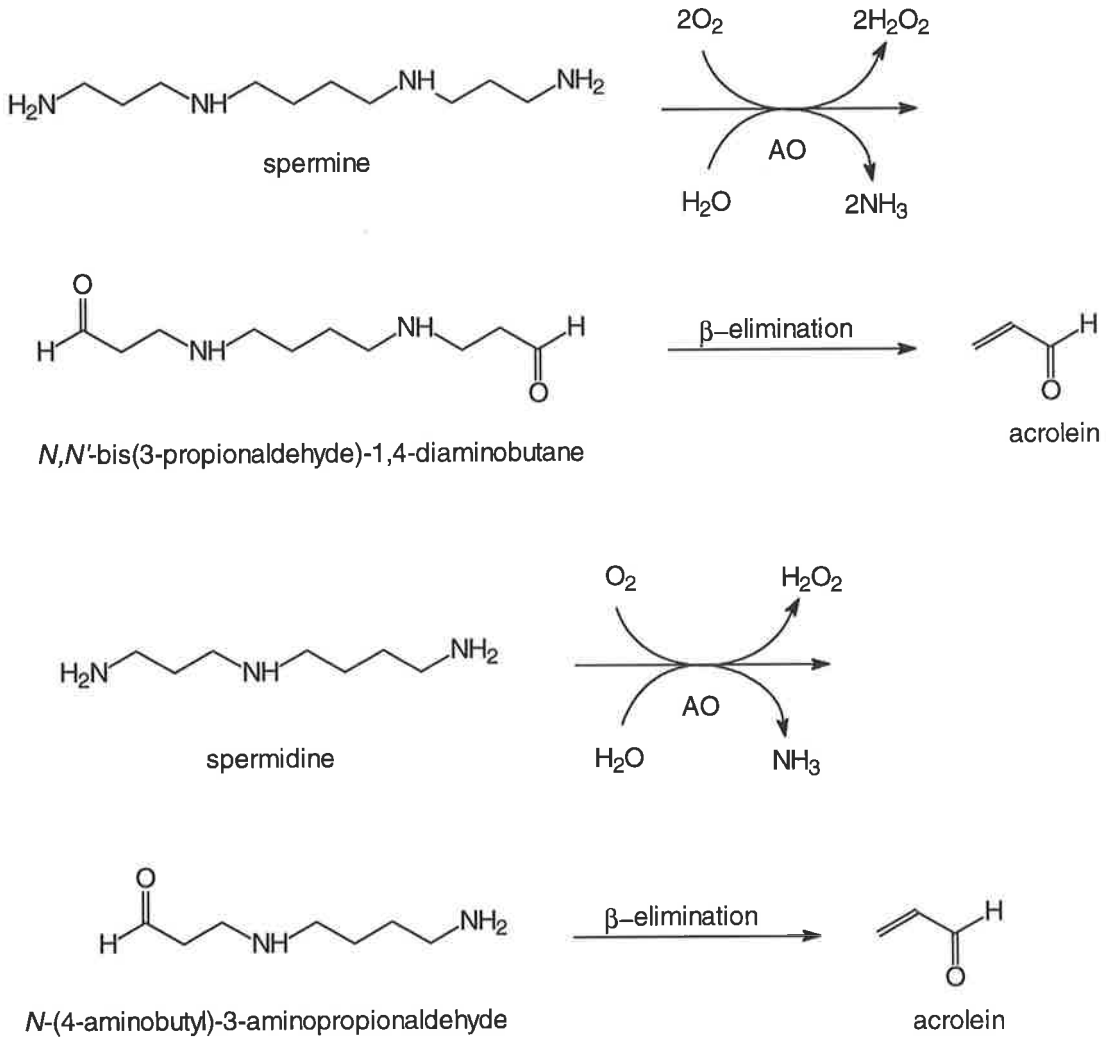


FIGURE 1.6. Polyamine Oxidation by Amine Oxidase at Primary Amino Groups

along with ammonia and hydrogen peroxide in the case of both substrates [168,213,214]. Acrolein may be formed from the aminoaldehydes by β-elimination [215-217] as shown in Figure 1.6. 1,5-Diazabicyclo[4,3,0]nonane has also been suggested as a product of enzymatic spermine oxidation [218]. Where oxidation occurs at secondary amino groups, interconversion of the polyamines occurs with the formation of 3-aminopropionaldehyde (Figure 1.7). Alternatively, a more internal cleavage would yield diaminopropane [6].

Aminoaldehyde products of polyamine oxidation may undergo further oxidation to their corresponding acids, in which the aldehyde moiety is converted into a carboxylic group by an aldehyde dehydrogenase (EC 1.2.1.3). A number of amino acids, which are oxidative metabolites of spermine and spermidine have been detected in mammalian tissues and urine [219,220]. *In vivo,* 3-aminopropanal is probably oxidized further to the amino acid β-alanine under most conditions [221]. Similarly, the aldehydes formed from the oxidation of spermidine and spermine

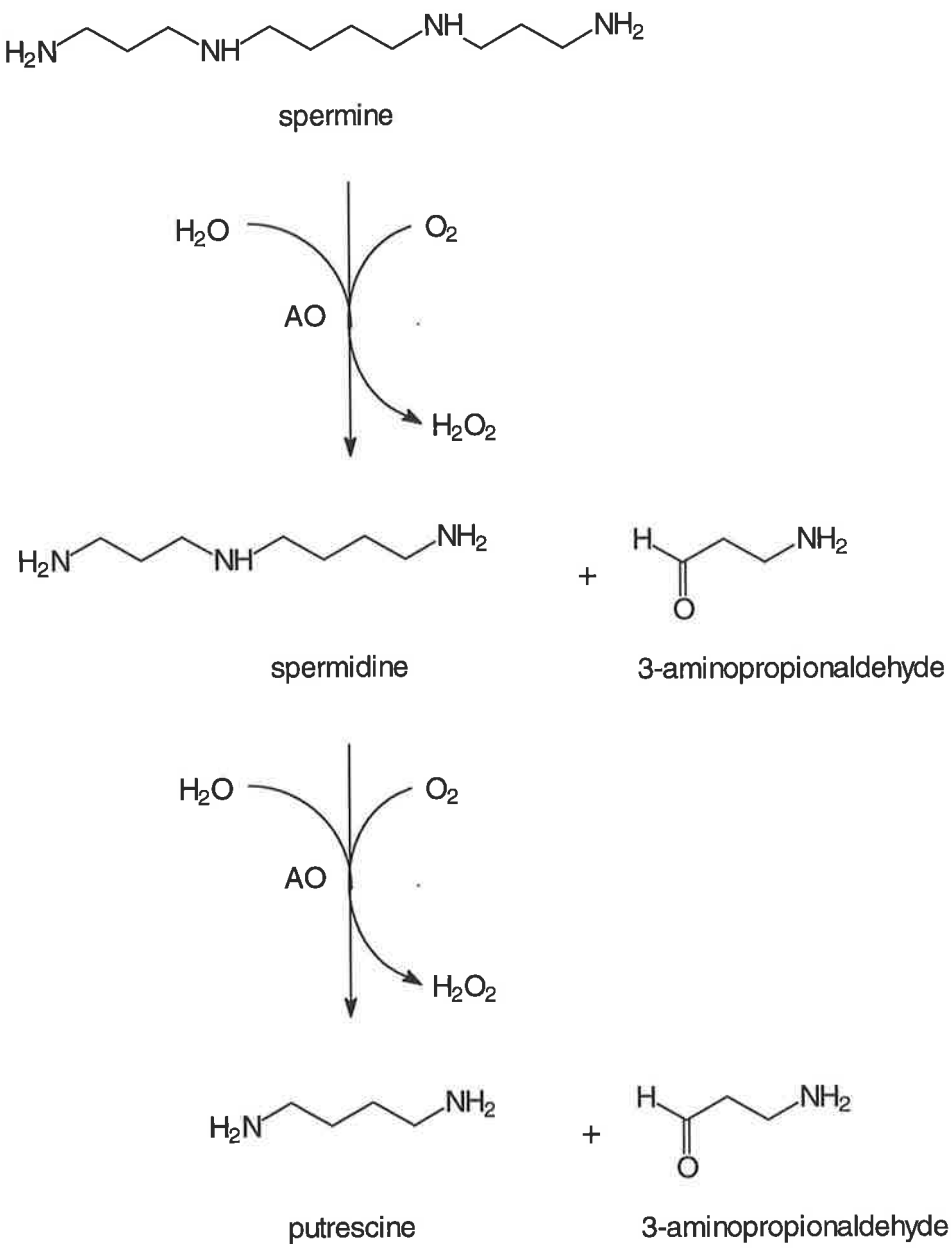


FIGURE 1.7. Polyamine Oxidation by Amine Oxidase at Secondary Amino Groups

at primary amino groups would be oxidized further, to putreanine (*N*-(4-aminobutyl)-3-aminopropionic acid) [220] and spermic acid [56] respectively.

Aminopropionaldehyde is highly reactive and unstable under non-physiological conditions, which has presented difficulties in its isolation and identification. Under physiological conditions no acrolein is formed [222-224], but under non-physiological conditions β-elimination occurs readily, resulting in the formation of acrolein and ammonia during isolation procedures such as rotary evaporation. Furthermore, reaction of acrolein with putrescine can lead to the formation of 3-(4-aminobutylamino)propanal and 3,3'-(butane-1,4-diamino)dipropanal, which has been a source of further confusion in identification of amine oxidase reaction products.

Involvement of human liver aldehyde dehydrogenase in the metabolic oxidation of biogenic aldehydes arising from biogenic amines and polyamines has been suggested [225]. Aldehyde dehydrogenase is involved in the formation of GABA from γ-aminobutyraldehyde formed by the oxidation of putrescine as previously mentioned. Alternatively, the aldehyde products of polyamine oxidation can be converted to γ-lactams when the aminobutyl moiety of spermidine is oxidatively deaminated [219,226] (Figure 1.8).

Acetylation of the polyamines, putrescine, spermidine and spermine has already been discussed. This acetylation occurs intracellularly in mammalian systems. Transglutaminases that can incorporate polyamines into proteins and protein or peptide linked polyamines have been reported but not fully characterized [107].

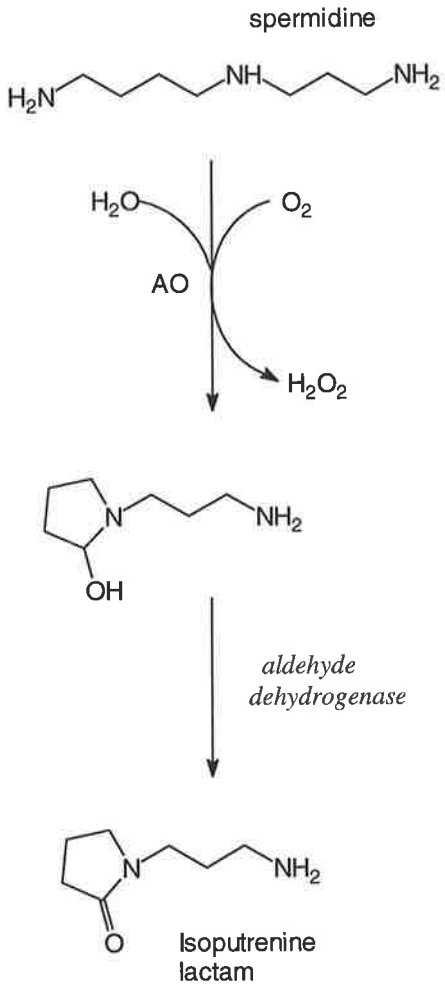


FIGURE 1.8. Conversion of Aldehyde Products of Polyamine Oxidation to γ-Lactams

1.2.6 Polyamine Physiology

1.2.6.1 Growth

Polyamines play an important role in growth. Their metabolites, such as β-alanine, may also serve as growth factors [227]. In 1948, Herbst and Snell identified putrescine as a factor essential for the growth of *Haemophilus parainfluenzae* [228]. Since then, polyamines have been shown to be stimulatory to (or essential for) the growth of various organisms, including bacteria [229-231], fungi [232], and cultured mammalian cells [126,233].

Polyamines are considered to be essential for cell division [75,91,142,234,235]. Their concentrations vary throughout the cell cycle [236-240], polyamines are capable of stimulating the transition of quiescent G_0 cells to the S phase and this effect may be mediated by their affect on growth-regulated gene expression [241].

The cellular concentrations of polyamines are markedly increased in various stages of tissue development [233,242] and embryonic growth [110,243,244]. Raina observed that a peak in the concentration of polyamines was coincident with that of nucleic acids during the development of the chick embryo [110]. Similar correlations have been observed for other developing systems [233,242,243,245,246]. When levels of polyamines in cultured cells are reduced using specific inhibitors, cell proliferation is inhibited and changes in differentiation are observed [247-249]. When the polyamine depleted cells were supplemented with polyamines, they resumed a normal growth rate. The importance of polyamines for cell proliferation was clearly illustrated by a mutant chinese hamster ovary cell line in which ornithine decarboxylase activity was deficient and could not grow in the absence of exogenous polyamines [250]. Dietary polyamines are considered to be important in human cell regeneration and growth [251-255].

On the other hand, high concentrations of polyamines have been shown to inhibit cell proliferation in a number of cell lines [256]. Polyamine toxicity is thought to be mediated by oxidases that generate reactive aldehydes [215] (*qq.v.* §1.4.4 and 1.5 *et seq.*). Polyamine toxicity may be due in part to intracellular events [257,258]. Wallace *et al.* have observed a dose-dependent inhibition of cell growth in BHK-21/C13 cell growth [222,259].

1.2.6.2 Differentiation

Besides their role in cell proliferation, polyamines have been shown to play an important role in differentiative processes [107,260-262]. It is difficult to separate the growth and differentiative effects, as many differentiative processes require cell division. The affects of polyamine depletion on embryonic differentiation have been reviewed [261]. In many experiments, inhibitors of polyamine synthesis were used to alter polyamine levels in model systems and the affect on the

expression of various differentiated phenotypes was observed. Inhibitor targets have included ornithine decarboxylase and *S*-adenosylmethionine decarboxylase. In some cell types the polyamines act as positive effectors of cell differentiation, in others as negative effectors. This dichotomy is probably a reflection of the complexity of polyamine functions and perhaps the selective expression of PAOs.

1.2.6.3 Polyanion Binding

Polyamines are known to have a high affinity for negatively charged compounds and molecules, e.g. the phospholipids of cell membranes [263-266], nucleic acids and proteins [267-280]. Polyamines bound to DNA stabilise its structure against digestion by nucleases and denaturation [13,74,81,86,116,126,127,281,282]. Furthermore, the levels of polyamines required for this stabilisation are much lower than the concentrations of Mg^{2+} necessary for comparable effects [283]. X-ray diffraction amongst other techniques has suggested several possible conformations for the complex formed between the polyamines and double-stranded DNA [270,284-286]. In all of these configurations polyamines are attached in a histone-like manner along the DNA molecule. The tight binding of polyamines, as compared with inorganic divalent cations, is probably due to their charge separation by methylene carbons. It seems likely that the interaction of polyamines with macromolecules, especially nucleic acids and structures containing nucleic acids, is involved in their ability to modulate cell function.

1.2.6.4 Interaction with Membranes

The association of polyamines with membranes has been demonstrated *in vitro*, suggesting that they may play a role in biological membrane function. Membrane stabilizing effects of polyamines and regulatory effects on membrane bound enzymes have been reported [263]. Numerous publications have indicated that polyamines influence the cell membrane properties and functions [287-291]. The exact mechanisms of these actions are not clear. Some authors suggest that the stabilizing properties of polyamines may be due to their interactions with the negatively charged residues of the membrane-bound proteins [287-289] or with acid phospholipids [63,264,265]. The polyamine induced change of the surface charge may influence some of the membrane-bound enzymes [292] as well as the biosynthesis of the membrane lipids [290,293] and glycoproteins [294]. Polyamines may be involved in transmembrane signalling processes [295-297]. Polyamines modulate the *N*-methyl-D-aspartate receptor found on nerve cell body membranes (*q.v.* §1.2.6.9). Electrostatic interactions probably have an important contribution to the intermolecular interactions of the polyamines. The binding specificity of the polyamines, which is not matched by inorganic cations, suggests a more specific interaction based

on the partial aliphatic nature of the polyamines. Because the polycationic polyamines have their positive charges distributed at fixed lengths along a conformationally flexible carbon chain, they are able to bridge discrete distances, allowing more specific interactions than the single point charges of inorganic cations.

1.2.6.5 Cellular replication, transcription and translation

Polyamines play a role in DNA replication, transcription and translation. *In vitro*, polyamines directly stimulate various DNA and RNA polymerases, methylases, nucleotidyltransferases, hydrolases and ribonucleases and affect reactions involving tRNA, ribosomal RNA, and mRNA molecules, contributing to polyribosomal protein synthesis [13,78,81,116]. At a transcriptional level, polyamines influence strand selection and chain initiation, extension, and termination. At the translational level, polyamines can activate tRNA, facilitate aminoacetylation of tRNA, messenger binding to the ribosomal subunits, translation of mRNAs, and assembly of ribosomal subunits [64,70,71,79,116]. Polyamines stabilise the structures of tRNA, ribosomes, and polysomes [13,79,116,126,127]. Indeed, high affinity binding sites with conformational influences exist on tRNA molecules [86,298-307].

Polyamines are included in molecular biology protocols to stabilise the crystallization of nucleic acids (DNA, RNA), improve endonuclease reactions and supplement reticulocyte lysate systems [308]. Spermine, spermidine and putrescine were reported to increase by 3- to 5-fold the protein synthesis by adenovirus, globin and bacteriophage mRNA in a cell-free system [309]. DNA synthesis of type C retroviral DNA polymerase was increased 10-fold by millimolar concentrations of polyamines [310].

1.2.6.6 Hormones

Numerous hormones increase polyamine biosynthesis in their specific target tissues, suggesting that polyamines may function as mediators of hormone action [311,312].

1.2.6.7 Toxicity

Although early work by Poehl advocated the use of spermine as a therapeutic agent (*q.v.* §1.2.1), polyamines have been found to be quite toxic. The toxicity of polyamines is primarily related to renal tubular necrosis [45,313,314]. Toxic effects of polyamine metabolites are discussed in latter sections (*q.v.* §1.4.4 and 1.5). In addition to the nephrotoxic effects of polyamines mentioned above, reported effects of spermine and spermidine include sedation, hypothermia, anorexia, convulsions and paralysis [315,316], lethargy, ataxia and cardiorespiratory failure [316]. Pharmacological effects of polyamines have been reviewed in [46].

1.2.6.8 Antioxidant effects

Polyamines have an antioxidant effect. They protect polyunsaturated fatty acids, Vitamin E, and carotenoid pigments against oxidation [317], scavenge aqueous peroxy radicals [295], superoxide radicals [318-321] and hydroxy radicals [322].

1.2.6.9 Other Effects of Polyamines

Polyamines have been shown to inhibit functional characteristics of cells. They have been shown to inhibit gastric acid secretion [323-325], inhibit platelet aggregation [326], possibly inhibit secretion from polymorphonuclear leukocytes [327], and inhibit exocytosis in sea urchin eggs [328]. There is evidence that polyamines may participate in the regulation of insulin [329,330] and neurotransmitter secretion [331].

Spermidine and spermine may affect free intracellular calcium ion levels [332-336], which are essential in intracellular activation processes [332]. In addition, polyamines appear to inhibit protein kinase C [329], an enzyme implicated in intracellular activation processes [337].

Polyamines have been found to regulate the *N*-methyl-D-aspartate (NMDA) receptor [338]. The NMDA receptor mediates the action of the excitatory amino acid neurotransmitters glutamate and aspartate in the central nervous system [339]. The receptor complex contains a specific recognition site for polyamines. Endogenous polyamines may modulate excitatory synaptic transmission by acting at the polyamine recognition site of the NMDA receptor [340].

In plants and micro-organisms polyamines are important in growth (*q.v.* §1.4.8). They may also have more specialized roles as the precursors of various alkaloids or insect pollinator attractants (*q.v.* §1.4.7).

1.2.7 Polyamine Pathophysiology

Most disease states are characterized not by new products, but by an attenuation or amplification of biochemical pathways. Pathways of polyamine synthesis and degradation have been extensively studied in this regard [58,341]. Some clinical conditions associated with changes in polyamine metabolism are summarized in Table 1.2 and a selection of these are discussed below.

Many disease states can be recognized by abnormal concentrations of circulating or urinary constituents that can be used as markers of pathological processes. Increased levels of polyamines in blood (hyperpolyaminemia) are commonly seen where there is increased production, retention, and failure of degradation. The elevated blood polyamine levels seen in pregnancy and malignancy reflect active cell proliferation [87,342]. Elevated levels are also seen in chronic renal failure and advanced liver disease [343,344]; and when tissue damage is induced by surgical

TABLE 1.2. Clinical Conditions with Disturbances in Polyamine Metabolism

Etiology	*Clinical Condition*	*Reference*
Increased production	Pregnancy	[363,364]
	Fetus and newborn	[342]
	Acromegaly	[365]
	Malignancies	[58,88-93,366-368]
	Psoriasis	[369-373]
	Muscular dystrophy	[374]
Decreased biodegradation	Liver insufficiency	[344]
Retention	Renal insufficiency	[375]
Enzyme dysfunction	ornithine transcarbamylase deficiency	[376]
Mixed etiologies	Cytotoxic drugs	[345]
	Burns	[351]
	Exercise	[377]
	Infection	[344]
	Radiation	[346]
	Rheumatoid arthritis	[352,353]
	Systemic lupus erythematosus	[354-356]
	Transplant rejection	[378-381]
	Trauma/surgery	[347,349,350]
	Cystic fibrosis	[382-385]
	Schizophrenia	[386-389]
Altered renal transport	Cystinuria, Fanconi's syndrome	[376]
	Lowe's syndrome (Oculocerebrorenal Syndrome)	[390]
Impaired production	Intrauterine growth retardation	[342,391]
	Hormone dependent dwarfism	[362]
	Beckwith–Wiedemann syndrome	[392]

trauma, irradiation, burns, or cytotoxic drugs [345-351]. Similar changes are associated with immunoinflammatory diseases such as systemic lupus erythematosus and rheumatoid arthritis [352-356]. Hyperpolyaminemia is usually associated with increased urinary polyamine secretion. However, uremia appears to be an exception [357].

Normal tissue levels of spermine and spermidine in mammals are in the range of 1 to 2 μmole/g wet weight [127] (a concentration of 1–2 mM). Putrescine is generally present in concentrations about an order of magnitude lower except in tissues that have a high rate of cell proliferation [87]. Concentrations of spermine are lowest in skeletal muscle and brain (about 50–200 μM) and highest in the spleen and prostate (about 24 mM) [358]. Nevertheless, spermine may have profound effects in the brain due to its action on the NMDA receptor. In contrast, normal plasma and serum polyamine levels are in the high nM – low μM range [359]. When plasma and red blood cell polyamine levels were determined from single samples and compared, red blood cell polyamine levels were one or two orders of magnitude higher. Normal body fluid values have been determined [58,359-361] and it has been reported that in humans there is a significant decline in circulating and excreted polyamines with age [362]. The concentration of spermine in seminal plasma can be very high (30 mM [58]).

1.2.7.1 Malignancy

Intra and extracellular concentrations of polyamines are known to increase in several malignancies [88-93,367,368]. Patients with certain tumours excrete increased concentrations of polyamines in their urine and these increases may serve as an indicator of tumour proliferation [393]. Polyamine concentrations in extracellular fluids have been proposed as rapid indicators of efficacy of therapy in cancer and other disorders that involve altered cell growth and loss of cell components [46,58].

1.2.7.2 Psoriasis

Elevated levels of polyamines have been found in the blood and skin of patients with psoriasis [369,370]. With clinical improvement in treated psoriasis, lowered cutaneous and urinary levels of polyamines were found [370-373].

1.2.7.3 Cystic fibrosis

Polyamines may be of etiologic significance in cystic fibrosis (CF). An altered metabolism of polyamines in CF is suggested, though the relationship between the altered metabolism and CF is unclear. Studies of urinary concentrations of polyamines indicated that children with cystic fibrosis excreted elevated levels of putrescine, spermidine, and spermine compared to their heterozygote siblings or normals [385]. Elevated spermidine/spermine ratios were detected in the whole blood of both cystic fibrosis patients and their heterozygote siblings [383], especially in erythrocytes [382]. The level of circulating polyamines is elevated in individuals homozygous and heterozygous for the CF gene [394]. Abnormally high circulating DAO activity was found in 30% of CF homozygotes and heterozygotes [394]. Cystic fibrosis patients have detectable levels of a DAO in their saliva. Because oxidation products of endogenous polyamines are potentially capable of inhibiting sodium transport this was thought to be significant in CF etiology [395]. Oxidase activity is up-regulated in monocytes that are heterozygotic and homozygotic for the CF gene. This finding supports the hypothesis that the mononuclear phagocytes of CF heterozygotes have a significantly increased ability to kill intracellular microbes and this may confer a selective advantage to the host [396]. On the other hand, no difference in the specific activity of DAO was found between normal and CF fibroblasts [397], nor was any differential toxicity of polyamines to CF fibroblasts in culture found when compared with normal cell lines [384]. It is of interest that the structural gene for the DAO has been mapped to human chromosome 7q34–q36. This region flanks the region implicated in cystic fibrosis (7q32) [398].

1.2.7.4 Growth Disorders

Polyamines have been demonstrated to be a requirement for growth (*q.v.* §1.2.6.1). Growth hormone stimulates polyamine production in rat liver and ornithine decarboxylase is known to be induced [399]. Polyamine content of pancreatic β-cells was increased in response to growth hormone, platelet-derived growth factor, and insulin-like growth factor I [400]. Administration of growth hormone to growth hormone deficient and hypopituitary patients resulted in a significant and rapid increase in blood polyamine concentrations [401,402]. Acromegaly, a pituitary disorder involving the excessive release of growth hormone, has been reported to be associated with increased urinary excretion of polyamines [365]. On the other hand, Beckwith–Wiedemann syndrome, a congenital growth disorder, was found to have essentially undetectable 24 hour spermidine and spermine excretion rates [392]. These studies highlight the rapid stimulation of polyamine synthesis and accumulation by growth-stimulating hormones.

1.2.7.5 Uremia

In uremia, polyamines accumulate in the blood, indicating the importance of the kidney in polyamine homeostasis. Elevated concentrations of polyamines have been found in the serum, red blood cells and tissues during uremia [343,357,375,403]. A significant increase in DAO activity is also associated with uremia [404] and this may be due to release of the enzyme from damaged cells or perhaps a response to the sustained hyperpolyaminemic state. Deterioration of renal function may be due to the result of proliferative glomerular responses to polyamines [405] or toxic effects of oxidized polyamines (*qq.v.* §1.2.1, 1.4.4 and 1.5 *et seq.*).

1.2.7.6 Liver Disorders

The affects of hepatic malignancy, hepatitis and cirrhosis on body fluid polyamine levels have been examined [344]. High levels (5–30 times normal) have been reported in liver failure. Acute liver cell injury, as occurs during infectious hepatitis, would be expected to release polyamines into the circulation. Chronic parenchymal damage, reducing effective liver function, would be expected to compromise biodegradation of polyamines. Together the two effects may promote progressive hepatic damage. The liver is known to be an important source of PAOs [48,164,406-412] (which may *also* be released by liver damage [413-416]) and probably plays an important role in polyamine homeostasis.

1.2.7.7 Pre-eclampsia

In pregnancy, a profound yet physiological hyperpolyaminemic state exists [342,364]. Plasma spermine and spermidine levels peak early (10 to 16 weeks gestation) and late (34 to 38 weeks

gestation). Plasma concentrations follow the concentrations in amniotic fluid, as do urinary concentrations [417,418]. During normal pregnancy, extracellular concentrations of polyamines reflect rapid growth periods, relating to a large number of cells undergoing mitosis and high cell loss known to occur during embryogenesis. Fetal tissues [110], the placenta [419], and amniotic fluids [417] are all rich in polyamines. An enzymatic barrier, containing polyamine oxidizing enzymes is found in the decidual portion of the placenta [420] and may serve to reduce the maternal polyamine load [405,421]. It has been suggested that pre-eclampsia may result 'from placental polyamine overflow at a time when the accretion of fetal mass is rapidly approaching a physiological limit.' [405], although it is likely that other mechanisms exist [422] (*vide* §1.6.9).

1.2.7.8 Schizophrenia

The dramatic and accidental discovery behavioural side effects exhibited by tuberculosis patients treated with the monoamine oxidase inhibitor iproniazid lead to an explosive interest in monoamine metabolism and studies on the possible involvement of amines in a variety of mental disorders (reviewed in [423-426]). There is evidence to suggest an involvement of polyamines in schizophrenia [388,389,427,428]. The antimalarial drug, hydroxychloroquine, which contains a spermidine moiety, is effective in the treatment of schizophrenia [386]. Dopamine receptor blocking activity has never been demonstrated with the drug and it has been proposed that its site of action is a polyamine receptor [possibly the polyamine binding site on the NMDA receptor]. Since its analogues hydroxychloroquine and propranolol have been found to be effective in schizophrenia treatment and since the effect of these drugs is reversed by polyamines [387], it is possible that the polyamines are involved in the etiology of schizophrenia.

1.2.7.9 Conclusions

To draw conclusions about the physiological functions of polyamines based on the thousands of papers published about them is difficult because of the myriad of disparate effects exerted by them. However, polyamines do appear to be a prerequisite for eukaryotic cell proliferation. Polyamines probably have a multifunctional role in physiological processes and have been considered a potential 'Rosetta Stone for medicine' [405].

Manipulation of endogenous concentrations of polyamines may be used to validate observations made with *in vitro* (or model) systems and for the physiological responses elicited by exogenously added polyamines. Promising approaches appear to be the use of polyamine auxotrophic cells, the production of polyamine depletion by means of specific chemical intervention of their biosynthesis (with compounds like α-difluoromethylornithine (DFMO)), and augmentation of polyamines by inhibition of their metabolism; the development of specific

polyamine oxidase inhibitors will be important in this regard. However, it is often difficult to judge what the primary effects in response to polyamine manipulation are.

Little real progress has been made linking changes in extracellular polyamines to any particular pathological condition in human beings [9]. The initial enthusiasm for clinical applications of polyamine research in the area of cancer has been tempered somewhat because the experimental work with tumour bearing animals could not be directly translated into clinical practice. Nevertheless, polyamine antimetabolites may be useful in the treatment of some parasitic diseases, notably African trypanosomiasis (because of the unique ornithine decarboxylase that the parasites contain) and *Pneumocystis carinii* [9,15,429].

1.3 Amine Oxidases

1.3.1 Classification

Enzyme Commission (EC) nomenclature [430] includes those oxidoreductases (EC 1.4) acting on the CH–NH_2 group of donors, primary amino groups as are found in monoamines, diamines and polyamines. When oxygen is the electron acceptor the classification becomes EC 1.4.3. EC 1.5 nomenclature indicates that CH–NH is the donor or oxidation at secondary amino groups, as are found in polyamines. It appears that some enzymes are capable of acting as EC 1.4 or 1.5 subclass enzymes depending on the substrate being oxidized and this has led to confusion of their EC classification. The same enzyme may fit into a number of different EC 1.4.3 or 1.5.3 groups depending on the substrate being used to assess their enzymic action. On the other hand, enzymes from different sources, e.g. bacterial, plant or animal are classified into the same entry. A consequence of the adoption of the chemical reaction as a basis for the classification of enzymes is that a certain name designates a group of proteins with the same catalytic property even though they may have distinct physicochemical differences. This has contributed to confusion within the amine oxidase EC nomenclature.

The trivial nomenclature is even more confusing. The polyamines, putrescine, spermidine and spermine are able to be oxidized by various enzymes from a wide variety of different sources including mammalian tissues, bacteria, plants, ruminant serum, pregnancy serum, seminal plasma and amniotic fluid (*q.v.* Appendix A). Confusion is a consequence of the *ad hoc* naming of the same enzyme by different names, for example 'histaminase', 'diamine oxidase', 'semicarbazide-sensitive amine oxidase', 'serum amine oxidase', 'benzylamine oxidase', 'serum monoamine oxidase', 'plasma amine oxidase' and 'polyamine oxidase'. Conversely, different enzymes with similar substrate specificities and different sites of enzyme action are frequently referred to by the same name of 'polyamine oxidase' or 'diamine oxidase'. In some cases the same abbreviation may indicate different meanings, e.g. PAO [plasma- or poly-amine oxidase], SAO [serum or

semicarbazide-sensitive amine oxidase]. Zeller stated in 1979 that 'Anarchy is reigning supreme in the nomenclature of amine oxidases.' [431]. Since then the situation has not improved significantly.

In this thesis, those enzymes capable of oxidizing the polyamines, spermine and spermidine will be collectively referred to as 'polyamine oxidases', irrespective of whether they also act on mono- or diamines, following Morgan's nomenclature [432]. Enzymes acting on diamines, such as putrescine, may be referred to as 'diamine oxidases' and this nomenclature is synonymous with PAO where the enzyme acts on both types of substrates (*vide* §1.2). Sometimes the more general term amine oxidase is used, but is generally intended to exclude the classical monoamine oxidases in its meaning (*vide infra*).

The Enzyme Commission Nomenclature handbook [430] lists four enzymes oxidizing polyamines (two more than the previous edition [433]): EC 1.4.3.4 amine oxidase (flavin-containing), EC 1.4.3.6 amine oxidase (copper-containing), EC 1.4.3.10 putrescine oxidase, and EC 1.5.3.11 polyamine oxidase. Other mammalian enzymes with the potential to oxidize polyamines include ceruloplasmin, EC 1.16.3.1, and lysyl (protein–lysine 6-) oxidase, EC 1.4.3.13.

Historically, the amine oxidases have been divided into two distinct families: (1) the monoamine oxidases [MAO; deaminating, amine:oxygen oxidoreductase EC 1.4.3.4], which act on primary, secondary and tertiary amines and (2) diamine oxidases [DAO; diamine:oxygen oxidoreductase; EC 1.4.3.6], which act on aliphatic diamines, including histamine, polyamines and primary monoamines. There are differences in substrate affinities and specificities of the DAO enzyme(s) between species and between tissues.

The amine oxidases have been reviewed in [2,6,10,50,56,59,61-63,105,108,109,161,170,172,431,432,434-473]. Early work on amine metabolism has been reviewed by Blaschko [445]. The first clear indication that amines are catabolised by deamination reactions was reported in 1877 by Schmiedeberg who examined the metabolites of benzylamine in dogs [474]. Direct demonstration of a histamine (β-imidazolylethylamine) metabolizing enzyme was first made by Eustis in 1915 who demonstrated a histamine-inactivating substance in liver extract from the turkey buzzard, *Chathartes aura* [475], though this finding has been generally overlooked. In the late 1920s Hare demonstrated the presence of tyramine oxidase in liver preparations [476] and Best reported a histaminase in the lung [477]. Histaminase was also found in dog and pig kidney [478-480], human placenta [481], and in the blood plasma of pregnant women [482].

The naming of the EC 1.4.3.4 and EC 1.4.3.6 enzymes originates from the simultaneous reports of Blaschko, Richter and Schlossman [483] and Pugh and Quastel [484] in 1937, who

found that the tyramine oxidase also acted on catecholamines, such as adrenaline; 5-hydroxytryptamine and aliphatic monoamines. The enzyme was called amine oxidase by both groups. However, the enzyme did not appear to act on aliphatic diamines such as putrescine and cadaverine and became known as 'monoamine oxidase'. In 1938, Zeller reported a histaminase from pig kidney which oxidized the diamines putrescine and cadaverine [485] and proposed the nomenclature 'diamine oxidase' for this type of enzyme [486]. The histaminase from human placenta [487] and pregnancy plasma [488] was also found to oxidize aliphatic amines. This data led to the conclusion that just one enzyme, DAO, attacks both diamines and histamine [485]. The enzyme was partially purified in 1951 and the co-identity of DAO and histaminase in the pig kidney was demonstrated [443,489]. It became clear that there were two separate enzymes: DAO (EC 1.4.3.6), which is identical to histaminase, and monoamine oxidase (EC 1.4.3.4), which differentiates between biogenic monoamines and diamines [446,490]. Another enzyme acting on polyamines has been recognized and has been called polyamine oxidase (EC 1.5.3.11) [161,164,165,491], this enzyme is thought to be distinct from the EC 1.4.3.4 and 1.4.3.6 type enzymes although it may also act on primary amino groups and oxidize monoamines and diamines in an EC 1.4.3 manner.

As an example of how confusing classification can arise, the bovine serum amine oxidase oxidises spermine, spermidine and N^8-acetylspermidine efficiently but putrescine, acetylputrescine and N^1-acetylspermidine less efficiently [223]. The enzyme has been said to act on primary amino groups of polyamines, those forming part of an aminopropyl moiety being more readily attacked [492]; and also primary amines [49,493] leading to its classification as an EC 1.4.3.6 enzyme [430]. This classification was supported by evidence for enzymatic oxidation of spermidine and spermine to aminomonoaldehyde [*N*'-(4-aminobutyl)aminopropionaldehyde] or a dialdehyde [*N,N*'-bis(3-propionaldehyde)-1,4-diaminobutane], ammonia and hydrogen peroxide [168]. The aminoaldehyde products are not recycled and enter into 'terminal pathways' of polyamine catabolism (*qq.v.* §1.2.3 and 1.2.5). The conclusion that 'terminally' oxidized polyamines are the major reaction products of the enzyme was based on comparison of R_f values on TLC plates, melting points, elemental analyses and IR spectra of $NaBH_4$ reduction products. However, such analyses are now considered inadequate for unambiguous characterization. Furthermore, they are in conflict with other results [44,49,56,214,223,494] that indicate a sequential conversion of spermine into spermidine and spermidine into putrescine. The bovine serum amine oxidase is now known oxidize polyamines at the secondary amino group acting as an EC 1.5.3 enzyme.

The relationship of monoamine oxidases with neurophysiology that was recognized in the early 1950s has led to an expanded interest in the monoamine oxidases. Monoamine oxidases can

be further subdivided. The most widely investigated enzymes of this group, the mitochondrial monoamine oxidases, are primarily involved in the metabolism of the biogenic monoamine neurotransmitters. The mitochondrial enzymes, located in the outer membrane of mitochondria, have been classified into two forms, A and B, on the basis of their selective inhibition by clorgyline and deprenyl [495]. The monoamine oxidases have been reviewed elsewhere [423-426,438,496-499] and will not be considered in detail here.

Studies of the substrate and inhibitor specificities of purified enzymes showed that the simple division of amine oxidases into PAOs, MAOs and DAOs is an oversimplification. Individual enzymes lack strict substrate specificity, although the relative catalytic efficiency of individual enzymes with monoamines, diamines and polyamines may be different (*vide* §1.3.6). Because of the difficulties associated with this type of nomenclature Blaschko *et al.* proposed the adoption of a new classification system based on the sensitivity of the enzymes to inhibition by carbonyl group reagents such as semicarbazide and hydroxylamine [500] (*vide* §1.3.7). This classification system divided the amine oxidases into two classes, viz. carbonyl reagent sensitive enzymes, including DAO, benzylamine oxidase and some PAOs, and carbonyl reagent resistant enzymes including the monoamine oxidases and other PAOs, which may be flavin–adenine dinucleotide (FAD)-dependent. Since some carbonyl reagents could inhibit enzymes from both classes, Zeller suggested that 'carbonyl reagent' be replaced by 'semicarbazide' as this (carbonyl group) reagent was more selective [501]. This has lead to the adoption of the 'semicarbazide-sensitive amine oxidase' (SSAO) nomenclature (*vide* Callingham and Barrand [502,503] and references [472,473,504-508]). In this thesis the general term 'amine oxidase' is intended as a general term like SSAO [503], and excludes the classical monoamine oxidases unless specified.

Since different enzymes may be affected by the same inhibitor, the source of the enzyme in question should be stated. Enzyme preparations from different sources in the same organism or from the same source in different species may be different. The same tissue from one species may have several different types of amine oxidase activity. Individual enzymes do not usually have unique substrate and inhibitor specificities. All of these circumstances create difficulties in the classification of amine oxidases.

In view of the difficulties associated with classification of the amine oxidases according to EC or trivial nomenclature, the following amine oxidase classification criteria were suggested by Bachrach in 1985 [509]:

(1) substrate specificity,
(2) mode of oxidation,
(3) sensitivity to inhibitors,
(4) structure of the enzyme and cofactor requirement.

The first three criteria are based on phenotypic properties of the enzyme that may be altered by

environmental factors or by procedures used for their assay. The fourth criterion, on the other hand, is based on intrinsic properties of each enzyme. Mondovì [1] suggested a division of the amine oxidases into two groups: the 'flavin' amine oxidases containing flavin adenine dinucleotide (FAD) as a cofactor and the 'copper' amine oxidases, containing copper and an organic prosthetic group. There appear to be a large number of enzyme proteins showing structural heterogeneity within each of these two groups.

1.3.2 Distribution and Localization

Amine oxidases are widespread in mammals, fish, insects, plants, and both prokaryotic and eukaryotic micro-organisms: their distribution in life forms appears ubiquitous (*q.v.* Appendix A). Localization of the amine oxidases has mostly been established using traditional biochemical assay methods, histochemical and immunohistochemical techniques. More recently, cDNA probes have been used in the localization of amine oxidase genes [411,649,982]. The enzymes may be intracellularly or extracellularly located. Ultrastructural studies are rare and have mostly depended on biochemical analysis of subcellular fractions obtained after differential centrifugation of crude homogenates. Each of the localization methods has its relative merits and disadvantages. For example, immunohistochemical methods may indicate the presence of enzyme protein with high sensitivity, and Northern blot analyses may indicate the expression level of mRNA; but neither detects catalytic enzyme activity. On the other hand, histochemical techniques that detect enzyme activity, may lack sensitivity and specificity. Amine oxidases from a wide variety of animal and plant tissues as well as various bacteria and fungi have been purified and characterized. The primary structure of a number of amine oxidases has been determined and the atomic structure of at least two amine oxidases has been solved [529,650]. Publication of third atomic structure is imminent [519].

Describing the source and localization of a particular amine oxidase often gives it a unique identity. However, a particular source may contain more than one type of amine oxidase or the same enzyme known by different names; the pregnancy-associated enzymes are among the most confusing in this regard. Furthermore, amine oxidases may exist as a number of different forms or isozymes.

1.3.2.1 Bacteria

The amine oxidases of micro-organisms have been reviewed [105,432,471]. Oxidative degradation of spermine and spermidine by bacteria (*Escherichia coli*) was first observed by Silverman and Evans in 1944 [523]. Subsequently, oxidation of polyamines has been observed with intact cells or extracts of other bacteria (*q.v.* Appendix A). The oxidative activities for these

amines usually increase when the bacteria are grown with the amines in growth medium, suggesting that the oxidative enzymes are inducible. Some of the amine oxidases, notably from *Micrococcus rubens* [534-538], *Serratia marcescens* [537,546,547], *Arthrobacter globoformis* [517,518], *Arthrobacter* P1 [512,514,516] and *Escherichia coli* [526-529] have been purified. The primary amino acid sequence of the enzymes from *Micrococcus rubens* [538], *Arthrobacter* P1 [516], *Arthrobacter globoformis* [518] and *Escherichia coli* [528] has been determined. Atomic structure of the amine oxidase from *Escherichia coli* has been solved and refined to a resolution limit of 2 Å [527,529]. More recently, the crystallization and preliminary X-ray analysis of PEAO from *Arthrobacter globoformis* have been reported [519].

1.3.2.2 Fungi and Yeast

PAO activity has been found in the mycelia of fungi belonging to *Aspergillus, Mucor, Penicillium, Rhizopus, Cylindrocarpon, Fusarium,* and *Gibberella* when grown on media containing spermine or spermidine as the sole nitrogen source [580]. *Aspergillus niger* produces an inducible amine oxidase that has been partially characterized [550-553,556,558]. An amine oxidase has been isolated from a *Penicillium* sp., which secretes an extracellular enzyme [575-577]. PAOs have been isolated from the yeast *Candida boidinii* [561-563] and *Pichia pastoria* [562,564] and purified to crystallization from *P. chrysogenum* and *A. terreus* [560,574]. Amine oxidase activity has also been found in yeast belonging to *Candida, Pichia, Trichosporon, Sporopachydermia, Hansenula* and *Kluyveromyces* spp. (*q.v.* Appendix A). It is of interest that *Saccharomyces cerevisiae* does not appear to have any endogenous amine oxidase activity [1045]. This well-characterized yeast is therefore a suitable expression host for amine oxidase genes. The cDNA amino acid sequence has been determined for the yeast amine oxidase *Hansenula polymorpha* [566,567,569].

1.3.2.3 Plants

The PAOs of plants have been reviewed [6,11,468-470]. PAOs from various Leguminosae, including pea (*Pisum sativum*), lentil (*Lens esculenta, Lens culinaris*), groundnut (*Arachis hypogea*), soybean (*Glycine max*), chick pea (*Lathyrus sativus*), broad bean leaves (*Vicia faba*), chick pea (*Cicer arietinum, Lathyrus cicera*), and *Phaseolus vulgaris*, have been partially purified and characterized (*q.v.* Appendix A). These enzymes catalyse the enzymatic oxidation of diamines and polyamines at their primary amino groups. In contrast, PAOs from the Gramineae: oats (*Avena sativa*), barley (*Hordeum vulgare*), and corn (*Zea mays*); oxidize polyamines at secondary amino groups. PAOs have also been found in other Gramineae: rice (*Oryza sativa*), wheat (*Triticum aestivum*), millet (*Setaria italica*), and rye (*Secale cereale*); though at lower

activities than oats, barley and maize. PAOs from other plants, including water hyacinth (*Eichhornia crassipes*), tubers of the Jerusalem artichoke (*Helianthus tuberousus*), the latex of the herbaceous Mediterranean perennial *Euphorbia characias*, *Hyoscyamus niger*, and the Solanaceae, *Nicotiana tabacum* and *N. rustica*, have also been partially purified and characterised.

The cDNA-derived amino-acid sequence of lentil seedling (*Lens culinaris)* amine oxidase has been determined [617]. Similarly, a pea seedling (*Pisum sativum*) amine oxidase has been isolated and sequenced. Hybridization analysis of Southern blots with a cDNA probe indicated that the amine oxidase gene was present in dicotyledons from the Leguminosae (lentil, pea, soybean), Solanaceae (tobacco, tomato, potato), Chenopodiaceae (sugar beet) and monocotyledons from the Gramineae (rice, wheat, barley) [649]. The pea seedling enzyme has been crystallized and X-ray diffraction structural studies have been reported [645,650], representing the first elucidation of a eukaryotic amine oxidase structure.

1.3.2.4 Parasites

PAOs from the free living pathogenic amoeba *Acanthamoeba culbertsoni* [1039] and from the parasitic nematode *Ascaris suum* [1041], have been partially purified and characterised. PAO has been identified in the parasitic nematode *Onchocerca volvulus* and in the filarial worm parasite *Dirofilaria immitis* [1043]. It is of interest that polyamine *N*-acetyltransferases are lacking from the nematodes and so PAO is solely responsible for polyamine interconversion and catabolism.

1.3.2.5 Animals

There are a large number of reports of amine oxidases from animals (*q.v.* Appendix A). In this dissertation attention is focused on those enzymes (EC 1.4.3.6 and 1.5.3.-) for which polyamines, diamines and histamine are substrates, although they may also oxidize monoamines.

a) Serum (Plasma) Amine Oxidase. Amine oxidase activity has been found in the serum or plasma of most species examined (*q.v.* Appendix A), although the distribution of enzyme activity is not uniform. The non-uniform distribution of serum enzyme activity between species suggests a specialized function. Ruminants and pregnant animals with deciduate placentae have the highest levels of serum amine oxidase activity. Non-ruminants normally have very low levels of serum amine oxidase activity, which can only be detected with the use of sensitive methods [955]. Intravenous heparin administration and various pathologic conditions cause a significant increase in the serum level of DAO in various species including humans as discussed in section 1.4.6 *et passim*. Various tissues, including liver, kidney, intestine, and placenta, may be the source of

DAO in serum.

The first recognition of serum amine oxidase activity was in the blood of pregnant women as histaminase [687-690]. The enzyme activity increases to high levels before parturition [686,690-692], followed by a rapid *post partum* decay during the puerperium. Pregnancy serum amine oxidase has been studied extensively in humans (*vide* Tables 1.5, 1.7, 1.8 and 1.9) and observed in other mammals with deciduate placentae [686,690,989,1014]. Pregnancy-associated amine oxidases are discussed in detail in section 1.6 *et seq.*

Subsequent to the discovery of pregnancy serum amine oxidase, the presence of an amine oxidase in sheep and bovine serum with high activity when spermine and spermidine were substrates was reported by Hirsch [856]. Extensive surveys of animal sera were carried out by Blaschko *et al.* who found the highest activity of amine oxidases in the serum of ruminants [458,500,819]. The amine oxidases from human pregnancy serum, bovine serum and pig plasma have been amoung the most extensively studied serum amine oxidases (for references see Appendix A). Serum amine oxidases have multisubstrate specificity which, in part, has led to their confusing nomenclature. They have variously been known as 'histaminase' [686,912], 'spermine oxidase' [856], 'plasma amine oxidase' [823], 'plasma monoamine oxidase' [661,665,666,1046], 'serum amine oxidase' [659,840], 'monoamine oxidase' [493,661,666,944,1046,1047], 'polyamine oxidase' [169,1048], 'spermidine amine oxidase' [832], 'benzylamine oxidase' [912] and 'semicarbazide-sensitive amine oxidase' [502]. It is most likely that authors are referring in many cases to the same structural phenotype although the substrate specificity and inhibitor sensitivity may differ from species to species. However, it is also possible that more than one type of circulating amine oxidase may exist in the same animal.

The bovine serum amine oxidase has been purified [49,451,493,756,825,832,835-838,849] and characterized (see purification references and [214,223,718,822-824,827,829-831,833,834,840,841,843-848,851,854,856,1049]). The enzyme has been cloned and its primary amino acid sequence has been determined [411]. A cDNA library screen revealed for the first time that bovine liver is the source of the bovine serum amine oxidase [411,853], though previous investigations have suggested that serum enzymes may arise from the small intestine [963] and vascular smooth muscle [767,933] (*vide infra et passim*).

Pig plasma amine oxidase was purified by Buffoni in 1964 [912] and has been extensively studied since [461,473,910,913,915-931,1050]. It has been reported that the enzyme oxidizes histamine but not spermine, spermidine or putrescine [914,915]. This substrate specificity needs re-examination using more sensitive techniques. Most studies have been carried out with benzylamine (or a similar aromatic amine derivative) as the substrate, since the oxidative deamination of benzylamine to form benzaldehyde can be conveniently followed by monitoring

the change in absorbance at 250 nm. In the case of other substrates, relatively insensitive manometric techniques were used.

Because of its activity on primary amines (such as benzylamine, tyramine, tryptamine and dopamine), which are also oxidized by the mitochondrial monoamine oxidases, MAO-A and MAO-B, human serum (mono)amine oxidase has also been referred to as human plasma monoamine oxidase [661,665,666,1046]. However, there is evidence of substantial differences between the serum enzyme and the mitochondrial amine oxidases [661,1046].

In general, serum amine oxidases from ruminants show a greater activity toward the polyamines. Whereas the serum enzymes from non-ruminants, such as pigs, show greater activity toward monoamines, such as dopamine and serotonin. A report describing the purification and characterization of non-ruminant serum amine oxidase from horse has been published [939].

b) Liver amine oxidase. Since the early observation of histaminase activity in the liver of the turkey buzzard [475], amine oxidase activity has been observed in the liver of many species (*q.v.* Appendix A). DAO activity is especially high in guinea pig liver [1017]. Rat liver 'polyamine oxidase' may be the best characterized liver amine oxidase (*sed confer infra*). Hölttä isolated and characterized an amine oxidase from rat liver that oxidizes spermine, spermidine and their acetylated derivatives [164,491]. This enzyme acts on secondary amino groups and is responsible for the interconversion of spermine to spermidine and spermidine to putrescine; it therefore acts as an EC 1.5.3 type enzyme and has been classified as EC 1.5.3.11 [430]. PAO has also been purified from porcine liver, like the rat liver enzyme it has a high affinity for N^1-acetylspermine and N^1-acetylspermidine [409]. Sequence analysis data suggests that the liver is probably the source of the bovine serum amine oxidase [411,853], in which case the bovine liver enzyme is the best characterized liver amine oxidase.

Liver amine oxidase has been localized to hepatocytes in the rabbit [952], rat [976], and guinea pig [1017], but is probably absent from rabbit Kupffer cells [952] in contrast to the localization of PAO activity to rabbit alveolar macrophages [955]. In rabbit, quail, and guinea pig, liver DAO activity was localized to the microsomal fraction [406,965,1017,1051] (known to be rich in peroxisomes [412]). DAO activity could be demonstrated in gerbil (sinusoidal endothelial cells), but not rat liver, using a histochemical method [48,976] except with the use of acetylated polyamines [976]. In rat liver PAO activity has been localized to peroxisomes [164,412,973,976], whereas in porcine liver, PAO was localized to a soluble cytoplasmic fraction [409]. In chicken liver, DAO was found to be distributed between the pellet and the supernatant after differential centrifugation [1028], however the *g*-forces used do not exclude its predominance in the microsomal fraction [952].

c) Kidney amine oxidase. DAO activity is relatively high in the kidney of many species (*q.v.* Appendix A). The enzyme from pig kidney cortex was discovered early by Zeller [485-487,778], who also observed DAO activity in the kidney of guinea pig, cow, and cat [778]). Since then, the porcine kidney enzyme has been purified [531,745,756,888,892,906,909,1052] and extensively studied (see purification references and [883,891,898,899,901,903-905,907,908,911,1053-1055]). The purification of the phenamil binding protein associated with the porcine renal Na^+ channel [906,909] led to the cDNA structure of the human kidney amiloride binding protein (ABP) [781] and its subsequent identification as diamine oxidase [47,411,757] (*vide* §7.2.8).

Amiloride is a K^+-sparing diuretic drug that exerts its action by blocking Na^+ channels in the apical membrane of renal distal and collecting tubules [1056]. It is described as potassium sparing since it increases the excretion of sodium accompanied by chloride as its counterion. It does not act by inhibiting aldosterone. Amiloride is a pyrazinoyl-guanidine (Figure 7.2) and its actions have been reviewed by Benos *et al.* [1057,1058]. Due to the presence of the guanidium moiety, amiloride can exist as a positively charged amine. Clinically amiloride has been used to treat edema and is marketed by Merck Sharp & Dohme under the trade name Midamor [1059]. Amiloride binding protein (ABP) was thought to be one of the components of the amiloride-sensitive epithelial sodium channel [1059]. Phenamil, an amiloride analogue, has been used to purify an ABP from pig kidney [906,909]. The purified protein (M_r 185,000) was a dimer of two identical glycoprotein subunits with an apparent M_r 105,000 crosslinked by disulphide bonds. Barbry *et al.* cloned the ABP from a human kidney cDNA library using synthetic oligonucleotide probes derived from partial sequences of the purified M_r 105,000 subunit from pig kidney [781]. The primary structure of ABP was deduced from the DNA sequence, reported in their paper [781], and deposited in the GenBank and EMBL data bases (accession no. 36335; EMBL entry M55602).

DAO had been previously been identified in [480,777,779] and purified from human kidney [753,780]. Immunofluorescent studies of pig kidney, localized DAO to the basal portion of epithelial cells of the (proximal) convoluted tubules [884,905]. In some species, e.g. rat [48,857,982,998,1060] and rabbit [857,953], kidney diamine/polyamine oxidase activity is reported as low. Nevertheless, in rats PAO activity has been localized to peroxisomes of proximal tubular (epithelial) cells [972,976]. In Northern blot analyses no signal was detected, but a PCR analysis revealed the presence of ABP (DAO) suggesting its restricted expression [982]. Sartori *et al.* localized more than 60% of the total DAO activity to the microsomal fraction of rabbit renal cortex [953], suggesting its subcellular localization in peroxisomes.

d) Intestinal amine oxidases. A high level of amine oxidase activity was found in the intestines of nearly all animals studied (*q.v.* Appendix A). Intestinal DAO has been purified from rats [999], humans [796] and a human colon cell line, Caco-2, which expresses some differentiated features typical of small bowel enterocytes [786]. The rat enzyme has been cloned and its primary sequence determined [982], although at the time the authors had targeted an amiloride binding protein. In general, amine oxidase activity is high in the small intestine, especially the ileum, and its activity decreases from the distal duodenum toward the pylorus [859]. In the rat intestine, DAO is found in the enterocytes of the villus tip rather than in the proliferating cells of the crypt [1061]. This also appears to be the case in humans [453,786], where it has been localized using immunohistochemical methods. Activity based histochemical methods have confirmed these findings [48,967,976]. As a general consideration, care needs to be taken in the interpretation of activity based histochemical findings since diaminobenzidine, a commonly used detection agent, has been found to both inhibit DAO activity and lead to nonspecific staining: 4-Cl-1-naphthol has been suggested as a suitable detection agent [1062]. Similarly, biochemical analysis based on subcellular fractionation techniques have suggested that DAO is predominantly located inside the villus cells, with about 60% linked to organelles and the rest free in the cytosol. Only a negligible activity was associated with the brush border membrane [952,1001]. Ultrastructural studies have revealed that PAO activity is localized to microperoxisomes in the duodenum of the rat [976].

e) Placental amine oxidase. The placenta contains very high levels of amine oxidase activity (*vide* Appendix A, Table 1.9). The localization of amine oxidase in the placenta was first examined by Swanberg [686], who dissected human placentae after delivery and measured DAO in the mechanically isolated components, demonstrating that the highest activity was in the maternal part of the placenta (i.e. the *decidua basalis).* This result was later confirmed by Southern *et al.* [684] and by Gunther and Glick [740], who measured enzyme activity in serial histologic sections. Illei and Morgan found significantly higher PAO activity in decidual tissue homogenates when compared to placental homogenates [420]. Enzyme activity has been observed in the fetal membranes (chorion, amnion) [684,760], but no significant amounts have been found in fetal plasma [420,684]. While the localization of the placental amine oxidases is discussed here and in section 1.6.6.1, their other characteristics are discussed in detail in section 1.6 *et seq, et passim.*

Lin *et al.* [749] using an immunofluorescent method, and Weisburger *et al.* [750] using an immunoperoxidase method, have demonstrated that the enzyme is present in the cytoplasm of decidual cells, while absent from trophoblasts. This observation should be confirmed using double labelling immunohistochemistry [1063,1064] to ensure the correct identification of the

different cell types (Yee Khong, Department of Histopathology, Queen Victoria Hospital, *personal communication*). Lin *et al.* noticed a specific fluorescence in the intercellular spaces of the decidua [749]. This extracellular fluorescence may represent active secretion of the enzyme as suggested by Argento-Cerù and Autuori [952], and be the reason for the high activity observed in retroplacental fluid [420]. Using an activity based histochemical method, Nakos and Gossrau have localized DAO activity to the cytoplasm of decidual cells of human, rat and mouse placenta, and possibly the surrounding extracellular matrix [48]. Bruun and Houen, using an enhanced chemiluminescence method for *in situ* detection of DAO activity on tissue sections from term human placentae, confirmed that enzyme activity was exclusively localized to the maternal side and was concentrated in vessels and fibrinoid areas [721]. Ryder *et al.* also found a high level of (benzyl)amine oxidase activity located in human placental blood vessels in their histochemical study [1065].

In vitro production of DAO by perfused human placentae has been demonstrated by Southern *et al.* [739]. Without surrounding decidua, no significant amounts of enzyme were produced by the placental preparations. Similarly, activities in the supernatant media of human endometrium in organ culture were generally below the limits of detectability, whereas decidua secreted large amounts of DAO into the organ culture medium. Furthermore, serum factors caused a marked increase in DAO levels [754].

The observations described above, and studies of physicochemical similarities to enzymes described in following sections, point to the decidua as the most likely the source of the pregnancy-associated amine oxidases observed in pregnancy serum, amniotic fluid [678,684,739,760,815,816,1066] and retroplacental serum [420]. This maternal enzyme source stands in contrast to the general perception that placental proteins, such as alkaline phosphatase, human chorionic gonadotropin and placental lactogen, are expressions of fetal or trophoblastic genomes [748,1067,1068].

Exceptionally, monoamine oxidase is discussed in this section since this study focuses on pregnancy-associated amine oxidases. Bardsley *et al.* isolated two amine oxidases from homogenates of human placenta [712,744]. One was characterized as a classical DAO, and the second was described as a monoamine oxidase, inhibited by iodoacetamide but not by aminoguanidine, and with a rate of oxidation of adrenaline an order of magnitude higher than putrescine. The 'monoamine oxidase' was also active with spermidine, spermine, histamine and benzylamine. Monoamine oxidase activity of human placental extracts has been recognized since the late 1940s [1069]. Prior to the reports of Bardsley *et al.*, Youdim and Sandler reported the isolation of a placental monoamine oxidase that appeared to exist as two isoenzymes [1070], although the proteins were not particularly well characterized. MAO-A or the clorgyline-sensitive

MAO, is the major form of monoamine oxidase expressed in human placenta [497,1071] (probably of trophoblast mitochondrial origin), and this enzyme has been more recently purified by Weyler and Salach [1072,1073]. The placental monoamine oxidase is FAD-containing [1072-1075], has a subunit M_r ~64,000 and probably exists as a dimer [1074,1076]. Curiously, placental MAO-A peptides had sequences that were different [1076] from those deduced from cDNA sequences for the human MAO-A (liver) mitochondrial enzyme [1075]. The two enzymes appear to have different substrate specificities [1077]. On the other hand, a low but significant amount of MAO-B activity is consistently present in human placenta [1078,1079], especially in mitochondiral preparations. Cloning and sequencing of a copper- (and TOPA quinone-) containing 'monoamine oxidase' from human placenta has been reported [1080] and its sequence appears distinct from the human placental 'diamine oxidase' [47,758].

f) Prostate and seminal fluid. Very high levels of DAO activity are present in seminal fluid [751,770-776]. This activity is mostly of prostatic origin [19,771,772,776], although 5% of the total activity is associated with spermatozoa and cannot be removed by washing with buffers containing high concentrations of salts or with 1% Triton X-100 [774]. There are suggestions that DAO activity comes partly from the upper part of the male genital tract (testis and/or epididymis) [410,776], in addition to the prostatic gland secretion. The seminal plasma enzyme has been partially purified and characterized [751,772-775].

g) Connective tissue amine oxidases. Amine oxidases have been localized to connective tissues such as bone [507,988,1027], derma [810,1020], dental pulp [507,828,860,861,936,1020], cartilage [1081], and blood vessels (such as aorta and umbilical arteries) [505,507,767,768,815,816,855,857,864,933-935,942,948,964,966,967,979,983-987,1029,1030]. The relationships between connective tissue diamine/polyamine oxidase activity, benzylamine oxidase, semicarbazide-sensitive amine oxidase, and lysyl oxidase are confusing. Discrete enzymes (with quite similar enzymatic activities [751]) probably exist. Vascular smooth muscle cell membranes may be the location of most of the semicarbazide-sensitive amine oxidase activity in blood vessels [502,767,966,985,986]. The human serum and umbilical artery enzymes appear to be identical [505], but distinct from bovine and rat semicarbazide-sensitive amine oxidases. It is pertinent that vascular endothelial cells and (skin) fibroblasts express binding sites or receptors for DAO [811-814,963,1000,1082] that can bind DAO synthesized by and exported from enterocytes [786], placental decidual cells [811], neutrophils [813] and perhaps hepatocytes [411,853]. Bound DAO can be displaced by heparin implicating structural specificity of heparinoid glycosaminoglycan moieties in the enzyme binding [814,963] (*vide* §1.3.4.3, 1.4.6, 7.2.8).

h) Leukocytes

(i) Macrophages. There is evidence that macrophages (derived from rabbit alveoli and mouse peritoneum) contain an intracellular PAO and, when activated, release the enzyme into culture medium [955,1083]. It is likely, but not certain, that PAO exists in the phagolysosome of macrophages. Amine oxidase activity has also been identified in macrophages from human peripheral blood and characterized with a range of substrates: polyamine, diamine and monoamine oxidase activities were found [807].

(ii) Neutrophils and eosinophils (polymorphonuclear cells, PMN). DAO activity has been demonstrated in human eosinophils and neutrophils [755,799-806], but was not detected in monocytes, basophils or platelets [800]. The total cellular content of DAO can be released from human granulocytes in a non-cytotoxic manner, by opsonized zymosan or the calcium ionophore, A23187 [1084,1085]. Complement proteins are probably involved in the release [803], which could be inhibited by agents that disrupt microtubule function [1084]. Cytochalasin B, a fungal metabolite that affects the motility of polymorphonuclear leukocytes, inhibits the opsonized zymosan mediated release of DAO from eosinophils, but not neutrophils [1085]. PMN leukocyte derived DAO activity can be inhibited by histamine H-2 class antagonists [801]. The granulocyte DAO is physicochemically and functionally similar to the DAO isolated from human placenta [755,800] and was principally localized to the 27,000 × *g* granule rich fraction of eosinophil and neutrophil homogenates [800]. Human neutrophils activated with secretagogues preferential for the specific granule, such as A23187 in a limited concentration, phorbol myristate acetate (PMA), formyl-methionyl-leucylphenylalanine (fMLP), and concanavalin A, release DAO. Recruitment of azurophilic granules had no incremental effect on the DAO release. Subcellular fractionation of resting neutrophils by sucrose density gradient centrifugation to separate specific granules from two classes of azurophilic granules localized the DAO activity to the specific granule fractions [805]. A third compartment in human neutrophils characterized by high gelatinase activity has also been found to be enriched in DAO activity [806]. See also §1.5.3.4.

1.3.3 Localization of Amiloride Binding Protein/Diamine Oxidase messenger RNA

Although the bovine serum DAO was cloned from a liver library and human amiloride binding protein/diamine oxidase (ABP/DAO) from a kidney library, previous investigations suggested that serum DAOs may arise from the small intestine [963] and from vascular smooth muscle [767,933]. Indeed, preliminary immunohistochemical and immunocytochemical (ultrastructural) studies with the human (retro)placental serum enzyme suggested the presence of the enzyme on the epithelial lining of blood vessels (this work, data not presented). The expression of ABP/DAO mRNAs in human and rat tissues has been examined [982]. The ABP/DAO mRNA has been

identified in several gastrointestinal tissues in rat, such as the duodenum, proximal and distal colon, and in the liver, placenta, lung and haematopoietic tissues such as thymus and spleen. The ABP/DAO mRNA was not detected in rat salivary glands, testis, uterus, skeletal muscle, heart or brain. In rat kidney, no signal was detected by Northern-blot analysis, but a PCR analysis revealed the presence of ABP. In human tissues the ABP transcript was detected in kidney and placenta and, at a much lower level, in liver, fetal lung; but not in brain, muscle or pancreas. These results are in agreement with previous localization of DAO (*vide supra et* Appendix A). Chassande *et al.* [757] have also detected ABP/DAO mRNA in HT29D4 and T84 cells, two colonic epithelial cell lines, as well as in U937 cells, a human promyeloctic cell line, but not in HeLa, CHO, A7R5 (aortic smooth muscle), or C2C12 (skeletal muscle) cells. DAO has been previously found in the human epithelial colonic cell line Caco-2 [786]. Proximal promoter elements containing binding sites for nuclear transcription factors may participate in the control of the expression of the gene in monocytes and macrophages [757] and the PU-1 transcription factor consensus site near the palindromic E-PAL motif in the ABP/DAO proximal promoter sequence is thought to be a likely candidate.

The gene for amiloride-sensitive DAO has been mapped to human chromosome 7q34–7q36 by *in situ* hybridization with ABP cDNA [398].

1.3.4 Structure

1.3.4.1 Prosthetic Groups and Cofactors

a) Organic cofactor. The classification of amine oxidases according to their substrate specificity or inhibitor sensitivity has led to great confusion in their nomenclature because of the overlap in these properties between different enzymes. In 1951, Zeller reviewed together for the first time, the oxidative deamination of monoamines, diamines, and polyamines and divided the enzymes into two types, viz. monoamine oxidases and DAOs [447] (*vide* §1.3.1). Subsequently, a division of amine oxidases into two general classes according to the prosthetic groups present in the enzyme became vogue. Thus, the enzymes became known as FAD-containing amine oxidases and copper-containing amine oxidases. The DAOs were generally copper-containing amine oxidases and were considered to belong to EC 1.4.3.6, although they may also exhibit EC 1.5.3 behaviour when oxidizing polyamines [223]. The FAD-amine oxidases were considered to belong to EC 1.4.3.4 and were called monoamine oxidases, although they may also act on diamines and polyamines. A second type of FAD-containing amine oxidase, was classified as EC 1.5.3.11 and is called PAO (although it may show EC 1.4.3 behaviour with diamines and monoamines). It is thought to require a different metal cofactor, possibly Fe^{2+} [164,430].

(i) Copper-containing amine oxidases. In addition to copper at the active site, the copper-containing amine oxidases contain a covalently bound carbonyl cofactor capable of forming derivatives with carbonyl reagents such as semicarbazide and phenylhydrazine, which led to the early conjecture that pyridoxal was the organic cofactor [449,820,821,903,913,1086] (*q.v.* Figure 1.9). Carbonyl group reagents, such as semicarbazide, phenylhydrazines, hydrazine and hydroxylamine, are potent inactivators of some amine oxidases [449,451,1087]. However, unlike other pyridoxal phosphate requiring enzymes, the cofactor in copper-containing amine oxidases is not released upon protein denaturation; furthermore, the chemistry of these proteins fails to conform to properties predicted from simple pyridoxal models. Prior to 1984, a number of alternatives to pyridoxal phosphate in copper-containing amine oxidases had been proposed, including a modified flavin [1088] and a redox-active cysteine connected to an unknown residue [830,831].

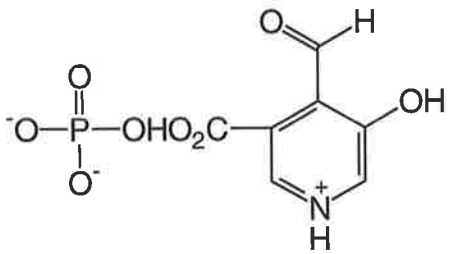


FIGURE 1.9. Structure of Pyridoxal Phosphate

In 1984, Ameyama *et al.* [1089] and Lobenstein-Verbeek *et al.* [841] proposed that the active site cofactor in bovine serum amine oxidase was a pyrroloquinoline quinone (PQQ), a tricyclic quinone (Figure 1.10) previously demonstrated in a number of prokaryotes [1090]. However, the evidence in support of a covalently bound PQQ was weak [457,844].

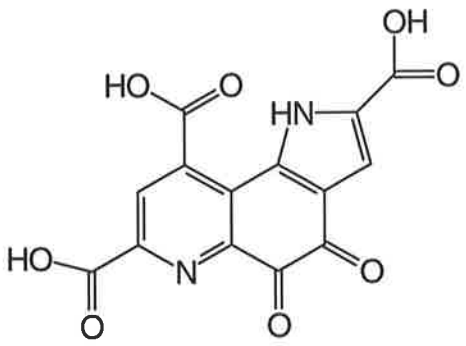


FIGURE 1.10. Structure of Pyrroloquinoline Quinone (PQQ)

Subsequently, Janes *et al.* clearly demonstrated that the bovine serum amine oxidase active site cofactor is the dihydroxy derivative of tyrosine, 2,4,5-trihydroxyphenylalanine quinone (6-hydroxydopa), or TOPA quinone (TPQ) [843] (Figure 1.11). Since then TPQ has been identified

biochemically, or its presence inferred from amino acid sequence similarity, in other copper-containing amine oxidases including those from *Hansenula polymorpha* [566,568,1091], *Klebsiella aerogenes* [1092], *Escherichia coli* [524,526-531], lentil [617,1093], pea [613,647,649], *Arthrobacter* strain P1 [514,516], *Arthrobacter globoformis* [518,520], *Aspergillus niger* [557], human kidney (first identified as amiloride binding protein; [47,411,757,781], porcine kidney [47,411,531,756,909], rat kidney [982], rat intestine [982,999], human placenta [47,756,758,1080], pregnancy serum [756], bovine liver [411,853], and equine plasma [939]. Nevertheless, some workers still insist that the organic cofactor in pig kidney and pig plasma DAO is pyridoxal phosphate [473,910,1094].

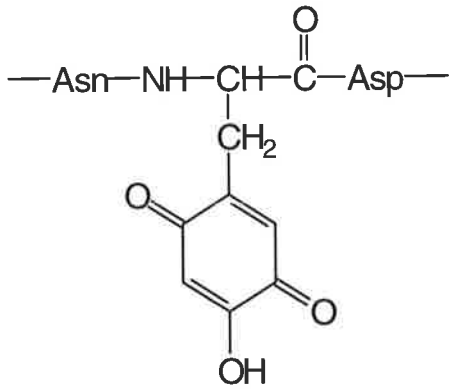


FIGURE 1.11. Structure of 2,4,5-Trihydroxyphenylalanine (TOPA) Quinone. TPQ is shown as a peptide amino acyl side group. TPQ has the potential to exist in a *para-* or *ortho-*quinonoid form.

There is evidence that the formation of the TPQ cofactor occurs through a novel self-processing mechanism in which a tyrosine residue is oxidized by protein-bound copper and oxygen [1095-1097]. Besides the biogenesis of the functional quinone cofactor, the copper is also required for the catalytic oxidation of amines in the mature enzyme. The dual function of the copper atom is a novel example of 'biological parsimony', and perhaps the result of two different, but closely related evolutionary selection criteria.

(ii) FAD-containing enzymes. The mitochondrial membrane-bound monoamine oxidases, MAO-A and MAO-B, contain covalently bound FAD as their cofactor [451,497,1098,1099]. Although the mammalian tissue 'polyamine oxidase' (from rat liver) appears to contain non-covalently bound FAD as a prosthetic group, the data supporting this is not convincing. The rat liver enzyme is strongly inhibited by quinacrine, a known inhibitor of several flavoproteins, however quinacrine lacks specificity and the enzyme is also sensitive to carbonyl group reagents [164]. Furthermore, the absorption spectrum of the purified enzyme did not show the typical three-banded spectrum for flavoproteins, nor was the activity of purified apoenzymes fully restored by exogenous FAD. Moreover, the amount of extractable FAD was quite low [164]. The plant 'polyamine oxidases' isolated from the monocots and water hyacinth are also thought to be FAD-

containing. However, the oat 'polyamine oxidase' does not appear to contain FAD. A 'monoamine oxidase' from *Aspergillus niger* contains a consensus sequence for a putative FAD binding site [557,559]. Putrescine oxidase from *Micrococcus rubens* and tyramine oxidase from *Sarcina lutea* contain non-covalently bound FAD (see Appendix A for references).

b) Copper Content. The presence of copper as a cofactor has been established for many oxidative enzymes. Copper always binds to amino-acid side-chains of copper-containing proteins. However, the nature of the ligands and their geometry around the metal confer three very different spectroscopic and chemical properties to the cupric copper. Copper proteins have been classified as Type-I, Type-II, and Type-III [430,1100]. Type-I have a single Cu atom at the active site and are often called the 'blue copper proteins' because of their intense absorption at about 600 nm. These proteins have unusually narrow hyperfine coupling in the electron paramagnetic (or spin) resonance (EPR) spectrum of the Cu^{II}, and high reduction potentials. Type-II proteins also have a single copper atom. They are characterised by low absorption in the visible range and do not have an unusual EPR spectrum. As such, they lack the intense cerulean colour of the Type-I proteins. Type-III proteins have a pair of copper ions with anti-ferromagnetically coupled spins and therefore do not show an EPR signal. Type-III copper is always present, together with the other two types, in multicopper oxidases such as ceruloplasmin [464]. The EC 1.4.3.6 type copper-containing amine oxidases in Table 1.3 have EPR detectable copper. Since their EPR spectra lack unusually narrow hyperfine coupling and were found to have low intensity absorption in their visible region spectra, they can be classified as type-II [464]. Their copper is associated with the TPQ cofactor and ligated by histidine residues [529,820,903]. A characteristic feature of EC 1.4.3 and 1.5.3 copper-containing amine oxidases is their 'peach' pink colour corresponding to a broad absorption maximum at around 480 nm. This chromophore appears unusual when compared to the blue or blue-green colour of simple copper salts. However, some copper(II) complexes, in which the ligands absorb in the (ultra)violet region, are red [1101]. Nevertheless, it is mostly the TPQ cofactor that gives rise to the 480 nm absorption band [1090] (*q.v.* §1.3.4.1c).

The amine oxidase from seedlings of the soybean *Glycine max*, is markedly inhibited by cuprizone but not by bathocuproine disulphonic acid [597], suggesting that Cu^{2+} is a cofactor [820]. This was later confirmed by low temperature EPR spectrum. Protein sequence analysis of yeast (*H. polymorpha*) and lentil seedling enzymes has led to the identification of three conserved histidines as ligands for the active site copper [617]. The histidine containing motifs are conserved in other amine oxidases. One His-X-His motif is highly conserved (*q.v.* §7.2.8). This may contain two of the histidine ligands to copper, since His-X-His motifs have been identified as ligands to type II or III copper in the crystal structures of several copper proteins [457].

The importance of the His-X-His motif in copper binding has been demonstrated for the *H. polymorpha* enzyme by mutagenesis studies [567]. The structure determination for the *E. coli* enzyme described by Parsons *et al.* identifies His^{689} as the third copper ligand, although the putative ligand, the conserved His^{440} is close to the active site suggesting that it plays an important role [529]. X-ray crystallography reveals that in the *E. coli* enzyme the three histidines that act as ligands to the copper are His^{524} and His^{526} of the His-X-His motif and the His^{689} that lies close to the C-terminal end of the sequence. All of these active site residues are well buried in the interior of the molecule. The bound copper lies 12 Å below the surface of the molecule. Crystal structure of *E. coli* copper-containing amine oxidase indicates a single atom of copper per subunit in the homodimeric structure [529]. The pea seedling enzyme has a similar structure [650] (*q.v.* §1.3.4.5).

The role of copper in the EC 1.3.4.6 class of enzymes is bifunctional. Besides the biogenesis of the functional quinone cofactor as mentioned above, it is involved in the catalytic mechanism. Copper is involved mainly in redox processes in living organisms; so amine substrates may be expected to reduce Cu^{2+} to Cu^{+} while oxygen would restore copper to the divalent state. However, the results of EPR investigations on all amine oxidases examined, including freeze–quench studies, show that almost all the copper remains divalent following anaerobic reaction with amine substrate, so the role of copper has thus been a puzzling feature of the enzyme. These studies led to the suggestion that copper(II) either acts as a Lewis acid, that it has an indirect role in catalysis, or that it may have a role in maintaining the tertiary structure of the enzyme [1]. The removal of the metal from the enzyme molecule results in an apoenzyme that is inactive, but whose activity can be restored upon readdition of copper, though not by other metals [840]. Evidence for the catalytic role of copper includes studies on the pig plasma enzyme where Lindström *et al.* [921] and later Baker *et al.* [929] who observed that azide both modified the Cu^{2+} EPR spectrum and inhibited enzyme activity. Evidence for the generation of a Cu^{I}-semiquinone state by substrate reduction of several amine oxidases under anaerobic conditions has more recently been presented [1102,1103]. This Cu^{I}-semiquinone intermediate would react rapidly with molecular oxygen to regenerate the Cu^{II}- TPQ form of the enzyme. As might be expected for enzymes that use radical chemistry [1097] (*q.v.* §1.3.5), the Cu–TPQ active site is not directly accessible from the solvent [529,650].

Varying estimates for the copper content of the amine oxidases are present in the literature. The variability is mainly a result of the M_r assumed for the enzyme, the method for calculating protein concentration and how rigorously adventitious copper has been removed. Table 1.3 summarizes the copper content of the enzymes from different sources. These data suggest that copper-containing amine oxidases (of the EC 1.4.3 and 1.5.3 types) have one copper atom per

TABLE 1.3. Copper Content of Amine Oxidases

Source	*Copper content mol/mol protein (dimer)*	*Reference*
Arthrobacter amine oxidase	2	[513,514]
Aspergillus niger	1.9,2.1	[556,1104]
Bovine plasma	2.2	[834]
Bovine lysyl oxidase	1.0 (monomer)	
E. coli	2.0	[529]
Hansenula polymorpha	2, 2.04	[566,567]
Hordeum vulgare	1.94	[604]
Human ceruloplasmin	6–8 (per molecule)	[1105]
Human placenta	2.0	[744]
Lentil seedlings	2	[618]
Pea seedling	2	[637,640,647,650]
Porcine plasma	2.1	[929]
Porcine kidney	2.0	[892,898]
Soybean seedlings	1.94	[600]
Vinca faba	2	[653]

subunit. This has been confirmed for the *E. coli* [529], and pea seeding [650] enzymes by X-ray crystallographic analyses.

c) UV-Visible spectrum. Spectroscopic methods are useful for characterising the nature of cofactors in amine oxidases. Flavin-containing amine oxidases have an easily identified visible absorption spectrum [535,548]. It is of interest that the absorption spectrum of the purified rat liver PAO, thought to contain flavin–adenine dinucleotide as a cofactor, did not show the typical three-banded spectrum for flavoproteins (*vide supra*). Only a very small peak at 456 nm, along with the major absorption peak at 275 nm, was observed for the native enzyme [164].

Purified preparations of copper-containing amine oxidases are a 'peach' pink colour because of a broad absorption maximum around 470–480 nm in the visible spectrum [1106] (*vide supra*). However, it is the organic cofactor, TPQ, rather than copper, which is thought to be the chromophore that gives rise to this absorption band [614,820,903,1090,1107,1108]. Evidence includes; bleaching of the 500 nm absorption band by substrate addition under anaerobic conditions not associated with significant EPR spectrum change [558,604,618,634,820,892,912,920,1104,1109]. (Cu^{II} gives a characteristic EPR spectrum [1106] (*q.v.* §1.3.4.1b)). Bleaching also occurs with FAD enzymes, though EPR spectra were not examined [535,536]. Procedures that markedly altered the EPR spectrum, such as azide or cyanide treatment, did not significantly alter the UV–visible spectrum [903,921,1110]. Copper removal under nonreducing conditions produces a metal-free apoenzyme with only a slight blue shift in the visible absorption peak [614,634] and no change in the UV region where the λ_{max} is 278 nm. Substitution of the Cu^{II} with Ni^{II} or Co^{II} only causes a slight red shift in the visible region absorption maximum [840]. Reduction of the chromophore with sodium dithionite causes disappearance of the absorption band [558,820,840,892,1111], although there is one report of the

absorption band of the pig kidney enzyme *not* being diminished by reduction with dithionite [903]; it is not clear whether sufficient reagent was used. In contrast, other copper-containing oxidases, such as ceruloplasmin, are sky-blue and show an absorption maximum around 600 nm (*q.v.* §1.3.4.1b). Published UV–visible absorption values for amine oxidases are presented in Table 1.4.

TABLE 1.4. UV–Visible Absorption Characteristics of Amine Oxidases

Source	λ_{maxima}	*Description*	*References*
Pig kidney diamine oxidase	500 nm	very broad absorption (half-width ≡ 90–100 nm)	[903]
	280 and 470 nm	–	[893]
	405 and 480 nm	'pink-yellow'	[892]
Porcine serum	278 and 470 nm	concentrated solutions of the enzyme are pink	[912]
Bovine serum	280 and 480 nm	–	[493]
		'peach coloured'	[848]
	480 nm	broad absorption band around 'pink type II copper protein'	[833]
Bovine aorta	280 and 480 nm	–	[855,863]
Aspergillus niger	280, 330, 410, 480 nm	–	[451]
	280, 480–500 nm, shoulder at 410 nm	–	[558]
Aspergillus terreus 'polyamine oxidase'	275, 380 and 457 nm	'flavoprotein with FAD "yellow" like the enzyme in rate liver' [*sic*]	[560]
Penicillium chrysogenum	275, 375, and 450 nm	yellow FAD containing polyamine oxidase typical three banded spectrum for flavoprotein	[574]
Escherichia coli K12	band around 480 nm as well as the protein absorption peak at 280 nm and	'pink-coloured' 'clearly lacks the intense absorbance bands at 370–360 nm and 460–470 nm, which are characteristic of bacterial flavin-containing amine oxidases'	[526]
Arthrobacter P1	480 nm	–	[1050]
Micrococcus rubens 'putrescine oxidase'	280, 380, and 460(458) nm	'yellow colour' FAD containing enzyme	[535,536]
Pea seedling	280, 480, 505 nm	–	[451]
	280 and 500 nm	–	[634,640,1111]
	279 and 490 nm	'pink'	[637]
	280 and 500 nm	'pink'	[647]
Lens esculenta seedlings	278 nm and 480(498) nm	'pink-red'	[614,840]
Oats (*Avena sativa*)	275, 350 nm and 460 nm	maxima	[584]
	276, 370 and 452 nm	'yellow enzyme', said to be typical of an oxidized flavoprotein	[586]
Glycine max (Soybean seedling)	280 nm	–	[600]

TABLE 1.4 (*continued*)

Source	λ_{maxima}	*Description*	*References*
Euphorbia latex	278, 490 nm broad absorption	'pink'	[593]
Eichhornia crassipes (Water hyacinth)	275 nm	though the concentrated solution was yellow-brown, it is thought to be a flavoprotein like the maize enzyme, results were equivocal.	[594]
Hordeum vulgare (Barley)	278 and 498 nm	'pink-red'	[604]
Maize (*Zea mays*)	278, 380, (456) 460 nm	'light yellow' polyamine oxidase. Under aerobic conditions the intensity of the visible region absorption bands were decreased by addition of sodium dithionite or spermidine. The flavoprotein nature of the maize PAO has been inferred by inhibition studies with acridine compounds such as quinacrine and rivanol. The yellow chromophore of the purified enzyme was extracted and identified as FAD as distinct from FMN or riboflavin	[654-656]
Hansenula polymorpha	280 and 472 nm	'pink'	[567]
Human plasma ceruloplasmin	280 and 605 nm	the strong absorption band at ~ 600 nm is responsible for 'intense blue' colour	[464,659,1100,1105,1112]
Bovine and porcine liver and brain	280, 450–455 nm, 490 nm	FAD containing monoamine oxidases. Unlike the typical flavoproteins they lack an absorbance maximum at 375 nm.	[451]
Human placental 'histaminase'	–	'solutions of highly purified placental enzyme are pale pink' and 'may be a property of placental histaminase just as it is that of pea seedlings'	[738]
Human retroplacental polyamine oxidase	280 and 407 nm	*not very pure and this is probably why the absorption spectrum did not show an absorption maximum around 480 nm and was unchanged after addition of dithionite	[726]

d) Structure of the active site. An early model of the active site in amine oxidases was based mainly on kinetic studies with pig kidney DAO using modified substrates. In this early model (illustrated in Figure 1.12), the amino group of the substrate to be removed binds as a nucleophile (unprotonated) to a positively charged enzyme group having a pK_a of about 8.8, close to the reactive carbonyl group of an organic prosthetic group [920,924]. A second negatively charged enzymic group is located 6–9 Å distant from this site for the binding of the second amino group of diamines in protonated form. The region between the binding sites is hydrophobic to facilitate binding of the aliphatic region of the substrates. The presence of a hydrophilic region surrounding the negatively-charged site is suggested by the inability of amine oxidases to oxidize compounds containing a trimethylammonium group [874]. Slight variations in active site architecture may be reflected by different substrate specificities.

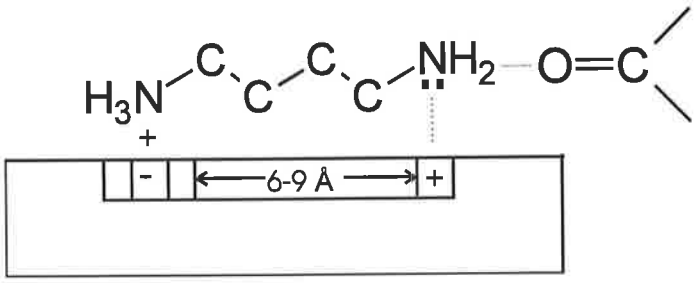


FIGURE 1.12. Schematic Structure of the Substrate Binding Site of PAO. Putrescine is represented. A protonated binding site for the binding of the substrate amino group to be removed is shown on the right hand side of the Figure, close to the reactive carbonyl of the organic cofactor. An anionic site surrounded by a hydrophilic region for the binding of the second amino group of diamines in the protonated form is located 6–9 Å distant from the protonated binding site. A hydrophobic region for binding of the aliphatic region of the substrate lies in the region between the two sites.

More recent crystallographic and spectroscopic evidence has lead to refinements of the amine oxidase active site model [466,529,645,650,848,1050]. The active site is now known to be buried within the enzyme structure, and substrate access to it appears to require a rearrangement of the polypeptide. Each subunit of the enzyme contains an active site consisting of an essential Cu^{2+} atom and the organic cofactor. The copper atom is coordinated by the imidazole groups of three conserved histidine residues ($His^{442,444 \text{ and } 603}$ in the pea seedling enzyme [650]) and by two water molecules in an approximately square-pyramidal geometry. The TPQ cofactor is close to the copper atom. A quinone oxygen ($O_{(2)}$) lies 6 Å from the copper atom but is not coordinated to it, all three oxygen atoms of TPQ are hydrogen bonded to neighbouring groups on conserved amino acid residues. The reactive electrophilic centre of TPQ is at the $C_{(5)}$ carbonyl [1113,1114] (*vide* Figure 1.11).

e) Other metal ions. The crystal structure of pea-seeding amine oxidase has revealed a second, octahedral metal-binding site [650]. The ligands are carboxylate groups of conserved $Asp^{(451,453,592)}$ residues, peptide carbonyl oxygens of Phe^{452} and Ile^{593}, and a water molecule. The electron density of the second metal site is lower than that at the Cu site and has been modelled as manganese, which is consistent with observations on eukaryotic amine oxidases. Specifically, Mn^{II} has been frequently observed in highly purified pea-seedling amine oxidase preparations, being identified by its EPR signal (David M. Dooley, Department of Chemistry and Biochemistry, Montana State University, Bozeman; unpublished observations). An identical site occurs in the crystal structure of *E. Coli* amine oxidase, and the possibility that this site is occupied by Mn^{II}, Ca^{II} or Mg^{II} under physiological conditions has been indicated [529].

It is likely that other amine oxidases with aspartic acid residues equivalent to those in the pea-seeding amine oxidase also have a second metal site that may be occupied by Mn^{II}. For example, there is evidence that the human placental enzyme, in which all three aspartic acid

residues of the second metal site in pea-seedling amine oxidase are conserved (*q.v.* Appendix E), contains Mn^{II}. This is consistent with early suggestions by Paolucci [742] and Crabbe *et al.* [744] that the placental enzyme might contain Mn^{II} in addition to Cu^{II}. EPR spectroscopy of partially purified enzyme preparations by Crabbe *et al.* revealed the presence of iron, presumably from contaminating haem-containing proteins. Their subsequent studies, using an enzyme preparation that had been passed through a column of Chelex-100 resin to remove any extraneously bound metal ions, strongly suggested that Cu^{2+} and Mn^{2+} were strongly bound to the human placental amine oxidase. Atomic absorption spectroscopy confirmed the presence of copper and manganese and indicated a 1.0 g-atom of copper and 1.2 g-atom of manganese per M_r 70,000 subunit [744]. The 0.4 g-atoms of iron per M_r 67,000 identified in Morgan's partially purified preparation of human retroplacental serum PAO by electrothermal atomic absorption spectroscopy may also have been due to contamination by haem containing proteins. Although the presence of iron in the rat liver PAO has been reported [491], evidence for this appears flimsy and consists of the partial restoration of enzymatic activity with ferrous ions (Fe^{2+}) in the absence of thiols, after treatment with cysteine and the iron chelator, *o*-phenanthroline [164].

1.3.4.2 Molecular Weight (Relative Molecular Mass, M_r) and Molecular Mass

The molecular weight (relative molecular mass, M_r) of amine oxidases ranges from about 32,000 (Lysyl oxidase [1115]) to 250,000 (*Aspergillus niger* [556]). Copper-containing amine oxidases are usually homodimers of M_r 60,000–110,000 subunits depending on the source. It is generally perceived that proteins with a preparative M_r > 50,000–100,000 may reveal subunit structure [1116], so the copper-containing amine oxidases fulfil this expectation. Most of these amine oxidases have a carbohydrate content of between 7 and 14% of total molecular weight. In contrast, the FAD containing amine oxidases, such as the rat liver PAO and some of the plant PAOs are said to be monomeric and have molecular weight range 53,000–85,000. The molecular masses and relative molecular masses of selected amine oxidases have been reviewed in the following section.

The formation of amine oxidase aggregates appears to be a common phenomenon [823,906,1117,1118]. The molecular weight of various amine oxidases, for example the pig kidney [899] and bovine serum [823] enzymes, remained controversial for years, in part, because of their tendency to undergo association–dissociation phenomena varying with the enzyme concentration and environmental conditions.

As with many proteins, the apparent relative molecular mass of some amine oxidases varies depending on the method used for its determination. Post-translational glycosylation of proteins may result in their anomalous behaviour during characterization studies, in particular during SDS-

PAGE and size-exclusion chromatography. Anomalously high apparent molecular weights may be indicated for glycoproteins by size-exclusion chromatography because of their disproportionately large hydrodynamic radii, due to the greater hydration of their carbohydrate chains, when compared to polypeptide chains of an equivalent molecular mass [1119-1121]. Glycosylation is also well known to cause the anomalous behaviour of glycoproteins in SDS-PAGE gels of different concentrations [1122,1123]. Glycoproteins consistently exhibit reduced mobility compared to nonglycosylated proteins of similar molecular weight, probably as a result of their diminished binding to SDS reducing their net charge to weight ratio. The difference between the apparent molecular weight of the glycoprotein and its actual molecular weight decreases with increasing acrylamide pore size. This may be compensated for, in part, by the construction of Ferguson plots [1124,1125]. It has been proposed that a more accurate molecular mass can also be estimated by plotting the apparent molecular weight of the glycoprotein at various acrylamide concentrations and extrapolating the value to an asymptotic minimum [1123]. Further confusion has been introduced by the designation of different molecular weights for the same molecular weight markers by different investigators. Variations in reported values for different molecular weight standards are presented in Table 2.2.

Where the amino acid sequence of an amine oxidase is available, an accurate estimate of its actual molecular mass (in daltons) may be calculated from the sequence. However, these estimates do not include the mass of post-translational modifications so the apparent relative molecular mass might be significantly higher.

a) Animal amine oxidases

(i) Bovine plasma amine oxidase. The relative molecular mass of the bovine plasma amine oxidase was initially reported to be 261,000 [822]. This was later shown to be in error and the result of association–dissociation of the enzyme [823] according to the following scheme:

monomer $\Leftrightarrow$ dimer $\Leftrightarrow$ trimer

Self-association of the enzyme was favoured by a neutral pH environment. Furthermore, time and high protein concentration favoured the irreversible association of the monomeric unit into polymeric forms. The M_r of the nonassociated whole enzyme, determined by gel filtration chromatography and sedimentation–equilibrium ultracentrifugation, was 170,000. Treatment of the enzyme with 5 M guanidium chloride containing 0.1 M 2-mercaptoethanol dissociated it into M_r 87,000 subunits as determined by analytical centrifugation [823]. This data, and peptide mapping of a tryptic digest, suggested that the enzyme was comprised of two equivalent subunits linked by disulphide bridges. The subunit M_r, analysed by SDS-PAGE under reducing conditions,

was subsequently reported as 85,000 [451]. Carbohydrate accounts for about 7–8% of the total enzyme M_r [827], although previously reported as 4.6% [822]. The molecular mass of 82,836 Da per subunit, calculated from the primary structure of bovine serum amine oxidase, is consistent with the estimate of glycosylation and the observed M_r of the mature enzyme subunit [411].

(ii) Bovine dental pulp. The approximate molecular weight estimated by Sephadex G-200 gel filtration is 170,000 [828,861].

(iii) Porcine kidney diamine oxidase. Early reports, based on sedimentation analyses, gave M_rs of 135,000, 129,600, and 119,500 for the pig kidney enzyme, although a minimum molecular weight of 87,000 was determined by copper analysis [892]. In the same year, a value of M_r 185,000, based on ultracentrifugation studies, was also published [893]. A subsequent study also found a value of M_r 185,000 and suggested that the enzyme is subject to association–dissociation change, particularly in anoxic media, with the determination of a M_r 725,000 (tetrameric) form [899]. The M_r 185,000 was broadly confirmed by Kueltz and Schmidt who reported a value of M_r 172,000 by sedimentation–diffusion and sedimentation–equilibrium ultracentrifugation analysis, and that the enzyme consists of two identical subunits of M_r 91,000 by SDS-PAGE under reducing conditions [898]. Sugars have been reported to account for approximately 10% [913] (11.1% [907]) of total enzyme mass.

Radiation inactivation analysis of pig-kidney amiloride binding protein (ABP), later shown to be DAO, found a target size of 90 $\pm$ 10 kDa [47]. SDS-PAGE under reducing conditions showed a single protein with an apparent M_r 105,000. Under non-reducing conditions the protein ran as a M_r 185,000 band; and aggregation to larger forms, especially on storage, was noted [906]. Faint bands with apparent M_rs of 70,000 and 80,000 copurified with the 105,000 band and tryptic digests indicated that these were likely to be degradation products of the 105,000 band, indeed their relative abundance increased on storage [909]. Deglycosylation of the purified enzyme caused a decrease in the apparent M_r of the major protein from M_r 182,000 to 176,000 when SDS-PAGE was carried out under non-reducing conditions and from M_r 105,000 to 102,000 under reducing conditions, indicating that sugars represented about 5% of the total protein mass. Ferguson analysis indicated a subunit M_r of 88,000. Protein aggregates were observed at the interface of the stacking and resolving gels [909].

(iv) Porcine plasma amine oxidase. The molecular weight of pig plasma amine oxidase determined by analytical centrifugation was 195,000 [912,929]. This was later confirmed as M_r 196,000 by gradient gel electrophoresis of the dimer by Falk *et al.* who also found that the enzyme had a single, 'somewhat broad' band [probably reflecting its glycoprotein nature], as seen by protein and periodic acid staining, with a M_r 95,000 under SDS-reducing conditions [931].

(v) Porcine liver. SDS-PAGE and gel filtration chromatography showed that the porcine liver

PAO is a monomeric protein with a M_r 62,000 [409].

(vi) Rat Liver. PAO (EC 1.5.3.11) from rat liver was reported as being a single polypeptide chain with a M_r 60,000 with no evidence for structural subunits [164,165,491]. However, the reducing agent, dithiothreitol, was used in the buffers used to purify the enzyme.

vii) Rat intestine, liver, placenta and thymus. The rat colon amiloride-sensitive amine oxidases have been cloned. A 2.7 kb transcript codes for an 85,021 Dalton protein subunit of the disulphide linked amine oxidase homodimer (calculated from amino acid sequence, Swiss-Prot Accession No. P36633). This transcript was detected in the proximal and distal colon, duodenum, liver, placenta and thymus by Northern blot analysis [982].

b) Human amine oxidases

(i) Kidney. SDS-PAGE studies by Suzuki and Matsumoto suggested that the molecular weight of the human kidney DAO subunit was 105,000. Size exclusion chromatography showed broad peaks in the range from void volume (V_0) to M_r 300,000 and indicated that, like the porcine kidney enzyme, the human kidney enzyme polymerizes to larger forms [780]. More recent studies demonstrated that the kidney amine oxidase has a glycoprotein subunit appearing as a single band at M_r 97,000 on SDS-PAGE, and migrating under nonreducing conditions as a dimer with a M_r 180,000. Inhibition of *N*-glycosylation caused a shift of the M_r 97,000 band to M_r 70,000. The calculated molecular mass of the mature subunit peptide is 78,886 Daltons [411,781].

(ii) Intestine. The human intestinal amine oxidase appeared as a single band with an apparent M_r of 95,000 in 7.5% acrylamide gels under reducing and denaturing conditions [786]. A more recent study showed that purified enzyme migrated with an apparent M_r of 100,000 on SDS-PAGE, whereas under non-reducing conditions it was at M_r 180,000. Other proteins were also present. The purified protein appeared to consist of two forms of virtually identical sizes but with slight differences in hydrophobicity and acidity [796].

(iii) Pregnancy associated. The molecular weights observed for pregnancy-associated amine oxidases are presented in Table 1.5. The primary sequence of the human gene amine oxidase is now known and molecular mass of the mature subunit peptide was calculated to be 83,415.6 Da. [47,757] (Swiss-Prot Entry P19801; EMBL Entry X78212). An apparent M_r of 'around 105,000' for the placental enzyme subunit was reported [47]. Subsequently, the molecular mass of a 751 amino acid peptide derived from translation of a cDNA variant sequence of human placental DAO was calculated to be 85,423 Da [758] *pao 1* (GenBank accession number U11862). More recently, the cDNA sequence of another placental amine oxidase has been reported, the mature protein would be comprised of 744 amino acids and have a molecular mass of 82,525 daltons [1080].

TABLE 1.5. Relative Molecular Masses (M_rs) of Human Pregnancy-Associated Amine Oxidases

Nomenclature	*Source*	*Molecular Weight*	*Subunit Molecular Weight*	*Reference*
diamine oxidase	placental homogenate	n.d.[a]	100–105 kDa[*sic*][b]	Novotny *et al.*, 1994 [47]
diamine oxidase	placental homogenate	180,000[c]	90,000[d]	Houen *et al.*, 1993 [756]
diamine oxidase	pregnancy serum	180,000[c]	90,000[d]	Houen *et al.*, 1993 [756]
diamine oxidase	placental homogenate	≤440,000[e]	84,000[f]	Morel *et al.*, 1992 [755]
diamine oxidase	placental extract	n.d.	95,000[g]	Denney, *et al.*, 1988 [759]
polyamine oxidase	retroplacental serum	67,000[h]	67,000[i]	Morgan, 1985 [726]
histaminase	placental extract	n.d.	110–91,000[j]	Lin *et al.*, 1981 [752]
diamine oxidase A	pregnancy serum	245,000[k]	n.d.	Tufvesson, 1978 [678]
diamine oxidase B	pregnancy serum	485,000[k]	n.d.	Tufvesson, 1978 [678]
diamine oxidase A	amniotic fluid	245,000 (240–255,000)[l]	109,000[m] range 103–110,000	Tufvesson, 1978 [763]
diamine oxidase B	amniotic fluid	485,000 (465–495,000)[l]	112,000[m] range 105–115,000	Tufvesson, 1978 [763]
histaminase	pregnancy plasma	n.d.	major band 100,000 (80%) minor band 120,000[n]	Baylin, 1977 [748]
diamine oxidase	placental homogenate	300,000[o]; 280,000[p]	170,000[q]	Hata, 1976 [745]
histaminase	placental extract	195,000[r]	93–97,000[s]	Lin & Kirley, 1976 [747]
diamine oxidase	placental extract	235,000[t]	70,000[u]; 69,500[v]; 60,000[w]; 82,000[x]	Crabbe *et al.*, 1976 [744]
histaminase	pregnancy plasma	≥200,000[y]	90,000 minor band[m] 70,000 major band (contaminant)	Baylin & Magorlis, 1975 [713]
diamine oxidase	placental extract	n.d.	90,000[u]	Bardsley *et al.*, 1974 [712]
histaminase	placental extract	125,000 ± 5000[z] 250,000 ± 5000 375,000 ± 5000 500,000 ± 5000	n.d.	Paolucci *et al.*, 1971 [742]
histaminase	placental extract	≥200,000[y]	n.d.	Smith, 1967 [741]

Notes to Table 1.5
a. Not done
b. SDS-PAGE, not described
c. SDS-PAGE under non-reducing conditions. SDS-PAGE 7.5% [1126].
d. SDS-PAGE reducing conditions [1127]
e. Superose 12 HR 10/30 FPLC
f. 5–15% SDS-PAGE [1128], Coomassie blue staining reveals several other bands in the lane containing the most highly purified active fraction from concanavalin A-Sepharose. Isoelectric focusing focuses this activity into two peaks pH 4.9 and pH 5.5
g. Immunoblotting
h. Sephacryl S-200
i. 4–27% SDS-Page [1127] one major band and three minor bands
j. SDS-Page [1129] on a range of gel concentrations 5–15% with the highest values at the lowest gel concentrations: extrapolation [1123] gives 90,000; @7.5% 97,000
k. gel filtration
l. Sepharose 6B 2.6 x 85 cm
m. SDS-Page 7.5% [1130]
n. SDS-PAGE 7.5% [1130,1131]
o. Sephadex G-200
p. SDS-PAGE 10% [1130] non-reducing conditions
q. SDS-PAGE 10% [1130] reducing conditions
r. 4–30% SDS-PAGE
s. SDS-PAGE 7% resolved into two peaks by DEAE-cellulose peak A slow moving, peak B fast moving (slightly lower Mwt.)
t. sedimentation–equilibrium
u. SDS-PAGE [1130]
v. SDS-PAGE from replot at various concentrations [1132]
w. S-300 Sephadex
x. dissociating sedimentation–equilibrium
y. Sephadex G-200, elutes just after void volume
z. BioGel A5M

Pregnancy-associated amine oxidases have an apparent molecular weight range of 180,000 to 250,000 by SDS-PAGE analysis under non-reducing conditions (Table 1.5). The enzymes are homodimeric, consisting of two apparently identical subunits. Evidence for association of the native enzymes to higher molecular weight aggregates has been seen. The apparently low molecular weight of 67,000 observed by Morgan for the retroplacental serum PAO in gel filtration experiments is curious, and may be the result of including a reducing agent (2-mercaptoethylamine) in the chromatography buffers causing the enzyme protein to dissociate into its subunits, and thus coelute with serum albumin [726]. Serum albumin is probably the major (80%) protein seen after gradient SDS-PAGE analysis, with a M_r 67,000, the amine oxidase may have been represented in one of the three minor bands observed. Estimation of M_r by gel filtration is notoriously imprecise and probably led to the assumption of the enzyme activity being associated with the M_r 67,000 band; an amine oxidase subunit with an apparent molecular weight of around 90,000–110,000 would elute in approximately the same volume on Sephacryl S-200 media. Crabbe *et al.* first reported a M_r 90,000 for the placental enzyme [712]; their subsequent report of a M_r 70,000 subunit [744] indicates that they may have been similarly mislead. Indeed, Baylin and Magorlis report a major contaminant with an apparent M_r 70,000 in their earlier preparations of the pregnancy serum enzyme [713]. Morel *et al.* failed to produce convincing

evidence that enzyme activity was associated with the M_r 84,000 band of their enzyme preparation purified 57-*fold* from placental homogenate [755]. Furthermore, although they state that the 'molecular mass of the [native] enzyme in the major peak [from gel filtration column eluate] was about 180 kDa', their data indicate that it was actually much higher at ≤440,000 and that the minor peaks were perhaps even larger aggregates of the native enzyme! Minor variations in the reported values probably reflect the use of different concentrations of acrylamide gels to determine M_r values, and perhaps differences in the molecular weight values used for marker proteins. The pregnancy-associated amine oxidase is a glycoprotein and glycoproteins have a well-known tendency to run anomalously in SDS-PAGE (*vide supra*). Lin *et al.* observed the apparent M_r of the placental enzyme to reduce from 110,000 to 91,000 with increasing acrylamide concentration [752].

(iv) Semen. An amine oxidase isolated from human seminal plasma was demonstrated to have an apparent M_r 185,000 by ultracentrifugation [773].

c) Plant amine oxidases. All of the plant copper-containing amine oxidases examined are homodimers. Carbohydrate usually makes up more than 10% of the enzyme molecular mass. In the plant kingdom, PAOs have been found in the Gramineae (*vide infra* vii–x) [469]. Except for the barley enzyme, the PAOs that have been characterized from this group are FAD dependent monomers.

(i) Pea seedling (Pisum sativum). The pea-seedling amine oxidase was found to have an apparent M_r of 185,000 by sedimentation–equilibrium centrifugation [637]; M_r 180,000 by gel filtration chromatography [640] (although 150,000 [590] and 142,000 [646]); and M_r 85,000 (although 72,000 [590] and 75,000 [646]) by SDS-PAGE under reducing conditions, suggesting that the enzyme is a homodimer. In 1993 Vignevich *et al.* reported a preliminary crystallographic characterization of the pea-seedling amine oxidase showing that the molecular mass of the asymmetric native molecule is 131 kDa [645], consistent with independent evidence that it has two identical ~66 kDa subunits [617]. The calculated molecular mass of the pea seedling amine oxidase subunit was 73,700 Da, SDS-PAGE showed a M_r 72,500 which shifted to 69,500 on deglycosylation suggesting that carbohydrate accounts for around 4% of the mass of the mature enzyme [649]. Similarly, more recent SDS-PAGE analyses indicated a relative molecular mass of 72,000 per subunit [647]. Highly purified enzyme (98%) was examined by laser desorbtion mass spectroscopy and found to have a subunit molecular mass of 75.7 ± 1 kDa, consistent with the calculated molecular mass determined from the translated gene sequence and the enzyme's carbohydrate content [647].

(ii) Lentil seedlings (Lens). SDS-PAGE of a purified DAO revealed a single band with M_r 78,000

under reducing conditions. Sedimentation velocity experiments gave a M_r 187,000, indicating that the native enzyme was a homodimer of the subunit observed by SDS-PAGE [618]. Later studies found that the subunit had a M_r 72,000 and that the M_r of the native enzyme by gel filtration was 150,000 [590]. The mature enzyme subunit polypeptide from lentil seedling has a calculated molecular mass of 64,321 Da, and glycosylation of this polypeptide would account for the higher apparent M_r observed on SDS-PAGE [617].

(iii) Vicia faba. The relative molecular mass of the DAO from the leaves of the broad bean plant, *Vicia faba*, was estimated by gel filtration on G-200 Sephadex, and found to be about 126,000. SDS-PAGE revealed a single band of *M*r 74,000 under reducing conditions, indicating that the *Vicia* amine oxidase is homodimeric [653].

(iv) Euphorbia characias. The native copper-containing amine oxidase from the latex of the *Euphorbia characias* shrub was M_r 140,000 on gel filtration [593] and 186,000 in sedimentation velocity experiments [469]. SDS-PAGE showed a single band with M_r 72,000 under reducing conditions. So, the *Euphorbia* DAO also appears to be a dimer of identical subunits [593]. Similarly, the amine oxidases from the chick-pea, *Lathyrus sativus*, and *Cicer arietinum* were characterized as M_r 150,000 homodimers of a 72,000 molecular weight subunit [590].

(v) Tobacco (Nicotiana). The purified amine oxidase from the roots of *N. rustica* had a M_r 54,000 by SDS-PAGE [627], which is smaller than the enzyme in *N. tabacum* described by Davies [625].

(vi) Soybean seedlings (Glycine max). The molecular weight of the purified amine oxidase as determined by gel filtration and SDS-PAGE was reported as 25,000 [598]. Another [more detailed] study of the amine oxidase [600] reported a M_r of 113,000 from gel filtration experiments. Denaturing SDS-PAGE of the purified enzyme showed a single stained band with an apparent M_r of 77,000. An EPR spectrum showed that the enzyme contained 0.109% copper. The enzyme is therefore now considered to be a homodimer with one atom of copper per subunit.

(vii) Maize (Zea mays). The maize amine oxidase appears to be a M_r 53,000 monomer by gel filtration (Bio-Gel P200 or SE-HPLC) and SDS-PAGE [656]. However, an earlier study found a slightly larger relative molecular mass of 65,000, as estimated by gel filtration, sedimentation analysis and SDS-PAGE [655].

(viii) Barley (Hordeum vulgare). The M_r of the enzyme determined by gel filtration chromatography was 85,000 [584]. Another study found that the protein eluted with a M_r of 150,000 on gel filtration, while SDS-PAGE showed a single band with an apparent M_r of 75,000 [604]. The purified enzyme contains 0.082% copper and on this basis a minimum molecular mass of 77,000 was estimated (M_r(Cu).100/%(Cu)). The enzyme appears, therefore, to be a dimer of identical subunits. Neutral sugars make up 14% of the total molecular mass.

(ix) Oats (Avena sativa). The M_r of the oat amine oxidase was 85,000 by gel filtration chromatography [584]. The enzyme had a M_r of about 63,000 by SDS-PAGE under reducing conditions [586] suggesting that it is a monomer.

(x) Millet (Setaria italica). The apparent M_r of the millet amine oxidase estimated by gel filtration was 80,000 [651]. Because the molecular weight has only been determined under non-denaturing conditions, it is not known whether the enzyme is comprised of subunits.

(xi) Water hyacinth (Eichhornia crassipes). The molecular weight of the PAO from water hyacinth was 87,000 as estimated by gel filtration, while SDS-PAGE under reducing conditions revealed a single band at M_r 60,000 [594]. The results suggest that the PAO is a monomeric, non-globular protein.

d) Micro-organisms.

(i) Yeast (Hansenula polymorpha). A protein band at a M_r 78,000, was seen in the expression extract of the enzyme in *Saccharomyces cerevisiae*, but not in the control extract [567]. The molecular mass of the protein corresponds to the expected molecular mass of the amine oxidase calculated from the protein sequence (77,533 Da) [566]. In crude extracts, the recombinant amine oxidase behaves as a homodimer [569].

(ii) Aspergillus niger. The M_r obtained by ultracentrifugal analysis has been reported as 252,000 [558]. Dissociation with guanidium chloride and 2-mercaptoethanol yielded subunits with a M_r 85,000 [552]. A later study found a M_r 255,000 for the native enzyme by gel filtration, a subunit M_r of 125,000 by SDS-PAGE under reducing conditions, and a M_r 137,000 by denaturing sedimentation studies, in contradiction to earlier results. The more recent results indicate that the native enzyme has a dimeric structure, consisting of identical M_r 125,000 subunits. Electron microscopy and carboxy-terminal analysis of the amine oxidase support this view [556].

(iii) Aspergillus terreus. The molecular weight of the PAO was determined to be about 130,000 by gel filtration. SDS-PAGE revealed a single band with a M_r 65,000 [560] indicating a homodimeric structure.

(iv) Penicillium chrysogenum. An 'agmatine oxidase' from *Penicillium chrysogenum*, considered to be in the same class as the copper-containing DAOs, was found to have a molecular weight of 160,000 by gel filtration, although a polymer of the enzyme was observed at a M_r 320,000. SDS-PAGE under reducing conditions revealed a single subunit M_r of 80,000 [573] indicating a homodimeric structure. Additionally a 'polyamine oxidase' from this mould showed a molecular weight of 160,000 by gel filtration and ultracentrifugal analysis. This enzyme was also considered to be a homodimer since SDS-PAGE under reducing conditions gave a M_r of 80,000 [574]. The two enzyme activities may have an identical phenotype.

(v) Candida boidinii. A 'polyamine oxidase' from the yeast, *Candida boidinii,* showed variations in molecular weight, dependent on the method used for its determination. Gel filtration indicated a molecular weight of 80,000 to 90,000 while a sucrose density gradient method gave a M_r of 111,000. Ferguson analysis by non-denaturing PAGE indicated a M_r of 200,000 for the native enzyme [563].

A 'benzylamine oxidase' showed a molecular weight of 288,000 by gel filtration, 136,000 by non-denaturing PAGE and a M_r of 79,000 by SDS-PAGE under reducing conditions suggesting that the enzyme is homodimeric in its native form [561].

A 'methylamine oxidase' was indicated to have M_r values of 150,000 and 510,000 by Ferguson analysis in nondenaturing PAGE and 257,000–286,000 by gel filtration. SDS-PAGE under reducing conditions revealed a M_r 81,000 subunit. The native enzyme is therefore most likely to be a disulfide bond linked homodimer of the 81,000 subunit, and form dimeric and tetrameric aggregates [561].

(vi) Arthrobacter. Methylamine oxidase from the gram-positive facultative methyltroph, *Arthrobacter,* is a M_r 170,000 homodimer. Molecular masses of 72,728 and 72,805 Da deduced by sequence analysis of two slightly different forms of the subunit [516] were consistent a molecular weight estimate of 80,000–82,000 for the subunit by SDS-PAGE [513]. The molecular mass of 70,644 Da per subunit, calculated from the amino-acid sequence of *A. globoformis*, is consistent with preliminary crystallographic data [519].

(vii) Klebsiella aerogenes. A 'monoamine oxidase' from the gram negative bacterium, *Klebsiella aerogenes,* was suggested to have a subunit M_r 80,000 by SDS-PAGE under reducing conditions. The molecular mass calculated from its amino acid sequence is 80,647 Da [1092].

(viii) Escherichia coli. The subunit molecular mass of 'tyramine oxidase' from *Escherichia coli,* calculated from the amino acid sequence, is 84,378 Da [529], identified as a M_r 80,000 band by SDS-PAGE under reducing conditions [526]. The native enzyme exists as a homodimer of the subunit [529].

(xi) Micrococcus rubens. Putrescine oxidase from *Micrococcus rubens* was characterized by ultracentrifugal studies to have a M_r 80,000 [535], and later as 90,000–82,000 based on a number of methods [536]. SDS-PAGE revealed a single band with an apparent M_r 46,000 [536] suggesting the enzyme is a homodimer. Okada *et al.* found two bands with M_rs 43,000 and 51,000, but could not explain the discrepancy [537]. The molecular mass of the subunit calculated from its amino acid sequence is 51,914 Da, suggesting that the M_r 43,000 peptide may have been an impurity [538].

1.3.4.3 Glycosylation

All amine oxidases that have been examined for carbohydrate have been demonstrated to be glycosylated [450], with the possible exception of the PAO from the water hyacinth, *Eichhornia crassipes* [594]. Glycosylation is a post-translational modification of proteins in which one or more carbohydrate chains are covalently linked to the polypeptide [1133,1134]. Glycoproteins occur in cells, both in soluble and membrane-bound forms, as well as in the extracellular matrix and in extracellular fluids. Glycosylation occurs without exception in integral membrane proteins and is quite common in secretory proteins. Almost all blood serum proteins are glycosylated [1133], it appears to affect the half-lives of serum proteins and is known to be involved in protein transport [1135].

Bovine plasma enzyme has been demonstrated to contain approximately 4–8% carbohydrate [451,827]. Fragments of pronase digestion of the enzyme were separated, and the major fragment was shown to contain asparagine, which binds carbohydrate to protein through an amide linkage to *N*-acetyl-D-glucosamine [827]. The carbohydrate content of the enzyme was also indicated by the enzyme's affinity to concanavalin A [833]. The enzyme–lectin interaction was used to show that the carbohydrate is not essential for activity and that the carbohydrate is covalently attached to the protein in a region distant to the active site. Yasunoubu *et al.* suggested that the observation of multiple enzyme forms may be a result of differences carbohydrate content [451].

The pig plasma enzyme has been reported to contain 9.8% carbohydrate [649]. The glycoprotein nature of the enzyme has been established and the enzyme was found to interact with concanavalin A. The heterogeneity of the pig plasma amine oxidase was suggested to be due to variable carbohydrate content [931]. The pig kidney enzyme was also reported to contain carbohydrate [907]. Deglycosylation by treatment with neuraminidase and endoglycosidase F or with neuraminidase and glycopeptidase F indicated that glycosylation accounts for at least 5% of the pig kidney enzyme mass [909].

In cells transfected with the human kidney cDNA for amiloride-sensitive copper-containing amine oxidase the extent of protein glycosylation was investigated by treating cells with tunicamycin, an inhibitor of the *N*-glycosylation process. The treatment led to the disappearance of a diffuse M_r 97,000 band and its replacement by a sharper M_r 70,000 band [781].

Glycosylation of human pregnancy-associated amine oxidases has been previously suggested by several pieces of evidence, including: the affinity of the human placental DAO for concanavalin A-Sepharose and that it can be released with α-methylglucoside [744]; the presence of isoenzymatic forms of very similar molecular weight as suggested by rocket immunoelectrophoresis [747] and ion exchange chromatography [747,763]; the appearance of the

purified enzyme as a diffuse band after SDS-PAGE [747,748,752,763]; and the observation of multiple components after isoelectric focusing of purified placental enzyme [752]. The possibility that placental histaminase contains carbohydrates was also suggested by the observation that the apparent molecular weight decreases with higher acrylamide concentration in SDS-PAGE [752]. This is typically seen in glycoproteins containing more than 10% carbohydrate [1123]. More recent evidence for glycosylation of the pregnancy-associated amine oxidases includes: the finding of potential *N*-glycosylation sites in the amino-acid sequence of the human placental enzyme [47] (*qq.v.* §6.2.9.2 and 7.2.8.3); and, by analogy, the results of the enzymatic deglycosylation of human kidney ABP, and tunicamycin treatment of cDNA expression systems [47,781,909].

The pea-seedling enzyme contains around 3–4% carbohydrate as judged by deglycosylation experiments [649], although previous determinations had found 13% neutral sugars. The large difference in the level of glycosylation of the pea seedling amine oxidase compared with the pig plasma amine oxidase (9.8%) probably accounts to some extent for differential success in crystallization studies. The pea seedling enzyme has been crystallized and its structure determined at 2.2 Å resolution [645,650]. This is in contrast to pig plasma amine oxidase where crystallization studies have resulted in only very small crystals of poor (X-ray crystallographic) quality. No significant improvement in crystal quality could be achieved even when native pig plasma amine oxidase was treated with a combination of glycopeptidase F and endoglycosidase. Such treatment produced a simpler but still heterogeneous population of species as judged by isoelectric focusing, presumably due to the inability of *N*-glycosidase to gain access to all *N*-linked carbohydrate sites in the native enzyme [649]. X-ray crystallography has revealed that all four potential glycosylation sites, $Asn^{131,334,364,\text{ and }558}$, are located on the surface of the pea-seedling amine oxidase molecule. There is evidence for sugars at all but the Asn^{364} site on electron-density maps [650].

Lentil-seedling amine oxidase has about 3% carbohydrate [617], though the content was previously thought to be 14% [618]. There are three putative glycosylation sites in its primary amino acid sequence, only two of which bear a carbohydrate chain. Inspection of a hydropathy plot of the amino-acid sequence suggests that Asn^{234} and Asn^{364} may be *N*-glycosylated, but not Asn^{130} since it sits in a hydrophobic environment [617] (*q.v.* §7.2.8.3).

The amine oxidases of other plants are also glycoproteins. The amine oxidase found in *Euphorbia characias* latex contains 12% carbohydrate as neutral sugars [593]. A copper-containing amine oxidase from *Hordeum vulgare* seedlings was found to contain 14% neutral sugar. FAD dependent PAO from *Zea mays* seedlings was found to contain 3% neutral sugars [655] (2.5% [656]). The *Glycine max* seeding enzyme is a glycoprotein since it binds strongly to

concanavalin A-Sepharose and is eluted with α-methylglucoside [600]. Isoelectric focusing experiments revealed a heterogeneity suggested to be a consequence of the carbohydrate moiety. The isoelectric heterogeneity of amine oxidases from the Leguminosae, *Lathyrus sativus*, *Cicer arietinum*, *Pisum sativum*, *L. culinaris* [615] and *Lens esculenta*, suggests the heterogeneity of carbohydrate chain substituents, as suggested for pig plasma amine oxidase by Falk *et al.* [931]. The DAO from *Vicia faba* leaves showed a diffuse band of enzyme activity after PAGE [653], typical of glycoproteins [1123]. However, carbohydrate was *not* detected in the PAO from water hyacinth [594] by the phenol–H_2SO_4 neutral sugars procedure of Dubois *et al.* [1136].

Glycosylation does not appear to have been examined in any of the amine oxidizing enzymes from micro-organisms, although for those enzymes for which the amino acid sequence is known, *E. coli* K12 [528], *Klebsiella aerogenes* [1092], *Arthrobacter* strain P1 [516] and *Arthrobacter globoformis* [518], potential *N*-glycosylation sites exist.

1.3.4.4 Isoelectric Forms

The isoelectric points reported for various amine oxidases are presented in Table 1.6. Evidence for differently charged forms has been reported for some amine oxidizing enzymes. Large isoelectric differences may be a consequence of enzyme aggregation and isoelectric microheterogeneity a reflection of glycosylation differences.

Yasunobu *et al.* have reported multiple forms of the bovine plasma amine oxidase [451,493]. Three major forms were consistently observed and could be resolved by DEAE-ion exchange and hydroxyapatite chromatography. A rabbit antiserum prepared against one form reacted with all forms of the enzyme. Isoelectric focusing showed the three forms had isoelectric points around 4.8 [451]. Mondovì's group demonstrated heterogeneity in the isoelectric focusing pattern. Preparative isoelectric focusing separated three main isoforms, each of which had the same *N*-terminal amino acid sequence for a stretch of 17 residues. They concluded that the molecular heterogeneity was due to differences in carbohydrate composition [845]. Similar conclusions were made about the pig-plasma amine oxidase [931], which was resolved into multiple forms by ion-exchange and hydroxyapatite chromatography [493]. Falk *et al.* found that each preparation of the pig-plasma amine oxidase could be resolved into at least two, and sometimes three, distinct peaks of activity on DEAE-cellulose and that the relative amounts of material in these peaks differed from batch to batch [931]. On SDS-PAGE all fractions migrated as a single broad band with a molecular weight of 95,000, as visualized by both protein and periodic acid carbohydrate staining. The copper content, amino acid composition, and Michaelis constants of the fractions, using benzylamine as the substrate, were not significantly different.

TABLE 1.6. Isoelectric Forms of Amine Oxidases

Source	*Enzyme*	*pI(s)*	*Description*	*Method*	*Reference*
human kidney	diamine oxidase	6.0	single peak	preparative chromatofocusing	[753,780]
human placenta	diamine oxidase	5.4 ± 0.2	single peak	preparative	[753]
human placenta	diamine oxidase	6.5	single peak	preparative	[744]
human placenta	histaminase	5.3 – 6.6	heterogeneous	analytical	[752]
human placenta	diamine oxidase	4.9, 5.5	two peaks	preparative	[755]
human amniotic fluid	diamine oxidase	4.0, 5.8	isolated forms	preparative	[763]
porcine kidney	diamine oxidase	6.0 ± 0.1	single peak	preparative	[753]
porcine serum	diamine oxidase	4.5 – 5.0	heterogeneous	analytical	[931]
bovine kidney	diamine oxidase	5.6	single peak	preparative	[753]
bovine serum	diamine oxidase	4.8	heterogeneous	preparative	[845]
rat liver	polyamine oxidase	4.9	single peak	preparative	[164]
Penicillium chrysogenum	polyamine oxidase	5.5	single peak	preparative	[574]
	agmatine oxidase	5.7	single peak	preparative	[573]
Pisum sativum					
epicotyls	diamine oxidase	7.4	single peak	preparative	[640]
seedlings	amine oxidase	7.3	one band	analytical	[647]
seedlings	diamine oxidase	7.2, 6.8, 6.5	heterogeneous	analytical	[590]
seedlings		7.35 ± 0.05		chromatofocusing and isoelectric focusing	[646]
Hordeum vulgare seedlings	amine oxidase	6.8	single peak	preparative	[604]
Lens esculenta seedlings	diamine oxidase	7.4	single band	analytical	[618]
		***	at least 6–7 bands	analytical	[615]
		6.1, 5.9, 5.5	heterogeneous	analytical	[590]
Lathyrus cicera	diamine oxidase	6.0, 5.8, 5.1	heterogenous	analytical	[590]
Cicer arietinum	diamine oxidase	6.0, 5.7, 5.2	heterogenous	analytical	[590]
Glycine max seedlings	diamine oxidase	7.10, 7.05, 7.00, 6.90	3 major, 1 minor bands	analytical	[600]
Vicia faba leaves	diamine oxidase	7.2	single peak	preparative	[653]
Hansenula polymorpha	diamine oxidase	5.55 – 5.67		analytical	[567]

However, there were significant differences in the carbohydrate content of the different forms. The chromatographically resolved multiple forms all yielded complex isoelectric focusing patterns. The p*I* of the isoelectrically focused bands ranged from 4.5 to 5.0, with each form showing a distinct pattern. These investigators suggested that the heterogeneity revealed by isoelectric focusing may be due to variable carbohydrate content of the enzyme.

Libby and Porter found that PAO from L1210 murine leukemia cells could be separated into two forms on hydroxyapatite [1023]. The two forms were found to have different molecular weights by Sephacryl S-300 chromatography and were proposed to be isozymes. The two isozymes showed slightly different kinetic characteristics with spermine, spermidine, N^1-acetylspermine, and N^1,N^{12}-diacetylspermine. They proposed that the enzymes are similar to the rat liver enzyme described by Hölttä [164], and that the isozymes were, by analogy, due to

cytosolic [165] and peroxisomal [164] forms.

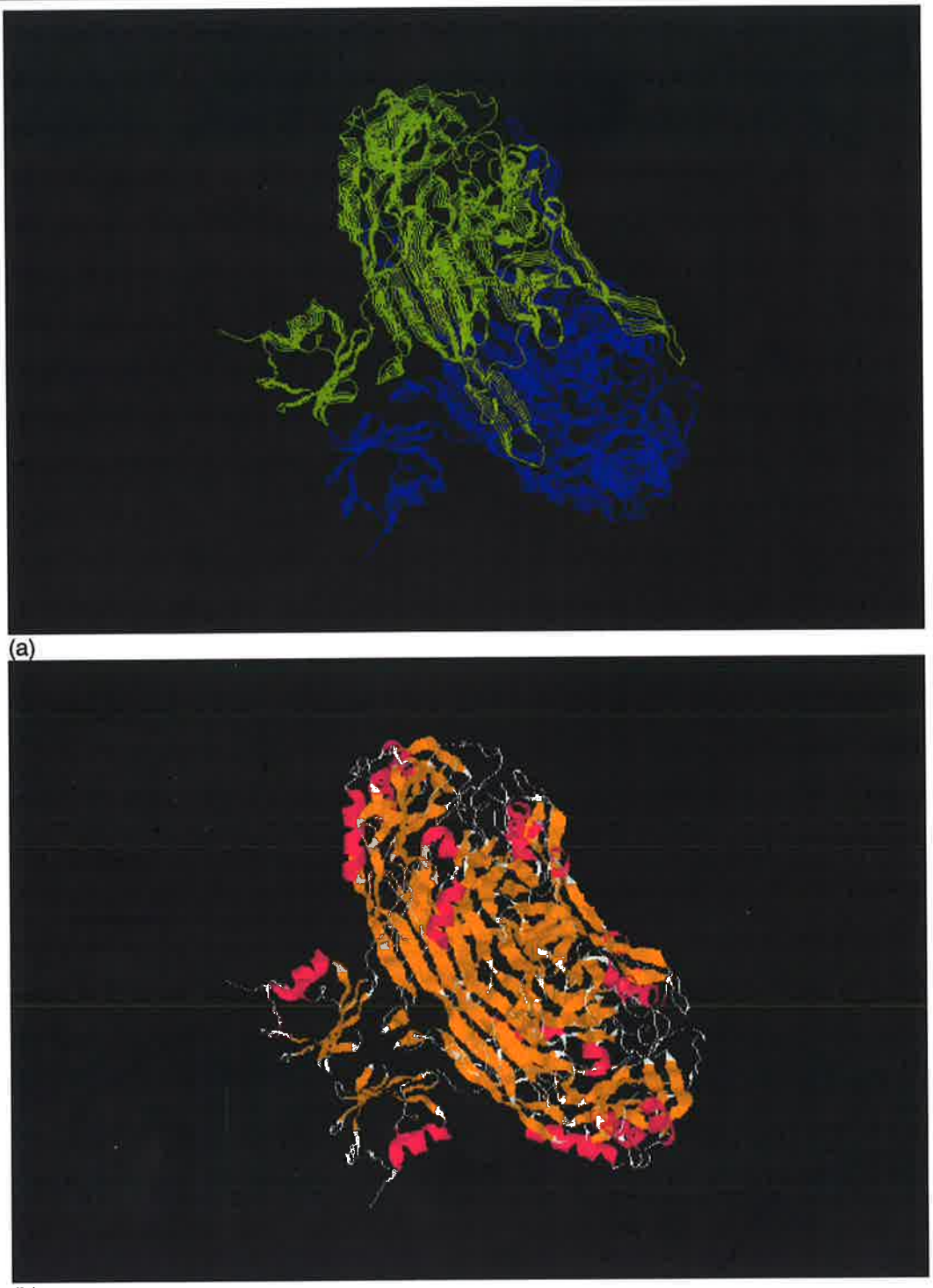
Four different human serum (mono)amine oxidase isoenzymes have been separated using chromatographic and electrophoretic techniques [1137-1140].

The human pregnancy-associated amine oxidases have also been observed as multiple enzymic forms. The placental enzyme has been resolved into two forms by DEAE-anion exchange chromatography [747,763] and by rocket immunoelectrophoresis [747]. The two forms of the amniotic fluid enzyme [763] were reported to have p*I* values of 5.8 and 4.0 and native molecular weights of 245,000 and 485,000 by gel filtration on Sepharose 6B. The two forms displayed similar substrate specificity and inhibitor sensitivity. SDS-PAGE indicated very similar subunit molecular weights (109,000 and 112,000), like those seen for the placental enzyme, which was considered to consist of two, possibly identical, subunits with M_r 95,000 [747]. Preparative isoelectric focusing of the human placental DAO resolved the enzyme activity into two peaks with p*I*s 4.9 and 5.5 [755]. Morgan observed the resolution of retroplacental serum amine oxidase into two forms after chromatography on Cibacron Blue Sepharose and after preparative electrophoresis of the native enzyme. Both chromatographic fractions had the same substrate specificity when the relative rates of spermine, spermidine and putrescine oxidation were examined, although inhibitor sensitivities appeared to be slightly different [442]. Amicosante *et al.* reported the p*I* for the placental enzyme to be 5.4 [753]. Crabbe *et al.* found that the active human placental enzyme focused at pH 6.5 in preparative experiments [744]. After isoelectric focusing of human placental 'histaminase' in polyacrylamide gels, Lin *et al.* demonstrated at least five major bands between pH 5.3 and 6.6 where most of the enzyme focused [752].

1.3.4.5 Molecular Structure of Copper-Containing Amine Oxidase

Mondovì's group have analysed the thermal unfolding of bovine serum amine oxidase by differential scanning calorimetry which revealed a homodimeric protein with a four domain structure. Each subunit consisted of two domains, comprising 60% and 40% of the polypeptide respectively. The calorimetric data for the bovine serum enzyme is supported by data from its primary structure suggesting that the morphology for DAO is similar to numerous other enzymes characterized by substrate binding sites at the interface of two domains. The structure of human DAO is consistent with this model since the active site peptide residues 453–477 [411] are flanked with about 60% of the polypeptide from the amino terminus and 40% from the carboxyl terminus. A better idea of mammalian enzyme tertiary structure awaits enzyme crystallization and the availability of atomic coordinates from X-ray diffraction studies.

A copper-containing amine oxidase from *Escherichia coli* has been crystallized and its structure determined to 2Å resolution [529]. Its coordinate data file was deposited at the



(a)

(b)

Figure 1.13. Three-Dimensional Structure of *E. coli* Copper Amine Oxidase.

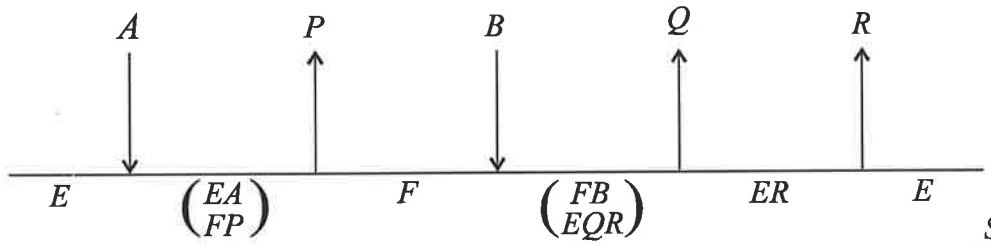
(a) The *E. coli* copper amine oxidase viewed perpendicular to the molecular dyad axis. The two subunit chains are shown as green and blue strands. The two β-strand 'arms' which extend from the green subunit along the surface of the blue subunit are clearly seen. (b) The amine oxidase is shown as 'ribbons' coloured according to a scheme which displays the protein secondary structure. α-helices are coloured magenta, β-sheets are yellow (both 1.52 Å), turns are pale blue and other residues are white (both 0.4 Å). The secondary structure was assigned from the PDB file; the Figure was generated using RasMol version 2.6 for Microsoft Windows, © 1996 R. Sayle, Biomolecular Structures Group, Glaxo–Wellcome Research and Development, Stevenage, Hertfordshire, UK obtained by anonymous FTP from ftp://ftp.dcs.ed.ac.uk /pub/rasmol/v2.6beta/. Coordinates (entry 1oac, version of February 1996) used for the amine oxidase [529] structure were obtained from the Protein Data Bank [1141,1142] at the Brookhaven National Laboratory, NY by gopher://pdb.pdb.bnl.gov:70/00/ FTP/fullrelease/uncompressed_files/oa/ pdb1oac.ent

Brookhaven Protein Data Bank [1141,1142], identification code 1OAC, and can be found on the Internet at <gopher://pdb.pdb.bnl.gov:70/11/PDB /Entries/.1oac>. The *E. coli* amine oxidase homodimer is mushroom shaped (*vide* frontispiece), with the 'stalk' comprising the first 85 amino acids of each polypeptide chain and the 'cap' the remaining 640. The 'cap' of the amine oxidase is roughly rectangular, with dimensions 60 Å × 100 Å, and 40 Å thick parallel to the molecular dyad axis. The bulk of the molecule, comprising the 440 amino acid C-terminal, folds into an extensive β sandwich, which contains the active site and mediates intersubunit interactions. At the periphery of the molecule, each subunit has a pair of highly conserved domains (D2 and D3) comprising residues 100–185 and 186–285 respectively (Figure 1.13).

More recently, the first molecular structure of a eukaryotic amine oxidase (from pea seeding) has been solved and refined at 2.2 Å resolution [650]. The atomic coordinates have been deposited in the Brookhaven Protein Data Bank, identification code 1KSI, tracking number T9156. The pea seedling amine oxidase is a dimer of two crystallographically independent but chemically identical subunits. The molecular dimensions are approximately 100 × 63 × 42 $Å^3$. Each subunit consists of three domains. These domains are similar to three of the domains in the structure of the *E. coli* amine oxidase. The *N*-terminal 'stalk' domain of the *E. coli* amine oxidase is missing from the pea seedling amine oxidase gene: so the two subunits of the pea seedling amine oxidase form the cap of the mushroom without a stalk. Nevertheless, there is considerable structural similarity between the two proteins.

1.3.5 Oxidation Mechanism

Chemical and kinetic data indicate a double displacement or 'Ping Pong Bi Ter' enzyme substitution mechanism for many amine oxidases (reviewed in [1,451,464,1086]), with the sequence:



Scheme 1.2

where *A* represents the amine substrate, *B* the second substrate, oxygen, *P* the aminoaldehyde product, *Q* H_2O_2 and *R* H_2NR^2 (cf. Scheme 1.1). *E* represents the 'free' enzyme and *F* is enzyme involved in complex formation. (see reference [1143] for Cleland nomenclature.)

Many of the transient and steady-state kinetics studies have been done with the bovine [824,847,851] and porcine plasma [916,917,919,920,924-927,930] amine oxidases. However,

steady-state kinetics studies with porcine kidney amine oxidase [875,876], pea-seedling amine oxidase [638], and human pregnancy-associated enzymes [712,716,763] indicate that these enzymes also obey a Ping Pong Bi Ter mechanism. Bardsely *et al.* have done extensive kinetic studies on the placental amine oxidase [712,716] and found steady-state kinetics consistent with the reaction scheme found by Tufvesson for the amniotic fluid enzyme [763]. Tufvesson showed that, with diamines, the enzyme had parallel Lineweaver–Burk plots for different oxygen concentrations, consistent with a Ping Pong mechanism. Anaeorbic incubation of the enzyme allowed the aminoaldehyde, but no H_2O_2, to be formed. This indicates that the enzyme first reacts with the diamine producing aminoaldehyde. Oxygen then reacts with the enzyme and H_2O_2 and NH_3 are formed. Ammonia was found to be a competitive inhibitor to putrescine indicating that NH_3 is the last product [1143-1145]. The observation that H_2O_2 is an uncompetitive inhibitor to putrescine is consistent with such a reaction scheme [1143-1145].

Although there may be some mechanistic variability, especially with regard to stereospecificity, the copper amine oxidases follow the general scheme:

$$E_{ox} + R'CH_2NHR'' \rightarrow E_{ox}\cdot R'CH{-}NHR'' \rightarrow E_{red}{-}N^+R''{=}CHR' \rightarrow E_{red}{-}NH\,R'' + R'CHO \qquad (1)$$

$$E_{red}{-}NHR'' + O_2 \rightarrow E_{ox} + H_2O_2 + R''NH_2 \qquad (2)$$

involving enzyme reduction by the substrate (equation 1), followed by enzyme reoxidation by molecular oxygen (equation 2). (Reviewed in [457,466,467].)

The substrate (*A*) recognises the active site of the free enzyme (*E*) and interacts with it by electrostatic and van der Waals forces (*EA*, Scheme 1.2). During the reductive half reaction (1), the enzyme reacts with substrate which binds covalently to the TPQ $C_{(5)}$ through the formation of a Schiff base complex (*FP*) [920,925]. An aminotransferase mechanism involving proton abstraction from an imine intermediate releases the product aldehyde (*P*) to leave an aminoquinol form of the enzyme (*F*). Following aldehyde release, the reduced cofactor is reoxidized by molecular oxygen (*B*) (in sequential one-electron steps) via a Cu^I–semiquinone radical intermediate [466,1102] with the release of H_2O_2 (*Q*), and TPQ is regenerated by hydrolysis with the release of R^2NH_2 (*R*) [1146].

Unlike the FAD-containing oxidases, where the pro-*R* proton is always abstracted, the stereochemistry of proton abstraction catalysed by copper-containing amine oxidases may or may not be stereospecific. With simple amines such as benzylamine the pro-*S* hydrogen is always removed, while with larger substrates, e.g. 2-phenylethylamine, proton abstraction at $C_{(1)}$ can be pro-*S*, pro-*R*, or non-stereospecific, depending on the the enzyme source [599,1147]. This property, together with variation in the solvent exchange at the $C_{(2)}$ position of substrates and characteristic substrate preferences between enzymes, infers subtle variations in their active sites.

1.3.6 Substrate Specificity

Because of the vast literature dealing with PAOs from a large number of animal species, and when within a single species, the many tissues and organs examined, it is a daunting task to draw an integral and comprehensive picture of their substrate specificity. The data is further complicated by the variation in assay conditions and techniques employed, making it difficult to come to definitive conclusions regarding substrate specificity of a particular enzyme. Nevertheless, the substrates of amine oxidases have four common features:

(a) an $C_{(1)}$-methylene residue,
(b) the amino group,
(c) hydrophobic residues consisting of aliphatic, aromatic or arylalkyl moieties,
(d) moieties attached to the amino group, side chain, or aromatic system.

The $C_{(1)}$-methylene residue appears to be the reactive centre of the substrate. The other three substrate components are involved in the binding and positioning of the substrate molecule in the active site. The amino group binds the substrate to the active site, positioning of the substrate depends on (c) and (d). Thus, it is not surprising that a considerable overlap occurs in substrate specificity. It may be possible to define a given amine oxidase if a number of pertinent amines are tested and the pattern of enzyme activity is compared with that of known amine oxidases. However, direct comparison of results is exceedingly difficult because of the many different assay methods and experimental conditions used in different studies.

Ad hoc naming of amine oxidases has often arisen because the substrate incidentally used for activity determinations has provided what might be termed 'epithetical nomenclature', e.g. histaminase, methylamine oxidase, spermine oxidase, spermidine oxidase, benzylamine oxidase, serotonin oxidase, putrescine oxidase. Perhaps this would have been acceptable if the old paradigm of one enzyme acting on one substrate was still valid. This old paradigm was derived from Fischer's famous *'schloss und schlüssel'* [lock and key] model for enzyme–substrate interactions [1148,1149] and seems slow to fade. In 1959, Koshland introduced the concept of induced fit of the substrate at the active site to explain multisubstrate specificities which had been observed for various enzymes [1150,1151]. Observed multisubstrate specificity is reflected in nomenclature such as 'monoamine oxidase', 'diamine oxidase' and 'polyamine oxidase'. However, amine oxidases have been found to have an even broader substrate specificity encompassing all of these catagories. A proper understanding of the amine oxidases and their inter-relationships has often been obscured by inadequate nomenclature.

The FAD-containing monoamine oxidases prefer primary aromatic substrates, but will also act on primary aliphatic, secondary and tertiary amines [497]. Plasma amine oxidases may also oxidase monoamines, purtescine and polyamines. Although, some (plasma) DAOs have little or

no activity towards monoamines such as tyramine and tryptamine. PAOs, oxidizing spermine, spermidine, and their acetylated derivatives, may or may not be FAD-containing, and may also oxidize diamines and monoamines [467]. It has been reported that some PAOs, namely those of the rat liver or cytoplasmic type, have a much higher activity with acetylated polyamines than the free polyamines spermine and spermidine [164,165,409,410,725,726,974]. Since at least some of these PAOs also have high activity with putrescine, a diamine, the amine oxidase nomenclature has become very confusing (*vide* §1.3.1). Benzylamine and phenylethylamine are metabolised almost universally, while tyramine is metabolized by most tissue bound amine oxidases and methylamine and allylamine appear to be substrates in plasma [352,502]. Since benzylamine is not thought to occur endogenously, it is unfortunate that the term 'benzylamine oxidase' (*vide* Lewinsohn [1152,1153]) has been used to describe tissue bound amine oxidase activity (*vide* [430] *Rule 6*), and this has lead to some confusion in the literature [503].

Historically, the human pregnancy-associated enzymes have been shown to be active with histamine and diamines, such as putrescine, it is now accepted that the two activities are a result of the same enzyme (*vide* §1.3.1). Subsequent studies have suggested a broad substrate specificity for the human pregnancy-associated amine oxidases [712,738], viz. acetyl-polyamines [717,725,881], histamine [686], putrescine [752,755], spermine [420,442,722,724,726,760] and spermidine [442,719,726]. It is difficult to compare the results of different authors since in many studies only a single substrate activity was examined or only a couple of substrates were used. In amniotic fluid the two enzyme forms were most active with putrescine and histamine, which both caused substrate inhibition. The enzyme showed little activity against benzylamine and butylamine and no activity with tyramine under the conditions used [763]. Similarly, benzylamine activity was not shown for the placental enzyme purified by Bardsley *et al.* [712], that purified from pregnancy plasma [713], or from retroplacental serum [1153]. However, benzylamine activity with amniotic fluid enzymes was found by Smith [741] and Lewinsohn [816,1153].

Some studies of amine oxidase activity have used synthetic substrates [744,873,875,1154], however their use may produce misleading results if the kinetic mechanism followed differs from that obeyed when a natural substrate is used. The use of synthetic substrates in direct assay methods may result in special difficulties such as substrate inhibition [1154]. Studies with synthetic substrates are also limited in that they do not provide quantitative information about natural substrates.

Because even minor alterations in any of the assay parameters, such as temperature, pH, buffer strength and composition, can have profound affects on measured catalytic activity, the direct comparison of absolute steady-state kinetics values between laboratories is difficult. The use of different assay conditions reported by different investigators may be a result of

TABLE 1.7. Kinetics Studies with Pregnancy-Associated Amine Oxidases[a]

spermine	*spermidine*	*putrescine*	*benzylamine*	*histamine*	*N^1-acetyl spermine*	*N^1-acetyl spermidine*	*cadaverine*	*Reference*
		8 μM						Novotny *et al.*, 1994 [47]
		+++						Morel, 1992 [755]
1 (K_m 0.3 mM)	4	20			10	10		Morgan, 1985 [726]
1	3	25						Morgan, 1983 [442]
	10.9 μM	2.5 μM						Gahl, 1982 [719]
	1 (pH 7)	24 (pH 7)						
	4 (pH 11)	1 (pH 11)						
1.0	2.0	100					106	Matsumoto *et al.*, 1981 [1155]
1.02	1.00	1.12	0.46	0.58		1.57	0.88	Gahl, 1981 [717]
		100 (4.0 μM)	8	30 (2.4 μM)				'DAO A' Tufvesson, 1978 [678]
		100 (3.6 μM)	6	30 (2.8 μM)				'DAO B' Tufvesson, 1978 [678]
		100 (3.4 μM)	10	28 (2.5 μM)				'DAO A' Tufvesson, 1978 [763]
		100 (3.4 μM)	9	29 (2.5 μM)				'DAO B' Tufvesson, 1978 [763]
		2.0×10^{-5}M		2.8 μM				Baylin, 1977 [748]
		+++						Lin & Kirley, 1976 [746]
				+++				Lin & Kirley, 1976 [747]
		+++	–	+++				Baylin & Margolis, 1975 [713]
50	140	100	67	49			84	PMAO; Bardsley *et al.*, 1976 [744]
32	0	100	0	30			147	Placental DAO [744]
0	106.5	100	0	45			129	pregnancy plasma DAO [744]
	270	140		50				Bardsley & Crabbe, 1973 [743]
6		100 (3.3 μM)	0	85 (6 μM)			69 (3.3 μM)	Paolucci *et al.*, 1971 [742]
		4.0 μM						Tufvesson & Tryding, 1969 [664]
		54	5	23			100	Smith, 1967 [741]
		++		+			+++	Kapeller-Adler, 1965 [738]
			+++					McEwen & Cohen, 1963 [659]

Notes to Table 1.7

a. K_m values are indicated; unitless numbers indicate relative rates of oxidation; +,++,+++ indicate relative rates of oxidation that were not quantitiated

improvements in the technology, made available as time has progressed, or perhaps a reflection of different resources available between laboratories. Different strategies and philosophies also contribute to the differences. Although it is difficult to compare the results of substrate specificities reported for pregnancy-associated amine oxidases because of the wide variety of assay conditions reported in the literature, the results of some previous studies are shown in Table 1.7.

1.3.7 Inhibition of Amine Oxidase Activity

Compounds inhibiting amine oxidase activity can be classified into different groups: substrate analogues, 'suicide substrates', time-dependent inactivators (including the carbonyl group reagents), copper chelating agents, active site analogues, and substrates at high concentrations.

Substrate analogue inhibitors are numerous and include: sulphonium and trimethylammonium compounds, which act as competitive inhibitors, and noncompetitive bis-onium compounds [732,777,871,872,874,876].

Enzyme-activated irreversible inhibitors, or 'suicide substrates', produce a time-dependent inhibition because they must be altered by enzyme catalysis after which they react covalently with the enzyme to produce inactivation. They include aminoacetonitrile, propargylamine, allylamine, *cis*-imidazolylallylamine, 1,4-diamino-2-butyne, 1,4-diaminobutyne-2,β-bromoethylamine and glycine phenyl esters [817,818,1156-1159]. These compounds have mainly used to examine enzyme mechanisms.

Time-dependent inactivators can be considered as irreversible inhibitors because they form a covalent bond with an essential group of the enzyme. They include the carbonyl group reagents, phenylhydrazine, semicarbazide, hydroxylamine, aminoguanidine, hydrazine and hydroxylamine [630,632,731,777,868,869,1160]. A collection of these types of compounds was summarized by Buffoni in 1966 [449]. Methylglyoxal bis(guanylhydrazone) (MGBG) and its analogues also fit into the category of carbonyl reagents [170]; as does the lathyrogen, β-aminoproprionitrile, which is known to inhibit lysyl oxidase, but also inhibits pregnancy-associated amine oxidases [751]. Copper-containing amine oxidases have TPQ at their active site as a prosthetic group. Carbonyl reagents react with this active site prosthetic group preventing binding of substrates. However, carbonyl reagents may also react with pyridoxal phosphate and PQQ, which has led to some confusion as to the true nature of the active site of amine oxidases [467] (*q.v.* §1.3.4.1a).

Monoamine oxidase and DAO can be divided into semicarbazide-sensitive and -insensitive enzymes [501] (*vide* §1.3.1). This distinction has formed the basis of the semicarbazide-sensitive amine oxidase nomenclature [502,503]. The broad term 'carbonyl reagent' sensitive amine oxidase should not be used, since some carbonyl group reagents also inhibit the classical FAD-containing monoamine oxidases. Hydrazine derivatives, monomethylhydrazine, phenyl-

ethylhydrazine, phenylisopropylhydrazine and α-methylbenzylhydrazine, which have been used for the inhibition of monoamine oxidase and for the separation of its activity from other enzymes, also strongly inhibit DAO, though by a different mechanism. Similarly, phenylhydrazine inhibits monoamine oxidase in addition to the copper-containing amine oxidases [1161]. Nevertheless, phenylhydrazine has been very useful in the study of reactive TPQ and may be used for its titration by monitoring loss of activity or increase in absorption at 450 nm [848,918,923,928,932]. Interpretation of any experiment using these types of inhibitors requires careful consideration as more than one enzyme may be involved. More selective, and potent inhibitors such as procarbazine (*N*-isopropyl-α-(2-methyl hydrazino)-*p*-toluamide hydrochloride or PCZ) [503,672] and the MDL compounds (*vide infra*) hold promise for further studies.

Seiler and his colleagues at Marrion Merril Dow synthesized substituted putrescine analogues that are assumed to be enzyme-activated irreversible inhibitors of PAO [179,968,1162]. The 2,3-butadienyl derivatives; N^1-methyl-N^4-(2,3-butadienyl)-1,4-butanediamine (MDL 72521) and N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527), were found to be most potent. Incubation of these compounds with partially purified PAO from pig or rat liver resulted in a time and dose dependent irreversible loss of enzyme activity [161,968]. The kinetic and inhibitory characteristics are compatible with the assumption that the butadienyl-1,4-butanediamines are enzyme activated irreversible inhibitors of this type of PAO [179]. They are thought to bind FAD during the enzymatic reaction, blocking the active site, though they may also bind irreversibly to TPQ. MDL 72521 and 72527 are thought to be specific and are said not to inhibit any other enzyme significantly at concentrations which are sufficient to activate PAO completely, both *in vitro* and *in vivo* [968]. Furthermore, they do not exhibit significant toxic effects in experimental animals [179]. These compounds, which are not commercially available, are reported to show a high selectivity for PAO [131]. It remains to be seen whether they inhibit pregnancy-associated amine oxidase, but this seems quite likely since this enzyme oxidizes polyamines.

Copper chelating reagents such as 1,10-phenanthroline, 2,2′-bipyridyl, and diethyldithiocarbamate, inhibit copper-containing amine oxidases by removing enzyme bound copper [897,922,1163]. Azide and cyanide may function as inhibitors by binding to copper [919,930,1050,1110]. Quinacrine [N^4-(6-chloro-2-methoxy-9-acridinyl)-N^1,N^1-1,4-pentanediamine] is a FAD analogue. At 0.1 mM it inhibits rat liver PAO activity. In contrast with copper chelators, the iron chelators, α,α-dipyridyl, 8-hydroxyquinoline, and *o*-phenanthroline are effective inhibitors of rat liver polyamine oxidase [164]. However, neither quinacrine nor the iron chelators are specific this enzyme.

Some substrates at high concentrations can cause substrate inhibition of amine oxidases. Substrate inhibition is pronounced with π-electron containing substrates such as histamine

[900,1053,1164].

A clear distinction between FAD- and copper-containing amine oxidases is also difficult to make with other monoamine oxidase inhibitors. The acetylenic inhibitors, such as pargyline (*N*-methyl-*N*-benzylpropargylamine), clorgyline, diprenyl, nialamide, iproniazid, and isoniazid, although considered to be classical monoamine oxidase inhibitors, are also able to inhibit some copper-containing DAO activity from human placenta, pig kidney [730,1015] and human kidney [777]. Pargyline also inhibits the tissue type PAO described by Hölttä [164] and Seiler [969], albeit weakly. Pargyline can also inhibit DAO at 10^{-3} M. So, with these inhibitors, and inhibitors in general, caution needs to be used in the interpretation of results. Even with apparently well discriminating inhibitors, pH differences may alter their effectiveness. Inhibitor sensitivities cannot therefore be regarded as absolute [1,2,131,159,170,439,496,509,1165,1166].

Previous studies of inhibitor action on pregnancy-associated amine oxidases are summarised in Table 1.8. These studies can mostly be regarded as preliminary and further careful work needs to be done to establish IC_{50} values, K_is and other kinetic data for individual enzymes.

Drugs normally administered for purposes other than the impairment of polyamine catabolism may also cause amine oxidase inhibition. The monoamine oxidase inhibitors isoniazid and iproniazid were originally developed because of their potent antitubercular action [1167]. The antimalarials, amodiaquine, quinacrine, and chloroquine, inhibit putrescine catabolism in the rat [1168]. Levamisole, an antihelminthic drug, also known as an inhibitor of sperm motility, is a potent inhibitor of both seminal and placental amine oxidases [769]. The antiparkinsonian drugs, carbidopa (methyldopa hydrazine) and benserazide possibly inhibit copper-containing amine oxidases during prolonged therapy [1169].

Amiloride and its analogues, clinically useful as diuretics and antihypertensive agents, inhibit DAO *in vitro* [47]. Perhaps amiloride inhibition of DAO contributes to its antihypertensive effects *in vivo*, and to some of its adverse side effects such as eczema [1170].

TABLE 1.8. Inhibitors of Pregnancy-Associated Amine Oxidases

Isoniazid	*Pargyline*	*Clorgyline*	*aminoguanidine*	*semicarbazide*	*MGBG)*	*quinacrine*	*amiloride*	*Reference*
			10 mM? = 100%				$K_{0.5}$ = 5.1 μM	Novotny *et al.*, 1994 [47]
			1 mM = 100%					Morel, 1992 [755]
	0.1 mM = 0%		0.1 mM = 50%		0.1 mM = 75%	0.1 mM = 100%		Morgan, 1985 [726]
			K_i(putrescine) = 3.0 μM					Gahl, 1982 [719]
			K_i(spermidine) = 0.8 μM					
0.05 mM = 29%	0.05 mM = 0		0.05 mM = 100%	0.05 mM = 94%				'DAO A' Tufvesson, 1978 [678]
0.05 mM = 27%	0.05 mM = 0		0.05 mM = 100%	0.05 mM = 93%				'DAO B' Tufvesson, 1978 [678]
0.05 mM = 31%	0.05 mM = 4%		0.05 mM = 100%	0.05 mM = 81%				'DAO A' Tufvesson, 1978 [763]
0.05 mM = 34%	0.05 mM = 0		0.05 mM = 100%	0.05 mM = 92%				'DAO B' Tufvesson, 1978 [763]
100 μM = 37%			0.10 mM = 100%	100 μM = 97%				Lin & Kirley, 1976 [746]
10 μM = 0			0.01 mM = 80%	10 μM = 87%				
			0.001mM = 38%	1 μM = 3%				
0.1 mM = 68%			0.02 mM = 100%	0.1 mM = 99%				Baylin & Margolis, 1975 [713]
				0.01 mM = 8%				
0.05 mM = 0	–	–	1.0 mM = 0	0.05 mM = 0	–	–	–	PMAO; Bardsley *et al.*, 1974 [712][a]
0.05 mM = 40%	–	–	0.01 mM = 100%	0.05 mM = 50%				Placental DAO [712]
0.1 mM = 42%	0.05 mM = 51%	–	0.05 mM = 68%	0.05 mM = 37%				pregnancy plasma DAO [712]
			0	0				Bardsley & Crabbe, 1973 [743]
			0.01 mM = 100%	0.01 mM = 27%				Paolucci *et al.*, 1971 [742]
1.0 mM = 44%			1.0 mM = 100%	1.0 mM = 100%				Smith, 1967 [741]
0.01mM= 24%			0.01mM = 100%	0.01mM = 56%				
0.1 μM = 0			0.1 μM = 63%	0.1 μM = 2%				

[a] Histamine as a substrate

1.4 Physiology and Pathophysiology of Polyamine Oxidases

1.4.1 Metabolism

In the tissues in which they are expressed, and fluids into which they are secreted, PAOs control the level polyamines, diamines, histamine, 5-hydroxytryptamine, dopamine and other biogenic amines. Because of their role in the regulation of polyamine levels the amine oxidases are implicated in the physiological and pathophysiological processes influenced by polyamines (*q.v.* §1.2.6 and 1.2.7).

1.4.1.1 Regulation of Intracellular Polyamine Levels

Besides uptake and excretion [180], intracellular polyamine concentration is controlled by regulating the activity levels of both the biosynthetic (ODC, SAMD) and degradative enzymes [1] (*vide* §1.2.3). In contrast to the regulation of biosynthetic decarboxylase activities, relatively little is known about the regulation of degradative oxidase activity.

Several observations demonstrate that the activity of both biosynthetic and degradative enzymes are correlated, in that a variation of ODC and SAMD activities is usually associated with a modification of amine oxidase activity [155,978,1003,1171-1176]. Experiments with regenerating rat liver after partial hepatectomy reveal a large increase in putrescine concentration in response to the induction of ODC [155]. An increase in DAO activity is also observed under the same experimental conditions [978]. Rapidly growing tissues; such as embryonic tissues [408,1177], rat kidney during compensatory hypertrophy after unilateral nephrectomy [1060], human and animal tumours [978,1176,1178-1180], transformed cells [1003], and regenerating tissues [1061], showed, together with increased polyamine biosynthesis, an enhanced amine oxidizing activity that correlated with increases in intracellular polyamine levels. Human central nervous system tumours showed a positive correlation between polyamine biosynthetic and degradative activities, and tumour growth. Both rates of polyamine biosynthesis and oxidation of putrescine were proportional to the degree of histologically determined malignancy of the tumours [1171,1176].

1.4.1.2 Regulation of Extracellular Polyamine Levels

Plasma amine oxidases are involved in the regulation of blood polyamine levels. They may also play a role in regulation of intracellular levels since the polyamines can be absorbed from extracellular compartments. Serum enzymes may also be involved in the regulation of biogenic amines such as dopamine and 2-phenylethylamine [448,942] and this may have important implications because of the roles of these amines in neurotransmitter or neuromodulatory action.

Similarly, histamine is an important substrate of serum amine oxidases and its role in inflammatory, allergic, and ischaemic phenomena is well documented [1181] (*vide* §1.5.3.4).

1.4.2 Detoxification

The monoamine oxidase (MAO-A) found in intestinal mucosal cells is thought to play an important role in the detoxification of xenobiotic amines (*vide* references [502,1182-1184]). Copper-containing amine oxidases are also thought to play a role in detoxification of xenobiotic amines or as scavengers of potentially harmful amines, perhaps present as a result of rapid cell division. The relatively high amine oxidase activity in the serum of ruminants has been suggested to be involved in the metabolism of potentially toxic bacterial or plant polyamines absorbed from the rumen [458].

DAO activity is also relatively high in the intestine of many mammals; it has been suggested the enzyme is involved in the detoxification of ingested histamine and other amines [449,1182,1185,1186]. Amine oxidases may have a more general role of regulating the action of xenobiotic or endogenous histamine on smooth muscle or other target sites [1185]. Histamine methyltransferase (HMT) may also play a significant role [1187-1189]. Histamine [1186] and polyamines [1190] are present in wine (especially red), beer, and certain foods. Amine oxidases have been implicated in hypersensitivity to these foods and beverages, and in headache pathophysiology [1186,1191,1192]. It is of interest that DAO is inhibited by alcohol [1191]. See also sections 1.4.5, 1.5.3.4 and 1.6.7.3.

The protective effect of amine oxidases as detoxification enzymes has been questioned since many metabolites of polyamines are even more toxic than the polyamines themselves. Complex mechanisms probably operate *in vivo*: aldehyde dehydrogenases and reduced glutathione may act to metabolise potentially toxic metabolites to more innocuous compounds (*vide* §1.5). Similarly aldehyde oxidase and xanthine oxidase (EC 1.2.3.2) also catalyse aldehyde oxidation [194-197] and may be important in the metabolism of aldehyde products of polyamine oxidation (*vide* §1.2.5).

1.4.3 Regulation of Enzyme Levels

Polyamine biosynthetic activity is generally induced in response to certain external stimuli, whereas the increase in amine oxidase activity appears to be a consequence of enhanced intracellular polyamine concentration. Activity of DAO follows an increase in putrescine concentration [977,1060,1193].

Increases of DAO activity may be due to synthesis of new protein, activation of a pre-existing enzyme, or slowing of enzyme turnover. The injection of protein and RNA synthesis

inhibitors, cyclohexamide and actinomycin D, into both partially hepatectomized and unilateral nephrectomized rats, completely prevented enhancement of DAO activity when given at the time of the operation; the half-life of the enzyme remained the same in both pathological and control animals [977,1060]. Thus, increases of DAO activity can be attributed entirely to *de novo* synthesis of mRNA coding for the enzyme [977,1060,1193].

1.4.4 Production of Oxidized Polyamines and Hydrogen Peroxide

Since amine oxidases are responsible for the production of oxidized polyamines, they are implicated in the processes involving these metabolites (*q.v.* §1.5 *et seq.*). Vascular semicarbazide-sensitive amine oxidase can metabolize the xenobiotic aliphatic amine, allylamine, to the cytotoxic aminoaldehyde acrolein and this has been linked to the ability of this amine to produce cardiovascular lesions in experimental animals, mimicking features of atherosclerotic disease [1071,1194]. SSAOs are associated with the smooth muscle cells of blood vessels and metabolise endogenous and xenobiotic amines, perhaps in a scavenging role. It has long been thought that environmental toxins might play an underlying role in vascular diseases such as atherosclerosis [1194]. Methylamine occurs endogenously as a breakdown product from a number of biochemical pathways and is metabolized *in vitro* to formaldehyde by semicarbazide-sensitive amine oxidase suggesting toxicological consequences upon cellular function if such conversions were to occur *in vivo* [472,1195-1198]. In particular, adrenaline is deaminated by MAO-A to form methylamine [1199]. Elevated methylamine levels are found in several pathologic states including uremia and diabetes mellitus [1196,1200], this may result in the overproduction of formaldehyde in tissues with high amine oxidase activity, especially blood vessels, with angiopathic consequences. Indeed, SSAO activity is elevated in diabetes mellitus [1201] and congestive heart failure [1202].

Hydrogen peroxide is also an important product of polyamine oxidation. Hydrogen peroxide appears to be involved in modulation of transmembrane signalling processes, perhaps by effecting the oxidation of sulphydryl groups on receptors or ion channels [1203-1210]. It may also act in intracellular signal pathways as a messenger to control particular cellular processes [1211]. On the other hand, H_2O_2 may cause peroxidative damage to biological molecules in the absence of scavengers such as reduced glutathione and catalase [1203,1212]. Indeed, the destructive power of hydrogen peroxide (and aminoaldehydes) appears to have been harnessed and used by organisms to destroy both invading pathogens and neoplastic cells; and has important implications in apoptosis (*q.v.* §1.5 *et seq.*). The role of hydrogen peroxide in signal transduction is therefore enigmatic, and perhaps relates to fine regulation of its production.

GABA is a potentially important product from the oxidation of putrescine (*q.v.* §1.2.5).

1.4.5 Implications of Amine Oxidases in Etiology

Because of their roles in the regulation of polyamine levels the amine oxidases are implicated in diseases hallmarked by an alteration of polyamine levels. Altered levels of amine oxidase activity may play an important role in the pathophysiology of the disease, or possibly reflect a key metabolic derangement of amine oxidase metabolism due to its altered level or dysfunction in the disease state (*vide supra*). PAO determinations do not presently play a direct diagnostic role in any human disease. Serum DAO activity is relatively low (4 mU/l) in all physiological conditions [664], except pregnancy (*q.v.* §1.6 *et seq.*). Serum DAO levels are significantly lower in adolescents when compared with adults [664]. An inherited familial plasma DAO hyperactivity, without any associated disease, has been described [1213]. Age-related physiological changes in human serum (mono)amine (benzylamine) oxidase activity (*q.v.* §1.3.2.5a) have been reported. The enzyme activity is increased in childhood, falls during adolescence and reaches a minimum in young adults [1214], followed by a slow increase with aging [1214-1218]. Small gender-related differences in human serum amine oxidases are controversial [666,1214,1215,1218,1219]. In pregnancy, human serum (mono)amine oxidase was found between normal limits [659,666,1219].

Elevated levels of DAO activity are associated with human cancers (reviewed [453,761,1181,1220]). A high DAO activity was found in human medullary thyroid carcinoma [1179,1221], endometrial carcinoma [1222], ovarian cancer [1223] and other neoplastic disorders [1224,1225]. The activity was enhanced not only in the neoplastic tissue, but for some patients with this type of pathology and particularly those having metastases, also in the blood [1179,1226,1227]. Subsequently, small cell carcinoma of the lung, which frequently shows endocrine cell properties, was demonstrated to have an enhanced DAO activity [1178,1228,1229]. Other lung cancers of different histologic types also had increased DAO levels [1180,1230]. Histaminase was found in the cytoplasm of cancer cells of primary and metastatic tumour of the lung, thyroid gland, pancreas, stomach, esophagus, ovary, pharynx and in carcinoids, dysgerminomas and in melanoma. No significant difference was found by histologic type or by degree of cellular differentiation and histaminase detection was rare in benign conditions [1231]. Increased DAO activity also was detected in ascites fluid of patients having ovarian carcinoma, which appeared to be its source [1232], and in pleural effusions of patients with stomach and colon carcinoma [668,1223,1224]. On the other hand, a decrease of SSAO activity (but increase in MAO-A activity) was observed with increasing malignancy of solid breast tumours induced in rats [1233]. It has been confirmed that tumour cells are the source of DAO in the circulation and effusion of patients with cancer [1228,1234]. The levels of DAO and PAO are higher in transformed cells [790,797,1003]. Initially, it was thought that circulating levels of DAO might be a useful indicator of clinical disease in patients with tumours. However, subsequent studies have

shown that the release of DAO into the circulation from tumours appeared too variable to use DAO as a marker [1228,1229]. Nevertheless, the presence of DAO in cancer and its etiology remains an important issue.

There appears to be some immunologic and structural identity between the DAO found in human tumours and that located in noncancerous human tissue, especially placenta [746-748,752,1224,1235]. A placental 'monoamine oxidase' cDNA variant has been localized to the q21 region of human chromosome 17, where *BRCA1,* a gene associated with hereditary breast and ovarian cancer, has been mapped; and there is evidence that it is perhaps expressed in breast and ovarian cancers [1080]. It is possible that the increased activity in neoplastic cells represents an aberrant derepression of nonfetal gene expression in cancer cells with no particular function in their metabolic activity. However, the possibility of a closer link cannot be ignored [1236,1237], especially in view of the reported abnormalities of polyamine levels in patients with cancer (*q.v.* §1.2.7.1). Indeed, the balance between polyamine degradation and biosynthesis appears to be disengaged from the control exerted by DAO and ornithine decarboxylase in human colorectal cancer [1238].

Increased serum DAO levels have been reported in patients with chronic renal failure [1239] and urine DAO decreased in uremia [404]. The relationship between amine oxidases and kidney damage has been discussed (*q.v.* §1.2.7.5).

The role for DAO in the metabolism of histamine has been considered in pathophysiological states involving allergic reactions. The relationship between anaphylaxis in guinea-pigs and DAO has been examined [1018,1240]. Increased serum histaminase has been described in bronchial asthma [1241], other allergic conditions [1242,1243] and in anaphylactic shock [449,677,761]. The correlation between DAO activity and the allergic condition has been investigated by Herman, who evaluated DAO activity in human eosinophils. An increase in eosinophil DAO in patients with active asthma, urticaria, and parasitic infection was reported [804] (*vide* §1.5.3.4).

The roles of amine oxidases in vascular endothelial cell damage, and the implications for diseases such as congestive heart failure and diabetes mellitus have been discussed (*vide* §1.4.4).

PAO levels are elevated during infection [1244], and in hepatitis [955] (*q.v.* §1.2.7.6 and 1.5.1). Reduced liver DAO levels have been reported to result from chronic ethanol feeding in rats [981].

There is evidence for an alteration of PAO levels in the sera of schizophrenic patients [427,428,1245] (*q.v.* §1.2.7.8).

Human serum (mono-/benzyl-)amine oxidase levels in various diseases have been widely investigated. They were reported to be significantly increased in chronic liver diseases with

fibrosis (including Wilson's disease) [1137,1139,1246-1251], congestive heart failure [1252], diabetes mellitus [1214,1252], agromegaly [1251], and progressive systemic sclerosis [1251]. In severely burned patients [1253] and in patients with rheumatic fever [1254], low human serum amine oxidase levels were reported.

The relationships between amine oxidases and their substrates, and etiological factors, pathogenic factors and expression of pathological conditions are complex. It is difficult to find direct causal relationships between them. For most diseases in which some derangement of polyamine metabolism has been implicated there are other factors that could produce the same disease. Furthermore, the disease may exist without any alteration in polyamine metabolism.

1.4.6 Release of Amine Oxidase by Heparin

After synthesis in the enterocytes of the villus tip, DAO is transported to glycosaminoglycan binding sites [963,1000] on intestinal microvascular endothelial cells [1082] or basolateral membranes of villus epithelial cells [786,790]. Heparin administered by different routes induces a rise in plasma DAO activity in humans [674,677,682] and in many animal species [945,946,961-963,998,1000,1024,1255,1256]. Except in guinea pig [1257], and possibly bovine [411] and equine [1255] subjects where the enzyme may be released from the liver, the postheparin plasma amine oxidase activity is thought to arise from displacement of the enzyme from the binding sites on the intestinal endothelium (but see §1.3.2.5g). At least in humans and rat, postheparin plasma DAO activity is thought to be a sensitive quantitative marker of small bowel mucosal damage, including intestinal injury from ischaemia, radiation, and chemotherapy [79,681,682,787,962,1255,1256,1258-1266]. A reduced postheparin DAO increase has been observed in patients with acute and chronic liver diseases [413], with chronic renal diseases [761,1267], during hyperlipoproteinemia [676], and after anaphylactic shock [1268]. Serum post-heparin DAO activity is altered in several intestinal disorders including celiac disease [680,1269], gastroenteritis [1270], and Crohn's disease [679,789,1271-1273].

Heparin induced DAO activity returns to normal with a much shorter half life than the *post partum* decay [676,706], even in pregnancy, and since there are physicochemical differences, it seems that pregnancy DAO and heparin induced DAO are distinct isoenzymes [706]. Although the purified enzymes showed similar kinetic properties and substrate specificity, post heparin blood serum contained only one active DAO fraction with a molecular weight of 185,000, whereas DAO from both pregnancy serum and amniotic fluid separated into two active fractions with apparent molecular weights of 245,000 and 485,000 [678].

1.4.7 Functions in Plants

Plant PAOs probably play a role in growth and development (including the cross-linking of molecules within cell walls), wound and resistance responses, and in secondary metabolism. Hydrogen peroxide, a product of amine oxidases, is known to play a key role in defence responses of plants. It is possible that in some plants amine oxidases have specialized functions such as the attraction of pollinating insects.

Plant amine oxidases and polyamines are found in the apoplast, loosely associated with the cell wall [1274-1276]. There is evidence, particularly from the Leguminoseae, of a role for amine oxidases in conjunction with cell wall peroxidases in lignification and cross-linking of cell wall components, both during normal growth, and in response to stress and wounding [589,1275-1279].

A correlation has been demonstrated between amine oxidase and peroxidase levels in chickpea tissues undergoing wound-healing [589,1278,1279]. Different levels amine oxidase have been observed in susceptible and resistant cultivars of chickpea upon infection by *Ascochyta rabiei*, suggesting the enzyme may also play a role in the defence response [1278]. Hydrogen peroxide and aminoaldehydes formed during polyamine oxidation may decrease plant damage by killing invading plant pathogens [1274,1278]. Wounding is a physical stress that increases the likelihood of attack by pathogens. An increase in amine oxidase and peroxidase in wounded internodes of chick-pea stems has been reported [589]. This increase in amine oxidase activity as a physiological response in wound-healing may also provide a plant defence against pathogens.

In other plants, such as tobacco [624,625] and *Hyoscyamus* [609], amine oxidases may play a role in alkaloid biosynthesis [11]. In tea, the enzyme is responsible for the production of flavour components such as theanine (*N*-ethyl-γ-L-glutamine) [587]. Amine oxidases are also involved in the biosynthesis of the principal auxin (plant hormone) indoleacetic acid [11,468].

During flowering, the rate of oxidative processes in the appendix of the spadix of the certain arum lilies is high enough to produce warming of the plant tissue by 10–15 °C over ambient air temperature. The oxidation is thought to be due to light-stimulated action of amine oxidase on plant (poly)amines. The increased temperature produced by amine oxidation volatiltizes plant amines and their oxidation products, which may then attract insect pollinators [126,1280,1281]. These arum lilies include the giant Sumatrum arum, *Amorphophallus titanum*, and the voodoo lily, *Sauromatum guttatum*. On flowering these plants give off a characteristic unpleasant odour, redolent of rotting flesh[1]. The distinctive cadaverous odour produced by the arum lilies is comprised of a cocktail of volatile compounds, including the polyamines cadaverine

1. The Wall Street Journal Europe, Vol. XIV No. 122 (July 25, 1996). Giant cannibal plant gets up British noses.

and putrescine, their oxidation products, volatile amines, free amino acids, ammonia, and skatole [1281], rather than just one particular or unusual compound. The heat and scent production are an elegant illustration of 'biological parsimony', because they are the result of the same oxidative process, thus simultaneous and inextricably linked. The volatile odoriferous base, *N*-γ-aminopyrroline, which is derived from spermine oxidation, probably contributes the odour associated with semen [126] and may also contribute to the curious odour of certain plants.

The widespread occurrence of amine oxidases in the plant kingdom suggests, that in addition to their specialist roles in a limited number of plants, that they have also a more fundamental role, probably associated with the regulation of polyamine levels, which are in turn implicated in macromolecular metabolism and growth [11,1282]. In lentil and pea, as well as in *Lathyrus* and *Glycine*, amine oxidase appears to be absent from ungerminated seeds, however activity increases during early periods of germination. The enzyme activity is greatest in the growing regions and decreases with maturity and senescence of the plant [469,602].

1.4.8 Functions in Micro-organisms

In bacteria and lower eukaryotes amine oxidases provide a route for the utilization of various amine substrates as nitrogen and carbon sources [105,550,561,563]. Furthermore they must be involved in the regulation of the levels of essential polyamine growth factors (*q.v.* §1.2.6.1).

1.5 Oxidized Polyamines and their Biological Effects

Products of polyamine oxidation (*q.v.* §1.2.5) have a number of biological effects including antimicrobial, antiproliferative, anti-inflammatory, cytotoxic, and immunoregulatory activity. Evidence points to the primary involvement of aminoaldehydes in these processes. In general, aldehydes are well known to be cytotoxic [1283]. The action of the aminoaldehydes is not clear though many putative actions have been suggested. It seems likely that they do not have a single mode of action.

The highly reactive aminoaldehydes may interact with a number of different molecule types and their particular effects probably depend on where they are produced. The aldehydes may act as reducing agents and react with essential protein SH^- groups [1284]. Aldehydes are known to bind reversibly with the basic residues of proteins, and to combine irreversibly with the sulphydryl groups of cysteine [1283]. Oxidized spermine binds to nucleotides and phosphoproteins [1285]. The carbonyl groups of the aminoaldehydes may react with the amino groups of the purines and pyrimidines in nucleic acids through the formation of Schiff bases. It is tempting to speculate that aminoaldehydes may affect microtubules or microfilaments in a manner similar to that of colchicine or cytochalasin B [1286] and that this may be the cause of the

immotility observed when they react with spermatozoa and trypanosomes (*q.v.* §1.5.1.4). Like other highly reactive compounds, such as azide, the toxicity of aminoaldehydes may also be mediated by their effects on cAMP or cGMP levels.

Aldehydes produced by polyamine oxidation can arrest cell growth and division through their action as inhibitors of protein and nucleic acid synthesis [227,1287]. The aldehydes may act as inhibitors of cell proliferation by means of their interaction with nucleotides and inhibit transcription and translation processes [1288,1289]. Oxidized spermine inhibits protein synthesis [1290]. It is possible that an interaction between oxidized spermine and nucleic acid causes this inhibition [1288,1289,1291-1295]. The aminoaldehydes resemble bifunctional alkylating agents, such as the nitrogen mustards, and form DNA adducts. They may also act like those antibiotics [such as actinomycin D] that inhibit DNA-directed RNA polymerase by binding to the DNA template [1291].

The toxicity of aminoaldehydes may be mediated through their reaction with membrane molecules in a manner analogous to the lipid oxidation products produced by oxygen stress in erythrocytes and other cell types [1296]. It has been reported that the dialdehyde resulting from spermine oxidation by bovine plasma PAO can crosslink cells [1297]. The aminoaldehydes may exert their toxicity through some change in membrane components that prevents the ingress of nutrients, or egress of toxic metabolites, or both [2]. The killing of intracellular parasites by the polyamine–PAO system without apparent damage to the host cell supports this hypothesis. Aminoaldehydes may interact with molecules exposed on cell membranes in a manner that inhibits signal transduction [1298]. The toxicity may be due to some gross alteration in membrane activity such as zeiosis and blebbing [1299].

Defences against the toxic products of polyamine oxidation may include several strategies. (1) Protection from aminoaldehydes: aldehyde dehydrogenases that metabolize the aminoaldehydes eliminate these reactive species [225,1300] (*q.v.* §1.2.5). Similarly, the molybdenum-hydroxylases, aldehyde oxidase (EC 1.2.3.1) and xanthine oxidase (EC 1.2.3.2) catalyse aldehyde oxidation [194-197] and may be important in the elimination of aldehyde products of polyamine oxidation. Non-protein sulphydryls, such as reduced glutathione, may form adducts with the polyamine derived aminoaldehydes [1301]. (2) Protection from H_2O_2 toxicity: the selenoenzyme, glutathione reductase/peroxidase, and reduced glutathione are intimately involved in the catabolism of H_2O_2 [1302,1303]; together with catalase [1304], they play a protective role against the potentially harmful effects of H_2O_2. They protect against the effects of H_2O_2 that has diffused through the phagocytic vacuole membrane into the cytosol. The glutathione system is probably more important than catalase for the concentrations of H_2O_2 occurring in leukocytes [1303].

Anti-oxidant defence systems (glutathione, catalase and superoxide dismutase) may be important in detoxifying reactive polyamine oxidation products and represent metabolic defence systems [1305,1306]. The physiological consequences polyamine oxidation will be dependent on the location (and timing) of both oxidative product formation [1307] and of native anti-metabolite defence systems that neutralise polyamine oxidation products.

Figure 1.14 illustrates a unified view of the polyamine–PAO system. Each of the items in boxes to the sides and top of the diagram contributes polyamines or polyamine oxidases (or both) to the system producing the effects indicated in the box at the bottom of the diagram (heavy arrow). These effects are discussed in the following sections.

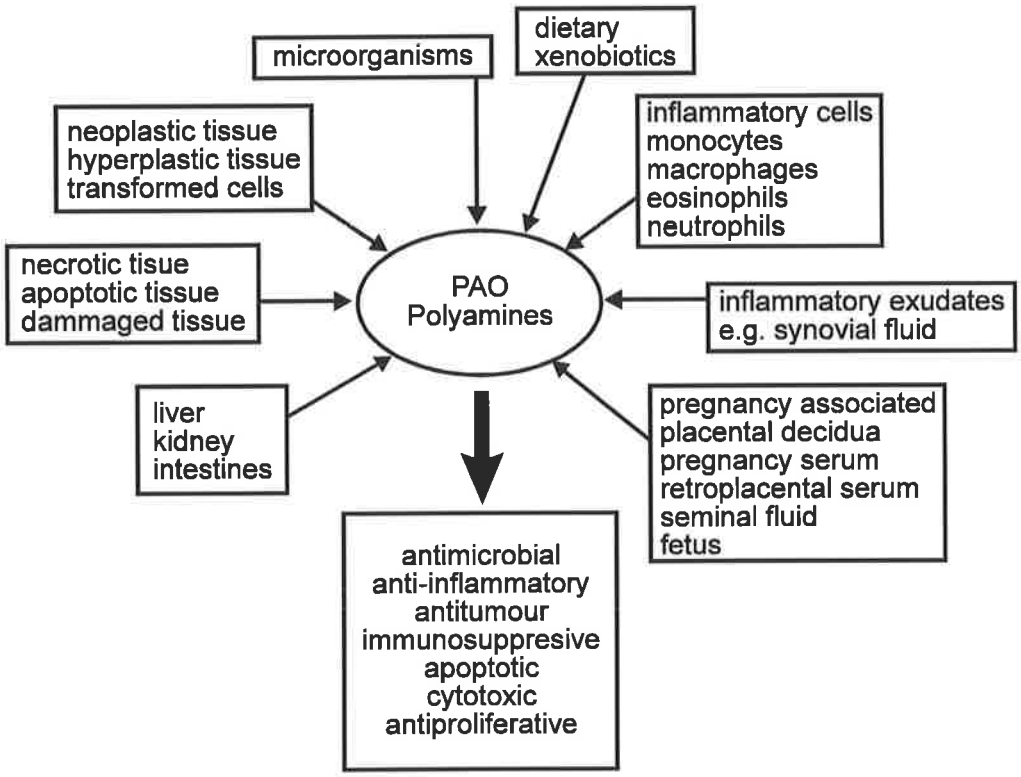


Figure 1.14. A Unified View of the Polyamine–PAO System and its Effects

1.5.1 Antimicrobial

1.5.1.1 Antibacterial

The products of polyamine oxidation have antibacterial effects. Oxidized polyamines inhibit the growth of tubercule bacilli [1308] and other bacteria, including *Staphlococci, E. coli, Bacillus subtilis, Proteus vulgaris, Salmonella, Shigella, Pseudomonas, Mycobacterium tuberculosis* and *Serratia* [44,49,227,1290,1309-1312]. It has been found that guinea pig alveolar macrophages kill *Mycobacterium tuberculosis in vitro,* independent of susceptibility to hydrogen peroxide or

triggering of the respiratory burst [1313]. It is generally held that the macrophages are the final effector cells of protective immunity against *M. tuberculosis* [1314]. Oxidized polyamines may form part of the macrophage antimicrobial armamentarium [955] (*qq.v.* §1.3.2.5h and 1.5.1.5).

Hirsch and Dubos found that spermine was extremely toxic for tubercle bacilli in the presence of bovine serum, or bovine plasma fraction V (albumin) [1308,1310]. These findings were later confirmed and extended by Tabor and Rosenthal [44]. It was realised since the investigations by Hirsch, that the presence of the amine oxidase in bovine serum, or as a contaminant of bovine plasma fraction V (albumin), was essential for the inhibitory or bactericidal effect of the polyamines [spermine 30 μM], and provided the earliest suggestion that oxidized polyamines may be involved in non-specific immunity [1310,1315]. At that time, it was also recognized that human semen and prostatic fluid, which both contain polyamines and an amine oxidase, had antibacterial effects [1309,1316]. The antimicrobial effect of semen had been reported by Poehl approximately 50 years before [43], and Glasser and Prinz had also described the bactericidal effect of spermine [1317]. Gurevitch *et al.* demonstrated that the bactericidal effect of human semen was due to spermine [1318].

1.5.1.2 Antiviral

Oxidized polyamines have antiviral effects. Bacteriophage, including coliphage of the T-odd series, RNA phage [227,1319-1325], plant viruses [1326] and animal viruses, including Sindbis, Newcastle, Influenza, West Nile, Vaccinia, and human immunodeficiency type 1 (HIV) viruses are all neutralized by oxidized polyamines [1320,1327-1332]. Oxidized polyamines are known to inhibit the replication and transcription of DNA [1292]. Virus neutralisation is thought to involve modification of viral nucleic acid by oxidized polyamines [1288,1322,1323,1325]. Moreover, oxidized polyamines also react with capsid/coat proteins [1331].

1.5.1.3 Fungicidal

The polyamine–PAO system forms a potent fungicidal and fungistatic weapon [1333]. Activated macrophages have been shown to kill fungi [1334-1336], and the polyamine–PAO system may contribute to the macrophage- and other effector cell-mediated killing of fungi (*qq.v.* §1.3.2.5h and 1.5.1.5). The polyamine–PAO system kills the yeast *Cryptococcus neoformans* and shows fungistatic activity toward *Candida albicans* and *Aspergillus fumigatus.* The products of the polyamine–PAO system, but not ammonia, killed *C. neoformans*. Catalase failed to inhibit killing, suggesting that aminoaldehydes were the principal toxic products [1333].

1.5.1.4 Antiparasitic

The activated macrophage and its products are important components of the human immune response to malaria. Macrophage oxidative processes can mediate killing of *P. falciparum* [1337]. The nature of the soluble non-antibody factor(s) responsible for intraerythrocytic cell death remain uncharacterized. It is pertinent that significant killing remains in the presence of activated macrophages that have been treated with catalase even though hydrogen peroxide has been implicated in the killing [1337,1338] (*qq.v.* §1.3.2.5h and 1.5.1.5). Since macrophages contain PAO [807,955], it is possible that macrophage PAO and its products may be candidates for the soluble factor(s) that inhibit the growth of malaria parasites and cause their intracellular death.

Oxidized spermine and spermidine can kill intraerythrocytic parasites without perceptible damage to the host cell [1244,1339,1340]. Bovine serum amine oxidase and spermine (125 μM) have been shown to be inhibitory to the intraerythrocytic stages of the hemoprotozoan parasites *Babesia rodhaini*, *B. microti* [1244,1339], *Plasmodium yoelii*, *P. falciparum* [1338,1341], *P. chaubaudi* [1338,1339,1341], which cause malaria and babesiosis infections. Treatment of the intraerythrocytic asexual blood stages with oxidized polyamines caused parasite destruction, possibly representing the crisis forms seen *in vivo* which are characteristic of non-specific immunity [1338,1342]. Experiments with human retroplacental serum PAO without added polyamines failed to demonstrate toxic or inhibitory activity against *B. rodhaini* [1244,1339], *P. chaubaudi* [1244] or *P. falciparum* [1338]. However, a reinvestigation using longer incubation times, and retroplacental serum samples with possibly higher PAO activity, demonstrated inhibitory activity of human PAO with 50 μM spermine against *P. falciparum in vitro* [1342]. The polyamine substrates for PAO are produced by all growing cells and are found in extracellular fluids [10], which could explain the inhibition seen in the presence of high concentrations of retroplacental serum even without the addition of exogenous polyamine.

Serum levels of PAO are elevated during some infections including babesiosis and hepatitis [432]. Furthermore, there is evidence that activated macrophages contain higher levels of the enzyme and release it into culture medium [807,955,1083]. Evidence, including the effect of catalase and superoxide dismutase (a free radical scavenger) supports the theory the amino-aldehydes are the *principal* mediators of cell death by the polyamine–PAO system and not ammonia, hydrogen peroxide or reactive oxygen intermediates such as the hydroxy radical ($OH^{\bullet}$) or superoxide (O_2^{-}) [1338,1343]. Superoxide was not detectable during the reaction of polyamines with PAO [440]. It is not that the other metabolites do not contribute to killing, but that the aminoaldehydes are particularly potent. Indeed, purified aminoaldehydes produced by polyamine oxidation had a lethal effect on the plasmodia within normal and glucose-6-phosphate-dehydrogenase (G6PD)-deficient erythrocytes (ID_{50} approx. 150–500 μM for the aldehydes).

Parasites cultivated in the G6PD-deficient erythrocytes were more sensitive to the effects of the aminoaldehydes. It has long been suggested that G6PD deficiency confers some degree of protection against malaria infection [1344,1345]. Oxidation of the highly reactive aldehydes (to putreanine, β-alanine and spermic acid) abolished the lethal effect. A similar effect of purified aminoaldehydes on bacteria has been reported [1346].

Oxidized polyamines inhibit the growth of the leishmanial parasitic protozoan, *Leishmania infantum*. This anti-leishmanial effect was from aminoaldehydes derived from maize [656] PAO and bovine serum amine oxidase [1347].

Oxidized polyamines can also damage larger multicellular helminth parasites: *Schistosoma mansoni* schistosomula, *Dirofilaria immitis* microfilariae, and although more resistant, exsheathed third-stage larvae of *Nematospiroides dubius* [1348]. Again catalase failed to abolish the PAO-damage to the worms, indicating that the effects were not entirely due to hydrogen peroxide. Furthermore, it has been reported that shistosomula were not sensitive to H_2O_2 [1349]. Polyamines were toxic at around 100 μM. Activated macrophages in cooperation with immunoglobulin E immunocomplexes mediate schistosomula cytotoxicity [1350,1351]. The polyamine–PAO system may contribute to schistosomula killing by activated macrophages. The principal effector cell involved in the killing of *D. immitis* is the neutrophil [1352]. This cell has also been shown to contain polyamine (diamine) oxidase [755,805] (*qq.v.* §1.3.2.5h, 1.5.1.5, 1.5.2, 1.5.3.3, 1.5.3.4 and 1.6.8.2).

A range of trypanosome species were sensitive to the products of the polyamine–PAO system at a polyamine level in the order of 50 μM, including *Trypanosoma musculi* [727], *T. lewisi* [1353], *T. equiperdum* [44], and the African trypanosomes, *T. brucei* [1354], *T. brucei, T. vivax, T. congolense* [1355]. Trypanosomes contain relatively high levels of polyamines [1356], which may be liberated during a specific immune response. The liberated polyamines may augment the immune response and contribute to nonspecific immune mechanisms in the presence of bovine serum or macrophage PAO. Indeed, African cattle resistant to trypanosomiasis have a higher level of PAO in their serum than do trypanosensitive cattle [1354]. Trypanosomes were also killed by 50 μM H_2O_2, but not by ammonia. Subsequent experiments showed that catalase did not prevent killing by the PAO–polyamine system, nor was the PAO mediated killing of trypanosomes in the presence of spermine inhibited by high concentrations of superoxide dismutase or the $OH^{\bullet}$ radical scavengers, mannitol, ethanol, or benzoic acid [1353]. These studies show that other products of polyamine oxidation besides H_2O_2 are toxic to the trypanosomes and that these are most likely to be the aminoaldehydes. The ammonia generated by the enzymatic reaction is unlikely to participate in the trypanosome killing because the parasites were found to be resistant to even high (>200 μM) concentrations of ammonia. Macrophages play an important

role in the killing of parasites *in vivo* (*q.v.* §1.5.1.5), and it is possible that PAO contributes to macrophage mediated trypanosome killing.

Although spermatozoa are not strictly parasites they are included here because the effects of oxidized polyamines are similar to those seen on trypanosomes [1357]. Enzymatically oxidized polyamines can cause a loss of spermatozoa motility and powerfully inhibit some metabolic processes in the spermatozoa [44,772,1358,1359]. Catalase did not inhibit the effects of polyamine–PAO system, nor was ammonia toxic to the spermatozoa; GSH and 2-mercaptoethanol had some antagonistic effect, preventing, but not reversing the immobilisation of the spermatozoa caused by spermine/amine oxidase, pointing to the involvement of aminoaldehydes.

1.5.1.5 Non-Specific Immunity

Macrophages are terminally differentiated cells (monocytes) and play an important role in defending the body against infections and malignancy [1360]. In response to various extracellular stimuli that are usually present at inflammatory sites, such as interferon-γ and bacterial lipopolysaccharide, they produce reactive oxygen metabolites and other toxins [1361-1365] used in the killing of micro-organisms and tumour cells [1366-1372].

Rabbit alveolar macrophages, obtained by bronchial lavage, contain a PAO, as do mouse peritoneal and human peripheral blood macrophages [807,955]. Activation of macrophages *in vivo* results in an increase in cellular PAO. Differential centrifugation of the activated macrophages showed that most of the activity was in the soluble fraction, though some was particulate [439].

Incubation of alveolar macrophages activated with zymosan, which is known to stimulate secretion of lysosomal enzymes by macrophages, did not significantly stimulate extracellular secretion or alter the intracellular level of PAO. Attempts to induce cultures of nonactivated mouse peritoneal cells to secrete PAO, with a number of compounds known to affect macrophage enzyme secretion, were also unsuccessful. However, medium from mouse peritoneal macrophages activated by long exposure to LPS did contain some PAO activity [439].

The concentrations of spermine and spermidine in human leukocytes have been estimated to be as high as 1 mM [382,1373], far exceeding the polyamine requirements for PAO–polyamine toxicity. There is also evidence for the existence of PAO in the phagolysosome of macrophages and other phagocytes (*q.v.* §1.3.2.5h). Clearly, *in vitro,* the PAO–polyamine system is involved in parasite killing. Any *in vivo* role must be cautiously extrapolated, since the exact nature of the macrophage enzyme and concentrations of polyamines in the microenvironments where killing may occur are not known. It remains to be established whether the PAO–polyamine system forms part of the antimicrobial armamentarium of the macrophage. If this system is, it may form an

important antimicrobial role.

The polyamine arm of the polyamine–PAO system appears to be mobilized in response to macrophage activation. In terminally differentiated cells there is evidence for the activation of ornithine decarboxylase and polyamine accumulation [1374]. It has been shown that ODC activity in a macrophage-like cell line can be induced by LPS and other immunoadjuvants [1375-1377]. Macrophage activation by LPS, tumour necrosis factor (TNF) and interferon-γ involves an elevated expression of the ODC gene resulting in ODC mRNA accumulation [1378,1379]. Specific inhibitors of polyamine biosynthesis, α-difluoromethylornithine (DFMO) and methyl-glyoxal-bis[guanylhydrazone] (MGBG), have indicated that polyamines are involved in the functional activation of human macrophages. Inhibition of ODC with DFMO can reduce the capacity of murine macrophages to phagocytose protozoan organisms [1380]. Furthermore, the inhibitors also diminished the respiratory burst activity of macrophages induced by bacterial lipo-polysaccharide (LPS) and interferon-γ. These inhibitory effects, particularly those of MGBG could be reversed by spermine [1381]. It is interesting that MGBG was the more potent inhibitor of macrophage functional response, since MGBG inhibits the formation of spermine and spermidine [247], which are thought to yield more potent aminoaldehydes [1382]. LPS has been shown to also increase collagenase production by macrophages. Collagenase production was inhibited by DFMO and this effect was reversed by putrescine [1376].

Cells of mononuclear phagocyte lineage secrete a number of substances, many of which can inhibit cell proliferation [1368]. Flescher *et al.* found that human monocytes secrete ammonia (a product of PAO activity) and that its production can be blocked by inhibitors of ornithine decarboxylase and PAO [1383]. They found that the addition of exogenous ammonia at levels produced by monocytes in their system (0.6 – 6 mM) significantly reduced lymphocyte viability. These levels of ammonia are high and it is noted that they maintained their monocyte cultures for 2 days to achieve these levels. Others have found similar decreases in viability and decreased mitogenic responsiveness of lymphocytes from various sources after addition of similar levels of ammonia [1384,1385]. Systems in which PAO is involved may be affected by the ammonia produced by this enzyme, although other investigators have not found any effect of ammonia at putative physiological concentrations after short term culture (200–1000 μM) [1333,1338,1353]. These studies require confirmation, though it seems likely that the aminoaldehydes are much more potent. Flescher *et al.* proposed that endogenous H_2O_2 production is a principal mediator in the inhibition of IL-2 production, contrary to other evidence indicating the importance of aminoaldehydes [1386]. That the interaction between PAO and putrescine, which also produces H_2O_2, did not suppress IL-2 production suggests specific roles for spermine and spermidine oxidation products.

1.5.2 Antiproliferative and Cytotoxic

The cytotoxic and cytostatic effects of oxidized polyamines *in vitro* have been well documented for a wide variety of cell types including tumour cells and a number of mammalian cell lines [227,257,454,1343,1387]. Tumour cells [256,257,1388] (including Shays chloroleukemia cells (W25), acute lymphoblastic leukemia (ALL), acute myoblastic leukemia (AML)), myeloma cells [1389], Ehrlich ascites cells [1287,1390], NS1 cells [1391], K562 and NALM/6 lines [727,1297,1392,1393], hepatoma cells [1394], baby-hamster kidney (BHK-21/c13) cells [1395], chinese hamster ovary (CHO) cells [1396-1398], human vascular endothelial cells [1382], fibroblasts and transformed fibroblasts [384,1399-1401], thymocytes [955,1400] and mitogenically transformed lymphocytes [241,1048,1400,1402-1406] have all been shown to be affected. Many of these observations were made in the presence of calf serum (which contains amine oxidase), oxidized polyamines prepared with the bovine serum enzyme, or bovine serum albumin in which PAO was present as a contaminant [384,1359,1387].

Human retroplacental serum in the presence of exogenous spermine significantly suppresses the *in vitro* incorporation of [^{3}H]thymidine by spontaneously growing human lymphocytes [1407]. The inhibitory effects of polyamines in the presence of PAO were reversed by the addition of aminoguanidine, an inhibitor of DAO, suggesting that DAO is responsible for the toxic effects of spermine and spermidine [1399,1408,1409]. Mitogen induced lymphocyte proliferation is also enhanced by inhibitors of DAO [1410]. Inhibition of serum PAO activity (with 1-hydroxybenzyloxyamine) can stimulate tumour cell proliferation *in vitro* [1409].

These observations suggest that the poor cell proliferation of several tumours *in vitro* may result from the reaction of polyamines (released from the rapidly dividing and/or dead cells in cell cultures) and PAO (from serum supplements or the tumour cell line itself), and that growth conditions may be optimized by adding nontoxic doses of enzyme inhibitor or by exchanging FCS for other growth factors or an amine oxidase free serum. It seems likely that there are differences in the levels of PAO found in fetal calf, newborn calf, cow and horse serum and this may in part explain the difference in usefulness of various sera as culture medium supplements (*vide* §1.3.2.5a). It is well known that fetal bovine serum batches vary greatly in their capacity to support cell growth [1409]. There may of course also be other reasons, such as different concentrations of hormones and growth factors, or other inhibiting factors. However, significant lot to lot variability in fetal bovine serum cytotoxicity has been observed in the presence of added spermidine [1391]. In general, the serum cytotoxicity increased with the age of the animal from which it was obtained, reflecting increases in enzyme activity that were also found to increase with the age of the animal [1391,1411]. Lymphoid cells contain nanomolar amounts of polyamines [1412]. For *in vitro* cultures supplemented with fetal bovine serum it is conceivable

that with the large amount of naturally occurring cell death, polyamines liberated from dying cells could react with PAO in the *milieu* and contribute to the self-limiting nature of the cultures.

Spermine and spermidine were found to be the most toxic polyamines in the presence of amine oxidases [1387]. Polyamines at micromolar concentrations were not toxic to mammalian cells in the absence of amine oxidase [215,256,1287,1387,1388,1413,1414]. However, polyamines in higher concentrations [>2 mM] may be directly toxic [1415]. Linde *et al* suggested that polyamines, present in ascites fluid as a result of tumour cell death during growth of Ehrlich ascites tumour, may act as a negative regulator of growth of this tumour *in vivo* [1416]. Indeed, immobilized pig kidney DAO injected intraperitoneally in Ehrlich ascites tumour-bearing mice inhibits tumour growth, probably through the release of toxic aldehydes derived from the oxidation of endogenous polyamines [1390]. It seems likely that the toxicity of inhaled spermine in rat lungs [1417] is due to the production of cytotoxic products of polyamine oxidation by PAOs present in alveolar macrophages, mast cells or eosinophils. Perhaps these cells are also the source of the enzyme found in (bovine) lung (*vide* Appendix A).

Gaugas and Dewey showed that oxidation of polyamines by serum PAO results in the arrest of mammalian cell proliferation in the G_1 phase of the growth-division cycle [1284,1418]. The effect was cytostatic rather than cytocidal [1402,1419], at least at low polyamine concentrations. Aminoaldehyde products, rather than by-products of oxygen-dependent free radicals ($O_2^{-\bullet}$, $OH^{\bullet}$), were implicated as the principal causative agents [1284].

1.5.2.1 Apoptosis

The polyamine–PAO system may be involved in apoptosis, the ubiquitous biological phenomenon of intentional (programmed) cell death [1420-1423]. Polyamines have been implicated as the cytotoxic agent responsible for inducing programmed cell death in the mammalian blastocyst and the limb bud of the 14-day-old mammalian embryo. Parchment and Pierce found that the embryonic limb contains PAO activity and polyamines at the time of programmed cell death and that tumour cell growth by implanted melanoma cells is suppressed in this setting or in limb bud conditioned media [1389]. Inhibitors of PAO (aminoguanidine) and polyamine toxicity (2-mercaptoethanol) abolished the cytotoxicity.

PAO, found in blastocoel fluid of murine blastocysts, contributes to apoptosis in the inner cell mass of the blastocyst [1236,1420,1424-1426]. Selective killing occurs: cells with embryonic potential are resistant, trophectodermal cells die and are therefore not expressed, ensuring an absence of placental tissue in the embryo. Apoptotic cell death (vs. necrosis), mediated by PAO and perhaps dependent on the its developmental expression, rids the embryo of redundant stem cells with unneeded (pre-trophectoderm) phenotypes. The selective killing may be due to

developmental expression of glutathione-dependent or catalase-dependent (detoxification) protective mechanisms in the surviving cells [1236,1394,1424-1427] or perhaps the selective expression of aldehyde dehydrogenase, aldehyde oxidase or xanthine oxidase (*q.v.* §1.4.2). This model challenges the previous paradigm that cells 'suicide' and suggests that programmed cell death, in the blastocyst at least, is 'murder' and that the specificity of cell death is dependent on the ability of the target cells to prevent damage by detoxification mechanisms [1426-1429]. Insight into the mechanisms of apoptosis and negative regulation of cell growth would contribute to understanding ageing processes and the regulation of cell mass by morphogenic sculpting [1236].

1.5.2.2 Chalones

A paradigm can be envisaged whereby polyamines and their oxidized derivatives form part of an integrated biochemical system regulating cell proliferation, by means of stimulating substances (polyamines) and inhibiting substances (oxidized polyamines) [93]. The oxidized polyamines have been considered as 'chalones'. Chalones are defined as endogenous cell-specific but species non-specific inhibitors of cell proliferation [1430], and have potential use in the control of neoplasia [1431,1432]. Allen *et al.* identified a thymic inhibitor of lymphocyte transformation as a spermine complex and suggested that its activation by amine oxidase produced an inhibitory factor, suggested to be a lymphocyte 'chalone' [1433]. This idea has been challenged on the basis that the inhibition by endogenous aminoaldehydes is unlikely to be cell specific [1419]. Nevertheless, the findings suggest the possibility that the oxidized polyamines might have chalone-like properties [1434]. Inhibition of cell proliferation by aminoaldehydes may be prevented by selective expression of aldehyde dehydrogenase or other detoxification mechanisms (*q.v.* §1.5 *et seq.*). The lability of the aminoaldehydes formed by the action of PAOs could partly explain the difficulties experienced in isolating chalones from biological materials. The low molecular weight of the aminoaldehydes and their tendency to bind to proteins [1283] could explain the progressive decrease in the reported molecular weight of lymphocyte chalone preparations, from 170,000 to less than 1,000 [1435].

1.5.3 Anti-inflammatory

Polyamines have been observed to have anti-inflammatory activity. Their effects may be mediated by products of their oxidation or by the polyamines themselves. In some situations the action of PAOs seems to be important in limiting the inflammatory response. The effects of amine oxidases appear to be two-fold. They are involved in the production of oxidized polyamines which may in turn downregulate cytokine production or affect leukocytes. On the other hand, they may limit the

effect of inflammatory mediators such as histamine.

1.5.3.1 Glucocorticoid Effects

Polyamines appear to have biological actions similar to those of glucocorticoid anti-inflammatory drugs (e.g. dexamethasone) [1436]. Anti-inflammatory effects of polyamines were observed in a model of inflammation involving serotonin and carrageenan induced paw oedemata in rats [1436]. Vascular permeability may be affected by polyamines in a manner similar to the effects of glucocorticoids through the induction of the putative vascular permeability inhibitory protein 'vasoregulin' [1436,1437]. Their observed actions are not simply due to release of glucocorticoids, since adrenalectomized rats responded in the same way as normal rats [1436]. Polyamines can stabilise the mRNA of proteins such as vasoregulin and this may be a mode of anti-inflammatory activity. Theoharidies suggested that spermine and spermidine could inhibit calcium-dependent inflammatory processes through interference with calcium fluxes [85]. The role of polyamines is postulated to be as in Figure 1.15. The shortlasting inhibitory actions of polyamines make them suitable as second mediators of glucocorticoids. It has been demonstrated that polyamines inhibit the activation of neutrophils, possibly by interfering with the production of second messengers in the hydrolysis of poly-phosphoinositides [1438,1439].

Intraperitoneal injection of polyamines prior to subcutaneous injection of turpentine, a chemical inflammatory agent, partially counteracted an increase in the inflammatory marker, serum α_2-macroglobulin. It is possible that polyamines (or their metabolites) inhibit the release of inflammatory mediators, so that anti-inflammatory proteins are not synthesized in the liver [1440]. Other studies also suggest the inhibition of inflammatory mediator release by polyamines as an anti-inflammatory mechanism [1441].

1.5.3.2 Rheumatoid Arthritis

Polyamines released as a consequence of tissue damage [128,353,1443,1444] and polyamine oxidizing enzymes found in synovial fluid from patients with rheumatoid arthritis [798] may contribute to the anti-inflammatory activity of these [1445] and other inflammatory exudates. PAO may be released from inflammatory macrophages and/or pannus tissue associated with the affected joints of rheumatoid arthritis patients.

Il-2 production by normal and rheumatoid arthritis peripheral blood mononuclear cells is downregulated by products of polyamine oxidation [1386,1444]. IL-2 is made by most T lymphocytes during the early stages of the immune response, it plays a central role in the

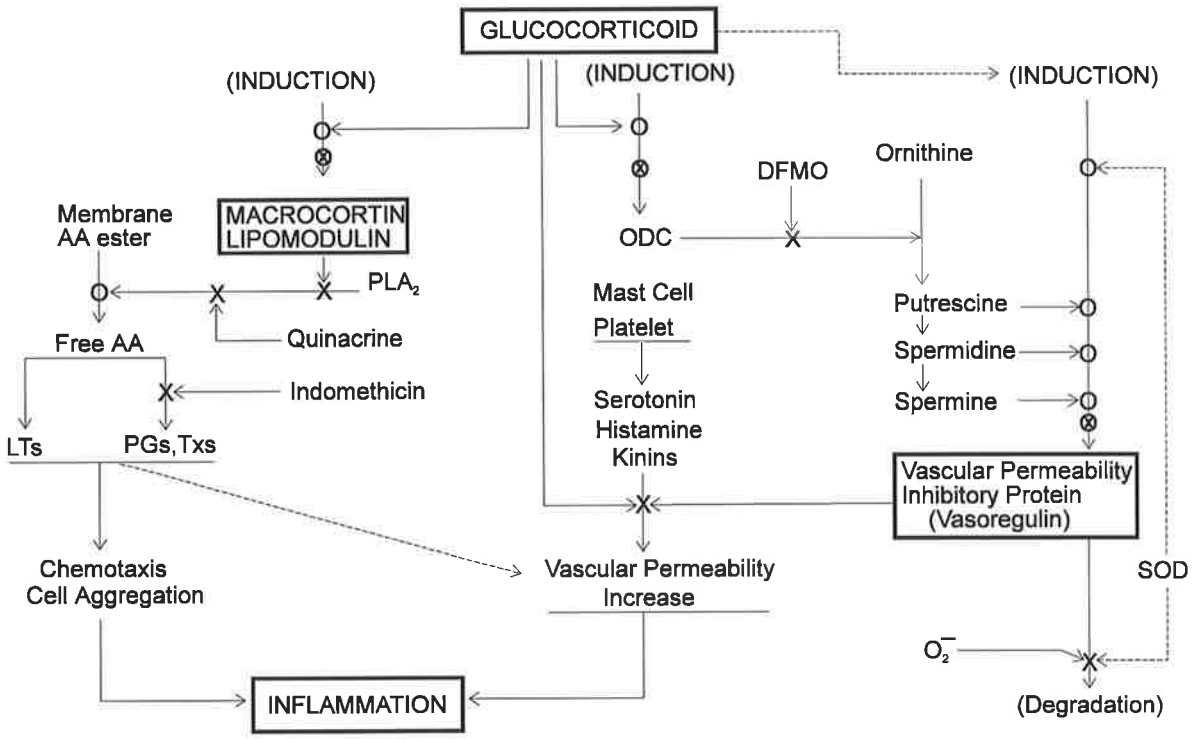


FIGURE 1.15. Possible Role of Polyamines and Glucocorticoids in Inflammation. ODC: ornithine decarboxylase, DFMO: α-difluoromethylornithine, AA: arachadonic acid, PGs: prostaglandins, Txs: thromboxanes, LTs: leukotrienes, PLA_2: phospholipase A_2. (o) shows stimulation, (×) shows inhibition, (⊗) is a site that can be blocked by cyclohexamide. Glucocorticoids provoke the induction of ODC to form putrescine, spermidine and spermine which increase the synthesis of vascular permeability protein (vasoregulin). Direct induction of vasoregulin should not be overlooked. The level of vasoregulin is also maintained by superoxide dismutase (SOD). Cycloheximide blocks the anti-inflammatory effects of glucocorticoids, polyamines and SOD. The postulated roles of PLA_2 inhibitors, macrocortin and lipomodulin, are also presented. Direct inhibition of vascular permeability by glucocorticoids may also exist. The proportion of direct and indirect participation by glucocorticoids is not clear. (After Oyanagui [1436].) In another report SOD is reported as an inactivator of vasoregulin rather than stimulating the synthesis of this protein [1442].

expansion of antigen selected lymphocytes and in the induction of their effector functions [1446]. In a collagen-induced arthritis model [1447], a link between rheumatoid arthritis and polyamine synthesis in peripheral blood mononuclear cells has been reported [1448]. Flescher *et al.* found that blocking polyamine production with ornithine decarboxylase inhibitors resulted in increased Il-2 production by peripheral blood mononuclear cells from rheumatoid arthritis patients (RA PBMC) [1444]. Moreover, PAO inhibitors increased the Il-2 production by RA PBMC [1444,1449]. The concentrations of polyamines are elevated (2–20 *fold*) in rheumatoid arthritis blood and synovial fluid mononuclear cells [1444], and significant levels of PAO have been found in rheumatoid arthritis synovial fluid [798]. Monocytes are activated in rheumatoid arthritis synovial effusions and have been shown to be a source of PAO activity in mononuclear cell populations [1444]. Thus, both the enzyme and its substrates are present at the rheumatoid arthritis inflammatory site and their interaction may contribute to the decreased Il-2 production

characteristic of rheumatoid arthritis synovial fluid mononuclear cells (RA SF MNC). The production of IL-2 by RA SF MNC is inversely correlated with the concentration of polyamines in these cells [1449]. So, in rheumatoid arthritis, polyamine dependent inhibition of IL-2 production may contribute to the inhibition of T cell proliferation. This might be a compensatory and protective mechanism designed to limit the T cell-driven destructive rheumatoid process [1444]. Furthermore, spermine inhibits the depolymerisation of hyaluronic acid and may protect against inflammatory damage of the joints [318]. One of the characteristics of inflammatory arthritis is the loss of viscosity of the synovial fluid due to the depolymerisation of hyaluronic acid; the synovial fluid loses it lubricating properties and causes friction in the joint [1450]. It is thought that in synovial fluid, the 'antioxidant' ceruloplasmin protects joints against inflammatory damage [1451]. Ceruloplasmin has enzymatic activity similar to that of other amine oxidases; it is possible that mechanisms similar to those described above occur. Further characterization of the amine oxidases in synovial fluid appears necessary to clarify this point. Polyamine oxidation products may also play a role in the T cell hyporesponse seen in RA patients.

1.5.3.3 Neutrophil Activity

At subcytotoxic concentrations, products of the polyamine–PAO system are thought to inhibit the respiratory cell burst (of hexose monophosphate (HMP) shunt activity [1372]) of stimulated polymorphonuclear leucocytes (neutrophils, PMN) [1452]. This may limit inflammatory reactions by depressing oxidative activity and locomotion of cells at inflammatory sites. The inhibition of the burst in HMP shunt activity was attributable to aminoaldehydes. Catalase, added to remove H_2O_2, failed to prevent inhibition of the respiratory burst.

The respiratory burst in neutrophils is associated with membrane changes as a consequence of the interaction of opsonised particles with membrane components [1372]. Neutrophils release a variety of inflammatory mediators in response to membrane perturbation. Many of these substances cause host tissue damage when released in excess. PAO and polyamines, which have been found in inflammatory exudates, may function to limit excessive tissue damage by regulating neutrophil activity. Moreover, oxidized polyamines inhibit human neutrophil migration, both random and chemotactic, at noncytotoxic concentrations [1453]. These observations may explain the anti-inflammatory activity found in inflammatory exudates, such as arthritic rat plasma, human pregnancy serum, human peritoneal fluids, extracts from regenerating liver following damage, sponge exudates in rats and human rheumatoid synovial fluids [1445,1454-1460]. It has been observed that spermine and putrescine are anti-inflammatory *in vivo* in the carrageenan-induced oedema rat model and in the adjuvant induced arthritic rat model [327]. Oxidized polyamines may, by depressing the oxidative metabolism and locomotion of

neutrophils, limit the inflammatory reaction. Macrophages, with their capacity to secrete PAOs on stimulation [955], could inhibit excessive neutrophil damage upon their later arrival at inflammatory sites. Oxidized polyamines may arise as a consequence of tissue damage and serve to further limit neutrophil accumulation, concomitantly trapping cells already present at inflammatory sites (*q.v.* §1.5.3.2).

The regulation of inflammation by the PAO–polyamine system is supported by the findings of Flescher *et al.* who obtained evidence suggesting that the polyamine–PAO system is involved in the down-regulation of immune reactivity associated with rheumatoid arthritis [1444] (*vide supra*). The depression of cytokine production along with the capacity of oxidized polyamines to inhibit the migration and oxygen radical production of neutrophils [1452,1453] could contribute to the anti-inflammatory activity of synovial fluid from rheumatoid arthritis patients [1445], arthritic rat plasma [1455,1456] and other inflammatory exudates.

Other observed anti-inflammatory activities of polyamines include: the formation of aminoaldehydes by the polyamine–pregnancy PAO system that inhibit lymphocyte proliferation *in vitro*, and may contribute to the protection of feto–placental allograft *in vivo* [1404,1407]; spermidine substitutes for hydrocortisone in enhancement of milk protein synthesis by cultured mammary epithelium *in vitro* [1461]; and suppression of human platelet aggregation by spermine and spermidine [326,1462].

1.5.3.4 Histamine Regulation

Histamine is well documented as a mediator of inflammatory processes such as acute hypersensitivity and allergic reaction [1187,1463,1464]. Responses to histamine can be terminated by metabolic transformation through the action of histamine methyltransferase (HMT) (EC 2.1.1.8) [1465] and DAO/histaminase (EC 1.4.3.6) [1466]. HMT catalyses the nonoxidative *N*-methylation of histamine to 1,4-methyl histamine. Histamine methyl transferase is not blocked by the DAO specific inhibitor aminoguanidine [737,1467] and is thought to be the main normal route of histamine metabolism in humans [1465]. Histamine is oxidized to imidazole acetaldehyde by DAO [1463] (and then to imidazole acetic acid by aldehyde dehydrogenases).

Several control mechanisms involving granulocytes have been described for mediators of inflammatory processes [1468]. Some of these control mechanisms depend on enzymes that have capacity to inactivate inflammatory mediators of mast cells and basophils [1468], such as histamine. Mast cells are known for their involvement in allergic reactions [1469] during which they secrete many biologically active molecules upon stimulation by IgE [1470].

Histaminase activity (EC 1.4.3.6) has been demonstrated in human eosinophils and neutrophils [800]. Increased eosinophil histaminase content has been found in acute inflammatory

diseases, including asthma, urticaria and parasitic infection [804]. The increased levels of histamine that have been found in acute anaphylaxis; as well as histamine metabolites in patients with asthma [1471-1473], may reflect an early activation of histamine metabolizing enzymes in acute inflammatory states. An increase in the level of blood DAO has been observed in different animal species following anaphylactic shock [1257,1474,1475].

Heparin causes a marked increase in plasma DAO in humans [674,675,700]. In animals release of DAO is mediated by heparin [1018,1240,1257]. The concomitant release of histamine and heparin from mast cells may provide the basis of a simple regulatory control for limiting the effects of histamine [961,1257].

Eosinophils and neutrophils release histaminase during incubation with zymosan opsonized with normal human serum [803]; zymosan prepared with heated serum does not induce histaminase release [1084,1085]. Noncytolytic release of histaminase from polymorphonuclear cells is dependent solely on particle bound complement fragment C3b [803], and was a result of the interaction of particle bound C3b at the cell surface. However, phagocytosis of the C3b coated particles was not required. In contrast, histaminase release from eosinophils was dependent on phagocytosis of 'opsonized' particles; though other proteins as well as C3b, were able to opsonize particles. These studies suggested a dual role for complement (C3) in modulating a component of vascular permeability: release of a vasoactive mediator, histamine, by the action of C3a and C5a on mast cells; and the release of histaminase, from neutrophils and eosinophils to inactivate the mediator by C3b.

Histaminase thus represents a potential homeostatic control mechanism for limiting the action of histamine [449,1018,1257]. The induction of eosinophil histaminase and/or other controlling enzymes, such as histamine methyltransferase, may contribute to the modulation of histamine-mediated inflammatory reactions. The data do not exclude a role of the eosinophils or other leukocytes in modulating inflammatory reactions by other mechanisms. The finding of histaminase activity in neutrophils suggests that these cells may limit the effects of histamine at sites of inflammatory exudates.

Leukocyte histaminase is physicochemically and functionally similar to histaminase isolated from human placenta [799,800] (*q.v.* §1.6.8.2). However, only limited studies have been done in this area. The neutrophil and eosinophil enzyme activities had similar elution volumes from Sephadex G-200 with histaminase partially purified from human placenta by the method of Paolucci *et al.* [742]. The enzymes eluted from the column at a relative molecular mass stated by the authors to be approx. 160,000, although recalculation from their data indicates a higher M_r, closer to 230,000. The enzymes were found to have similar K_ms (in the order of 2×10^{-6} M for histamine) and similar K_is (in the order of 5×10^{-9} M) for aminoguanidine. Morel *et al.* report

that on the basis of substrate specificity, effects of specific inhibitors, and optimal physicochemical conditions for enzyme activity (pH, ionic strength, temperature) that functional similarities exist between leukocytes and placental histaminase [799]. Human leukocyte histaminase was principally located to the 27,000 × *g* specific granule rich fraction of eosinophil and neutrophil homogenates by subcellular fractionation of resting cells on sucrose density gradients. It is released from neutrophils in response to secretagogues preferential for the specific granule, such as calcium ionophore A23187, phorbol myristate acetate (PMA), formyl-methionyl-leucyl-phenylalanine (fMLP), and concanavalin A [805]. The finding of eosinophil and neutrophil histaminase in granule rich fractions stands in contrast to reports of histaminase localization to microsomes in rabbit liver [950], cytoplasm in dog and rat intestine [992] and in peroxisomes in rat liver [164,972,973]. These differences suggest that control of release of histaminase from granulocytes and its physiologic function may differ from that of the corresponding enzyme found in other tissues (*q.v.* §1.3.2.5hii). In contrast, histamine methyltransferase is detected in monocytes, but not in granulocytes, eosinophils, lymphocytes or platelets, and is localized to the 100,000 × *g* cell sap supernatant fraction [800].

Work in guinea pigs showed that serum levels of histaminase are increased during experimental inflammation induced by subcutaneous injection of turpentine [1476,1477] and during anaphylactic shock [1257]. Spermine and spermidine inhibited the immunologically (IgE) stimulated mast cell secretion of both histamine and serotonin in a time- and concentration-dependent, non-cytotoxic manner [1441]. This effect occurred only in the presence of calf serum and could be attributed to the aldehydes produced from spermine and spermidine by the action of PAO [168]. Inhibition of PAO blocked the polyamine effect [1441]. Aldehydes derived from other amines metabolized by the same enzyme (e.g. benzaldehyde) did not have the same effect as those derived from spermine and spermidine. This suggests that the inhibition of mast cell secretion is therefore specific for the aldehydes of the naturally occurring polyamines and is not a general aldehyde effect or a non-specific effect on the plasma membrane. Other products of polyamine oxidation, ammonia and hydrogen peroxide, do not appear to play a role in the observed inhibition since benzylamine, which also produces these products, did not cause inhibition. The aldehydes formed by enzymatic polyamine oxidation must bind to the cells and cause the observed effects, since washing the cells and resuspending them in a polyamine and calf serum free medium did not abolish the inhibition nor did subsequent addition of the enzyme inhibitor phenylhydrazine. Simultaneous addition of phenylhydrazine blocked the inhibitory effect of the polyamines. The results suggest that the polyamines regulate mast cell secretion through the metabolic products of PAO providing another arm to the control of histamine.

The ability of polyamine metabolites to inhibit mast cell secretion may be of therapeutic

importance in other pathophysiological conditions where mast cells appear to be involved, such as migraine [1192] and multiple sclerosis [1478]. Syndromes such as multiple sclerosis and intestinal cystitis have been associated with mast cell activation [1479,1480]. It is noteworthy that allergic and other autoimmune syndromes improve considerably during pregnancy [1481,1482].

1.5.4 Immunoregulatory

There is evidence that the PAO–polyamine system may be involved in immunosuppressive processes. Cells, tissue extracts, and fluids shown to be immunosuppressive are known to contain high levels of polyamines and PAO [751,771,772]. These include sperm and seminal plasma [1483-1493], amniotic fluid [494,1494,1495], placental extracts [1496], tumour extracts [1497], serum from tumour bearers [1498,1499], ascites from tumour bearers [1500,1501], extracts of thymus and spleen [1433], and supernatants from both normal [1502,1503] and neoplastic [1504,1505] cells cultured *in vitro.*

The PAO–polyamine system can reversibly inhibit *in vitro* parameters of immunity [1402]. Potent reversible suppression of the mitogenic transformation of lymphocytes occurs at subcytotoxic concentrations of oxidized polyamines *in vitro*. Suppression of the mitogenic transformation was measured by inhibition of the lymphocyte DNA synthetic response to mitogens, determined by [^{125}I]iododeoxyuridine (IUdR) incorporation. The later the polyamine was added after inception of the lymphocyte cultures, the greater the amount required to obtain the same level of inhibition [1402,1403]. This contradicts acute toxicity as a mechanism [1287], but is consistent with an affect on an early event in cell differentiation. Inhibitors of polyamine oxidation enhance mitogenically induced lymphocyte proliferation [1402].

Theoretically, mitogen stimulation may be blocked by a factor that blocks receptor sites on the lymphocyte or inactivates the mitogenic property of the mitogen. Autologous plasma from patients with alcoholic cirrhosis of the liver inhibited phytohaemagglutinin (PHA) stimulated lymphocyte transformation [1506]. Similar results were obtained with plasma from patients with allergic rhinitis [1506], suggesting the release of a humoral immunoregulatory factor; perhaps amine oxidase, from sensitised cells [1507] or damaged liver cells [955]. A similar reduction of PHA stimulated lymphocyte transformation was seen in patients with breast cancer, both using lymphocytes from cancer patients with autologous serum and lymphocytes from normal individuals cultured in serum from cancer patients [332]. It is well known that there is an increased level of PAO in the serum of cancer patients (*q.v.* §1.4.5). Again, in patients with uremia [1508], a disease that is associated with a suppression of the immune response [1509,1510], uremic plasma has an inhibitory effect on PHA stimulated lymphocyte transformation *in vitro* [1508]. Perhaps kidney PAO is liberated by renal damage in this disease.

The proliferative response of lymphocytes to antigens, measured by the incorporation of [^{3}H]thymidine into DNA, has been used to quantitatively assess immunological responsiveness [1511]. The *in vitro* DNA synthetic response of lymphoid cells stimulated by mitogens or irradiated allogenic cells can be blocked by micromolar quantities of polyamines spermine and spermidine in the presence of amine oxidase containing serum [1403]. Lower doses of polyamine suppressed responses to an optimal dose of the B cell mitogens bacterial lipopolysaccharide (LPS) or pokeweed mitogen (PWM), whereas higher doses of polyamine were required to suppress the response obtained with optimal doses of the T cell mitogens phytohaemagglutinin (PHA) and concanavalin A (con A). The presence of oxidized polyamines inhibits the ability of murine lymphocytes to recognise irradiated allogenic cells; or in mixed lymphocyte reaction (MLR) to mount a noncomplement dependent cytolytic response [1402].

The immunological response of lymphocytes is also affected by variations in histamine concentration [1512].

Antigenic challenges which fail to elicit an appropriate immune response include neoplasia and conception. Neoplastic cells [155], seminal fluid and spermatozoa [21], fetal and placental cells [1513] possess specific antigens but avoid immunological rejection. All contain or produce polyamines in relatively high concentration. It has been postulated that the placenta accomplishes its fetal protective role by elaborating an immunosuppressive material that 'switches off' potentially harmful maternal lymphocytes in the microenvironment of the placenta. An immunosuppressive factor has been isolated from the placenta and characterized as spermine [1403] (*vide* 1.6.7.1).

By inhibiting the development of immune responses in their early stages, polyamines could allow the initial development and continued growth of neoplastic cells. *In vitro* fertilisation supernatants contain low molecular weight substances found to be inhibitory to the proliferation of con A stimulated lymphocytes in the presence of non-heat treated FBS [1514]. Further studies indicated that the polyamines spermine and spermidine were most likely to be responsible for the suppressor activity [1515,1516]. These polyamines have been shown to correlate with successful implantation and are essential for proliferation of eukaryotic cells, including those of the early embryo [1515,1517,1518]. Spermine at a concentration of 9 μM produced almost complete suppression of cell lymphoproliferation while spermidine was about 10× less potent. DAO-containing mouse amniotic fluid converts polyamines into immunosuppressive products that inhibit the LPS stimulated mitogenesis of mouse spleen cells [494]. PAO may play a role in the immunosuppression associated with pregnancy (*q.v.* §1.6.7.1). Immunosuppression is proposed to be due to oxidized polyamines acting at a cellular level decreasing Il-2 production (*q.v.* §1.5.3.2). Cells involved in the immune response were more susceptible to inhibition of proliferation by

oxidized polyamines than other cells such as fibroblasts.

Suppression of graft vs. host disease in a histoincompatible mouse model by *ex vivo* treatment of bone marrow with oxidized spermine in *in vitro* assays showed: that the T cell proliferation on con A stimulation and natural killer activity of spleen cells were differentially more sensitive to the spermine dialdehyde than myeloid cells required for reconstitution [1519]. The myeloid cells have higher levels of aldehyde dehydrogenase, which probably confers a protective effect [1520].

Oxidized polyamines inhibited IgM production from murine lymphocytes stimulated with LPS. LPS selectively stimulates B lymphocytes inducing proliferation and antibody production [1521].

It is pertinent that several compounds that exhibit an immunomodulatory effect are inhibitors of DAO [1410]. In particular, levamisole and imidazole [732,769] are both immunomodulators [1522,1523]. Cimetidine, which possesses immunomodulatory effects [1524], is also an inhibitor of human placental DAO [1525]. The mechanisms by which these immunotherapeutic reagents enhance the immune response are not yet clear but the immunomodulating effect may be linked to enzyme inhibition of both DAO and alkaline phosphatase [1410]. Aminoguanidine, a potent DAO inhibitor, increases the proliferative T cell response as measured by thymidine incorporation (DNA replication) in tetanus toxoid-stimulated normal human lymphocytes [1526]. Enzyme inhibitors that were found to enhance con A-induced lymphocyte proliferation also inhibit DAO suggesting a negative role for DAO in the proliferation process [1410].

Human oocytes cultured *in vitro* release a substance with nonspecific immunosuppressive and antiproliferative activity that has been correlated with successful implantation [1514]. The activity is thought to be attributable to polyamines in association with PAO [1514,1515,1518,1527] (*vide supra*).

It seems likely that polyamine oxidation contributes to immunosuppressive activity. However, whether polyamine oxidation has any immunosuppressive function *in vivo* is not yet clear.

1.6 PREGNANCY-ASSOCIATED AMINE OXIDASES

1.6.1 Recognition in the Placenta and Pregnancy Serum

Amine oxidase activity associated with human pregnancy has been recognized since 1937 when Danforth and Gorham reported histaminase activity in term placentae during their search for histamine, which was thought to have oxytocic properties [481,736]. Since then, amine oxidase activity, identified as histaminase, diamine or polyamine oxidase activity has been reported to be associated with pregnancy [442]; early studies include [485-488,490,683,688,689,691,694-696,702,885]. The human term placenta contains higher levels of the amine oxidase than any other normal tissue analysed [449], although liver, intestine, and kidney also contain high levels of amine oxidase activity [410] (*q.v.* §1.3.2.5).

In 1938 Marcou *et al.* reported an increased level of histaminase in the blood of the uterine cavity [688]. A dramatic increase in maternal serum amine oxidase is observed with increasing gestational age. This progressive increase in activity is detectable as early as 9–28 days post conception [714] and increases steadily until about 21–23 weeks, then plateauing until term. Plasma levels of the enzyme are elevated as much as 500–1000 *fold* over basal levels during pregnancy [686,690]. Considerable individual variation in activity is observed [663,722,1528] (Figure 1.18). *Post partum*, the activity declines to basal levels within several days (72 h) [690,692,698,709]. After abortion enzyme activity returns to basal levels within a similar interval [699]. In children, men and non-pregnant women blood serum amine oxidase activity is normally very low [664,686,690,709] (cf. §1.4.5). Similar findings have been made in rats [206,989,1529,1530] and guinea pigs [1014].

The pregnancy-associated amine oxidase activity has been reported as: histaminase [456,686,689-695,699,714]; DAO [488,663,684,697,698,700-711,715-718,720,1528,1531]; spermidine oxidase [719] and PAO (spermine as a substrate) [169,439,722,724]. It is likely that all of these observed activities are due to the same enzyme. All increases of amine oxidase activity during pregnancy are similar (*vide* Figure 1.16). Re-evaluation of the linear increase in PAO [169,439,724] indicated that, like 'histaminase' activity, this activity also plateaus. The correlation coefficient between a radiochemical method for determining DAO and a biological method for determining histaminase [690] in 100 samples of pregnancy plasma was 0.98 [702].

The increase in serum amine oxidase activity reflects the growth of the feto–placental unit [1067]. The curve of DAO concentration throughout pregnancy has a sigmoid shape when plotted on an arithmetic scale [1528]. This conceals the exponential rise in concentration when the levels are plotted on a logarithmic scale (Figure 1.16) [690,702]. The logarithmic plots clearly illustrate that the rate of increase in concentration is maximal in early pregnancy and decreases with time.

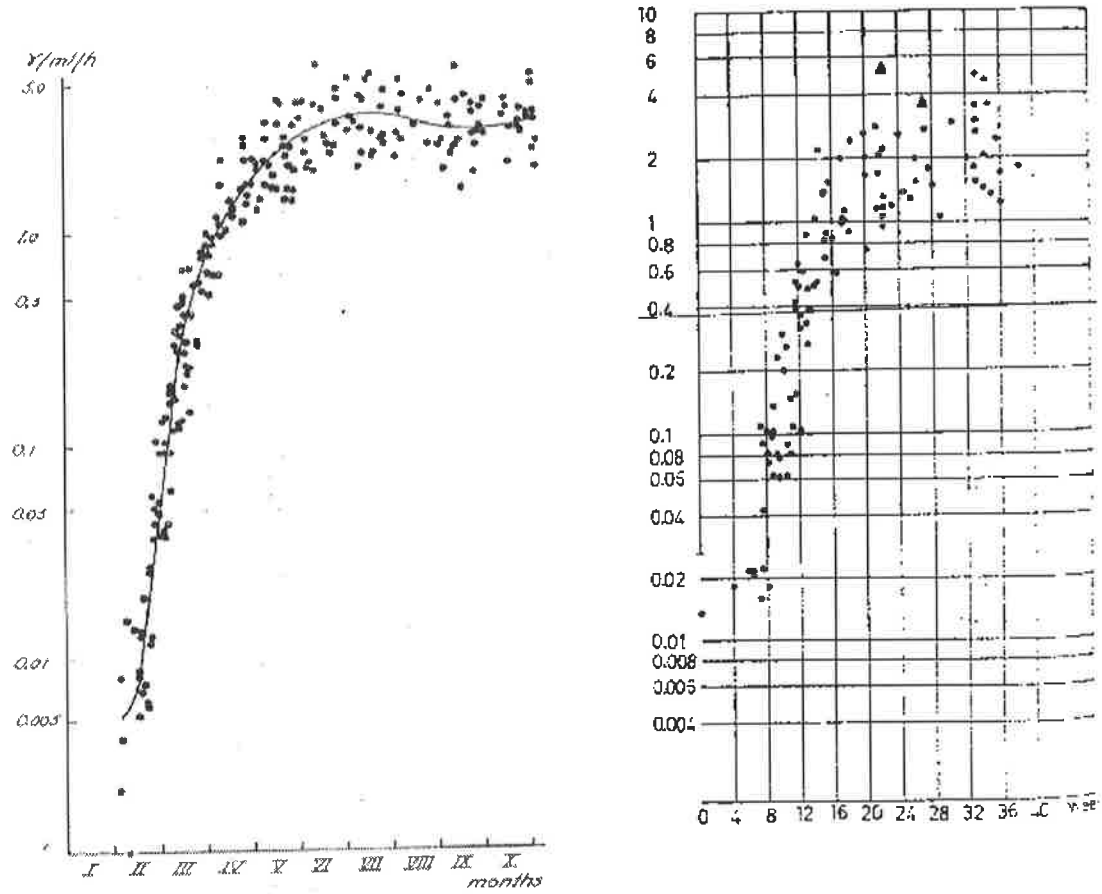


FIGURE 1.16. Amine Oxidase Activity in Plasma of Normal Pregnant Women. Histaminase activity expressed as γ (μg) histamine inactivated per ml plasma per hour is shown on the left hand side of the Figure (from Ahlmark [690]). Months of pregnancy calculated from the first day of last menstruation are indicated on the abscissa. DAO activity expressed as U/l by the method of Tryding [700] is shown on the right hand side of the Figure (from Tryding and Willert [702]). Weeks of pregnancy calculated from the first day of last menstruation are indicated on the abscissa. (▲ = twin pregnancies.)

Benzylamine oxidase behaves differently [659,712,815,816]. No correlation was found between benzylamine oxidase values in maternal blood and fetal growth. Benzylamine oxidase [816,1153] appears to be a distinct enzyme although its inhibitor sensitivities resemble those of the pregnancy-associated amine oxidase, in particular its sensitivity to quinacrine and aminoguanidine. Location of the activity of the enzyme in fetal lung and gut, well-known sites of human DAO activity, is another similarity. Benzylamine oxidase was also found to act on polyamines. Clarification of this point awaits a more thorough characterization of the enzymes.

1.6.2 Different Amine Oxidases in Pregnancy

The broad substrate specificity of pregnancy-associated amine oxidases has lead to their confusing nomenclature (*qq.v.* §1.3.1 and 1.3.6). Because of its action on the secondary amino groups of polyamines, the enzyme isolated from human placenta does not fit precisely into either the classical monoamine oxidase class (EC 1.4.3.4) or DAO class (EC 1.4.3.6) [744]. Reports of amine oxidase activity have often been made using a single substrate, which may merely reflect

the use of a conveniently available assay or what was known about the enzyme at the time. It is now apparent that a number of oxidases are capable of acting on a single amine and that a single enzyme associated with human pregnancy is capable of acting on histamine (histaminase), putrescine and cadaverine (diamine oxidase) or spermine, spermidine and their acetyl derivatives (PAO) and that the trivial nomenclature used in the literature is *ad hoc*.

Pregnancy serum oxidizes histamine [690], putrescine [726], benzylamine derivatives [1154], spermine [722,726] and acetylated polyamines [717,726]. There may be a family of amine oxidases responsible for these activities as suggested by Crabbe [444]. However, it remains to separate these activities. It appears likely that a single enzyme could account for all of the observed activities, although cDNA sequences for variant forms of placental amine oxidizing enzymes have been reported [758,1080]. The available data is not always sufficient to make direct comparisons between the results from different laboratories. However, the pregnancy-associated polyamine oxidase appears to be distinct from other amine oxidases such as ceruloplasmin, benzylamine oxidase and lysyl oxidase. In contrast to the pregnancy-associated amine oxidase, ceruloplasmin has a sky-blue colour and a different molecular weight [659]. Moreover its amino acid sequence is different [1532]. Benzylamine oxidase (monoamine oxidase?) serum levels in pregnant women were not statistically different from levels found in non-pregnant women [449,659], and lysyl oxidase has a molecular weight and amino acid sequence that are both different to that of the pregnancy-associated enzyme [1115,1533]. Methods in which benzylamine or its derivatives are used as the substrate may only measure monoamine oxidase activity [659,712,815]. Monoamine oxidase has been reported in placental tissue [1069,1078-1080] and soluble mitochondrial monoamine oxidase [1070] is also present (*qq.v.* §1.3.2.5a,e). DAO is distinct from benzylamine oxidase [659], the soluble monoamine oxidase from human plasma [659] and monoamine oxidase from human placenta [712,1070] by its failure to deaminate benzylamine or tryptamine. It may be that a placental monoamine is copper- and TPQ-containing, but the rest of the cDNA sequence is distinct and does not code for the same amino acid sequence as reported for the copper-containing amiloride-sensitive diamine oxidase whose *N*-terminal sequence is different [47,758]. The purified pregnancy-associated enzyme is completely inhibited by low concentrations of aminoguanidine and is less affected by semicarbazide or iproniazid [712].

1.6.3 Co-Identity of Amine Oxidases in Pregnancy

It is most likely that the human pregnancy-associated enzymes 'histaminase', 'diamine oxidase' and 'polyamine oxidase' are identical (*vide supra*). Support for this includes the inability to separate the putrescine, spermidine and spermine oxidizing activity during purification [726] and

multisubstrate specificity of purified enzyme. All show a similar increase during pregnancy (*q.v.* §1.6.1). Evidence has been reported for the identity of histaminase and DAO [702,741,1516]. Histaminase oxidatively deaminates histamine and aliphatic amines such as putrescine and cadaverine [443]. DAO has been identified with spermidine oxidase [719]; DAO and spermidine oxidase activities could not be separated and appeared to copurify. No preparation of spermidine oxidase has allowed the product putrescine to accumulate. The products observed from spermidine oxidation appeared to be products of putrescine oxidation. The enzymes had identical pH and temperature optima. Aminoguanidine inhibited enzyme activity with both spermidine and putrescine as substrates [719]. The separation of spermine, spermidine, putrescine, acetyl PAO activity was not achieved by Morgan [726] or Gahl [1534]. Perhaps the different enzymes are so physicochemically similar that their separation has not been able to be accomplished.

1.6.4 Substrate Pattern

A strong pH dependency of the Michaelis constant for pregnancy-associated amine oxidases has been reported [719,763], this suggests that the enzyme reacts with the unprotonated form of the amino group of the substrate. In view of the wide variation of conditions used to determine K_ms by various groups, comparison of these values is difficult. Many authors have merely reported the relative reaction velocities for various substrates. Again, with the variety of conditions used to measure these rates, often only one substrate concentration has been used and substrate inhibition not taken into account. A summary of steady-state kinetics for some representative substrates with purified enzymes is presented in Table 1.7 (*q.v.* §1.3.6).

The site of polyamine cleavage by pregnancy-associated enzymes is at secondary amino groups [439,442,726].

1.6.5 Inhibitor Pattern

Aminoguanidine, a carbonyl group reagent, is considered a specific inhibitor for DAO, [1015] and inhibits both DAO and spermidine oxidase activity of partially purified pregnancy serum enzyme [719], distinguishing its activity from that of monoamine oxidase. Isoniazid, an inhibitor of 'pyridoxal phosphate' containing enzymes, inhibits the amine oxidase in sheep and bovine serum [49], but not the flavin-containing rat liver polyamine oxidase, which is inhibited by quinacrine [164]. Yet quinacrine, an inhibitor of flavin containing enzymes, inhibits the pregnancy serum enzyme strongly. The pregnancy serum enzyme is also inhibited by isoniazid, but not as potently as with quinacrine [722] (*qq.v.* §1.3.7 and Table 1.8). The lack of inhibitor specificity makes it difficult to distinguish the different amine oxidases by their sensitivity to different inhibitors.

1.6.6 Localization of Pregnancy-associated Amine Oxidase Activity

1.6.6.1 Placenta

The maternal decidual component of the placenta is considered to be the source of the elevated serum amine oxidase activity associated with pregnancy (*q.v.* §1.3.2.5e). Swanberg, who used gross tissue dissection techniques followed by biological assay methods, demonstrated high enzyme activity in the *pars materna* (*decidua basalis* adjacent to the uterine wall) of the placenta and little activity associated with the *pars foetalis* (fetal trophoblastic) portion [686]. The relationships between the placental parts may be found in references [1064,1535-1539]. Clean separation of fetal and maternal parts of the placenta by gross dissection is technically difficult because of the intimate contact of the maternal and fetal elements and the septae that extend from the decidua into the fetal placenta. Some of the chorionic villi are firmly attached to the decidua. Decidual cells in the basal plate are in close relation to the trophoblast and trophoblast cells may migrate into the decidua [1064], so incomplete separation of the two tissue types cannot be excluded. A high concentration of amine oxidase is found in the maternal decidua compared with the myometrium [663,686,689] (*q.v.* §1.3.2.5e).

Several pathologic observations suggest that the source of elevated serum amine oxidase during pregnancy is not trophoblastic. In cases of choriocarcinoma and hydatidiform mole (a trophoblastic tumour), the maternal plasma contains normal or near normal nonpregnant levels of amine oxidase [660,1540], although increased levels of serum DAO activity above normal in have been reported in patients with hydatidiform moles. In these patients the amine oxidase activity was considered to correspond to expected activity from a normal placenta of the same gestational age [686,690,707]; Swanberg [690] noted that the mole itself contained no activity. Patients with trophoblastic disease (choriocarcinoma) did not generally show any increase of DAO activity above normal [707]. It pertinent that the level of serum DAO continued to rise in cases where the fetus was expelled and the placenta was retained, and even the retention of a small piece of placenta was found to sustain enzyme formation [1540,1541]. After the placenta is completely removed the enzyme level promptly falls.

The localization of pregnancy-associated amine oxidase activity to the decidua has been confirmed by a number of studies. PAO activity in the decidua is significantly higher than in placenta [420]. Southern localized amine oxidase to the decidua by a biochemical analysis of the enzyme in maternal and fetal plasma and tissues at parturition [684]. Gunther and Glick assayed enzyme activity in microtome sections of the placenta and observed the highest activities in Nitabuch's membrane and the decidua [740]. Immunohistological methods have localized amine oxidase to decidual cells and placental septae, but not trophoblasts [750]. Serum remaining in the

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placenta resulted in lakes of staining in the intervillous spaces. Significant amounts of enzyme immunoreactivity were found in the intercellular space surrounding the decidual cells [749]. Immunohistochemical studies are limited in that they do not demonstrate the catalytic behaviour of the enzyme concerned. Nakos and Gossrau [48] have developed a cerium-diaminobenzidine-H_2O_2-cobalt method and have localized DAO to the decidual cells of the human, mouse and rat placenta. Activity was localized to the cytoplasm of decidual cells and in the surrounding extracellular matrix. Lin *et al.* estimate that a term placenta, which weighs 500 g and contains 50 g protein, may have as much as 20 mg histaminase [752]. If it is assumed that the enzyme is produced by the decidual cells, which make up less than 1% of the total placental mass, then the amount of histaminase produced by those cells is equal to about 4% of the total cellular proteins. This indicates that the production and excretion of histaminase is one of the major cellular functions of the decidual cells of the placenta.

In vitro perfusion studies of human placentae indicate an active production by the perfused placenta *in vitro* [663]. Holinka and Gurpide examined DAO activity in human endometrium at different stages of the menstrual cycle and in decidua from first trimester pregnancies [754]. Enzyme activity in decidual tissues was significantly higher than in the endometrium. Activities in endometrial tissue culture supernatants were generally below the limit of detection. However, the decidual cultures secreted large amounts of enzyme into the supernatant medium, greatly exceeding the initial tissue activities, suggesting *de novo* output of the enzyme rather than merely the release of stored enzyme. Serum factors stimulated DAO secretion.

Other evidence too, suggests a decidual cell origin for the amine oxidase. Histaminase is absent from nondeciduate placentas (swine, horse and dog) and present in deciduate placentas (rabbit, guinea pig and rat) containing maternal elements [686,690]. The histaminolytic activity in the placentas was in proportion to the amount of decidual tissue present.

1.6.6.2 Amniotic Fluid

Pregnancy-associated amine oxidase activity is found in amniotic fluid and its level increases as pregnancy progresses [463]. Conflicting reports exist regarding the actual levels of enzyme activity [463,663,684,703,708,716,725,760]. One study found a two-*fold* greater concentration in amniotic fluid when compared with serum at term [684]. Another found no significant difference [708], though in second trimester the amniotic concentration was lower than in serum, in agreement with Swanberg, who found lower levels in amniotic fluid than in serum at term [686]. Yet another study [711] found DAO levels in amniotic fluid were lower in fluid than in serum, but as pregnancy progresses the pattern is reversed. PAO activity in amniotic fluid parallels that found in peripheral blood serum, but values were lower throughout pregnancy [760].

Human pregnancy serum amine oxidase appears to be biochemically identical to the amniotic fluid enzyme [678]. It is not likely that activity exists in fetal urine or faeces, since fetal defecation ceases around mid-gestation [816]. Fetal pharyngeal aspirate is reported to contain benzylamine oxidase [816]. DAO has been found in fetal liver in both guinea pigs [408] and humans [783], and possibly in fetal lung and gut [816]. Enzyme levels found in fetal membrane homogenates, especially the amnion, are higher than those of placental homogenate, but not as high as the levels found in the decidua [760]. A transmembrane passage of the enzyme from the decidua is postulated to be a mechanism for the presence of the relatively large amounts of enzyme in amniotic fluid [684]. Tracer techniques show a rapid interchange of amniotic fluid with maternal circulation and this exchange is thought to occur primarily through the membranes [684]. The concentration of enzyme in the placenta is always higher than the parturition plasma level. Movement of the enzyme occurs down a concentration gradient by diffusion [716]. Indeed a number of studies suggest that the majority of proteins in amniotic fluid are serum type [1066]. However, maternal fluid of non-serum origin can enter the amniotic fluid from the uterine decidua and myometrium [1066]. The activity in the amniotic fluid most likely arises from the diffusion of the enzyme from the decidua through the membranes and into the amniotic fluid [708].

1.6.6.3 Serum

The dramatic increases in serum amine oxidase activities during pregnancy have already been discussed. A high degree of physicochemical similarity exists between the purified placental [712] and plasma enzymes [713]. The PAO found in maternal serum probably represents a diffusion of the enzyme, rather than a secretion into the circulation (Figure 1.17).

1.6.6.4 Retroplacental Fluid

Retroplacental fluid, which is comprised mainly of intervillous blood with admixture of placental and decidual interstitial fluid [1542], contains PAO activity 20 to 30 times higher than that found in pregnancy peripheral blood or uterine venous blood sera [420], corresponding to the high levels seen in the retroplacental decidua [663,684].

1.6.6.5 Fetal Circulation

Only very low PAO activity, relative to that found in maternal circulation, has been found in human fetal cord blood sera [420,663,698,708,719]. The differential is even more striking when it is considered that equivalent secretion into both circulations would yield higher levels in the fetus because of its smaller volume. Accepting that the enzyme source is decidual, the differential must

be the result of the barrier between the decidua and the fetal blood, consisting of basement membrane and capillary endothelium, whereas no barrier exists on the maternal side (Figure 1.17).

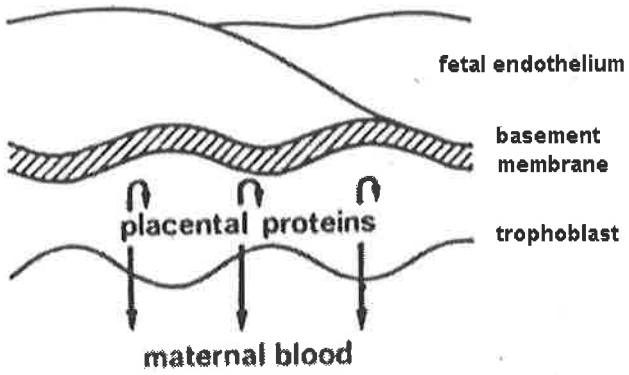

FIGURE 1.17. The Basement Membrane Barrier. The barrier prevents the entry of placental proteins into the fetal circulation. There is no such barrier to maternal blood and placental proteins are secreted exclusively into the mother (after Gordon and Chard [1068]).

1.6.7 Role of Pregnancy-associated Amine Oxidase

It would seem reasonable that an enzyme that is virtually absent from blood serum except during pregnancy should have a specific physiological function(s) in the gravid state. It is generally assumed that the enzyme plays some sort of protective role.

1.6.7.1 Protection of the Feto–Placental Allograft

Because of paternally inherited histocompatibility antigens, the conceptus can be considered as an intrauterine semiallograft that is not rejected by the mother: this constitutes the feto–placental allograft paradox [1538]. Among the mechanisms suggested for the survival of the feto–placental allograft is the production of an immunosuppressive factor during pregnancy. There is evidence of diminished immune responsiveness during pregnancy, including the depressed *in vitro* lymphocyte response to mitogens [1543,1544] (cf. §1.5.2). During pregnancy the size of lymph nodes draining the uterus is decreased due to depression of their germinal centres and a significant reduction in their cellular content [1545].

Human pregnancy serum inhibits mitogen-stimulated lymphocyte proliferation [1546-1548]. Crude human chorionic gonadotropin (hCG) preparations also inhibit mitogen stimulated lymphocyte proliferation [1549], but highly purified hCG did not influence the lymphocyte mitogenic response, suggesting that the lymphocyte suppression observed was due to additional, as yet unidentified, substances in the crude hormone preparations [1550,1551].

Maternal peripheral serum taken at early stages of pregnancy failed to inhibit PHA induced lymphocyte proliferation or the *in vitro* MLR, while late pregnancy serum had markedly suppressive effects [1547,1548]. Lymphocytes separated from uterine blood during the first trimester were significantly less responsive to PHA stimulation than lymphocytes separated from the peripheral blood of the same patients [1552]. This suggests that in early pregnancy an immunosuppressive factor is operating within the uterus, but is not yet detectable in the peripheral circulation. Human pregnancy serum (after about 15 weeks) but not non-pregnant human serum, will inhibit spontaneous lymphocyte proliferation (measured as *in vitro* uptake of [^{3}H]thymidine) in the presence of polyamines [1404].

Polyamines and PAO in bovine serum produce non-cytotoxic inhibition of cell proliferation [1402,1419,1433], in particular the inhibition of the mitogen stimulated DNA synthesis in murine lymphocytes, arresting proliferation in the G_1 phase, but not the S phase (DNA synthesis) of the cell cycle [1418]. This inhibition may be an immunosuppressive mechanism [494]. Retroplacental serum will inhibit lymphoproliferation in the presence of spermine (proportional to PAO content) [1407]. It has been suggested that the polyamine–PAO system contributes to the inhibition of mitogen stimulated lymphocyte proliferation observed during pregnancy [1404,1544,1548,1553]. Partially purified human retroplacental PAO causes a similar reduction of [^{3}H]thymidine uptake by cultures of lymphoid cells [455] (*q.v.* §1.5.4).

The placenta is rich in spermine [417,419,1554]. There is an increase in circulating polyamines in maternal blood [417] during pregnancy and increased levels of polyamines are found in urine [1555]. The increases in the concentration of polyamines and their metabolites in urine and amniotic fluid are dependent on gestational age [364,1556]. Furthermore, there is a high level of PAO in retroplacental blood [420], in the region where the first and most intimate contact between fetal and maternal surfaces occurs, suggesting that the principal site of polyamine–PAO interaction is in the placental bed. The inhibitory effect of the polyamine–PAO interaction on mitogen stimulated lymphocyte proliferation may operate *in vivo* to contribute to the protection of the conceptus from maternal immunological onslaught. Thus, the interaction of polyamines and PAO may contribute to the putative immunological barrier in the placental bed that protects the feto–placental allograft from maternal immune rejection, through a local immunosuppressive effect on maternal cellular immunity. The system may 'mediate a subversion of the host immune system in those individuals bearing tumours and outbred fetuses' [1402].

The PAO activity found in the maternal circulation is probably as a result of 'leakage' or diffusion (*vide supra*) rather than active secretion into the maternal circulation and may not be high enough to cause a generalized suppression of the maternal cellular immunity although some decrease occurs (*q.v.* §1.5.4). Since the mature placenta consists of a syncytial arrangement of

trophoblasts, the placenta is protected from the cytostatic G_1-phase arrest of cell proliferation. Further evidence comes from the observation that the polyamine–PAO system will inhibit the ability of murine lymphocytes to recognize irradiated allogenic cells and also the induction, but not the expression of the cytolytic lymphocyte response [1403]. This may occur very early in pregnancy (*q.v.* §1.5.4).

1.6.7.2 Control of Inflammation

Histaminase may act as an anti-inflammatory agent, produced by the decidua, to suppress an inflammatory response by the host during the invasive growth of the placenta and fetus (*q.v.* §1.5.3).

1.6.7.3 Detoxification of Amines

Polyamine oxidase may be protective for the mother in detoxifying excess histamine and polyamines produced by the fetus [712] (cf. §1.4.2). High concentrations of histamine and of the polyamines putrescine, spermidine and spermine, together with high polyamine synthesizing activities, have been reported in embryos and larvae of the toad [1557], in the chick embryo [110,1558,1559], in the rat fetus [1560], and in human placenta [1554]. In rats, the fetus forms large amounts of histamine and the excretion of histamine in the urine is increased [1561-1563]. High levels of histamine found in the rat and mouse fetus are produced by the high histidine decarboxylase activity in the fetal liver and kidneys [1564,1565]. Guhna and Jänne have indicated that the activity of ornithine decarboxylase is raised in the placental and fetal tissue of rats [1566]. Circulating polyamine levels in rats are elevated with the largest increase toward parturition and then falling after pregnancy [1567].

Histaminase catabolises the excess histamine [and other amines] produced by the fetus during pregnancy, preventing a return of histamine [and other amines] into the maternal circulation [743,890,1568]. Histidine is thought to pass through the placenta from the maternal into the fetal circulation where it is converted by histidine decarboxylase to histamine, which is thought to be required for fetal growth. An analogous situation occurs with ornithine, which is converted to putrescine by fetal ornithine decarboxylases. Excess amines not used for fetal growth may be catabolised by histaminase/DAO/PAO produced by the decidua, protecting the mother from the toxic effects of these amines.

Polyamine oxidase may be viewed as an adaptive enzyme, produced by the mother in response to a stimulus from the fetus. PAO is produced in response to the increased production of amines (histamine, putrescine and polyamines) by the fetus (Figure 1.18) [421,1569]. The serum level of PAO in pregnancy may therefore reflect the production of fetal amines and the responsive

synthesis of the enzyme by the placenta. Because of its broad substrate specificity, the enzyme may have a generalized protective function in the normal physiology of pregnancy, protecting both mother and fetus from elevated concentrations of amines that might occur as a result of the challenge presented to the mother's homeostatic mechanisms by the feto–placental unit.

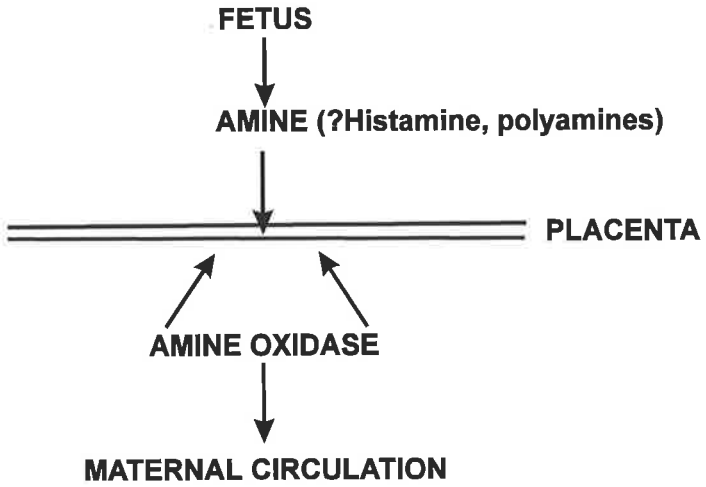

FIGURE 1.18. Hypothesis for Amine Oxidase Production During Pregnancy in Response to Fetal Stimulation. Amine oxidase in the maternal circulation represents a spillover of that portion not utilized in amine metabolism (after Weingold *et al.* [421]).

In vitro perfusion studies of human placentae indicate an active production of DAO by the perfused placenta *in vitro* [663]. The replacement of fetal perfusate with fresh fluid had a suppressive effect on placental DAO production suggesting that the fetal circulation contained a factor that can simulate enzyme production by the placenta. This factor may be the hypothetical fetal amine described by Kahlson [1568].

DAO is *not* elevated in cases of trophoblastic tumours [660,707]. The implication is that the intact feto–placental unit is required for enzyme production. On the other hand, this may also be because the enzyme is produced by decidual cells. The appearance of increased histaminase activity in decidua in rabbits from the nonpregnant uterus and decidua in rabbit pseudopregnancy suggests that fetal elements are not required for the formation of the enzyme [686]. Furthermore, histamine challenge did not induce the formation of the enzyme in non-pregnant women [1570].

If histaminase activity in pregnant rats is inhibited to at least 50% by aminoguanidine, the maternal and fetal mortality is high [1571]. If the dose of aminoguanidine is reduced so as to have no harmful effect on rat pregnancy, but to still inhibit histaminase, excretion of histamine is increased [1561]. In another study on urinary excretion of polyamines, pregnant rats excreted slightly more polyamines than non-pregnant rats showing peak excretions shortly before parturition. Aminoguanidine treated pregnant rats excreted significantly more polyamines than untreated pregnant rat controls and showed peak excretions shortly before parturition. It seems that polyamine synthesis is greatly increased in pregnancy, but that DAO metabolises most of the

polyamines produced [1572]. Similar findings were reported by Andersson *et al.* [1573]. Aminoguanidine treated pregnant rats had fetuses that exhibited organic and skeletal abnormalities, but in this case no fetal death was observed. In particular, the abnormalities included lung, head and liver haematomas, and abnormal ossification [1574].

In cancer, it may be that the increase of plasma diamine oxidase, like that seen in endometrial adenocarcinoma [1222], is a reaction of the body to increased formation of histamine and polyamines in the cancerous tissue. This may be analogous to the increased production of histamine and polyamines in fetal tissue and the rise of diamine oxidase in the plasma of pregnant women.

1.6.7.4 Regulation of Growth and Differentiation

The widespread occurrence of PAO in living forms indicates that it also has more generalized functions. Several lines of evidence suggest a role for the enzyme in the regulation of growth and differentiation (*q.v.* §1.2.6). The role of polyamines in organization of tRNA structure [86] is well recognized. Polyamine synthesis is increased with rapid cell proliferation [75,91,144,417] and polyamines are present at elevated concentrations in fetal [1513] and neoplastic [155,1575] tissues. Polyamine levels are elevated in the urine and amniotic fluid of women with normal pregnancies [417,418]. Polyamine conjugates in amniotic fluid are detected during pregnancy [1495] and substantial increases in the polyamine content of the placenta occur during gestation [417]. The levels are higher in early pregnancy than at term. This may be due to higher levels of amine oxidases in the decidua near term. A rapid decline in urinary putrescine concentrations is also observed after the 13th week of gestation [417] at a time when marked increases in the enzyme levels occur. The presence of polyamines is accordant with an *in vivo* function in the regulation of embryonic, fetal, and placental growth and development.

Pregnancy-associated amine oxidases may act as a regulators of tissue levels of amines [460] as part of the mechanisms regulating cell growth [367,439,1576]. PAO may influence cell proliferation by modulating the intracellular levels of polyamines in processes that are associated with normal and neoplastic growth [367]. Furthermore, this regulation may limit the placental invasion of the myometrium [456].

Histamine is important in the metabolism of all rapidly growing tissue and is known to be present in high concentrations in fetal tissue as well as in healing wounds and tumours. Histamine is active as an intracellular substance in growth tissues with primary action in the microcirculation of damaged tissues. The trophoblastic–decidual interface is such a situation [1563,1577]. Relatively high concentrations of histamine are observed in the umbilical and carotid arteries of the fetus in sharp contrast to absence of histamine in the maternal plasma [1561].

DAO activity in the maternal part of the rodent placenta is high and putrescine concentrations are low; in the fetal part of the placenta DAO activity is low and putrescine concentration is high [1566,1578], suggesting that putrescine is actively eliminated in the maternal system, but conserved in the fetus [754].

1.6.7.5 Placental Collagen Synthesis

Placental DAO can use collagen, tropocollagen and lysyl peptides, e.g. lysyl vasopressin, lysyl–arginine, which are of importance in collagen biosynthesis, as substrates [744]. Polyamine oxidases may act to catalyse the crosslinking of collagen precursors regulating the biosynthesis of this protein [744]. This may occur *in vivo* and be of importance to feto–placental growth. It is known that amine oxidases exist in connective tissues [504,828,861] (*qq.v.* §1.3.2.5g and Appendix A). Because of the similarities between placental DAO and lysyl oxidase, it may be involved in collagen crosslinking using similar mechanisms [751].

1.6.7.6 Epilogue

The biological effect produced by an enzyme will depend on the relative local concentrations of a particular enzyme and a particular substrate at a given time. Perhaps all of the proposed effects of enzymatic polyamine oxidation are significant during pregnancy. Polyamine oxidase may adapt to its different roles according to its localization and concentration and that of its substrates. Perhaps it performs as yet unrecognized functions.

A simple hypothesis has been proposed, that DAO *per se* has no specific biological function in the sense of being essential for the mother or the fetus. That it is simply a by-product of a more fundamental process concerned with the basic functioning and maintenance of the placenta as an individual organism, i.e. the production of the placental enzyme indicates nothing other than that the placenta is there [1067].

An association between fetal death and low maternal DAO activity would have a few important implications. The hypothetical result of inadequate PAO activity would be the immune rejection and subsequent abortion. Therefore inhibition of polyamine degradation with, for example, aminoguanidine, should prompt immune rejection of the feto–placental unit. Monoamine oxidase inhibitors are known to cause abortions [1579]. If aminoguanidine is used to inhibit plasma histaminase activity in pregnant rats, both maternal and fetal mortality is high [1571]. However, in another rat study using aminoguanidine to inhibit amine oxidase activity, fetal abnormalities were observed but no fetal death was seen [1574]. Like the clinical observations with MAO inhibitors and those correlated with changed amine oxidase levels during pregnancy (*q.v.* §1.6.9), the *in vivo* experimental observations are inconclusive.

1.6.8 Similarities and Differences with Other Amine Oxidases

1.6.8.1 Tumour Histaminase

Biochemical and immunological studies suggest the histaminase in tumour tissues and malignant effusion fluids has identity with the placental enzyme and may therefore be an onco–placental enzyme [746,752,1224]. Tumours appear to escape the defensive mechanisms of their host, perhaps through the impairment or evasion of immunological surveillance (for neoplastic growth) [746,1580-1582], a situation seductively analogous to the gravid state. It has been suggested that the enzyme acts to suppress inflammation during invasive growth of tumours (*q.v.* §1.6.7.4, cf. §1.5.3). Studies of placental enzymes and cancer protiens may offer insight into the processes of neoplastic transformation (*vide* §1.3.2.5e).

In ovarian cancer, elevation of histaminase activity coincides with an increase in the concentration of the Regan isoenzyme, a placental-type alkaline phosphatase associated with a number of human cancers [1583,1584]. The Regan isoenzyme is considered to be an expression of an embryonic gene in neoplasia [1585], i.e. a derepression of a fetal or trophoblastic gene by the malignant process. Polyamine oxidase may represent an example of tumour expression of fetal trophoblastic peptides such as placental alkaline phosphatase, human chorionic gonadotropin or placental lactogen. However, the site of histaminase (polyamine oxidase) production is the decidual tissue of the *pars maternalis* of the placenta. Since purified placental histaminase and tumour histaminase showed immunochemical identity with human kidney DAO, it is suggested that the tumour enzyme is an expression of a mature, not fetal, genome [748]. This appears to be supported by the possible expression of a human placental amine oxidase in breast and ovarian tissue [1080].

1.6.8.2 Eosinophil and Neutrophil Amine Oxidases

There are suggestions that the placental enzyme is similar to the enzyme demonstrated in eosinophils and neutrophils [800,805]. It seems unlikely, however, that the protein purified from human placental homogenates showing immunological identity with a protein secreted from fMLP stimulated neutrophils is actually DAO. The enzyme activity isolated from placental homogenates by Morel *et al.* [755] was only purified 57-*fold* and a correlation of enzyme activity was not demonstrated with the protein they choose to electroelute from a preparative SDS-PAGE of their partially purified preparation. The molecular mass of the 84,000 dalton protein that they isolated is not in agreement with the molecular weight range of the placental (glyco)protein (M_r 105,000) that has an NH_2-terminal amino acid sequence identical to that of a cloned human kidney protein expressing DAO activity [47].

1.6.8.3 Macrophages

It seems likely that the pregnancy enzyme is similar to an enzyme found in macrophages [807,955] (*qq.v.* §1.3.2.5h and 1.5.1.5). Evidence from paper electrophoresis of radioactive spermine oxidation products suggests that, like the placental enzyme, macrophage PAO cleaves spermine at secondary amino groups. Macrophage PAO activity is maximal at pH 8 [955,1083]; that for pregnancy serum is optimal at pH 7.5, in contrast to the liver enzyme's pH optimum of 10 [164].

1.6.8.4 Kidney, Intestine and Liver

Purified placental histaminase and tumour histaminase showed immunochemical identity with human kidney DAO [748]. Human intestinal histaminase crossreacted strongly, but to a lesser extent. The intestinal enzyme has a lower apparent M_r (95,000) than the placental enzyme, but a similar inhibitor sensitivity [786,790]. Unlike the rat liver enzyme, the human liver enzyme has not been well characterized. The human liver enzyme appears to have a different inhibitor sensitivity to the placental enzyme [725] (*q.v.* §1.3.2.5d).

1.6.9 Diagnostic Value of Amine Oxidase in Pregnancy

Although some limited increase in plasma DAO activity has been described in neoplasia, disorders of the gastrointestinal tract [1061], allergic states [803] and cystic fibrosis [394] (*q.v.* §1.4.5 and 1.4.6), no other physiological condition besides pregnancy causes such a dramatic and sustained rise in the blood titre of DAO [697]. It may be that low or falling amine oxidase activity might be a factor in the etiology or symptomatology of clinically important disorders of pregnancy.

The diagnostic value of serum amine oxidase levels during pregnancy have been investigated. Plasma levels of the DAO may be an index of fetal well-being [714]. Maternal blood levels of the enzyme have been measured in an attempt to correlate them with placental function [660,684,688,1586-1588].

DAO serum levels have been examined in cases of threatened abortion, missed abortion [684,686,690,1528], habitual abortion [684], cervical incompetency syndrome [1589], poor obstetric history and diabetic gravida [1587]. Some authors have suggested that falling plasma DAO levels indicate fetal stress, intrauterine death or high risk situations [1587,1588]. Investigations, based on serial determinations of DAO in a small number of subjects, suggested that low DAO levels are associated with subsequent stillbirths or spontaneous abortions [710,714,1587]. Outlier values of DAO were found in 73% of spontaneously aborting patients who presented with threatened abortion [1540]. Serum levels of amine oxidase in women with

clinical signs of threatened abortion, who aborted spontaneously between 11 and 22 weeks gestation, were found to have lower serum PAO activity than women with normal pregnancies of the same gestational age [690,710,720,723]; though the clinical significance of this is not clear [723]. Patients with pregnancies resulting in fetal death had significantly lower serum DAO levels than patients with pregnancies of the same gestational age resulting in live births. Moreover, the probability of fetal death was much higher for pregnancies in patients with low serum DAO levels than for those with normal serum DAO levels [720]. Serial determinations of DAO in women at high risk for fetal death have been shown to assist in the management of several obstetric conditions including habitual abortion, recurrent prematurity, late fetal loss and neonatal death [421]. A large proportion [83%] of patients with fetal death showed abnormal DAO curves.

In some cases of severe Rhesus iso-immunization, the levels of DAO in amniotic fluid are increased while in others the levels remain unaltered [711]. It is not known whether the increases were a result of an active process or a consequence of tissue damage.

It has been suggested that histaminase may play a role in the etiology of pre-eclampsia (pre-eclamptic toxaemia) in protecting the mother from increased levels of polyamines and histamine which could damage the liver and kidneys [691]. Diminished levels of histaminase were seen in pre-eclamptic toxaemia and only trace activity in *hyperemesis gravidarum* or more severe cases of pre-eclampsia and eclampsia. In patients with hypertensive-pre-eclampsia, the curve was below normal in early pregnancy [703,1528].

However, conflicting reports have appeared concerning the potential usefulness of plasma DAO measurements as a fetal monitoring method. Resnik and Levine found that patients with 'high risk' pregnancies had serum DAO values well within the normal range during pregnancy [705]. Other studies also, do not support the view that DAO levels fall during fetal distress [709]. In one study no correlation was found between certain fetal wastage categories and DAO curves [421].

Continued production of enzyme and substrate after fetal death and retention *in utero* during late pregnancy have been observed [709]. It is possible that proteolytic activity after the fetus is dead may provide precursors of diamines in increased amounts. There is an appreciably higher level of DAO in twin pregnancy compared to normal singleton pregnancies and the difference may be related to a higher placental mass [710]. However, a significant negative correlation has been found between placental enzyme concentration and birth weight [1531]. On the other hand, no correlation has been found between the level of activity and the duration of pregnancy, the size, or the sex or ethnicity of the neonate [686,696,724].

Ward *et al.* found that the wide scatter of normal values, especially later in pregnancy limits the value of serial DAO as a placental function test [710]. Extremely high and extremely low

values can be found in normal pregnancy with no detectable clinical consequences [716]. The values found in abnormal pregnancies are not appreciably distinct from normal because of the extremely wide spread of normal values [707,716]. Other studies show false negatives [709]. Besides the possible effects of prescribed drugs during pregnancy [809], it may be difficult to establish normal physiological levels of placental enzymes as their levels may be induced or inhibited by maternal lifestyle variables (including illicit drug-taking, smoking, drinking).

The curve of DAO concentration throughout pregnancy has a sigmoid shape when plotted on an arithmetic scale. The logarithmic plot shows that the rate of increase in concentration is maximal in early pregnancy and decreases with time. This makes the assessment of any falling levels in an individual quite difficult, especially later in pregnancy (*vide* §1.6.1).

The utility of plasma DAO assays for predicting the outcome of high risk pregnancies appears uncertain. Where there is a normal DAO curve but a neonatal death, it may be that, whatever the cause of eventual perinatal loss, the fetal growth and maternal–placental environment were not compromised before the loss [421]. DAO activity may hint at a pre-existing, subclinical, uteroplacental insufficiency [703]. In the case of false positives (abnormal curve, normal pregnancy) the implications to long term paediatric outcome have not yet been evaluated [421].

Thus, alterations in enzyme activity may reflect alterations in the stimulus production (fetal) or decidual synthesis (placental) and therefore variations in fetal growth and environment. It may be that falling and persistently low levels of plasma DAO in early pregnancy indicate fetuses living at 'high risk' *in utero* even though the pregnancy results in a live birth [663]. These surviving infants may develop subnormal neurologic or other signs postnatally. This remains to be examined [1588,1589]. Fetuses developing in a subclinically compromised environment may demonstrate the stigma of this compromise later, as neonates or during paediatric development. The lack of an objective method of assessing the baby after delivery is difficult because of the multifactorial etiology of perinatal morbidity and mortality. Effects may be subclinical, compounding the difficulties of assessment and recognition of abnormalities.

The absolute risk associated with altered DAO levels appears low, so clinical intervention with anti-abortifacients would appear unwarranted. However, the detection of increased relative risk of fetal death might suggest obstetrical precautions late in pregnancy.

Fetal environmental monitoring should probably be directed toward multiple indices which can be used for screening of environmental compromise. Their validity can be evaluated through the addition of other factors such as urinary estriol and ultrasonography as a battery of tests. Plasma DAO may be applicable as a screening tool. However, little information is available on the prognostic significance of a single low DAO level early in pregnancy. In view of the number

of high false positive values of DAO found in the prediction of a compromised fetus, serum DAO levels are probably only useful as a screening test for fetal risk. It is difficult to draw any conclusions regarding the prognostic value of serum DAO measurements. Serum DAO levels may be of value in identifying the infant who will require intense postnatal observation, in predicting sublethal stigma of a stressful environment or subclinical abnormality.

Only a small proportion of perinatal morbidity is due to placental dysfunction in late pregnancy [1067]. It is possible to assess the value of a placental function test and to compare it with other parameters if an entire obstetric population is examined so that one can assess true and false positive and negative results: the ratio of true positive to false negative providing the basis of the relative risk. However, with current technology it is unlikely that the measurement of DAO activity will ever compare with ultrasonic measurement of the fetus or with antenatal fetal heart monitoring in assessing the risk of perinatal morbidity in late pregnancy, since DAO is an indirect index.

DAO levels may be of some value in predicting gestational age [715] or diagnosing ruptured membranes [762]. Indeed, the use of DAO for the diagnosis of ruptured amniotic membranes may have advantages over other methods in certain situations [1590]. However, precautions may need to be observed to avoid false negative results, such as consideration of the effects of chlorhexidine containing obstetric creams [1591].

The serum DAO level may be used as a test for pregnancy [696,697]. Although the DAO method is not as accurate as the hCG levels in early pregnancy from the 5th to 8th week after the last menstrual period, it will give no false positives or negatives from 60 days after the last menstrual period until term [663,696] (*vide* Figure 1.16).

Serum DAO activity in early pregnancy has been proposed as a date of confinement predictor [715]. Although the estimated date of delivery can be calculated from the first day of the last menstrual period, it is not possible to do this in all cases [1592]. The date of the last menstrual period may be unknown or uncertain; the patient may have irregular bleeding; conception may occur before the return of menses after the previous delivery or abortion. From the 6th to 16th week of gestation, measurement of serum DAO is clinically useful for predicting confinement dates. However, this does not discount the utility of other methods.

1.7 Purification of Human Pregnancy-associated Amine Oxidases

Numerous attempts have been made to purify and characterize the pregnancy-associated amine oxidase(s). These have been summarized in Table 1.9. Although many similarities appear between the different studies, reports often appear to lack consensus, particularly in regard to the relative molecular mass of the enzyme (Table 1.5), which poses difficulties in identification of the enzyme protein. Further confusion has been contributed by the different nomenclature used by the various authors (*qq.v.* §1.6.2 and Table 1.5). It is difficult to reconcile the findings of the different characterization studies of human pregnancy-associated amine oxidases because of the apparently conflicting results reported by different authors.

The different results reported by different investigators may be a consequence of using different starting materials for their purifications, each containing a different amine oxidase. On the other hand, the same enzyme may exist in the different sources, placenta, retroplacental serum, pregnancy plasma, pregnancy serum and amniotic fluid. It is also possible that the same enzyme with different post-translational modifications exists in different sources. Perhaps a number of different polyamine oxidizing enzymes exist in each source. It seems likely that the same enzyme is present in each source and that different results have been reported as a consequence of the different purification and analytical methods used. In some cases enzyme activity may have been incorrectly attributed to a protein devoid of such activity.

1.7.1 Purification and characterization of human retroplacental serum polyamine oxidase

Studies of PAOs are gaining wider importance as further evidence of its role in a range of physiological and pathophysiological functions is generated. Advances in the study of PAO as a pregnancy protein, as an enzyme in the nonspecific killing armamentarium of macrophages, as a granule specific enzyme in neutrophils and eosinophils, as an onco–placental protein and as an enzyme involved in immunosuppressive and anti-inflammatory mechanisms would be aided by the availability of purified enzyme and methods that are more sensitive and specific in the detection and quantitation of this enzyme in cells, tissues, sera or fluids. Discrete substances and their interactions must be understood before more complex phenomena can be explained. Therefore, the identification and characterization of isolated PAOs would facilitate an understanding of their biological role and of their complex biochemical interactions in intact cells and organisms.

The aims of enzyme characterization generally include identification its primary gene structure and the three dimensional end product it encodes, its specific catalytic properties, and its functional significance. Purification and characterization of an enzyme may ultimately lead to the

TABLE 1.9. Attempts to Purify Human Pregnancy-Associated Amine Oxidases

PAO Source	Specific Activity of starting material		Specific Activity of the final preparation		Purification factor	Reference
	Units described	*Equivalent Units (mU/mg[a].)*	*Units described*	*Equivalent Units (mU/mg)*	*-fold*	
Placental	not reported	–	ND	–	ND	Novotny *et al.*, 1994 [47]
Placental extract	not reported	–	ND	–	ND	Houen *et al.*, 1993 [756]
Pregnancy serum	not reported	–	ND	–	ND	Houen *et al.*, 1993 [756]
Placental homogenate	30 ng putrescine/h/mg	0.006	1714 ngputrescine/h/mg	0.32	57	Morel *et al.*, 1992 [755]
Retroplacental serum	4.817 mU/ml[b.]	1.96	not quoted	–	3000	Morgan, 1985 [726], 1979 [420]
Retroplacental serum	not reported	–	–	–	400	Morgan, 1983 [442]
Placental homogenate	not reported	–	n.d.	–	ND	Denney *et al.*, 1988 [759]
Pregnancy serum	5×10^{-6} IU/kg[c.]	0.30	2550 IU/kg	153	500	Gahl *et al.*, 1982 [719]
Placental extract	2.98 U/mg[d.]	0.564	5466 U/mg	1033	1835	Lin *et al.*, 1981 [752]
Placental homogenate	not quoted	0.305[e.]	45.7 nmole/mg/30 min	1.52	5.4	Matsumoto *et al.*, 1981 [1155]
Decidual tissue	5.442 mU/g[b.]	0.108	ND			Morgan, 1979 [420]
Pregnancy serum	0.266 mU/ml[b.]	0.106	ND			Morgan, 1979 [420]
Amniotic fluid	540 mU/g[f.]	0.54		209	387	Tufvesson, 1978 [763]
Amniotic fluid			'A' 350×10^3 mU/g	350	650	Tufvesson, 1978 [763]
Amniotic fluid			'B' 200×10^3 mU/g	200	350	Tufvesson, 1978 [763]
Placental homogenate	170 U/mg[g.]	–	150,923	–	900	Baylin, 1977 [748]
Placental homogenate	7.8×10^{-3} U/mg/h[h.]	–	9.4 U/mg/h[h.]	–	1200	Hata, 1976 [745]
Placental extract	1.48 U/mg[d.]	0.153	4151 U/mg	429	2803	Lin & Kirley, 1976 [747]
Placental homogenate	0.214 mU/mg[i.]	–	7,000 mU/mg	–	[j.]30,800	Crabbe *et al.*, 1976 [744]
Pregnancy plasma	12.4 U/mg[g.]	–	38,245	–	3000	Baylin & Margolis, 1975 [713]
Placental homogenate	0.214 mU/mg[i.]	–	1,000 mU/mg	–	3906	Bardsley *et al.*, 1974 [712]
Placental homogenate	0.017 mU/mg[i,k.]	–	1,500 mU/mg	–	1,000,000	Bardsley & Crabbe, 1973 [743]
Placental homogenate	0.75 mU/mg[l.] (0.0286[k,m.])	–	7,200 mU/mg (14 mU/mg[m.])	–	9,600	Paolucci *et al.*, 1971 [742]
Placental extract	0.40 SA[n.] (0.0286[k,m.])	–	206 SA (6 mU/mg[m.])	–	500	Smith, 1967 [741]

Notes to Table 1.9

a. mU ≡ 1 nmole H_2O_2 produced/min with putrescine as the substrate
b. Substrate = spermine; relative oxidation rates spermine : putrescine = 1 : 20, Morgan, 1985 [726]
RPS = 50 mg protein/ml (estimated)
c. Specific activity = 1 IU = 1 mole putrescine/sec/kg at 37 °C, pH 9.0
d. 1 U = 1 μg putrescine hydrolysed/hr at 37 °C, pH 7.5
e. Substrate putrescine at 37 °C, pH 7.8
f. Substrate putrescine
g. 1 U = 1 pmole β[^{3}H]histamine/hr at 37 °C, pH 7.4
h. U = disappearance of 1 μmole histamine per h
i. 1 U = 1 μmole *p*-dimethylaminobenzidine/min at 37 °C, pH 7.0
j. 32,710 × calculated
k. 0.3 Units/placenta; four placentae = 70,000 mg [712,744]
l. 1 unit = 1 pmole histamine per min at 37 °C, pH 6.8
m. Bardsley & Crabbe, 1973 [743]
n. 1 SA (specific activity) Unit = 1 SU enzyme units/ml/E_{280}
1 SU = 1 spectrophotometric unit (modified indigodisulphonate method with cadaverine as a substrate, pH 6.8, 37 °C, a decrease of 1.0 in absorbance value at 610 nm per 24 hours
ND. not done

development of more specific enzyme inhibitors, facility for augmentation of enzyme activity through allosteric mechanisms or exogenous supplementation, and the identification of its reaction products. These things may in turn be used in the investigation of the enzyme's biological significance.

The generation of specific markers such as monoclonal antibodies and monospecific antisera is facilitated by the availability of purified enzyme, which may be used in immunizations and for the development of screening assays used in monoclonal antibody production. Antibodies to the enzyme may be used as immunological tools for the establishment of immunoassays or as biological inhibitors. The antibodies may also be used as immunological probes in the specific and sensitive cellular and subcellular localization of the enzyme or in the screening of cDNA expression libraries [1593,1594].

Purified enzyme may also be used to isolate its gene or cDNA using oligonucleotide probes generated from partial protein amino acid sequences [1595]. The complete amino acid sequence can then be determined from recombinant DNA clones. Enzyme DNA sequence allows the development of sensitive assays (e.g. PCR, Northern- and Southern-blot analyses) and probes for the localization and expression of enzyme genes. Expression of enzyme gene as a homogenous polypeptide population is the confirmatory synthesis that allows the unambiguous assignment of a particular enzymatic activity to a specific protein. This information, coupled with comparison to previously characterized enzyme(s), may be used to gain insight into the structural features of the enzyme. Purified enzyme also provides a starting point for its crystallization, which in turn allows elucidation of its three dimensional structure by X-ray crystallography.

Characterization of enzymes allows their comparison with enzymes isolated from other tissues and species. It seems likely that the amine oxidase activities associated with human tumours, pregnancy, leukocytes, kidney, intestine and liver are found in enzymes similar, if not

identical to the retroplacental serum PAO (*qq.v.* §1.6.8 and Table 1.9). If they are not, this is also of interest. The identification and characterization of the PAO(s) in retroplacental serum would be greatly assisted by their purification and would allow their direct comparison with other polyamine oxidizing enzymes. The biological tools generated from purified retroplacental PAO would also facilitate its comparison with other enzymes. If the enzymes are identical, their study will be facilitated by the tools generated from the relatively more abundant retroplacental enzyme and the purified enzyme may then also be used to investigate a wide range of biological effects without the necessity of its purification from less abundant sources. Because of the relative ease with which human retroplacental serum can be obtained and enzyme's relative abundance in this source it was desirable to produce a highly purified PAO from this source.

Lack of available reagents and definitive characterization of the human PAOs have placed limitations on our understanding of these enzymes. Neither monoclonal antibodies nor a sequence for the human retroplacental serum polyamine oxidase have previously been available so pursuit of these goals formed major components of this work. The project involved the establishment of an assay for the oxidase. The enzyme protein was then purified using classical biochemical and novel affinity methods to provide enzyme for detailed structural and functional analyses, and to provide purified enzyme for monoclonal antibody production. The monoclonal antibodies were useful tools for the further purification of the oxidases on immunoaffinity columns. It is likely that the mAbs will be useful in establishing immunoassays for PAO and for immunohisto- and immunocyto-chemical studies. The immunoaffinity purification of the human retroplacental PAO allowed the identification of the protein responsible for the enzyme activity and provided purified enzyme from which a limited *N*-terminal protein sequence was derived. The protein sequence was then used to confirm the molecular nature of the enzyme and provided the key to the identification of its complete primary structure. This in turn allowed further characterization of human retroplacental amine oxidase through examination of its protein sequence, and by considering analogies with similar and previously characterized amine oxidases.

However, this reductionist approach to protein purification may have a number of drawbacks for solving biological problems [1596,1597]. They include:

(1) The possibility of overlooking complex associations. The protein of interest may be functionally associated with other proteins to form a heteromeric multiprotein cluster. In this scenario the separation and characterization of all components of the enzyme complex is of particular importance. A powerful way of achieving this is to use an immunological approach to affinity purify the enzyme as was done here.

(2) Difficulty in the integration of seemingly unrelated efforts until the molecular identity of proteins is established. The same protein species may be characterized from different

perspectives. This was exemplified here when protein sequence analysis revealed that human retroplacental PAO was identical to an amiloride binding protein. To a lesser extent this problem also existed here because the literature is replete with confusing nomenclature regarding the human pregnancy-associated amine oxidase(s). Considering the apparent enzyme specificities or properties of the enzyme, human pregnancy-associated PAOs have been assigned names including benzylamine oxidase, human serum monoamine oxidase, semicarbazide-sensitive amine oxidase, plasma amine oxidase, diamine oxidase, and histaminase. It now seems likely that all of these enzymes, except for perhaps the benzylamine/monoamine oxidases, are identical to amiloride-sensitive copper- and TPQ- containing human placental amine oxidase.

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Chapter 2

MATERIALS AND METHODS

2.1 Introduction

General methods used in the purification and characterization of human retroplacental polyamine oxidizing enzymes are reported here. Methods specific to particular experiments or procedures are reported in later chapters.

2.2 Protein Assays

2.2.1 Sample preparation

Where necessary, protein samples were purified from interfering substances before protein assay. This was accomplished by protein precipitation, which was also useful for concentrating dilute protein solutions.

2.2.1.1 DOC–TCA precipitation

Sodium deoxycholate (DOC; 0.1 ml of 0.15% (w/v) solution) was mixed with 1.0 ml of protein sample and allowed to stand at room temperature for 10 min [1598]. One millilitre of 72% (w/v) trichloroacetic acid (TCA) was added and the mixture centrifuged for 10 min at 1000 × *g* (at 20 °C) in a JB-6 centrifuge fitted with swing–out buckets (Beckman Instruments, Fullerton, CA). After centrifugation, the supernatant was carefully aspirated using a Pasteur pipette attached to a suction device. Assays were performed directly on the precipitated protein.

2.2.2 Folin–Phenol Protein Assay

This method is based on reduction of the phosphomolybdic–tungstic mixed acid chromogen in Folin and Ciocalteu's reagent. The mixed acid chromogen ($3H_2O.P_2O_5.13WO_3.5MoO_3.10H_2O$ and $3H_2O.P_2O_5.14WO_3.4MoO_3.10H_2O$) is reduced by a rapid reaction with the aromatic amino acids, tyrosine and tryptophan, and a slower reaction with copper chelates of the peptide chain and polar side chains. The reduced species have a characteristic blue colour (λ_{max} 745–750 nm) [1599].

Peterson's [1600,1601] modification [1599] of Lowry's [1602] original method was used. The modifications included: (a) changes to the reagents to permit the convenient addition of only

two reagents. In the first step the protein sample was mixed with copper ions in an alkaline medium (Biuret reaction). In the second step, the Folin–Ciocalteu reagent was reduced. The reagents are stable and more concentrated. (b) SDS was included to ensure rapid solubilization of proteins and to make the procedure compatible with detergents. (c) An optional DOC–TCA precipitation technique [1598], allowing the simple, rapid and quantitative recovery of proteins from even very dilute solutions and the removal of interfering substances.

2.2.2.1 Reagents

CTC reagent, 0.1% (w/v) $CuSO_45{\cdot}H_2O$, 0.2% (w/v) sodium (+) tartrate, (since potassium dodecyl sulphate is insoluble, Rochelle's salt (sodium potassium tartrate) is not used) [1603]. The sodium carbonate was dissolved in about one half of the final volume and added slowly to a stirred solution of copper-sulphate-tartrate. The solution kept indefinitely under refrigeration.

Sodium dodecyl sulphate (SDS), 5% (w/v)

Sodium hydroxide, 0.8 M

Folin–Ciocalteu phenol reagent, 2.O *N* [1604]. The exact molarity of the commercially supplied reagent was determined by titration against standard 0.10 *N* NaOH as follows: Folin's reagent (1.0 ml) was added to 50 ml water. Phenolphthalein (0.5 ml) 0.1% (w/v) in ethanol was added as an indicator and the solution was titrated to the endpoint (colourless grey) with about 20–25 ml standardized 0.1 *N* NaOH (Merck, Titrisol). The colour change is yellow-green ⇒ grey (end point) ⇒ violet.

Sodium deoxycholate (DOC), 0.15% (w/v).

Trichloroacetic acid (TCA), 72% (w/v).

Standard. Bovine serum albumin (BSA) standard was prepared from dried crystalline bovine serum albumin. BSA was heated to 80 °C for 1 h and cooled in a vacuum desiccator over hydroscopic silica gel beads. A 2.0 mg/ml solution was prepared in saline and its absorbance checked in an SP8-100 UV–visible spectrophotometer (PYE Unicam, Cambridge, UK) using matched quartz cuvettes (Starna, Essex, UK) and a saline blank. The protein concentration was found to be 1.89 mg/ml using an extinction coefficient $\varepsilon_{280}^{1\,mg/ml}$ 0.66 [1605]. A new solution, 423 mg/200.00 ml saline, was prepared, filtered to 0.45 μm and the protein concentration found to be 2.02 mg/ml. This 2.02 mg/ml solution was aliquoted and stored at –20 °C to be used as the standard. As an alternative protein standard (especially for determining immunoglobulin concentrations), lyophilised bovine γ-globulin (Bio-Rad protein Standard I) was made up to 2.00 mg/ml.

Reagent A. CTC reagent, 5% SDS and 0.8 M NaOH were mixed 1 : 2 : 1 respectively. The

reagent was stable for 2–3 weeks at room temperature and longer at 4 °C. A white (SDS) precipitate appeared at low temperatures, but redissolved quickly upon warming. The accumulation of a dark precipitate indicated that the solution should be discarded.

Reagent B. 2.0 *N* Folin–Ciocalteu phenol reagent, diluted 1 : 5 with water. This reagent was stable for several months at room temperature.

2.2.2.2 Assay Procedure

Samples containing 5–100 µg protein were made up to 1.0 ml with water. Reagent A (1.0 ml) was added and the reaction mixture allowed to stand for 10 min at room temperature. Reagent B (0.5 ml) was added and mixed in immediately on a vortex mixer. It was essential to mix in the Folin phenol reagent immediately after each addition. After 30 min at room temperature, the absorbances at 750 nm were read on a PYE Unicam SP8-100 UV–visible spectrophotometer fitted with a flow cell and sipper sampling accessory. Standards were run in triplicate and water was included as a reagent blank. Standards were 5, 10, 20, 40, 60, 80, 100 µg protein from the 2.0 mg/ml stocks. The relationship of protein concentration and absorbance for the Folin–phenol protein method is nonlinear [1600]. Calculations of unknowns were made by comparison to a standard curve produced using computer software for nonlinear curve fitting (IMI Graph Pack II, version F, curve fitter ©P.K. Warme, Interactive Microware, Inc., State college, PA).

2.2.3 Bicinchoninic Acid Protein Assay

Protein was measured using the bicinchoninic acid (BCA; 4,4´-dicarboxy-2,2´-biquinoline) method described by Smith *et al.* [1606]. BCA substitutes for the Folin–phenol reagent of Lowry's method [1602] as the basis of the analytical method measuring cuprous ion produced in the reaction of protein with alkaline Cu^{2+} (Biuret reaction). BCA forms a purple complex with cuprous ion (Cu^{1+}) in an alkaline environment.

This method has the advantage of being a single step technique, with greater tolerance of interfering substances such as simple buffer salts, for example Tris, which were encountered during protein manipulation.

2.2.3.1 Reagents

Reagent A. 1% (w/v) BCA-Na_2, 2% (w/v) Na_2CO_3 (anh.), 0.16% (w/v) disodium (+) tartrate, 0.4% (w/v) sodium hydroxide and 0.95% (w/v) sodium bicarbonate; salts were dissolved with gentle heating and pH adjusted to 11.25 with 2 *N* NaOH.

Reagent B. 4% (w/v) copper sulphate pentahydrate.

Reagents A and B were stable indefinitely at room temperature.

Standard working reagent (SWR). was prepared as required from Reagent A and Reagent B, 100 : 2. SWR was an apple-green colour and stable for about a week, giving progressively higher blank readings as it aged.

2.2.3.2 Assay Procedure

Protein sample (100 µl containing 5–100 µg protein) was mixed with 2.0 ml SWR and incubated at 37 °C for 30 min. The samples were cooled to room temperature and their absorbances measured at 562 nm in a PYE Unicam SP8-100 UV–visible spectrophotometer. The concentration of unknowns was determined from a standard curve as described for the Lowry method. Where necessary the Bensadoun and Weinstein DOC–TCA precipitation technique [1598,1600] (*q.v.* §2.2.1.1) was used to avoid the effect of interfering substances or concentrate dilute solutions.

2.2.4 *o*-Phthalaldehyde Protein Assay

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of 2-mercaptoethanol to form a fluorescent OPA adduct with an excitation maximum at 450 nm [1600,1607]. With intact proteins, OPA reacts with the NH_2-terminal amino acid and the ε-amino group of lysine residues. The presence of Brij 35 was essential for maximum fluorescence of the lysine adduct. The OPA method was a sensitive and relatively simple procedure for determinations where the amount of protein is limited. Less than 10 ng protein can be measured using this method. The determination of total protein by the OPA reaction with intact proteins was about 10 times more sensitive than the BCA or Folin–phenol methods, but is more variable with different proteins since the fluorescence is primarily due to only one amino acid (Lys). The method used was an adaptation of the procedure published by Viets *et al.* [1608] for protein quantitation using intact proteins as presented by Peterson [1600].

2.2.4.1 Reagents

Stock OPA solution, after Lee *et al.* [1607]. OPA (120 mg) was dissolved in 1.5 ml of methanol and added to 100 ml of 1.0 borate buffer (1.0 M boric acid titrated to pH 10.4 with 10 M KOH. The boric acid was only slightly soluble in water but dissolved on addition of the KOH with gentle (<40 °C) heating and stirring. The pH was adjusted at room temperature and the solution filtered to 0.22 µm followed by the addition of 600 µl of 30% (w/v) Brij 35. The solution was

stored in an amber bottle at room temperature for no longer than three weeks.

NaOH, 0.5 M.

OPA reagent. At least 30 min before use, 3 µl of 2-mercaptoethanol was added per ml of OPA stock solution.

2.2.4.2 Assay Procedure

Sample containing 100–2000 ng of protein was brought to 10 µl with water. OPA reagent (100 µl) was added to each sample. After 15 min incubation in the dark, 1.5 ml 0.5 M NaOH was added and the fluorescence (λ_{ex} 340 nm; λ_{em} 450 nm) of each sample was measured immediately using an LS 50 Luminescence spectrophotometer (Perkin–Elmer, Beaconsfield, UK). BSA standards were determined at 100, 500, 1000, 1500, 2000 ng per tube.

2.2.5 UV Absorption Protein Determination

Absorbance at 280 nm has been widely used to estimate protein concentration since the method was reported by Warburg and Christian [1609]. $\varepsilon_{280}^{1\,mg/ml}$ is 0.4–1.5 for most proteins. The A_{280} method provides a rapid and sensitive means of estimating total protein concentration, moreover, it is non-destructive. A_{280} was routinely used to estimate relative protein concentrations in chromatography eluates using an 8300 Uvicord II UV-analyser LKB, Bromma, Sweden), a 163 variable wavelength detector (Beckman Instruments, Fullerton, CA) or a PYE Unicam SP8-100 UV–visible spectrophotometer with 10 mm path-length matched quartz cuvettes (Starna, Essex, UK).

Absorption in the far-UV around the peptide absorption band is used as a more sensitive method and is much less affected by protein amino acid composition [1610,1611]. Technical difficulties usually prevent the measurement of the actual peak of absorption around 190 nm. Moreover, the most consistent values for extinction coefficient measurements are obtained on the side of the band at 205 nm where the extinction coefficients for nearly all proteins fall in the range 28.5 – 33 [1610]. By making a correction for the tyrosine and tryptophan content (by also determining A_{280}) the extinction coefficient at 205 nm can be predicted within 2% of the actual value [1612]. Thus, with the 280/205 nm method developed by Scopes, absolute protein concentrations can be obtained with substantial reliability.

The formula used was:

$$\varepsilon_{280}^{1\,mg/ml} = 27 + 120 \times (A_{280}/A_{205})$$

The absorbance at 205 nm was determined in 50 mM potassium sulphate containing 5 mM potassium dihydrogen phosphate, adjusted to pH 7.0 with potassium hydroxide since most other salts absorb at this wavelength. Potassium sulphate helps prevent adsorption of protein to the cuvette. Brij 35 was also added to prevent protein adsorption [1613]. The protein was measured in the range 5–25 μg/ml at 205 nm and 50–1000 μg/ml at 280 nm.

2.3 AMINE OXIDASE ASSAY

2.3.1 Fluorometric Amine Oxidase Assay

The fluorometric assay for amine oxidases offers greater sensitivity and selectivity than conventional colorimetric methods, e.g. those based on coupled oxidation of chromogenic compounds with peroxidase, *o*-dianisidine [662,740,882,889,943,1062,1614] or indigo disulphonate [661]. The indigo disulphonate method is not specific because histamine and other amines may themselves oxidize the indigo chromogen [443]. The *o*-dianisidine method is not specific and is sensitive to superoxide radicals [444,1615]; *o*-dianisidine may also act as an inhibitor of enzyme activity [878]. Furthermore, the method using *o*-dianisidine as an oxygen acceptor may give false results when haemoglobin, which is also an oxygen acceptor, is present in the sample. Moreover, *o*-dianisidine may be directly oxidized by ceruloplasmin [1614].

The fluorometric method permits the use of a variety of substrates, which is of value in characterization and identification of amine oxidases; and permits continuous monitoring of the enzyme reaction, which is important for steady-state kinetic studies. Radiometric methods are limited to the use of single substrates and the measurement of product formation at one time point. Manometric methods or measurement of oxygen uptake with a Clark electrode (polargraphic method) allow the use of multiple substrates but are relatively insensitive and technically more difficult. Coupled ammonia measurement is relatively insensitive [635] and limited to amine oxidases acting at primary amino groups. The fluorescent method described here is comparable in sensitivity to the radiometric methods [673] for amine oxidases and is relatively simple to perform.

Amine oxidase activity was measured fluorometrically using a modification of the method first described by Guilbault *et al.* [1616,1617] and Snyder and Hendley [996,1618], in which hydrogen peroxide formed in the amine oxidase reaction is measured fluorometrically by coupling it to the oxidation of homovanillic acid (HVA; I) which dimerizes to the fluorescent biphenyl structure (II) [1619-1621] in the presence of horseradish peroxidase (donor : H_2O_2–oxidoreductase; HRPO; EC 1.11.1.7) [1617] (Figures 2.1 and 2.2). HVA can act as an electron acceptor (hydrogen donor) with peroxidase [1616]. HVA solutions are stable and therefore the method has advantage over the diacetylfluorescine method of Keston and Brant [1622], or the

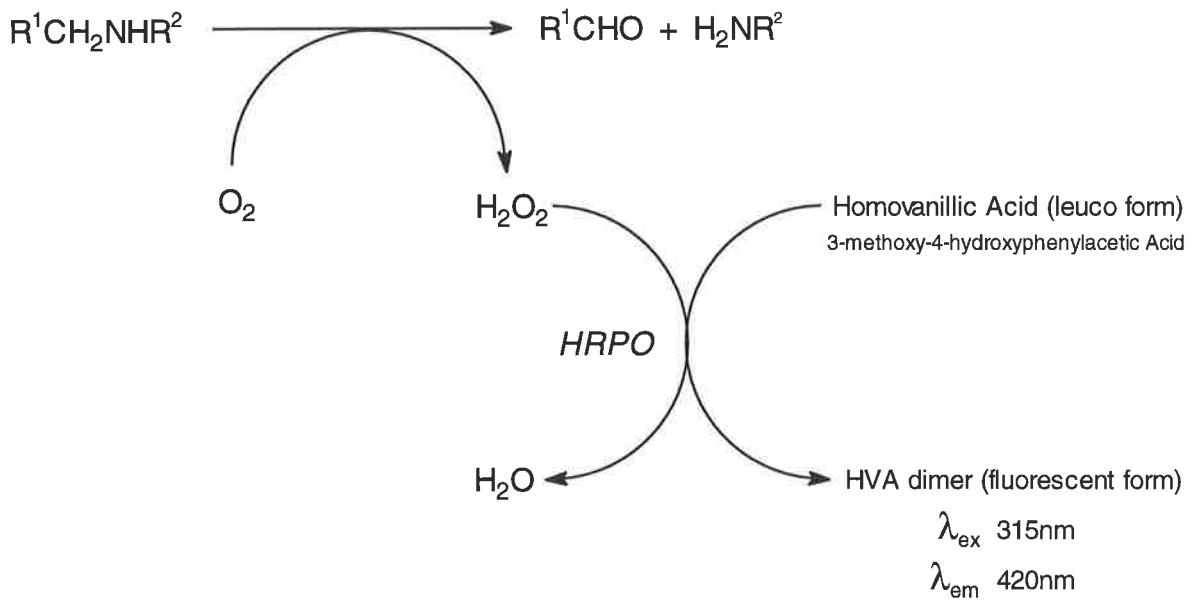


FIGURE 2.1. Principle of the Fluorometric Amine Oxidase Assay

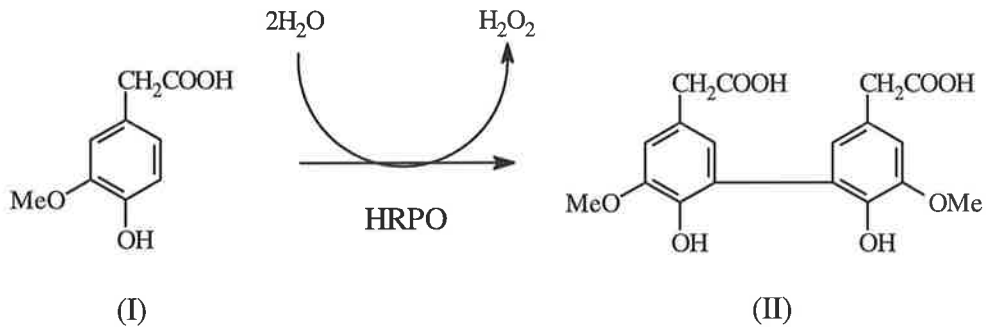


FIGURE 2.2. Formation of the Homovanillic Acid Dimer

procedure of Andreae [1623] in which the extinction of fluorescence after scopoletin (6-methoxy-7-hydroxy-1,2-benzopyrone, 7-hydroxy-6-methoxycoumarin) oxidation is measured [1624-1627]. Furthermore, the HVA method is more sensitive. However, unlike other more sensitive fluorogenic substrates, HVA does not inhibit polyamine oxidase [1628,1629] and the fluorescent dimer is more stable [765].

2.3.1.1 Oxidation of Hydrogen Donors by the Peroxidase (HRPO)–H_2O_2 Complex

The HRPO reaction will underestimate H_2O_2 concentration when hydrogen donors (e.g. serum albumin, reduced haemoglobin or reducing agents such as 2-mercaptoethanol or DTT [1630] that compete for HRPO–H_2O_2 (compound III) are present in the reaction mixture [1625,1627,1631-1633]. Serotonin (5-hydroxytryptamine) [1632], other hydroxyindoles, aromatic amines, phenolic compounds, such as phenol red, hydroquinones and hydroquinoid amines can act as hydrogen donor substrates and will compete in the reaction [1629,1634-1642]. Although the enzyme is

specific for the hydrogen acceptor/electron donor, H_2O_2 [1635], the specificity of horseradish peroxidase for the hydrogen donor is quite low and rate constants have wide variation [1635,1636]. Sometimes a lag phase is observed in the assay, during which time it is thought that endogenous hydrogen donors are oxidized by the H_2O_2 being produced by the amine oxidase reaction. Upon exhaustion of the endogenous hydrogen donors, the H_2O_2 then oxidizes the HVA and H_2O_2 production rates can be measured more reliably [1625].

Hydrogen peroxide formed in the reaction would normally be destroyed by catalase in serum samples. However, in the presence of low concentrations of H_2O_2 and a suitable acceptor, catalase will act peroxidatically [1643,1644]. Nevertheless, the presence of peroxidase ensures that negligible H_2O_2 will react with catalase due to the large differences in affinities (K_m) of catalase and peroxidase for hydrogen peroxide [1645].

The mechanism of HRPO oxidation, as proposed by Chance [1646] and later supported by George [1647] is:

$$\overset{Fe^{1+}}{HRP} + H_2O_2 \longrightarrow \overset{Fe^{2+}}{HRP\text{-}H_2O_2(III)}$$

$$\overset{Fe^{2+}}{HRP\text{-}H_2O_2(III)} + \underset{\text{(hydrogen donor)}}{AH_2} \longrightarrow AH^{\cdot} + \overset{Fe^{3+}}{HRP\text{-}H_2O_2(IV)}$$

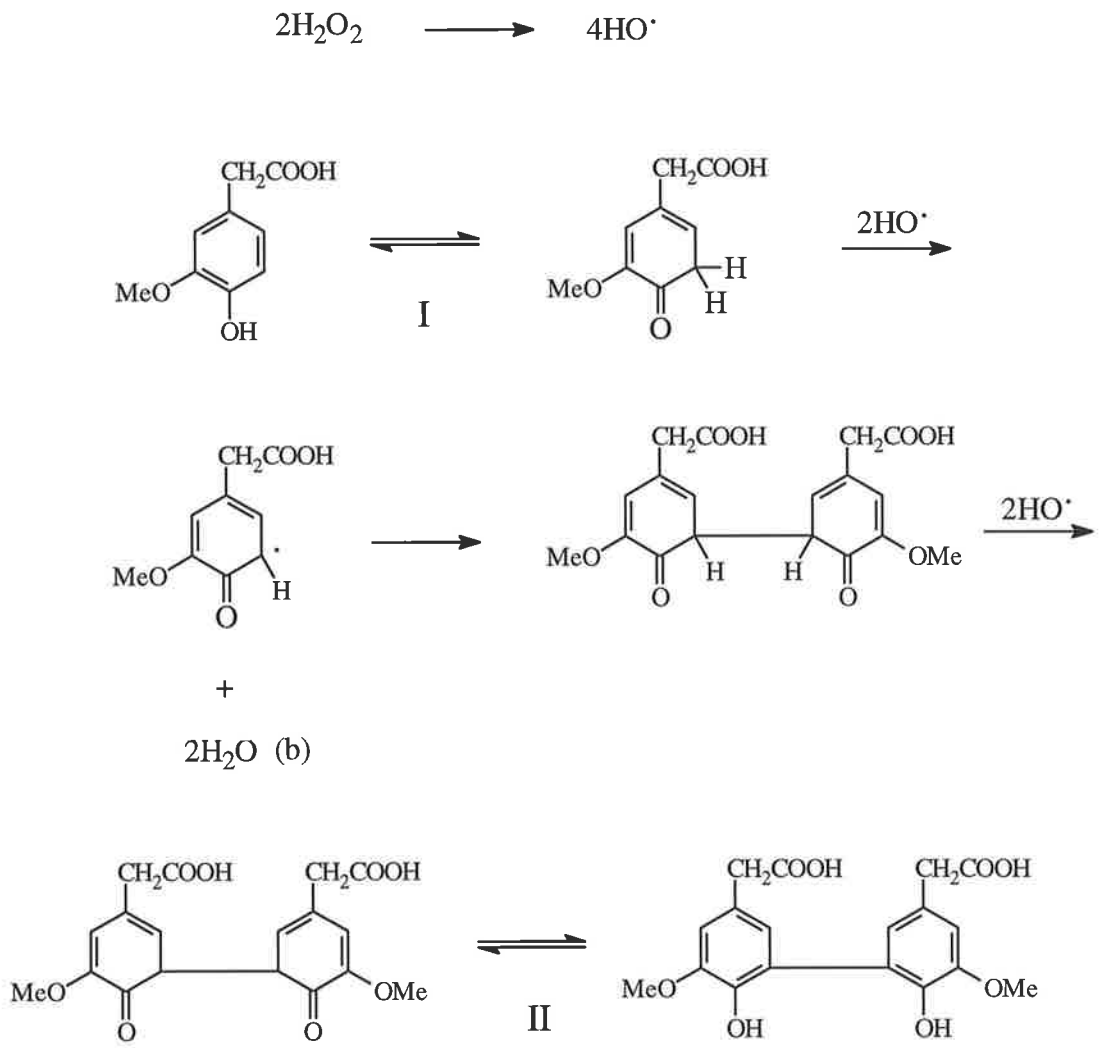
$$\overset{Fe^{3+}}{HRP\text{-}H_2O_2(IV)} + AH^{\cdot} \longrightarrow \overset{Fe^{1+}}{HRP} + A + 2H_2O$$

where AH_2 represents any oxidizable substrate and $AH^{\cdot}$ the free radical formed by the reaction. If HVA and H_2O_2 are considered as substrates in a three substrate (Ter Bi) Ping Pong type reaction [1648,1649], the model predicts that a HVA radical is generated. The coupling of the radical produces the fluorescent dimer, thus:

$$H_2O_2 + FH_2 \underset{HRPO}{\Rightarrow} 2H_2O + F$$

(FH_2 = fluorogen (leuco dye); F = fluorophore)

The oxidation of homovanillic acid (I), 3-methoxy-4-hydroxyphenylacetic acid, to form the fluorescent compound (II), 2,2´-dihydroxy-3,3´-dimethoxybiphenyl-5,5´-diacetic acid, by peroxidase is believed to proceed by the following mechanism [1616]:



Free radical hydroxyls are formed by the action of hydrogen peroxide [1616]. These radicals then attack HVA (I) to yield the fluorescent compound (II) via a free radical mechanism. The structure of (II) was elucidated by Corrodi and Werdinius [1619], and similarly identified as 2,2´-dihydroxy-3,3´-dimethoxybiphenyl-5,5´-diacetic acid by Guilbault *et al.* [1616]. The slight increase in fluorescence of blank solutions is probably due to oxidant impurities in the HRPO, most likely Fe^{3+}. Homovanillic acid (I) in aqueous stock solutions was stable at –15 °C.

Optimal assay conditions were carefully selected. The high velocity of the HRPO reaction with H_2O_2 and homovanillic acid is well established [1616] and sufficient HRPO was included in the assay to accurately reflect H_2O_2 production by the amine oxidase system [1629]. Homovanillic acid was titrated (100 µM – 10 mM) against different HRPO concentrations (0–500 U/ml) in the presence of 200 µM H_2O_2 (a concentration below the rate limiting value [1650]). Following the suggestion by Guilbault *et al.* [1616], it was found that 1 mM HVA in the assay gave maximal fluorescence response whilst maintaining a relatively low reagent blank. The assay was linear for initial H_2O_2 concentrations up to 100 µM. A pH optimum of 7.4 was determined for the retroplacental polyamine oxidase by a radiochemical method [169] and falls in the

physiological range for serum enzymes. HVA was used at 1 mM (0.1822 mg/ml) compared with 467 μM (0.085 mg/ml) used by Guilbault *et al.* [1616]. With a HVA concentration greater than 1 mM, blanks are increased with no net increase in the fluorescent product formed with H_2O_2, as also found by Snyder and Hendley [996].

Although partial substrate inhibition of peroxidase has been observed with H_2O_2 greater than 5 mM [765,1649], the peroxide generated by the amine oxidase reaction is consumed by the peroxidase before reaching inhibitory concentrations. A polyamine substrate concentration of 100 μM was used, which was sufficient to prevent adverse (>20%) substrate depletion during the course of the assay, except perhaps in the most active samples. Substrate inhibition and competing reactions may occur when longer chain polyamines (spermine, spermidine) are used, but these effects were of no serious consequence for most assays. The metabolism of longer chain polyamines is favoured before the reaction with shorter polyamines, and this helps to keep the reaction linear. Apart from the consideration of substrate inhibition from high substrate concentrations, excessively high concentrations of substrates were avoided, since substrate preparations are likely to contain enzyme inhibitors as contaminants.

Neufeld and Chayen quote an unpublished observation of Crabbe that the coupled peroxidase method is unreliable [765]. They found that in amniotic fluids taken at delivery, the activity of the sample was not proportional to the volume of the fluid. With the method of Guilbault *et al.* [765] they found lag times of 5–20 min at the beginning of the reaction and a lag phase of 10–60 min with the method of Paolucci *et al.* [735] due to the presence of peroxidase inhibitors (the hydrogen donors which compete for the chromogen/fluorogen in the peroxidase catalysed oxidation). Although with unpurified enzyme the assay results must be treated with caution because of these lag times, we have found the assay to be quite satisfactory for examining chromatography eluates where large numbers of samples must be examined in a short time in a rapid and convenient way. This avoids delays during the isolation of the enzyme, a requirement of enzyme purification. This stopped (discontinuous/indirect) method provides a single determination endpoint, which is suitable for the screening of large numbers of samples as incubation times are simultaneous. The system was examined for any reaction in the absence of enzyme and none was evident.

The peroxidase coupled method avoided time consuming and unreliable separation and counting steps of radiochemical methods. It also had the advantage that it may be used with a wide variety of substrates. In radiochemical methods, such as that described by Neufeld and Chayen [765], variations in the extraction of reaction products and other caveats have been noted [673]. No true internal standard is available to provide a calculation of extraction and counting efficiencies (η) of the reaction products [1615] and there is the possibility of interference by

aldehyde metabolizing enzymes [193].

2.3.1.2 Stock Solutions

×10 Buffer (×1 buffer = 0.05 M Tris–HCl, pH 7.40 at 37 °C). Tris–HCl (28.60 g) and Tris-Base (8.30 g) were made up to 500 ml with water, filtered to 0.45 μm and stored at room temperature. The pH was not adjusted. Tris is an appropriate buffer for the reaction [1616] since phosphate complexes with polyamines and sodium-phosphate buffers have been reported to inhibit amine oxidases [1615,1651]. Tris was found not to have an appreciable effect on blanks, cf. reference [996].

Horseradish peroxidase (HRPO). HRPO ((Type II, Cat. No. P-8250, Sigma Chemical Co., St Louis, MO); Reinheiszahl (A_{403}/A_{275}) was 2.0; Activity was 210 purpurogallin units/mg at 25 °C, and 1 purpurogallin unit [1652] is equivalent to approx. 18 IU/min at 25 °C) was stored at –15 °C as a desiccated powder. This was equilibrated at room temperature just prior to reconstitution to 1.06 mg/ml (*4000 IU/ml*) in 0.05 M Tris–HCl, pH 7.4, 0.45 μm filtered, aliquoted and stored at –15 °C.

In the final assay mixture HRPO was predicted to be 40 IU (2.23 purpurogallin units; 10.6 μg) per ml. However, under the conditions of the assay it was found that the maximal velocity, V_{max} of the HRPO was 3.55 ± 0.093 μM.s^{-1}, equivalent to 213 mIU/ml.

$K^{app}_{mH_2O_2}$ = 17.3 ± 1.4 μM (at 1.0 mM HVA).

K^{app}_{mHVA} = 1.67 mM (at 200 μM H_2O_2), though there is some controversy about the validity of K_m for a hydrogen donor.

The lag time to measure 0.98 *v*, the experimental velocity of the amine oxidase, with this amount of coupling (auxiliary) enzyme under these conditions was calculated to be 19 s [1653,1654] after which time the PAO catalysed reaction was rate limiting.

Homovanillic acid. A stock solution of 100 mM HVA was prepared by dissolving 18.22 mg HVA/ml water with gentle warming and stirring. The HVA solution was 0.45 μm filtered, and stored in 2 ml aliquots at –15 °C.

Substrates. 10 mM stock solutions were prepared as follows:

Substrate	M_r	*per 100 ml*
Putrescine.2HCl	161.1	16.1 g
Spermidine.3HCl	254.6	25.5 g
Spermine.4HCl	348.2	34.8 g

Reaction mixture

Buffered HRPO Stock solution	*per assay (µl)*	*[final]*	*per 100 assays (ml)*
0.5 M Tris–HCl (×10 stock)	100	0.05 M	10.0
water	590	–	59.0
HRPO (4000 U/ml stock)	10	40 U/ml (10.6 µg/ml); 2.23 purpurogallin units/ml; 213 mU/ml	1.0
	700		
Substrate solution:			
HVA (100 mM)[a.]	10	1.0 mM	1.0
Substrate (10 mM)	10	100 µM	1.0
Water	180	–	18.0
	200		
Sample (1 to 100 µl + saline balance)	100		
	1000		
Stop reagent (1.0 M NaOH)	100		

[a.] warmed in hot water to ensure crystals are dissolved

Hydrogen peroxide. 10 mM stock was freshly prepared. Approximately 97 µl of 30% (w/v) ('100 volumes') hydrogen peroxide, was diluted to 100.00 ml. The actual concentration of the 30% (w/v) hydrogen peroxide stock solution was initially determined by titration with ceric(IV) sulphate solution that had been standardized with anhydrous arsenious oxide (*q.v.* §2.3.1.7). Extinction coefficients of hydrogen peroxide were determined using a PYE Unicam SP8-100 UV–vis spectrophotometer. ε_{230} was 62.42 $M^{-1}.cm^{-1}$ in agreement with data from Nelson and Kiesow [1655] (*q.v.* §2.3.1.6). Actual concentrations of '10 mM' H_2O_2 working stock solutions were determined spectrophotometrically. Hydrogen peroxide standards in the range 10–100 nmole were included in each assay.

2.3.1.3 Assay Procedure

Isotonic buffer or saline was added to all assay sample tubes so that the final sample volume would be 100 µl. Samples of an appropriate volume are added to each tube using a positive displacement pipette (Microman M25, Gilson Medical Electronics, Villers-le-Bel, France). Standards, controls and reagent blanks were added to assay tubes (100 µl each) followed by 200 µl substrate solution and then 700 µl buffered HRPO solution, using a rapid-repeat dispensing pipette (4780 Multipette, Eppendorf Gerätebau Netheler + Hinz GmbH, Hamburg, Germany). The reagents were incubated for 10 min at 37 °C and 200 µl substrate solution then added to each

sample to initiate the reaction. The reactions were allowed to proceed with constant shaking at approximately one cycle per second in a waterbath set at 37.0 °C. After 30 to 60 min (as appropriate for the anticipated activity of the samples) the tubes were removed from the waterbath and reactions immediately terminated with 100 µl 1.0 M NaOH.

After the assays had equilibrated to room temperature, their fluorescence was measured using an LS 50B luminescence spectrophotometer (Perkin–Elmer, Beaconsfield, UK) fitted with an autosampler accessory and a No. 96 Wratten 0.6 OD neutral density attenuating filter (Kodak, Rochester, NY) on the emission window. Fluorescence settings were excitation λ_{315}; emission λ_{420} with 5.0 nm slits. Wavelengths were determined by examination of excitation and emission spectra of the homovanillic acid dimer generated by the enzymatic reaction.

2.3.1.4 Microplate Format

The fluorogenic peroxidase coupled amine oxidase assay was adapted to a 96-well microtitre plate format to facilitate screening of large numbers of samples, though this method is less sensitive than the standard method described above.

To each microtitre well in a MicroFLUOR 'W' plate was added 10 µl sample, 190 µl Tris buffered HRPO solution, and 50 µl substrate solution. A standard curve from 0 to 100 nmoles H_2O_2 was included in the assay and an 'autozero' (reagent) sample and an 'autoconc' sample (usually 10 nmoles H_2O_2) were included in the well plate dialogue of the LS 50B Fluorescence Data Management instrument software.

After initiating the reactions, plates were incubated at 37 °C for an appropriate period (30–90 min). Reactions were terminated with 1.0 M NaOH (25 µl) stop solution. After equilibration to room temperature, samples were read using an LS 50B Luminescence spectrophotometer fitted with a microplate reader. Instrument settings were: λ_{ex} 315 nm, λ_{em} 420 nm; slits 10 nm/15 nm; without an emission filter.

2.3.1.5 Continuous Coupled Enzyme Assay

Where a more accurate determination of enzyme activity was required (such as for the determination of the activity of pooled fractions), continuous production of hydrogen peroxide was followed. The formation of hydrogen peroxide by the oxidase reaction was followed by coupling it to the formation of a fluorophore from the hydrogen donor, homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid), in the presence of donor : hydrogen peroxide oxidoreductase (horseradish peroxidase, HRPO; EC 1.11.1.7) as described for the (NaOH) stopped assays.

Procedure. The formation of the fluorophore was measured using a Perkin–Elmer LS-50B

luminescence spectrophotometer equipped with a four position, water thermostated, stirred-cuvette holder. Temperature control was by means of a waterbath fitted with a Braun Thermomix 1420 water recirculating immersion thermoregulator. Reaction mixtures were contained in matched 10 mm path length quartz fluorometer cuvettes (Starna, Essex, UK). Typically, 2.950–2.935 ml of reaction mixture containing Tris–HCl buffered HRPO and HVA was preincubated in a stirred cuvette until it was thermally equilibrated at 37.0 ± 0.1 °C (measured in the cuvette). The polyamine substrate was added in a total volume of 30 µl and the mixture incubated for a further few minutes to achieve thermal equilibrium. Recording was commenced to control for blank rate (Fluorescence Data Manager software 'time drive' dialogue) and then 5–50 µl of enzyme preparation added to initiate the reaction. Fluorescence (λ_{ex} 315 nm; λ_{em} 420 nm) was monitored continuously for at least 5 min at 1.00 s intervals. In the final reaction mixture (3.0 ml), substrate concentration was 10 µM (approximately 10 × K_m) or as appropriate for the determination of kinetic parameters; HVA concentration was 1.00 mM, HRPO concentration was 2.23 purpurogallin units (10.6 µg)/ml in 0.05 M Tris–HCl, pH 7.43 ± 0.05 (measured in the cuvette at 37.0 °C). The fluorometric data was related to hydrogen peroxide production by adding 0.10 nmole and 0.50 nmole increments to Tris buffered HVA/HRPO to obtain a measure of fluorescent units per nmole hydrogen peroxide production. Hydrogen peroxide concentration had been accurately determined (*qq.v.* §2.3.1.2, 2.3.1.6 and 2.3.1.7).

Under the conditions of the assay, the maximal velocity, V_{max} of the auxiliary (coupling) enzyme, horseradish peroxidase, was 3.55 ± 0.093 µM.s^{-1} and $K^{app}_{mH_2O_2}$ = 17.3 ± 0.4 µM ([HVA] 1.0 mM), thus, the time lag to measure 0.98*v*, the experimental velocity of the amine oxidase, was 19 s [1653,1654] after which time the amine oxidase catalysed reaction was rate limiting. The rates of the enzyme reactions were obtained from analysis of the slopes of the initial linear portion of progress curves of the coupled enzyme reaction, which were generally followed for at least 5 min. Initial velocities were determined from the slope of a least squares linear regression to the linear portion of the progress curve between t = 20 and 80 s using the Obey software (©Perkin–Elmer) for the LS 50B Luminescence spectrophotometer. During this time substrate depletion was less than 5%. The blank rate was subtracted from the initial rate measurements for each substrate concentration to correct for the formation of the fluorophore in the absence of the oxidase. Control experiments indicated that inactivation of the oxidase was not apparent during the course of the measurements.

2.3.1.6 Spectrophotometric Hydrogen Peroxide Determination

Hydrogen peroxide concentrations were measured routinely on a PYE Unicam SP8-100 UV–visible spectrophotometer (PYE Unicam Ltd, Cambridge, UK). Reported values of molar absorption coefficients (ε) for hydrogen peroxide have wide variance, Table 2.1.

TABLE 2.1. Reported Molar Absorption (Extinction) Coefficients for Hydrogen Peroxide

Extinction Coefficient ($M^{-1}.cm^{-1}$)	*Reference*
ε_{240} 40	[1636] p.439
ε_{240} 45.34 ± 1.13	[1636] p.495
ε_{240} 43.6	[1656]
ε_{240} 39.4 ± 2	[1644,1655]
ε_{240} 41	[1657] p.165
ε_{240} 40 ± 1	[1657]
ε_{240} 40.0	[1658]
ε_{240} 39.27 ± 1.62	[1635]
ε_{230} 81	[1639,1659]
ε_{230} 72.4	[1647]
ε_{230} 59.5	[1660]
ε_{240} 34.34	[1660]

To determine H_2O_2 concentration spectrophotometrically, the absorption characteristics of H_2O_2 were required (Figure 2.3). The molar absorption coefficients ± SD found under these conditions were:

ε_{230} 62.43 ± 1.10 $M^{-1}.cm^{-1}$

and ε_{240} 39.22 ± 0.56 $M^{-1}.cm^{-1}$

in agreement with more recently reported values [1655].

For a solution of approximately 10 mM H_2O_2 about a 1/1000 dilution of 10.299 M stock (~30% w/v '100 volumes' H_2O_2) was required. At this concentration A_{230} gives an absorbance value that can be conveniently and accurately measured.

2.3.1.7 Redox Titration of Hydrogen Peroxide with Ceric(IV) Sulphate

Hydrogen peroxide concentrations were accurately determined by titration with a 0.1 *N* ceric(IV) sulphate solution standardized by reaction with arsenious acid in the presence of Ferroin redox indicator and 'osmic acid' [1661].

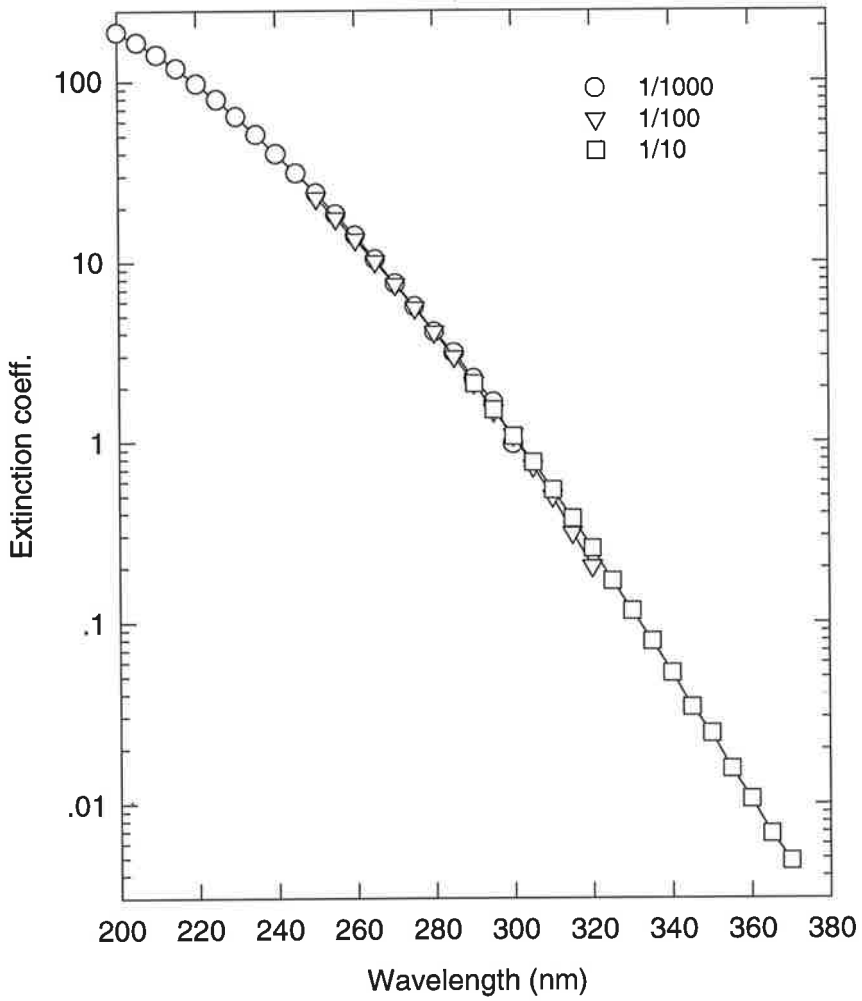


FIGURE 2.3. Absorption Characteristics of Hydrogen Peroxide in Water
The concentration of hydrogen peroxide stock solution was determined by titration with ceric(IV) sulphate that had been standardized with arsenious oxide, then molar absorption coefficients from 200–400 nm were measured. Matched quartz cuvettes, cleaned as described by Beers and Sizer [1660]. The stock solution was 10.299 M. Bandwidth 1 nm.

2.3.2 Steady-State Kinetics Measurements

2.3.2.1 Enzyme Purity for Kinetics Studies

Ideally, a pure, stable enzyme preparation is required for kinetics studies. However, no enzyme is purified to the point of absolute homogeneity. Even when other proteins constitute less than 1% of the purified enzyme, there are likely to be millions of foreign molecules in a reaction mixture. In the measurement of steady-state enzyme kinetics most important aspects of purity are the elimination of: contaminating activities that may compete for the substrates, contaminants that interfere with the detection system or remove the product that is being measured, and inhibitors of the enzyme or its detection system. Enzymes that may interfere with the assay of polyamine oxidase include: those which may compete for the substrates, e.g. monoamine oxidases or other enzyme isoforms; those which may interfere with the detection method, e.g. catalase; and those

which may remove the products, e.g. aldehyde dehydrogenases.

Purification removes particulate matter that may interfere with the assay by reflecting or refracting light. Purification also removes inhibitors such as those found in amniotic fluid [765] and hydrogen donors (*q.v.* §2.3.1.1), which cause lag times at the beginning of the progress curve. Indeed, a variable lag phase was observed when working with unpurified retroplacental serum samples. Unpurified enzyme preparations are also likely to contain endogenous substrate. Purification of enzyme and determination of enzyme purity also facilitate the evaluation of various kinetic parameters that depend on a knowledge of enzyme concentration.

It may be impossible to eliminate a side reaction that arises from the intrinsic properties of the enzyme as occurs with polyamine oxidase, which produces substrates as products of the enzymatic reaction; e.g. when spermine is metabolised to spermidine, spermidine is also a substrate for the enzyme. However, by ensuring that the measured initial rate period is kept to a minimum (less than 10% of substrate consumption is the often quoted 'rule of thumb' [1662]), these effects remain negligible.

The use of highly purified enzymes may be a problem because of the potential for very large amounts of enzyme to be included in the assays. If a slow reaction is observed where a large amount of enzyme is used, it is difficult to be certain that the reaction is due to the enzyme being studied and not to a very small amount of another enzyme present as a contaminant.

It is important to ensure that the purification procedures used do not lead to alterations in the properties of the enzyme. Polyamine oxidase appears to be subject to polymerization in concentrated form and this could potentially lead to inhibition of activity or steric hinderance.

High dilution of enzymes into an assay mixture may cause enzyme instability or adsorption of the enzyme onto surfaces. This can sometimes be overcome by using protective agents such as detergents, glycerol, PEG, PVA, or enzymatically inactive proteins. The presence of non-enzymatic protein in the enzyme preparation may help to stabilize the enzyme and reduce inaccuracies caused by enzyme adsorption to surfaces. Careful consideration and investigation were necessary to ensure that these protective agents do not interfere with enzyme kinetics (e.g. detergents, changing substrate affinities or albumin, adsorbing substrates [1663]) or interfere with the detection system (i.e. fluorescence).

Partially purified enzyme was used for steady-state kinetics studies reported here. Coincidently the enzymatically inactive protein impurities conferred a stabilizing effect on the enzyme. Two polyamine oxidase isoforms were separated by anion exchange chromatography and samples were taken from peak fractions. The purity of the enzyme was assessed by analytical methods including SDS-PAGE followed by Coomassie staining and the number of catalytic sites was estimated in this way.

2.3.2.2 Enzyme Concentration

It is useful to have a measure of enzyme concentration. Enzyme concentration is usually expressed in terms of catalytic rate, i.e. specific activity (Units/mg protein), which is adequate for comparison purposes, e.g. between tissues, or for monitoring changes or differences in the enzyme levels in samples in response to different treatments, developmental stages or disease states. In contrast, protein and immunochemical methods measure the total concentration of enzyme molecules, both active and inactive. Indeed, immunoassays may measure fragments of the enzyme or partially processed forms. The enzyme concentration in terms of the number of subunits and the number of catalytic sites that exist on each molecule of the enzyme can be used to calculate the stoichiometry of the enzyme–substrate interaction. $[E]_0$, which may be written as $[E]_t$ or $[E]_{stoich}$, is the total or stoichiometric concentration of catalytic centres. This corresponds to the total enzyme concentration if there is a single catalytic centre per molecule [1664]. So consideration of the subunit structure and polymeric forms of the enzyme is important.

A direct measure of the enzyme catalytic site concentration may be made by the titration of the active site of the enzyme; for example, by active site labelling, in which a suitably labelled analogue is incorporated into the active site region of the protein to form a stable adduct [1665]. This may be done even when the enzyme is only partially purified. The label is usually a substrate analogue or potent competitive inhibitor which may be radioisotopically labelled [1666,1667]. For amine oxidases, methods have included the use of radiolabelled benzaldehyde, but this may not be an appropriate method for the pregancy-associated enzyme as suggested in section 1.3.6. It is an assumption that every molecule of a particular enzyme will have the same intrinsic activity as any other molecule. Since proteins are subject to post-translational modification as part of a natural maturation process it is possible that molecules at different stages of their processing (or degradation) may differ in their specific activities. Furthermore, it is possible that not all the active sites are used during a catalytic turnover, for example in situations of negative co-operativity [1665].

The carbonyl group reagent phenylhydrazine has been used for the active site titration of pig-plasma benzylamine oxidase [918,923,928] and can form a chromophoric complex. For the pig-plasma enzyme a single reactive prosthetic group (catalytic site) per enzyme molecule (dimer) and at least two copper molecules were reported to be present in the enzyme [918,928]. However, more recent studies by Klinman *et al.*, who used phenylhydrazine in titration of yeast amine oxidase enzyme activity, found that, like the bovine serum enzyme [848], there were two moles of titrated TPQ per enzyme dimer [567]. Indeed, this is consistent with the structure of the amine oxidases from *E. coli* and pea-seedlings, which are dimers of identical subunits each containing an active site [529,650].

The total or stoichiometric concentration of catalytic centres, $[E]_0$, for enzyme preparations in this study were estimated from enzyme protein purity and specific activity calculations assuming two catalytic centres per enzyme dimer.

2.3.2.3 Substrate Purity for Kinetics Studies

Along with enzyme purity, impurities in substrates must also be considered as sources of experimental error. The presence of contaminants in substrates may result in errors in the determination of kinetic parameters if they lead to inaccurate concentrations of substrate solutions or if any of the impurities are inhibitory [1668]. Impure substrate preparations are likely contain other substrates of the enzyme as contaminants, which may compete for the enzyme. A non-hyperbolic dependence of initial velocity on substrate concentration is observed if a substrate is contaminated by even a small amount of another substrate that has a much higher affinity for the enzyme but is broken down much more slowly [1662,1668,1669].

a) Substrate preparation and purity. The purest substrates commercially available were used in this study. Their purity was confirmed by reversed phase ion-pairing HPLC with dansyl chloride precolumn derivitization [1043]. The spermine.4HCl and spermidine.3HCl obtained from Calbiochem–Novabiochem were found to conform to the manufacturer's specifications of 99% and 99.5% pure (by GC). Putrescine was found to be >98% pure in accordance with the Sigma's specification (Figure 2.4). N^1-acetylspermine.3HCL, N^1-acetylspermidine.2HCl and histamine.2HCl were >99%, approx. 99% and >99% pure respectively by thin layer chromatography (t.l.c.) of underivatized compounds followed by ninhydrin analysis according to the manufacturer and were not analysed further.

b) Purity of polyamine substrates by HPLC of dansyl polyamines

Dansylation of polyamines. Dansyl chloride (5-dimethylaminonapthalene-1-sulphonyl chloride) has been widely used for derivatization of polyamines prior to their separation by t.l.c. or HPLC (reviewed in references [1670-1672]). Dansyl chloride reacts stoichiometrically with primary and secondary amines to form highly fluorescent derivatives. Each amino group is derivatized by the reagent [1673].

Polyamines were obtained as hydrochloride salts, made up to 10 mM in water and stored in polypropylene tubes at –70 °C. Since polyamines have been found to adsorb to glass [61,1674,1675], the plastic vials were used and subsequent manipulation was performed in siliconized glass tubes. Polyamines have been shown to be stable under these conditions [1675-1677].

Derivatization of the polyamines was performed as follows. High-purity dansyl chloride

was dissolved at 5 mg/ml in dry acetone and stored below 0 °C in the dark. (Impure dansyl chloride produces poor results [1678].) A 200 µl aliquot of the 10 mM polyamine solution was added to 500 µl of the dansyl chloride in acetone and 300 µl saturated Na_2CO_3 solution added to provide an alkaline medium for labelling, since the reaction produces hydrochloric acid [1673,1677]. The reaction mixture was vortexed for 30 s and dansylation allowed to proceed overnight at room temperature in the dark. Excess dansyl chloride was removed from the reaction by adding 100 µl L-proline 100 mg/ml, vortex mixing for 30 s, followed by a 30 min incubation at room temperature in the dark [1677]. Dansylated proline remains in the aqueous phase after extraction. Dansylation procedures were performed in the dark or under subdued laboratory light, since the derivatives are light-sensitive [1282,1677,1678]. Dansyl polyamines were extracted into 5 ml toluene by vortex mixing for 30 s. Toluene was used, since the peroxides in diethyl ether, an alternative solvent, are likely to generate free radicals which may cause chemical cleavages. The organic phase was removed, placed in a siliconized glass tube and dried under a stream of filtered N_2 in a heating block (Techne Dri–Block, Duxford, UK) at 35 °C. The residue was dissolved in 200 µl methanol (HPLC grade) containing 5% (v/v) HPLC grade acetic acid. The sample was then ready for analysis. If samples were not immediately analysed, they were stored at –15 °C where they have been shown to be stable [1043,1677,1679]. Side products may be produced under conditions of dansylation [1670] and decomposition of dansylated polyamines may occur at elevated temperatures, [1677] for example, during extraction and drying.

HPLC separation of dansylpolyamines. Separation of the dansylpolyamines was achieved on an Ultrasphere ODS reversed phase C-18 column (250 mm × 4.60 mmØ) with 5 µm packing. Instrumentation consisted of a Beckman Model 344 HPLC system equipped with 114M pumps controlled by a Model 421 programmable digital computer, a 165 variable wavelength detector and a 340 organizer consisting of a 410A sample injection valve fitted with a 20 µl sample loop and a high pressure mixing chamber (Beckman Instruments, Fullerton, CA). Fluorescence detection was with an LS-50 Luminescence Spectrophotometer (Perkin–Elmer, Buckinghamshire, UK), fitted with a 1.3 mm (26 µl) flow cell λ_{ex} 334 nm, λ_{em} 520 nm. Optimal excitation and emission wavelengths were determined by scanning each spectrum of dansyl polyamines in mobile phase A. Mobile phases were freshly prepared and consisted of the ion pairing reagent octane-1-sulphonate 20 mM, pH 3.5 in water with glacial acetic acid; acetonitrile-190 made up the balance of the mobile phase [1043]. Solvent A contained 50% v/v acetonitrile and solvent B contained 90% v/v acetonitrile, solvents were degassed by vacuum filtration to 0.22 µm and helium purging.

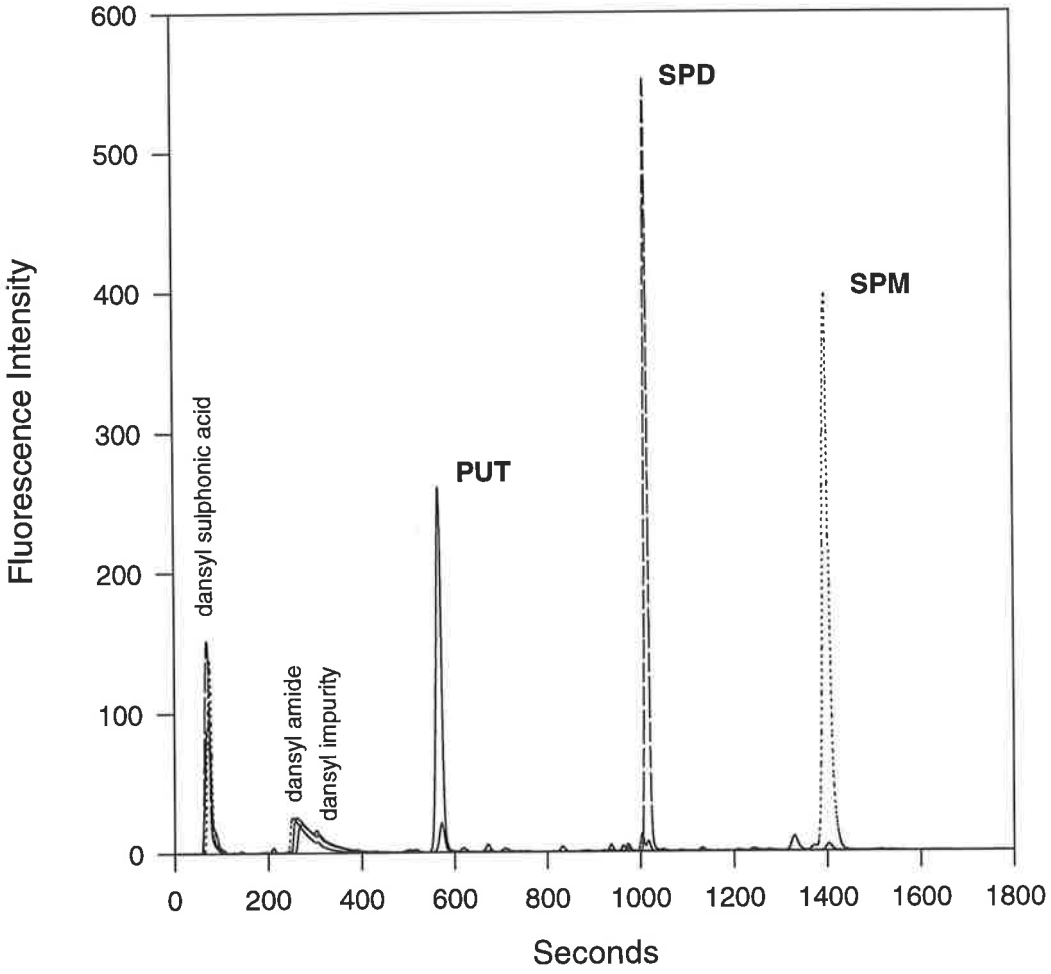


FIGURE 2.4. Reversed Phase HPLC Separation of Dansylpolyamines. Analyses were made as described in the text. The graph shows the results of individual analyses of dansyl-putrescine (PUT), -spermidine (SPD) and -spermine (SPM) on an Ultrasphere ODS reversed phase column (250 mm × 4.60 mmØ) with 5 µm packing (Beckman Instruments, Fullerton, CA). Dansylated polyamines were separated at 1.60 ml/min using ion-pairing HPLC according to file 20 after at least 5 min pre-equilibration before injection (File 20 = time (min):%B; 0:35; 5:38; 6:30; 15:90; 20:90; 25:100; 30:100; 31:35). Good resolution of dansyl polyamines and their *N*-acetyl derivatives has been shown by this method [1043].

2.3.2.4 Enzyme Kinetics

Steady-state kinetic parameters of polyamine oxidation by the multiple enzyme forms were determined using the horseradish peroxidase (HRPO) linked assay system as described above. Peak fractions from the anion exchange chromatographic separation of the multiple enzyme forms were used as the source of enzyme. Sufficient HRPO (40 U/ml) was included in the assay system to accurately reflect the true initial rate of polyamine oxidations [1629,1653]. Reactions were in 0.05 M Tris–HCl, pH 7.4 at 37 ± 0.1 °C. Reaction mixtures were pre-equilibrated at 37 °C and the reactions initiated by the addition of enzyme. Initial rate experiments were conducted using polyamine substrates in the range 0–1000 µM in reaction mixtures equilibrated with atmospheric oxygen.

Michaelis constants, K_{mA}, for various polyamines and histamine were calculated by fitting the Michaelis–Menton equation, modified to compensate for the observed substrate inhibition (which was indicated for the reactions by plots of Lineweaver–Burk transformations), to the data. The equation takes the form Equation 2.1 [1664,1680],

$$v = \frac{V_{max}[A]}{K_{mA} + [A] + [A]^2 / K_{iA}} \qquad \textit{Equation 2.1}$$

where K_{iA} is the substrate inhibition constant, to untransformed data sets of at least 10 initial rates (v) at varying substrate concentrations [A] by non linear regression analysis using 'Enzfitter' software from Elsevier–Biosoft (Biosoft, Cambridge, UK; ©R.J. Leatherbarrow, 1987) that uses the Marquardt iterative algorithm [1681] to determine the parameters for which reduced Chi-squared ($\chi^2.v$, the sum of the squares of the differences between the dependant variable in equations and the observations) is minimized by a reiterative process. Data were weighted by a 'robust' weighting using the algorithm of Mosteller and Tuckey [1682] as implemented by Duggleby [1683] in which each squared residual is multiplied by a 'bi squared' weight, (b_w). The 'bi squared' weighting detects and reduces the effects of the observations that deviate markedly from the fitted equation.

2.4 Radiochemical Amine Oxidase Assays

2.4.1 [^{14}C]Spermine Oxidation

2.4.1.1 Radiolabelled Substrate

One ml [^{14}C]spermine (50 µCi/ml; approx. 100 mCi/mmole) was added to 7.5 mM unlabelled spermine (5.2 ml) to give a final concentration of 6.37 mM spermine at 1.26 µCi/µmole. Buffered substrate solution was prepared with 50 µl labelled substrate diluted with 1.0 ml of 0.05 M Tris–HCl buffer, pH 7.4 (at 37 °C) containing 0.1% (v/v) Triton X-100. Spermine concentration was 303 µM with a specific activity of 1.26 µCi/µmole in this solution.

2.4.1.2 Procedure

Polyamine oxidase activity was measured using the radiochemical method described by Morgan and Illei [169], with modifications described by Ferrante *et al.* [798]. A 100 µl sample was added to 200 µl buffered substrate solution, the final spermine concentration was 202 µM. The reaction mixture was incubated in a waterbath at 37.0 ± 0.2 °C for 90 min with shaking. The reactions were terminated by addition of 50 µl trichloroacetic acid (50% w/v). Precipitated protein was sedimented by centrifugation at 1000 × g for 10 min. Aliquots (200 µl) of the reaction mixture

supernatant were applied to columns of Dowex 50W X2 (200–400 mesh, H^+ form; 4 cm × 0.8 cmØ in Econocolumns, Bio-Rad, Richmond, CA) followed by 200 µl water. Reaction products and substrate were separated by stepwise elution with standardized hydrochloric acid (*q.v.* 2.4.1.3) 1.9 M (10 ml) and 4.0 M (10 ml). The eluates were collected as two fractions into polypropylene tubes, triplicate 1.0 ml aliquots of each fraction were transferred to polyethylene scintillation vials and mixed with 12 ml Ready-Solv EP scintillation cocktail. DPM per sample were determined by counting in a Mark III Liquid Scintillation counter (Searle Analytic, Des Plains, IO), and percent substrate conversion calculated. Controls and blanks were included in each assay. The separation of substrate and reaction products by this method was confirmed by dual channel scintillation counting in an LS 3801 Liquid scintillation counter (Beckman, Fullerton, CA) using [^{3}H]spermidine and [^{14}C]spermine.

2.4.1.3 Standardization of Hydrochloric Acid

Concentrated hydrochloric acid, supplied as a ~36% (w/w) solution, density, $\rho \equiv 1.18$ g/ml, approximately 11.5 M, was standardized by volumetric analysis against recrystallized borax (sodium tetraborate.decahydrate) using methyl red as an indicator [1661]. Solutions of accurately known concentration were prepared after determination of the true HCl density by picnometry. The absolute density of water at ambient temperature for picnometric calculations was obtained from standard tables.

2.4.2 Reaction Products

Potential reaction products of polyamine oxidation have been discussed in section 1.2.5. Ion-pairing reversed-phase HPLC was used to identify the enzymatic cleavage sites of spermine and its metabolites when oxidized by retroplacental serum polyamine oxidase. Spermine was specifically labelled with [^{14}C] at internal carbons (*N,N′*-bis-(3-aminopropyl)-[1,4-^{14}C]tetramethylene-1,4-diamine). HPLC is a particularly useful technique in this case because it allows the simultaneous identification of multiple reaction products during a reaction [1684].

2.4.2.1 HPLC Separation of Polyamine Oxidase Reaction Products

Products of enzymatic polyamine cleavage were identified in a time course study of the retroplacental polyamine oxidase reaction, by separation of the ^{14}C labelled spermine substrate and its reaction products using HPLC. Reactions were in 0.05 M Tris–HCl, pH 7.4 [at 37 °C] with 50 µM [^{14}C]spermine (*N,N′*-bis-(3-aminopropyl)[1,4-^{14}C]-tetramethylene-1,4-diamine) as the substrate (specific activity 50 mCi/mmol, i.e. 2.5 µCi of [^{14}C]spermine (50 µCi/ml; 110 mCi/mmol stock) and 27.5 nmol additional cold spermine in a total assay volume of 1.00 ml).

The reactions were initiated by the addition of approx. 0.9 mU enzyme (determined by the method of Okuyama and Kobayashi [877], using [^{14}C]putrescine as the substrate) purified by pentyl-/ω-aminohexyl-agarose affinity chromatography (*q.v.* §5.2.1.2). Reaction mixtures were incubated at 37 °C and reactions terminated by adding 100 µl 72% (w/v) TCA. Precipitated protein was sedimented by centrifugation at 10,000 × *g* for 5 min (Eppendorf microcentrifuge). [^{14}C]Spermine and its reaction products were separated by reversed-phase, ion-pairing HPLC on a C_{18} column (Spherisorb ODS-2, 150 mm × 4.6 mmØ, Phase Separations, Deeside, UK) with a gradient of methanol:water in 20 mM potassium hydrogen orthophosphate containing 20 mM sodium octane-1-sulphonate, pH 3.0 (adjusted with phosphoric acid). The gradient comprised: solvent B (methanol : water – 70 : 40) 75% to 100% over 10 min, held for 10 min at 100% B, returned to starting conditions over 10 min and equilibrated for at least 15 min. Solvent A was methanol : water – 40 : 60. Solvents were delivered at 1.00 ml/min using a Beckman Model 344 liquid chromatography system as described in section 2.3.2.3. Aliquots of the reaction mixtures were diluted 1:4 in solvent A before injection (100 µl). The [^{14}C]polyamine standards mixture included 1.25 µCi [^{14}C]spermine, 0.75 µCi [^{14}C]spermidine and 0.05 µCi [^{14}C]putrescine per ml. Eluate was collected as 0.20 ml fractions using a Frac-100 fraction collector (Pharmacia, Bromma, Sweden), 4.0 ml of OptiPhase 'HiSafe' 3 scintillation fluid was added to each fraction and the radioactivity determined by scintillation counting (Model LS-3801, Beckman Instruments, Fullerton, CA).

2.4.3 Radiometric Diamine Oxidase Assay

Diamine oxidase (EC 1.4.3.-) activity was measured using a modification of the method first reported by Okuyama and Kobayashi [877] using [^{14}C]putrescine as a substrate, as developed by Tryding *et al.* [664,700,702]. Oxidation of [1,4-^{14}C]putrescine by amine oxidase leads to the formation of γ-[^{14}C]aminobutylaldehyde which rapidly and spontaneously undergoes an internal cyclization to form the internal aldimine ring compound, Δ^1-[^{14}C]pyrroline. The pyrroline may undergo further spontaneous polymerization [489]. Unlike putrescine, which is very soluble in water, Δ^1-pyrroline and its polymers are soluble in toluene at alkaline pH and may be easily separated from the substrate by solvent extraction. As shown in Figure 1.5, γ-aminobutylaldehyde may be alternatively oxidized by aldehyde dehydrogenases (EC 1.2.1.3) to γ-aminobutyric acid (GABA) [193,212,1016] in biological mixtures. The formation of 4-amino-1-butanol is also possible through reduction by aldehyde dehydrogenase and/or alcohol dehydrogenase [193,1685] which prevents cyclization. Other fates of putrescine in biological mixtures include its acetylation to acetylputrescine [208,212,1686] by *N*-acetyltransferase and its subsequent oxidation by monoamine oxidase (EC 1.4.3.4) [191,212].

Putrescine may also be metabolized to spermidine by spermine synthase (EC 2.5.1.6) [947,1025]. The Δ^1-pyrroline level *decreases* in all of the scenarios described above and may be interpreted as low DAO activity. Thus, results with this radiochemical method must be regarded with caution if biological mixtures are assayed. A number of authors have used enzyme inhibitors to check the validity of the assays [193,947,978,980,1016,1025]. However, these inhibitors may also influence DAO activity [978]. It is pertinent to this study that plasma lacks enzymes that degrade γ-aminobutylaldehyde [733]. The assay system has been critically reviewed by a number of authors [673,733,765,782,998].

2.4.3.1 Procedure

A substrate stock solution was prepared with 250 µl [^{14}C]putrescine (50 µCi/ml; 118 mCi/mmole) and 500 µl of 10 mM unlabelled putrescine made up to a total volume of 5.00 ml. One hundred microlitres of substrate stock, to give a final assay concentration of 102 µM putrescine at 2.45 mCi/mmole, was added to 750 µl water and 100 µl 0.5 M Tris–HCl buffer stock (pH 7.4 at 37 °C) in screwcap siliconized glass reaction tubes. The buffered substrate was preincubated for 10 min at 37 °C. Reactions, in the presence of atmospheric oxygen, were initiated by the addition of a 50 µl enzyme sample and allowed to proceed for 60 min in a 37.0 °C waterbath with shaking before they were terminated by addition of 100 µl saturated sodium carbonate solution. Reaction products were immediately extracted into 5.0 ml toluene containing 1,4-di(2-(5-phenoxazolyl))-benzene (POPOP) 0.1 g/l and 2,5-diphenoxazole (PPO) 6.0 g/l. The mixtures vortexed for 30 s to selectively extract reaction products [673] and then centrifuged at 400 × *g* for 5 min to facilitate phase separation (JB-6 Centrifuge, Beckman Instruments, Fullerton, CA). The aqueous phase was frozen and the organic phase decanted into miniature polypropylene vials for scintillation counting in a Beckman LS-3801 spectrometer. A 100 µl aliquot of the aqueous phase was also counted with 5 ml scintillation cocktail. Controls included a reagent blank and a sample blank in which enzyme activity was terminated at time zero by the addition of 100 µl 72% (w/v) trichloroacetic acid.

2.5 Preparation of Retroplacental Serum

Retroplacental blood (36 ± 22 ml per placenta, n = 297; mean ± SD) was collected as described by Klopper and Hughes [1542] from human placentae after both Caesarean section and vaginal deliveries at a mean gestational age of 38.27 weeks (range 33.5 – 42 weeks). Placental membranes were inverted over the maternal (decidual) surface of the placentae, and retroplacental blood, which is comprised mainly of intervillous blood with admixtures of decidual and placental interstitial fluid from the placental–endometrial interface, collects at

the placental margin and between the cotyledons on the placental surface. The retroplacental blood was syringed up and placed in serum collection tubes. After the blood clotted, serum was separated by centrifugation at 2,000 × *g* and stored at –70 °C. Placentae were obtained from the labour theatres of the Queen Victoria and Calvary Hospitals, Adelaide; usually within 20 min *post partum*. Retroplacental serum polyamine oxidizing activity was determined using radiochemical and/or fluorometric methods (*qq.v.* §2.3.1 and 2.4.1).

Retroplacental blood collections were carried out in accordance with the National Health & Medical Research Council statement on human experimentation, the guidelines recommended by, and with the approval of the Ethics Committees of the Queen Victoria Hospital, the Women's Children's Hospital (formerly the Adelaide Children's Hospital) and The University of Adelaide.

2.6 Chromatographic Procedures

2.6.1 Low Pressure Chromatography

Chromatographic media was generally packed into Pharmacia K-columns. Chromatography was conducted at 4 °C using a 2120 Varioperpex II pump (LKB, Bromma, Sweden) connected to an LKB 2070 UltroRac fraction collector. Glass fraction collecting tubes, treated with dimethyldichlorosilane to reduce protein adsorption, or polypropylene tubes were used. All buffers were filtered through Millipore 0.45 μm membrane filters under reduced pressure. During chromatography protein concentration in column eluates was monitored by absorbance at λ_{280} in an LKB 8300 Uvicord II UV-analyzer with output to an LKB 6520 chopper bar recorder. Protein concentration in column eluates was measured by absorbance at λ_{280} in a PYE Unicam SP8-100 UV–visible spectrophotometer. Absorbance values >3.0 were not measured and are indicated as 3.0. Unless stated otherwise enzyme activity in column fractions was determined fluorometrically using putrescine as the substrate (*q.v.* 2.3.1). Salt gradients were measured by chloridimetry using a CMT Chloride Titrator (Radiometer, Copenhagen, Denmark) or by osmolality using a Digital Micro-Osmometer (Roebling, Germany).

The pH of Tris–HCl buffered solutions has a large temperature coefficient and the values quoted are those at 4 °C.

2.6.2 Size-Exclusion High Pressure Liquid Chromatography (SE-HPLC)

SE-HPLC was conducted at ambient room temperature. Instrumentation consisted of a Beckman 344 HPLC system equipped with 114M pumps controlled by a Model 421

programmable digital computer, and a Model 340 organizer consisting of a 410A sample injection valve fitted with a 250 µl sample loop (Beckman Instruments, Fullerton, CA). SE-HPLC buffer was 100 mM sodium phosphate containing 0.30 M NaCl, pH 6.80 (I = 0.50)) and was prepared from stock solutions that had been purified by sequential chromatography through AG-1-X8, Chelex 100 resin and a C_{18} SepPak to remove impurities which might interfere with spectrophotometric protein detection and contaminate the enzyme preparation [1687]. The buffer was filtered through a 0.45 µm Millipore HA-membrane under reduced pressure and dissolved air was removed by purging with helium gas. Column eluate was monitored at $A_{280 \text{ or } 205}$ using a Beckman Model 163 variable wavelength detector fitted within standard flow cell, interfaced with a pen recorder (Rikadenki Kogyo, Japan), 0.20 ml fractions were collected using a Frac-100 fraction collector (Pharmacia, Bromma, Sweden) and their polyamine oxidase activity determined fluorometrically (*q.v.* §2.3.1). Glass fraction collecting tubes, treated with dimethyldichlorosilane to reduce protein adsorption, or polypropylene 'Eppendorf' microtubes were used.

2.7 Polyacrylamide Gel Electrophoresis

In this study, protein containing samples were resolved in polyacrylamide (PA) slab gels using discontinuous electrophoresis methods for the high resolution analytical separation of protein mixtures based on techniques reviewed in [1688-1691]. The sodium dodecyl sulphate (SDS) system of Laemmli [1127] was used. This method separates protein subunits on the basis of their molecular weight [1130,1692]. SDS greatly reduces conformational and charge density differences between proteins and so reduces the effect of variability in partial specific volume and hydration [1690]. Using the slab gel technique [1126,1693,1694], multiple samples, including molecular weight marker proteins, could be electrophoresed under identical conditions in a single gel such that the band patterns produced could be directly compared.

2.7.1 Stock Solutions

2.7.1.1 Acrylamide/bis-Acrylamide Monomer

A 30% (w/v) Acrylamide/bis-acrylamide monomer stock solution was prepared according to Hjertén nomenclature, viz. 30% T, 2.67 %C [1695]. Acrylamide (29.2 g) and *N,N'-bis*-methylene-acrylamide (0.8 g) were made up to 100 ml with water, filtered to 0.22 µm and stored at 4 °C in an amber coloured bottle in the dark, since polymerization of acrylamide monomers can be initiated by light. During storage of acrylamide solutions some hydrolysis occurs yielding acrolein, acrylic acid and ammonia, so acrylamide stock solutions were not kept beyond two months.

2.7.1.2 Resolving Gel Buffer

Resolving gel buffer stock of 3 M Tris–HCl, pH 8.9 was prepared by dissolving 36.33 g high purity Tris base in 48.00 ml 1.0 M HCl, the solution was made up to 100 ml with water. The buffer was filtered to 0.45 μm and stored at 4 °C. The pH, approx. 8.9, was not adjusted. HCl concentration was determined by titration against borax with methyl red as an indicator (*q.v.* §2.4.1.3).

2.7.1.3 Stacking Gel Buffer

Stacking gel buffer, 0.5 M Tris–HCl, pH 6.80, 0.4% (w/v) SDS, was prepared by dissolving 6.06 g high purity Tris-base in 40 ml of water, this was titrated to pH 6.80 with approximately 48 ml 1.00 M HCl, 4 ml of 10% (w/v) SDS stock was added and the solution made up to 100 ml with water. The solution was filtered to 0.45 μm and stored at 4 °C.

2.7.1.4 Sodium Dodecyl Sulphate

A 10% (w/v) sodium dodecyl sulphate solution was prepared by dissolving SDS [C_{12} > 99.5%, protease free] in water. It is important that SDS with a high C_{12} content is used [1696]. The binding of SDS to most proteins under denaturing reducing conditions results in the formation of complexes with a constant charge per unit mass (1.4 g SDS per 1 g protein at SDS concentrations > 8×10^4 M [1697-1699] so protein separations occur on the basis of their molecular weight. The presence of contaminating C_{13}, C_{14} alkyl sulphates is known to change the binding ratio and consequently the mobility of detergent protein complexes. Variability in the alkyl chain composition not only affects the estimation of molecular weight by electrophoresis but will make interpretation and comparison of complex gel patterns difficult [1700]. Furthermore, longer chain alkyl sulphates, especially ≥ C_{16}, bind tenaciously to proteins inhibiting their renaturation [1696], e.g. for *in situ* activity staining or sequencing.

2.7.1.5 Bromophenol Blue Solution

Bromophenol Blue (100 mg), a sulphonphthalein dye in acid form, was triturated with 2 ml of 0.1 *N* sodium hydroxide and made up to 100 ml with water to give a 0.1% (w/v) solution.

2.7.1.6 Ammonium Persulphate Catalyst Solution

A 10% (w/v) ammonium persulphate solution was prepared by dissolving 100 mg of ammonium persulphate (APS) in 900 μl water. This solution is unstable and was made just before use.

2.7.1.7 SDS Running Buffer

A ×5 buffer stock was prepared by dissolving 30.3 g Tris base, 144.0 g glycine, and 10.0 g SDS in 2 l water without adjusting the pH. Buffer stock was stored at 4 °C. On dilution the ×1 buffer was 0.025 M Tris, 0.192 M glycerol and 0.1% (w/v) SDS, pH 8.3.

2.7.1.8 SDS Overlay Solution

SDS overlay solution, 0.375 M Tris–HCl, pH 8.8; 0.1% (w/v) SDS, was prepared by mixing 12.5 ml resolving gel buffer stock with 1.0 ml 10% (w/v) SDS and 86.5 ml water.

2.7.1.9 Sample Buffer Stock

A ×2 sample buffer stock of 0.125 M Tris HCl, 4.6% (w/v) SDS (pH 6.8), was prepared by dissolving 1.515 g Tris base in water, adjusting the pH to 6.8 with 1.0 N HCl, and adding 4.6 g SDS. The solution was made up to 100 ml with water, filtered to 0.22 µm, and stored at room temperature.

2.7.2 Gel Preparation for Discontinuous SDS-PAGE Slab Gels

Discontinuous SDS-PAGE slab gels were prepared essentially as described by Laemmli [1128] with modifications according to Ames, Reid and Bieleski [1126,1693] and refinements as described in the text. The volumes of gel solutions required for slab gels run in a Protean II vertical slab gel electrophoresis cell (Bio-Rad, Richmond, CA) were calculated and used.

2.7.2.1 Single concentration gels

a) Stacker gel. Monomer solutions for stacker gels with a final composition of 3.75% acrylamide in 0.125 M Tris–HCl, (pH 6.8) and 0.1% SDS were prepared by mixing 3.75 ml of 30% acrylamide/bis stock, 7.5 ml of stacking gel buffer and 18.75 ml of water. Solutions for 3.0% gels were prepared by appropriately reducing the amount of acrylamide and increasing the water volume. The monomer solutions were degassed under vacuum at room temperature for 15 minutes. This procedure avoids bubble formation in the gel and removes oxygen, which inhibits polymerization by acting as a free radical scavenger. Immediately before use, 30 µl TEMED and 90 µl freshly prepared 10% APS were added to initiate polymerization. The polymerization catalysts were mixed in carefully to avoid reintroducing air into the mixture.

b) Resolving gel preparation. Monomer solutions for resolving gels of varying final acrylamide concentrations (5, 6, 7.5, 8.5, 10 and 12.5%) in 0.375 M Tris HCl, (pH 8.8) and 0.1% SDS were prepared by mixing varying amounts of 30% acrylamide/bis stock solution with varying amounts

of water and constant amounts of 10% SDS and resolving gel buffer stock. Immediately before use, the buffered monomer solutions were degassed for 15 min and polymerization catalysts, APS (final concentration 0.025% w/v) and TEMED (final concentration 0.025% v/v) were added.

c) Resolving gel solutions for linear–exponential gradient gels. Resolving gel monomer solutions for linear–exponential gradient gels were prepared as essentially described by Kelly and Luttges [1125,1689,1701,1702]. 'Heavy' (17.5%) acrylamide solutions were prepared by mixing 7.30 ml 30% acrylamide/bis monomer stock solution with 1.563 ml resolving gel buffer stock, 125 µl 10% SDS, and 3.52 ml 50% (w/v) sucrose. 'Light' (10 or 5%) acrylamide solutions were prepared with appropriate volumes of 30% acrylamide/bis monomer stock solution mixed with 3.125 ml resolving gel buffer, 250 µl 10% SDS, and water to a final volume of 25.0 ml. Immediately before use the monomer solutions were degassed for 15 min and APS (final concentration 0.02% w/v) and TEMED (final concentration 0.025% v/v) were added to initiate polymerization.

2.7.3 Sample Preparation for SDS-PAGE

Overloading and underloading of gels was avoided by using about 1–10 µg of each protein (per band) to give optimal results. So for a complex mixture 50–100 µg protein was required.
Where necessary, precipitation of the protein samples was used to remove interfering compounds and to concentrate dilute protein samples [1125,1688,1703]. Samples were heated in a boiling waterbath for 3–5 minutes to ensure denaturation of the protein, reduction, and complete reaction with SDS. After boiling, the samples were cooled to room temperature and centrifuged for 5 minutes in an Eppendorf bench centrifuge at 10,000 × *g*.

2.7.3.1 Sample Buffer

Samples with a final volume of 25–60 µl contained 62.5 mM Tris–HCl (pH 6.80), 2.3% (w/v) SDS, 10% glycerol (v/v), 5% (v/v) 2-mercaptoethanol and 0.0018% (w/v) bromophenol blue (and 0.009% (w/v) phenol red) titrated with < 3 µl of 1.0 M Tris base if necessary [1125,1127]. 2-mercaptoethanol was omitted under nonreducing conditions.

2.7.4 Electrophoresis Procedure for Laemmli Slab Gels Using the Bio-Rad Protean II Gel Apparatus

2.7.4.1 Assembly of the glass plate sandwich

The glass plate sandwich was assembled and placed into a Protean II slab gel casting stand according to instructions recommended by the manufacturer [1704]. Spacers were smeared with a thin layer of grease (Celloseal, Hoefer Scientific, San Francisco, CA) to prevent leaks. This

technique, as recommended by Ames [1126], is more effective and technically less difficult than sealing the plates with hot agarose solution.

2.7.4.2 *Casting Discontinuous Gels*

Resolving gel monomer solution of an appropriate acrylamide concentration was quickly and smoothly poured into the space between the plates allowing room for the stacking gel and comb. The monomer solution was immediately overlaid with buffer of the same composition as the buffer in the gel mixture. Care was taken to ensure that the overlay was added gently to prevent mixing with the monomer solution. Mixing at the surface could be detected because of the different refractive indices of the overlay and acrylamide solutions. After undisturbed polymerization (45 min to 1 h), the gel surface was promptly rinsed with resolving gel buffer and well drained. The rinse removes residual acrylamide dissolved in the overlay buffer, which may polymerize in an irregular manner [1126]. A very sharp liquid–gel refractile interface was seen when the monomer had polymerized. Gels were left undisturbed for about 10 min after the appearance of the refractile boundary to ensure that polymerization of the monomer was complete. Alternatively, the monomer solution was overlaid with water saturated *n*-butanol [1704,1705]. Mixing was generally less of a problem when using the *n*-butanol overlay because of the much higher density of the monomer solution. The overlays spread evenly across the surface of the gels to give them flat surface and prevent inhibition of polymerization at the gel surface by excluding oxygen. Immediately after polymerization, the alcohol overlays were poured off, the surface of the gels were rinsed with water, and the overlay replaced with resolving gel overlay buffer. The alcohol overlays were not permitted to remain on the gels longer than about 1 h to avoid the risk of gel surface dehydration.

2.7.4.3 *Casting the Stacking Gel*

The stacking gel monomer solution was prepared as previously described and a small volume used to rinse the surface of the resolving gel. The stacking gel mixture was then poured into the remaining space between the plates. The comb was then placed into the monomer solution being careful not to trap any bubbles below the teeth of the comb. Oxygen would inhibit polymerization and cause a local distortion of the gel surface at the bottom of the wells. When the comb was properly aligned, more stacking gel solution was added to fill the sandwich completely.

The monomer solutions were allowed to polymerize undisturbed for 30–45 min. Once the stacking gel had polymerized in place, the gel was used immediately before mixing of the pH interface occurred, since the pH discontinuity of the stacking gel, resolving gel and electrode buffer is essential for stacking and sharp unstacking of the samples. After polymerization of the

stacking gel, the comb was removed and the wells rinsed with reservoir/tray buffer. The wells were then left full of electrode buffer to prevent their surfaces dehydrating.

2.7.4.4 Assembling the Upper Buffer Chamber

The gel sandwich was assembled to the central cooling core according to instructions for the Protean II slab gel apparatus recommended by the manufacturer [1704]. A recirculating water cooler (Julabo F40, Seelbach, Germany) was connected to the central cooling core and set to 10 °C. Half a litre of reservoir buffer was reserved for the upper buffer chamber and the remainder (1.5–3.0 l) poured into the lower buffer tank. A large, teflon coated, magnetic stirring flea was added to the lower buffer tank so that the buffer could be circulated. More efficient cooling was maintained if the buffer tank was topped up with additional buffer or water. Dilution of the lower buffer by up to 1 : 2 should have no adverse effect on resolution.

2.7.4.5 Loading the Sample Wells

Pre-prepared samples were conveniently loaded using Multiflex MT1379 pipette tips (Multitechnology Inc, Salt Lake City, UT). Loading was done as quickly and carefully as possible to avoid diffusion of the sample and of the discontinuous pH boundaries, and to avoid mixing between adjacent wells or with the electrode buffer. The glycerol in the samples provided the density required to underlay the electrode buffer without mixing. Each well contained equal volumes of sample or blank sample buffer. When all the samples were loaded, the upper buffer chamber was filled.

2.7.4.6 Running the Gel

The power supply, Model 2103 (LKB, Bromma, Sweden) or an EPS 500/400 electrophoresis power supply (Pharmacia, Uppsala, Sweden), was run at 25 mA constant current per gel (i.e. 50 mA/two gels) beginning at about 85 volts. When the bromophenol blue tracker dye, indicating the Kohlrausch boundary [1689,1706], had penetrated the resolving gel, the current was increased to 30–35 mA/gel. During the run (total time approximately 4–5 h) the voltage delivered increased to about 0.35 kV. When the refractile schlieren boundary [1707], which runs just ahead of the Kohlrausch boundary, reached the bottom of the plates the electrophoresis was complete.

2.7.4.7 Removing the Gel

The gel sandwich was disassembled by removing the clamps from the glass plates. One of the spacers was used to gently pry the plates apart, so that the upper glass pulls slowly away from the gel, which usually remained on the lower plate. The gel was lifted from the plate and immediately

placed into fixative for silver staining or dye solution for Coomassie Blue staining.

2.7.5 Casting Discontinuous Linear–Exponential Gradient SDS Polyacrylamide Gels

Linear–exponential gradient SDS-polyacrylamide gels were prepared using the Bio-Rad model 385 gradient forming apparatus according to the manufacturer's instructions [1702]. The gel overlaid with resolving gel buffer overlay solution as previously described (*q.v.* §2.7.4.2).

2.7.6 Relative Molecular Mass Protein Standards

Bio-Rad high and low molecular mass markers and Amersham prestained Rainbow markers were used. Molecular weights reported for proteins examined by SDS-PAGE often vary sightly according to the values used for the molecular weight markers. We have used molecular weights most appropriate for determining the relative molecular mass (M_r) values in SDS-PAGE (*q.v.* Table 2.2), viz. Myosin, 200,000 [1708]; β-galactosidase, 116,250 [1709]; phosphorylase-b, 97,400 [1710]; bovine serum albumin, 68,000 [1130,1711]; ovalbumin, 45,000 [1712]. These values may vary slightly from molecular mass values (m, in daltons) calculated from amino acid sequences. In particular BSA and ovalbumin, which are glycosylated, run at higher apparent M_r values. M_r values were calculated from first order polynomial regressions to the data given by the markers.

TABLE 2.2. Variations in Reported Molecular weight, M_r (Relative Molecular Mass) Values of Protein Standards used in Electrophoresis

Protein	*Subunit M_r*	*Reference*	*Comments*
myosin	200,000	[1708]	rabbit skeletal muscle by sedimentation velocity
β-galactosidase	116,250	[1709]	*E. coli* subunit molecular mass calculated from protein sequence 116,248 Da
phosphorylase b	97,000	[1710]	rabbit muscle glycogen phosphorylase calculated from the protein sequence 97,412 Da
	92,500	[1713]	sedimentation velocity ultracentrifugation analyses
	94,000	[1130,1714]	ultracentrifugation analyses
	100,000	[1715]	SDS electrophoresis
albumin	67,000	[1689,1716-1718]	bovine serum physical methods
	68,000	[1130,1711,1719]	relative molecular mass by electrophoretic methods
	66,267	[1720,1721]	bovine serum albumin calculated molecular mass by sequence analysis
	69,000	[1722]	gel electrophoresis
Ovalbumin	45,000	[1707,1712,1723-1725]	the molecular mass of ovalbumin based on amino acid sequence of its polypeptide chain is 42,699; however ovalbumin contains 4.29% carbohydrate [1707,1724,1725] and the glycoprotein has an apparent molecular weight of 45,000 when determined by physical methods [1712]

2.7.6.1 Amersham Rainbow Markers

Individually coloured protein relative molecular mass markers, prestained with covalently bound triazine dyes were used to provide an internal control for Western blots and to allow the protein separation to be monitored in real time. The dyes can cause significant shifts in apparent molecular mass of the proteins and were therefore not used for accurate relative molecular mass determinations.

2.7.7 Protein Detection

2.7.7.1 Coomassie Blue R Staining

Protein staining using Coomassie Brilliant Blue R was based on adaptation of methods reviewed by several authors [1726-1732]. Coomassie Brilliant Blue R-250 staining solution was prepared as 0.125% (w/v) in 50% (v/v) methanol, 10% (v/v) acetic acid [1130,1733,1734] (The dye belongs to the magenta family, R indicates a reddish hue, λ_{max} 550 nm, CI 42660; 250 indicates the dye strength is ×2.5 that of the 'normal' supply; Sigma Chemical, Cat. No. B-0149; dye content is stated as 70–75%). Gels were bathed in the stain with gentle shaking overnight for maximum sensitivity (although clearer backgrounds were obtained with only 2 h staining). The gels were rinsed with water and destained in 50% (v/v) methanol, 10% (v/v) acetic acid for one hour (fast destaining, as recommended by Wyckoff, Robard & Chambach [1735] and Matsuidaria & Burgess [1736]) followed by final slow destaining in 5% (v/v) methanol, 7% (v/v) acetic acid [1705,1733]. A few grams of Dowex 50 ion exchange resin was added to the destaining solution to absorb the dye as it diffused from the gel during slow destaining [1734]. The gels were stored in water or 7% (v/v) acetic acid [1737]. During gel storage the intensity of the band staining increased [1737]. The detection limit using this stain was 0.2–0.5 µg protein per band [1688,1719] and staining was linear to around 50 µg for at least some proteins [1738-1740].

2.7.7.2 High sensitivity Coomassie Brilliant Blue G Staining

The high sensitivity Coomassie Brilliant Blue G (Xylene Brilliant Cyanin G; CI 42655; G indicates a greenish hue λ_{max} 595 nm) staining procedure was adapted from that of Neuhoff *et al.* [1728,1741], who had developed the method using the colloidal properties of the dye in the presence of ammonium sulphate.

a) Purification of the dye. Coomassie Brilliant Blue G was purified before use because commercially available dyes contain impurities that increase background staining and decrease sensitivity [1719,1726,1728,1732]. The dye was purified using the method described by Neuhoff

b) Staining. The staining method described by Neuhoff [1741] was followed. After electrophoresis the gel was soaked in 12.5% (w/v) trichloroacetic acid for 1 h to immobilize proteins and wash out gel components that may cause background staining (Tris, glycine, SDS [1728]).

Staining solution was prepared from 80 ml of stock staining solution and 20 ml methanol. A stock staining solution of 0.1% (w/v) purified CBB G in 2% (w/v) phosphoric acid, 10% (w/v) ammonium sulphate, was prepared by dissolving ammonium sulphate in 2% (w/v) phosphoric acid, followed by addition of CBB G-250 from a stock solution (1 g purified dye in 20 ml water), after the ammonium sulphate had completely dissolved. The stock solution was shaken to disperse large colloids before use.

The gel was incubated overnight in the staining solution with gentle shaking. After staining, the gel was briefly rinsed in 25% (v/v) methanol [1728], then stored in 20% (w/v) ammonium sulphate, which fixes the stain. Before drying, the ammonium sulphate was washed from the gel by soaking it once for 30 min; and twice for 15 min in changes of 0.5% (v/v) acetic acid containing 3% (v/v) glycerol. The detection limit of this method is about 0.05 µg per band [1719] or 0.7 ng/mm^2 for BSA [1728] and staining intensity is linear to around 20 µg.

2.7.7.3 Silver Staining

Silver stain methods have been reviewed in [1727,1729,1731,1742-1745].

a) Merril silver staining. Silver staining was performed using a kit available from Bio-Rad (Richmond, CA) based on Merril's nitric acid/dichromate/silver nitrate silver-staining method [1746]. The method is highly sensitive with a detection limit of around 0.01 ng/mm^2 [1746] or 1–10 ng/band [1719]. The method is not quantitative and has a linear range of 0.02 to 2.0 ng/mm^2. Silver staining is an order of magnitude more sensitive than CBB R-250 staining [1733] and has an oxidation step, making it applicable to glycoproteins, which can be difficult to stain [1744]. The grade of methanol used during the fixation step can affect the sensitivity [1747]. Fixation of gels with ultrapure methanol (e.g. HPLC grade) may lead to poor staining. Ajax AnalaR grade methanol was used here and contains trace amounts of formaldehyde that facilitate the staining reaction and fixation of proteins. Fixation time was usually extended to 10–16 h to ensure removal of buffer components from the gel; and the water wash steps were extended to 15 min each to minimise oxidizer carryover. A third ethanol/acetic acid step was included.

b) Gottlieb and Chavako silver staining. The Bio-Rad Silver Stain Plus kit based on the method of Gottlieb and Chavako [1748] was used in later experiments as the method is simpler than the Merril based method, more sensitive and provides a clearer background.

2.7.7.4 Drying Gels

Gels were dried under heat and vacuum for transmission densitometry and storage, using a Bio-Rad model 443 slab gel drier with a procedure based on the manufacturer's instructions [1749]. Before drying, gradient gels or gels ≤ 10% acrylamide were soaked for 1–3 h in 3% (v/v) glycerol, 40% (v/v) methanol, and 10% (v/v) acetic acid *or* 3% (v/v) glycerol, 0.5%(v/v) acetic acid for CBB stained gels. The gels were dried between cellophane sheets, moistened (with soaking solution) and supported on prewetted 3MM filter paper (Whatman, Kent, UK). The gel dryer was attached to a rotary vacuum pump, protected by a cold finger cooled with liquid nitrogen in a Dewar flask. Since resolution may be diminished during the drying step and since the gels were prone to cracking, they were photographed before drying using a fine grain panchromatic film.

2.7.7.5 Densitometry

Stained bands were quantitated using an LKB 2202 UltroScan Laser densitometer interfaced with an LKB 2190 GelScan data acquisition and manipulation software package (Version 5.2, 1986; LKB Produkter AB, Bromma, Sweden).

2.8 Cell Culture

Cell culture techniques were used following general aspects of the techniques that have been reviewed in references [1750-1753]. Where appropriate, manipulations were conducted in laminar flow cabinets using aseptic technique.

2.8.1 Cell Culture Media

2.8.1.1 Roswell Park Memorial Institute 1640 medium (RPMI 1640)

RPMI 1640 [1754] without L-glutamine was obtained from Flow Laboratories (Irvine, UK); from Cytosystems (Castle Hill, Australia); or prepared from powdered media (Cytosystems or Sigma Chemical Co.). Medium prepared from powder had undetectable endotoxin levels by the gel-clot *Limulus* amoebocyte lysate method (< 0.125 endotoxin units/ml).

Medium supplements, which have a short shelf life at 4 °C: L-glutamine (200 mM) ×100 stock; penicillin (5000 IU/ml) and streptomycin (5000 µg/ml) ×50 stock, and fetal bovine serum FBS were obtained from Flow Labs or Cytosystems, aliquoted and stored frozen at –70 °C. FBS was treated at 56 °C for 30 min after thawing to inactivate complement proteins (ΔFBS). Before use RPMI 1640 was supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin (PSG) and 5–20% (v/v) ΔFBS. RPMI 1640 plus PSG is known as complete medium

or complete RPMI 1640. All cell culture media and medium supplements were sterile filtered to 0.45 or 0.22 μM.

2.8.1.2 ×100 HAT Stock

Hypoxanthine 10.0 mM, Aminopterin 0.048 mM, Thymidine 1.6 mM. Aminopterin, 1.9 mg, (4-amino-pteroylglutamic acid) was dissolved in approximately 40 ml sterile pyrogen free water made alkaline with 0.5 ml 1 *N* NaOH. Similarly, hypoxanthine (6-hydroxypurine, Sigma) 136 mg, and thymidine (Sigma) 38.8 mg, were dissolved in water with approximately 2 ml 1 *N* NaOH was added by heating in a hot water bath (70 °C). The two solutions were mixed and made up to 100 ml with water. The ×100 stock was aliquoted and stored at –20 °C. Complete RPMI 1640 supplemented with HAT is known as HAT medium.

2.8.1.3 ×100 HT Stock

HT solution was prepared as described above, omitting the aminopterin, made up to 100 ml with water and stored in aliquots. The method was subsequently refined to ensure solubility of the HT in culture medium and maintain the medium's osmolality: hypoxanthine 136 mg (10 mM) and thymidine 38.8 mg (1.6 mM) were dissolved in RPMI 1640 medium by adding 1–2 pellets of solid NaOH to dissolve the solutes at room temperature and made up to 100 ml with medium, 0.22 μm filtered and stored at –70 °C. Complete RPMI 1640 supplemented with HT is known as HT medium.

2.8.1.4 Gey's Haemolytic Medium

Gey's haemolytic medium was used to lyse erythrocytes in spleen cell suspensions. This buffer causes less damage to lymphoid cells than alternative haemolytic solutions such as isotonic ammonium chloride [1755].

Gey's stock A

Reagent	*Source*	*Amount (g)*	*mM*	*[Final] mM*
Ammonium chloride	(Merck GR)	35.0	654	130
Potassium chloride	(Ajax AR)	1.85	25	5
$NaH_2PO_4.12H_2O$	(BDH AnalaR)	1.50	4.2	0.42
KH_2PO_4	(Ajax AR)	0.119	0.874	0.174
Phenol Red	(BDH)	0.05	0.005%	–
Gelatin	(Difco 'Bacto')	25.0	2.5% (w/v)	0.5% (w/v)

Reagents were dissolved in 1000 ml water. Gelatin dissolution required heating the solution to 60–70 °C. The solution was cooled and filtered through AP15 depth filters. Aliquots (20 ml) were

dispensed into 25 ml wide-necked McCartney bottles, autoclaved for 15 min at 121 °C and stored in the dark at room temperature.

Gey's stock B

Reagent	*Source*	*Amount (g)*	*mM*	*[Final] mM*
$MgCl_2.6H_2O$	(Ajax AR)	2.1	20	1
$MgSO_4.7H_2O$	(Merck GR)	0.7	5.7	0.25
$CaCl_2.2H_2O$	(Merck GR)	2.25	30	1.5

Reagents were dissolved in 500 ml water dispensed as 10 ml aliquots into 15 ml narrow-necked McCartney bottles, autoclaved for 10 min and stored in the dark at room temperature.

Gey's stock C (sodium bicarbonate 5.6% (w/v)). Sodium bicarbonate (5.6 g) was dissolved in water.

Gey's solution. (Made up no more than 30 min before use)

Sterile pyrogen free water	14.5 ml
Gey's stock A	4.0 ml
Gey's stock B	1.0 ml

Gey's Stock C was added dropwise ~0.5 ml until the colour of the indicator showed the pH to be 7.2–7.4 (orange). The final solution was sterile filtered.

2.8.1.5 PEG/DMSO Fusogen

Polyethylene Glycol/DMSO fusogen was obtained as a gift from Dr D.A. Brooks, Department of Chemical Pathology, Women's & Children's Hospital, Adelaide. It was prepared by weighing 10 g polyethylene glycol 4000 (BDH) into a screwcap glass bottle and autoclaving (15 psi/15 minutes) to liquefy the PEG. While liquid, 14 ml of a solution prepared by adding DMSO (3 ml) to 17 ml sterile PBS (Dulbecco's without Ca^{2+} and Mg^{2+}) was added and mixed in. The pH of the solution was checked and adjusted to between 7.2 → 8.0. The final solution, containing 42% (w/v) PEG and 15% (v/v) DMSO was stored at 4 °C.

2.8.2 Viable Cell Counting

The vital dye, trypan blue, was used in a dye exclusion procedure for viable cell counting.

2.8.2.1 Trypan Blue in Isotonic Saline

Trypan blue, 1.0 g, (direct blue 14; CI 23850; dye content approx. 50%) was triturated in a glass

mortar with a few millilitres of 1.0 M NaOH and saline, the solution was made up to about 50 ml, neutralised with 1.0 M HCl and made up to 100 ml with saline to give a concentration of (0.5% w/v). The solution was filtered through AP15, AP25 and AW03 filters (Millipore) on an AP10 support pad and separated by AP32 dacron mesh spacers. The solution was dispensed as 2 ml aliquots and stored frozen at –20 °C.

2.8.3 Parent Myeloma

P3-x63-Ag8.653 (P3–653), a HGPRT deficient, non-immunoglobulin expressing myeloma cell line derived from BALB/c mice [1756] was obtained as a gift from Dr D.A. Brooks, Department of Chemical Pathology, Women's & Children's Hospital, Adelaide, Australia. This cell line was used as the parent myeloma in hybridoma production. The P3–653 cells were thawed as described in section 2.8.4.2, cultured in 24-well cluster plates, and expanded to 50 ml cultures grown in 75 cm^2 cantered-neck tissue culture flasks. The cells were maintained between 5×10^4 and 2×10^6 viable cells/ml in complete RPMI 1640 [1754] containing 10% ΔFBS, buffered with 5% CO_2/95% air at 37.0 ± 2 °C in a humidified incubator. Cultures were maintained in volumes below 50–60 ml to avoid the risk of contamination of larger volumes of culture and the use of more expensive larger culture flasks.

Ideally the cultures were maintained in log phase between 5×10^4 and 5×10^5 cells per ml. At 1×10^6 cells per ml viability was around 85% and was generally not allowed to fall below this level. The absolute limit for growth was 6×10^6 cells/ml and if cultures grew beyond this density they were discarded. The culture was in lag phase at around 10^4 cells/ml, into log phase growth at about 10^5 cells/ml and plateaus around 10^6 cells/ml. The split ratio for subculture/passage was 1 : 10–20 every 2–3 days. The myeloma doubling time was usually 14–16 h although the range may be extended to 12–24 h depending, among other factors, on the state of cells when the culture was split.

Prior to fusion of the myeloma and spleen cells, the culture was scaled up and given 50% fresh medium (split from around 5×10^5 cells/ml) on the day before the fusion so that the cells would be at a density of $2.5 - 5.0 \times 10^5$ viable cells per ml at >90–95% viability on the day of fusion. In this state the cells will be in the best log phase position, with as many cells as possible in the G_2 + M phase of the cell cycle. Healthy cells had a rounded 'fat' appearance with smooth membranes, not granular or ruffled. Typical yields from mouse spleens were around 10^8 cells, so 10^7 myeloma cells or 20 ml culture at 5×10^5 cells/ml was required to give a ratio of 10 spleen cells : 1 myeloma cell).

2.8.3.1 Growth of Myeloma Cells

As mentioned above typical myeloma cell density doubling times are 14–16 h. If the cells are in exponential growth, the doubling time (t_D) may be calculated from the equation:

$$t_D = \frac{0.693t}{\log_e \frac{N}{N_0}}$$

where t = elapsed time

N_0 = starting number of cells

N = final number of cells

e = 2.7183

Similarly, if the doubling time was known, and a certain number of cells are required, one may arrange the initial number of cells and the culture time according to the formula:

$$\frac{N}{N_0} = e^{\frac{0.693t}{t_D}}$$

A consequence of exponential growth is that cells appear to be growing extremely slowly at low densities, but rapidly at high densities. As the cultures become very dense, they start to look unhealthy (ruffled cell membranes and granular appearance), and viability drops. This may happen quite suddenly; if the cultures are too dense on one day by the next day most of the cells will certainly be dead [1757].

2.8.4 Cryopreservation of Cells

The cryopreservation method of Zola *et al.* was followed [1755,1758].

2.8.4.1 Freezing Cells

Myeloma cells or myeloma/spleen cell hybridomas were taken from healthy suspension cultures (viability > 80%), split to 3×10^5 viable cells per ml the previous day. Optimal recoveries were obtained from cells harvested from late logarithmic to early stationary phases of growth. For example, if 5×2 ml vials were required at 3×10^6 cells/ml, then 50 ml of culture at 6×10^5 viable cells/ml were used. Cells were centrifuged at $200 \times g$ using an Accuspin centrifuge (Beckman Instruments, Fullerton, CA), fitted with a swingout bucket rotor and operated at 1100 rpm, and resuspended at 6×10^6 ml in HT medium as appropriate containing 50% ΔFBS (5 ml; i.e. 1 ml cell suspension per ampoule). An equal volume of complete RPMI 1640 containing 30% v/v

dimethyl sulphoxide was added dropwise to the cell suspension at a rate of less than 1 ml/min, with continuous gentle mixing. The cell suspension was aliquoted into prelabelled 1.8 ml cryotube freezing vials (internal thread, silicone washer). Aliquoted cells were frozen at a controlled rate: with the general aim of reducing the temperature from ambient to –10 °C at 1 to 2 °C/ min, accelerating the cooling rate at –10 °C to absorb the latent heat of fusion as the mixture freezes, continuing the cooling rate at 1 to 2 °C/min to –25 °C, and then increased it gradually to a rate of 5 to 10 °C/min. When the temperature reached –80 °C, the vials were transferred to the vapour phase of a liquid nitrogen Dewar container (–156 °C). An aliquot of surplus suspension was diluted and viable cells counted. The freezing down procedure involved incubation for 30 min at 4 °C, 30 min (vials on a 45° angle) at –20 °C, and overnight at –70 °C after which the vials transferred to liquid N_2 storage.

2.8.4.2 Thawing Cells

The procedure is described using media appropriate for hybridoma cells. Ampoules were removed from a liquid nitrogen Dewar flask and placed in a 37 °C waterbath until the ice had just melted. The cell suspension was then diluted by slow dropwise addition of an equal volume of HT medium at room temperature. The HT medium (2 ml) was added over a period of 10 min with gentle mixing. When necessary the contents of the cryotube were gently transferred to a 10 ml tube. The cell suspension was allowed to stand at room temperature for 15 min, and diluted further with 6 ml HT medium added dropwise over 10 min. The cell suspension was again left to stand at room temperature for 15 min. The cells were centrifuged at 200 × *g* for 5 min, resuspended in 10 ml HT medium containing 10% v/v HECS and placed in the wells of a pregassed (5% CO_2/95% air) and warmed 24-well cluster plate. Cluster plates containing the cell suspensions were incubated in a humidified incubator at 37 °C in an atmosphere of 5% CO_2/95% air. A viable cell count by Trypan blue exclusion was performed at this stage.

2.8.5 Hybridoma Production

2.8.5.1 Splenectomy

Spleens were required for 'feeder cells' [1755,1759], and to provide 'immune' spleen cells for hybridization with myeloma cells. Spleens were removed from mice using aseptic technique. Mice were killed by cervical dislocation and pinned to a dissecting board, ventral side up, and swabbed with 70% alcohol to sterilize the dissection field. Abdominal skin over the mouse's spleen was pinched up and a small incision made. The skin was torn and reflected, revealing the abdominal muscles and peritoneal wall through which the spleen was visible. The peritoneal wall

over the spleen was lifted and an incision made, taking care to avoid the gut. The spleen was gently delivered through the incision, released by cutting its mesentery and placed into complete RPMI 1640 in a petri dish.

The mice were killed by cervical dislocation since death is instantaneous by this method. During ether anaesthesia the glucocorticosteroid level in the blood promptly rises (up to six-fold) and the T lymphocyte function will be temporarily impaired due to the blockage of interleukin production. So the use of ether and other non-instantaneous forms of death, e.g. CO_2 asphyxiation, were therefore avoided for spleen ablation, since the spleen was required for *in vitro* lymphocyte cultivation [1760].

2.8.5.2 Feeder Cell Preparation

Lymphoid cells often grow poorly or die when grown at low density. Feeder cells, which may be normal allogenic spleen cells (10^5/ml) [1755,1759] (though other cells and strategies have been adapted [1755,1757,1761]), provide soluble growth factors for lymphoid cell growth, e.g. cytokines. Feeder cells were prepared on the day before fusion of spleen cells and myeloma cells, allowing the feeders time to establish themselves and condition the medium. Spleen cells make convenient feeders; they have a limited lifespan and so will provide the emerging hybridomas with growth factors and then die. The phagocytic cells such as macrophages in the spleen cell preparations may contribute to the removal of dead cell debris after aminopterin treatment [1761].

Feeder cells were prepared on the day preceding hybridization of spleen cells and myeloma cells following procedures adapted from those described by Zola and others [1755,1757,1758,1761].

(1) Two unimmunized BALB/c mice were killed by cervical dislocation and swabbed liberally with 70% alcohol. The mice were transferred to a laminar flow cabinet and their spleens removed using aseptic technique as described above [1755,1759].

(2) Spleens were trimmed of excess fat and placed in a 100 mm diameter sterile plastic tissue culture petri dishes containing about 10 ml of complete RPMI 1640 with 10% ΔFBS. The petri dishes were transferred to a second laminar flow cabinet where the spleens were injected with medium and then teased apart releasing cells. Rapidly and gently, smaller spleen fragments were also teased apart, releasing more cells.

(3) The spleen cell suspensions in the petri dishes were transferred to a sterile 50 ml V-bottomed centrifuge tube, avoiding fragments of spleen capsule and tissue. The spleen pieces and petri dishes were washed once with 10 ml complete RPMI 1640 containing 10% ΔFBS, and the medium, containing remaining cells, transferred to the centrifuge tube. The cell suspension was allowed to stand for a few minutes, during which time clumps of cells and tissue were allowed to

sediment. The cell suspension was transferred to a new sterile 50 ml V-bottomed centrifuge tube, leaving behind the clumps, and the cells centrifuged at 200 × *g* for 5 min. (Beckman Accuspin centrifuge fitted with swingout buckets operated at 1100 rpm.) During this time 40 ml Gey's haemolytic medium was prepared. Since it is unstable, Gey's medium was used within 30 min of preparation.

(4) Supernatant was carefully removed without disturbing the spleen cell pellet, which was then loosened by 'flicking' the tube. The spleen cells were resuspended in Gey's haemolytic medium at 20 ml/spleen (i.e. 40 ml) at room temperature. The suspension was allowed to stand at room temperature for exactly 5 min and immediately centrifuged at 200 × *g* for 5 min. The cell pellet was resuspended in 20 ml complete RPMI 1640.

(5) Cells were counted, typically 1.25×10^8 cells per 2 spleens were obtained at 95%+ viability.

(6) Cells were washed by centrifugation at 200 × *g* for 5 min and the pellet resuspended in 100 ml complete RPMI 1640 containing 10% ΔFBS, therefore at 1.25×10^6 cells/ml.

(7) The cell suspension was evenly distributed at ~ 1 ml well into 24-well cluster plates, which were incubated in a humidified incubator at 37 °C in an atmosphere of 5% CO_2/95% air.

The method of spleen cell preparation described is gentle to obtain optimum cell viability. Procedures such as forcing cells through a wire mesh may give better yields, however viability is poorer [1755]. Gey's was used as the haemolytic medium to reduce damage to lymphoid cells.

2.9 ELISAs

The general methods used in the ELISA protocols were based on those described in references [1757,1758,1761-1770].

2.9.1 Buffers

2.9.1.1 50 mM Carbonate/Bicarbonate Buffer

Anhydrous Na_2CO_3, 1.59 g and 2.93 g $NaHCO_3$ were each dissolved separately in less than 500 ml and the solutions mixed, the pH was adjusted to 9.6 if necessary by the addition of carbonate or bicarbonate solution as was appropriate and the volume then adjusted to 1 litre. The buffer, 15 mM sodium carbonate/35 mM sodium bicarbonate, pH 9.6, was filtered to 0.22 μm and stored at 4 °C.

2.9.1.2 Dulbecco's Phosphate Buffered Saline

Phosphate buffered saline without Ca^{2+} or Mg^{2+} (PBS) was prepared as described by Dulbecco and Vogt [1771] for their solution 'a': 8000 mg NaCl, 200 mg KCl, 1150 mg anhydrous Na_2HPO_4,

200 mg anhydrous KH_2PO_4 were dissolved in water and made up to one litre. The pH was between 7.1 and 7.4.

2.9.2 Screening for Anti-Amine Oxidase Antibodies in Serum

Mouse sera were screened for anti-amine oxidase antibodies using an ELISA similar to that illustrated in Figure 4.3. The ELISA design was based on antigen detection ELISAs [1761,1762]. Purified enzyme antigen of each enzyme form, viz. PAO I and II, was used to coat microtitre plates. The antigens had been purified through the SE-HPLC step (*qq.v.* §3.2.6,7). Wells in microtitre plates were coated with 100 ng antigen in 50 µl, 50 mM carbonate/bicarbonate, pH 9.6, per well, for 5–16 h at 4 °C, washed with Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+} (PBS) containing 0.05% Tween 20 (PBS–T), and subsequently blocked with 200 µl PBS–T containing 10% (v/v) sheep serum (blocking buffer) for 3 h at 37 °C in a humidified environment. After washing with PBS–T; sera from groups of mice immunized with either type I or II PAO, diluted 1/1000–1/64,000 in blocking buffer (50 µl per well), were tested in checkerboard titrations against both antigens; using preimmune serum diluted 1/1000 as a control. Plates were incubated for 3 h at 37 °C. Species-specific affinity-purified goat anti-mouse.β-galactosidase conjugate diluted 1/1000 in blocking buffer (50 µl per well) was incubated in the wells for 5–16 at 4 °C, after washing with PBS–T. Unbound conjugate was washed from wells with PBS–T and the assay developed using 3 mM *o*-nitrophenol-β-D-galactopyranoside (ONPG) dissolved in phosphate buffered saline containing 1 mM 2-mercaptoethanol and 10 mM magnesium chloride [1761] (100 µl/well). After incubation for 1–3 h at room temperature, the reaction was stopped with 1.0 M sodium carbonate (50 µl/well), which inactivates the enzyme and intensifies the colour reaction. Absorbances were quantitated using a Titertek Multiskan II microtitre plate reading spectrophotometer (Flow Laboratories, Irvine, UK) at λ_{405}.

The substrate solution was prepared from 30 mM ONPG stock (9 mg/ml in PBS dissolved at 40–50 °C) and PBS–T containing 10 mM $MgCl_2$ (1.0165 g $MgCl_2.6H_2O$/500 ml PBS–T). The 30 mM ONPG stock was stable for 3 days in the dark at 4 °C and was diluted 1 : 10 to 3 mM in PBS–Tween, Mg^{2+}/2-mercaptoethanol just before use. PBS–T containing 10 mM Mg^{2+} and 1 mM 2-mercaptoethanol was prepared by adding 35 µl 2-mercaptoethanol to 50 ml PBS Tween 20, 10 mM $MgCl_2$. 2-Mercaptoethanol alone stimulates the cleavage of ONPG, but inactivates the enzyme, while 10 mM Mg^{2+} confers better heat stability to the enzyme and impedes the inactivating effect of 2-mercaptoethanol.

2.9.3 Substrate Solution for Enzyme Capture ELISA

Stock solution was prepared by mixing 2.00 ml 10 mM ABTS (2,2[1]-azino-bis(3-

ethylbenzthiazoline-6-sulphonate) [548.7 mg/100 ml], 200 µl 4000 U/ml HRPO, 200 µl 10 mM substrate (e.g. putrescine), 15.6 ml H_2O, and 2.00 ml 0.5 M Tris buffer, pH 7.4 [at 37 °C] to give 20.0 ml 1 mM ABTS, 40 U/ml HRPO, 100 µM polyamine in 0.05 M Tris–HCl.

2.9.4 Monoclonal Antibody Isotyping Two-site ELISA

Microtitre-plate wells were coated with 500 ng affinity purified sheep anti-mouse immunoglobulin in 50 µl, 50 mM carbonate/bicarbonate buffer, pH 9.6, for 5–16 h at 4 °C. Wells were washed with PBS–T and remaining binding sites blocked with PBS–T containing 1% BSA (blocking buffer/200 µl well) for 3 h at 37 °C. Undiluted hybridoma supernatants were incubated in columns of wells for 5–16 h at 4 °C after washing with PBS–T. Unconditioned HT medium containing 5% ΔFBS and 0.1% normal mouse serum in blocking buffer were included as negative and positive controls respectively.

Wells were washed with PBS–T and rabbit anti-mouse immunoglobulin subclass specific antisera were incubated in rows of wells (50 µl/well) for 3 h at room temperature. Wells were washed again and bound subclass specific rabbit antibodies were detected by the addition of sheep anti-rabbit.horseradish peroxidase conjugate (1/1000 in blocking buffer; 100 µl/well) (incubation 5–16 h at 4 °C); followed by 56 mM citrate–87 mM phosphate (0.1 M sodium citrate/phosphate buffer), pH 4.2, containing 1 mM 2,2[1]-Azino-bis(3-ethylbenzthiazoline-6-sulphonate) (ABTS), 3.5 mM H_2O_2 (ABTS–H_2O_2 substrate solution [1772]) after washing with PBS–T.

Absorbances were quantitiated as the chromogenic reaction proceeded at room temperature, using a Model MR-7000 microtitre plate reading spectrophotometer (Dynatech, Alexandria, VA) at λ_{414} interfaced with a PC data acquisition system via an RS-232C line. Assay results were calculated using a polynomial algorithm with 5 degrees of freedom by 1224 Multicalc immunoassay data reduction and analysis software (Pharmacia/Wallace, Oy, Finland).

2.9.5 Quantitative Mouse Immunoglobulin ELISA

Mouse immunoglobulin was measured using a two-site ELISA method based on those described previously [1755,1773-1775]. Microtitre-plate wells were coated for 5–16 h at 4 °C with 500 ng affinity purified sheep anti-mouse immunoglobulin in 50 µl 50 mM carbonate/bicarbonate buffer, pH 9.6 [1776-1778]. Wells were washed with PBS–T and remaining binding sites were blocked with 1% BSA blocking buffer at 200 µl per well for 2–3 h at room temperature. Wells were washed again with PBS–T and 50 µl samples, diluted in blocking buffer, were added. Mouse myeloma IgG_1 or purified mouse monoclonal IgG_1 were used as reference standards. Plates were incubated for 2–3 h at room temperature. Wells were washed with PBS–T and affinity purified

sheep anti-mouse.HRPO conjugate diluted 1/8000 in 50 µl blocking buffer were added per well. Plates were incubated for 2–3 h at room temperature. Wells were washed with PBS–T and 100 µl ABTS–H_2O_2 substrate solution (described in §2.9.4) was added to each well. After incubation at room temperature for approximately 20 min, enzyme reactions were stopped with 2% (w/v) oxalic acid (50 µl/well). Absorbances were quantitated on a microplate reading spectrophotometer at λ_{414} (Dynatech Model MR-7000) interfaced with a PC data acquisition system. Assay results were calculated using 1224 Multicalc immunoassay data reduction and analysis software as described above.

2.9.6 Quantitative Sheep Immunoglobulin ELISA

Sheep IgG was measured using an ELISA method similar to that described for mouse IgG with extended incubation times and modifications as noted. Microtitre plate wells were coated with affinity purified donkey anti-sheep IgG (Silenus) (cross reacting antibodies were adsorbed out on a mouse mAb IgG_1 column) in coating buffer (500 ng/50 µl/well). Remaining binding sites in wells were blocked with 0.5% EIA [enzyme immuno-assay] grade gelatine in PBS–T (gelatine blocking buffer). Samples were diluted in gelatine blocking buffer and incubated in the wells after washing with PBS–T. Sheep IgG was used as the reference standard. Mouse IgG and bovine IgG were included in the controls. Wells were washed with PBS–T and affinity purified donkey anti-sheep.HRPO conjugate diluted in blocking buffer were added per well. Wells were washed with PBS–T and the assay developed by adding ABTS–H_2O_2 substrate solution to the wells. Results were quantitiated as described above.

2.9.7 Quantitative Bovine Immunoglobulin ELISA

Bovine immunoglobulin was measured using an ELISA similar to that described for quantitation of sheep immunoglobulin except that affinity purified sheep anti-bovine immunoglobulin was used as the capture antibody and sheep anti-bovine.HRPO was the conjugate. EIA grade gelatine (0.5% w/v) was included in the blocking buffer and mouse IgG_1 and sheep Ig were used in the controls. The ELISA was shown to be specific for bovine immunoglobulin.

2.10 Two-Dimensional Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to separate proteins in one dimension according to their isoelectric points (p*I*) using isoelectric focusing (IEF) after reduction of disulfide bonds, followed by separation in a second dimension according to their molecular weights using SDS-PAGE, as defined by O'Farrell [1779]. Two-dimensional PAGE allows the resolution of a complex protein mixture into more discrete components than 1D-PAGE

since it separates on the basis of protein charge in addition to molecular weight, this provides an indication of protein microheterogeneity. Furthermore, 2D-PAGE, especially when combined with sensitive detection techniques such as silver staining, provides a powerful method for establishing protein purity.

2.10.1 2D-Electrophoresis Method

The method used for two dimensional electrophoresis was based on modifications of protocols [1689,1704,1705,1780,1781] derived from the original method described by O'Farrell [1779].

2.10.1.1 Stock Solutions

Acrylamide/bis-acrylamide monomer stock was as for one dimensional SDS-PAGE (*q.v.* §2.7.1.1)
Nonident NP-40 (Boheringer–Mannhiem, Germany) was prepared as a 10% (v/v) solution in water.
Overlay buffer was prepared from 2.0 ml 10% Nonident P-40, 0.05 ml Biolyte 3–10 (Bio-Rad, Richmond, CA), 0.45 ml Ampholine 5–7 (LKB–Bromma, Sweden), made up to 10 ml with water.
IEF monomer solution was prepared from 48.6 g urea, 28.8 ml water, 11.8 ml acrylamide monomer stock, 20.3 ml 10% Nonident P-40, 0.50 ml Biolyte 3–10, 4.50 ml Ampholine 5–7, and stored as 5.5 ml aliquots at –70 °C.
Sample buffer was prepared from 6.25 ml 0.0625 M Tris–HCl (pH 6.8), 10.0 ml 10% SDS, 10.0 ml 50% glycerol, 2.5 ml 2-mercaptoethanol and 50 ml water.
Electrolytes

Catholyte: 0.1 *N* NaOH
Anolyte: 0.06% Phosphoric Acid

2.10.1.2 IEF Gel

IEF monomer solution (5.5 ml) was degassed for 15 min. Freshly prepared 10% APS (7.25 µl) and 5.5 µl TEMED was added. Glass tubes (1.5 mmØ) were prepared by sealing their ends with parafilm and placing them in a casting stand. The monomer solution was poured into the tubes with the aid of a long needle attached to a 5 ml syringe, carefully overlaid with overlay buffer and polymerization allowed for 1 h. After polymerization the gel surfaces were rinsed with water. Fresh overlay buffer (~10 µl) was added to each gel and the tubes inserted into a Protean II tube gel adaptor which was in turn attached to the cooling core. The top of the tubes were topped up with catholyte and the bottom of the tubes rinsed with anolyte after removing the parafilm.
Sample preparation. Solid high purity urea was added to a 40 µl sample of immunoaffinity purified PAO at 2250 µg/ml until it was saturated (approximately 9.0 M urea); 50 µl IEF sample buffer (9.5 M urea, 2% NP-40, 2% ampholytes, 5% 2-mercaptoethanol) was then added. The

sample was centrifuged at 10,000 × *g* for 5 min and 20 µl aliquots loaded onto the gels.

Running the gels. The lower reservoir was filled with anolyte and the upper chamber filled with catholyte. Gels were run at 400 Volts constant current overnight and at 800 Volts for a further two hours.

2.10.1.3 Second dimension gel

A 7.5% acrylamide resolving gel was cast according to standard protocols (*qq.v.* §2.7.4.1–3). Space for a 1.5 cm stacker gel was left remaining. A plate with a bevelled edge was used as the short plate of the sandwich. After casting, the monomer solution was overlaid carefully with water saturated *n*-butanol. After polymerization (60 min), the water saturated *n*-butanol was poured off the gel surface, replaced with overlay buffer, and polymerization allowed to continue overnight. The overlay buffer was poured off the resolving gel surface, which was rinsed with distilled water followed by the stacking gel solution before pouring the stacker.

The stacker solution was prepared during the final 2 h/800 Volt period of IEF; poured above the resolving gel to the bottom of the bevelled edge of the short plate, and overlaid with water saturated *n*-butanol.

2.10.1.4 Preparation of the IEF Gel and Running the Second Dimension Gel

Power to the IEF apparatus was switched off and the gels in the tubes recovered. The IEF gels can be difficult to recover, but were extruded from the tubes as follows. The end of the tube gel was rimmed with a 26 gauge short needle attached to the end of a 5 ml disposable syringe filled with water. The other (sample) end was washed briefly with H_2O. A plastic Eppendorf micropipette tip attached to a 5 ml syringe filled with water was inserted into the tube at the sample end, light pressure was applied so that the gel was gently extruded [1782]. The gels were collected into 50 ml beakers containing freshly prepared second dimension sample treatment buffer.

The extruded gels were equilibrated with sample treatment buffer as the stacking gel set (approx. ½ hour). As the stacker sets it shrinks slightly creating a groove 1~2 mm deep along the top of the gel, just below the bevelled edge. The gel surfaces were rinsed with H_2O followed by running buffer.

The treated IEF tube gels were poured onto a piece of parafilm, into which a groove had been scored approx. ½~1 cm from a straight edge. Excess treatment buffer was discarded and blotted from the parafilm. The tube gel was then transferred to the top of the stacking gel and sealed into place using an agarose stacker solution overlay which had been melted in a boiling water bath (Prosieve Gel Stacker, FMC BioProducts, Rockland, ME).

The gels were clamped to the cooling core and placed in the electrophoresis tank with

running buffer in the usual way (*qq.v.* §2.7.4.4,6). Approximately 200 µl of 0.1% bromophenol blue was added to the upper reservoir as a tracker dye to indicate the schlieren line that runs just ahead of it. Gels were run at a 20 mA constant current per gel, which was increased to 50 mA per gel when the tracker dye entered the resolving gel, with cooling at 10 °C. The current was discontinued when the tracker dye approached the bottom of the resolving gels. The gels were subsequently stained using standard protocols. One gel was silver stained using the Merril silver staining method and a replica gel was stained with Coomassie Blue R.

2.11 Isoelectric Focusing

Isoelectric focusing separates proteins on the basis of surface charge as a function of pH. Separation of proteins was achieved in the presence of carrier ampholytes (amphoteric electrolytes), which established a pH gradient increasing from anode to cathode. Theoretical and practical aspects of IEF are well documented and can be found in references [1688,1689,1783-1785].

IEF is a high resolution technique generally carried out under nondenaturing conditions (but not in the 2D technique described above). The resolution of proteins differing in their p*I* values by only 0.02 pH unit, or less, is possible. Because of this high resolution, protein samples that appear to be homogeneous when tested by other methods, can often be separated into several components by IEF. Such microheterogeneity may indicate differences in primary structure, conformational isomers, differences in the kinds and numbers of post-translational modifications, or denaturation.

2.11.1 Preparative Isoelectric Focusing

A Rotofor cell [1786] (Bio-Rad Laboratories, Richmond, CA) was used for preparative isoelectric focusing of amine oxidases. The Rotofor cell apparatus allows focusing in free solution and incorporates a cylindrical focusing chamber with an internal ceramic cooling finger. Two electrode assemblies hold the anolyte and catholyte solutions. Ion exchange membranes and gaskets isolate the electrolytes from the sample in the focusing chamber while allowing electrical contact with the sample. The anolyte used was 0.1 M H_3PO_4 and the catholyte was 0.1 M NaOH. Separation of proteins occurred within the annulus formed by the inside wall of the chamber and the cooling finger. A series of woven polyester screens divided the focusing chamber into 20 discrete compartments, each holding one fraction. The focusing chamber was rotated at 1 r.p.m. around the horizontal focusing axis to avoid thermal and gravitationally induced convection, maintain even cooling, and prevent the screens from becoming clogged by precipitated protein. After focusing, the solution in each compartment was rapidly collected by aspiration through lines

connecting each of the 20 compartments with corresponding tubes in a vacuum chamber that forms the harvesting apparatus. The rapid sample collection minimises remixing of the fractions by diffusion after focusing is stopped.

2.11.2 Flatbed Isoelectric Focusing

A Model 111 Mini IEF Cell (Bio-Rad Laboratories) was used for analytical isoelectric focusing. The Model 111 IEF Cell uses two graphite electrodes and the gel was run inverted, in direct contact with the electrodes. Thin (0.4 mm) gels were run to ensure good heat dissipation.

Polyacrylamide gel was used as the IEF support matrix. Polyacrylamide gels are suitable for focusing proteins up to about M_r 500,000 [1784]. Polymerization of acrylamide monomer and the crosslinking comonomer, *N,N′*-methylenebisacrylamide, was initiated by combined use of APS, TEMED, and illuminated riboflavin. The photochemical initiator, riboflavin, was included because the APS–TEMED system is inefficient at low pH. The three phase catalyst system ensures polymerization with a minimum of ionic contamination. This is important since residual ions will effect the final attainable voltage, and can cause distortions in the gel. The IEF matrix must be nonsieving and mechanically stable. Five percent (w/v) total monomer (acrylamide and bis-acrylamide) with the cross-linker, bis-acrylamide, accounting for three percent (w/w) of the total monomer was used. Thus, 5% *T*, 3% *C*. This formulation gave a non-sieving gel suitable for proteins up to 10^6 Daltons, that was still rigid enough to handle conveniently.

2.11.2.1 Stock Solutions for Polyacrylamide IEF Gels

Monomer stock solution (25% T, 3% C). Acrylamide (24.25 g) and bis (0.75 g) were dissolved in water, made up to a final volume of 100 ml and the solution was filtered to 0.45 µm.
Monomer–ampholyte solution was prepared by mixing 2.0 ml monomer stock solution, 5.5 ml water, 2.0 ml 25% (w/v) glycerol, and 0.5 ml 40% Bio-Lyte 3/10 ampholytes.

2.11.2.2 Preparation of the Gel

A few drops of water were placed on a clean glass plate. The hydrophobic side of a gel support film for polyacrylamide (Bio-Rad, Richmond, CA) was placed against the plate and rolled flat to remove excess water so that the film would adhere securely to the plate. The glass plate was placed on the casting tray so that it rested on the spacer bars with the gel support film facing downwards. After degassing the monomer–ampholyte solution for 5 min, catalyst solutions were added (15 µl 10% (w/v) APS, 50 µl 0.1% (w/v) riboflavin-5′-phosphate, and 5 µl TEMED and the mixture was pipetted into the casting tray so that it flowed evenly under the glass plate. The monomer was polymerized under a fluorescent desk lamp for 45 min, after which the plate and

gel were lifted gently from the casting tray, inverted, and irradiated for a further 15 min to eliminate unpolymerized monomer on the gel surface.

2.11.2.3 Sample Preparation

Immunoaffinity purified PAO samples were desalted on Sephadex G-25M [1787], supplemented with 2% Tween 20, 2% ampholytes and saturated with urea to 9.6 M.

Application. A sample application template was placed on the polymerized gel, 0.7 µl aliquots of samples and standards were applied and allowed to diffuse into the gel for 5 minutes.

2.11.2.4 Running the Gel

The gel with the adsorbed samples was inverted and carefully placed directly on the graphite electrodes of the Model 111 IEF cell. A voltage was applied across the gel; ramping to 100 V for the first 15 min, 200 V for the next 15 min followed by 450 V for an additional 90 min during which time the current decreased to 0.5 mA.

2.11.2.5 Band Detection

The proteins were fixed using a solution containing 4% sulphosalicylic acid, 12.5% trichloroacetic acid, 30% methanol for 60 min. Ampholytes, which interfere with silver staining, were removed by soaking the gel in a solution of 12% TCA, 30% Methanol (2 × 60 min, 1 × 30 min). Followed by 3 × 15 min washes in 10% ethanol/5% methanol. Followed by Merril [1746] silver staining (*q.v.* §2.7.7.3). Oxidizer, 3 min; oxidizer removal, 3 × 5 min H_2O; silver stain, 15 min; H_2O rinse, 30 sec; developer 3 × 150 ml, 30 s, 2 min, 3 min; stopped with 5% acetic acid, 5 min; H_2O rinse, 5 min. The stained gel was air dried.

2.12 Immunoblotting

The immunoblotting technique used here was based on those described in several books and reviews [1788-1791]. Proteins were separated by SDS-PAGE and then electrophoretically transferred (Western transfer [1792]) to a nitrocellulose membrane, where they are probed with antibodies to locate antigens [1791].

2.12.1 Electrophoresis

SDS-PAGE was performed using a method based on modifications of Laemmli's method [1127] as described above. A wide comb (1.5 mm × 140 mm × 5 mm) with an additional reference well, for protein M_r standards, was used. Immunoaffinity purified retroplacental poly-amine oxidase (400 µg), in standard sample buffer, was loaded in the wide well created by the comb.

2.12.2 Western Transfer

Electrophoretic transfer of proteins from the polyacrylamide gel a nitrocellulose sheet was accomplished with a method based on that described by Towbin *et al.* [1793] using Trans-Blot Cell apparatus (Bio-Rad Laboratories, Richmond, CA). After electrophoresis the gel was soaked in transfer buffer, 25 mM Tris–192 mM glycine/20% methanol at pH 8.3, for 1 h. The gel was then sandwiched, together with a nitrocellulose membrane (0.45 μm pores) (Bio-Rad Laboratories) that had been pre-wet with transfer buffer, between several sheets of 3MM blotting paper (Whatman, Kent, UK). The transfer sandwich was assembled in the TransBlot apparatus between Scotchbrite pads such that the membrane was closest to the anode. Transfer was conducted at 250 mA for 10 hours with cooling at 5 °C.

The transfer sandwich was disassembled and the gel silver stained (Merril method) to control for protein transfer. The portion of nitrocellulose membrane containing the molecular weight markers and some of the antigen containing portion, was cut from the membrane and the remainder soaked in blocking buffer (1% w/v ovalbumin (Sigma Chemical Co, St Louis, MO) in 0.05 M Tris–HCl, 0.5 M NaCl, 0.1% Tween 20) for three hours in preparation for immunoprobing.

2.12.3 Immunoprobe

The blocked membrane was mounted in Miniblotter MN45 apparatus (Immunetics, Cambridge, MA) according to the manufacturer's instructions. The Miniblotter System allows for parallel screening of different antibodies on a single Western blot without having to cut the blot into strips. Multiple channels function as separate incubation trays and a clamping system prevents cross-contamination between channels. Hybridoma supernatants and culture medium (complete RPMI 1640 with 10% ΔFBS), used as negative controls; and immune mouse serum positive controls (immune mouse serum diluted 1/500 in culture medium), were introduced into the Miniblotter channels and the apparatus was agitated on a rocking platform for one hour. The antibody solutions and controls were aspirated from the channels and the membrane washed with 500 ml 0.05 M Tris–HCl, 0.5 M NaCl, 0.1% Tween 20 (TST buffer) through manifolds connected to the channel openings. Affinity purified sheep anti-mouse immunoglobulin.alkaline phosphatase conjugate, diluted 1/400 in 0.05 M Tris–HCl, pH 8.0 containing 0.1 M NaCl, 5 mM $MgCl_2$, 0.1 mM $ZnSO_4$ and 1% ovalbumin was incubated in the channels for one hour at room temperature with rocking. The secondary antibody conjugate was then aspirated from the channels and the membrane briefly washed with TST buffer through the manifolds. The apparatus was disassembled and the nitrocellulose membrane was washed with three changes of TST buffer for a total of 10–15 minutes. The bound conjugate was detected with a substrate solution of 0.1

mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt; 0.1 mg/ml nitroblue tetrazolium chloride (both from Sigma Chemical Co., St Louis, MO) and 4 mM magnesium chloride in bicarbonate–carbonate buffer, pH 9.6, for 30 minutes at room temperature. The portion of the membrane (which had not been immunoblotted) containing molecular weight markers was stained with india ink using the method described by Hancock and Tsang [1719,1794]. The membrane was washed with 0.4% Tween 20 in PBS with two changes and then incubated in ink solution (Pelikan Fount india drawing ink (Pelikan AG, Hannover, Germany) diluted 1/1000 in PBS containing 0.3% Tween 20) overnight at room temperature. The membrane was then destained with several changes of PBS, rinsed with water and allowed to air dry.

2.13 Protein *N*-Terminal Amino Acid Sequencing

High resolution purification and high sensitivity structural microanalysis of polyamine oxidase was based on methods reviewed in references [1597,1795-1802]. The *N*-(amino)terminal amino acid sequence of polyamine oxidase from human retroplacental serum was examined using a 475A automated protein sequencer (Applied Biosystems, Foster City, CA). This instrument uses Edman chemistry [1803,1804]: the amino group of the terminal amino acid residue was reacted with phenylisothiocyanate under basic conditions to produce an anilinothiazolineone amino acid. Gaseous phase trifluoroacetic acid was then used to cleave the derivatized amino acid from the end of the peptide chain. Another reaction converted the anilinothiazolineone amino acid to a phenylthiohydantoin (PTH) amino acid, which was then identified by reversed phase chromatography on a C_{18} column using an on line HPLC. The design and operation of the gas phase sequencer is described in references [1801,1805]. Applied Biosystems operating procedures and programs for both the sequencer and the on-line PTH–amino-acid analyser were used.

Edman protein sequence analysis is dependent on obtaining highly purified protein compatible with Edman degradation procedures. Mixtures of peptides will generate several PTH-amino acids at each cycle of the Edman degradation procedure. If the peptide of interest is not relatively pure it is extremely difficult to assign sequence information to a particular peptide in the mixture. If the *N*-terminus of the peptide of interest is blocked by an amino acid modification, either endogenous or artifactual, then the sequences seen may arise from contaminating proteins. For successful protein sequence analysis by automated Edman degradation, the sample must be free from Tris, glycine, SDS, acrylamide, primary amines, oxidizing agents, buffer salts, and other contaminants that will either affect the performance of the sequenator, or clutter the chromatograms with large artefact peaks. Sufficient material too, must be available. *N*-terminal modification, blocking the sequencing, and sample loss during purification (electrophoresis and electroblotting) lead to uncertainty in the actual amount of sequencable sample. So the efficient

purification of enough protein for partial sequence analysis therefore presents a considerable technical challenge. Sequence analysis of proteins separated by PAGE link the descriptive patterns provided by gel electrophoresis to the molecular characterization of the separated polypeptides.

2.13.1 Sequencing Sample Preparation

In this study, sample preparation was based on electrophoretic transfer of proteins to a chemically inert support [1806,1807]. After SDS-PAGE of immunoaffinity purified enzyme under carefully controlled conditions, the electrophoretically separated proteins were transferred to a PVDF membrane by Western blotting. Bands associated with enzyme activity were excised and processed in an ABI 475A Protein Sequencer.

Proteins prepared by SDS-PAGE can potentially be damaged by this procedure. However, precautions were taken to minimize events that reduce the success of protein sequence analysis. These events include, *N*-terminal blockage of the proteins, alteration of amino acid side chains, cleavage of proteins into smaller fragments and introduction of contaminants. The purity of reagents used in PAGE and subsequent steps was important, particularly that of acrylamide, SDS and Coomassie Blue [1799,1801]. When preparing samples for electrophoresis, excessive heating was avoided as it may cause hydrolysis of sensitive peptide bonds, especially Asp–Pro bonds [1808,1809]. Excessive sample heating may also cause loss or oxidation of the reducing agent, with the possible reformation of disulphide bonds. The possibility of *N*-terminal modification, preventing sequencing (i.e. blocking), potentially a major problem for *N*-terminal protein sequence analysis, was reduced by using the following precautions: use of a reducing agent in the protein solution [1796]; pre-electrophoresis; degassing gel solutions to reduce the amount of catalyst needed for polymerization and thus free radical formation; use of thioglycolate in the cathodal tank buffer; use of ultrapure SDS [1696,1801,1810]; extended gel polymerization times; and avoiding urea in sample buffers. A possible reactant in gels that can lead to blocked *N*-termini is free acrylamide (monomer) [1811], the procedures just described minimize its concentration. The potential problem of tryptophan, histidine and methionine side chain destruction by free radicals or oxidants in the gel matrix [1796,1799] was minimized by using sodium thioglycolate in the cathodal buffer reservoir as a scavenger. The thioglycolate travels at the dye front during electrophoresis and scavenges destructive species in the gel.

2.13.1.1 Materials

Materials specific to sample preparation for protein sequencing included: electrophoresis grade acrylamide and Coomassie Blue R-250 (CI 42660) for sequencing (Bio-Rad, Richmond, CA),

AnalaR grade hydrochloric acid (BDH, Poole, UK), Ultrol grade CAPS (3-(cyclohexylamino)-1-propane sulphonic Acid) (Calbiochem, San Diego, CA), thioglycolate (Sigma Chemicals (Cat. No. T-0632), St Louis, MO), HPLC grade methanol (Ajax Chemicals, Auburn, Australia), Immobilon–P^{SQ} (or P) PVDF membranes (Millipore, Bedford, MA).

2.13.1.2 SDS-PAGE

Electrophoresis was performed as previously described by Laemmli [1128], with modifications as suggested by Moos *et al.* 1988 [1796], Yuen [1810], Hunkapiller *et al.* [1801] and Clements [1812] to avoid NH_2-terminal blockage and oxidation of susceptible amino acids. Briefly, fresh acrylamide stocks were prepared and 7.5% resolving gels were cast according to standard protocols (*q.v.* §2.7) at least one day in advance to allow complete polymerization of acrylamide and reduce the amount of free radicals in the gel [1796]. The gels were pre-electrophoresed in resolving gel buffer overnight at 7 mA per gel [1810]; 0.1 mM thioglycolate was added to the cathodal reservoir. Samples were prepared in SDS sample buffer (0.125 M Tris–HCl, pH 6.8; 10% glycerol; 5% 2-mercaptoethanol, and 2% SDS) and placed in a boiling water bath for 3 min *after* which 1–2 µl bromophenol blue tracker dye was added [1810]. A conventional Laemmli stacking gel (3%) was cast and allowed to set for 1 hour from the initiation of polymerization [1812]. The extended setting time was allowed to reduce the amount of acrylamide monomer in the gel. The samples were loaded and electrophoresis performed at 25 mA per gel until the tracker dye neared the end of the resolving gel (approx. 4.5 h).

2.13.1.3 Electrotransfer

Western blotting to Immobilon PVDF membranes was performed according to a protocol modified from that of Towbin *et al.* [1793] using Trans-Blot Cell apparatus. Unlike the standard Towbin buffer, the transfer buffer was glycine free, since glycine would interfere with the protein sequencing. The concentration of methanol in electroblotting transfer buffer has been discussed by Shannon *et al.* [1800] and 10% methanol as suggested by Mozdzanowski and Speicher [1798], was used in this study. After electrophoresis the gels were soaked in transfer buffer [1807] (10 mM CAPS in 10% methanol, pH 11.0) for 20 min to reduce the amount of Tris and glycine in the gel. The Immobilon–P^{SQ} (or P) membrane was placed in methanol for a few seconds, soaked in water for 5 min, then soaked in transfer buffer. The gel was sandwiched between the PDVF membrane and several sheets of blotting paper. The transfer sandwich was then assembled in the TransBlot apparatus between Scotchbrite pads such that the membrane was toward the anode. Transfer was conducted at 150 mA for 40 min followed by 200 mA for 2.5 h with cooling to 4 °C [1812].

2.13.1.4 Staining of Electroblotted Proteins

The transfer sandwich was disassembled and the membrane washed in water for 5 min, stained with 0.2% Coomassie Blue R-250 in 50% methanol for 5 min and destained in 2 changes of 50% methanol, 7% acetic acid for 5–10 min [1807]. The membrane was finally rinsed in water for 5–10 min. While still wet, stained bands for sequencing (approx. 2 mm × 4 mm) were excised using scalpel blade, air dried and stored at –20 °C until sequencing. Destaining times were kept to a minimum, since sensitive bonds, such as Asp–Pro, can be partially fragmented by exposure to low pH during staining and destaining [1799,1801], reducing the yield of sequencable protein [1813].

The transfer gel and a replica gel were stained with Coomassie Blue R-250 using a protocol adopted from Hoefer and others (*q.v.* §2.7.7.1) and scanned using an LKB Laser densitometer in order to estimate the amount of protein transferred (*qq.v.* §2.7.7.4,5). Gels were stained 4–8 h in 0.2% (w/v) Coomassie Blue R-250 in 50% methanol, 10% acetic acid and destained in 50% methanol, 10% acetic acid for 1 h followed by destaining in 5% methanol, 7% acetic acid with several changes over several hours. Gels were photographed, sandwiched between cellophane sheets and dried under vacuum before densitometry.

2.13.1.5 Loading Samples

Samples attached to PVDF membrane were loaded into the sequencer. Selection of the PVDF membrane has been discussed in references [1800,1814,1815]. In practice, the Immobilon–P^{SQ} membrane caused a greater amount of precipitate formation compared to Immobilon–P membrane. Precipitate formation tends to block the sequenator plumbing, for example from the cartridge to the flask, and the collect line; sometimes only intermittently. This caused a premature end to the sequencing cycles or a drop off in all PTH-amino acid chromatogram peaks in the case of partial blockages, interfering with the reading and sequence determination for these cycles. The increased protein binding of the Immobilon–P^{SQ} did not compensate for the technical problems caused by increased precipitate formation. In any case the capacity of Immobilon–P is quite high, at about 172 μg/cm^2 [1816], so in a 4 × 9 mm strip there could potentially be approximately 61 μg protein.

The Immobilon–P (or P^{SQ}) membrane strips containing the protein band of interest were sandwiched between the Teflon seal and a glass fibre filter in the cartridge block of the ABI model 475A protein sequenator. Long slits were cut along the band giving the membrane a comb-like appearance, facilitating access of solvents and reagents to the sample during sequencing. The arrangement of the strips of PVDF membrane is illustrated in references [1806,1816] and the cartridge block is illustrated in references [1597,1817].

The signal size of a protein isolated by the electroblotting process for sequencing has reported to be as high as 40–50% of the amount of the protein applied to the gel (unless it is completely blocked at the *N*-terminus) [1597], in this study the figure was closer to 30%, perhaps due to *N*-terminal blocking during the extensive purification procedures or hydrolysis during sequencing. On the other hand, others report that the amount of sequence obtained averages only about 25% of the sample loaded on the gel [1818].

2.14 Copper Analysis

The copper content of immunoaffinity purified retroplacental serum polyamine oxidase was determined by analysis in a Perkin–Elmer 5100PC Zeeman thermal atomic absorption spectrophotometer against a plasma trace elements control (Utak Laboratories, Valencia, CA). The sample was filtered through a Chelex disk (Bio-Rad Laboratories, Richmond, CA) to remove any loosely bound adventitious copper.

To remove extraneous metal ions, all reusable apparatus was immersed overnight in a mixture of 50 g/l Pyroneg detergent and 20 g/l disodium EDTA in water followed by at least six rinses in water. Auto-analyser cups and lids, micropipette tips and containers for standards and quality controls were also soaked in 20 g/l disodium EDTA solution, followed by thorough rinsing in water before air drying in a clean room.

Standard. Utak high level trace elements control #66815 was used (47.3 μmol copper/l). Seronorm trace element – 210710 Nycomed was used as the quality control.

Procedure. Water (50 μl), 50 μl of ¼,½ and whole UTAK plasma, 50 μl of Seronorm control and 50 μl sample were pipetted into autoanalyser cups. Triton X-100 (500 μl of 0.2%) was added to the standards and samples. The Perkin–Elmer HGA 600, Zeeman 5100 unit and AS60 Autosampler were set up to measure copper at $\lambda_{324.8}$, and the atomic absorption spectrophotometer operated according to the manufacturer's recommendations [1819].

Chapter 3

PURIFICATION AND BIOLOGICAL PROPERTIES OF HUMAN RETROPLACENTAL POLYAMINE OXIDASE

3.1 Introduction

Polyamine oxidase was purified from human retroplacental serum to further its biological and biochemical characterization and to identify the molecules responsible for pregnancy-associated polyamine oxidation in humans. The degree of confidence with which this can be achieved increases concomitantly with increasing enzyme purity [509,1669,1820]; it was therefore desirable to have the enzyme(s) as pure as possible. The degree (*fold*) of purification of a substance to homogeneity is intrinsically dependant on the specific activity of the crude starting material, with higher degrees of purification being obtained from starting materials with lower specific activity (*q.v.* §1.7). However, to obtain a high degree of homogeneity (*purity*), starting materials with a high specific activity may give the best results. Extracts of term placentae have shown considerable variability in specific activity [747,752], and it has been observed that more homogeneous amine oxidase preparations were obtained with extracts of higher specific activity [752]. Most previous purifications of pregnancy-associated amine oxidase have used placental extracts, pregnancy serum or amniotic fluid as the enzyme source (*q.v.* Table 1.9). It is not certain whether the pregnancy associated amine oxidases from the different sources are identical; however this seems likely (*q.v.* Chapter 1 *passim*). Human retroplacental serum [1542] is known to be the richest source of pregnancy associated polyamine oxidase activity [420]. Because of the relative abundance of polyamine oxidase in human retroplacental serum, and the relative ease

with which the serum can be obtained, it was appropriate to produce highly purified human polyamine oxidase from this source. This work has been preceded by Morgan's attempts to purify polyamine oxidase from human retroplacental serum [442,726].

Morgan observed the resolution of retroplacental polyamine (spermine) oxidase activity into two peaks when it was subjected to affinity chromatography on Cibacron Blue-Sepharose. A separation of enzyme activity was also observed after polyacrylamide gel electrophoresis under conditions that maintain the enzyme(s) in its native state [442]. The distribution of the activity was similar by both methods, about 80% in the major peak and 10–15% in the minor peak. Both fractions oxidized spermine, spermidine and putrescine at rates in the ratio 1 : 3 : 25. The resolution of activity into two forms is similar to some earlier studies of pregnancy-associated amine oxidases from placenta [747] and amniotic fluid [763]. In other studies, resolution of the activity into multiple forms was not attempted. Ammonium sulphate fractionation of retroplacental serum followed by gel filtration on Sephadex G-100 media gave only a single peak of enzyme activity that appeared to have eluted at the exclusion limit indicating a relative molecular mass >150,000 [442]. In a later study [726], Morgan eluted a narrow peak of polyamine oxidase activity from Sephacryl S-200, after purification of retroplacental serum on Cibacron Blue-Sepharose and DEAE-Sephacel, corresponding to molecules with M_r 67,000. Gradient SDS-PAGE indicated a major band accounting for 80% of the protein (M_r 67,000) and three minor bands (*q.v.* 1.3.4.2biii). Relative rates of oxidation (estimated by a fluorometric method [1822] (*q.v.* §2.3.1)) were spermine 1, spermidine 4 and putrescine 20. Retroplacental serum appeared to contain an FAD-containing polyamine oxidase.

Preparation of highly purified enzymes using standard biochemical procedures often presents considerable difficulties, particularly when the enzyme protein only represents a very small fraction of the total protein in the starting material. The final stages of purification are often the most difficult, often resulting in poor yields and contamination of the enzyme preparation with proteins not easily separated by standard biochemical techniques. This was found after purification of the retroplacental serum polyamine oxidase using reported methods [726]. So, in addition to standard biochemical techniques, affinity chromatography was used here for the purification of retroplacental polyamine oxidase. Using affinity chromatography, specific molecules can be isolated from complex mixtures of biological materials on a functional rather than a physicochemical basis [1823-1826]. Affinity chromatography has been used for the bioselective purification of enzymes using their specific reactivity with certain immobilized substrates. This strategy was applied here through the use of ω-aminoalkylagaroses to selectively bind the target enzymes. Even so, these media also have a tendency to adsorb irrelevant proteins that may contaminate final enzyme preparations.

The biological effects of oxidized polyamines have attracted interest because of their important implications in antimicrobial (e.g. non-specific immunity), antitumour, anti-inflammatory and immunosuppressive function (*q.v.* §1.5 *et seq.*). Further evidence is presented here that the retroplacental serum polyamine oxidase generates aminoaldehydes with these properties.

3.2 Purification of Multiple Enzyme Forms from Human Retroplacental Serum

3.2.1 Characterization of Polyamine Oxidase Activity by Size Exclusion Chromatography

Retroplacental serum (*q.v.* §2.5) was filtered and injected onto a Bio-Sil TSK-250 (G 3000 SW) column preceded by a Bio-Sil TSK-400 (G 4000 SW) guard column. The TSK column was eluted (*q.v.* 2.6.2) and polyamine oxidase activity was determined fluorometrically using putrescine as a substrate (Figure 3.1). In other experiments, when using spermine as a substrate the activity profile was similar (data not shown).

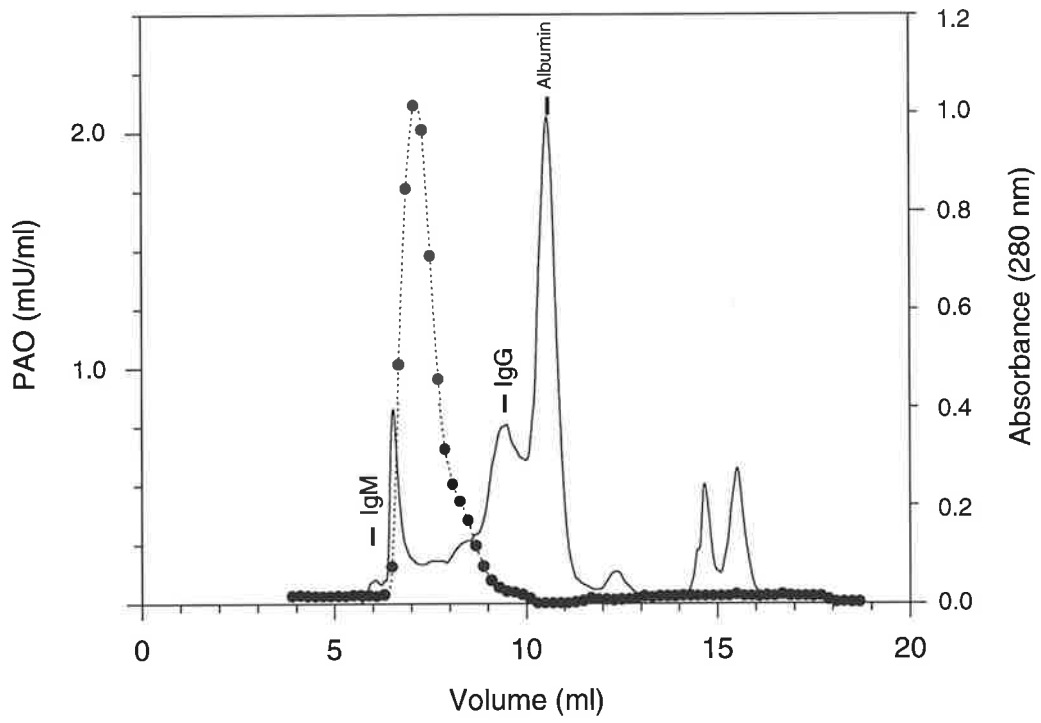


Figure 3.1. Chromatography of Human Retroplacental Serum on TSK (3000) sw. Retroplacental serum (*q.v.* §2.5) was filtered through a 0.45 µm Millex HA filter and a 50 µl aliquot injected onto a Bio-Sil TSK-250 (G 3000 SW) column (300 mm × 7.5 mmØ) preceded by a Bio-Sil TSK-400 (G 4000 SW) guard column (75 mm × 7.5 mmØ). The TSK column was equilibrated with 0.3 M KCl in 50 mM sodium/potassium phosphate buffer, pH 6.00 (I = 0.55), and eluted with the same buffer at a flow rate of 1.0 ml/min collecting 0.20 ml fractions (*q.v.* §2.6.2). Polyamine oxidase activity indicated by the solid circles (●---●) was determined fluorometrically using putrescine as a substrate. Serum proteins in each fraction were measured by laser nephelometry on a Beckman ICS II immunochemistry system (Beckman Instruments, Brea, CA) and used as internal molecular weight markers. Peak concentrations are indicated in the Figure. Absorbance of the column eluate was monitored at 280 nm indicating the protein profile (solid line).

The chromatogram indicated that polyamine oxidase activity was associated with molecules with a relative molecular mass greater than 150,000. The activity appears as a major peak with $M_r \cong$ 500,000 followed by a shoulder $M_r \cong 250{,}000$ indicating that the enzyme activity exists as at least two forms of differing molecular weight. This data is in agreement with that seen after low pressure chromatography of retroplacental serum on the size exclusion media including Sephacryl S-200 and Fractogel TSK HW 55 S. Rate nephelometric analysis of S-200 fractions of retroplacental serum in a Beckman ICS II immunochemistry system (Beckman Instruments, Brea, CA) showed that ceruloplasmin eluted with a different elution volume to that for polyamine oxidase activity (data not presented).

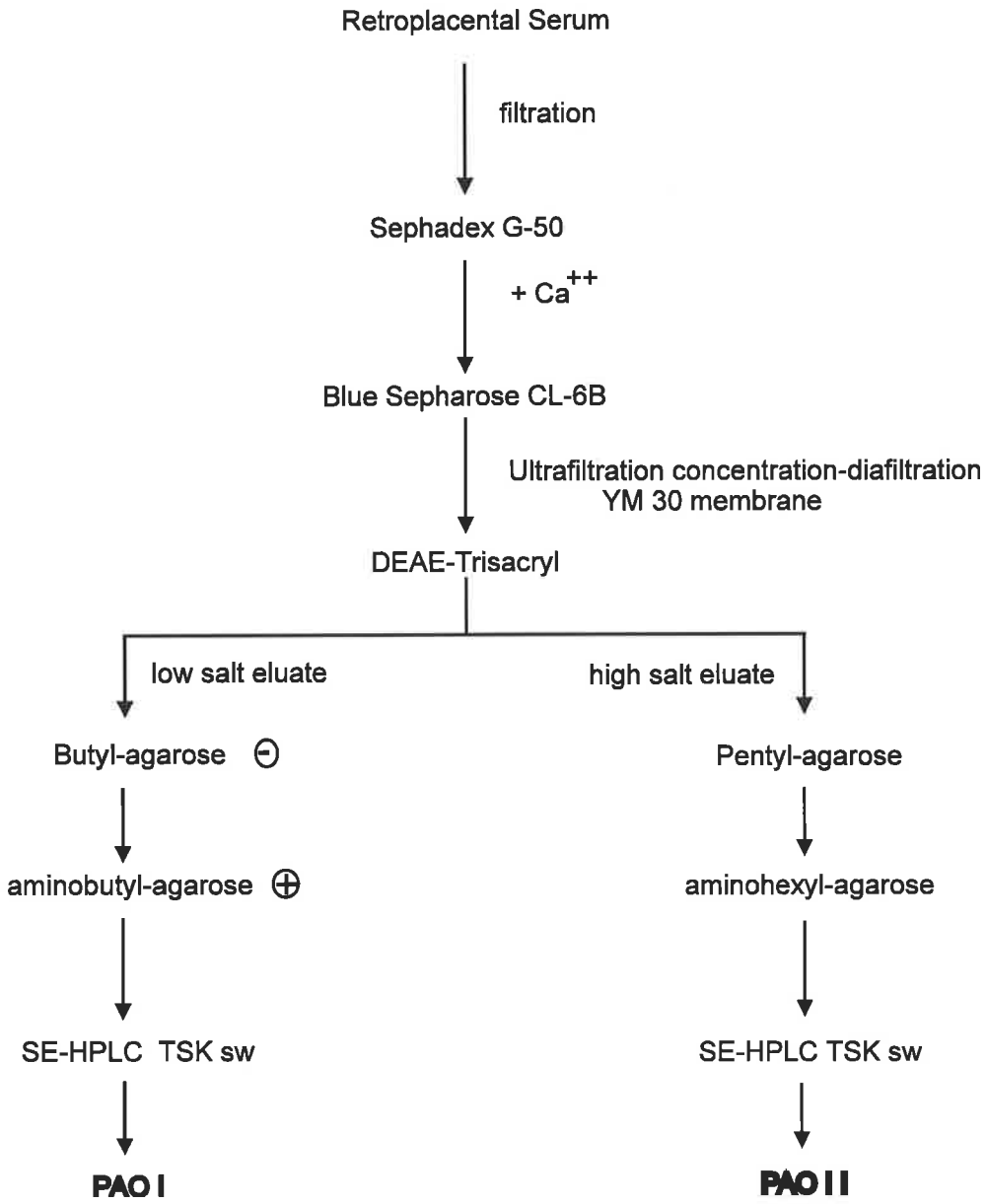


FIGURE 3.2. Isolation of Multiple Forms of Human Retroplacental Serum Polyamine Oxidase
PAO I and PAO II were separated and purified using this scheme. Ca^{2+} was added to promote binding of the enzyme to Blue-Sepharose. The alkylagaroses act as media with 'negative selectivity' and the aminoalkylagaroses as affinity media with 'positive selectivity', adsorbing the enzymes.

Preliminary experiments indicated that the two molecular weight forms could be resolved by anion exchange chromatography on DEAE media, but not by dye affinity chromatography on Blue-Sepharose. Spermine, N^1-acetylspermine, spermidine, and putrescine activity could not be resolved. Both molecular weight forms were active with each substrate, and the relative rates of oxidation by each molecular weight form were in approximately the same ratio. Both molecular forms were insensitive to 0.1 mM pargyline at pH 7.4, but were completely inhibited by 0.1 mM semicarbazide at pH 7.4 using both putrescine and spermine as substrates.

Using data accumulated from small scale pilot studies (data not presented, but described in this Chapter *passim*), a large scale purification scheme was designed for the preparative purification of the two polyamine oxidase forms. A flow diagram of the purification scheme is shown in Figure 3.2.

3.2.2 Gel Filtration Chromatography on G-50 Sephadex

Specific activity of retroplacental serum showed wide variation. When assayed using spermine as the substrate (*q.v.* §2.4.1) activity was 1.683 ± 0.868 U/l (n = 50; mean ± SD); and 12.48 ± 5.62 U/l (n = 250) when assayed fluorometrically using putrescine as the substrate (*q.v.* §2.3.1).

Retroplacental serum samples (*q.v.* §2.5) with relatively high specific activity were passed through a series of borosilicate glass microfibre prefilters (AP 10 and AP 15, Millipore, Bedford, MA) supported on cellulosic support discs (AP 10) followed by membrane filtration to 0.22 µm (Millipak 40/60, Millipore). The filtrate was diluted 1 : 1 with 25 mM Tris–HCl containing 30 mM NaCl, pH 8.0 [4 °C]. Diluted retroplacental serum filtrate was applied to a column of Sephadex G-50 Coarse, which was eluted as described in Figure 3.3.

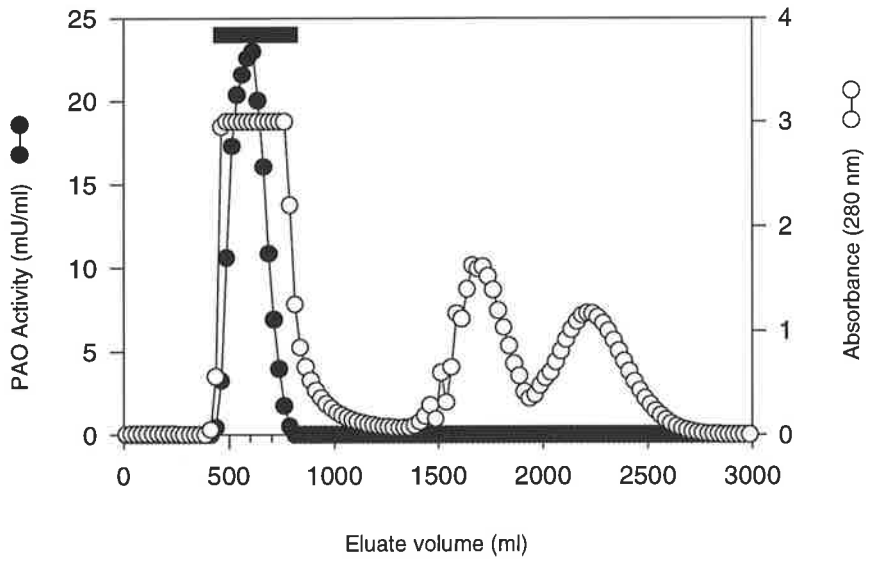


FIGURE 3.3. Gel Filtration of Retroplacental Serum on Sephadex G-50. Diluted retroplacental serum filtrate (200 ml) was applied to a column of Sephadex G-50 Coarse (95 cm × 5.0 cmØ) that had been equilibrated with 30 mM NaCl in 25 mM Tris–HCl, pH 8.0. The column was eluted with the same buffer at

200 ml/h collecting 25.0 ml fractions. Absorbance was monitored at 280 nm (o) and the enzyme activity of each fraction determined using the fluorometric method (•) (*qq.v.* §2.6.1, 2.3.1, Table 3.1 footnote b). The bar indicates pooled fractions. Absorbance values >3.0 were not measured and are indicated here as 3.0.

Polyamine oxidase activity eluted just after the void volume indicating a molecular weight, M_r >30,000. The Sephadex G-50 chromatography transfers the enzyme into a buffer appropriate for the subsequent dye affinity chromatography and removes low molecular weight (M_r <30,000) contaminants. The observed increase in total activity (*vide* Table 3.1) may be attributed to the removal of endogenous inhibitors of enzyme activity and/or the assay systems, for example hydrogen donors, which compete in the HRPO–H_2O_2 reaction [1625,1631-1633].

3.2.3 Dye Ligand Affinity Chromatography on Blue Sepharose CL-6B

In previous studies [442,726], Cibacron Blue Sepharose 4B [1827] was used for the purification of retroplacental PAO using a strategy of 'negative' selectivity in which the enzyme activity was not bound to the stationary phase. We found that a significant, but variable amount of the activity bound to the Blue Sepharose CL-6B using this method.

Enzyme binding to other dyes [1828] was examined using a Dyemâtrex screening kit [1829]. Enzyme preparations with the highest specific activity were obtained where the enzyme had been bound to the Mâtrex Blue A gel (Mâtrex Blue A ≡ Blue Sepharose CL-6B ≡ Cibacron F3GA-Sepharose), and eluted with a high salt buffer. Enzyme binding to the other dye ligands was variable, being strongest to Green A and Red A and weaker to both Orange A and Blue B. The effects of pH, ionic strength and the presence of Mg^{2+} and Mn^{2+} on the various dye affinity chromatography media were systematically examined; Blue A was still found to be the most appropriate ligand. The presence of the divalent metal ions was found to enhance the binding of the activity to the columns.

Binding of the activity to Blue Sepharose CL-6B was optimized. Blue Sepharose CL-6B was chosen; because it has better flow properties than Mâtrex Blue A, which was desirable for scaled up procedures, and it yet possesses an identical ligand, Cibacron F3GA. Matrices containing the Cibacron F3GA dye also have the advantage of possessing a high capacity and selectively for serum albumin. Albumin represents more than 50% of total plasma protein and is often a major contaminant in isolation of other serum proteins. It is difficult to remove from serum protein preparations by methods such as salt fractionation and ion exchange chromatography, because of its relatively high concentration and physicochemical similarity to other serum proteins, including the target enzyme, polyamine oxidase. When albumin was removed with Blue Sepharose prior to fractionation of other serum components, the quantity of material for subsequently processing was reduced, allowing higher resolution of minor components. Unlike precipitation methods, Blue Sepharose affinity chromatography is mild, so

the risk of enzyme denaturation is reduced.

Since the introduction of Blue Sepharose CL-6B, dye adsorbents have become widely used in protein purification [1827-1836]. Blue Sepharose CL-6B and similar dye ligand adsorbents have been used for the non denaturing fractionation of human plasma proteins and the almost quantitative removal of serum albumin [1827,1837-1843]. The selective removal of albumin from plasma was first developed by Travis and Pannell, using a Sepharose–Blue Dextran conjugate [1837]. This procedure was then used for the purification of a human plasma α-1-proteinase inhibitor [1838]. They subsequently improved the efficiency of their system using dye coupled directly to Sepharose as is the case with Blue Sepharose-CL 6B. The albumin can be quantitatively recovered by desorption with NaSCN [1839]. Angel and Dean examined the effect of pH on the adsorption of plasma proteins by Cibacron Blue-Sepharose [1841]. Gianazza and Arnaurd examined the effect of pH and ionic strength on the fractionation of plasma proteins on immobilized Cibacron Blue F3-6A [1843], and systematically studied the fractionation of plasma components [1840]. The three step procedure used here was modelled on their method: (1) a low molarity buffer eluted non-binding protein, mostly IgG, IgA and transferrin; (2) a linear gradient followed by a limit buffer wash eluted a complex mixture of protein and some albumin, which leaches from the column; (3) finally, tenaciously bound proteins such as albumin and lipoprotein were desorbed with NaSCN, freeing binding sites on the column for another purification cycle.

Blue Sepharose CL-6B columns were prepared and conditions for chromatography optimized with respect to pH and ionic strength. In general, a lower pH weakened the binding of proteins and a higher pH strengthened it. Blue Sepharose CL-6B can act as a weak cation exchanger; to minimize this non-selective attraction, elution buffer ionic strength was maintained above 0.05 M. The use of a nonphosphate buffer increases column capacity. Tris–HCl was ideal for the desired pH (8.0 [4 °C]). All of the bound enzyme activity was desorbed with 1 M NaCl at pH 8.0.

Low concentrations of metal ions, particularly those of the first row transition series (Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}) and group IIA ions (Ca^{2+}, Mg^{2+}), promote binding of proteins to a number of immobilized triazine dye adsorbents [1831]. Manganese(II) ions promoted binding of polyamine oxidase activity to Blue Sepharose CL-6B and all the other dyes tested [1829]. Optimum binding was promoted by an ion concentration of about 5–10 mM; higher concentrations did not appear to promote significantly more binding. Promotion of enzyme binding to Blue Sepharose CL-6B columns by Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Al^{3+}, Ca^{2+} and Zn^{2+}, was systematically examined. Binding was promoted in the order: $Mn^{2+}<Co^{2+}<Mg^{2+}<Ca^{2+}<Ni^{2+}$; Al^{3+} and Zn^{2+} caused problems due to precipitation of their salts. The specific activity of the

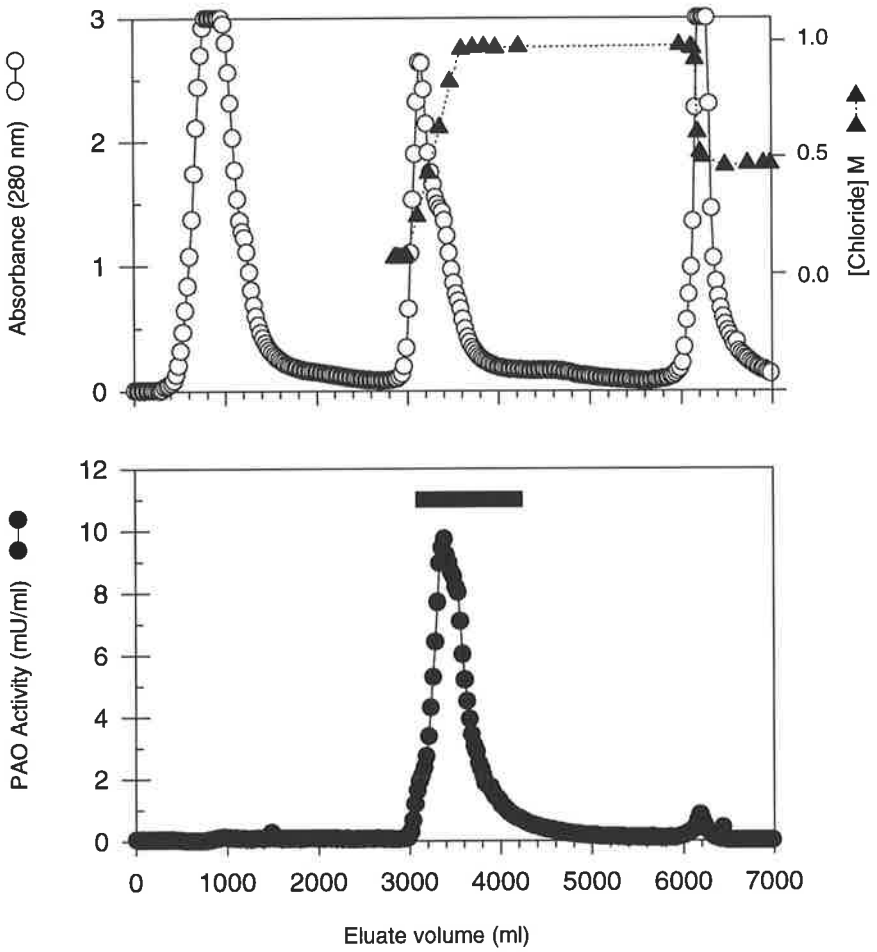


FIGURE 3.4. Chromatography of Pooled Sephadex-G 50 fractions on Blue Sepharose CL-6G. Enzymatically active Sephadex G-50 fractions were pooled (320 ml), supplemented to 10 mM Ca^{2+} with 1 M $CaCl_2$, and applied to a column of Blue Sepharose CL-6B (45 cm × 5.0 cmØ) that had been equilibrated in 25 mM Tris–HCl containing 30 mM NaCl, pH 8.0, and primed with one column volume of the same buffer containing 10 mM Ca^{2+}. The column was eluted with the Ca^{2+} containing buffer at 150 ml/h collecting 25.0 ml fractions. When A_{280} indicated that most of the unbound protein had eluted, enzyme activity was desorbed with a 600 ml linear (0.03 – 1.00 M) NaCl gradient and concomitant reverse Ca^{2+} gradient (10–0 mM) in 25 mM Tris–HCl, pH 8.0. Following further elution with limit buffer until the enzyme activity had been desorbed, 0.5 M NaSCN in 25 mM Tris–HCl, 20 mM EDTA, pH 8.0 was used to remove more tenaciously bound proteins. Absorbance was monitored at λ_{280} (o), enzyme activity (•) using a fluorometric assay, and [NaCl] (-----) by chloridimetry (*q.v.* §2.6.1). The bar indicates the pooled fractions.

desorbed enzyme fraction increased in the reverse order indicating that the binding of other proteins was promoted by the metal ions non-specifically. Calcium(II) and nickel(II) promoted the most selective enzyme binding to the columns. Calcium(II) was chosen to promote binding because of the stability of its 2+ oxidation state (cf. Mn^{2+}) and it is not coloured like Co^{2+}, which also interferes with the enzyme assay system. Furthermore, Ca^{2+} occurs endogenously in serum and is therefore more physiological. Highest specific activities were obtained when the columns were eluted with 1.0 M NaCl. The chromatography was scaled up, and the flow rate and gradient conditions optimized. Differential binding was selected such that enzyme activity bound to the column, non-adsorbing proteins passed straight through, and tenaciously bound proteins such as

albumin remained bound to the column under conditions that eluted most of the enzyme activity. Enzyme activity, along with other proteins, was eluted with an NaCl gradient; and finally, the most tenaciously bound proteins such as albumin, were stripped from the column using 0.5 M NaSCN in a buffer containing 20 mM EDTA, liberating binding sites and thus regenerating the column for subsequent purification cycles.

Fractions from the chromatography of RPS on Sephadex G-50 containing enzyme activity were pooled, supplemented with Ca^{2+}, and applied to a column of Blue Sepharose CL-6B that had been equilibrated in 25 mM Tris–HCl containing 30 mM NaCl, and primed with one column volume of the same buffer containing 10 mM Ca^{2+}. The column was eluted with the Ca^{2+} containing buffer. When A_{280} indicated that most of the unbound protein had eluted, enzyme activity was desorbed with a linear NaCl gradient and concomitant reverse Ca^{2+} gradient in 25 mM Tris–HCl as described in Figure 3.4. Following further elution with limit buffer until the enzyme activity had been desorbed, 0.5 M NaSCN was used to remove more tenaciously bound proteins, such as serum albumin (indicated by rate nephelometric analysis of fractions using a Beckman ICS II immunochemistry system and by radial immunodiffusion (Behring RID plates, Germany)) and lipoproteins [1839,1841]. The columns were immediately re-equilibrated in the start buffer *sans* Ca^{2+} to prevent dye being stripped from the matrix.

3.2.4 Resolution of Two Enzyme Forms on DEAE-Trisacryl

Enzymatic activity, eluted from the Blue Sepharose column, was resolved into two peaks by chromatography on DEAE-Trisacryl M. Active fractions from Blue Sepharose chromatography were pooled (1000 ml) and concentrated over a YM30 membrane (nominal cut off M_r 30,000). The osmolality of the enzyme sample was adjusted by pressure dialysis with 30 mM NaCl in 25 mM Tris–HCl, pH 8.0 to 127 mOsmol (Micro-osmometer, Roebling, Germany). Subsequently, the enzyme sample was applied to a column of DEAE Trisacryl M that was eluted to remove unbound protein, followed by elution with a linear NaCl gradient, which resolved the polyamine oxidizing activity into two separate peaks designated PAO I and PAO II (Figure 3.5).

PAO I eluted first from the anion exchange column under the salt gradient, and PAO II, representing approximately 90% of the activity, eluted next. The data presented in Figure 3.5 indicates that each of the polyamines; putrescine, spermidine and spermine, was oxidized by each form. The relative rates of oxidation of spermine, spermidine and putrescine by both forms were approximately 1 : 3 : 5.

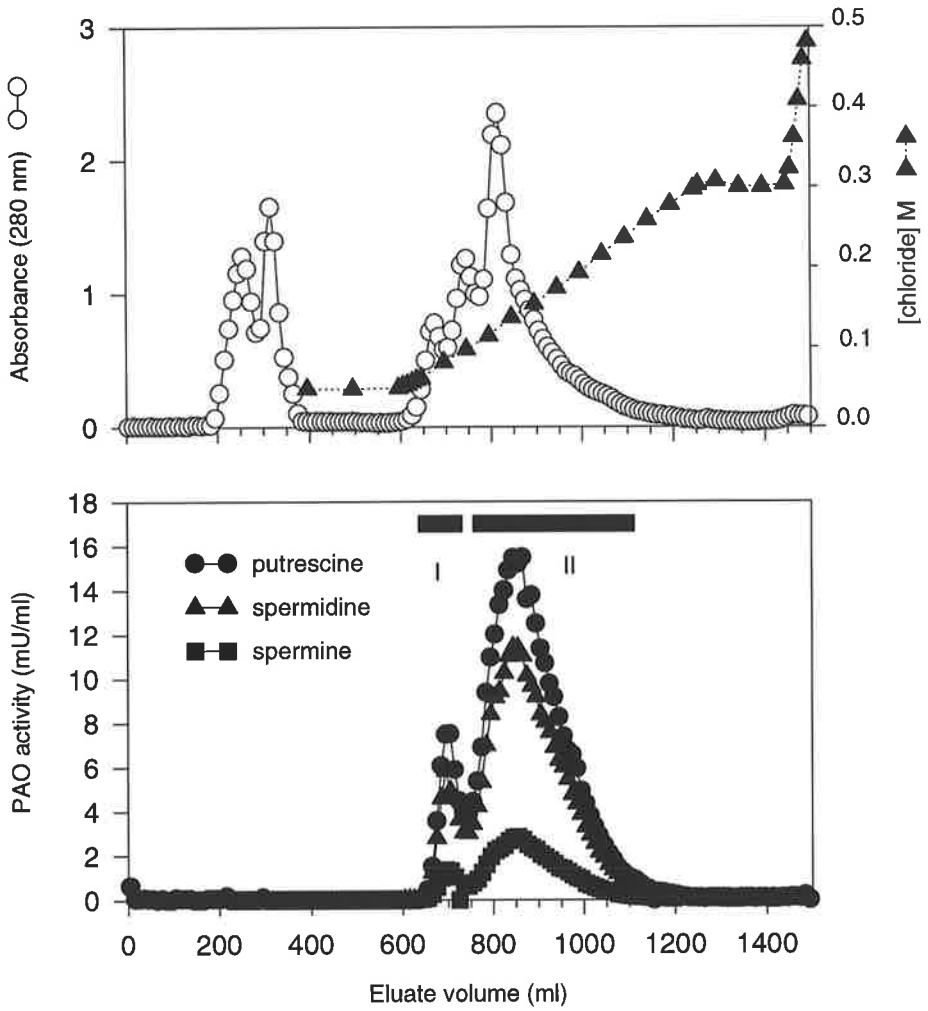


FIGURE 3.5. Chromatography of Pooled Blue Sepharose Fractions on DEAE-Trisacryl M. The concentrated and dialysed Blue Sepharose enzyme preparation was applied to a column of DEAE Trisacryl M (92 cm × 2.6 cmØ) that had been equilibrated with 30 mM NaCl in 25 mM Tris–HCl, pH 8.0. The column was eluted with the same buffer at a flow rate of 33 ml/h followed by a linear NaCl gradient, (0.03 – 0.30 M; 500 ml (----)); that desorbed the polyamine oxidizing activity in two separate peaks designated PAO I and PAO II. Elution was continued with limit buffer and finally 0.5 M NaCl in 25 mM Tris–HCl, pH 8.0. Absorbance was monitored at 280 nm (o). Enzyme activity of each fraction was determined by a fluorometric method using putrescine (●), spermidine (▲) or spermine (■) as substrates (*q.v.* §2.3.1). Bars indicate pooled fractions containing the multiple enzyme forms, PAO I and PAO II.

3.2.5 Differential Hydrophobic Interaction/Affinity Chromatography

The two enzyme forms derived from the ion exchange column were individually processed using hydrophobic interaction and affinity chromatography on alkyl-agarose and ω-aminoalkyl-agarose columns arranged in tandem. Affinity chromatography for the purification of diamine oxidase was first introduced by Baylin *et al.*, who reported the purification of diamine oxidase from pregnancy plasma using a cadaverine- (diaminopentane-) Sepharose column [713,748] prepared using a method described by Cuatrecasas [1844]. Subsequently the cadaverine-Sepharose purification method was modified and used by others for the isolation of human pregnancy-associated amine oxidases [678,746,747,752,763,1224] and bovine serum amine oxidase [836]. Bovine serum amine oxidase has also been purified using aminohexyl-Sepharose [835]. Polyamine oxidases

have been affinity purified from bacteria [537] and water hyacinth [594] using octamethylenediamine-Sepharose. ω-Aminopentyl-agarose [561] and ω-aminobutyl-agaroses [563] have both been used to purify polyamine oxidase from the yeast, *Candida boidinii*, and octyl-Sepharose has been used to purify oat polyamine oxidase [586]. However, ω-aminoalkyl agaroses and alkylagaroses had not been systematically examined for the purification of human placental polyamine oxidases. The commercial availability of alkylagarose and ω-aminoalkylagarose homologous series [1845], which are identical in all structural respects except for the length of their alkyl chains [1845-1849], facilitated the optimization of hydrophobic/affinity chromatography of polyamine oxidizing enzymes from retroplacental serum. The alkyl- and aminoalkyl-agaroses selectively adsorb protein by a mechanism that mainly involves hydrophobic interaction between the hydrocarbon chain of the alkyl-agaroses and hydrophobic sites ('crevices' or 'patches') on the protein surface [1845,1846,1849]. These sites vary in their architecture and therefore in their hydrophobicity [1845-1851]. The binding strength of a particular protein generally increases with increasing length of alkyl chain on the agarose. The binding sites may include biorecognition sites or enzyme active sites ('reactive clefts'). Indeed, the substrate binding domain of diamine oxidases is thought to comprise a protonated site for the binding of the substrate amino (alkylamino) group to be cleaved (as a nucleophile, thus unprotonated) close to the reactive carboxyl of the organic cofactor, and an anionic site for the binding of a second amino group in protonated form. A 6–9 Å long hydrophobic region, for the binding of the methylene/alkyl chain of the substrates, is thought to exist between the two charged sites, as shown in Figure 1.12. Given the nature of the active site, it seemed reasonable to speculate that the aminoalkylagaroses would selectively recognize the enzymes. The systematic homologous series approach allowed the selection of adsorption strength that avoided an exceedingly tenacious enzyme retention, requiring harsh desorbtion conditions, possibly leading to loss of enzyme activity upon its recovery from the column. It was recognized that affinity chromatography using a polyamine as a ligand (spermine-Sepharose) led to the isolation of serum albumin from human placental extracts, and pregnancy and bovine serum [1663].

Although it has been observed that charged and noncharged series of alkylagaroses display a qualitative and quantitative similarity in their adsorption and resolution properties [1845-1847], the addition of an amino ($-NH_3^+$) group to the terminal (ω-) end of the carbon chain, changes the adsorption profile. The charged group at the tip of the hydrocarbon chains allows the column to participate in ionic interactions with negatively charged residues (or sites) on the protein and reduces the hydrophobic character around the ionised group. Thus, aminoalkyl members of the ω-aminoalkylagarose series have characteristics of shorter alkyl chain members of the corresponding alkylagarose series.

Homologous series of ω-aminoalkylagaroses and alkylagaroses whose ligands were attached through terminal α-amino groups to cyanogen-bromide-activated agarose (Figure 3.6) were screened in a systematic manner to find the most appropriate ligands, optimizing adsorption and desorption under mild conditions, to give preparations of greatest specific activity and yield for each enzyme form.

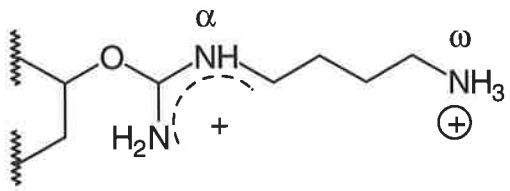


FIGURE 3.6. α,ω-Diaminobutane Attached to Cyanogen-Bromide-Activated Agarose

ω-aminobutyl agarose

Aliquots of peak activity fractions of PAO I and PAO II from the anion exchange chromatography were applied to a homologous series of ω-aminoalkylagarose and alkylagarose columns that had been equilibrated in 10 mM Tris–HCl containing 0.5 M NaCl, pH 8.0. The columns were eluted with the same buffer followed by 1.0 M KCl in 10 mM Tris, pH 8.0, then 1.5 M NaSCN in 20 mM Tris buffer, pH 8.0. Thiocyanate was chosen as a desorbing agent since enzyme activity was retained when it was used. It probably acts as a chaotrophic agent, changing the conformation of the enzyme and therefore the availability of hydrophobic sites [1845,1849], weakening enzyme binding. Gradient elution with NaSCN was used to select optimal release conditions.

Not only did the binding of enzyme activity to the ω-aminoalkylagaroses increase as longer alkyl chain members of the series were used, but the binding of other (hydrophobic) proteins was also promoted. Although the binding of the other proteins increased progressively through the series, the binding of enzyme activity did not. The ω-aminobutylagarose and ω-aminohexylagarose appeared selective. The most appropriate columns from the series for each enzyme were ω-aminobutylagarose for PAO I and ω-aminohexylagarose for PAO II.

Homologous series of alkylagarose columns were systematically screened to determine which column would be most suitable for differential negative chromatography of the ion exchange fraction, i.e. to select columns from the alkylagarose homologous series that would adsorb proteins of a similar hydrophobicity to those binding to the ω-aminoalkylagarose columns used for the affinity chromatography of the enzyme. The alkylagarose columns were used to adsorb hydrophobic contaminants from partially purified enzyme preparations that might otherwise bind to the ω-aminoalkylagarose columns. Enzyme activity was much less strongly retained on complementary columns of alkylagarose series and only adsorbed to hexylagarose and higher numbers of the series. Selective enzyme binding to complementary members the ω-aminoalkylagarose series suggests that biospecific recognition is involved in the binding of the

enzymes to some members of the ω-aminoalkylagarose series. The α,ω-diaminoalkane ligands on the agarose probably mimic endogenous substrates of the enzyme (Figure 3.6). Alkylagarose columns were found to have adsorptive properties similar to the members of the ω-aminoalkylagarose next highest in the series, as an expected consequence of the reduction of their hydrophobic character by the charged group [1849].

Under the conditions used for screening, the most suitable differential tandem column combinations were butyl-/ω-aminobutyl-agarose for PAO type I and pentyl-/ω-aminohexyl-agarose for PAO type II. Column arrangement was similar to that shown for the columns in Figure 4.8. The differential affinity chromatography resulted in approximately 50-fold purification of each enzyme form with good recovery (Table 3.1).

3.2.5.1 Chromatography of PAO I on Butyl-/ω-Aminobutyl-Agarose

Pooled fractions from the peak of enzyme activity that eluted first from the ion exchange column (PAO I) were applied to a column of ω-aminobutylagarose, arranged in tandem with a butyl-agarose column. The columns were eluted to remove unbound proteins. Bound PAO activity was desorbed from the ω-aminobutylagarose column (which had been uncoupled from the butylagarose column) with 1.5 M NaSCN (Figure 3.7).

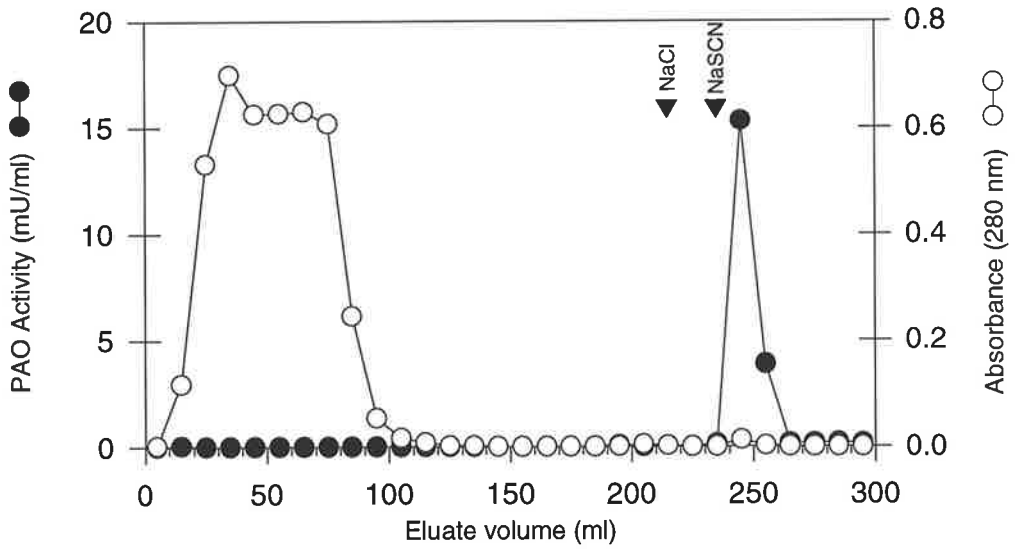


FIGURE 3.7. Affinity Chromatography of PAO I on Butyl-/ω-Aminobutyl-Agarose. Pooled fractions (65 ml) from the peak of enzyme activity that eluted first from the ion exchange column (PAO I) were applied to a column of ω-aminobutylagarose (6 cm × 1.6 cmØ), arranged in tandem with a butylagarose column (1 cm × 1.6 cmØ) and equilibrated with 50 mM NaCl in 25 mM Tris–HCl, pH 8.0. The columns were eluted with the same buffer followed by 0.5 M NaCl in 25 mM Tris–HCl, pH 8.0. Bound PAO activity was desorbed from the ω-aminobutylagarose column (which had been uncoupled from the butylagarose column) with 1.5 M NaSCN in 50 mM Tris–HCl, pH 8.0. Arrows indicate 0.5 M NaCl elution, the point of uncoupling, and 1.5 M NaSCN elution of the ω-aminobutylagarose. Absorbance was monitored at 280 nm (o), and activity of each fraction by the fluorometric method (●).

3.2.5.2 Chromatography of PAO II on Pentyl-/ω-Aminohexyl Agarose

Pooled fractions of the second peak of enzyme activity from the DEAE column (PAO II) were applied to a column of ω-aminohexylagarose arranged in tandem with a pentylagarose column. The columns were eluted to remove unbound protein and polyamine oxidizing activity was desorbed from the ω-aminohexylagarose column with 1.5 M NaSCN (Figure 3.8).

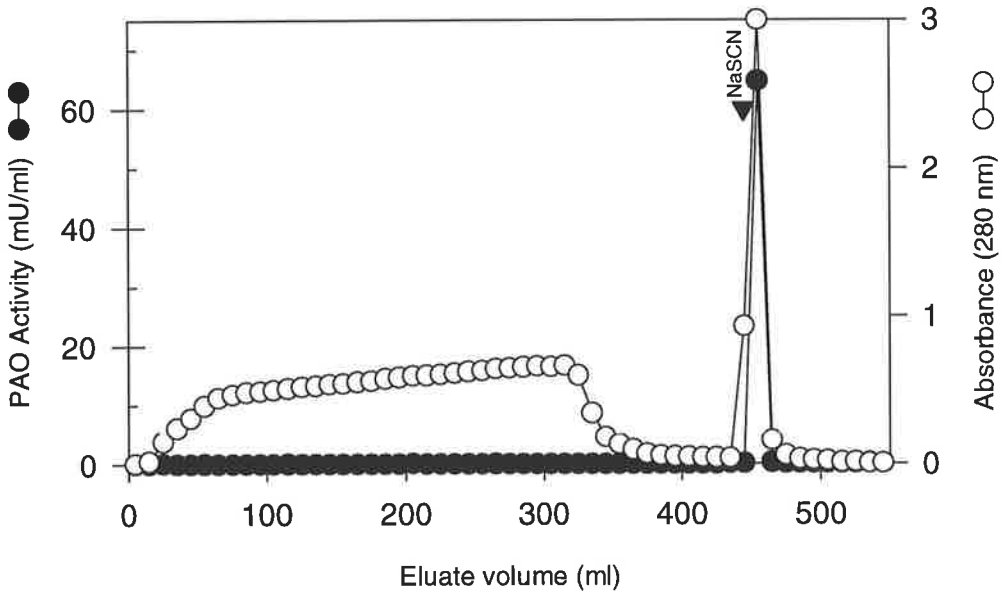


FIGURE 3.8. Affinity Cromatography of PAO II on Pentyl-/ω-Aminohexyl-Agarose. Pooled fractions (320 ml) of the second peak of enzyme activity from the ion exchange column (PAO II) were applied to a column of ω-aminohexylagarose (7 cm × 1.6 cmØ) in tandem with a pentylagarose column (1 cm × 1.6 cmØ) and equilibrated with 150 mM NaCl in 25 mM Tris–HCl, pH 8.0. The columns were eluted with the same buffer. Polyamine oxidizing activity was desorbed from the ω-aminohexylagarose column with 1.5 M NaSCN in 50 mM Tris–HCl, pH 8.0. The arrow indicates the point of uncoupling and elution of the ω-aminohexylagarose column with NaSCN. Absorbance was monitored at 280 nm (o) and activity of each fraction by the fluorometric method (●).

3.2.6 Size-Exclusion HPLC of PAO on Tandem TSK Columns

The two enzyme forms were individually purified on Bio-Sil TSK (3000 + 4000) sw columns, active fractions were pooled and recycled on the same columns. Fractions containing activity desorbed from the ω-aminoalkylagarose columns were separately pooled (PAO I and PAO II). The fraction pools individually concentrated over YM 30 membranes. The concentrates were diluted with SE-HPLC buffer, centrifuged 10,000 × *g* for 5 min and aliquots injected onto the TSK sw columns, which were connected in tandem and preceded by a TSK guard column. The columns were eluted with sodium phosphate buffer containing NaCl as described in Figure 3.9 (*vide* §2.6.2).

PAO I had an apparent molecular weight of 325,000 (Figure 3.9) and PAO II an apparent molecular weight of 489,000 (Figure 3.10). Solutions of the purified enzymes were a pink-orange colour. Electrophoresis indicated that although good purity had been achieved for the relatively

more abundant PAO II enzyme preparation, the affinity purified PAO I preparation was still a heterogenous protein mixture (Figure 3.11). The PAO II preparation contained a protein that ran as a diffuse band at an apparent relative molecular mass of 108,000 under reducing conditions. The PAO I preparation also contained the M_r 108,000 band, however it also contained other protein bands with different relative abundances. Enzyme preparations were diluted 1 : 1 with 50% glycerol and stored at –15 °C.

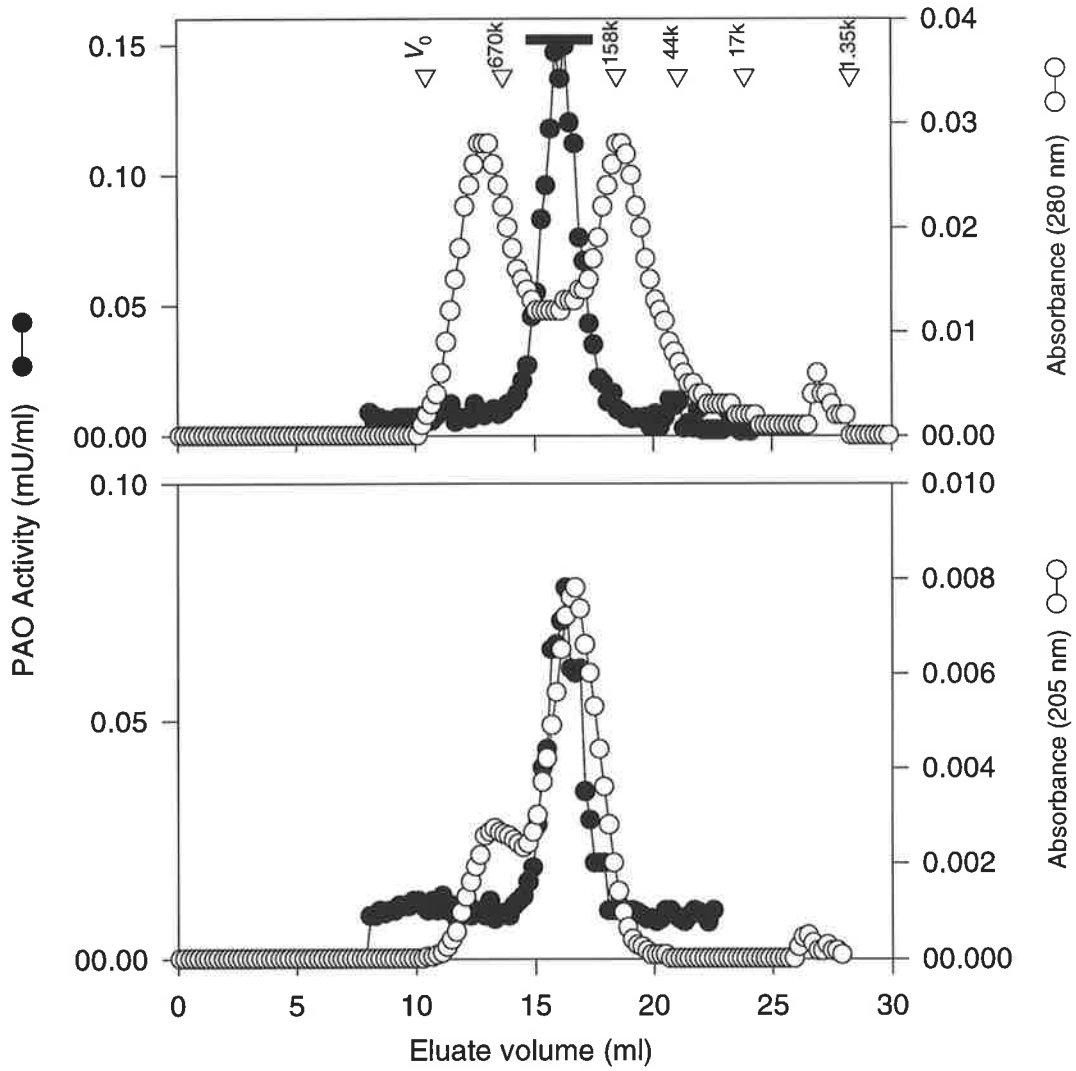


FIGURE 3.9. Chromatography of Affinity Purified PAO I on Bio-Sil TSK (3000 + 4000) sw. Pooled fractions were concentrated over YM 30 membranes in Centricon-30s. The concentrates were made up to 300 µl with SE-HPLC buffer, centrifuged 10,000 × *g* for 5 min and 250 µl aliquots injected onto the TSK (4000 + 3000) sw columns ((300 mm + 300 mm) × 7.5 mmØ) connected in tandem and preceded by a TSK guard column (75 mm × 7.5 mmØ). The columns were equilibrated in SE-HPLC buffer, 0.30 M NaCl in 100 mM sodium phosphate buffer, pH 6.80 (I = 0.50)) and eluted at a flow rate of 0.8 ml/min (*q.v.* §2.6.2). The columns had been calibrated with gel filtration standards for size-exclusion chromatography (Bio-Rad, Richmond, CA), which eluted in positions indicated by the triangles in the upper panel. (Nominal molecular weights were thyroglobulin, M_r 670,000; bovine γ-globulin, 158,000; ovalbumin 44,000; equine myoglobulin 17,000; and cyanocobalamin 1,350). V_0 was indicated by protein aggregates. Absorbance of the column eluate was monitored at 280 nm (o) and enzyme activity of each fraction (●) was determined using the fluorometric assay. Active fractions indicated by the bar, were pooled and recycled on the columns, the chromatographic profile is shown in the lower panel. Absorbance was monitored at 205 nm (o).

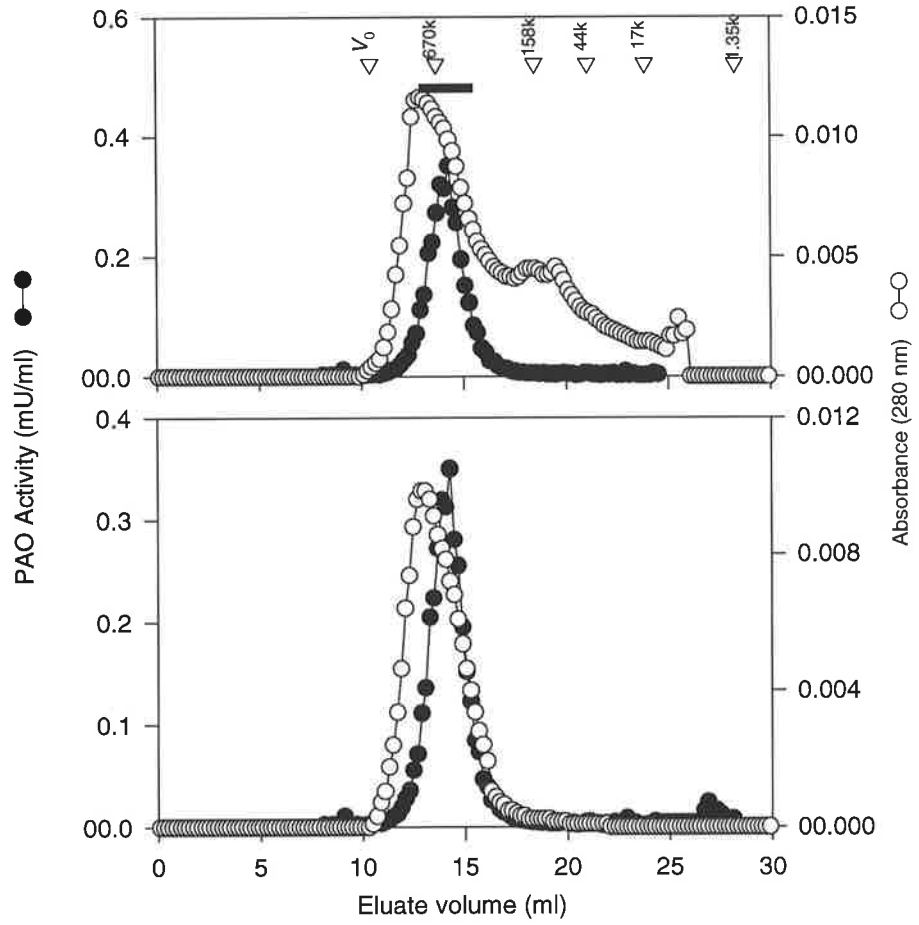


FIGURE 3.10. Chromatography of Affinity Purified PAO II on Bio-Sil TSK (3000 + 4000) sw. Pooled fractions were concentrated and injected onto the TSK HPLC columns, which were eluted as described in Figure 3.9. Protein molecular weight marker positions were as for Figure 3.9. Absorbance of the eluate was monitored at 280 nm (o), enzyme activity (●) using the fluorometric assay. Active fractions, indicated by the bar in the upper panel, were pooled and recycled on the columns. The chromatographic profile of the recycled fractions is shown in the lower panel.

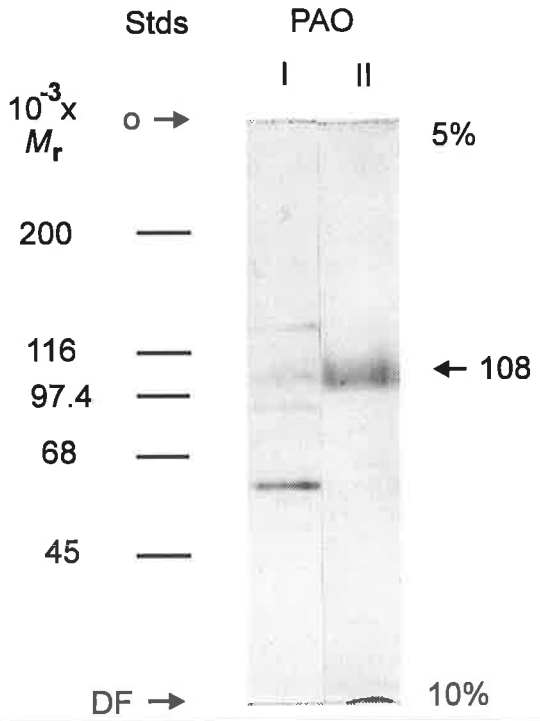


FIGURE 3.11. SDS-PAGE of Peak PAO Activity Fractions. Electrophoresis was under reducing conditions (*q.v.* §2.7). Peak PAO activity fractions were from recycling chromatography of PAO forms I and II on tandem Bio-Sil TSK (3000 + 4000) sw columns, as described in the text. The gel was poured as a 5–10% linear–exponential gradient (*q.v.* §2.7.5). Molecular weight standards (M_r = 200,000; 116,250; 97,400; 68,00; 45,000); *Left lane,* peak fraction PAO I (1.6 µg); *Right lane,* peak fraction PAO II (2 µg). Resolving gel origin (o); Dye front (DF). The gel was silver stained using Merril method described in section 2.7.7.3.

3.2.7 Summary

The purification of the polyamine oxidizing enzymes is summarized in Table 3.1.

TABLE 3.1. Purification of Multiple Forms of Polyamine Oxidase from Human Retroplacental Serum

Purification Step	Volume	Activity	Protein[c]	Specific Activity	Purification	Yield
	ml	*units*[b]	*mg*	*mU/mg*	*-fold*	*%*
1. Retroplacental serum[a]	100	4.262	4440	0.96	1.00	100
2. Sephadex G-50	325	5.400	4030	1.34	1.40	127
3. Blue Sepharose CL-6B	1000	4.394	947	4.64	4.83	103
4. YM-30 Ultrafiltration	65	4.079	329	12.4	12.9	96
5. DEAE-Trisacryl		3.002	180			70
(PAO I)	66	0.301	20.5	14.7	15.3	7
(PAO II)	320	2.704	160.0	16.9	17.6	63
6. ω-Aminoalkyl agarose		1.996	2.31			46.5
butyl-/ω-aminobutyl agarose (PAO I)	20	0.192	0.26	740	770	4.5
pentyl-/ω-aminohexyl agarose (PAO II)	10	1.804	2.05	880	916	42
7. TSK (3000 + 4000) sw		0.420	0.227			9.9
(PAO I)	2.6	0.031	0.021	1460	1520	0.73
(PAO II)	2.4	0.389	0.206	1890	1968	9.13

Notes to Table 3.1

[a] Six placentae

[b] 1 μmole/min production of H_2O_2 by putrescine [100 μM] oxidation at 37 °C in 0.05M Tris buffer, pH 7.40 detected by HRPO (40 U/ml) coupled oxidation of 3-methoxy-4-hydroxyphenyl acetic acid (homovanillic acid) to a fluorescent dimer.

[c] Protein was determined by methods described in section 2.2

3.3 Biological Activity of Purified Retroplacental Polyamine Oxidase

3.3.1 Purification of Human Retroplacental Polyamine Oxidase

Polyamine oxidase was purified from human retroplacental serum using various chromatographic media, Blue Sepharose CL-6B, DEAE Sephacel and Fractogel TSK HW-55 S, essentially as described by Morgan [726] (chromatographic data not presented). The activity from the multiple enzymatic forms observed was pooled. Relative molecular mass data obtained from the size exclusion chromatography (*q.v.* §3.2.1) were not in agreement with Morgan's findings and PAGE of active fractions followed by silver staining (Merril method) (*qq.v.* §2.7.5 and 2.7.7.3) showed many bands indicating a heterogeneous protein mixture. Nevertheless, an 83-*fold* purification was achieved (Table 3.2).

Table 3.2. Purification of Polyamine Oxidase from Human Retroplacental Serum

Purification Step	Volume	Activity	Protein[b]	Specific Activity	Purification	Yield
	ml	*F. units*[a]	*mg*	*mFU/mg*	*-fold*	*%*
1. Retroplacental serum	47	3615	2350	1.54	1.00	100
2. Blue Sepharose CL-6B	60	564	108	5.22	3.39	16
3. DEAE-Sephacel	220	154	4.8	31.98	20.77	4.25
4. Fractogel TSK HW-55 S	2.45	49	0.38	128.84	83.66	1.35

Notes to Table 3.2

[a] Arbitrary fluorescence units of H_2O_2 by putrescine oxidation at 37 °C detected by HRPO coupled oxidation of 3-methoxy-4-hydroxyphenyl acetic acid (homovanillic acid) to a fluorescent dimer.

[b] Protein was determined by Peterson's modification of Lowry's method, described in section 2.2.2

3.3.2 Trypanocidal Activity of Purified Polyamine Oxidase

Trypanosoma musculi (LUMP 1189) were maintained through bi-weekly passage in specific pathogen free female C3H/He mice. Trypanosomes were prepared by diluting infected blood, obtained from anaesthetized mice by cardiac puncture, 5- to 10-fold with medium 199 containing 10 IU/ml heparin. The diluted blood was centrifuged at 150 × *g* for 10 min, and the supernatant, containing trypanosomes, was collected. This was layered onto Hypaque–Ficoll medium, at 1.070 g/ml, and centrifuged at 150 × *g* for 30 min. During centrifugation residual red blood cells sedimented, and the trypanosomes banded at the interface between the media. The trypanosomes were removed, washed three times with medium 199 at 1000 × *g* for 5–10 min, and were adjusted to the required concentration.

Trypanocidal assays were conducted in flatbottomed wells of Linbro microtitre plates in triplicate using 2.5×10^5 parasites per well in a total volume of 200 µl as previously reported [1353]. The plates were incubated at 37 °C in an atmosphere of 5% CO_2/95% air with high

humidity. Death of trypanosomes was indicated by their lysis and assessed by examination with an inverted phase contrast microscope at ×200 magnification. Lysis was evident when tryanosomes showed loss of motility, loss of internal cellular organelles, transparency, and disruption of cells leaving only a skeleton, comprised of the flagellum and the network of microtubules.

Polyamine oxidase in the presence of polyamines was shown to be trypanocidal after 6 h incubation. Catalase did not prevent the PAO–polyamine mediated trypanosome damage (Table 3.3).

TABLE 3.3. Killing of Trypanosomes by Purified Polyamine Oxidase and Polyamines

Medium supplements	*Trypanocidal effect*
PAO (2.5 mU/ml[a])	>95% trypanosomes viable and parasites were in division phase of multinucleated forms
Spermine (20 μM)	as above
PAO + Spermine	No viable trypanosomes visible
PAO + Spermine + Catalase (5000 U/ml)	No viable trypanosomes visible

[a]PAO activity determined using a radiometric method (*q.v.* §2.4.1) and expressed as International Units (μmol product formed per min at 37 °C, therefore 1 nmol/min = 1 mU)

3.3.3 Anti-Inflammatory Activity of Purified Polyamine Oxidase

Purified retroplacental polyamine oxidase in the presence of spermine inhibited neutrophil (PMN) hexose monophosphate (HMP) shunt activity stimulated by zymosan (Figure 3.12).

Neutrophils were prepared from the blood of healthy human donors which had been drawn into tubes containing lithium heparin [1852]. The blood was layered onto a solution of Ficoll–Hypaque, at 1.114 g/ml. After centrifugation at 400 × *g* for 20–30 min at room temperature, the leucocytes resolved into two distinct bands. The mononuclear leukocytes appeared at the interface and the PMNs banded about one centimeter below. Erythrocytes sedimented to the bottom. PMNs were recovered in high yeilds at >96% purity and >99% viablity as assessed by trypan blue exclusion. The cells were washed three times in glucose-free Earle's medium with 25 mM HEPES and resuspended in glucose-free Earle's medium containing 2% human ΔAB^+ serum.

The HMP shunt activity of basal and zymosan stimulated PMN was measured by measuring $^{14}CO_2$ evolved from D-[6-^{14}C]glucose [1853] as described in the legend to Figure 3.12.

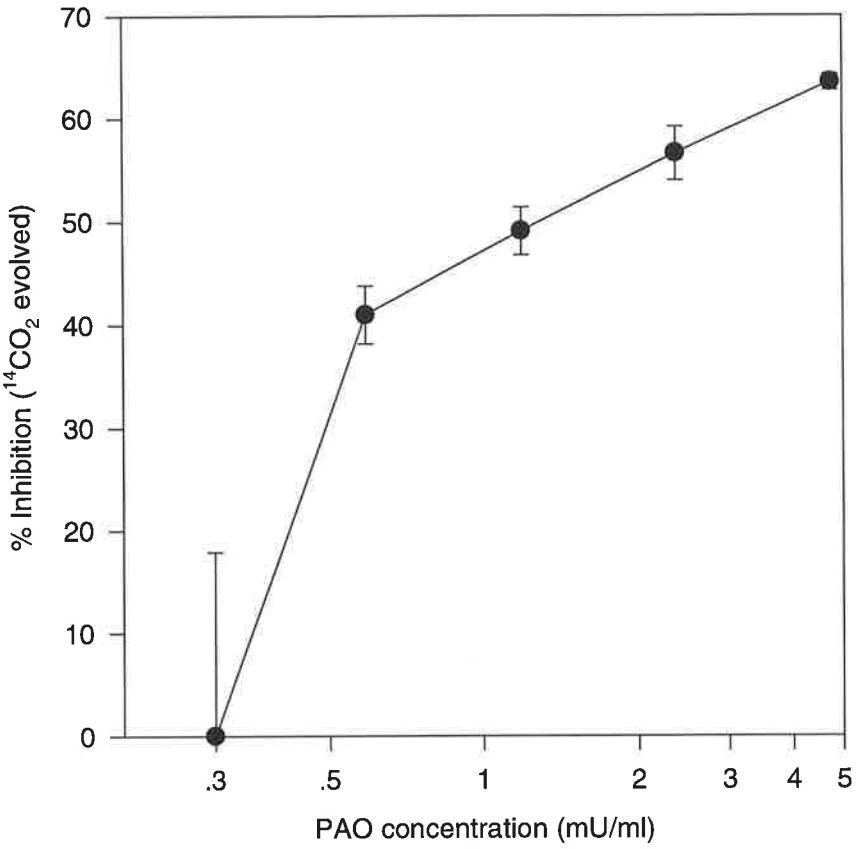


FIGURE 3.12. Suppression of Human Neutrophil HMP Shunt Acitvity by Purified Retroplacental Serum Polyamine Oxidase and Spermine.
The hexose monophosphate (HMP) shunt activity of PMN was measured by measuring $^{14}CO_2$ evolved from D-[6-^{14}C]glucose [1853]. To each outer vessel, a 20 ml scintillation fluid vial, 2 x 10^6 PMN, 0.24 µCi D-[6-^{14}C]glucose (3.96 mCi/mmol), and 4% human serum with or without 0.4 mg of opsonized zymosan was added. All solutions were made up in glucose-free Earle's medium and the final volume in each outer vessel was 1.0 ml. The inner vessels, 5 ml scintillation fluid vials, each contained 0.1 ml of 5 N NaOH to absorb the $^{14}CO_2$ evolved. The vessels were incubated in a shaking waterbath at 37 °C for 2 h, after which the radioactivity in the NaOH was determined by liquid scintillation counting (LS-3801, Beckman Instruments, Fullerton, CA). No effect on the basal HMP shunt activity was observed. Results are presented as % inhibition related to the control response. Error bars ± SD.

3.3.4 Antitumor Activity of Purified Polyamine Oxidase

Purified polyamine oxidase inhibited the proliferation of both K562 and NALM/6 cells in the presence of polyamines (Figure 3.13).

Cell proliferation assays were conducted using the erythroid myeloid leukemia cell line, K562 and the human acute lymphoblastic leukemia cell line, NALM/6 [1854]. Cells were maintained in log phase culture in complete RPMI 1640 containing 10% ΔFBS (cf. §2.8.3). They were washed three times in medium 199 containing 2% human ΔAB^+ serum and resuspended to desired concentrations in complete RPMI 1640 containing 5% human ΔAB^+ serum before use. Proliferation was assessed by [*methyl*-^{3}H]thymidine ([^{3}H]TdR) uptake [1855] as described in the legend to Figure 3.13.

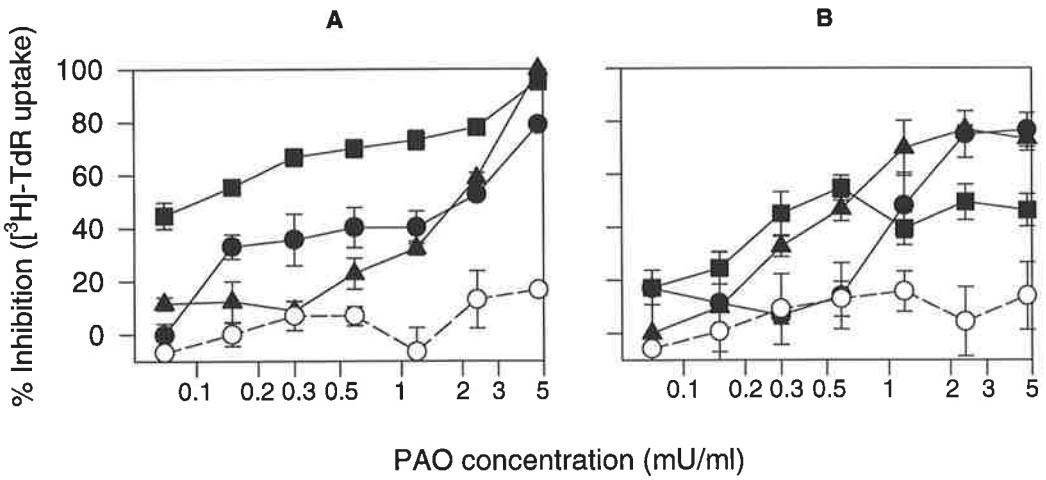


FIGURE 3.13. Suppressive Effects of Purified Retroplacental Polyamine Oxidase and Polyamines on (A) K562 and (B) NALM/6 Proliferation. Assays in triplicate were conducted in round bottomed wells of Linbro microtitre plates using 2×10^5 cells per well. Cell viability was assessed before assay by trypan blue dye exclusion and cultures with >98% viability used. Complete RPMI 1640 containing a final concentration of 2.5% human ΔAB^+ serum from non-pregnant donors was used as the culture medium. Cells were incubated in a humidified incubator at 37 °C in an atmosphere of 5% CO_2/95% air for 18 h, pulsed for 6 h with 1 μCi/well [^{3}H]TdR, before harvesting onto a glass-fibre filter mat using a Titertek D-001 cell harvester (Flow Laboratories, Irvine, UK). Radioactivity in harvested cells was assessed by liquid scintillation counting in an LS-3801 spectrometer (Beckman Instruments, Fullerton, CA). (O) No polyamines added, (●) spermine, (▲) spermidine, (■) putrescine. Polyamines were used at 250 μM. The polyamines in the absence of PAO had no apparent effect on cell proliferation. Error bars ± SD.

3.4 DISCUSSION

The data suggest a similarity between retroplacental serum polyamine oxidase and other pregnancy-associated amine oxidases. Purified retroplacental serum polyamine oxidase oxidized polyamines to products that were toxic to parasitic protozoa (trypanosomes), inhibitory to PMN respiratory activity, and inhibitory to leukemia cell line proliferative activity *in vitro*. In this respect the retroplacental serum enzyme appears similar to the bovine plasma amine oxidase (serum diamine oxidase).

The purification of the retroplacental serum polyamine oxidase described here refines and extends previous purification studies. Enzyme preparations with higher specific activities (% purity) than previously reported were obtained, although the degree of purification was not always as high as reported by other groups who have purified pregnancy-associated amine oxidases (Table 1.9). Since the degree of enzyme purification to a particular specific activity is dependent on the specific activity of the starting material, higher degrees of purification are obtained with starting materials of lower specific activity. It has been noted that greater purity and specific activity of the final enzyme preparation are obtained using a starting material with higher specific activity [752]. Starting materials used in previous purifications of pregnancy-associated amine oxidase include placental homogenates and extracts, pregnancy sera, and amniotic fluid, which

have specific activities more than an order of magnitude less than that of retroplacental serum [420]. Specific activities of enzyme preparations obtained from these purifications ranged from 429 mU/mg [747] [2803-*fold* from a placental extract] to 153 mU/mg [500-*fold* from pregnancy serum] and 1.5 mU/mg [1155]. Some reports [712,713,741,742,744,748] used units that could not be transformed to standard enzyme units (1 U ≡ amount of enzyme to oxidize 1 μmole substrate per min under standard conditions [1856]), so it is difficult to compare the results of these studies.

Further evidence that the retroplacental polyamine oxidase activity exists as multiple forms is presented here extending Morgan's earlier studies [442,726]. The multiple forms have different molecular weights as indicated by size exclusion chromatography and different charge as indicated by their behaviour on ion exchange media (DEAE-Trisacryl). The high molecular weight form, designated PAO II, represented about 90% of the total activity of retroplacental serum. Analysis of the two forms by SDS-PAGE under reducing conditions indicated a positive correlation of polyamine oxidizing activity with a protein band $M_r \cong 108{,}000$ (Figure 3.11), at least for the PAO II form, suggesting that the higher M_r forms observed by size exclusion chromatography (Figures 3.9 and 3.10) were probably multimers (or aggregates of multimers) of this subunit. The nature of the heterogeneity revealed by SDS-PAGE in the PAO I preparation may have included contaminating proteins, proteolytic degradation, or heteromeric subunits (*vide infra*). However, the possibility that any of these bands may be associated with enzyme activity could not be discounted.

Both enzyme forms showed activity when examined with the polyamines putrescine, spermidine, spermine (Figure 3.5) and N^1-acetylspermine (data not shown). The relative rates of oxidation of spermine, spermidine and putrescine by both forms were in agreement with Morgan's observations [442,726] given the extended incubation times used here, which would lead to non-linear reaction progress curves. The insensitivity of both enzyme forms to pargyline is also agreement with Morgan's findings for retroplacental polyamine oxidase. The sensitivity of the enzyme to the carbonyl group reagent semicarbazide, is similar to Morgan's findings for aminoguanidine [442,726]. The carbonyl group reagent sensitivity indicates the possible involvement of TPQ or perhaps pyridoxal phosphate at the active site (*qq.v.* §1.3.4, 1.3.7). All of these observations are not only similar to those found for pregnancy-associated amine oxidases, but also similar to those for other amine oxidases from different species (*qq.v.* §1.3.6, 1.3.7, 1.6 *et seq.*).

The resolution of retroplacental polyamine oxidase activity into multiple forms is in agreement with Morgan's earlier findings for the retroplacental serum enzyme [442] and studies of pregnancy-associated amine oxidases from other sources. Lin and Kirley first reported the

resolution of human placental histaminase (diamine oxidase) activity into two components by chromatography on DEAE cellulose [747] and later the separation of histaminase into multiple bands by isoelectric focusing [752]. Tufvesson reported the separation of amniotic fluid diamine oxidase into two forms by chromatography on DEAE-Sephadex A50, corresponding to multiple forms composed of $M_r \cong 110{,}000$ subunits in agreement with our results for retroplacental serum polyamine oxidase [763]. Furthermore, they predicted an identity between diamine oxidase from amniotic fluid, placenta, and pregnancy plasma [678]. It is of interest that multiple forms of the bovine plasma enzyme that can be separated by DEAE chromatography have also been observed [451] (*q.v.* §1.3.4.4).

The high molecular weight forms of the retroplacental enzyme observed in this study (apparent molecular weights 489,000 and 325,000 by size exclusion chromatography) are in contrast to Morgan's report of a narrow peak of retroplacental polyamine oxidase activity corresponding to M_r 67,000 after gel filtration on Sephacryl S-200 media [726] (*q.v.* §1.3.4.2biii). On the other hand, Morgan's earlier study indicated that retroplacental polyamine oxidase activity was associated with molecules with a relative molecular mass >150,000 [442]. High molecular weight forms of placental amine oxidase have also been reported by Paolucci *et al.* who observed four active molecular forms of the enzyme after chromatography on BioGel-A5m. These appeared to be multiples of a 125,000 ± 5000 molecular weight unit [742]. Smith too, observed that histaminase from incompletely desanguinated human placentae eluted in the first peak (void volume) from a Sephadex G-200 column suggesting a relative molecular mass greater than 200,000, though the molecular weight was not characterized [741]. This was also observed by Baylin and Margolis [713]. Lin and Kirley reported a 195,000 molecular weight species after examining oligomeric placental histaminase using PAGE, and a subunit $M_r \cong 95{,}000$ after SDS-PAGE under reducing conditions [747]. Similarly, Bardsley *et al.* demonstrated a M_r 235,000 species of placental diamine oxidase by native sedimentation–equilibrium ultracentrifugation [744], which appeared to be comprised of M_r 90,000 subunits [712] when examined by discontinuous SDS-PAGE [1130,1733,1734]. The $M_r \cong 70{,}000$ protein they observed by SDS-PAGE [744] was most likely a contaminant, probably albumin, which has been observed by others in their preparations [713,726,748,763]. Under dissociating conditions they observed an 82,000 molecular weight subunit by sedimentation–equilibrium ultracentrifugation, which is closer to the subunit molecular weight presented here for retroplacental polyamine oxidases (M_r 108,000); and that reported by others for pregnancy-associated amine oxidases using SDS-PAGE under reducing conditions, viz. 84,000 [755], 90,000 [713,756], 95,000 [747], 100,000 [748], 105,000 [47], 110,000 [763], and 90,000–110,000 [752]. Tufvesson observed multiple high

molecular weight enzyme forms in amniotic fluid [763], 'DAO A' eluting first from a DEAE column with a M_r 245,000 and a major form 'DAO B' eluting later from a DEAE column with a M_r 485,000. These findings are very similar to the findings presented here for the retroplacental serum enzymes. Baylin's group observed a large form of human placental diamine oxidase, proposed to be an aggregate of six 90,000 molecular weight subunits, by electron microscopy [1220]. This group reported that other multiple forms, which differed in size and charge, were observed during their purification studies and suggested that they represented aggregates of the subunits. The suggestion that high molecular weight multiple forms are a result of concentration-dependent aggregation [1118] is supported by kinetic evidence for aggregation of the enzyme [744]. Indeed, the molecular weights of the porcine kidney and bovine plasma enzymes remained controversial for some time because of their tendency to show association–dissociation phenomena [823,898,899] (*q.v.* §1.3.4.2 *et seq.*).

The retroplacental amine oxidases isolated here appeared distinct from ceruloplasmin and lysyl oxidase with which their amine oxidase activity might be confused [751,1115,1614,1857-1862]. They lacked the intense sky-blue colour that is observed when ceruloplasmin is purified (*qq.v.* §1.3.4.1b,c). Moreover, the molecular weight of proteins associated with the enzyme activity did not correspond to that of ceruloplasmin [1532] or lysyl oxidase [1533].

The degree of purification of human retroplacental polyamine oxidase obtained following Morgan's three-step chromatographic method (essentially that in Table 3.2) was not as high as was previously reported [726]. In this study the various steps of the purification scheme were optimized and an additional affinity step included as described in section 3.2. Although the enzyme purification scheme described here using optimized affinity and conventional biochemical techniques resulted in highly purified enzyme preparations, the purified enzyme preparations were not homogenous when examined by silver stained SDS-PAGE, particularly the relatively less abundant 'PAO I' form (Figure 3.11). Without further analysis, it is difficult to unequivocally attribute enzyme activity to a particular protein band as the enzyme activity may arise from one of the minor components. Furthermore, yields were poor at the final stages of enzyme purification (Table 3.1). Nevertheless, the new purification scheme presented here did provide sufficient enzyme antigen for the immunization of mice and development of screening assays for the production of monoclonal antibodies as described in Chapter 4.

It was desirable to use the most purified enzyme preparations as antigen [1863] for the production of monoclonal antibodies (*qq.v.* §1.7.1 and 4.1.1). Purified antigen also provided valuable material for the development of screening assays. A major dilemma faced was to decide how far to purify the antigen, given that one will probably approach 100% purity asymptotically

with a concomitant decrease in yield. To obtain sufficient purified protein for both characterization studies and immunization presented a considerable technical challenge.

Results presented here extend the previous findings using retroplacental serum and bovine serum [1353], by showing that purified polyamine oxidase from retroplacental serum caused trypanosome killing in the presence of polyamines and that the lytic effect continued in the presence of the H_2O_2 reductant, catalase. This evidence strongly suggests that it is the polyamine oxidase mediated generation of aminoaldehydes that is responsible for the antimicrobial effects of bovine serum, retroplacental serum and seminal plasma (*q.v.* 1.5.1). The possibility that the retroplacental serum enzyme may be similar to that released by macrophages has important implications for its possible contribution in non-specific immunity (*qq.v.* 1.5.1.5 and 1.6.8.3).

The products of polyamine oxidation by purified polyamine oxidase also inhibit leukemia cell line proliferation. In this respect, the effect of the purified enzyme is similar to the effects of retroplacental serum and the bovine serum enzyme on mitogenic lymphocyte transformation and lymphoid cell line proliferation (*q.v.* §1.5.2). Our results also demonstrate that purified retroplacental serum polyamine oxidase, like bovine plasma amine oxidase, inhibits the burst of HMP shunt activity of PMN during phagocytosis (*q.v.* §1.5.3). The implications of these findings remain speculative. However, retroplacental serum polyamine oxidase may represent a localized immunoregulatory mechanism at the trophoblastic–decidual interface that may contribute to the protection of the feto–placental allograft and limit placental invasion of the myometrium (*qq.v.* §1.5.3, 1.5.4 and 1.6.7).

The purification of human retroplacental serum polyamine oxidase was optimized and refined by the addition of an affinity step. Multiple enzyme forms with apparent molecular weights 489,000 and 325,000 by size exclusion chromatography representing 90% and 10% of the enzyme activity respectively were purified. The two forms showed different affinities to anion exchange media at pH 8.0, the larger form eluting with higher salt concentrations suggesting a higher p*I*. Similar relative rates of oxidation were seen with each form at pH 7.4, approximately 1 : 1 : 3 : 5 with spermine, N^1-acetylspermine, spermidine and putrescine respectively at extended incubation times. Both molecular forms were insensitive to 0.1 mM pargyline at pH 7.4, but were completely inhibited by 0.1 mM semicarbazide at pH 7.4 using both putrescine and spermine as substrates, indicating that neither form was a monoamine oxidase and that both forms were sensitive to carbonyl group reagents. The higher molecular weight form, PAO II, appeared to be comprised of M_r 108,000 subunits when analysed by SDS-PAGE under reducing conditions. The results presented here indicated that the less abundant low molecular weight form, PAO I, may also be comprised of the same subunits, although a number of other proteins were observed in the

preparation on SDS-PAGE followed by silver staining. The heterogeneity of the preparation could indicate heteromeric enzyme species. However, the different relative abundances of the proteins suggests that they were probably contaminants of the enzyme preparation.

The retroplacental serum enzyme appears similar if not identical to the pregnancy-associated amine oxidases found in placental extracts, pregnancy serum and amniotic fluid. The biological activity of the purified retroplacental enzyme is similar to the activity observed with both bovine plasma amine oxidase and retroplacental serum indicating a possible similarity between the two enzyme forms and provides further evidence for the involvement of polyamine oxidase in these effects.

Chapter 4

MONOCLONAL ANTIBODIES TO POLYAMINE OXIDASES

4.1 Introduction

Immunoaffinity chromatography, using immobilized antibody as a ligand, extends the affinity technique by adding the selectivity of immunological reactions to the separation process [1864-1869]. Although polyclonal antisera may be used to purify various enzymes, in general these reagents are not ideal for the immunoaffinity purification because of the variable affinity of the antibodies present in the polyclonal mixture and possible presence of crossreacting antibodies. In this study, monoclonal antibodies were used to purify the human retroplacental serum polyamine oxidase. Since monoclonal antibodies to the enzyme were not available at the commencement of this work, their production comprised an important component of this research and is reported in this Chapter.

4.1.1 Preparation of Enzyme for Monoclonal Antibody Production

The relative immunogenicity of proteins in a mixture is not readily predictable. In some cases the target (enzyme) antigen may be poorly immunogenic and not elicit a strong immune response; or may even be immunosuppressive. The immune response to the target antigen may be suppressed by the presence of other antigens in the antigen preparation. In generating polyclonal serum, even from highly purified preparations of enzyme, some impurity antigens may be immunodominant and elicit a strong immune response even when present in only trace amounts, perhaps undetectable by conventional methodology [1870]. This may subsequently be a problem in its use for immunodetection and immunoaffinity purification because the antiserum may then recognise the very impurities one is trying to avoid. When used on immunoaffinity columns the antibodies recognizing impurities would combine with their antigens and the observed selectivity of the column would be much poorer than expected.

The introduction of monoclonal antibodies has greatly advanced the immunoaffinity purification technique [1866,1867]. The exquisite specificity of monoclonal antibodies provides a powerful strategy for separating target enzymes from complex mixtures. Production of monoclonal antibodies enables the preparation of antibodies to an antigen that is difficult to purify from a complex mixture by conventional biochemical techniques. A unique advantage of

monoclonal antibody production is that impure antigens can be used to produce specific antibodies. Because hybridomas are single cells cloned before use, the immune response can be dissected and monospecific antibodies can be produced after immunizations with even complex mixtures of antigens. Given a specific selection strategy, enzyme preparations less than 100% pure are sufficient to produce the monoclonal antibodies to make affinity columns that in theory provide absolute selectivity for the enzyme. However, the spleen cell – myeloma cell fusion products will in general reflect the immunodominance of the serum antibodies [1761]. If the immunizing mixture contains a large number of strong antigens then these will tend to mask the minor antigenic components and the number of clones that produce antibody reacting with minor components will be greatly reduced. So the idea that there is no need to purify the antigen used for immunization to produce monoclonal antibodies is a misconception. At least some sort of preliminary purification of the target antigen is highly desirable. An approach involving the partial purification of the target antigens using novel affinity and conventional biochemical techniques, described in Chapter 3, was adopted in this study.

4.1.2 The Advantages of Monoclonal Antibodies to Polyamine Oxidase

A major drawback in the use of polyclonal antisera is crossreactivity. In polyclonal sera there may be antibodies that react with antigens unrelated to the antigen of interest. This type of crossreactivity, resulting from the presence of antibodies of several specificities, should be absent when using monoclonal antibodies unless the monoclonal antibodies are contaminated with crossreacting antibodies from ascites fluids or from serum supplements to tissue culture media. A single antibody can react with unrelated antigens if they possess a shared antigenic determinant or one with a very similar structure; though the latter reactions will be of lower affinity. This type of crossreaction may be observed with both monoclonal and polyclonal antibody preparations. The specificity of monoclonal antibodies derives from their immunological purity, but does not preclude the type of crossreactivity due to recognition of similar antigens, or identical determinants on different antigens. Common antigenic determinants expressed in different molecules might include an identical or similar sequence of amino acids or a carbohydrate moiety. For example, an antibody against the carbohydrate moiety of a particular glycoprotein may exhibit lectin-like properties due to carbohydrate related crossreactions. Carbohydrate structures are widely shared between different glycoproteins, and such an antibody may crossreact with other glycoproteins even if the polypeptide portions are totally unrelated. With polyclonal antiserum the crossreacting antibody subpopulation may be removed by adsorption with the crossreacting antigen(s), providing they are known and available. The specificity of a monoclonal antibody may be a problem if the enzyme antigen exhibits polymorphism unless the antibody is

directed towards an invariant determinant.

One major advantage of monoclonal antibodies over polyclonal serum is their potentially perpetual availability at a standard titre without variation in specificity, once a clone is established. This perpetual availability was exploited here to produce large amounts of antibodies for the construction of immunoaffinity columns for polyamine oxidase purification.

The monoclonal antibody approach to immunoaffinity purification offers an advantage over the use of polyclonal serum where a range of affinities are present. With the use of polyclonal serum elution quite harsh methods may be needed for complete desorption of bound antigen possibly causing its denaturation. Monoclonal antibodies will have a uniform affinity for the target enzyme. Monoclonal antibodies of relatively lower affinity may be selected allowing more gentle elution procedures that increase the probability of releasing the enzyme from the immunoaffinity column in its native (active) form.

Monoclonal antibodies to polyamine oxidases would, through their selectivity and specificity, provide unique immunochemical reagents that may not only be used for the further purification of the enzymes, but also for their characterization. In immunodetection studies they may be used to localize enzymes on Western blots, identify enzymes in immunoprecipitation studies, and localize enzymes in histologic and ultrastructural studies. They may be used to establish sensitive immunoassays, such as ELISAs that would be able to detect the presence of very low levels of enzyme. In crossreactivity studies they may be used to determine similarities between enzymes. The monoclonal antibodies may also be used for the screening of DNA expression libraries [1593,1594].

Potentially, monoclonal antibodies may also be used to specifically block enzyme activity. However, the reaction of a monoclonal antibody with an enzyme only very rarely results in full inhibition of enzyme activity [1762]. This is most likely because the enzyme active site is only a very small region of the enzyme molecule and the chances of it acting as the determinant site are low. Indeed, even with polyclonal sera, Baylin found that the major antigenic determinant of placental histaminase (diamine oxidase) was not in the active site area [748]. One approach has been to produce antibody to the enzyme substrate and then produce anti-idiotypic antibodies to these proteins [1871]; the recognition site of these antibodies would potentially mimic the substrate and block enzyme activity by binding to the enzyme active site.

4.1.3 Production of Antibodies to Human Pregnancy-Associated Amine Oxidases

4.1.3.1 Polyclonal Antibodies

Polyclonal antibodies have been prepared against human placental diamine oxidase and used for its localization [749,750] (*q.v.* §1.3.2.5e). Other studies have used antisera to compare placental

diamine oxidase with diamine oxidase activities from various sources using immunochemical techniques [745-748,752,755,1235].

Novotny *et al.* have purified human placental diamine oxidase according to Baylin's cadaverine-Sepharose affinity method [748] and used the purified enzyme to produce rabbit polyclonal antibodies that were in turn used to immunoaffinity purify more placental enzyme, which was then further purified on con A-Sepharose [47]. The NH_2-terminal sequence was found to have a high degree of similarity with the published sequence of human kidney amiloride-binding protein [781]. A high level of diamine oxidase activity was shown in the culture medium from transfected 293 cells expressing the human amiloride binding protein, (whereas no activity was detected in control non-transfected cells). The diamine oxidase activity in the conditioned medium could be inhibited by aminoguanidine, amiloride and amiloride analogues. Equivalent inhibition was seen when purified placental diamine oxidase is substituted for the conditioned medium.

4.1.3.2 Monoclonal Antibodies

Denney *et al.* isolated a monoclonal antibody that immunoprecipitated histamine- and putrescine-oxidizing activity in extracts of human placenta [759]. They obtained the antibody by immunizing mice with a preparation of human placental diamine oxidase partially purified by cadaverine-Sepharose chromatography. Hybridomas were screened and selected for production of an antibody that immunoprecipitated diamine oxidase in the presence of secondary immunoglobulin reagents. Immunoblotting of human placental enzyme with a monoclonal antibody indicated a polypeptide with a M_r 95,000, consistent with their expected molecular weight of diamine oxidase. They demonstrated that their monoclonal antibody competitively inhibited placental diamine oxidase by up to 75% when assayed at pH 7.3, 37 °C. Inhibition was observed with diamine oxidase from human intestine and a human ileal carcinoma grown in nude mice, but not rat or dog intestinal diamine oxidase, suggesting that the antibody was species specific. At pH 7.4, diamine oxidase in placental extracts bound to Affi-Gel 10 columns containing bound monoclonal antibody, and enzyme activity was eluted in the active form by exposing the column to the application buffer adjusted to pH 9.4. However, the nature of the immunoaffinity purified protein and the extent of purification of the enzyme were not reported.

Houen *et al.* have also produced monoclonal antibodies to diamine oxidase from human placental extracts and pregnancy serum [756]. They used enzyme purified by ammonium sulphate precipitation followed by affinity chromatography on aminohexyl-Sepharose and con A-Sepharose, with a final purification step using a Mono-Q ion exchange column. They report that both the placental and pregnancy serum enzymes showed a monomer and dimer 'M_rs of 90 kDa

and 180 kDa' [*sic*] respectively (data was not shown), no separation of enzyme activity after ion exchange chromatography was reported. The screening method used for identification of 'positive cultures' was not reported. Monoclonal antibodies were produced in ascites fluid from which they were non-specifically purified on Protein A-Sepharose and used for the small scale immunoaffinity purification of the enzymes. The results of the immunoaffinity purifications were not shown except to say that strong crossreactivity was observed between human placental, human serum and bovine serum diamine oxidases; and that peptide maps of these immunoaffinity purified enzymes were very similar. No sequence data were reported for the immunoaffinity purified enzymes, although it was stated that individual peaks were collected for subsequent sequence analysis. Where sequence data from peptides obtained from digests of bovine serum amine oxidase purified by conventional methods was obtained no 'homology' to known sequences were observed. The cDNA structure for human amiloride-binding protein had been reported two years previously [781].

Monoclonal antibodies have been raised against the pig kidney amiloride-binding protein (diamine oxidase) [47] and to the diamine oxidase produced by the intestinal cell line Caco-2 [786] and used for their immunoprecipitation.

4.1.4 The Approach Used to Making Monoclonal Antibodies

In this work, purified human retroplacental polyamine oxidase preparations obtained from procedures described in Chapter 3 were used to produce monoclonal antibodies. The mAbs, produced in large quantities in tissue culture and specifically purified using anti-mouse IgG immunoaffinity columns, were used to construct immunoaffinity columns for the further purification of the retroplacental polyamine oxidases.

4.2 Methods

4.2.1 Immunization of Mice with Human Retroplacental Polyamine Oxidase

The amount of antigen necessary to induce a strong immune response will depend on the individual antigen and the host animal. Enzymes are an example of soluble protein antigens and can yield strong responses and good monoclonal antibodies with doses of as low as 1 μg per injection. More commonly, 5–50 μg protein is used for primary immunizations and boosts [1761]. Quantities of this order were used here, with a small amount of antigen for the priming immunization (15 μg per mouse), a larger amount for the boost immunization (50 μg) and the largest amount in the final boost (86 μg). Coincidently, the purity of the human retroplacental polyamine oxidase antigen was progressively increased through the immunization regime as proficiency in its purification increased. This increase in antigen purity probably conferred an

advantage during boosting as the impurities were more likely to be adsorbed by circulating antibodies and rapidly cleared following boosts.

In a typical immune response, an increase in B cells bearing surface antibodies specific for the inoculated antigens will be first detected 5–6 days after the primary injection of antigen [1719]. Antibody will be detected in the serum from around 7 days after the injection and persist at a low level for a few days, reaching peak titre around day 10. Primary responses are often very weak, particularly with readily catabolized, soluble protein antigens. The production of monoclonal antibodies to soluble antigens such as enzymes appears to be a particular challenge as only very low numbers of antigen-specific hybridomas per total number of hybridomas have been found using conventional immunization techniques [1872]. The subcutaneous and intraperitoneal immunization routes, used here in the priming and first boost immunizations respectively, provide a depot of antigen especially when it is mixed with an oil based adjuvant such as Freund's [1873]. In addition to their depot effect, prolonging the antigen's duration in the immunized animal, adjuvants are nonspecific stimulators of the immune response. Adjuvants raise cytokine levels stimulating the activity of antigen presenting cells and causing a local inflammatory reaction at the site of the injection. Freund's complete and incomplete adjuvants were used here in the priming and first boost immunizations respectively. Details of Freund's adjuvants have been reported elsewhere [1719,1873]. Two to four volumes of adjuvant were used for each volume of aqueous antigen, providing a stable water-in-oil emulsion [1874]. The choice of adjuvant may influence the subclass of antibody produced, and Freund's tends to evoke an IgG_1 response [1757]. Indeed, the monoclonal antibodies produced here after immunizations with Freund's adjuvant were predominantly of the IgG_1 subclass. This was desirable as IgG_1 antibodies are conveniently purified, relatively stable and easily coupled to affinity matrices.

The response to the second injection of the same antigen is usually dramatically different to the priming immunization. The number of B cells bearing antigen-specific cell-surface antibodies typically increases exponentially after the secondary injection, reaching a peak between 3 and 4 days post injection. Antibodies in the serum are also detectable at this time but peak levels are usually achieved in around 10–14 days. High levels of antibody generally persist for 2–4 weeks after the second injection. A delay was therefore required before reintroducing the enzyme antigen into the primed animals. The interval between secondary and tertiary injections was extended to allow the circulating level of antibody to drop enough to prevent rapid clearance of the newly injected antigen. For mice the secondary and later boosts should be spaced at a minimum of 2–3 weeks. Some authors recommend 5 weeks or more [1875] and it is *advantageous to use an even greater interval*, as was done here, indeed mice will remain primed for at least a year after receiving the first injection. The booster injections can consist of a lower

dose of antigen than the primary, since the secondary response is more vigorous, however this may lead to the production of higher affinity antibodies [1719,1755]. Since lower affinity antibodies were the objective, the dose given was larger; this also avoids rapid clearance by any circulating serum antibodies.

The response to subsequent immunizations broadly mirror that of the secondary injection. However, higher titres of antibody are reached, moreover the character of the antibodies in the serum changes. Sera from primary (priming) immunizations will contain a substantial portion of IgMs, whereas the sera from later immunizations contain more IgG. Hyperimmune sera often have higher levels of IgG, a substantial amount of which is specific antibody. Levels of 1 mg of antigen-specific IgG/ml are possible, equivalent to approx. 10% of the total IgG content of serum [1719]. The average affinity of antibodies for an antigen increases with repeated injections. This change continues through multiple rounds of immunization. A typical antibody response to multiple injections with a good immunogen is illustrated in Figure 4.1 [1719]. Antibody affinity affects the performance of many immunochemical procedures. For immunoaffinity purification it is desirable that the antibody not be so tenacious as to make release of the antigen difficult. The antibody chosen for the immunoaffinity column should have an affinity that permits stable binding of the antigen at neutral pH and subsequent dissociation of the complex with reagents that allow enzyme activity to be retained. For these reasons, only a single boost in Freund's incomplete adjuvant was given before the final boost immediately preceding spleen cell harvesting.

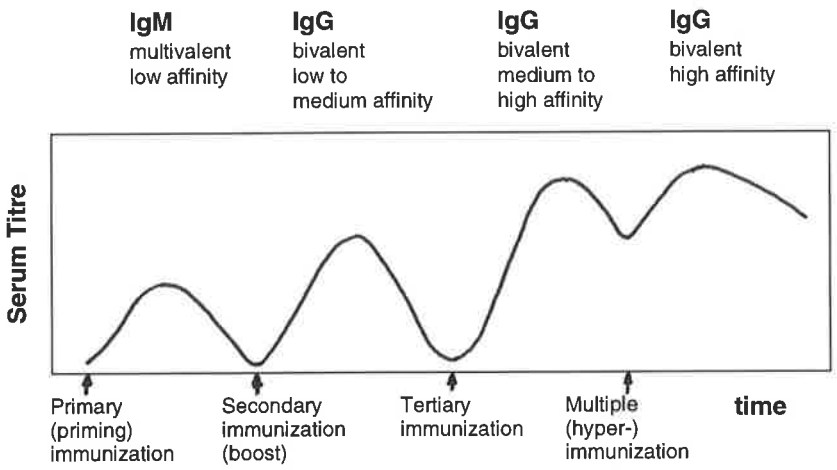


FIGURE 4.1. Kinetics of a Typical Immune Response

The decision of which mice to use for hybridoma production was based on the results of the screening assay used to determine the titre of the antibodies against the antigen. Serum titre relates primarily to the number of immunoglobulin producing cells and gives a strong indication

that fusion of splenocytes with myeloma cells will produce monoclonal antibodies of desired specificity [1761]. However, the antibody producing cells may not be the most suitable partners for fusion. In particular, the presence of rapidly dividing plasmablasts, which do not themselves secrete large amounts of antibody, has been correlated with a high frequency of antigen specific hybridoma production. Spleen cells that fused with myeloma cells tended to be larger than average, and had recently undergone antigen stimulation and blast formation [1876]. The inducement of these rapidly dividing plasmablasts has been achieved by giving large doses of antigen intravenously without adjuvant on each of the 3–4 days preceding the fusion using an animal which has been previously immunized [1872]. However, the scarcity of the antigen precluded such a strategy here. Although the final boost is sometimes given intravenously, this procedure was not adapted here because direct intravenous injection may induce (pulmonary) embolisms or lethal anaphylactic shock. A protocol was chosen in which a mouse with a high serum titre was boosted intraperitoneally without adjuvant 4–5 days before fusion; serum titre would not have reached a maximum at this time and was therefore not measured after the final boost.

The overall response of an individual animal to an immunogen may range from strong to very weak. This will be reflected in the proportion of hybrid clones producing antibody of desired specificity. A number of mice were therefore immunized and their serum response screened. Those giving a stronger response were likely to have more stimulated B cells available for fusion. From a practical point of view, the use of a number of animals also hedges against casualties.

The two purified retroplacental polyamine oxidase forms obtained by procedures described in Chapter 3 were used to immunize two groups of mice. Five 8- to 9-week-old BALB/c mice were each given a priming immunization of approximately 15 µg PAO I emulsified in Freund's complete adjuvant, divided amongst at least three subcutaneous sites. Similarly, another group of five mice were immunized with PAO II.

Antigen solution in saline was mixed with complete Freund's adjuvant [1719,1873] using two glass syringes with Leur lock fittings connected by a double hubbed 19G needle [1873]. The *aqueous phase was injected into the oil,* and the mixture then passed rapidly back and forth between the syringes until a stable water-in-oil emulsion formed. Subcutaneous injections of not more than 100 µl were made in dorsolateral areas of the neck and shoulder. A 26G needle was used to minimize loss of the inoculum through injection sites. Multiple site injections have the advantage of presenting the antigen by a variety of routes, provoking an enhanced immune response. Material injected into the shoulder regions will drain into the local lymphatic system and become concentrated in the lymph nodes close to the injected sites; these will ultimately drain to the spleen.

The mice of each group were subsequently given a second (boost) immunization with approximately 50 μg purified PAO I or PAO II antigen each as appropriate. The secondary immunization was given in Freund's *incomplete* adjuvant to avoid possible hypersensitivity reactions to the mycobacteria. The antigen emulsion was administered intraperitoneally using a maximum inoculum volume of 200 μl. Antigens injected into the peritoneum will drain into the thoracic lymphatics and then into the vena cava.

4.2.1.1 Test Bleed

Seven days after the second immunization, a blood sample was taken from each mouse. These 'test bleeds' provided small samples of sera which were titred to monitor the development of the response to antigen. The sera were compared to preimmune serum controls collected from the mice before the primary immunization.

Mice were bled from the retrobulbar sinus ophthalmic venous plexus (which lies at the back of the orbit) [1757,1760,1877,1878]. The blood was allowed to clot at room temp for 30–40 min and retraction allowed for several hours or even overnight at 4 °C. Mouse blood is prone to haemolysis and so clotting and retraction are performed at room temperature rather than at 37 °C. The blood was then centrifuged (2000 × *g* for 10 min) and the serum collected. For mice 200–400 μl was the usual size for most test bleeds, providing 100–200 μl serum. The sera were screened for antibodies to the two human retroplacental serum polyamine oxidase forms using the screening assays described in section 2.9.2. (cf. §4.2.3 *et seq.*).

4.2.2 Hybridoma Production

After immunizing mice with the partially purified enzyme forms, cell fusion was used as a method of immortalizing cells expressing the transitory function of antibody production. The hybrids of a cell fusion will coexpress genotypic and phenotypic characteristics of both parental cells. A refinement of the monoclonal antibody technique first described by Köhler and Milstein [1879] was used, involving the selection of a mutant strain of the myeloma parent line that did not secrete antibody. It was essential to choose a myeloma parent that was vulnerable to certain specified cell culture conditions so that it could not survive unless it had participated in a fusion.

The common hybridoma selection procedure devised by Littlefield [1880] was chosen. This strategy uses a myeloma parent that lacks hypoxanthine guanine phosphoribosyl transferase (HGPRT), an enzyme in the salvage pathway of nucleotide metabolism, and essential to cells growing in the presence of dihydrofolate reductase inhibitor aminopterin, which blocks the *de novo* pathway for nucleotide synthesis. Cells lacking HGPRT die in medium containing aminopterin, even when hypoxanthine and thymidine are included (HAT selection medium),

because both the *de novo* and salvage pathways to nucleotide production are blocked. However, hybrids between HGPRT deficient myeloma cells and spleen cells, which contain the wild type salvage pathway enzyme that complements the missing HGPRT in myeloma cells, will survive and therefore be selected from their parents in HAT containing medium. The HAT selection system is referred to as being half selective since only one of the fusion partners is selected against by the medium. Spleen cells will not continue to grow in culture without special stimuli so there was no need to select against unfused spleen cells. Among the hybrids, some expressed and secreted desired antibodies to the polyamine oxidases. Such hybrid cells were selected, individually isolated, and grown as clones secreting the specifically selected antibody. Expansion of the cultures allowed the antibody to be produced in quite large amounts such as were required for the construction of preparative immunoaffinity columns. Furthermore, the hybridomas could be cryopreserved and recovered later, thus providing a potentially unlimited and perpetual supply of the antibody without variation in specificity. Books and reviews have been written about monoclonal antibody production and include references [1719,1755,1757-1759,1761,1762,1881-1885], many the strategies used for monoclonal antibody production in this work have been drawn from these. The procedure that was adopted here for monoclonal antibody production is summarized in Figure 4.2.

4.2.2.1 Fusion of Spleen Cells and P3/653 Myeloma Cells

The fusion method used in this study was based on that described by Zola and Galfrè *et al.* [1755,1758,1759,1886]. Spleen cells were derived from the mice immunized with partially purified polyamine oxidase forms. A single cell suspension was required using procedures that were gentle and rapid, as the viability of the spleen cell preparation would be a major determinant in the success of the fusion. Although the cells could be used without any enrichment, any degree of enrichment would probably be beneficial because it would increase the possibility of B lymphocytes, particularly B cell blasts, coming into contact with myeloma cells. For this reason, erythrocytes were removed with Gey's haemolytic medium. The benefits of specific enrichment are probably only minor, and would only increase the relative proportion of useful hybrids that would be distinguished in screening assays in any case. As specific enrichment also risks losses of potentially useful cells it was not attempted.

The actual fusion procedure is probably not ideal. The conditions used were intended to facilitate fusion without causing excessive cell damage. The dilution process is critical; if it is too vigorous the yield of hybrids will be reduced. Incipient hybrid cells are particularly fragile, so it was important to keep centrifugation and resuspension steps to a minimum. On the other hand, it

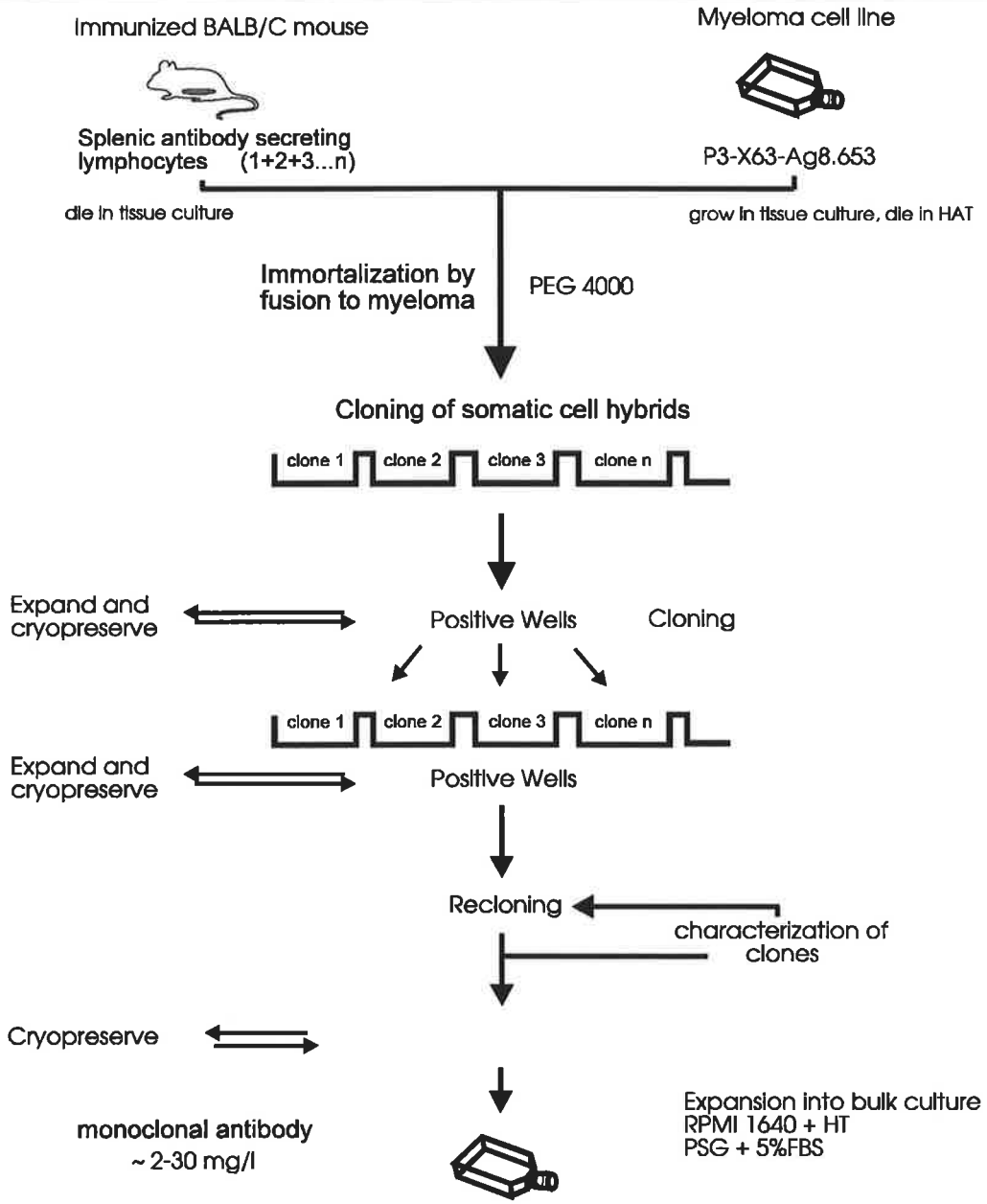


FIGURE 4.2. Steps in Derivation and Cloning of Antibody Producing Hybridomas

was important to reduce the concentration of PEG and DMSO. So the procedure used here was a compromise, with only a single wash immediately after dilution, followed by a longer period in medium, during which DMSO can diffuse out of cells and the fusion process can continue in cells that have started to fuse [1755,1758].

Westerwouldt has demonstrated a pH effect on the successful outcome of heterokaryon formation and showed that slightly alkaline pH (8.0 to 8.2) favours successful fusion; whereas at lower pH, premature chromosome condensation, which leads to cell death, is observed [1887]. However, premature chromosome condensation appears only to result when cells in mitosis fuse with interphase cells. Most myeloma and spleen cells will be in the G_0 or G_1 phases of the cell cycle. Zola *et al.* preferred neutral pH (7.2 to 8.0) to maintain cell viability [1755,1758]. Based on

these considerations, the pH of the media during fusion, as estimated from the colour of the phenol red pH indicator, was kept between these levels.

Immune mouse 2.II (serum titre 15,848, *vide* Table 4.1) was selected for hybridoma production on the basis that it had the highest titre of the Group II mice; its serum also contained antibodies to the PAO I preparation. PAO II was the most abundant form of the enzyme and therefore more readily available for immunizations. Mouse 2.II was immunized with an intraperitoneal injection of 86 μg purified PAO II in 1 ml of sterile saline four days before fusion.

On the day preceding the fusion, the P3-x63-Ag8.653 myeloma culture (*q.v.* §2.8.3) was counted (*q.v.* §2.8.2) and split from 9.4×10^5 cells/ml (96% viability) to 1.2×10^5 viable cells/ml; t_D was estimated at 12.65 h. Four × 24-well cluster plates containing 1.25×10^6 'feeder' spleen cells per well were prepared as described in section 2.8.5.

(1) On the day of the fusion the myeloma culture density was found to be 485,000 viable cells/ml with >95% viability.

(2) Immunized mouse 2.II was killed by cervical dislocation and its spleen removed (*q.v.* §2.8.5.1).

(3) Spleen cells were isolated, treated with 20 ml Gey's haemolytic medium (*q.v.* §2.8.1.4), and resuspended in 10 ml complete RPMI 1640 containing 10% ΔFBS (*q.v.* §2.8.1.1).

(4) During the Gey's haemolytic treatment of the spleen cell preparation the myeloma cells were prepared. A volume (40 ml) containing approximately 2×10^7 cells was transferred to a 50 ml centrifuge tube and centrifuged at 200 × *g* for 5 min. The myeloma cell supernatant was removed, the pellet loosened and resuspended to 10 ml with complete RPMI 1640 containing 10% ΔFBS.

(5) Immune spleen cells were counted and 11,280,000 cells at 97% viability were found.

(6) Myeloma cell concentration in the 10 ml suspension was found to be 1.68×10^6 cells/ml so the suspension was diluted with 5 ml complete RPMI 1640 containing 10% ΔFBS to 15 ml; the cell concentration was checked and found to be 1.2×10^6 cells/ml.

(7) Ten millilitres of the myeloma suspension was added to the 10 ml spleen cell suspension to give 1.2×10^7 myeloma cells and 11.3×10^7 immune spleen cells in 20 ml complete RPMI 1640 containing 10% ΔFBS, a spleen cell/myeloma cell ratio of approx. 10 : 1. The cell mixture was centrifuged to a common pellet at 200 × *g* for 5 min and the supernatant medium removed carefully and completely from the pellet. The tube was then 'flicked' to loosen the pellet. One ml of PEG 4000/DMSO fusogen (*q.v.* §2.8.1.5) at 37 °C was added to the cells and mixed with them using a pipette for exactly 1 min, after which time a dilution process was commenced.

(8) Three millilitres of complete RPMI 1640 at 37 °C containing 10% ΔFBS was added

dropwise over 10 min with gentle agitation to ensure even dilution. A further 10 ml complete RPMI 1640 at 37 °C containing 10% ΔFBS was added dropwise over 10 min with gentle mixing to prevent separation of the incipient hybrids. If the medium started to appear strongly alkaline the tube was briefly gassed with sterile CO_2 (from plugged Pasteur pipette attached, via tubing, to a side arm flask containing a few pellets of dry ice); bearing in mind that DMSO affects the indicator colour making the medium appear more alkaline than it actually is.

(9) The cells were then centrifuged at 200 × *g* for 5 min and resuspended in 20 ml complete RPMI 1640 at 37 °C containing 10% ΔFBS and placed in a warmed 75 cm^2 cantered-neck tissue culture flask gassed with a 5% CO_2/95% air mixture. The flask was then incubated in a humidified incubator set at 37.0 ± 2 °C in an atmosphere of 5% CO_2/95% air.

(10) After 3 h the hybridoma suspension was transferred to a conical bottomed 50 ml centrifuge tube.

(11) The hybridoma suspension was centrifuged at 200 × *g* for 5 min, and resuspended in 20 ml of double strength HAT medium (*q.v.* §2.8.1.2). This suspension was evenly split between two centrifuge tubes and each made up to 50 ml with ×2 HAT in complete medium. The myeloma cell concentration (based on the prefusion count) was 1.2×10^5 cells/ml [× 100 ml].

(12) The fusion mixture was dispensed as 1 ml cell suspension per well into 4 × 24-well cluster plates containing 1.25×10^6 'feeder' spleen cells/well in 1 ml medium, prepared on the previous day.

(13) Plates were incubated in a humidified incubator at 37 °C in an atmosphere of 5% CO_2/95% air.

4.2.2.2 Selection and Culture of Hybridomas

The objectives of this phase, covering the period from the plating of the fusion mixture, up to the point when the screened colonies are ready for cloning, were to encourage the growth of hybrids, to prevent the growth of anything else, and to maintain the hybridoma while their secreted antibodies were tested. Myeloma cells should grow normally in HT medium (*q.v.* §2.8.1.3), but should all die within 1–3 days in HAT medium [1757]. After a week in HAT medium, one can assume that all the parental myeloma cells will be dead, and that any growing cells will be hybrids. At this stage HAT selection may be concluded. However, the inhibition constant (K_i) of aminopterin for dihydrofolate reductase is less than 10^{-9} [1888], and the concentration of aminopterin in HAT medium is 4×10^{-7} M. So, the aminopterin concentration must therefore drop by more than 400-fold before the enzyme can regain activity. Unlike hypoxanthine and thymidine, aminopterin is metabolized extremely slowly; so the main route of elimination of

aminopterin from the cultures must be by dilution. Therefore, the recommendation of Zola and Brooks [1758] to grow HAT selected cells in HT medium permanently was followed.

The majority of fusion products die out in the first few days immediately after fusion [1755]. Hybrids do not start to divide immediately in culture, but have a variable quiescent period, many cells die without reaching the growth phase. Unfused myeloma cells die out within the first week because of the aminopterin nucleotide synthesis block, while the majority of spleen cells die out because they do not have the inherent capacity to divide or stay alive in culture without exogenous stimuli. Some macrophages and fibroblast-like cells were observed to adhere to the culture plates and divide slowly over the first week or so: as they grow much more slowly than the hybrids, overgrowth by these cells was not considered to be a problem. The new hybrids were on a knife-edge of viability so great care was taken in their maintenance to maximize their numbers.

(1) Fresh HT medium (*q.v.* §2.8.1.3) was added to the hybrid cultures seven days post fusion (day +7). Approximately 1 ml of culture supernatant medium was aspirated from each well of the 24-well cluster plates. Freshly prepared HT medium, warmed to 37 °C was very gently added at 1 ml per well. The medium had been recently supplemented to ensure a high glutamine level. The medium addition was gentle to avoid generating multiple colonies from a single parent colony, and to reduce the probability of mixing cells from adjacent colonies. However, it was necessary to compromise: if medium addition takes too long the medium becomes alkaline.

(2) Plates were checked daily after the day +7 medium addition. Plates were examined for colonies and yellowing of the phenol red medium pH indicator caused by the release of acid metabolites from cells, which may be hybrids, macrophages, fibroblast-like cells, or revertent myeloma cells. There was occasional fungal contamination, indicated by telltale filamentous mycelia ('the white fluffies') and identified as *Paecilomyces variotii*, that also yellowed the medium pH indicator. If this was detected, the medium was removed from the affected well, which was then rinsed in 70% alcohol to avoid the spread of the fungus to adjacent wells. Bacterial and mycoplasma contamination were not detected.

(3) Day +9. Many of the wells in the 24-well cluster plates were observed to have hybridoma colony growth. Circular colonies of round cells larger than small lymphocytes were seen using an inverted microscope with phase contrast optics. Macroscopically the colonies appeared as opaque white pinhead size disks.

(4) Day +11. The colonies were larger and readily visible, and the medium was yellowing. One ml of supernatant was removed from each well of the fusion plates and replaced with 1 ml of fresh HT medium at 37 °C. Supernatants were reserved for screening.

The recommendation of Galfrè and Milstein [1759] to use medium containing 20% ΔFBS for

selection and cloning of hybridomas was followed. As soon as a hybrid was selected the medium concentration was lowered to 10% ΔFBS. When the cells had adapted to growth in bulk culture, ΔFBS concentration was reduced to 5%.

4.2.3 Development of the Screening Assays

Development of a good screening assay is probably the most critical factor in the successful production of useful monoclonal antibodies. The assay system was required to be sensitive enough to detect the very small amounts of antibodies produced by the nascent hybridoma clones. Furthermore, the ultimate use of the monoclonal antibody for immunoaffinity purification of the enzyme had to be taken into careful consideration when designing the screening assay. It was also anticipated that only a few of the hybrids generated would secrete desired antibody to the enzyme. The screening assays had to be developed using the partially purified enzyme forms available.

Three general approaches have been used in the development of screening assays to detect anti-enzyme monoclonal antibodies [1762,1889]:

(1) Single-site indirect ELISA. In this strategy (enzyme) antigen is immobilized in the wells of plastic microtitre plates. After the application of hybridoma culture supernatants, monoclonal antibody bound to the antigen is detected using anti-mouse immunoglobulin.enzyme conjugate and the use of enzyme reagents in an ELISA. A caveat of this approach is: that if impure enzyme was used for immunisation of the mice and to coat the ELISA plates, then positive hybrids detected by this procedure may be producing antibodies that recognize the impurities, rather than the enzyme itself. Furthermore, binding of protein to a solid surface denatures it to some extent [1761], so enzyme conformation on the solid phase may be different to that of the enzyme in solution, or binding sites may sterically hindered through to binding of the enzyme to the plates (though this is likely to be a stochastic event). Therefore the method may not, or only poorly detect, the presence of antibodies to the enzyme [1890]. A corollary to this is, that this method may only detect enzyme distorted by binding to the plates. This may lead to the biased selection of monoclonal antibodies that bind to determinants not available in the native protein. Therefore, the hybridoma supernatants should be screened further by other complementary methods.

(2) Immunoprecipitation.

(3) Enzyme capture immunosorbent assay. In this approach, antibodies from hybridoma supernatants are immobilized in the wells of microtitre plates coated with anti-mouse immunoglobulin antibodies. Enzyme that is subsequently added in solution, will be captured in any wells containing immobilized specific antibody from the hybridoma supernatants. The immobilized enzyme may then be detected using an assay procedure specific for its activity. This

method depends on the retention of enzyme activity by the enzyme-(monoclonal) antibody complex. Where inhibition of the enzyme occurs, the method is still acceptable providing the inhibition is only partial. The occasional antibody that causes a complete inhibition of the enzyme activity would be missed by this assay but still be detected by the antigen detection assay (method 1). This enzyme capture method is particularly powerful as it allows the selection of specific antibodies without the necessity for pure (or well characterized) enzyme preparations, impurities are not likely to be detected.

The screening assays were designed to mimic the final use of the antibody. The state of the antigen should be as similar as possible in the screening assay as in the final use, i.e. in its native state. This was also considered for immunization. Two the approaches described above, namely methods 1 and 3, were used to screen hybridoma supernatants for specific monoclonal production (*vide* Figures 4.3 and 4). These complementary approaches were chosen as they would:

(a) select antibodies that were suitable for immunoaffinity purification of the native enzyme,

(b) detect hybridomas producing antibodies that potentially inhibit enzyme activity,

(c) detect hybridomas producing antibodies against impurities in our partially purified preparations, which may be subsequently useful for adsorbing out these impurities.

Other methods such as Western blotting and immunoprecipitation were not used since insufficient information was known about the molecular mass or subunit composition of the multiple retroplacental polyamine oxidase forms. Furthermore, in Western blotting, the enzyme would be denatured and probably not in a form recognized by antibodies useful for immunoaffinity purification of the native enzyme. Antibodies detecting enzymes on Western blots would most likely be directed against linear determinants. Similar problems would arise if immunohistochemical screening methods were used since the localization of the antigen (which may be denatured) was uncertain.

During the initial selection and screening of antibodies it is difficult to select for an antibody of chosen avidity. A strongly positive result in the screening assay may indicate an antibody of high affinity or simply the presence of a clone secreting large amounts of antibody. Interpretation of results may be further complicated if the antibody is inhibitory. Quantitative estimation of relative specific titres was subsequently used to improve interpretation (*q.v.* §4.3.6.5).

4.2.3.1 Theoretical Considerations

Antibody-antigen interactions are dependent on the concentration of both reactants and the conditions under which the reactions take place. The initial rate of association of an antibody with an antigen is described by the classic equation:

Rate of formation of the product = K_1 [antibody].[antigen]

With monoclonal antibodies, the effective antigen concentration is greatly reduced since there may only be one or perhaps a few determinants on each antigen molecule. The antibody concentration is also likely to be low, especially at early stages of screening. At low concentrations the antigen and antibody can be regarded as univalent. Thus, the parameters on the right hand side of the equation defining the association rate are likely to be several orders of magnitude below those prevailing when using polyclonal serum (such as the immune mouse serum used in the development of the screening assay). Therefore incubation times were extended beyond the usual 3 h conventionally used. The target antigen concentration was made as high as possible and the culture supernatants were tested several times during the growth of the hybrids. This was done with the consideration that the clones should not be overgrown, and that it is possible for antigen to leach from the plates if the assay time used is too long.

The dissociation rate of an antibody-antigen complex is defined by the equation:

Initial rate of dissociation = K_2 [antigen].[antibody]

With most antigen-antibody reactions, it is the dissociation rate rather than the association rate that determines the antibody affinity, the dissociation rate can vary over 8–9 orders of magnitude but is nearly always very much lower than the association rate. This indicates that conducing assays at lower temperatures, which would reduce the dissociation rate, would increase the probability of detecting positive samples, especially those containing antibodies of lower affinity. Therefore, a 5–16 hour incubation at 4 °C was routinely adopted.

4.2.3.2 Practical Considerations

Solid phase ELISAs were used for screening assays as large numbers of samples (as are produced during cloning stages of monoclonal antibody production) were easily manipulated by this method. The antigen or immunoglobulin were passively adsorbed onto the solid surface of polystyrene microtitre plates by (primarily) hydrophobic interactions. The capacity of various supports varies widely. After screening several plate types, Immulon IV plates (Dynatech, Chantilly, VA), which are described as high protein binding, were found to be suitable for the assays. Protein was bound from enzyme antigen solutions at 1–10 µg/ml. Binding capacity of the plate wells was 300 ng/cm^2; surface area of the well 0.38 cm^2 therefore protein binding will be ~100 ng/well.

The enzyme antigen or antibody coated on the microplate wells was as pure as possible so that potential (protein) binding sites on the plastic were not occupied by irrelevant protein, which

would result in a reduction of signal strength. So, if the first layer was a polyclonal antibody, such as sheep anti-mouse immunoglobulin, then an affinity purified preparation was used. If enzyme antigen was used it was as pure as possible, since this type of assay will indicate antibodies in the hybridoma supernatant reacting with the material on the plate and does not distinguish between the target antigen and an impurity (*vide supra et* Figure 4.3). If the surfaces of the wells are not fully saturated with the coating antigen/antibody they will adsorb protein nonspecifically in subsequent incubation steps. Proteins and Tween 20 were used to block remaining binding sites in the wells. Tween 20 also contributes to the prevention of nonspecific interactions.

4.2.3.3 Screening of the Hybridoma Supernatants for Specific Antibody

One of the objectives of the screening process was to identify useful hybrids and reject negative or uninteresting wells. Supernatant samples were collected as part of the spent medium replacement operation. If samples are taken too early a negative result will not be definitive. However, the colonies should not be allowed to overgrow, or the medium will become too acidic and the cells will die. Optimal sampling is obtained when the colony is about 25% of the well area, and two to three days after the last medium replacement [1755]. Wells that gave negative results were retested when the colony was larger and the medium more acid to ensure that they were indeed negative. Wells that scored negative twice were disregarded, and excluded from further screening. Wells that were positive were cloned as soon as possible. Weak positive results that could not be repeated on retesting may have resulted from antibody secretion by unhybridized lymphocytes.

The general methods used in the following protocols were based on others described elsewhere (*q.v.* §2.9 and references therein).

(i) Single-site indirect ELISA for anti-amine oxidase mAbs (Figure 4.3)

Alternate columns of microtitre plate wells were coated with 250 ng purified polyamine oxidase antigen in 50 µl, 50 mM carbonate/bicarbonate buffer, pH 9.6, per well. Remaining columns of control wells were coated with 250 ng BSA in 50 µl of the same buffer. Plates were incubated for 5–16 h at 4 °C. After washing wells with PBS–T, remaining binding sites in the wells were blocked with PBS–T containing 10% (v/v) sheep serum (blocking buffer) for 3 h at 37 °C. Wells were washed with PBS–T and undiluted hybridoma supernatants were incubated in wells coated with antigen and in the BSA coated control wells (50 µl/well) for 5–16 h at 4 °C. After washing with PBS–T, bound monoclonal antibodies were detected using an affinity purified, species specific, goat anti-mouse immunoglobulin.β-galactosidase linked whole antibody conjugate (incubation 5–16 hour at 4 °C) and its substrate, *o*-nitrophenol-β-D-galactopyranoside as

described for the serum screening assay (*q.v.* §2.9.2). Immune mouse serum, diluted 1/2000 in blocking buffer (or complete RPMI 1640 containing 10%ΔFBS), was used as a positive control. Serum from an unimmunized mouse, diluted 1/1000 in blocking buffer, and myeloma culture supernatant were included in the negative controls. Absorbances were quantitated on a Titertek Multiskan II microplate reading spectrophotometer (Flow Laboratories, Irvine, UK) at λ_{405}.

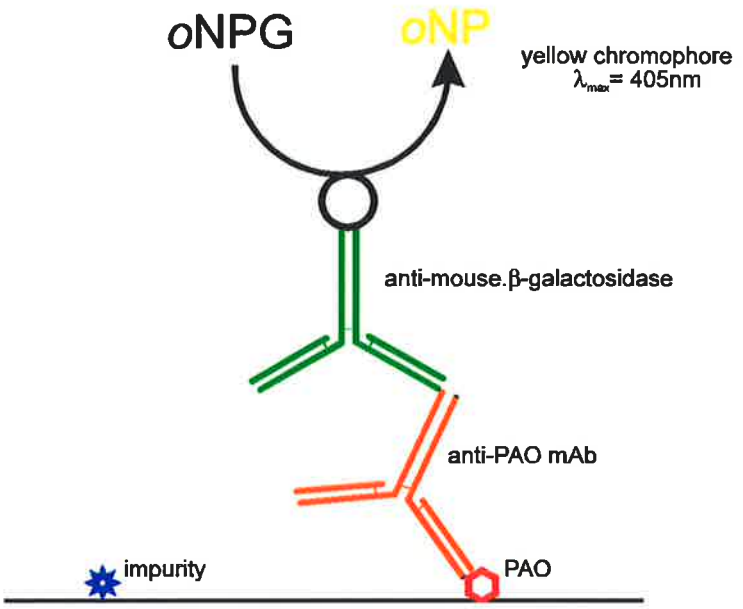


FIGURE 4.3. Single-Site Direct ELISA for Screening Hybridoma Supernatants
Wells were coated with purified polyamine oxidase antigen and alternate wells were coated with 250 ng BSA, remaining binding sites in the wells were blocked with PBS–T containing 10% (v/v) sheep serum (blocking buffer). Undiluted hybridoma supernatants were incubated in wells coated with antigen and in the BSA coated control wells. After washing with PBS–T, bound monoclonal antibodies were detected using an affinity purified, species specific, goat anti-mouse immuno-globulin.β-galactosidase linked whole antibody conjugate and its substrate, *o*-nitrophenol-β-D-galactopyranoside. Absorbances were quantitated on a microplate reading spectrophotometer at λ_{405}. Details are described in the text.

(ii) Enzyme capture immunosorbent assay (Figure 4.4)

Alternate columns of wells in microtitre plates were coated with 500 ng affinity purified sheep anti-mouse immunoglobulin in 50 µl 50 mM carbonate/bicarbonate buffer, pH 9.6. Wells in the remaining columns were coated with 500 ng BSA in 50 µl of the same buffer as controls. Plates were incubated for 5–16 h at 4 °C. Wells were washed with PBS–T and remaining binding sites in the wells were blocked with PBS–T containing 10% (v/v) donor sheep serum for 3 h at 37 °C. Subsequently hybridoma supernatants were added (50 µl/well). Wells were washed with PBS–T and PAO II solution of relatively high specific activity from an anion exchange purification step (*q.v.* 3.2.4) was added (50 µl/well). The moderate salt concentration in the ion exchange fraction (approx. 0.3 M) suppresses nonspecific adsorption of the enzyme. After incubation for 10–16 h at 4 °C wells were washed with PBS–T, and *in situ* enzyme activity detected by reaction with 1 mM ABTS, 40 IU/ml horseradish peroxidase (EC 1.11.1.7) and 100 µM putrescine in 0.05 M Tris–HCl, pH 7.4, at 37 °C (100 µl/well). Absorbances were quantified using a Titertek Multiskan II microplate reading spectrophotometer (Flow Laboratories) at λ_{415}.

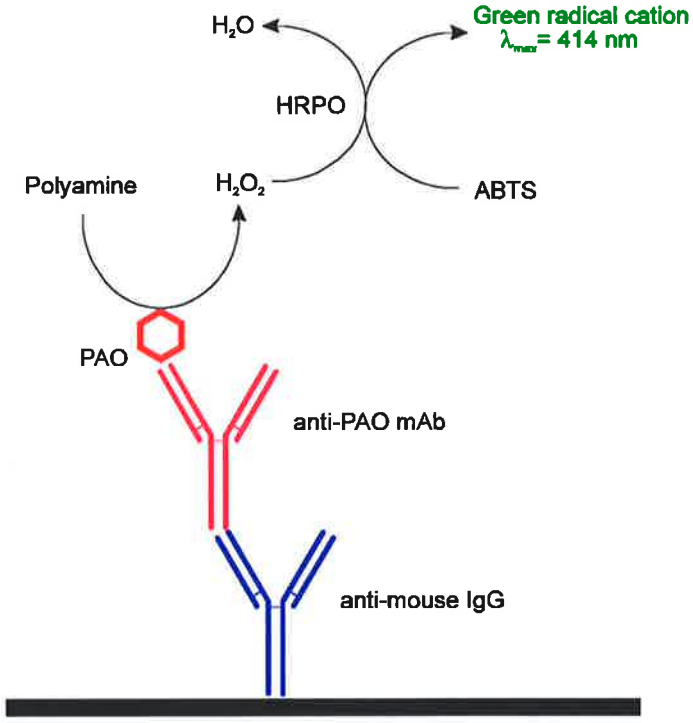


FIGURE 4.4. Enzyme Capture Immunosorbent Assay for Screening Hybridoma Supernatants
Wells in microtitre plates were coated with affinity purified sheep anti-mouse immunoglobulin. Subsequently hybridoma supernatants were added. Wells were washed with PBS–T and PAO II solution of relatively high specific activity from an anion exchange purification step (*q.v.* 3.2.4) was added. After incubation wells were washed again, and *in situ* enzyme activity detected by reaction with ABTS, HRPO and polyamine substrate. Absorbances were quantified using a microplate reading spectrophotometer at λ_{415}. Details are described in the text.

4.2.4 Hybridoma Cloning

Once a positive hybrid was identified, it was cloned as soon as possible to avoid overgrowth by other hybrids or nonproducing variants. The objective of cloning was to ensure that the cells producing antibody comprise a monoclonal population, that is that they have arisen from a single cell. Hybridoma cells in a single well of a cell culture plate may be descended from several fusion products, particularly in wells containing more than one colony. Furthermore, the loss of chromosomes during the first days after fusion may create heterogenous populations. Polyethlyene glycol fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a cell with a tetraploid number of chromosomes. The heterokaryon retains its nuclei until the nuclear membranes dissipate before mitosis. During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells. A mouse diploid cell has 40 chromosomes; thus a hybrid of two cells will have 80 chromosomes initially. Such a genome is unstable and the chromosomes may be lost during segregation if identical sets of chromosomes are not delivered to daughter cells [1757,1882]. If the chromosome that carries a functional immunoglobulin heavy or light chain gene is lost, production of the antibody will stop, resulting in a nonsecreting hybrid cell. If the chromosome that is lost also contains the gene used in drug selection then the growth of the cell will be unstable and it will die during selection. However, a cell which losses chromosomes may retain the gene used in drug selection and might be able to replicate its DNA faster than and therefore outgrow its neighbours. In a plasma lymphocyte as much as half the protein synthesising machinery may be devoted to the synthesis of

immunoglobulin. So hybrid cells producing antibody divert much of their energy into the process. Consequently they have less energy available for division and may be overgrown by cells not producing antibodies. Therefore it is important to clone early, so that antibody producing cells are not overgrown and lost. In mice, the hybridoma reversion rate to nonsecreting forms has been reported to be 10^{-3}/cell/generation [1761]. With established hybridomas however, reversion is a rare phenomenon and may be overcome by routine subcloning.

Different approaches to cloning may be used [1755,1757,1761]. In this work the limiting dilution strategy was chosen. Cloning involved the establishment of single cell cultures to give rise to a colony of identical cells. In this approach, hybridoma cells are plated out in individual culture wells such that, statistically, the most probable number of cells in any particular well is 1. If the cells are distributed at low densities, the fraction of wells with growth should follow the Poisson distribution [1757]:

$$f(0) = e^{-\lambda}$$

where $f(0)$ is the number of wells with no growth, and λ is the *average* number of clones per well. Poisson distribution statistics indicate that *if* the most probable number of cells per well is 1 (wells with growth containing single colonies), then 37% of the wells will have no colonies at all, i.e. if $\lambda = 1$, $f(0) = 0.37$. In practice, the cells have a cloning efficiency that is not only less than 100%, but also variable. This, along with the cell clumping phenomenon, leads to a degree of uncertainty of the monoclonality of the resulting colonies. If, in a series of cultures starting with the same inoculum, some have single colonies and some (>37%) have none, it is probable that the cultures with single colonies arose from single cells. If cloning results in a greater frequency of colonies then it would be unwise to interpret them as being derived from a single cell. To maximize the chances of obtaining satisfactory single cell clones with hybridoma cells of unknown cloning efficiency, cells cultures were initiated at several densities, e.g. 0.5, 1, 3 and 10 cells per well. This strategy will generally give the desired result of monoclonality at one of these levels. The cloned cells require feeders and/or conditioned medium.

First cloning was on day 16 postfusion. The target well contents were gently resuspended, counted and removed for cloning. Hybridomas were cloned in 24-well cluster plates, rather than 96-well microtitre plates, since the large wells give good control over growth conditions, longer time for screening, and lower risk of overgrowth. Cells were plated at limiting dilutions over feeder cells in HT medium. Feeder cells were at a final concentration of $2 - 0.5 \times 10^5$ cells per ml. Alternately, HT medium containing 10% ΔFBS and 20% HECS medium [1891-1894] (which contains 20% heat-inactivated human AB serum), or 10% hybridoma enhancing supplement (HES, a conditioned medium produced by a mouse macrophage-like cell line [1895] (Sigma Chemical Co., St. Louis, MO)), was set up in 24-well cluster plates the day before cloning.

Positive colonies, which were visible macroscopically 10–16 days after cloning (observed every 2 to 3 days, replacing the medium only when it started to yellow), were expanded in 24-well cluster plates then to 10 ml cultures and cryopreserved. Promising cell lines were recloned to increase the probability of the cell line being monoclonal. Monoclonality was suggested when all wells showing growth in the cloning plate screened positive. Cloning is not infallible, so while the cloned cells were grown up, the original (positive) wells were maintained by feeding after removing most of the hybrid cells, the uncloned populations were expanded and the cells cryopreserved, hedging against cell loss by cloning failure or contamination.

4.2.5 Monoclonal Antibody Production

Once a monoclonal cell line is established, the yield of monoclonal antibodies produced in tissue culture will be in the order of μg/ml. In ascitic fluid (and polyclonal sera) yields are in the order of mg/ml. However, the volumes obtained are small unless a large number of animals are used. Tissue culture medium will contain antibodies from fetal bovine serum and other serum supplements (e.g. human endothelial cell culture supernatant), and a large number of nonimmunoglobulin proteins. In principle, tissue culture produced mAbs will be free from contaminating mouse immunoglobulins. FBS has up to 6000 μg serum protein/ml, of which 1300–1500 μg/ml may be bovine IgG [1761]. The non-mouse IgG contaminating proteins can be separated by affinity chromatography. Ascitic fluid will contain 10–20 mg mouse protein/ml, of which 50–70% will be mouse immunoglobulins. Ascites always contains normal mouse immunoglobulin in addition the monoclonal antibody which will probably account for only 16–30% of the total immunoglobulin. Autoantibodies, particularly rheumatoid factor, anti-cytoskeletal antibodies and cross-reacting anti-carbohydrate specificities are likely to be present. These antibodies, if present on an immunoaffinity column, could easily result in contamination of the target enzyme especially when it is isolated from a complex biological mixture such as retroplacental serum. The non-specific antibodies are difficult to remove because they are from the same species. Therefore, the monoclonal antibodies used here for construction of the immunoaffinity columns were affinity purified from hybridoma culture media. This is in contrast to the studies by Houen *et al.* [756] and Denney *et al.* [759] in which ascites fluid derived antibodies were used.

Purification of monoclonal antibodies from tissue culture supernatants is technically demanding. The strategy used in the work described here was to immunoaffinity purify the selected monoclonal antibodies using a column of sheep anti-mouse Ig antibodies. This immunoaffinity method not only selects against the non-mouse antibodies from media supplements, but also selects against other proteins. If not removed by purification procedures,

the proteins from media supplements may potentially contaminate the immunoaffinity purified enzyme; since proteins covalently attached to insoluble supports can be released from the column, albeit usually in small amounts. So the more restricted the composition of the column (i.e. mouse mAb only), the more restricted the potential contamination of the purified enzyme, and the higher the certainty of its identification and characterization.

4.2.6 Immunoaffinity Chromatography

Immunoaffinity chromatography matrices should provide a stable covalent linkage between the support matrix and the antibody to prevent its release (bleeding) during chromatography. The *N*-hydroxysuccinimide ester linkages provided by the Affi-Gel and Affi-Prep affinity matrices fulfil this requirement. Carbohydrate binding ligands tend to destroy antibody activity and cyanogen bromide activated matrices have a tendency to release their attached antibodies. The *N*-hydroxysuccinimide ester linkage provides coupling under mild conditions so the specificity of the antibody is not altered on coupling. Affi-Gel and Affi-Prep affinity matrices provide a spacer arm that reduces steric inhibition of the antibody interaction by the matrix. For these reasons the Affi-Gel and Affi-Prep affinity matrices were chosen and used here.

The Affi-Prep 10 support polymer provides a stable chromatography matrix due to its rigid polymeric bead structure making it particularly suitable for the handling of the high flow rates. It is therefore more resistant to mechanical collapse than Affi-Gel 10, which is based on agarose beads, making it particularly suitable for mAb purification which involved the processing of large volumes of hybridoma cell culture supernatants and multiple cycles of use. Although Affi-Prep 10 has better flow properties it suffers from increased non-specific binding to the matrix when compared with Affi-Gel 10, possibly through hydrophobic interactions, it also has a lower ligand density; for these reasons Affi-Gel 10 (the matrix to which the monoclonal antibodies were ultimately bound) was used for the immunoaffinity purification of the enzyme where obtaining material of the highest possible purity was at a premium. With both matrices, high efficiency coupling to the antibodies was obtained through the *N*-hydroxysuccinimide ester link to their primary amino groups. A pH value of 6.5 – 8.0 gives the greatest coupling efficiency. HEPES was a particularly suitable buffer in this regard [1896]. Buffers that contain primary amino groups such as Tris will react with the uncoupled gel. When using highly activated matrices and low ratios of antibody per gel volume the recovery of antibody activity can be low. This may be because too many links are made between the antibody and the gel altering the antibody specificity. On the other hand too few covalent links to the antibody may result in increased losses of antibody from the column and subsequent contamination of immunopurified enzyme. Recovery of antibody activity is better at higher antibody : gel ratios, the suggested range of input is 5–30

mg/ml packed gel [1866], as one approaches 10 mg/ml gel relative capacity of the immunoaffinity matrix is considered optimal (D.A. Brooks, Department of Chemical Pathology, Women's and Children's Hospital, Adelaide, Australia; personal communication).

The capacity of a column is indicated by two observations; the depletion of the target antigen from the starting material and the recovery of purified antigen from the column. The capacity of the antibody column for its antigen may be determined by monitoring the antigen in the sample being applied to the column as well as that in the column eluate; when antigen appears in the eluate, the column is saturated. If column capacity is monitored, any deterioration of the antibody column can be detected and a new one prepared if necessary. A potential problem in the use of immunoaffinity columns is that the dissociating conditions used to desorb antigen from the column inevitably will lead to denaturation of the bound antibody, so it is desirable to minimise the time of the contact between the column and the dissociating buffer. An effective antibody column will bind about 10% of the antigen as expected from its theoretical capacity.

4.3 Results

4.3.1 Serum Screening for Anti-Amine Oxidase Antibodies

The serum screening assay for anti-amine oxidase antibodies was performed as described in section 2.9.2. PAO I was used to coat 'Plate 1'; and PAO II was used to coat 'Plate 2'. The antigen used to coat the plates had been purified through the size-exclusion HPLC step as described in Chapter 3. Mice from 'group I' had been immunized with PAO I and mice from 'group II' had been immunized with PAO II.

Table 4.1 Immune Mouse Serum Titres

Mouse	*Titre, Plate I*	*Titre, Plate 2*
group I		
1.I	7,998	1,513
2.I	10,000	2,884
3.I	31,695	6,309
4.I	21,877	<1,000
5.I	11,481	<1,000
group II		
1.II	<1,000	5,888
2.II	2,187	15,848
3.II	2,511	6,606
4.II	<1,000	7,943
5.II	<1,000	1,513

Results of the serum screening ELISA are presented in Table 4.1. Titres were arbitrarily designated as the serum dilution that would produce a response of 0.5 absorbance units at λ_{405} in the assay. The titres were calculated using computer software for nonlinear curve fitting to assay responses to serial dilutions of serum from 1/1000 to 1/64,000 (IMI Graph Pack II, version F, curve fitter ©P.K. Warme, Interactive Microware, Inc., State College, PA).

4.3.2 Hybridization Frequency

Colony growth was found at a frequency of around 4 clones per well in 4 × 24-well cluster plates. The hybridization frequency was therefore around 380/1.2 × 10^7 myeloma cells and 11.3 × 10^7 immune spleen cells.

4.3.3 Hybridoma Screening Assays

Histograms presenting the results of the hybridoma screening assays are shown in Figures 4.5 and 6.

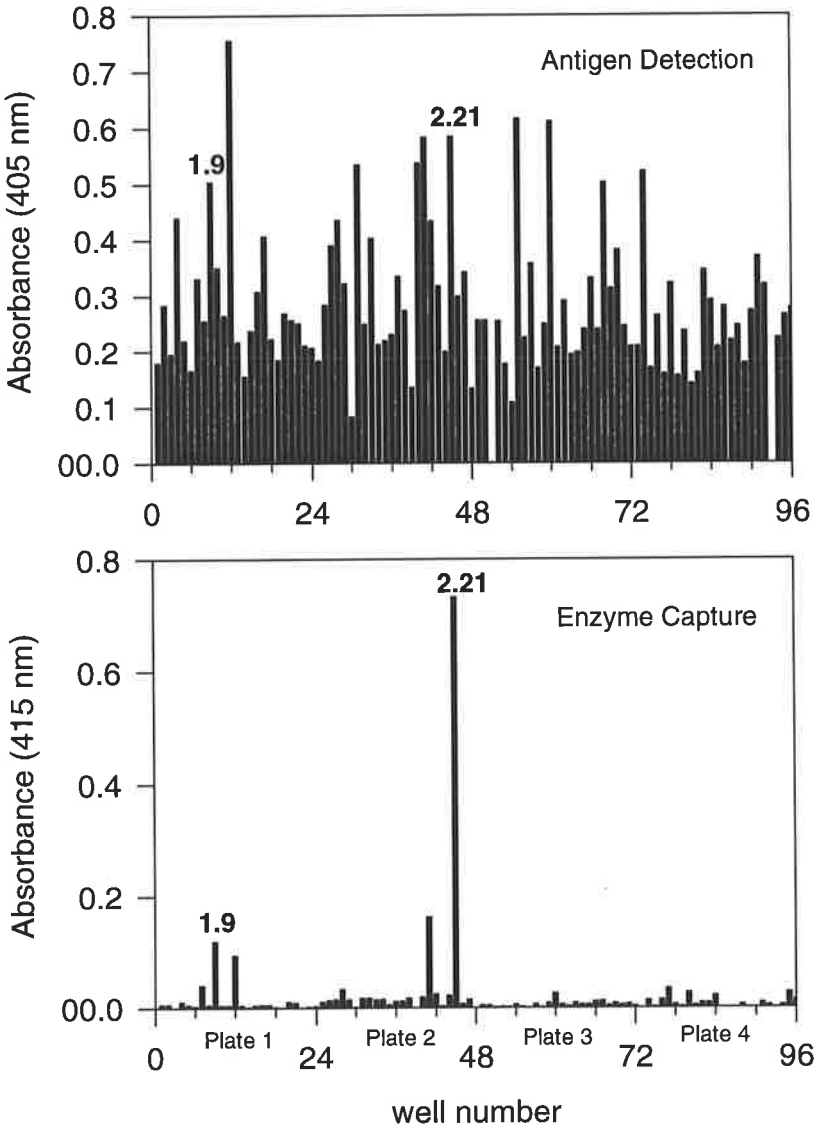


FIGURE 4.5. Hybridoma Screen 1 Results. The upper panel shows the results from the single-site indirect ELISA for anti-amine oxidase mAbs (*q.v.* Figure 4.3) and the lower panel results from the enzyme capture immunosorbent assay (*q.v.* Figure 4.4). Well numbers are indicated on the X-axis and correspond to samples from the 24-well cluster plates. Absorbances, corrected for control readings are indicated on the Y-axis.

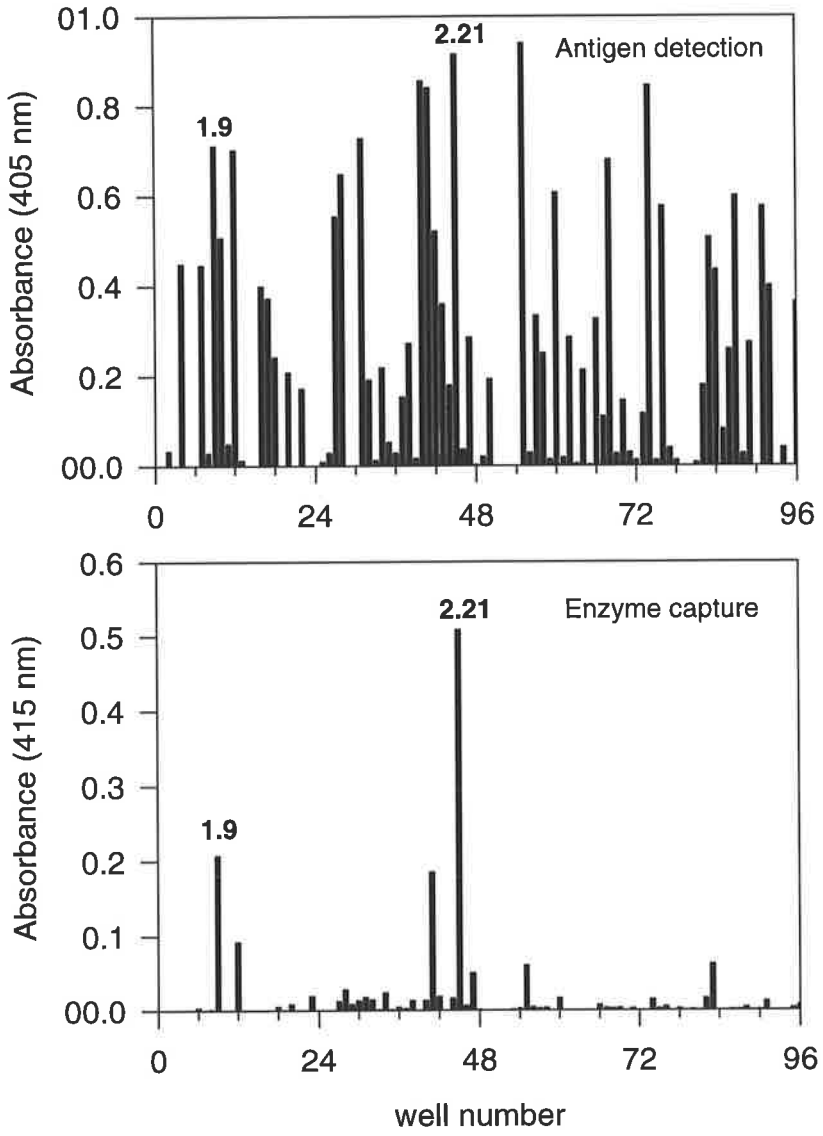


FIGURE 4.6. Hybridoma Screen 2 Results
As for Figure 4.5.

Some hybridomas yielded supernatants that gave positive reactions in both screening assays, 28 wells containing these hybridomas were selected for cloning. Some hybridoma supernatants were strongly positive in both assays in first and second screens, namely from wells 9 and 12 of plate 1 and wells 17 and 21 from plate 2. Cloning effort was concentrated on the hybridomas from these wells.

The mAb nomenclature derives from the plate/well origin of the clones (*ergo* pao-1.9, etc.).

4.3.4 Hybridoma Cloning and mAb Isotyping

The pedigree of the hybridoma cell lines is illustrated in Figure 4.7. At each cloning step positive hybrids were cryopreserved using procedures described in section §2.8.4. Monoclonal antibodies secreted by the positive hybrids were isotyped using a two-site ELISA described in §2.9.4. Isotyping results are shown in Table 4.2

Hybrid	*clone 1*	*clone 2*	*clone 3*
Plate 1			
1.4			
1.9			
	1.9A1		
		1.9A1B3	
			1.9A1B3A5
			1.9A1B3B4
			1.9A1B3C3
			1.9A1B3C5
		1.9A1B4	
		1.9A1B6	
	1.9C2		
1.12			
	1.12A1		
1.17			
Plate 2			
2.4			
	2.4A4		
	2.4D1		
2.9			
2.7			
	2.7D3		
2.10			
2.13			
	2.13B1		
2.16			
2.17			
2.18			
2.21			
	2.21B1		
		2.21B1C2	
			2.21B1C2A3
			2.21B1C2B5
		2.21B1C4	
			2.21B1C4A6
		2.21B1B6	
			2.21B1B6B5
Plate 3			
3.7			
3.12			
3.17			
3.19			
3.20			
3.21			
Plate 4			
4.2			
	4.2B3		
4.5			
	4.5A5		
4.11			
4.14			
4.15			
4.16			
4.19			
	4.19B2		
	4.19D5.1		
	4.19D5.2		
4.20			
4.23			
	4.23C2		
	4.23D2		

FIGURE 4.7. Hybridoma Cloning Pedigree.
The clone suffix nomenclature derives from the position of the clone on the cloning plates which were 24-well cluster plates with a 4 × 6 well matrix (*ergo* A1 for the first well in the first row, etc.).

TABLE 4.2. Monoclonal Antibody Isotypes[a]

mAb	*Subclass*
1.9	IgG_1
2.4	IgG_1
2.13	IgM
2.21	IgG_1
4.2	IgM

a. All antibodies had a κ- light chain isotype.

4.3.5 Bulk Culture of Hybridomas

A few of the cloned hybridoma cell lines identified as potentially useful for immunoaffinity purification were expanded into bulk culture. Namely 1.9A1B3, 1.9A1B3A5, 1.9A1B3C5; producing mAb 'pao-1.9' and 2.21B1B6B5; producing mAb 'pao-2.21', which had screened positive in both antigen detection and enzyme capture assays. These cultures were maintained in HT medium containing 5% ΔFBS at between 1×10^5 and 1×10^6 viable cells per ml. The reduced concentration of FBS: 'stresses' the cells inducing them to produce more antibody; economises on the use of FBS, which is an expensive component of the medium; and simplifies downstream processing by reducing the total protein concentration of the supernatant. Up to 50 × ~50 ml cultures were maintained at one time providing ~2000 ml conditioned medium per culture split. Tissue culture supernatants were harvested by centrifugation at 8000 rpm in a Beckman JB20B centrifuge using a J14 rotor fitted with 250 ml centrifuge tubes. Hybridoma supernatants were buffered by the addition of 50 ml 1 M Tris (pH 8.0) per 2000 ml, titrated with 3–5 ml 1 N HCl if necessary. Buffered supernatant was aliquoted (usually as 50 ml aliquots) and stored at –20 °C. Culture supernatants were routinely monitored for mouse mAb concentration by ELISA as described in §2.9.5.

Conditioned culture medium mouse monoclonal antibody concentrations were found to be in the range 2.5 – 50 μg/ml.

4.3.6 Immunoaffinity Purification of Monoclonal Antibodies to PAOs

4.3.6.1 Construction of an Anti-Mouse IgG Column for mAb pao-1.19 Purification

Columns for immunoaffinity purification of the monoclonal antibody were prepared as follows. Sheep anti-mouse IgG (30 mg specific antibody, 435 mg protein) was transferred into 100 mM HEPES, pH 7.5 by gel filtration on Sephadex G-25 F (21 cm × 2.6 cmØ) and coupled to 50 ml Affi-Prep 10 according to the manufacturer's instructions [1896]. Affi-Prep 10 matrix was washed with at least 50 volumes (2500 ml) of cold 10 mM sodium acetate, pH 4.5. Antibody solution in 100 mM HEPES, pH 7.5 was incubated with the Affi-Prep on a rocking platform, or roller, and

the reaction was allowed to continue overnight. Remaining active esters were blocked with 0.1 ml 1 M ethanolamine–HCl, pH 8.0, per ml of matrix. The coupled matrix was packed into a column (9.4 cm × 2.6 cmØ) and washed until a stable A_{280} baseline was obtained from the column eluate. Similarly 300 mg bovine γ-globulin was coupled to 42 ml Affi-Prep 10, for use as a guard column (8 cm × 2.6 cmØ). The columns were conditioned by precycling with 1 M ammonia, pH 11.5, followed by equilibration buffer (150 mM NaCl in 10 mM Tris–HCl, pH 8.0) to remove any loosely bound antibody ligand, thus avoiding its release into the monoclonal preparation.

4.3.6.2 Preparation of mAb pao-1.9 for Immunoaffinity Purification of PAO

Monoclonal Ab pao-1.9, of IgG_1 subclass and positive in both screening assays, was purified from bulk culture hybridoma supernatants by immunoaffinity purification (Table 4.3).

TABLE 4.3. Purification of Monoclonal Antibody pao-1.9

Purification step	Volume *ml*	Protein *mg*	Mouse IgG_1 *mg*	Specific Activity *mg IgG/mg protein*	Purification *-fold*	Yield *%*	Bovine IgG *mg*	Sheep IgG *mg*
Affinity column load	760	2020.84	22.85	0.0113	1	100	4.605	–
Concentrated pooled fractions	6	13.51	12.25	0.9067	80	54	<0.006	0.419
G25F pool	30	10.10	8.37	0.82	73	37	–	–
S-300 load	12.5	47.95	43.88	0.92	1	100	–	–
mAb pao-1.9 preparation	35	38.73	38.37	0.99	1.08[a.]	87[a.]	–	–

Notes to Table 4.3
a. from the size exclusion chromatography load.

Typically, supernatant from the cloned hybridoma line (pH 8.0 [4 °C]) was concentrated over a YM 30 membrane in an Amicon 2000 series stirred cell (Amicon Inc., Danvers, MA) by pressure dialysis. The concentrated culture supernatant was clarified by centrifugation followed by filtration to 0.45 μm, and loaded onto a sheep anti-mouse IgG—Affi-Prep 10 column (*q.v.* §4.3.6.1) arranged in tandem with a preceding bovine γ-globulin—Affi-Prep 10 column, which acts as a trap for proteins and lipids that may bind to the antibody column nonspecifically (Figure 4.8). After loading the culture supernatant, the columns were eluted with at least 1000 ml equilibration buffer. Subsequently the columns were uncoupled and 1 M ammonia, pH 11.5, was used to desorb the bound antibodies from the anti-mouse IgG affinity column (Figure 4.9). The antibody containing eluate was concentrated by ultrafiltration over a YM 30 membrane and neutralized with 1 M HCl. Hybridoma supernatants usually contained between 2.5 and 50 mg mouse IgG_1 per litre, as measured by ELISA, and approximately 10 mg of antibody was purified

per cycle. Column eluates containing monoclonal antibodies that had not bound were recycled on the affinity column until antibodies could no longer be detected in the eluate.

The affinity purified monoclonal antibody preparations were pooled, concentrated over a YM 30 membrane and transferred into 100 mM HEPES, pH 7.4 by gel filtration on Sephadex G-25 F (21 cm × 2.6 cmØ), the absorbance of the eluate was monitored at 280 nm and the antibody concentration by ELISA. The monoclonal antibody preparation was assayed for protein concentration. Mouse, sheep and bovine IgG concentrations were assayed using specific ELISAs. Bovine immunoglobulin was <0.2% by ELISA (*but* 3 mg/litre or approx. 30% total IgG in hybridoma supernatants) and sheep immunoglobulin <1%. Purity was checked by chromatography on BioSil TSK 3000 sw and finally assessed by SDS-PAGE under reducing conditions followed by Coomassie Blue R or silver staining. Monoclonal antibodies obtained by this method were at least 95% pure as assessed by scanning densitometry. These preparations were used to construct pilot columns for the immunoaffinity purification of human retroplacental PAO as described in the next section.

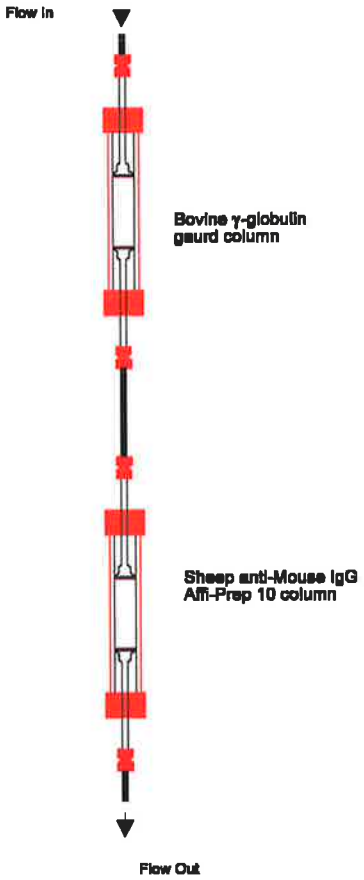


FIGURE 4.8. Tandem Arrangement of Bovine γ-Globulin and Sheep Anti-Mouse Columns

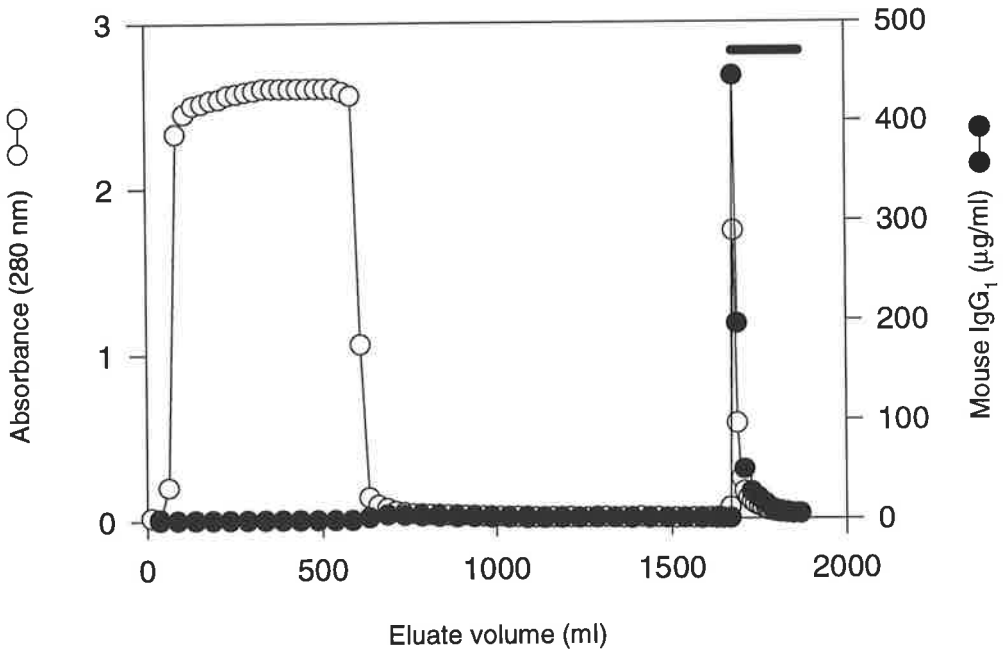


FIGURE 4.9. Affinity Chromatography of mAb pao-1.9 on Anti-Mouse—Affi-Prep 10. Concentrated clarified culture supernatant (500 ml) was loaded onto a sheep anti-mouse IgG—Affi-Prep 10 column (9.4 cm × 2.6 cmØ) arranged in tandem with a bovine γ-globulin—Affi-Prep 10 column (8 cm × 2.6 cmØ) (Figure 4.8) equilibrated with 150 mM NaCl in 25 mM Tris–HCl, pH 8.0. Columns were loaded at 25.0 ml/h collecting 25.0 ml fractions. After loading the culture supernatant, the columns were eluted with at least 1000 ml equilibration buffer. When the A_{280} of the eluate had fallen to baseline, the columns were uncoupled, the fraction volume changed to 10.0 ml, and 1 M ammonia, pH 11.5 used to desorb the bound antibodies from the affinity column. Fractions were pooled as indicated by the bar.

4.3.6.3 Construction of mAb pao-1.9 Columns for Immunoaffinity Purification of PAO

Affinity purified monoclonal antibodies were coupled to Affi-Gel 10 or Affi-Prep 10 according to the manufacturer's instructions. A pilot study, using mAb pao-1.9 bound to 2 ml Affi-Gel 10 at approximately 4 mg/ml gel showed that both forms of PAO could be bound by the column and desorbed using NaSCN with the retention of enzyme activity (data not shown).

Pooled pao-1.9 antibodies from later immunoaffinity preparations were concentrated (48 mg protein) and purified on a Sephacryl S-300 column, which also transferred the antibodies into 100 mM HEPES, pH 7.5 (Figure 4.10). Antibody containing fractions were pooled yielding a final preparation of 39 mg pao-1.9, better than 97% pure as indicated by silver-stained SDS-PAGE under reducing conditions (Figure 4.11) quantitated by scanning laser densitometry, with <1% sheep and <0.3% bovine immunoglobulins by a quantitative specific ELISA. This antibody was coupled to 8 ml of Affi-Prep 10 overnight. The coupling was shown to be >99% efficient by BCA protein determination indicating a concentration of 4.84 mg/ml Affi-Prep 10. Unreacted sites were blocked with ethanolamine at pH 8.0.

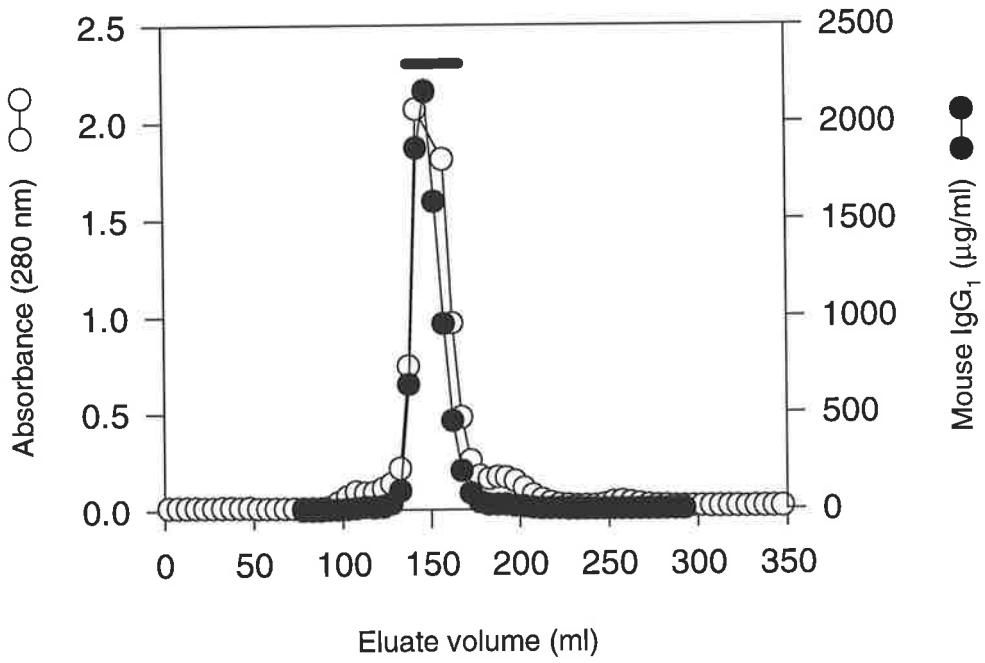


FIGURE 4.10. Size Exclusion Chromatography of mAb pao-1.9 on Sephacryl S-300
Pooled monoclonal antibody preparations were concentrated to 15 ml over a YM 30 membrane and loaded onto a Sephacryl S-300 superfine column (45 cm × 2.6 cmØ) run at 15 ml/h collecting 5.0 ml fractions. The column was equilibrated and eluted with 100 mM HEPES, pH 7.5. Fractions were pooled as indicated by the bar.

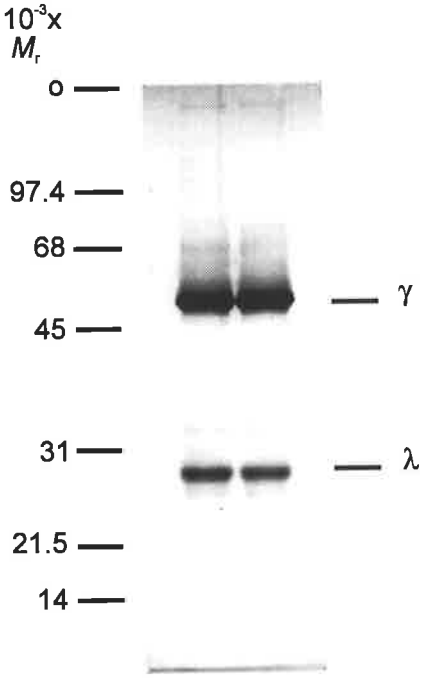


FIGURE 4.11. SDS-PAGE of mAb pao-1.19 under Reducing Conditions in 10% PA. Both tracks 6.64 µg. The heavy (γ) and light (λ) chains of the immunoglobulin were observed after silver staining.

4.3.6.4 Construction of an Anti-Mouse IgG Column for mAb pao-2.21 Purification

To improve the efficiency of mAb purification a new anti-mouse immunoaffinity column was constructed for mAb 2.21 purification. Sheep anti-mouse IgG (404 mg protein) was purified and transferred into 100 mM HEPES, pH 7.5 by gel filtration on a Sephacryl S-300 superfine column (Figure 4.12). The yield, 378 mg sheep anti-mouse IgG immunoglobulin protein, was coupled to

28 ml Affi-Prep 10 at 13.5 mg/ml gel. The coupled Affi-Prep was pooled with the 50 ml coupled Affi-Prep 10 used for the 1.9 mAb purification. A total of 10.16 mg protein/ml gel packed as a 15 cm × 2.6 cmØ column containing approximately 55 mg specific antibody. Again, the sheep anti-mouse IgG column was arranged in tandem with the bovine γ-globulin column that acts as a trap for nonspecifically binding proteins and lipids (Figure 4.8).

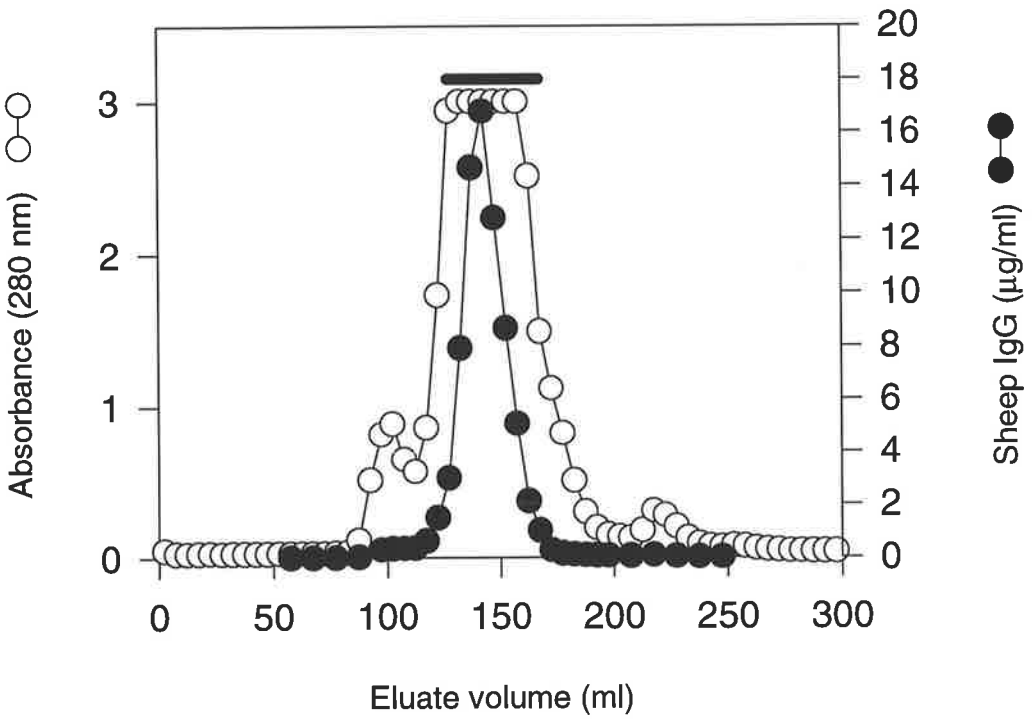


FIGURE 4.12. Sheep Anti-Mouse IgG Chromatography on Sephacryl S-300 Superfine
Sheep anti-mouse IgG load was 404 mg on a 45 cm × 2.6 cmØ column run at 15 ml/h collecting 5.0 ml fractions. The column was equilibrated and eluted with 100 mM HEPES, pH 7.5. Protein concentration was monitored by A_{280} and sheep immunoglobulin by specific ELISA.

4.3.6.5 Preparation of mAb pao-2.21 for Immunoaffinity Purification of PAO

Monoclonal antibody pao-2.21 was immunoaffinity purified from hybridoma supernatants on a column of sheep anti-mouse IgG coupled to Affi-Prep 10 (*q.v.* §4.3.6.4) as previously described for mAb pao-1.19 (*q.v.* §4.3.6.2). Typically, 2000 ml of hybridoma supernatant was neutralized with 3–5 ml 1 M HCl and concentrated by ultrafiltration over a 150 mm diameter YM 30 membrane in a 2.5 l stirred cell under pressurized high purity nitrogen to approx. 500 ml. The concentrated supernatant was filtered to 0.45 µm and loaded onto the sheep anti-mouse column arranged in tandem with a bovine γ-globulin guard column equilibrated with 150 mM NaCl in 25 mM Tris–HCl, pH 7.4. After loading, the columns were eluted with equilibration buffer to wash unbound protein from the columns. When the A_{280} of the eluate had fallen to baseline, the bovine γ-globulin guard column was uncoupled and bound antibodies were desorbed from the affinity column with 1 M ammonia, pH 11.5. The antibody preparation was neutralized with 1 N HCl and

concentrated over a 62 mm YM 30 membrane in a 200 ml stirred cell (Amicon 8000 Series). A typical round of mAb purification as described is summarized in Table 4.4. Eluate containing unbound antibodies was pooled and recycled with next batch of hybridoma supernatants (Figure 4.13). *A total of 194 mg purified pao-2.21 mAb was obtained from about 20 litres of hybridoma supernatant.*

TABLE 4.4. Purification of Monoclonal Antibody pao-2.21[a]

Purification step	Volume *ml*	Protein *mg*	Mouse IgG_1 *mg*	Specific Activity *mg IgG/mg protein*	Purification *-fold*	Yield *%*	Bovine IgG *mg*	Sheep IgG *mg*
Concentrated pooled supernatant	500	3600	13.5	0.0038	1	100	3.298	0.107
mAb pao-2.21	5.5	11.6	11.2	0.97	254	82	0.004	0.187

Notes to Table 4.4
a. typical

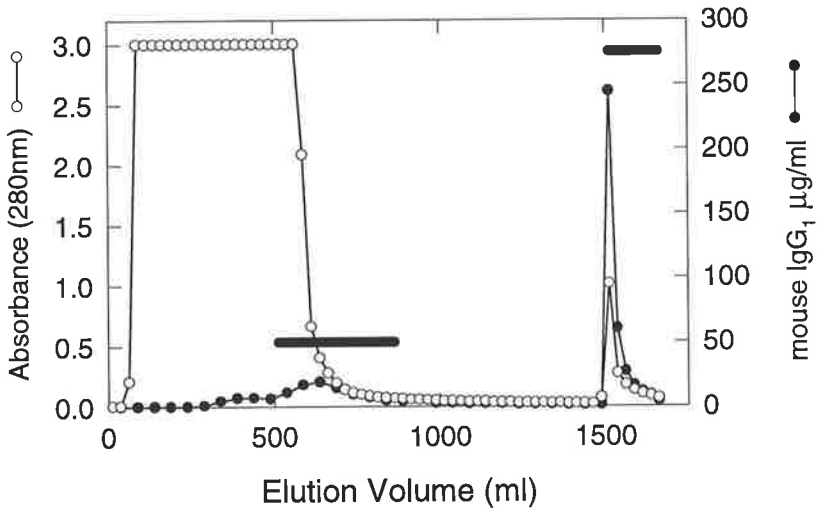


FIGURE 4.13. Affinity Chromatography of mAb pao-2.21 on Anti-Mouse—Affi-Prep 10.
Concentrated culture supernatant was filtered to 0.45 µm and loaded (500 ml) onto the sheep anti-mouse column (15 cm × 2.6 cmØ) arranged in tandem with a bovine γ-globulin guard column (8 cm × 2.6 cmØ) equilibrated with 150 mM NaCl in 25 mM Tris–HCl, pH 7.4. Columns were loaded at 25.0 ml/h collecting 25.0 ml fractions. After loading, the columns were eluted with equilibration buffer. When the A_{280} of the eluate had fallen to baseline, the bovine γ-globulin guard column was uncoupled, the volume of the fractions changed to 10.0 ml, and bound antibodies were desorbed from the affinity column with 1 M ammonia, pH 11.5. Fractions were pooled as indicated by the bars, the eluate 'breakthrough' fraction was recycled as described in the text.

Recovery of antibody activity in each preparation was monitored using the immunocapture ELISA (*q.v.* Figure 4.4) as described for the screening of hybridoma supernatants. Titre was defined as the dilution at which the maximum positive signal strength for a particular antibody was reduced by half.

The curve fitting algorithm used was:

$r = A + (D - A) / [(1 + (conc / C)^{B}]^{m}$

where

A = estimated blank

D = estimated reference (max absorbance)

B = slope factor

C = inflexion point

M = symmetry factor

r = response (y axis)

conc = concentration (x axis)

So $y = a + (d - a) / (1 + (x/c)^{B})^{M}$

to determine titre we needed to solve for x, where $y = ((d - a) / 2 + a)$

$$\frac{(y - a)}{(d - a)} = \frac{1}{\left(1 + \left(\frac{x}{c}\right)^{B}\right)^{M}}$$

rearranging

$$\left(\frac{d - a}{y - a}\right) = \left(1 + \left(\frac{x}{c}\right)^{B}\right)^{M}$$

and

$$\left(\frac{d - a}{y - a}\right)^{(1/M)} = \left(1 + \left(\frac{x}{c}\right)^{B}\right)$$

and

$$\left[\left(\frac{d - a}{y - a}\right)^{(1/M)} - 1\right]^{(1/B)} .c = x$$

so

$$f(y) = \left(\left(\left((d - a)/(y - a)\right)^{(1/M)} - 1\right)^{(1/B)}\right) * c$$

$$x = {}^{1}/_{f(y)}$$

Specific titre of the monoclonal antibody in the culture supernatants and in their corresponding purified monoclonal antibody preparations were determined to control for loss of antibody activity on purification. Specific activity was regarded as the titre divided by the monoclonal antibody concentration (determined by ELISA). *The specific titres of the purified monoclonal antibodies were of the order of 10,000/mg and indicated that, on average, greater than 83% of antibody activity was retained during its immunoaffinity purification.* Purified monoclonal

antibody protein concentrations were determined using the BCA protein method with purified bovine γ-globulin as the protein standard. Purity was assessed by SDS-PAGE (Figure 4.14). Monoclonal antibody preparations of the highest specific activity and purity were pooled (163 mg in 44 ml), concentrated over a YM 30 membrane to 12 ml; further purified and transferred into 100 mM HEPES, pH 7.5 by gel filtration on Sephacryl S 300 (Figure 4.15). Peak fractions were pooled and protein concentration of the monoclonal antibody preparation determined. Mouse, sheep and bovine IgG concentrations were assayed by specific ELISAs. Bovine immunoglobulin was <0.1% by ELISA and sheep immunoglobulin <1%. Purity was assessed by SDS-PAGE under reducing conditions (Figure 4.16).

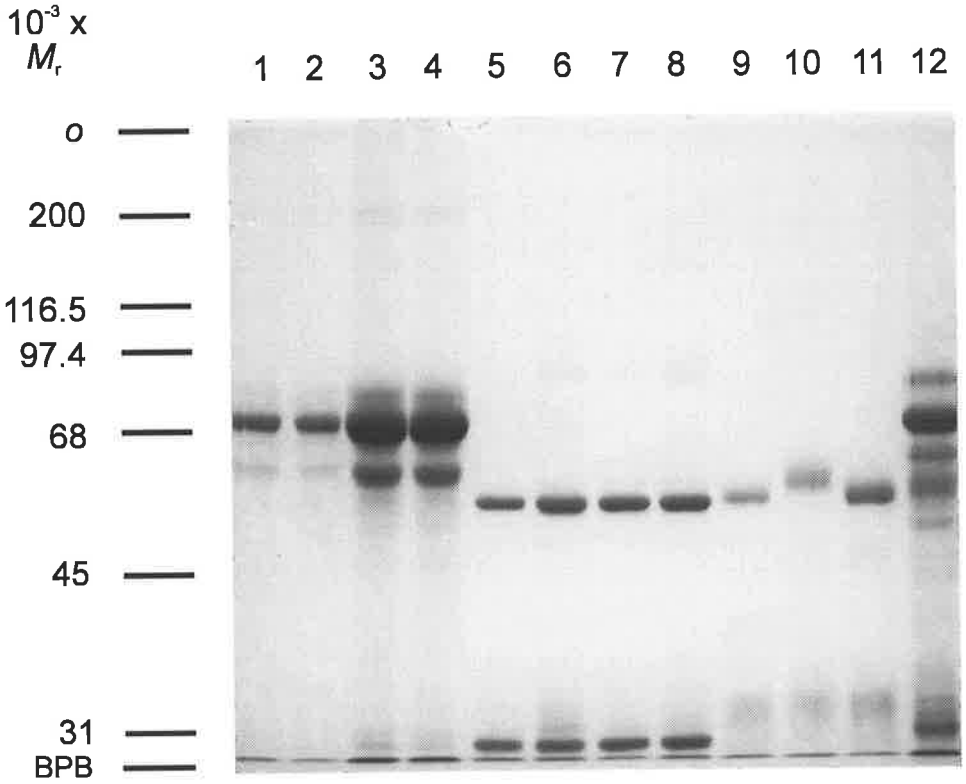


FIGURE 4.14. SDS-PAGE of mAb pao-2.21 Preparations, Hybridoma Supernatants and Antibodies on a 7.5% PA Gel Under Reducing Conditions. Lanes 1 and 2, hybridoma 2.21 supernatant; lane 3, concentrated hybridoma supernatant; lane 4, concentrated supernatant column eluate (*sans* mAb); lanes 5,6,7,8, mAb pao-2.21 preparations; lane 9, sheep IgG; lane 11, sheep anti-mouse IgG; lane 12, Meloy mouse myeloma IgG_1 myeloma standard. Coomassie Blue R staining. o, origin; BPB, bromophenol blue tracker.

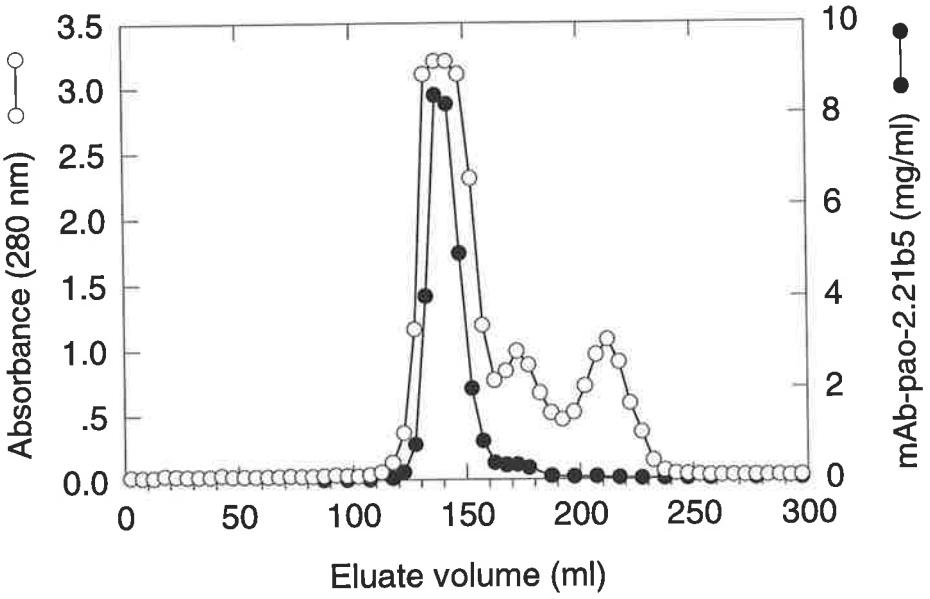


FIGURE 4.15. Size Exclusion Chromatography of mAb pao-2.21 Pool on Sephacryl S-300
Monoclonal antibody load was 163 mg in 12 ml on a 45 cm × 2.6 cmØ column run at 15 ml/h collecting 5.0 ml fractions. The column was equilibrated and eluted with 100 mM HEPES, pH 7.5. Protein concentration was monitored at A_{280} and mAb concentration by specific ELISA.

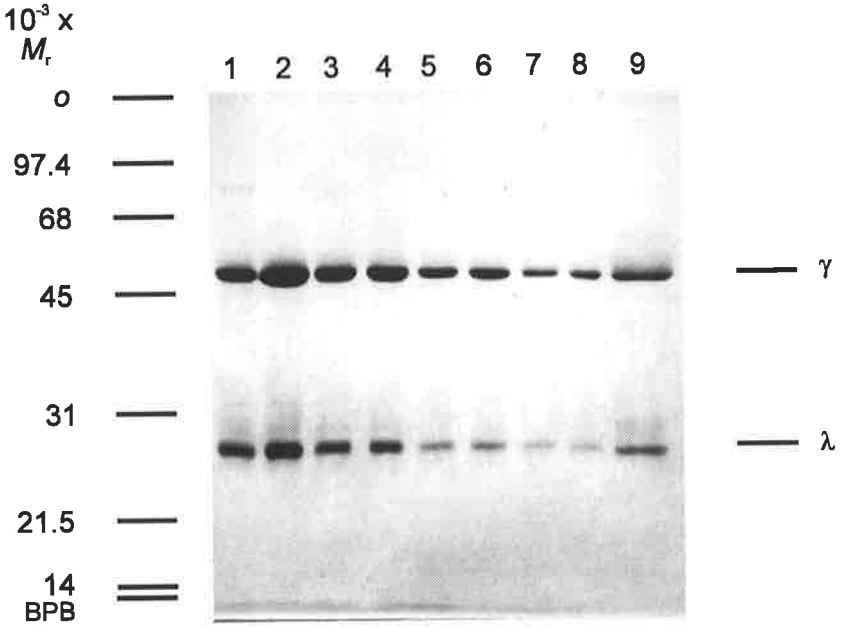


FIGURE 4.16. SDS-PAGE of Monoclonal Antibody pao-2.21 Preparation S-300 Fractions.
Lane 1, column load (10 µg); lane 2, purified mAb (20 µg); lanes 3,4 (10 µg); lanes 5,6 (5 µg); lanes 7,8 (2 µg); lane 9, peak side fractions 26,32 and 33 (10 µg). Coomassie Blue R staining. o, origin; BPB, bromophenol blue tracker.

4.3.6.6 Construction of a mAb pao-2.21 Column for Immunoaffinity Purification of PAO

Affinity purified antibodies were coupled to 8 ml of Affi-Gel 10 at 9.86 mg/ml gel according to the manufacturer's instructions, as previously described (overall coupling efficiency 98%). In this case unreacted active groups were not blocked with ethanolamine but allowed to hydrolyse spontaneously or react with Tris wash buffer.

4.3.6.7 Construction of a Bovine γ-Globulin Guard Column for Enzyme Purification

Bovine γ-globulin was purified and transferred into 100 mM HEPES, pH 7.5 by size exclusion chromatography on a Sephacryl S-300 column (Figure 4.17). Peak fractions were pooled (49.35 mg protein in 25 ml) and bovine γ-globulin coupled to 10 ml Affi-Gel 10 according to the manufacturer's instructions (coupling efficiency >98%). This column was used as a guard column for the mAb 2.21 column during enzyme purification.

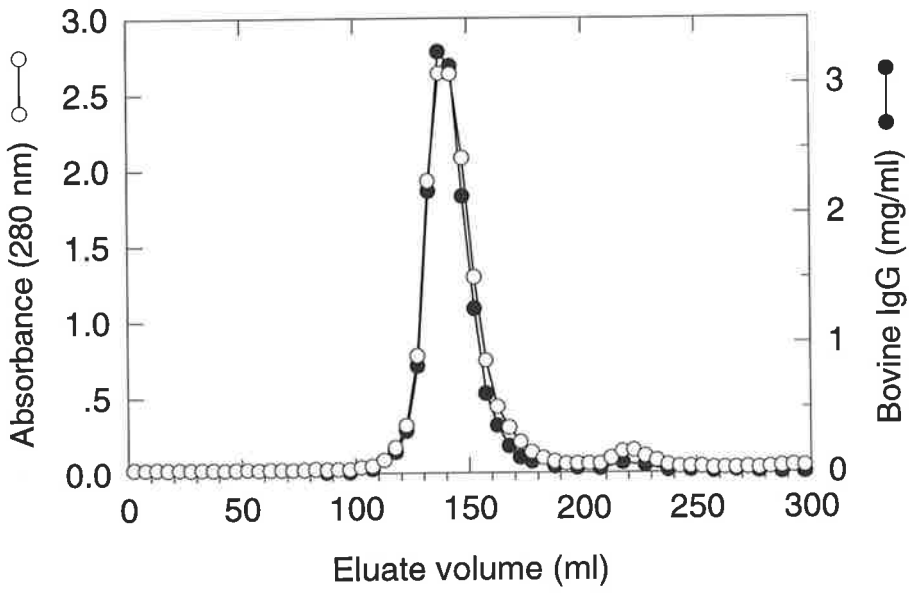


FIGURE 4.17. Size Exclusion Chromatography of Bovine γ-Globulin on Sephacryl S-300
Bovine γ-globulin load was 59.5 mg in 3.5 ml on a 45 cm × 2.6 cmØ column equilibrated and eluted with 100 mM HEPES, pH 7.5 at 15 ml/h collecting 5.0 ml fractions. Protein concentration was monitored at A_{280} and bovine immunoglobulin by specific ELISA.

4.3.7 Immunoblotting

The immunoblotting method was described in section 2.12.

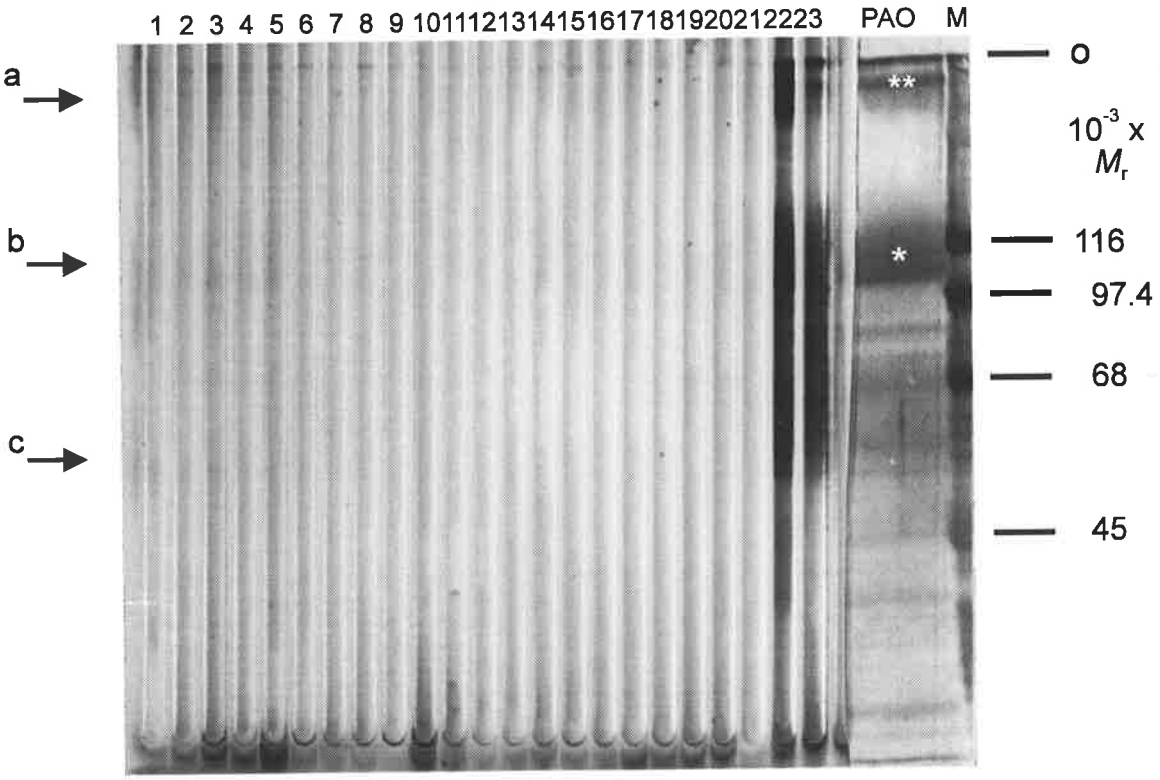


FIGURE 4.18. Immunoprobed Western Blot of Immunoaffinity Purified Polyamine Oxidase. Monoclonal antibodies (lanes 2–20, indicated by the numbers along the top of the nitrocellulose membrane) were raised against partially purified PAO in mice. Polyclonal serum from immune mice was also used on the blot (lanes 22 and 23). Negative controls of RPMI 1640 culture medium with 10% FCS were used in lanes 1 and 20. The unprobed section of the Western blot containing the reference lane of molecular weight markers (M) and a section of the wide comb lane of immunoaffinity purified polyamine oxidase sample were stained with india ink. The start of the resolving gel is indicated by **o**. Molecular weight markers indicated are as follows; β-galactosidase, 116,000; phosphorylase b, 97,000; bovine serum albumin, 68,000; ovalbumin, 45,000. The single asterisk indicates the position of the PAO subunit and the double asterisks indicate the position of the enzyme dimer. Faint staining of the subunit and dimer by the monoclonal antibodies can be seen at the M_r positions indicated by the arrows labelled **a** and **b**. Faint staining is also observed at the M_r position indicated by the arrow labeled **c**; the staining around this region is probably of partially processed or degraded enzyme forms. The antibodies used in each lane are indicated Table 4.5.

TABLE 4.5. Legend to the Immunoprobed Western Blot of Immunoaffinity Purified PAO.

Lane	*Sample*	*Lane*	*Sample*	*Lane*	*Sample*
1	RPMI 1640/FCS	9	2.17	17	2.21B1B6B6
2	1.9A1	10	4.2B3	18	2.21B1C2B5
3	1.9A1B6	11	4.5A5	19	2.21B1C2A3
4	1.9A1C2	12	4.19B2	20	2.21B1C2A6
5	1.9 purified	13	4.23C2	21	complete RPMI 1640/ΔFCS[a]
6	2.4	14	4.23D2	22	immune mouse 3.II serum 1/500
7	2.4D1	15	2.21	23	immune mouse 5.II serum 1/500
8	2.4A4	16	2.13		

Notes to Table 4.5.
The alpha–numeric codes refer to cloned monoclonal antibody producing hybridoma culture supernatants (*q.v.* Figure 4.7). The immune mice sera were diluted in complete RPMI 1640 medium containing 10% ΔFBS[a].

4.4 Discussion

4.4.1 Serum Screening for Anti-Amine Oxidase Antibodies

Serum screening assays indicated that the mice responded to immunization with what is a potentially immunosuppressive enzyme in its native form (*q.v.* §1.5.4). The variability of the serum titres illustrate the idiosyncratic responses of the mice to immunization. The results indicated that the group of mice immunized with PAO I produced serum antibodies that recognized PAO II and *vice versa* suggesting that the major antigenic determinants of the multiple enzyme forms were very similar or identical. Since serum titre relates strongly to the number of immunoglobulin producing cells, a mouse with high titre was selected for hybridoma production. As a single-site indirect ELISA method was used for polyclonal serum screening it is possible that the assay would detect antibodies to impurities common to both enzyme preparations. None of the immunized mice produced polyclonal sera that gave a strong result in the enzyme immunocapture assay, and this may have been because the proportion of serum antibodies directed to the polyamine oxidase did not produce a signal that could be discriminated from background (data not shown).

4.4.2 Hybridization Frequency

In general, hybridization of mouse myelomas with splenic lymphocytes should yield hybridization frequencies in the range 1–100 clones per 10^7 lymphocytes. Frequency of fusion will depend on the ratio of the two types of fused cells and the nature and purity of the lymphocytes used to partner the myeloma in the fusion. About 380 colonies/11.3 $\times$ 10^7 immune spleen cells were obtained from the fusion products in this study, which was within the anticipated hybridization frequency range; of these 187 produced antibodies positive in the antigen detection screening assay and 28 produced antibodies positive in the enzyme capture assay. The 28 wells containing hybridomas that produced antibodies positive in both assays were selected for cloning. Ten of these wells produced cloned hybridomas; two cell lines producing antibodies strongly positive in both screening assays were recloned twice and expanded into bulk culture.

4.4.3 Immunoaffinity Purification of the Monoclonal Antibodies

One of the aims of monoclonal antibody production to polyamine oxidase was to generate antibodies suitable for the construction of immunoaffinity columns with which to further purify the enzyme. Because of the difficulties associated with the removal of non-specific antibodies from the same species, which may reduce the selectivity of the immunoaffinity columns (*q.v.* §4.2.5), monoclonal antibodies to the polyamine oxidase forms were produced by cell culture.

Monoclonal antibodies were purified from hybridoma culture supernatants using specific immunoaffinity techniques to avoid their contamination with xenotypic antibodies and other proteins introduced through medium supplements (*q.v.* §4.2.5).

Monoclonal antibodies were produced in good yields in culture supernatants at approx. 2.5 to 50 µg/ml. The lower concentrations of mAbs may reflect isotypic or idiotypic variation in the immunoglobulin used as the ELISA reference standard and actually be an underestimate of the true concentration of antibody in the culture supernatant (*qq.v.* §2.9.5 and Figure 4.14, lane 12). The mAbs were obtained in high purity after immunoaffinity purification (>95%), as indicated by SDS-PAGE and size exclusion chromatography. The yields of mAbs from the immunoaffinity columns were good and a high degree of antibody specific activity was retained. More than 99.8% of bovine IgG was removed during immunoaffinity purification of the mAbs and the level of sheep IgG detected in the final preparations was very low, indicating minimal 'bleeding' of sheep anti-mouse IgG from the immunoaffinity columns. That sheep IgG was apparently detected at low levels in concentrated culture supernatants before processing suggests that the immunoglobulin detected may mostly be a result of crossreactivity in the ELISAs. The monoclonal antibodies were produced in quantities sufficient for the large scale (in the order of 10 mg quantities per cycle based on a 10% column efficiency) immunoaffinity purification of human retroplacental polyamine oxidase.

4.4.4 Immunoblotting

Immunoblotting was used in an attempt to identify monoclonal antibodies recognizing Western blotted retroplacental polyamine oxidase. If identified, these monoclonal antibodies could be used to locate and identify the enzyme in SDS-PAGE. Furthermore, they would be good candidates for use in immunohistochemical studies.

However, immunodetection of the antigen on Western blots by the monoclonal antibodies was not very definitive or sensitive. This may be due to a number of reasons, including that: (i) the monoclonal antibodies were of very low affinity. However, this does not appear to be true when the antibodies are used in affinity chromatography or ELISA. (ii) The quantity of antigen on the nitrocellulose was too small. However, silver staining of the gel after protein transfer and india ink protein staining of a strip-section of the membrane indicated that the protein had transferred successfully. (iii) The enzyme antigen had been denatured by SDS and 2-mercaptoethanol and that the determinants depend on its native conformation, which was not restored during transfer to the nitrocellulose, i.e. it is likely that the mAbs were directed against conformational, and not linear determinants. This is not particularly surprising since the mAbs were screened for recognition of native active enzyme. A degree of renaturation probably occurs

during the transfer, and subsequent washing and blocking, so monoclonal antibody may still bind accounting for the faint staining seen on Figure 4.18. For a monoclonal antibody there are likely to be only one or just a few determinants per enzyme molecule (*q.v.* § 4.2.3.1). In contrast, the mouse polyclonal antiserum will contain antibodies recognizing many different determinants, including conformation-independent (linear) and denaturation resistant determinants, increasing the density of the signal. Since the polyclonal sera were raised against partially purified polyamine oxidase, they were likely to contain antibodies that recognizing the impurities, which may be responsible for the widespread staining seen in lanes 22 and 23 in Figure 4.18.

Although Houen *et al.* have produced monoclonal antibodies to bovine serum amine oxidase that react with Western blotted bovine serum amine oxidase immunoaffinity purified using a mAb to the same enzyme; their mAbs to purified human pregnancy serum and human placental amine oxidase did not cross react with the bovine serum enzyme. They do report that the mAbs reacted with 'dot blotted' purified human pregnancy serum and human placental enzymes [756]. These results reinforce the concept that only mAbs that have been produced against native enzyme will recognize native enzyme determinants.

In their study of human placental diamine oxidase, Morel *et al.* excised and electroeluted a 84,000 dalton [*sic*] peptide band, which was thought to be diamine oxidase, from a SDS-PAGE gel of their purified preparation [755]. The electroeluted protein was used to raise antisera that reacted with proteins of M_r 84,000 from purified placental protein, amniotic fluid, and supernatants recovered from fMLP stimulated neutrophils on Western blots. However, there was no definitive correlation of the M_r 84,000 protein with enzymatic activity and it may well have been an impurity in their preparation (*vide* §1.3.4.2biii).

4.4.5 mAb Immunoaffinity Columns

Pilot studies with pao-1.9 bound to 2 ml Affi-Gel 10 at approximately 4 mg/ml gel indicated that both forms of PAO could be bound by the column and desorbed using NaSCN with the retention of enzyme activity, indicating antigenic determinant on the isoforms was invariant, as suggested by the polyclonal serum screening.

The mAb pao-1.9—Affi-Prep 10 and mAb pao-2.21—Affi-Gel 10 columns that had been constructed using affinity purified monoclonal antibodies to human retroplacental polyamine oxidase and the bovine γ-globulin guard column were used for the preparative scale immunoaffinity purification of the enzyme as described in Chapter 5.

Chapter 5

IMMUNOAFFINITY PURIFICATION AND CHARACTERIZATION OF HUMAN RETROPLACENTAL SERUM POLYAMINE OXIDASE

5.1 Introduction

Conventional affinity and biochemical techniques have presented difficulties in the preparation of highly purified polyamine oxidase forms from retroplacental serum. The purification described in Chapter 3 resulted in poor yields and contamination of the enzyme preparations with other proteins. The preparative scale immunoaffinity purification of two forms of polyamine oxidase observed retroplacental serum is described in this Chapter. A previously reported purification suggested the presence of multiple polyamine oxidase forms in retroplacental serum but failed to isolate and identify them [442]. A subsequent attempt to purify polyamine oxidase from retroplacental serum resulted in a contaminated preparation, and failed to resolve the multiple polyamine oxidase forms or identify the protein associated with enzyme activity [726]. The use of monoclonal antibodies in the immunoaffinity purification described here enabled a high degree of enzyme purity to be attained.

At the onset of research for this thesis no monoclonal antibodies had been produced to any of the polyamine oxidases. During the course of this work, Denney *et al.* [759] and later Houen *et al.* [756] reported the production of monoclonal antibodies against human placental and pregnancy serum diamine oxidases and their use in analytical scale purification of human pregnancy-associated diamine oxidase. However, as discussed in section 4.1.3.2, they did not report characterization of multiple enzyme forms or unequivocally identify the protein responsible for enzyme activity. Daniele and Quaroni reported the production of monoclonal antibodies to an intestinal diamine oxidase from Caco-2, a human colon carcinoma cell line, and their use in the small scale immunoaffinity purification of this enzyme [786].

The work described here represents the first report of production of monoclonal antibodies to the human retroplacental serum polyamine oxidase. This investigation extends the findings of Denney *et al.* and Houen *et al.* through the large scale preparative immunoaffinity purification of multiple forms of human retroplacental serum polyamine oxidase using two novel monoclonal antibodies and the identification and further characterization of the enzyme. In contrast to the previously reported studies, which used antibodies produced in ascites fluid, monoclonal

antibodies used in this study were specifically purified from cell culture supernatants, increasing the probability of correctly identifying the immunoaffinity purified target protein (*q.v.* §4.2.5).

5.2 Methods and Results

5.2.1 Purification using mAb pao-1.9—Affi-Prep 10

Anti-polyamine oxidase mAb pao-1.9 coupled to Affi-Prep 10 was used in this investigation to purify the multiple polyamine oxidase forms in an immunoaffinity step. The purification is summarized in Table 5.1. In an initial step, differential hydrophobic interaction/affinity chromatography was used (Figure 5.1) to reduce the load on the anion exchange column that was used to resolve the multiple enzyme forms (Figure 5.2). Intermediate ultrafiltration and size exclusion chromatography steps were used to concentrate and desalt the sample before anion exchange separation. After immunoaffinity chromatography (Figure 5.3), the multiple forms were applied to SE-HPLC columns arranged in tandem. Fractions from the TSK (3000 + 4000) sw columns, determined to be active using the fluorometric enzyme assay (Figure 5.4), were pooled and recycled on a BioSil SEC 400 column (Figure 5.5); peak and shoulder fractions were examined by silver stained SDS-PAGE under reducing conditions and indicated an enzyme molecular weight ≅ 108,000 (Figure 5.5), suggesting that the native enzyme probably exists as multimers of this M_r 108,000 subunit.

Table 5.1. Purification of Polyamine Oxidase Using mAb pao-1.9—Affi-Prep 10

Purification Step	Volume	Activity	Protein	Specific Activity	Purification	Yield
	ml	*units*[a.]	*mg*[b.]	*mU/mg*	*-fold*	*%*
1. Retroplacental Serum	600	22.88	30000	0.76	1	100
2. Pentyl-/ω-aminohexyl agarose	122	18.45	244	75.6	99	81
3. Sephacryl S-300	310	17.43	186	93.7	123	76
4. DEAE-Trisacryl		14.15	117	121.9	160	62
(I)	50	1.428	5.00	286	376	6
(II)	280	12.72	112	113	148	56
5. mAb 1.9—Affi-Prep 10		6.85	6.85	1481	1948	44
(I)	70	0.730	0.730	2121	2790	6
(II)	210	6.12	6.12	1406	1850	38
6. TSK (3000 + 4000)sw						
(I)	6		0.0375	–	–	–
(II)	10		0.1875	–	–	–

Notes to Table 5.1
a. One µmole/min H_2O_2 production with 10 µM putrescine as the substrate (*q.v.* §2.3.1).
b. By the method of Smith *et al.* [1606].

5.2.1.1 Preparation of Retroplacental Serum

Retroplacental serum samples (with relatively high specific activities) from 13 placentae (600 ml), collected as described in section 2.5, were pooled, diluted 1 : 1 in 25 mM Tris–HCl containing 150 mM NaCl, pH 8.0 and centrifuged at 20,000 × *g*. Supernatants were passed through a series of glass microfibre prefilters (AP 10 and AP 15) on cellulosic support discs (AP 10) followed by membrane filtration to 0.22 μm (Millipak 40/60 filter).

5.2.1.2 Affinity Chromatography on ω-Aminohexylagarose

Diluted retroplacental serum was prepared as described in section 5.2.1.1 and applied to an ω-aminohexylagarose column in tandem with a pentylagarose column as described in Figure 5.1. The columns were subsequently washed with equilibration buffer to remove unbound proteins, after which the ω-aminohexylagarose affinity column was uncoupled from the pentylagarose column. PAO activity was desorbed from the affinity column with an NaSCN gradient (Figure 5.1). Enzymatically active ω-aminohexylagarose fractions were pooled, concentrated over a YM 30 membrane and filtered to 0.45 μm.

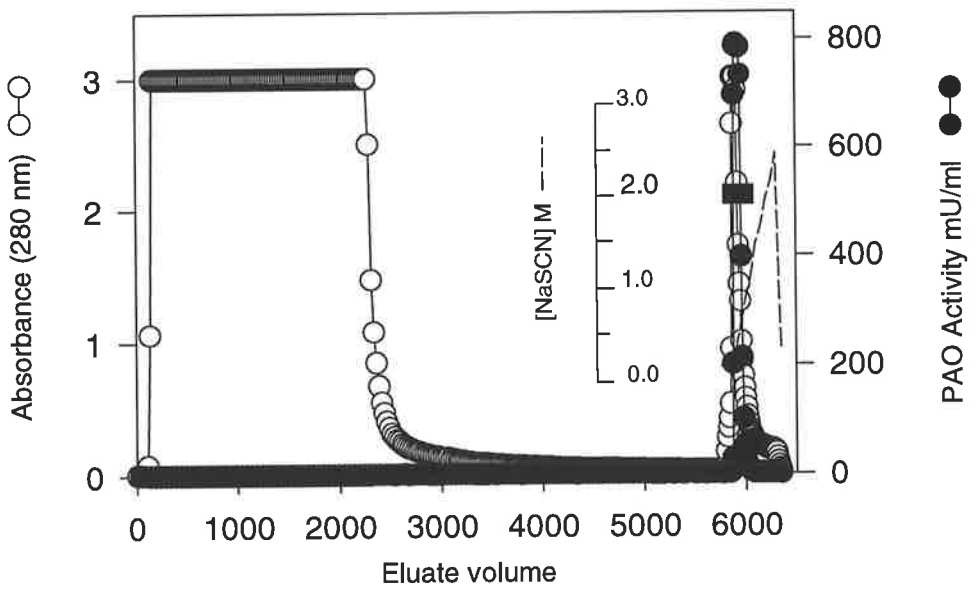


FIGURE 5.1. Affinity Chromatography of Human Retroplacental Serum on ω-aminohexylagarose. Diluted retroplacental serum was prepared as described in section 5.2.1.1 (1100 ml), and made up to 2200 ml with 150 mM NaCl in 25 mM Tris–HCl, pH 8.0 and applied at 50 ml/h to an ω-aminohexylagarose column (21 cm × 2.6 cmØ) in tandem with a pentylagarose column (7 cm × 2.6 cmØ) using chromatographic procedures described in section 2.6.1. The columns were subsequently washed with the Tris buffered NaCl (3500 ml) at the same flow rate, after which the ω-aminohexylagarose column was uncoupled from the pentylagarose column. PAO activity was then desorbed from the affinity column in the reverse flow direction with an NaSCN gradient (0.0 – 3.0 M) in 25 mM Tris–HCl. The active fraction pool is indicated by the bar. Fraction volume was changed from 25 to 10 ml at the start of the NaSCN gradient.

5.2.1.3 Gel Filtration

The chaotrophic salt, NaSCN, was removed from the sample by gel filtration at 50 ml/h on Sephadex S-300 (45 cm × 2.6 cmØ) equilibrated in a low ionic strength ion exchange buffer (30 mM NaCl in 25 mM Tris–HCl, pH 8.0).

5.2.1.4 Anion Exchange Chromatography

S-300 fractions containing PAO actuvity were pooled, diluted in low ionic strength ion exchange buffer and applied to a DEAE-Trisacryl M column. The column subsequently was eluted with a low ionic strength buffer to remove unbound protein and a linear NaCl gradient was applied, resolving the enzyme activity into two separate peaks, designated PAO I and PAO II (Figure 5.2).

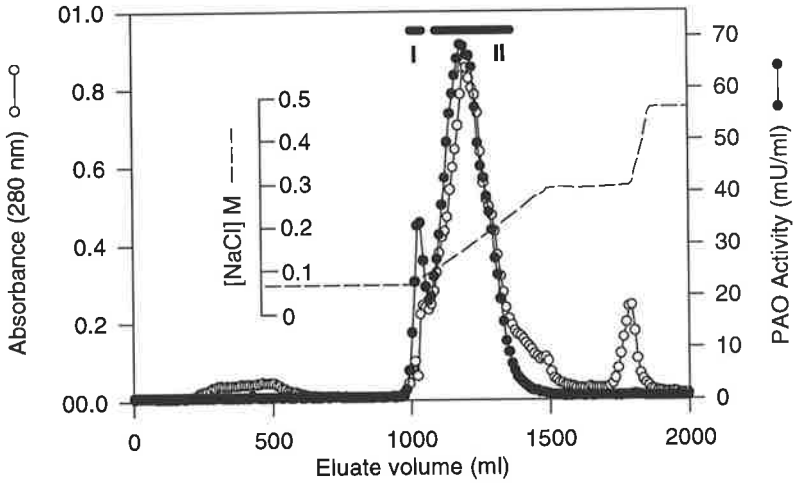


FIGURE 5.2. Resolution of Multiple Polyamine Oxidase Forms on DEAE-Trisacryl M.
S-300 fractions containing PAO activity were pooled, diluted 1 : 1 in low ionic strength buffer (30 mM NaCl in 25 mM Tris–HCl, pH 8.0) and applied to a DEAE-Trisacryl M column (94 cm × 2.6 cmØ) equilibrated in the same buffer. The column was eluted with the low ionic strength buffer followed by a 500 ml linear NaCl gradient (0.03 – 0.3 M in 25 mM Tris–HCl, pH 8.0), resolving the enzyme activity into two separate peaks, designated PAO I and PAO II. Fraction pools containing the multiple forms PAO I (**I**) and PAO II (**II**) are indicated by the bars. Elution was continued with limit buffer followed by 0.5 M NaCl in 25 mM Tris–HCl, pH 8.0 to remove tightly bound protein, regenerating the column.

5.2.1.5 Immunoaffinity Chromatography

Fractions from the first peak of enzyme activity eluting from the ion exchange column (PAO I) were pooled and applied to the mAb pao-1.9—Affi-Prep 10 column arranged in tandem with a bovine γ-globulin—Affi-Prep 10 column. The columns were eluted with equilibration buffer to remove unbound protein. PAO activity desorbed from the immunoaffinity column with 1.5 M NaSCN (Figure 5.3A). The PAO II from second peak of enzyme activity emergent from the ion exchange column was similarly purified (Figure 5.3B).

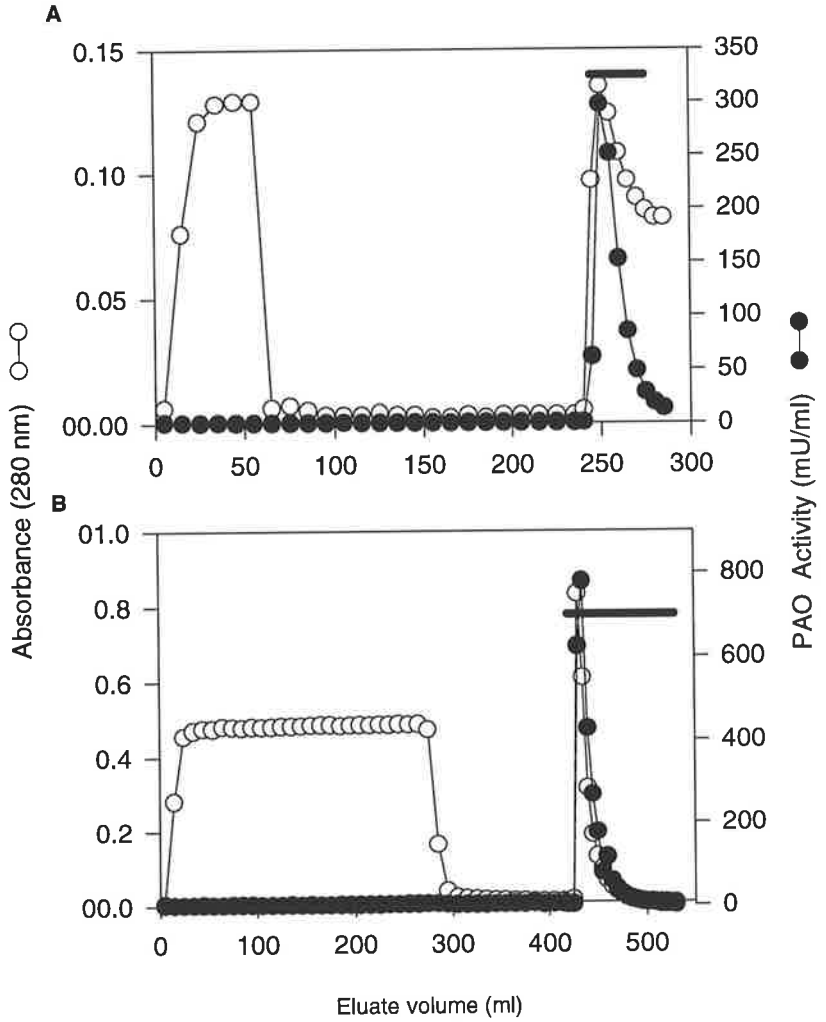


FIGURE 5.3 Immunoaffinity Purification of Polyamine Oxidase on mAb pao-1.9—Affi-Prep 10. The PAO I fraction pool (50 ml) from the DEAE column was applied to the mAb pao-1.9—Affi-Prep 10 column (4 cm × 1.6 cmØ) in tandem with a bovine γ-globulin—Affi-Prep 10 column (3.5 cm × 1.6 cmØ). Both columns had been precycled and equilibrated with 250 mM NaCl in 25 mM Tris–HCl, pH 8.0. The columns were eluted with the same buffer at 10 ml/h until A_{280} had fallen to baseline (approx. 20 column volumes). The columns were uncoupled and PAO activity was desorbed from the immunoaffinity column by elution in the reverse flow direction with 1.5 M NaSCN in 25 mM Tris–HCl, pH 8.0 (5.3A). PAO II from the second fraction pool from the ion exchange column was similarly purified (5.3B). Pooled immunoaffinity fractions are indicated by bars.

5.2.1.6 Size-Exclusion HPLC

The multiple enzyme forms from the immunoaffinity columns were independently applied to the SE-HPLC columns which had been arranged in tandem and run as described in section 2.6.2. Active fractions from the tandem SE-HPLC columns were pooled as PAO I and PAO II (Figure 5.4), concentrated and recycled on a BioSil SEC 400 column (Figure 5.5).

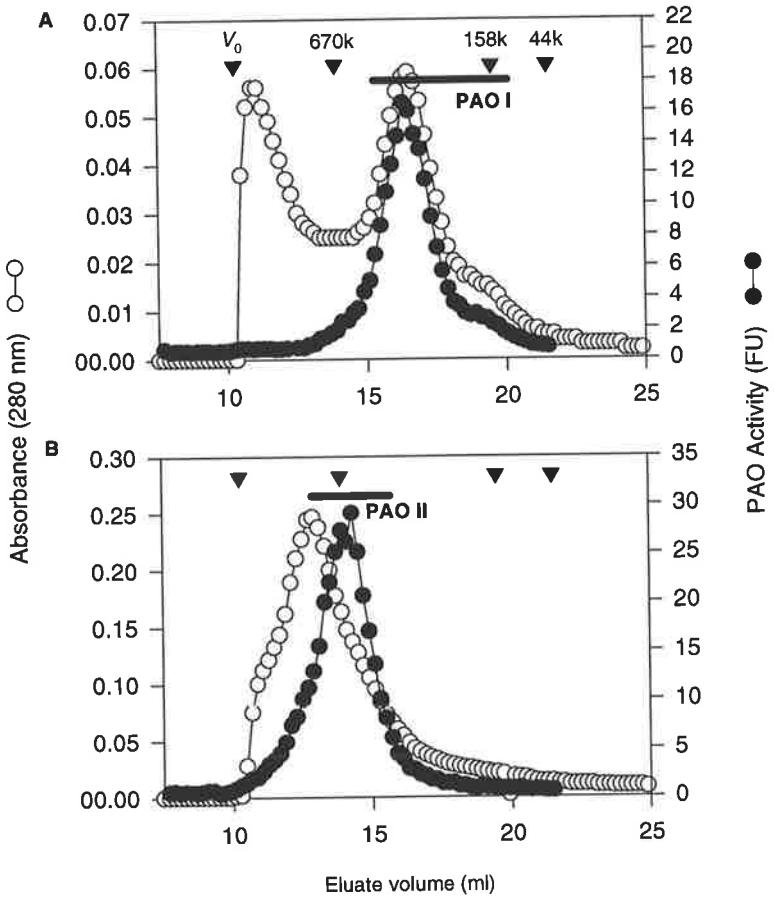


FIGURE 5.4. Size-Exclusion HPLC of Immunoaffinity Fractions on TSK Columns.
BioSil TSK-250 and TSK-400 (3000 + 4000) sw columns (300 mm + 300 mm × 7.5 mmØ) were arranged in tandem, preceded by a BioSil TSK guard column [3000 sw] (75 × 7.5 mmØ). The multiple enzyme forms from the immunoaffinity columns were independently concentrated over YM 30 membranes (Centricon 30) and applied to the SE-HPLC columns which had been equilibrated in 100 mM sodium phosphate buffer [1897] containing 300 mM NaCl, pH 6.80 (I_t = 0.5), prepared as described in section 2.6.2. SE-HPLC was conducted at 0.80 ml/min. Fractions were collected in siliconized glass tubes containing an equivalent volume of 50% (v/v) glycerol to stabilize the enzymes. Active fractions from the tandem SE-HPLC columns were pooled separately as indicated by the bars; 5.4A, PAO I; 5.4B, PAO II. The columns were calibrated using Bio-Rad gel filtration standards for size exclusion chromatography, elution positions are indicated by inverted triangles. Thyroglobulin M_r = 678,000; bovine γ-globulin, 158,000; ovalbumin, 44,000. Void volume, V_0, was indicated by protein aggregates. PAO activity was determined fluorometrically (*q.v.* §2.3.1) and is indicated in arbitrary (Fluorescence) units.

5.2.1.7 SDS-PAGE Analysis

Fractions from the recycling SE-HPLC of the multiple PAO forms on BioSil SEC 400 were analysed by SDS-PAGE using a discontinuous buffer system followed by silver staining. Results are illustrated in Figure 5.5.

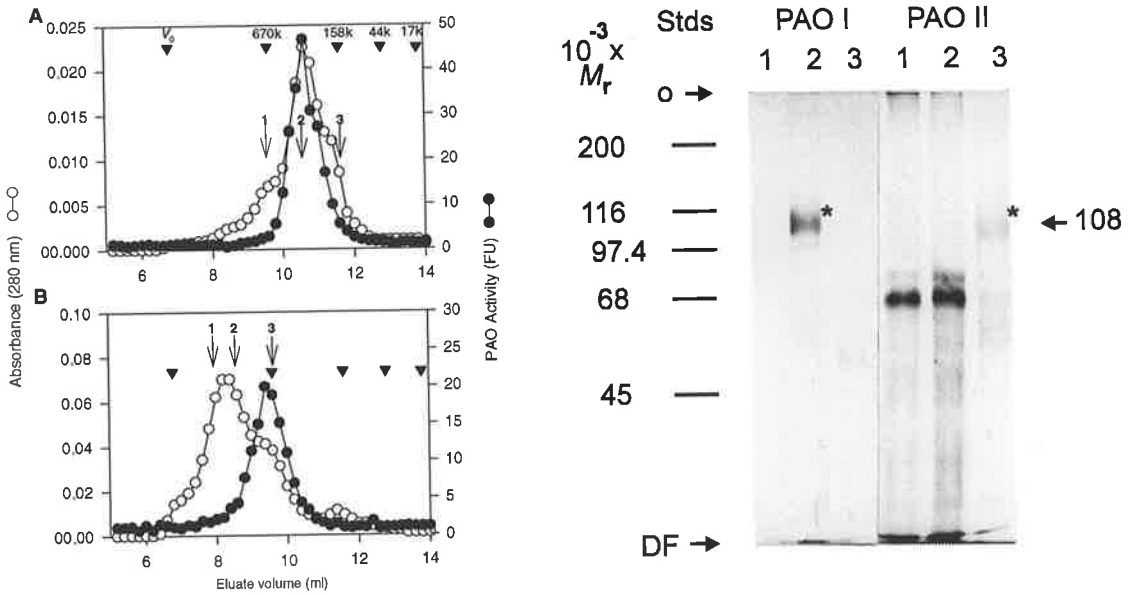


FIGURE 5.5. Recycling Size-Exclusion HPLC of Multiple PAO Forms. Pooled fractions from TSK SE-HPLC were concentrated over YM 30 membranes (Centricon 30s) and recycled on BioSil SEC 400 (75 mm + 300 mm × 7.8 mmØ) columns under the conditions described for the tandem TSK columns. The columns were calibrated with Bio-Rad gel filtration standards as described for Figure 5.4. The upper left hand panel, 5.5A, shows chromatography of PAO I; the lower left hand panel, 5.5B, chromatography of PAO II. The right hand panel shows electrophoretic analysis of samples from the fractions indicated by the numbered arrows in the chromatograms. Sample protein was determined using the *o*-phthalaldehyde method (*q.v.* §2.2.4). Prior to electrophoresis, samples were concentrated by deoxycholate/trichloroacetic acid precipitation and washed with cold ether/ethanol before dissolving in sample buffer. Electrophoresis was in 7.5% polyacrylamide gels (with a 3.75% stacker gel) as described in section 2.7. Proteins were silver stained using the method described by Merril, *et al.* (*q.v.* §2.7.7.3). Samples: PAO I chromatography (Figure 5.5A), *lane 1, 3.3 µg; lane 2, 5.2 µg; lane 3, 3.9 µg.* PAO II chromatography (Figure 5.5B), *lane 1, 4.5 µg; lane 2, 5.8 µg; lane 3, 4.7 µg.* The band revealed by silver staining which correlated with enzymatic activity shown in the left hand panel is indicated with an asterisk (*). Molecular weight markers are described in section 2.7.6

5.2.2 Purification using mAb pao-2.21—Affi-Gel 10

5.2.2.1 Preparation of the Retroplacental Serum

Retroplacental serum samples (with relatively high specific activity) from 10 placentae were prepared as described in section 5.2.1.1.

5.2.2.2 Affinity Chromatography on ω-Aminohexylagarose

Diluted retroplacental serum was prepared as described in section 5.2.1.1 and applied to an ω-aminohexylagarose column preceded by a pentylagarose column. The columns had been equilibrated with 150 mM NaCl in 25 mM Tris–HCl, pH 8.0 and were eluted with the same buffer. PAO activity was desorbed from the ω-aminohexyl-agarose column with 1.5 M NaSCN (Figure 5.6). Active fractions were pooled, concentrated and the NaSCN removed by gel filtration on Sephadex S-300 (45 cm × 2.6 cmØ) with 30 mM NaCl in 25 mM Tris–HCl, pH 8.0.

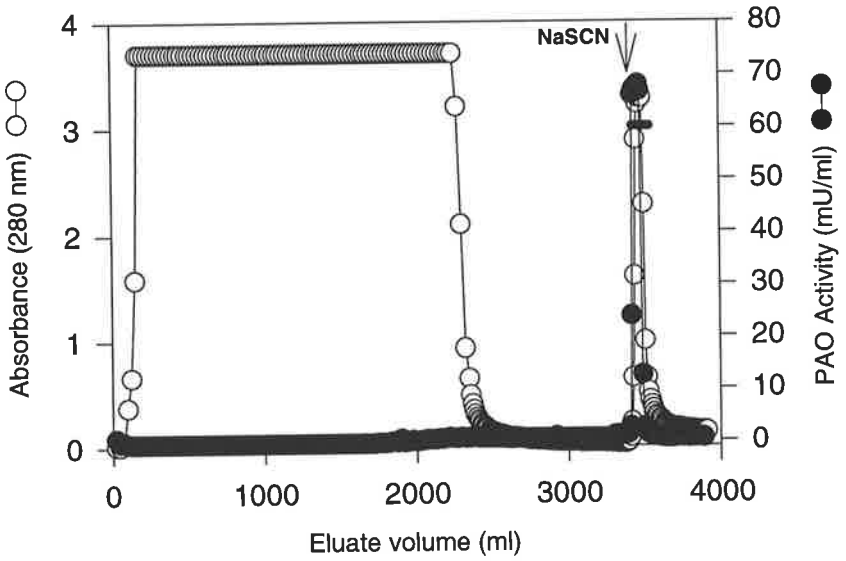


FIGURE 5.6. Affinity Chromatography of Human RPS on ω-Aminohexyl-agarose. Diluted retroplacental serum was prepared as described in section 5.2.1.1 (1000 ml) diluted further to 2000 ml with 150 mM NaCl in 25 mM Tris–HCl, pH 8.0 and applied at 50 ml/h to an ω-aminohexyl-agarose column (21 cm × 2.6 cmØ) preceded by a pentylagarose column (7 cm × 2.6 cmØ). The columns had been equilibrated with 150 mM NaCl in 25 mM Tris–HCl, pH 8.0 and were eluted with the same buffer. PAO activity was desorbed from the ω-aminohexyl-agarose column with 1.5 M NaSCN in 25 mM Tris–HCl, pH 8.0. Pooled fractions are indicated by the bar. NaSCN elution is indicated by the arrow. Fraction size was changed from 25 ml to 10 ml after NaSCN elution was commenced.

5.2.2.3 Anion Exchange Chromatography

Enzymatically active fractions from gel filtration of the affinity purified enzyme were pooled and the multiple enzyme forms, PAO I and PAO II, were resolved by chromatography on DEAE-Trisacryl M as previously described (*q.v.* §5.2.1.4) (Figure 5.7).

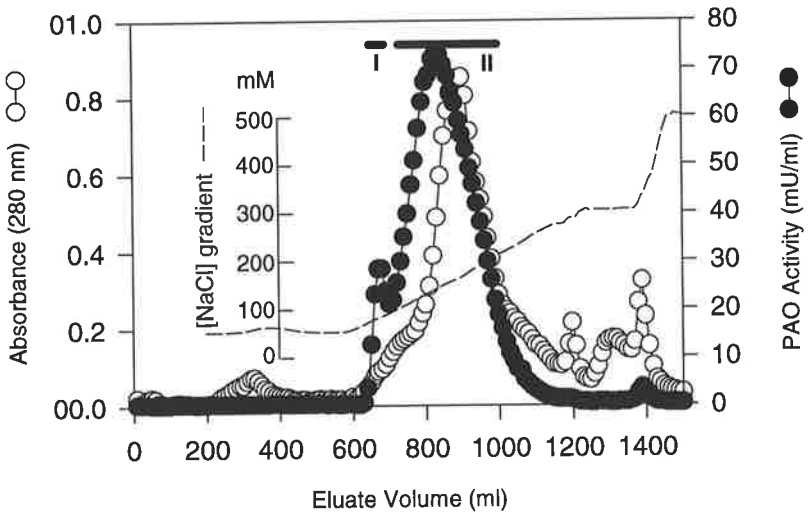


FIGURE 5.7. Resolution of Multiple PAO Forms on DEAE-Trisacryl M. PAO which had been affinity purified on ω-aminohexylagarose was exchanged into a low ionic strength buffer by gel filtration on Sephadex S-300 and applied to the DEAE column. Pooled activity is indicated by the bars.

5.2.2.5 Immunoaffinity Chromatography and Size-exclusion HPLC

Fractions from the DEAE column containing PAO I were pooled and applied to the mAb pao-2.21—Affi-Gel 10 column that had been arranged in tandem with a bovine γ-globulin—Affi-Gel 10 column to protect the immunoaffinity column and adsorb nonspecifically binding proteins. PAO activity completely adsorbed to the immunoaffinity column which was then eluted with 250 mM NaCl in 25 mM Tris–HCl, pH 8.0, followed by a more rigorous wash with 1.0 M NaCl in 25 mM Tris–HCl, pH 8.0 to remove nonspecifically adsorbed protein. PAO I activity was desorbed from the immunoaffinity column with 1.5 M NaSCN (Figure 5.8A), and further purified by HPLC on Bio-Sil SEC 400 (Figure 5.9A) as described for the TSK columns (*q.v.* §5.2.1.6). PAO II from the second ion exchange fraction pool was similarly purified (Figures 5.8B, 5.9C and D).

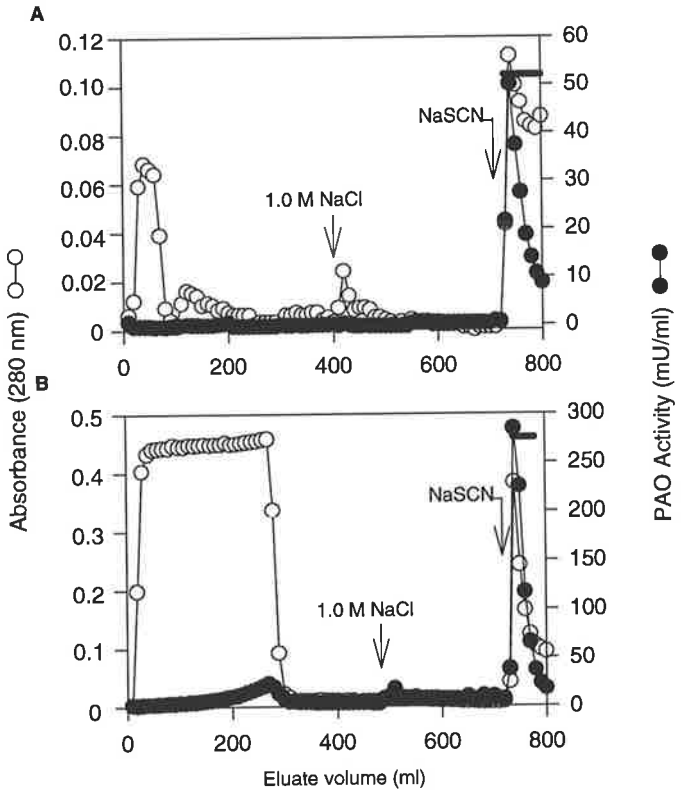


FIGURE 5.8. Immunoaffinity Purification of PAO on mAb pao-2.21—Affi-Gel 10. Pooled DEAE column fractions containing PAO I were applied to the immunoaffinity column (prepared as described in section 4.3.6.6 with mAb pao-2.21 and 8 ml Affi-Gel 10 at 9.86 mg/ml gel) preceded by a bovine γ-globulin—Affi-Gel 10 column (5 cm × 1.6 mmØ). PAO activity completely adsorbed to the immunoaffinity column (4 cm × 1.6 cmØ) which was eluted with 250 mM NaCl in 25 mM Tris–HCl, pH 8.0, followed by 1.0 M NaCl in 25 mM Tris–HCl, pH 8.0 (*arrow*). PAO I activity was desorbed from the immunoaffinity column with 1.5 M NaSCN in 25 mM Tris–HCl, pH 8.0 (*bent arrow*) (*panel* A), and further purified by HPLC on BioSil SEC 400 (Figure 5.9A). PAO II in the second ion exchange fraction pool was similarly purified (*panel* B and Figure 5.9C and D). Pooled fractions are indicated by the bars.

5.2.2.5 SDS-PAGE Analysis

SE-HPLC fractions of PAO I and PAO II were analysed by SDS-PAGE under reducing conditions in 7.5% PA as described in section 2.7 (Figure 5.9B,E).

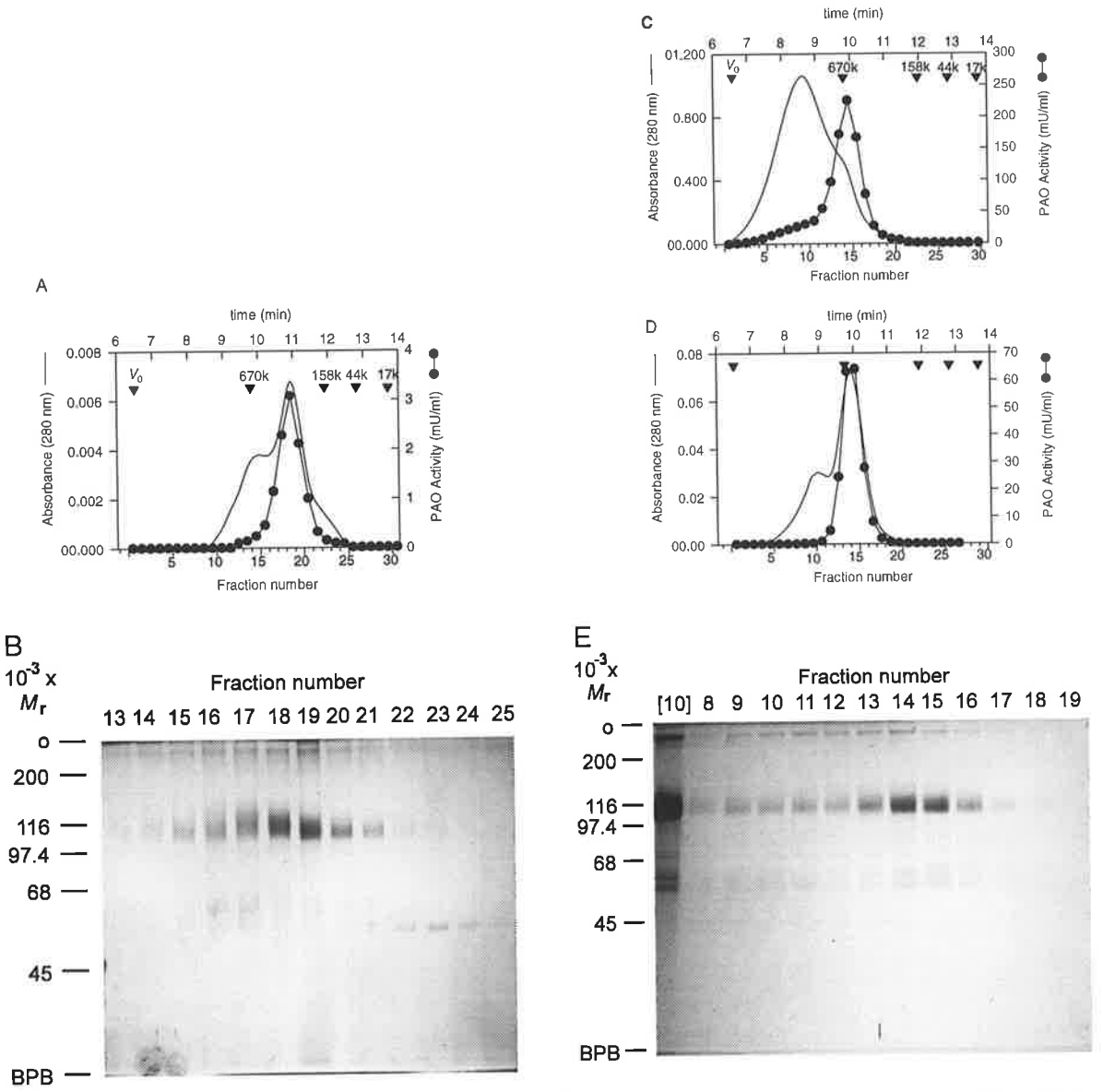


FIGURE 5.9. Size-Exclusion HPLC of Multiple PAO Forms on Bio-Sil SEC 400. Immunoaffinity purified PAO fractions (Figure 5.8A and B, indicated by bars) were concentrated using a Micro-ProDiCon ultrafiltration system (25,000 MWCO, Spectrum Medical Industries, Los Angeles, CA) and applied to a BioSil SEC 400 column set (80 mm + 300 mm × 7.8 mmØ). Columns were calibrated using Bio-Rad SEC standards and eluted at 1.0 ml/min as described for Figure 5.4, collecting 0.25 ml fractions. *Panel A,* PAO I; *Panels C and D,* PAO II. *Panel B,* SDS-PAGE of PAO I SE-HPLC fractions (panel A) under reducing conditions in 7.5% PA (*q.v.* §2.7); proteins were silver stained using the method described by Gottlieb and Chavako (*q.v.* §2.7.7.3). *Panel E,* SDS-PAGE of PAO II SE-HPLC fractions; panel C fraction 10, [10] and panel D fractions (without square brackets) under reducing conditions as described above. Proteins were stained using the highly sensitive CBB G (CI 42655) based method described by Neuhoff *et al.* (*q.v.* §2.7.7.2). *o* = Origin and BPB = bromophenol blue tracker dye.

Immunoaffinity purification of PAO I and PAO II using mAb pao-2.21—Affi-Gel 10 is summarized in Table 5.2.

TABLE 5.2. Purification of Polyamine Oxidase Using mAb-pao 2.21—Affi-Prep 10

Purification Step	Volume	Activity	Protein	Specific Activity	Purification	Yield
	ml	*Units*[a.]	*mg*[b.]	*mU/mg*	*-fold*	*%*
1. Retroplacental Serum	500	41.7	20780	2	1	100
2. Pentyl-/ω-aminohexylagarose	115	27.5	164	168	84	66
3. Sephacryl S-300	116	37.9	162	234	116	91
4. DEAE-Trisacryl						
(PAO I)	47	2.3	3.0	78	39	6
(PAO II)	260	24.4	79	308	153	59
5. mAb-pao-2.21–Affi-Gel 10						
(PAO I)	37	0.41	0.3	1385	689	1
(PAO II)	50	7.52	2.7	2838	1412	18
6. Concentration 25,000 MWCO						
(PAO I)	0.50	0.24	0.1	2424	1206	0.6
(PAO II)	0.52	4.88	1.4	3437	1710	12

Notes to Table 5.2

a. One μmole/min H_2O_2 production with 10 μM putrescine as the substrate (*q.v.* §2.3.1).

b. By the method of Smith *et al.* [1606].

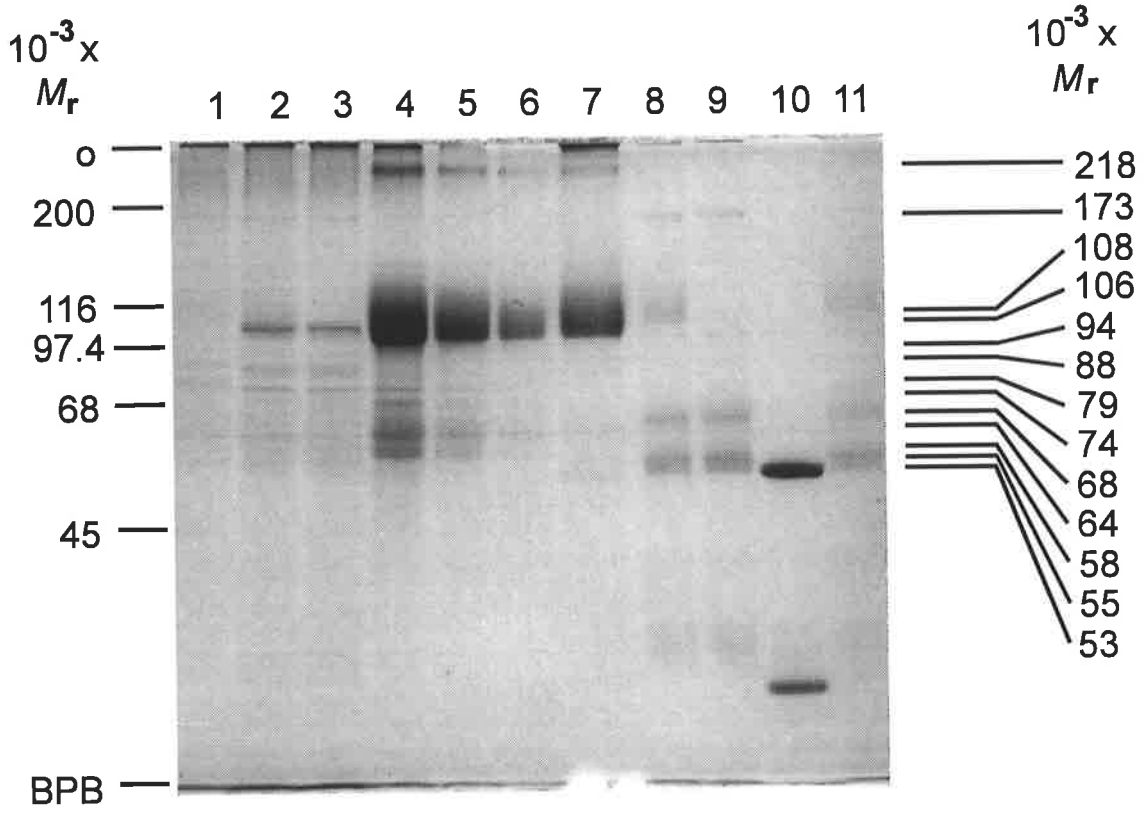


FIGURE 5.10. SDS-PAGE Analysis of Immunoaffinity (mAb pao-2.21) Polyamine Oxidase Purification. SDS-PAGE was conducted under reducing conditions in 7.5% PA resolving gels using the discontinuous buffer system of Laemmli [1127] (*q.v.* §2.7). Protein bands were stained overnight with Coomassie Blue R as described in section 2.6.7.1. *Lane 1*, DEAE-Trisacryl eluate polyamine oxidase type II (PAO II) activity peak fraction; *lane 2*, DEAE-Trisacryl PAO II fraction pool; *lane 3*, inactive immunoaffinity column eluate from PAO II purification (Fig. 5.8, *panel B*) fraction 5 (40 – 50 ml); *lanes 4,5 and 6* immunoaffinity purified PAO II (11.34 µg, 5.67 µg and 2.83 µg respectively). *Lane 7*, immunoaffinity purified polyamine oxidase type I (PAO I); *lane 8*, DEAE-Trisacryl eluate PAO I fraction pool; *lane 11*, DEAE-Trisacryl peak fraction. *Lane 9*, inactive immunoaffinity column eluate from PAO I purification (Fig. 5.8, *panel A*) fraction 4 (30 – 40 ml). *Lane 10*, monoclonal antibody pao-2.21.

Proteins from the purification steps were examined by SDS-PAGE under reducing conditions (Figure 5.10); enzyme activity correlated with a M_r 108,000 band. Other bands, with M_rs 218,000; 79,000; 74,000; 64,000; 58,000 and 53,000 were also observed in our preparations. The M_r 218,000 band vanished after deglycosylation (Figure 5.11) and remained under nonreducing conditions (Figure 5.12) suggesting that it is an unreduced dimer of a M_r 108,000 subunit. The faint M_r 53,000 band in the PAO I immunoaffinity preparation (Figure 5.10, *lane 7*) was probably a trace of mAb heavy chain (Figure 5.10, *lane 10*) which had bled from the column during the immunoaffinity purification. The band at M_r 79,000 in PAO II (Figure 5.10, *lanes 4,5,6*) was also seen in immunoaffinity column eluates from which all enzyme activity had been adsorbed (Figure 5.10, *lane 3*). The remaining bands, M_rs 74,000; 64,000; and 58,000 were not present or appeared in variable amounts in other immunoaffinity preparations (not shown). The sharp band at M_r 106,000 (Figure 5.10, *lanes 2 and 3*) does not appear to underlie the diffuse M_r 108,000 band in Figure 5.10, *lanes 4 to 7*. (cf. Figure 5.9B,E) nor did it correlate with enzyme activity (Figure

5.10, *lane 3*). The M_r 108,000 band is enriched after immunoaffinity purification (Figure 5.10, *lanes 2 to 6* and *8 to 7*) and depleted after immunoadsorption of enzyme activity (Figure 5.10 *lanes 2 to 3* and *8 to 9*).

Immunoaffinity purified PAO I activity eluted from the size-exclusion HPLC columns with an apparent molecular weight, M_r 336,000 (Figure 5.9A). The protein in the fractions correlating with enzyme activity ran as a diffuse band M_r 108,000 after SDS-PAGE under reducing conditions (Figure 5.8B). PAO II activity eluted with an apparent M_r 644,000 as a shoulder on the major protein peak (Figure 5.9C). The protein peak (fraction 10) from the first fractionation on BioSil SEC 400 (Figure 5.9C) had a similar SDS-PAGE profile (Figure 5.9E, *lane* [10]) to that of the peak activity fractions from the recycling run (Figure 5.9E; fractions 14 and 15) suggesting that the major protein peak with large apparent molecular weight was mostly inactive aggregates of the PAO II species. Indeed, a shoulder of residual polyamine oxidase activity is associated with this peak (Figure 5.9C). Protein in the fractions correlating with enzyme activity ran as a diffuse band M_r 108,000. The purification process inactivates a proportion of the enzyme resulting in reduced active site purity. The activity losses are indicated by data in Table 5.2. Formation of inactive enzyme aggregates is suggested by the size-exclusion HPLC and electrophoretic data.

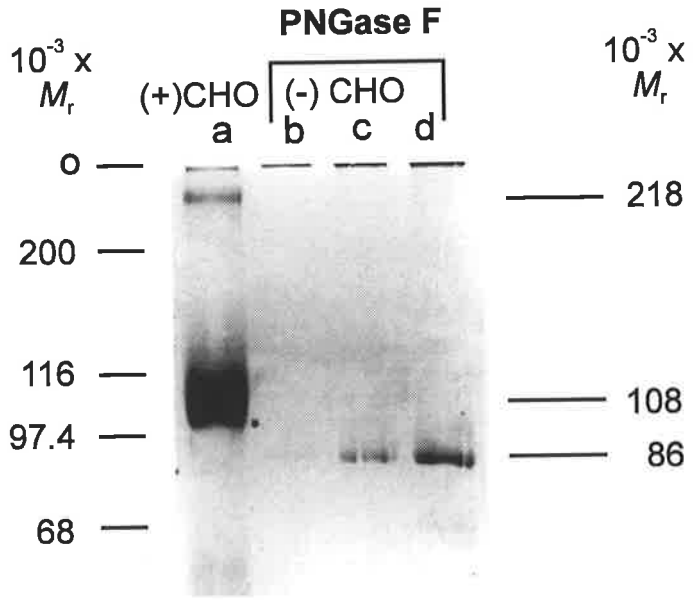


FIGURE 5.11. Enzymatic Deglycosylation of Polyamine Oxidase
An immunoaffinity purified preparation of polyamine oxidase (20 µg, forms I and II combined) was denatured at 100 °C (10 min) in the presence of 0.5% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. The denatured protein was treated with 2000 units (0.16 µg) of recombinant peptide:*N*-glycosidase F (EC 3.5.1.52) [PNGase F [1898], New England Biolabs] for one hour at 37 °C in 0.05 mM sodium phosphate buffer, pH 7.5 containing 1% NP-40 (v/v). *Lane a*, immunoaffinity purified PAO (4.2 µg) *before* PNGase digestion. *Lanes b,c,d*, immunoaffinity purified PAO *after* carbohydrate (CHO) removal, 1.86 µg, 4.65 µg and 9.3 µg respectively. The proteins were stained with Coomassie Blue R (CI 42660) followed by diffusion destaining [1734] (*q.v.* §2.7.7.1).

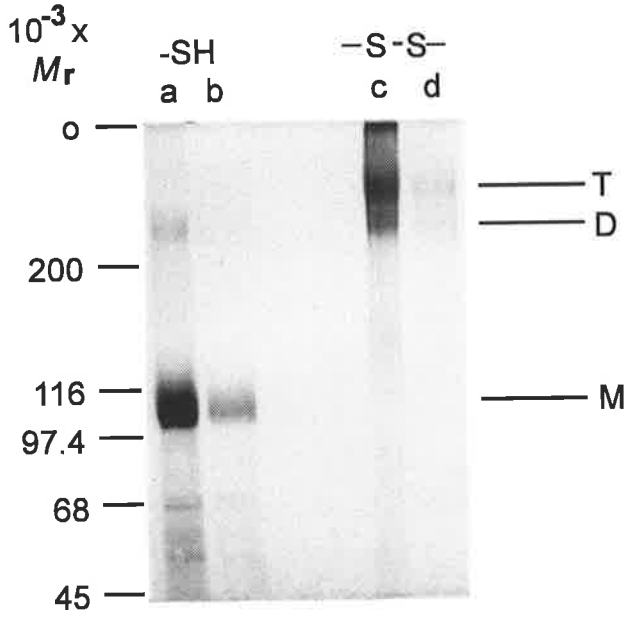


FIGURE 5.12. SDS - 5% PAGE of Polyamine Oxidase under Reducing (–SH) and Nonreducing (–S-S–) Conditions. *Lanes a and c,* immunoaffinity purified enzyme; *lanes b and d* pooled activity from size-exclusion HPLC of immunoaffinity purified enzyme. M, monomer; D, dimer; T, trimer/tetramer. The gel was stained with Coomassie Blue R as described for Figure 5.10.

Indeed protein aggregates could be observed at the interface of stacking and resolving gels Figures 5.9–12, even under reducing conditions which decreased their relative abundance (Figure 5.12 cf. lanes a and c).

5.3 DISCUSSION

A large scale preparative immunoaffinity purification of two polyamine oxidase forms from human retroplacental serum was achieved using monoclonal antibodies specifically purified from cell culture supernatants. Higher yields, higher specific activities and higher purity preparations were obtained using this immunoaffinity method compared with those obtained with conventional affinity and biochemical methods. Both strategies result in the isolation of two forms of polyamine oxidase that differed in apparent relative molecular mass, M_r, as indicated by size-exclusion HPLC; and charge, as indicated by their behaviour on anion exchange media. The high molecular weight form, PAO II (apparent M_r 644,000) with higher affinity for the anion exchange matrix, accounted for about 90% of the total activity of retroplacental serum. Analysis of the different forms by SDS-PAGE under reducing conditions showed the polyamine oxidizing activity of both forms I and II to be associated with a protein band with an apparent M_r 108,000. Under nonreducing conditions higher molecular weight species were observed, possibly a dimer and tetramer of the M_r 108,000 subunit held together by disulphide bonds. SE-HPLC of the multiple polyamine oxidase forms and analysis of the eluate fractions by reducing SDS-PAGE suggested that the high molecular weight forms observed were aggregates of a native dimeric enzyme. The anomalously high apparent relative molecular masses of the native enzyme dimer and its aggregates indicated by size-exclusion HPLC is probably due, in part, to their large hydrodynamic

radii as a consequence of the increased the hydration of the glycoprotein carbohydrate chains compared to the smaller hydrodynamic radii of similarly sized polypeptide chains alone [1119-1121]. Moreover, if the enzyme shape is not spherical, as suggested by X-ray crystallographic studies of other amine oxidases [529,650], this would contribute to an anomalously high apparent molecular weight for the enzyme indicated by size-exclusion HPLC.

The formation of enzyme aggregates is in agreement with the observation of aggregate formation by other amine oxidases (*q.v.* §1.3.4.2). In particular, the bovine plasma enzyme appeared to self-associate and this was favoured by time and high protein concentration [823]; conditions such as would be encountered during the purification of the retroplacental serum enzymes. The porcine kidney diamine oxidase was also found to be subject to association, particularly in anoxic media (such as encountered here during chromatography and electrophoresis), and a tetrameric form has been observed [899]. In SDS-PAGE studies of the porcine kidney enzyme (ABP), aggregation to larger forms, especially on storage, was noted [906], and protein aggregates were observed at the interface of stacking and resolving gels [909]. The aggregates may be equivalent to aggregates of placental diamine oxidase observed by electron microscopy [1220].

The contaminants observed in our preparations after immunoaffinity purification may have been inactive partially processed enzyme forms or degradation products of the enzyme bearing determinants recognized by the mAbs. Radioactive pulse–chase labelling studies may in future provide information about enzyme processing. Faint protein bands, seen after SDS-PAGE, like those observed in our preparations at M_rs 79,000, 64,000 and 58,000, have been observed in preparations of the pig kidney enzyme (ABP); tryptic digests indicated that they were likely to be degradation products of the enzyme subunit, indeed their relative abundance was observed to increase on storage [909]. On the other hand, the faint protein bands seen in the retroplacental serum enzyme preparations may have been proteins strongly adherent to the polyamine oxidase, nonenzyme proteins bearing a determinant recognized by the mAb, or proteins with strong nonspecific binding to the immunoaffinity column.

The diffuse nature of the M_r 108,000 band correlated with enzyme activity appears to be related to its glycosylation. The relative molecular mass of the deglycosylated enzyme polypeptide, M_r 86,000, is in close agreement with the calculated molecular mass of 85,357.6 Da for the human (amiloride-sensitive) copper-containing amine oxidase subunit (*q.v.* §6.2.9.2) and to the molecular mass of 83,415.6 Da calculated for the mature peptide subunit of human gene diamine oxidase [47,757] (Swiss-Prot Entry P19801; EMBL Entry X78212).

The losses of enzymatic activity during the purifications reported here may have been partially avoided by applying a different strategy to the desorbtion of polyamine oxidases from the

affinity columns. Thiocyanate has been reported as an inhibitor of other amine oxidases [1050,1110]. This may be through its binding to an equatorial coordination position on the Cu^{II}, with consequent displacement of H_2O (*q.v.* §1.3.4.1d). The inhibition may be avoided in future studies by the use of a pH change to effect the desorbtion of the active enzyme from the column, such as used by Denney *et al.* [759]. pH 9.4 may be sufficient to effect the desorbtion. However, higher pH has been shown to reduce enzyme activity. High pH may be quickly neutralized by including a concentrated buffer in the collecting tubes. Nevertheless, the thiocyanate was quickly removed from the preparations described in this study and may have only had a minor affect on enzyme activity.

It is difficult to reconcile the findings of Houen *et al.* [756] and Denney *et al.* [759] with our results. Denney *et al.* reported that immunoblotting (SDS-PAGE, reducing conditions) of a polypeptide revealed a protein of M_r 95,000 with a monoclonal antibody that immunoprecipitates human placental diamine oxidase activity, and adsorbs and releases diamine oxidase activity on immunoaffinity columns [759]. Material from the immunoaffinity columns, prepared using proteins from an ammonium sulphate cut of ascites fluid, appeared as a number of bands on SDS-PAGE although this was not reported (Dr Richard Denney, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galverston, TX; *personal communication*). It is possible that the immunoblotted protein was recognized by other antibodies in the preparation of monoclonal antibody from ascites used in the immunoblotting (*vide* §4.2.5). Houen *et al.* raised monoclonal antibodies to human placental and pregnancy serum enzymes which were both said to be dimers (M_r 180,000) of a M_r 90,000 subunit (data was not shown). The monoclonal antibodies were used in turn to purify amine oxidases from the sources used for monoclonal antibody production. However, molecular weight and purity data for the immunoaffinity purified enzymes were not reported. It is possible that the differences between the monomer molecular weight found in this study, M_r 108,000, and the other studies reflect differences in the gel concentrations or nominal molecular weight of the markers used in the different studies, although the molecular weight marker used at M_r 97,400 (phosphorylase b, *q.v.* §2.7.6) should not create such an ambiguity. Alternately, the differences may reflect different extents of post-translational modification, such as glycosylation, between the different enzyme sources or observed in the different laboratories.

Glycosylation is well known to cause anomalous behaviour of glycoproteins on gels of different concentrations [1122,1123]. Glycoproteins behave anomalously even when SDS and thiol reagent are in excess. It is implicit in the linear relationship of $\log_{10}M_r$ and mobility for SDS-PAGE that all polypeptides bind a constant mass ratio of SDS. Glycoproteins probably bind SDS to only the protein part of the molecule. The reduced net change resulting from reduced SDS

binding lowers the polypeptide mobility during electrophoresis, resulting in an artificially high M_r estimation. However, with increased polyacrylamide gel concentration, molecular sieving predominates over the change effect and the apparent molecular weights of glycoproteins decrease and approach their true molecular weights [1123,1129,1690]. It is possible that in those laboratories where lower molecular weights were observed higher concentrations of acrylamide were used in the resolving gels. Polyacrylamide concentration gradient gels can serve to sharpen diffuse glycoprotein bands as the glycoproteins encounter progressively decreasing pore sizes [1125]. The use of gradient gels may also lead to an apparent reduction of glycoprotein molecular weight.

On the other hand, association of human retroplacental serum polyamine oxidizing activity with a M_r 108,000 protein under reducing conditions is in agreement with the molecular weight 'of around' 105,000 reported by Novotny *et al.* for the human placental (amiloride-sensitive) copper-containing amine oxidase subunit [47] and the M_r 110,000 reported for the different amniotic fluid diamine oxidase forms [678,763] (*q.v.* §3.4).

Chapter 6

PROTEIN SEQUENCING AND FURTHER CHARACTERIZATION OF HUMAN RETROPLACENTAL SERUM POLYAMINE OXIDASE

6.1 Introduction

Immunoaffinity purification of human retroplacental serum polyamine oxidase resulted in the isolation of two forms of polyamine oxidase that differed in relative molecular mass and charge. Analysis of the two forms by SDS-PAGE under reducing and nonreducing conditions showed the polyamine oxidizing activity of both forms to be associated with a M_r 108,000 subunit of the native homodimeric enzyme.

Because of the controversial nature of molecular weight of the human pregnancy-associated amine oxidase subunit (including that of the retroplacental serum enzyme) and the limitations of SDS-PAGE analysis in protein identification, there was a need for further characterization of the retroplacental enzyme forms. Amino acid sequence analysis extends the investigation of the enzymes at a molecular level. Even short partial sequences may allow the identification of a protein. Where the entire primary amino acid sequence of a protein is unknown, partial protein sequences provide an ideal starting point for the isolation of its gene. Once its gene is isolated, the gene's sequence and thus the entire primary structure of the protein can be established. Expression of the enzyme gene as a homogenous polypeptide population provides the confirmatory synthesis that allows the unambiguous assignment of a particular enzymatic activity to a specific protein. At the commencement of research for this thesis a primary amino acid sequence was not available for any polyamine oxidase. During the progress of the research the lentil seedling amine oxidase sequence became available followed by that for the human placental amine oxidase. The expression of the human gene for human amine oxidase certified its identification.

Since it had been proposed that there are a range of placental amine oxidases [444], and since multiple enzyme forms with similar phenotypes had been identified in both amniotic fluid [763] and placenta [747]; it was of interest to determine whether or not the two enzyme forms observed in retroplacental serum were similar. To unequivocally identify the major band seen in the preparation of each retroplacental enzyme form, *N*-terminal protein sequencing was used. Furthermore, *N*-terminal protein sequencing was used to identify the protein band observed after

SDS-PAGE analysis that was proposed to be the enzyme homodimer, and to identify major impurities found in the enzyme preparations. SDS-PAGE and protein sequence analysis are complementary in that, gel electrophoresis provides the high resolving power for the separation of small amounts of proteins and sequence analysis allows the identification of each separated protein species at a molecular level. Sequence analysis of proteins separated by SDS-PAGE therefore provides a powerful link between the descriptive patterns provided by gel electrophoresis and the molecular characterization of the separated polypeptides.

Protein sequencing not only identifies proteins, but provides the key to the determination of the complete primary protein structure. The primary protein structure can reveal much about the protein. In this particular case the entire protein structure had already been determined already from the perspective of the protein's amiloride-binding properties [781]. Protein sequence analysis may identify sequence segments of special functional significance, such as sites of post-translational modification, binding sites, or active sites. The primary protein sequence was analysed here to provide information about the (retro)placental enzyme's molecular mass, secondary structure, antigenicity, hydrophobicity and consensus patterns. Furthermore, the amino acid sequence of a particular protein allows the identification of proteins with similar sequences. Homologies between different proteins can often be suggested on the basis of limited sequence data, so that in many cases even *N*-terminal sequence analysis can identify related proteins. The amino acid sequence of (retro)placental polyamine oxidase was compared with the sequences of other proteins to identify related proteins and classify the enzyme.

An important part of any detailed enzyme study is a measurement of its steady-state kinetic parameters. A knowledge of enzyme kinetics suggests how efficiently an enzyme in a metabolic pathway will operate with the steady-state levels of metabolites that are available to it and how the enzyme will respond to any changes in the metabolite levels. Furthermore, kinetics are a starting point in elucidating the detailed chemical mechanism of enzyme action. The studies reported here have been based on consideration of detailed discussions of enzyme kinetics which are available in review articles including [1143,1653,1654,1664,1667,1668,1899-1907], books including [1650,1666,1669,1908-1916], and in the scientific literature. To identify the substrate specificity of the retroplacental polyamine oxidase and similarities or differences between the two enzyme forms isolated here and between these forms and other amine oxidases, the steady-state kinetic parameters of the enzymes were examined with a number of substrates. Past studies of the retroplacental serum polyamine oxidase, and indeed other pregnancy-associated amine oxidases, have been hampered by inappropriate methods or have not systematically examined steady-state kinetic parameters for a range of substrates.

Because the mode of polyamine cleavage by amine oxidases has been controversial, the

products of enzymatic spermine oxidation were examined here. Identification of the oxidation products should allow more certain classification of the human retroplacental serum enzyme.

Knowledge of which substances cause inhibition of amine oxidase activity may be used to suggest how the retroplacental serum polyamine oxidase should be classified by suggesting information about its prosthetic groups, cofactors, and mechanism of enzyme action. Similarly, the UV–visible spectrum and copper content of the enzyme provide information about active site cofactors. These attributes of the retroplacental serum enzyme were examined and reported here.

SDS-PAGE provides a high resolution description of the retroplacental serum polyamine oxidase protein. This description can be compared to the descriptions provided by SDS-PAGE of other amine oxidases to suggest similarities and differences. A limitation of the method is that it does not allow for the identification of the isoelectric point of the enzyme or distinguish any microheterogeneity. Furthermore, the resolving power of SDS-PAGE does not allow the separation of proteins of very similar molecular weight that may have the appearance of a single protein on the one-dimensional gels. Since anion exchange chromatography suggested the native enzyme exists as at least two major isoelectric forms, retroplacental serum polyamine oxidase was subjected to preparative and analytical isoelectric focusing in an attempt to characterize their isoelectric points. Two-dimensional electrophoresis provides a further degree of resolution and was used to establish the purity of the major band associated with enzymatic activity, identifying the presence, if any, of contaminants with similar molecular weight to the enzyme.

The results of studies characterizing the human retroplacental serum polyamine oxidase are presented here. Methods and reviews of previous work have been described in preceding chapters. The results presented here are discussed in Chapter 7.

6.2 Results

6.2.1 Molecular Weight (Relative Molecular Mass, M_r)

M_r values were calculated from first order polynomial regressions to the data given by the molecular weight marker proteins (*q.v.* §2.7.6).

This study showed that:

(a) in SDS-PAGE gels (7.5% acrylamide), run under reducing conditions as described in section 2.7, the apparent M_r of the purified glycosylated polyamine oxidase subunit was: 108,215 ± 2,844 (n=11) ± SD. The glycoprotein behaved anomalously in gels of different acrylamide concentrations.

(b) The dimeric form of the enzyme ran at a M_r 219,824 ± 12,845 (n=17) ± SD on SDS-PAGE

gels (various single acrylamide concentrations from 5 to 12.5%).

(c) The deglycosylated enzyme subunit ran at a M_r 86,000 (2nd order polynomial regression) in a 7.5% SDS-PAGE gel (Figure 5.11).

(d) The two high molecular weight forms of the purified enzyme observed by SE-HPLC (*q.v.* Figure 5.9) were also observed in unpurified retroplacental serum both by SE-HPLC [727] and in gel filtration experiments (*q.v.* §3.2.1). The apparent M_rs by SE-HPLC were 544,000 ± 86,000 for the more abundant (approx. 90% relative abundance) higher molecular weight form and 303,000 ± 46,000 for the lower molecular weight form.

6.2.2 Isoelectric focusing

6.2.2.1 Preparative Isoelectric Focusing

A preparative isoelectric focusing cell was used as described in section 2.11.1 to determine the p*I*s of multiple isoelectric forms of polyamine oxidases observed when RPS is chromatographed on DEAE ion exchange columns. Retroplacental serum (25 ml) (*q.v.* §2.5), was desalted by gel filtration. The sample was diluted further and 2% (w/v) Biolyte 3–10 ampholytes added so that the final volume of the sample was 60 ml. Approximately 55 ml of this solution (9.8 mg/ml protein; 25.6 mU/ml activity) was focused in a Rotofor cell for 4 h at 12 W using a BioRad Model 3000Xi power supply. Voltage increased from 350 V to 850 V during the run. After focusing, analysis of the harvested fractions showed a peak of enzymatic activity at pH 5.4 (58 mU/ml) with a shoulder extending to pH 8.0 (Figure 6.1).

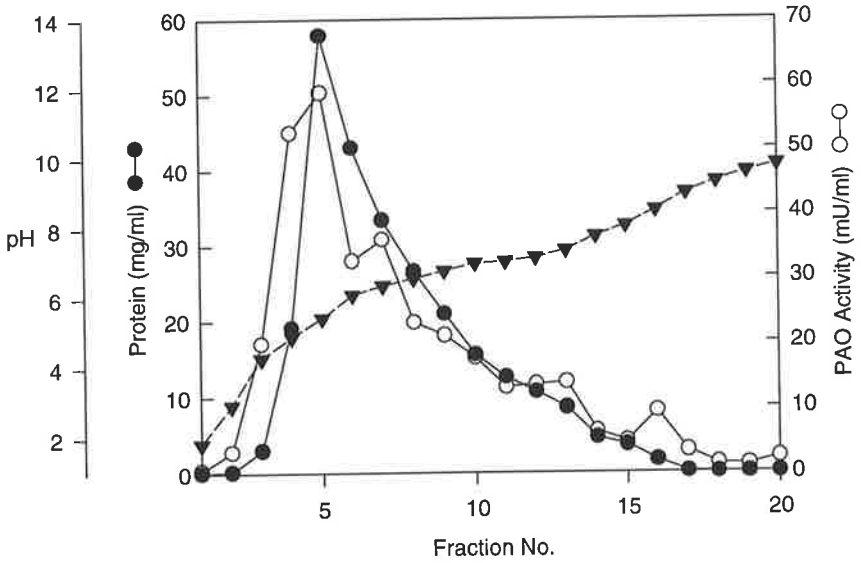


FIGURE 6.1. Isoelectric Focusing Profile of Retroplacental Serum.
Sample of RPS contained 2% (w/v) Biolyte ampholytes (3/10). Inverted triangles indicate the pH gradient.

Fractions containing enzyme activity (pH 4.5 – 8.05) were pooled, diluted to 35 ml with water

and reloaded into the Rotofor cell. The ampholytes in the pooled samples provided a relatively narrow pH range increasing the resolution of the proteins. Focusing was started at 640 V with 12 W constant power and was continued for 4 h, final voltage was 1355 V. The higher voltage of the run reflects the lower ionic strength of the sample. Focused fractions were harvested and assayed for enzyme activity. Figure 6.2.

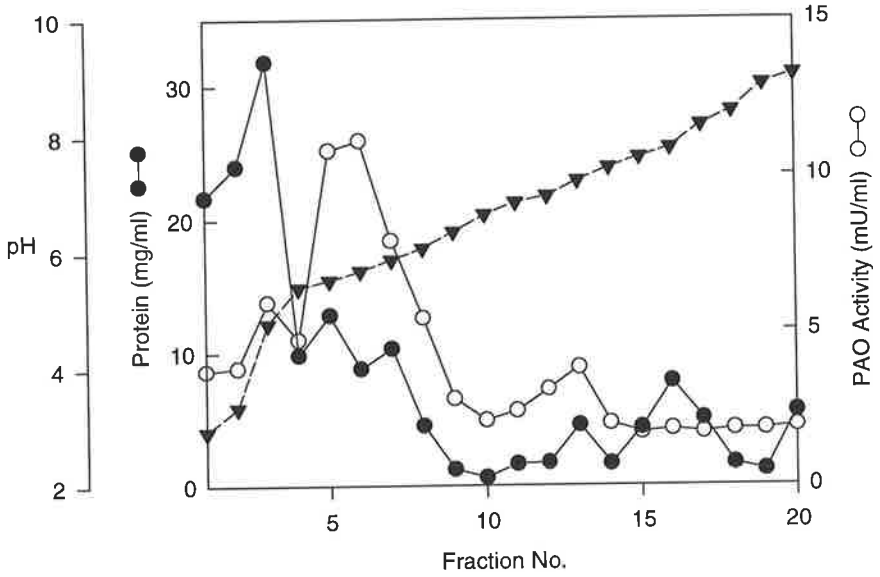


FIGURE 6.2. Refractionation of RPS in the Rotophor cell. Using pooled fractions pH 4.50 – 8.05 from the initial fractionation in Biolyte 3/10 ampholytes 2% (w/v). Inverted triangles indicate pH.

6.2.2.2 Analytical Isoelectric Focusing

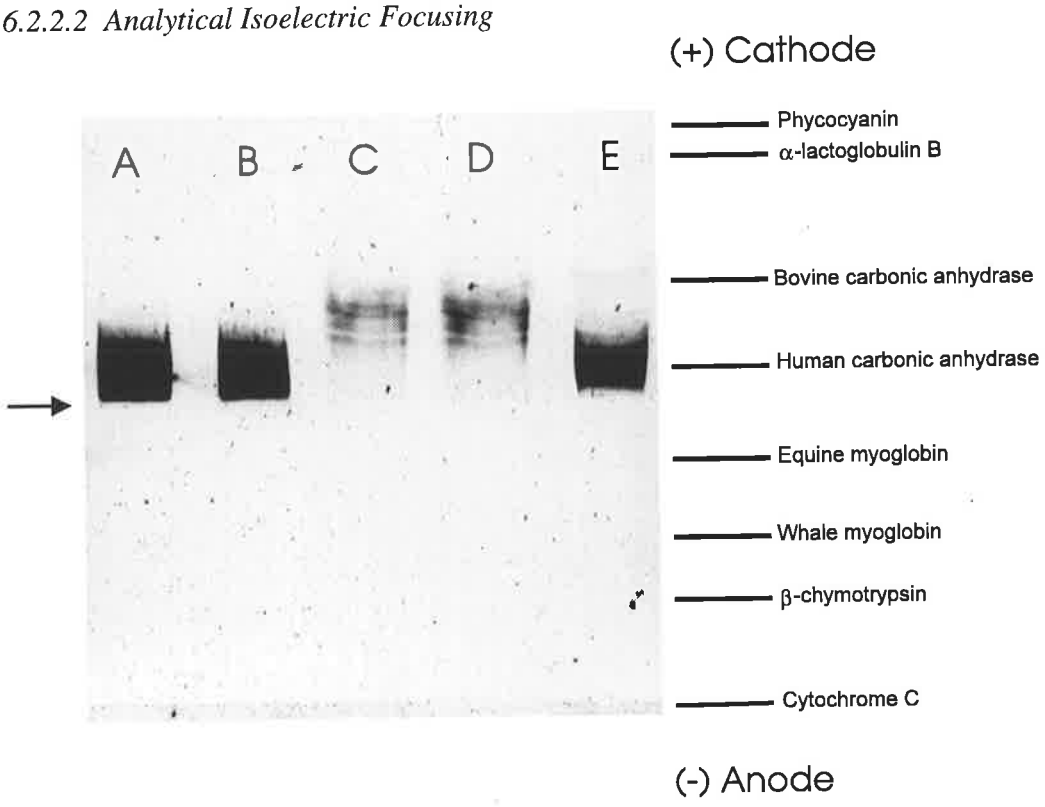


FIGURE 6.3. Horizontal Analytical Isoelectric Focusing of Human RPS Polyamine Oxidase Multiple forms were applied to a 0.4 mm 5% polyacrylamide slab gel and focussed according to the protocol described in section 2.11.2. Ampholyte range was pH 3–10. Samples in: lanes **A** and **B**

were PAO II (0.5 µl, 0.39 µg each lane); lanes **C** and **D** were PAO I (1.0 µl, 1.42 µg); lane **E** contained a mixture of PAO I and II (0.2 µg and 0.7 µg respectively). Bio-Rad broad range IEF standards, p*I* 4.6–9.6 were used for p*I* calibration, their positions are indicated on the right hand side of the gel. The *pI* values that the constituent proteins focus at are stated by Bio-Rad to be determined by direct measurement with a surface pH electrode and are: phycocyanin, 4.65; β-lactoglobulin B, 5.10; bovine carbonic anhydrase, 6.00; human carbonic anhydrase, 6.50; equine myoglobin, 7.00; whale myoglobin, 8.05; α-chymotrypsin, 8.80; and cytochrome c, 9.60. The arrow indicates the position of sample application.

6.2.3 High Resolution Two-Dimensional Electrophoresis

The silver stained gel (Figure 6.4) indicates that retroplacental amine oxidase exhibits microheterogeneity after high resolution two-dimensional (2D) electrophoresis (*q.v.* §2.10). The diffuse nature of the amine oxidase band at 1 shows one major isoelectric form, 1a, and at least 6 minor isoelectric forms toward the anode. The spot at 2 was an unknown. The sharp M_r 106,000 band observed in Figure 5.10 (lanes 2,3) did not appear to be present in the immunoaffinity preparation. The 2D-electrophoresis would have revealed any proteins underlying the M_r 108,000 band (cf. §5.2.2.5). The trails of spots at 3 and 4 were unknown proteins in the enzyme preparation. The group of spots at 5 were probably isoforms of mouse IgG heavy chain stripped from the immunoaffinity column during desorbtion of the enzyme. A Coomassie Blue stained replica gel showed a similar pattern though the staining was much fainter with the less sensitive staining (results not shown).

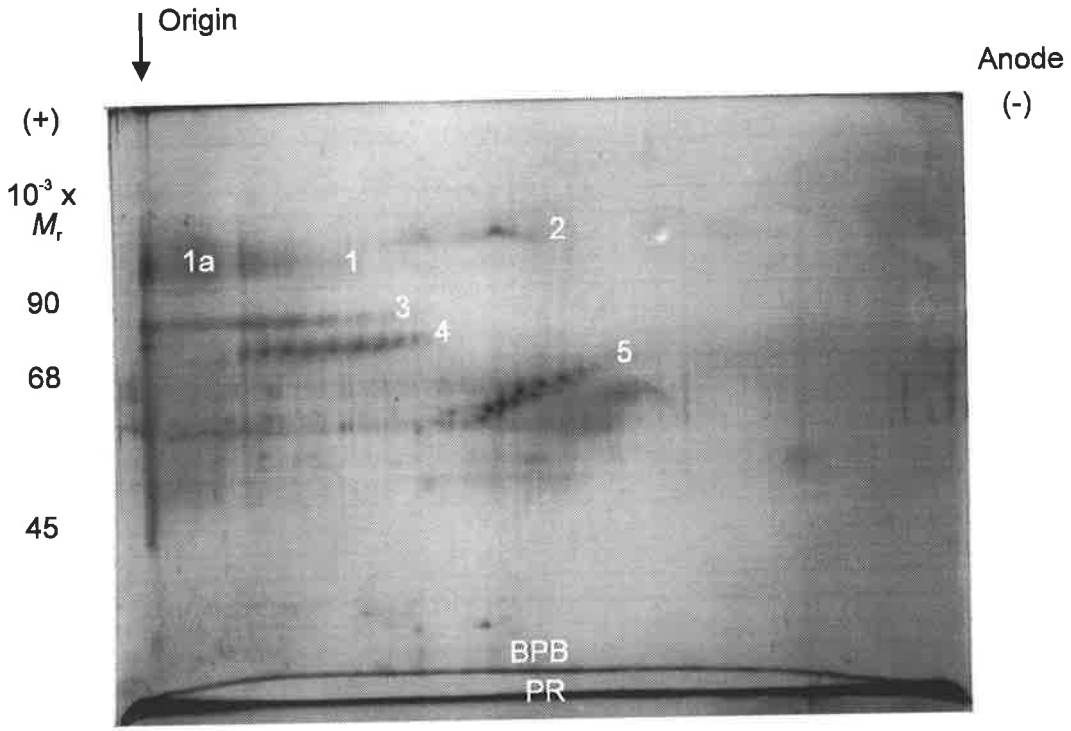


FIGURE 6.4. High-resolution Two-Dimensional Polyacrylamide Electrophoresis of Immunoaffinity Purified Retroplacental Amine Oxidase. 2D-Electrophoresis of the 22.5 µg sample was as described in the text (*q.v.* §2.10.1). The proteins were silver stained using the Merril method (*q.v.* 2.7.7.3). The two lines at the bottom of the gel indicate the positions of the tracker dyes bromophenol blue (BPB) and phenol red (PR). The acidic end of the IEF gel is toward the left, and the higher pH values toward the right. The second dimension is oriented such that relative molecular mass decreases toward the bottom of the gel.

6.2.4 Steady-State Kinetic Parameters for Enzymatic Polyamine Oxidation

Steady-state kinetic parameters, K_{mA}, K_{iA}, were determined for each substrate using methods described in section 2.3.2 and in the notes to Table 6.1. Initial rate data, displayed as Michaelis–Menton plots and Lineweaver–Burk transformations, are presented in Appendix C.

Notes to Table 6.1

1. $[O_2]$ in the kinetics assays was considered to be that for air saturated solutions at standard atmospheric pressure (101.325 kPa), 210 µM [1917]. When the assay mixture was saturated with pure oxygen no increase in reaction velocity, v, was observed.

2. [a] 26.29 pM, [b] 52.58 pM, [c] 105.2 pM, [d] 22.19 pM, [e] 44.38 pM, [f] 88.76 pM.
$k_{cat} = V_{max}/[E]_0$; where $[E]_0$, the stoichiometric concentration of active catalytic centres, was based on estimates of the active site concentration in the peak ion exchange fractions used in the kinetics assays. The purification process will inactivate a proportion of the enzyme resulting in a reduced active site purity in the enzyme. These activity losses are suggested by the data in Table 5.2. Activity recovery from the immunoaffinity purification step and enzyme purity based on electrophoretic data and SE-HPLC chromatograms indicate that the active site purities of the final enzyme preparations were 13.9% and 17.5% respectively for PAO I and PAO II. So if 100% of the activity were retained, the specific activity of the PAO I and PAO II preparations would be 17,353 mU/mg and 19,640 mU/mg respectively for the conditions indicated in Table 5.2. The specific activities of the peak ion exchange fractions used in the steady-state kinetics assays were 504.7 mU/mg for PAO I, DEAE Trisacryl fraction no. 67 (58.56 µg/ml) and 217.8 mU/mg for PAO II, DEAE Trisacryl fraction no. 83 (diluted ×2.5 in 25 mM Tris-HCl, containing 30 mM NaCl pH 7.4; 129.8 µg/ml). Thus, the active site concentrations in the assays were determined, assuming a single catalytic site per M_r *108,000* subunit in agreement with studies of the bovine serum enzyme [848] and more recent structural studies on the *E. coli* [529] and pea seedling enzymes [650]. More precise values for the concentration of catalytic centres remain to be determined by active site titration, with, for example, phenylhydrazine as described for pig plasma amine oxidase [923,928] and bovine serum amine oxidase [848] or with an enzyme-activated irreversible inhibitor such as MDL 72527 [968] (*q.v.* §2.3.2.2).

3. Initial rate velocities were determined for substrate concentrations in the range 0.01 – 1000 µM and Michaelis parameters were calculated for concentrations in the range 0.2 – 50 K_{mA}. Initial velocities were determined by analysis of the slopes of the initial, linear portion of progress curves of the coupled enzyme reaction, which was generally followed for at least 5 min. Initial velocities were determined from the slope of a least squares linear regression to the initial linear portion of the progress curve from a nominal lag time of t = 20 s, until t = 30–70 s using the Obey software (Ver. 3.50 ©Perkin–Elmer, 1988) bundled with the LS-50B luminescence spectrophotometer. During this time, substrate depletion was generally less than 5%, except at the lowest substrate concentrations where depletion did not exceed 10%. The assay period was restricted to avoid competitive reactions, and so that substrate depletion and product accumulation were negligable.

4. Values were ± standard error (*vide* Appendix C)

TABLE 6.1. Steady-state Kinetic Parameters for Enzymatic Polyamine Oxidation at pH 7.43 ± 0.05, 37.0 ± 0.1 °C, 0.05 M Tris–HCl.

[1]A	K_{mA}, μM		K_{iA}, μM		[2]k_{cat} s^{-1}		k_{cat}/K_{mA}, $M^{-1}.s^{-1}$		$\frac{k_{cat}/K_{mA}}{k_{catPutrescine}/K_{mPutrescine}}$	
Substrate	I	II	I	II	I	II	I	II	I	II
Putrescine	1.30 ± 0.08	1.14 ± 0.1	51 ± 3.6	61 ± 7	43.1[a]	44.1[d]	3.3×10^7	3.8×10^7	1.000	1.000
Spermidine	5.51 ± 0.23	5.50 ± 0.3	337 ± 58	438 ± 133	43.4[b]	40.5[e]	7.9×10^6	7.3×10^6	0.239	0.192
Spermine	1.86 ± 0.11	2.11 ± 0.1	137 ± 42	410 ± 207	6.97[c]	6.65[f]	3.7×10^6	3.2×10^6	0.112	0.084
N^1-Acetylspermidine	4.87 ± 0.36	5.05 ± 0.4	314 ± 57	774 ± 251	15.3[b]	14.4[b]	3.1×10^6	2.8×10^6	0.094	0.074
N^1-Acetylspermine	0.16 ± 0.01	0.14 ± 0.0	nd	nd	2.36[b]	2.52[b]	1.5×10^7	1.8×10^7	0.455	0.474
Histamine	0.30 ± 0.02	0.27 ± 0.0	12 ± 3	26 ± 4	25.6[b]	24.2[e]	8.4×10^7	8.8×10^7	2.545	2.316

K values are shown ± standard error (see Appendix C).

6.2.5 Enzymatic Reaction Products

Figure 6.5 shows the results of a time course study of the human retroplacental serum polyamine oxidase reaction (*q.v.* §2.4.2), in which the enzymatic production of spermidine from spermine, putrescine from spermidine (and subsequently, it can be assumed 4-aminobutyraldehyde and Δ^1-pyrroline from putrescine) is demonstrated. The use of a [^{14}C]spermine radiolabelled at the internal putrescine moiety (*N,N'*-bis(3-aminopropyl)-[1,4-^{14}C]tetramethylene-1,4-diamine) clearly indicates that the retroplacental enzyme cleaves polyamines at their secondary amino groups with the formation of aminopropionaldehyde or ammonia as one of the reaction products (*q.v.* Figure 1.7).

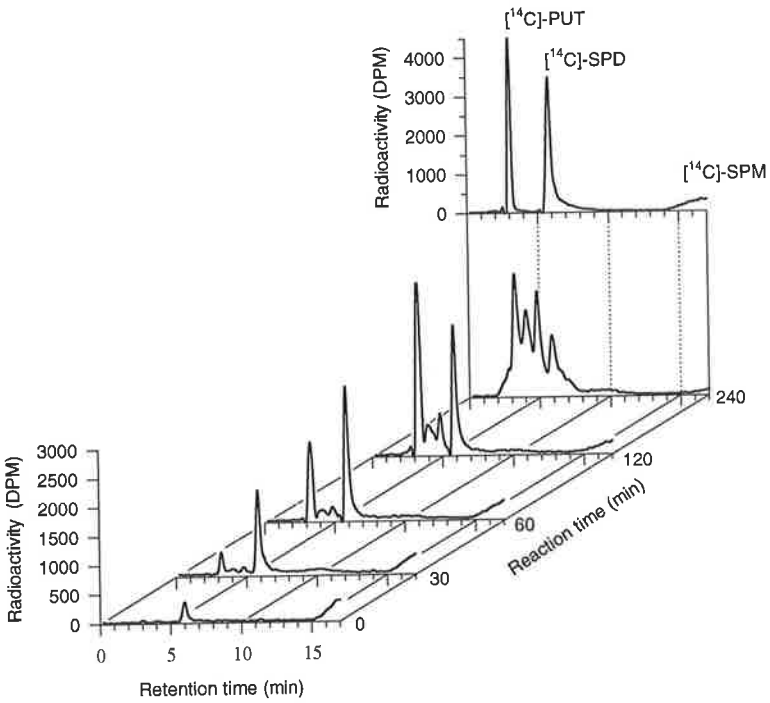


FIGURE 6.5. Analysis of Polyamine Oxidase Reaction Progress. Reaction progress with [^{14}C]spermine as the substrate for an affinity purified retroplacental serum enzyme preparation after the initiation of the reaction: sequentially at 30 s and 30, 60, 120, and 240 min. Reversed phase, ion-pairing HPLC and other experimental procedures are described in section 2.4.2. The time dependent decrease of spermine, and increase in spermidine, putrescine and its metabolites are shown on the chromatograms. The uppermost chromatogram shows the retention times of radiolabelled polyamine standards. PUT, putrescine; SPD, spermidine; SPM, spermine.

6.2.6 UV–Visible Absorption Spectrum

Concentrated immunoaffinity purified enzyme solutions were a pink-orange colour. The absorption spectrum of purified polyamine oxidase sample showed the characteristic protein absorption peak at 280 nm (0.144 AU) and a broad less intense peak (0.012 AU) around 450–500 nm.

6.2.7 Copper Content

The thermal atomic absorption analysis method described in section 2.14 indicated 2.09 copper atoms per M_r 218,000 dimer.

6.2.8 Inhibition Studies

Retroplacental polyamine oxidase (diamine oxidase activity) was strongly inhibited by quinacrine, aminoguanidine and MGBG. Pargyline and clorgyline were not inhibitory at 1.0 mM and isoniazid 22% inhibitory at 1.0 mM (Table 6.2).

TABLE 6.2. Polyamine Oxidase Inhibition

Inhibitor	*[Inhibitor] mM*	*% inhibition*[b.]
quinacrine	1.00	99.7
	0.10	97.6
	0.01	91.5
isoniazid	1.00	22.1
	0.10	3.0
pargyline	1.00	–3.4
	0.10	–9.0
semicarbazide	1.00	93.8
	0.10	49.3
	0.01	2.3
aminoguanidine	1.00	99.8
	0.10	99.7
	0.01	96.0
clorgyline	1.00	–2.4
	0.10	–4.1
MGBG[a.]	1.00	99.7
	0.10	98.8

Notes to Table 6.2

a. methylglyoxal-bis-(guanylhydrazone)

b. Measured in 0.05 M Tris–HCl, pH 7.4 using a procedure based on the method of Okuyama and Kobayashi [877,1918] as described in Chapter 2 (*q.v.* §2.4.3). Briefly, [^{14}C]putrescine was diluted with cold putrescine to give a final concentration of 102 µM in the assay with a specific activity of 2.45 mCi/mmole. Inhibitors were included in the assay mixture and the reactions were initiated by the addition of approx. 0.45 mU of the enzyme (in 50 µl) that had been purified to the end of the ω-aminohexylagarose chromatography step, *v.* Table 5.2). Reactions were allowed to proceed for 60 min at 37.0 °C, and then terminated with 100 µl saturated sodium carbonate solution. Reaction products were immediately extracted into a scintillation cocktail. The aqueous phase was frozen and the organic phase decanted for scintillation counting. Inhibitors were titrated with the enzyme substrate system and the percentage inhibition of the unfettered reaction determined.

6.2.9 *N*-Terminal Sequencing

Chromatograms from analyses of PTH amino acid derivatives generated by the Applied Biosystems 475A protein sequenator are presented in Appendix D along with tables showing the interpretation of the chromatograms.

PAO I (Ref:PSE 265)

A band corresponding to that in Figure 5.10, lane 7 at M_r 108,000 (PAO I) was excised from a PVDF membrane and the *N*-terminal amino acid sequence of the electroblotted protein determined as described in section 2.13. The signal consisted of one major sequence (shown below) which had an initial yield of 15 pmoles and made up >70% of the total protein signal. What appeared to be minor sequences or contamination were probably a result of the sample being slightly acid labile. Proteins with similar molecular weight may have contributed to the minor protein signals, although the presence of contaminating proteins of similar molecular weight was not suggested by two dimensional electrophoresis (*q.v.* §6.2.3). The *N*-terminal amino acid sequence was:

Glu-Pro-Ser-Pro-Gly-Thr-Leu-Pro-Arg-Xxx-Ala-Gly-Val-Phe-Ser-Asp-

PAO II (Ref:PSE 267)

Similarly, a sample corresponding to the band in Figure 5.10, lanes 4,5,6 at M_r 108,000 (PAO II) was sequenced. The sample had an initial yield of 100 pmoles with one major sequence (described below) making up >80% of the total protein signal. Approximately 200 pmoles was loaded into each gel lane and 90 pmoles was calculated to be present in the stained bands of interest, transfer efficiencies were approximately 80% [1597], so the initial yield efficiency for the 3 bands loaded was 46%, which was within the range of expected values (*vide* §2.13.1.5 and references therein). The *N*-terminal amino acid sequence was:

Glu-Pro-Ser-Pro-Gly-Thr-Leu-Pro-Arg-Lys-Ala-Gly-Val-Phe-Ser-Asp-Leu-Ser-
Asn-Gln-Glu-Leu-Lys-Ala-Val-His-Ser-

A FASTA [1919] search of the Swiss-Prot protein sequence data bank [1920] (release 32, November 1995; current March 1996, Amos Bairoch, Department of Medical Biochemistry, University of Geneva, Switzerland; *personal communication*) through FastA@ebi.ac.uk gave a best score of 118 with Abp_Human Entry #P19801, amiloride-sensitive amine oxidase [47,757,781]. Corresponding to a 100.0% identity in a 27 amino acid overlap. The FASTA search was made by submitting the sequence to the EBI (European Bioinformatics Institute) EMBL (European Molecular Biology Laboratory) outstation at FastA@ebi.ac.uk. A FASTA search submission form for submitting searches on a WWW page was found at http://www.ebi.ac.uk/searches/fasta.html.Access to the Swiss-Prot entries was through the Internet at:

http://www.ebi.ac.uk/htbin/swissfetch?P19801

or through the ExPASy server [1921] @http://expasy.hcuge.ch/cgi-bin/sprot-search-ac?P19801

The EMBL (release 45, December 1995) entries were found at:
http://www.ebi.ac.uk/htbin/emblfetch?M55602 [781]and
http://www.ebi.ac.uk/htbin/emblfetch?X78212 [757].

PAO A (Ref: PSE 279)

A sample corresponding to the band in Figure 5.10, lanes 4,5,6 and 7 at M_r 218,000 (potentially a dimer of PAO subunits) was sequenced. The sequencing cycles were terminated at cycle 10 since the same sequence as that for PAO I and II was found. The *N*-terminal amino acid sequence was:

Glu-Pro-Xxx-Pro-Gly-Thr-Leu-Pro-Arg-Lys-

PAO (Ref: PSE 201)

As described above, a protein corresponding to the band in Figure 5.10, lanes 4,5,6 and 7 at M_r 108,000 (a combination of PAO I and PAO II) was detected as having an initial yield of 10 to 15 pmoles (about 25%). An estimated 300 pmoles was loaded onto the gel and by laser densitometry of a replica gel an estimated 66 pmoles was present in the stained band to be sequenced. There was one major sequence and apparently some background contaminants, a lot of which were probably a result of baseline movement.

At that time a Swiss-Prot (release number 22.0) FASTA [1919] GenBank search indicated that the sequence (described below) matched that of ABP_HUMAN, human amiloride-binding protein precursor, with a score of 87 and a 73.1% identity in 26 amino acid overlap. The next best match, outer capsid protein VP2, was much poorer with 37.5% identity in a 16 amino acid overlap. Apparent mismatches precluding 100% identity with the human amiloride-binding protein precursor could be attributed to low yields for PTH amino acid derivatives during the Edman sequencing. The *N*-terminal amino acid sequence was:

Ser-Pro-Xxx-Pro-Gly-Thr-Leu-Pro-Arg-Lys-Ala-Gly-Val-Phe-Ser-Asp-Leu-Ser-Asn-Gln-Glu-

A major contaminant (Ref: PSE 204), resolved by SDS-PAGE analysis of the sample used above (for Ref: PSE 201) was found to be mouse IgG heavy chain (precursor). The protein was detected as having an initial yield of approximately 2 to 5 pmoles. There was one major sequence (described below). A Swiss-Pro Blast FASTA Search indicated that the sequence match was definitely that of IgG heavy chain. This was considered to be a likely contaminant through bleeding of the mAb from the immunoaffinity column. The *N*-terminal amino acid sequence was:

Pro-Val-Gln-Leu-Val-Glu-Ser-Gly-Xxx-Gly-Leu-Val-Gln-Pro-Gly-Gly-Ala-Leu-Glu-Xxx-Ser-Gly-Val-Gly-Val-

6.2.9.1 Sequencing Summary

Both forms PAO I and PAO II had 100% NH_2-terminal sequence identity with human amiloride binding protein for at least 16 amino acids (Figure 6.6). Furthermore, what was apparently a homodimer of the enzyme that appeared at M_r 218,000 on gels (PAO A) had an NH_2-terminal sequence identical to amiloride binding protein for the 10 residues identified. The human amiloride-binding protein has been characterized as amiloride-sensitive copper-containing amine oxidase (associated with human pregnancy) [47].

```
humanABP.seq    MPALGWAVAAILMLQTAMAEPSPGTLPRKAGVFSDLSNQELKAVHSFLWSKKELRLQPSS
PAOII.SEQ       ------------------EPSPGTLPRKAGVFSDLSNQELKAVHS--------------
PAOI.SEQ        ------------------EPSPGTLPRXAGVFSD------------------------
PAOA.SEQ        ------------------GPXPGTLPRK----------------------------
PAO.SEQ         ------------------SPXPGTLPRKAGVFSELSNQEXFSVPN--------------
                                  * ******
```

FIGURE 6.6. CLUSTAL W (1.5) Multiple Sequence Alignment
The CLUSTAL W (version 1.5, April 1995) multiple sequence alignment program [1922,1923], used for the sequence comparison was obtained over the Internet by anonymous ftp from <ftp.ebi.ac.uk/pub/software/dos/clustal$.exe>. Sequences were converted from text to FASTA format files [1919] using the BBSeqF (version 1.2) MS-Windows program available from the SimTel mirror @ <archie.au/micros/pc/SimTel/win3/biology> by anonymous ftp. FASTA files were concatenated for entry into the CLUSTAL multiple sequence alignment program using the multiple sequence alignment editor of the SeqPup program (version 0.4, July 1995 release) for MS-Windows written by D. Gilbert and available by anonymous ftp from <ftp://iubio.bio.indiana.edu/molbio/seqpup/>. "*" is used to indicate identical residues.

The initiation site of the amiloride-sensitive copper-containing human amine oxidase, i.e. residue number 1, was assigned to the methionine, which starts a 19-amino acid hydrophobic stretch that shows all the typical features of a signal sequence [1924]. The *N*-terminal sequence identified in this work by Edman degradation appears at residue 20 (Figure 6.6) and is situated just after the putative signal sequence.

6.2.9.2 Sequence Analysis

The human (amiloride-sensitive) copper-containing amine oxidase sequence was analysed here using several software packages available at various ftp sites on the Internet: The sequence (Swiss Protein entry P19801; EMBL entry X78212) was obtained from screening a human genomic library [757] (Figure 6.7).

```
SQ   SEQUENCE   751 AA;  85363 MW;  3CFA8045 CRC32;
     MPALGWAVAA ILMLQTAMAE PSPGTLPRKA GVFSDLSNQE LKAVHSFLWS KKELRLQPSS      60
     TTTMAKNTVF LIEMLLPKKY HVLRFLDKGE RHPVREARAV IFFGDQEHPN VTEFAVGPLP     120
     GPCYMRALSP RPGYQSSWAS RPISTAEYAL LYHTLQEATK PLHQFFLNTT GFSFQDCHDR     180
     CLAFTDVAPR GVASGQRRSW LIIQRYVEGY FLHPTGLELL VDHGSTDAGH WAVEQVWYNG     240
     KFYGSPEELA RKYADGEVDV VVLEDPLPGG KGHDSTEEPP LFSSHKPRGD FPSPIHVSGP     300
     RLVQPHGPRF RLEGNAVLYG GWSFAFRLRS SSGLQVLNVH FGGERIAYEV SVQEAVALYG     360
     GHTPAGMQTK YLDVGWGLGS VTHELAPGID CPETATFLDT FHYYDADDPV HYPRALCLFE     420
     MPTGVPLRRH FNSNFKGGFN FYAGLKGQVL VLRTTSTVYN YDYIWDFIFY PNGVMEAKMH     480
     ATGYVHATFY TPEGLRHGTR LHTHLIGNIH THLVHYRVDL DVAGTKNSFQ TLQMKLENIT     540
     NPWSPRHRVV QPTLEQTQYS WERQAAFRFK RKLPKYLLFT SPQENPWGHK RSYRLQIHSM     600
     ADQVLPPGWQ EEQAITWARY PLAVTKYRES ELCSSSIYHQ NDPWHPPVVF EQFLHNNENI     660
     ENEDLVAWVT VGFLHIPHSE DIPNTATPGN SVGFLLRPFN FFPEDPSLAS RDTVIVWPRD     720
     NGPNYVQRWI PEDRDCSMPP PFSYNGTYRP V                                    751
//
```

FIGURE 6.7. The Human (Amiloride-Sensitive) Copper-Containing Amine Oxidase Sequence

a) Molecular mass calculation

Amino acid composition and molecular mass of P19801 (Table 6.3) were determined using the aacomp.exe file of the FASTA program package ver 2.0x by W.R. Pearson of the University of Virginia, Charlottesville, VA available for IBM-PC/DOS @ <uvaarpa.virginia.edu/pub/fasta/dos/fa20u316.zip> by anonymous file transfer protocol (ftp).

TABLE 6.3 Amino Acid Composition and Molecular Mass of P19801 (Human Amiloride-Sensitive Copper-Containing Amine Oxidase)

Amino Acid		*No.*	*mole%*	*wt%*
A	Ala	48	6.39	4.00
C	Cys	7	0.93	0.85
D	Asp	32	4.26	4.32
E	Glu	43	5.73	6.51
F	Phe	42	5.59	7.24
G	Gly	55	7.32	3.68
H	His	36	4.79	5.78
I	Ile	22	2.93	2.92
K	Lys	24	3.20	3.60
L	Leu	69	9.19	9.15
M	Met	13	1.73	2.00
N	Asn	28	3.73	3.74
P	Pro	62	8.26	7.06
Q	Gln	30	3.99	4.50
R	Arg	44	5.86	8.05
S	Ser	46	6.13	4.69
T	Thr	45	5.99	5.33
V	Val	53	7.06	6.16
W	Trp	18	2.40	3.93
Y	Tyr	34	4.53	6.50
B	Asx	0	0.00	0.00
Z	Glx	0	0.00	0.00
X	?	0	0.00	0.00

751 aa; molecular mass: 85,357.6 Da

b) Hydrophobicity Profile

The hydrophobicity profile of M55602 was determined and is displayed in Figure 6.8 as a hydropathy plot. The hydrophobicity profile of a protein was first described by Kyte and Doolittle [1925] and consists of averaging the hydrophobicity index of individual amino acids.

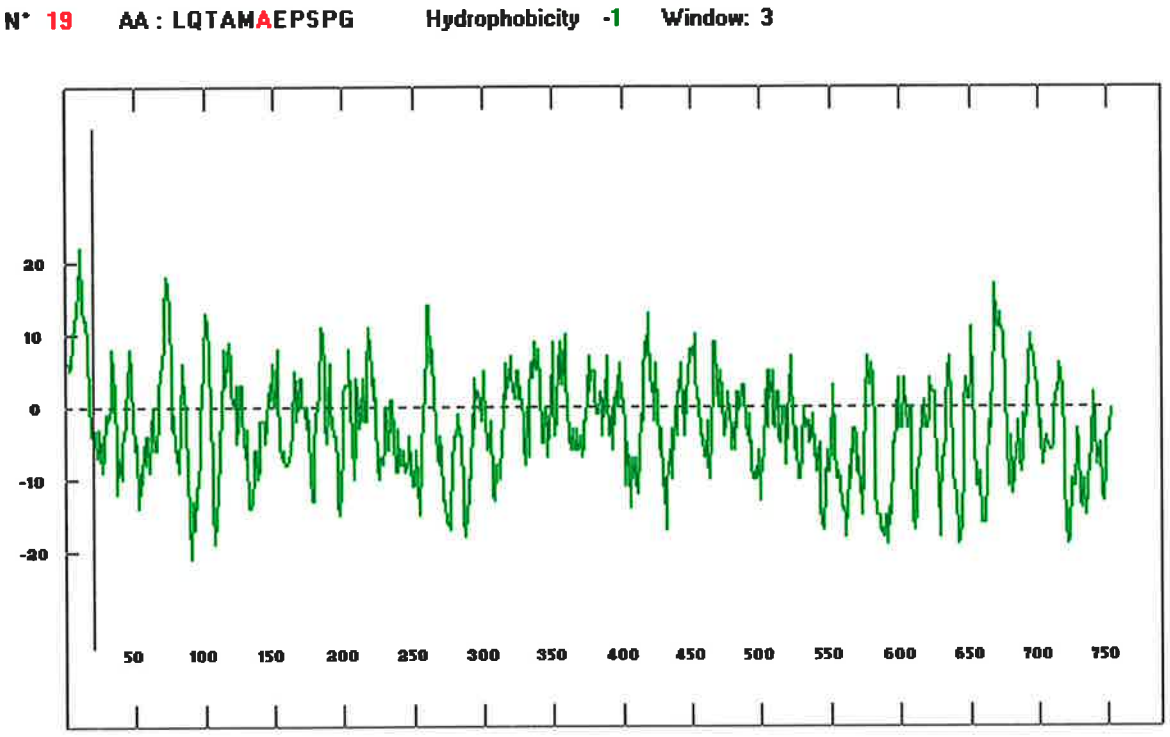


FIGURE 6.8. Hydropathy Plot of the Human Amiloride-Sensitive Amine Oxidase. (Copper-containing) precursor protein sequence [781], EMBL entry X78212; Swiss-Prot entry P19801. Note the highly hydrophobic signal sequence at the amino terminus (residues 1–19). Numbering starts from the first methionine residue, indicating a putative signal sequence. The cursor is placed at residue 19. Values indicating hydrophobicity and hydrophilicity are above and below zero respectively. The hydropathy plot for human amiloride-sensitive amine oxidase was generated using ANTHEPROT software for Windows, release 1.0, by G. Deleage from the Institut de Biologie et Chimie des Proteins, Lyon available from <ibcp.fr/pub/ANTHEPROT/windows/anthepro.exe + prosite.exe + swiss.exe> by anonymous ftp.

c) Secondary Structure

Profiles of human amiloride-sensitive copper-containing amine oxidase secondary structure were determined using ANTHEPROT software (*q.v.* Figure 6.8 caption) and are consistent with that of a soluble globular protein (Figures 6.9 and 10). The predicted accuracy of the two methods is about 59% and 62% respectively against the Kabsch and Sander database [1926] though it is generally agreed that the best predictions result from averaging over several methods [1927,1928].

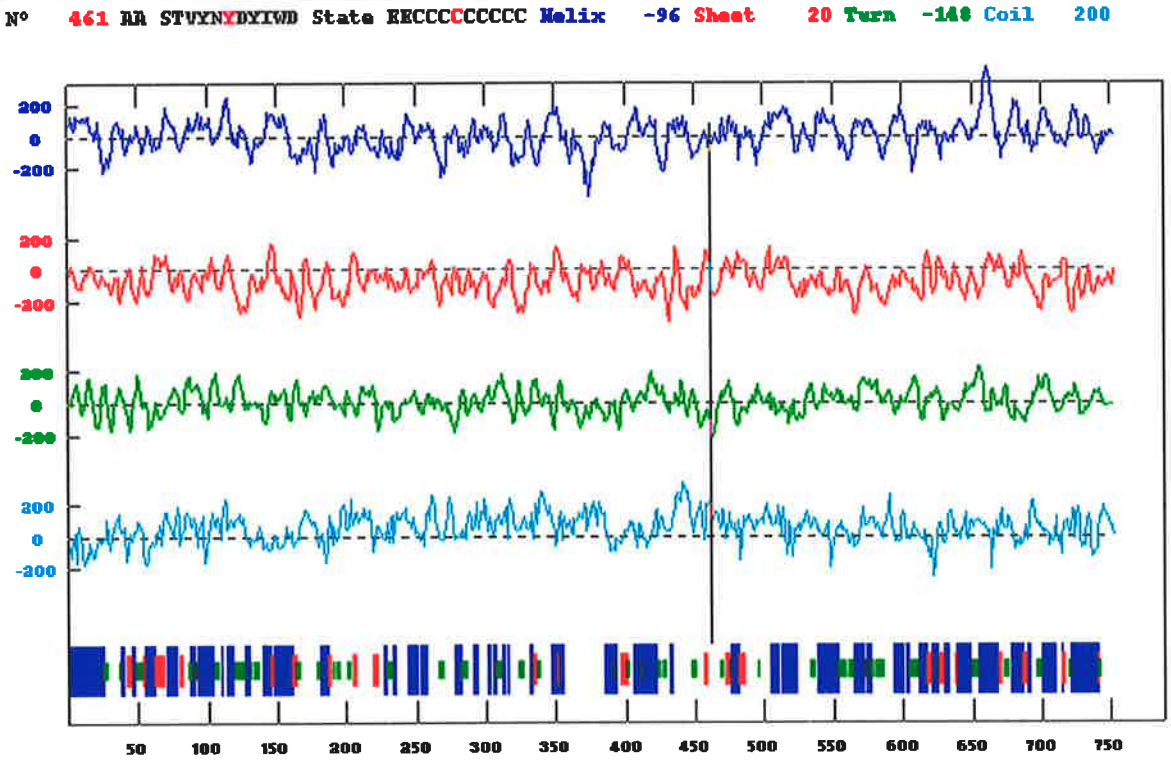


FIGURE 6.9. Secondary Protein Structure Prediction Human Amiloride-Sensitive (Copper-Containing) Amine Oxidase. Precursor protein sequence EMBL entry X78212; Swiss-Prot entry P19801.] using the Garnier–Robson [GOR I] method [1929] (as part of the ANTHEPROT software suite) with optimized decision constants, viz. helix 40; sheet 55; turn 30; coil 0; which gave a whole protein predicted content of helix 33%, sheet 6%, turn 14%, coil 48%.

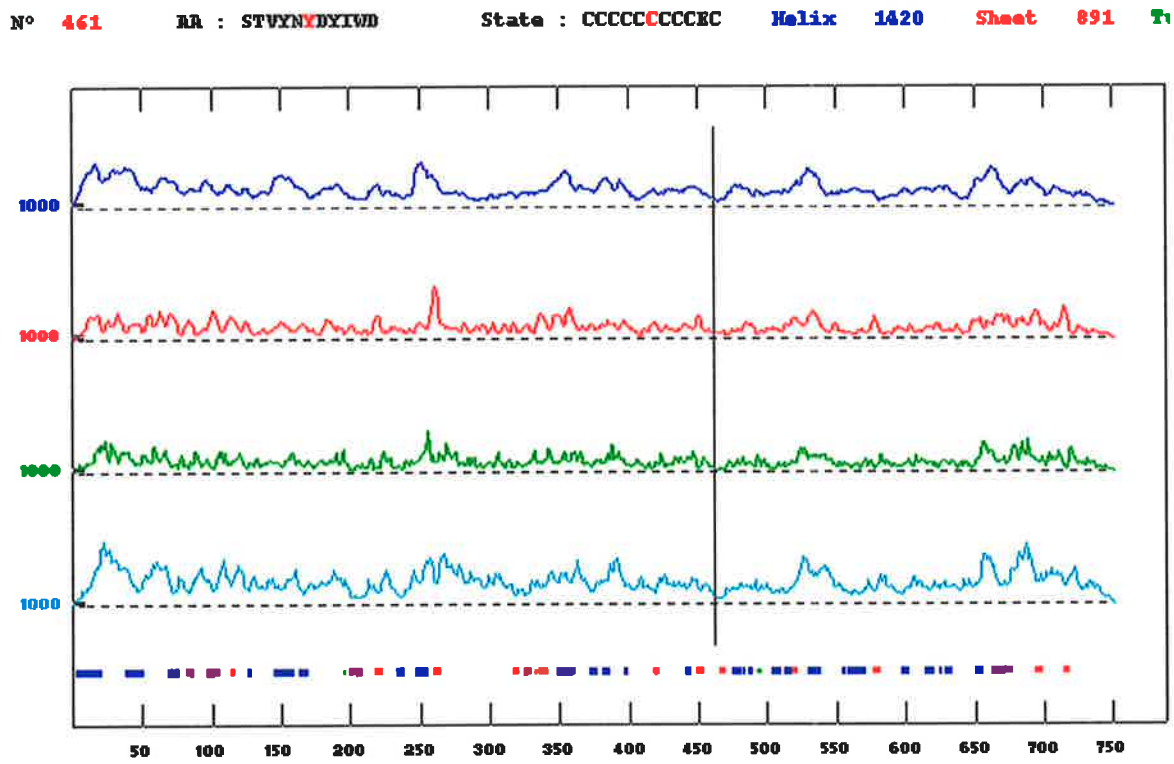


FIGURE 6.10. Homologue Secondary Protein Structure Prediction. Using the Levin method (as part of the ANTHEPROT software suite), which uses similarity with proteins of known secondary structure [1930], a whole protein content of helix 20%, sheet 8%, turn 1%, coil 71% was predicted.

d) Antigenicity Profile

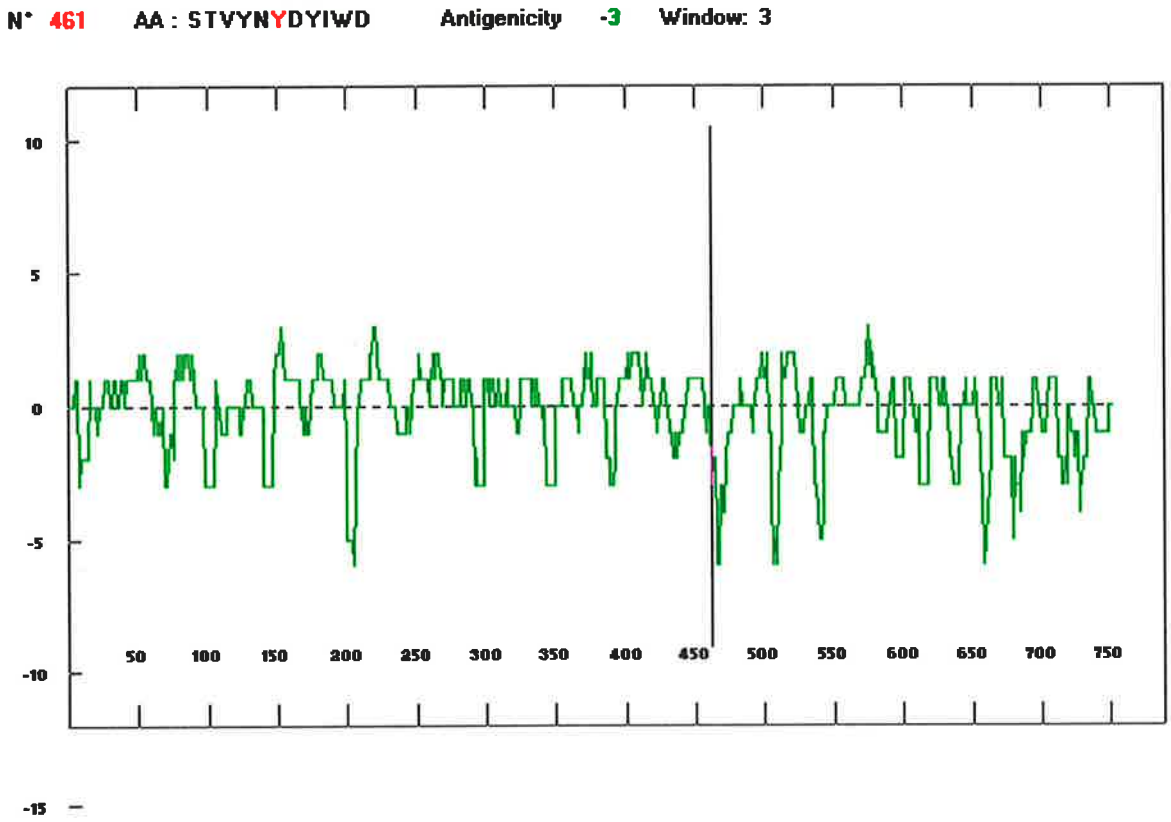


FIGURE 6.11. Antigenicity Profile Prediction. By the method of Welling *et al.* (as part of the ANTHEPROT software suite) that calculates antigenic preferences for each amino acid from known antigenic sites in sequences and plots the potentials [1931]. It is of interest that region surrounding the active site (residues 453–477; see cursor) is not predicted to be particularly antigenic, since this was suggested by Baylin [748] (*q.v.* §4.1.2).

e) Consensus Patterns

A Prosite search for consensus patterns in the sequence for human amiloride-sensitive amine oxidase, using the Prosite.Dat database [1932] from the EMBL EBI library (release no. 12, November 1995), identified a number of interesting regions that are presented below.

Similarity percentage 100
Number of mismatches allowed 0

|B|1N-glycosylation site|b|0 |8|gPS00001|0
N-[P]-[ST]-[P]
Theoretical frequency: 4.97E-003

```
Site :    110  to   113  NVTE   Observed frequency: 1.15E-005
Site :    168  to   171  NTTG   Observed frequency: 1.17E-005
Site :    538  to   541  NITN   Observed frequency: 5.80E-006
Site :    745  to   748  NGTY   Observed frequency: 6.68E-006
```

|B|1Protein kinase C phosphorylation site|b|0 |8|gPS00005|0
[ST]-x-[RK]
Theoretical frequency: 1.44E-002

```
Site :     50  to    52  SKK   Observed frequency: 4.45E-003
Site :    129  to   131  SPR   Observed frequency: 3.16E-003
Site :    284  to   286  SHK   Observed frequency: 4.45E-003
Site :    544  to   546  SPR   Observed frequency: 3.16E-003
Site :    592  to   594  SYR   Observed frequency: 3.16E-003
Site :    747  to   749  TYR   Observed frequency: 2.81E-003
```

|B|1Casein kinase II phosphorylation site|b|0 |8|gPS00006|0
[ST]-x(2)-[DE]
Theoretical frequency: 1.50E-002

```
Site :     37  to    40  SNQE   Observed frequency: 4.19E-003
Site :     50  to    53  SKKE   Observed frequency: 4.19E-003
Site :    144  to   147  STAE   Observed frequency: 4.19E-003
Site :    154  to   157  TLQE   Observed frequency: 3.73E-003
Site :    173  to   176  SFQD   Observed frequency: 3.75E-003
Site :    215  to   218  TGLE   Observed frequency: 3.73E-003
Site :    245  to   248  SPEE   Observed frequency: 4.19E-003
Site :    275  to   278  STEE   Observed frequency: 4.19E-003
Site :    351  to   354  SVQE   Observed frequency: 4.19E-003
Site :    396  to   399  TFLD   Observed frequency: 3.34E-003
Site :    581  to   584  SPQE   Observed frequency: 4.19E-003
Site :    599  to   602  SMAD   Observed frequency: 3.75E-003
```

|B|1N-myristoylation site|b|0 |8|gPS00008|0
G-[EDRKHPFYW]-x(2)-[STAGCN]-[P]
Theoretical frequency: 1.60E-002

```
Site :    191  to   196  GVASGQ   Observed frequency: 1.68E-005
Site :    195  to   200  GQRRSW   Observed frequency: 2.35E-006
Site :    224  to   229  GSTDAG   Observed frequency: 3.51E-005
Site :    473  to   478  GVMEAK   Observed frequency: 3.25E-005
Site :    494  to   499  GLRHGT   Observed frequency: 2.93E-005
Site :    507  to   512  GNIHTH   Observed frequency: 4.31E-006
Site :    524  to   529  GTKNSF   Observed frequency: 1.16E-005
Site :    689  to   694  GNSVGF   Observed frequency: 1.04E-005
```

Heparin binding site

A consensus heparin binding domain [1933], RFKRKLPK is located at R^{569} in human diamine oxidase.

Chapter 7

DISCUSSION AND CONCLUSIONS

7.1 Introduction

The ubiquity of polyamine oxidases and their role in a wide range of physiological and pathophysiological functions indicate the importance of these enzymes. Their identification and characterization are paramount in facilitating an understanding of their biological role. The literature is replete with confusing nomenclature of the human, especially pregnancy-associated, polyamine oxidases. Our understanding of these enzymes have been limited by a lack of reagents to and deficient characterization of these enzymes. Characterization of enzymes allows their definitive identification and comparison with enzymes isolated from other sources. Because of the relative abundance of polyamine oxidase in human retroplacental serum, and the relative ease with which this human enzyme source can be obtained, it was desirable to use this source as a starting point for the characterization of a human enzyme. Using polyamine oxidase purified from retroplacental serum, this study found that the retroplacental serum enzyme had the biological activity previously associated with unpurified retroplacental and pregnancy serum; indicating the potential importance of enzymes of its class in antimicrobial, immunosuppressive and anti-inflammatory mechanisms. It was of interest to determine whether the retroplacental serum polyamine oxidase was similar or identical to the other pregnancy-associated amine oxidases, and better characterized diamine oxidases from other species (of which the bovine serum enzyme is archetypical) *or* whether it was a similar to the flavin-containing polyamine oxidases such as that from the rat liver enzyme as reported in the literature [442,726]. It also remained to establish the identity of the multiple enzyme forms that had been observed in retroplacental serum [442].

Neither monoclonal antibodies nor a sequence for the human retroplacental serum polyamine oxidase have previously been available and therefore comprised major components of this work. During the course of this research monoclonal antibodies to placental and pregnancy serum enzymes were produced by others and used in attempts to immunoaffinity purify the enzymes. These studies failed to recognize the different molecular forms of the enzymes identified by others in these sources, and in retroplacental serum as observed in this study. The landmark discovery that the human placental diamine oxidase has a protein sequence virtually identical to the human amiloride-binding protein provided the conclusive evidence that the

retroplacental serum polyamine oxidase belongs to the class of amiloride-sensitive copper-containing amine oxidases containing TPQ as an active site cofactor. This Chapter includes a general discussion of the characterization of the human retroplacental serum polyamine oxidase concentrating on the results presented in Chapter 6 and draws conclusions about the nature of the enzyme.

7.2 Purification of the Human Retroplacental Serum Polyamine Oxidases

Like the multiple enzyme forms found in amniotic fluid [763] and placental extracts [747,755], the retroplacental enzyme appeared to exist as at least two molecular forms [442]. The possibility of multiple forms of the human plasma enzyme was suggested in a study by Hansson [706]. The findings are consistent with the observation of multiple forms of other amine oxidases, such as those in bovine serum [451] and mouse leukaemia cells [1023] (*q.v.* 1.3.4.4).

The isolation of two forms of human retroplacental serum polyamine oxidase that differed in their overall charge and relative molecular mass have been discussed in sections 3.4 and 5.3. Both forms of the human retroplacental serum enzyme, PAO I and PAO II, were comprised of identical M_r 108,000 subunits, which form homodimers held together by disulphide bonds in the enzyme's native state. Higher molecular weight forms, PAO II and larger inactive forms, appeared to be trimers, tetramers or aggregates of the enzyme subunit or the enzyme homodimer. PAO I and PAO II had identical *N*-terminal amino acid sequences for at least the 16 amino acid stretch determined for PAO I, as similarly demonstrated by Mondovì's group for multiple bovine serum enzyme forms [845]. Moreover, the *N*-terminal amino acid sequence of the polyamine oxidases isolated from human retroplacental serum showed 100% identity with human amiloride-sensitive copper-containing amine oxidase [47,757,781] for the 27 amino acid *N*-terminal sequence determined for PAO II.

Even using powerful resolving techniques including immunoaffinity chromatography and SDS-PAGE electrophoresis, 100% purity of the human retroplacental enzymes was not attained; as indicated by sensitive protein detection systems including silver staining. While purification of the enzyme to homogeneity (100% purity) facilitates its characterization, in practice enzymes are not purified to the point of absolute homogeneity. Even should other proteins constitute less than 1% of the purified protein there are likely to be millions of contaminating molecules present. However, such contaminants are not likely to be of any practical consequence, unless they interfere with the enzyme property being studied.

7.2.1 Relative Molecular Mass

The relative molecular mass of the human retroplacental serum polyamine oxidase homodimer subunit (M_r 108,215 ± 2,844), falls within the range of expected molecular weight values for copper-containing amine oxidases (*vide* §1.3.4.2). In contrast, the FAD-containing polyamine oxidases such as the rat liver enzyme and the polyamine oxidases of the Gramineae (e.g. maize, oats) are monomeric and have molecular weight range 53,000 – 85,000. Like other amine oxidases, the retroplacental serum enzyme is glycosylated, and has a carbohydrate content of apparently around 20% of its total molecular weight, which is slightly higher than the 7 to 14% carbohydrate content reported for most other amine oxidases. The anomalously high apparent molecular weight of the glycosylated polypeptide determined by SDS-PAGE in 7.5% PA gels may be a reflection of its large carbohydrate content.

The aggregation of the retroplacental serum enzyme to higher molecular weight forms is similar to that observed for the bovine serum enzyme, the pig kidney enzyme, and other pregnancy-associated enzymes during size exclusion chromatography as discussed elsewhere (*qq.v.* §3.4 and 5.3). The relatively less abundant lower molecular weight form (representing approximately 10% of the activity observed in human RPS), M_r 303,000 ± 46,000, may represent the enzyme homodimer; whereas the M_r 544,000 ± 86,000 form, PAO II, may be an aggregate of two dimers or a tetrameric form. The large standard deviations in the relative molecular masses reflect the different columns used in the M_r determinations (viz. TSK 3000 sw, TSK 3000 + 4000 sw and BioSil SEC 400), and the different calibration curves produced by these columns. The M_r 303,000 ± 46,000 form seen by SE-HPLC (PAO I), is probably identical to the M_r 219,000 ± 12,845 form seen by SDS-PAGE. As discussed in section 5.3, the anomalously high apparent molecular weight indicated by SE-HPLC is most likely a consequence of hydration of the glycoprotein's carbohydrate chains, confering a disproportionately high hydrodynamic radius on the molecule. Moreover, X-ray crystallographic studies of other amine oxidases indicate that the enzyme homodimer is not likely to be spherical which would contribute to an anomalously high apparent M_r by SE-HPLC analysis.

A commonly recognized limitation of SDS-PAGE is its inability to accurately predict the molecular weight of proteins containing various post-translational modifications, particularly glycosylation. Glycoproteins consistently exhibit reduced mobility compared to nonglycosylated proteins of closely similar molecular weights, probably as a result of their diminished binding to SDS resulting in reduced net charge per weight. Another feature of glycosylated proteins is their diffuse appearance on gels due to the microheterogeneity produced by variations in glycosylation. Because the difference between observed and actual molecular weight values decreases with increasing acrylamide pore size [1122,1123], it has been proposed that more accurate molecular

weights may be estimated by plotting apparent molecular weights of glycoproteins at various acrylamide concentrations, and extrapolating the apparent relative molecular masses to the asymptotic minimum value [1123]. Although the characterization of the retroplacental serum enzyme by Ferguson plots was attempted, penetration of the high concentration polyacrylamide gels was insufficient for meaningful results to be obtained in this study. Extended electrophoresis times, permitting further penetration of the gel by the enzyme subunit and allowing the Kohlrausch boundary (and lower molecular weight proteins) to elute from the gel should be the strategy of a further study. Thereby using apparent relative molecular mass for the plots, as described above, rather than relative mobility (R_f) values as required for linear Ferguson plots ($\log R_f$ vs. $\%T$).

The value of M_r 108,000 reported here for the retroplacental enzyme falls within the expected range of values reported for other human pregnancy-associated amine oxidases (90,000–115,000) as indicated in Table 1.5. As previously discussed, observations outside the stated range of values are likely to be a result of the incorrect attribution of enzyme activity to a contaminating protein. The variations in the reported values for subunit molecular weight can be attributed to the change in the subunit's apparent relative molecular mass in the different concentrations of acrylamide gels. The enzyme is a glycoprotein and, as discussed above, glycoproteins have a notorious tendency to run anomalously in SDS-PAGE. Indeed Lin *et al.* observed a decrease in the apparent M_r of the placental enzyme from 110,000 to 91,000 with increasing acrylamide concentration [752]. This tendency was confirmed by the observation that the enzyme subunit migrates in different positions in gradient and single concentration resolving gels, and at different positions in gels of different single concentrations. The use of slightly varying M_r values for marker proteins, probably contributes to the variation in reported subunit molecular weight. The relative molecular mass of 86,000 reported here for the deglycosylated retroplacental serum enzyme subunit is in close agreement with the minimum values reported for the human pregnancy-associated enzyme subunit and close to the molecular mass of 83,415.6 Da calculated for the 732 amino acid mature peptide subunit of human gene amine oxidase [47,757] (Swiss-Prot Entry P19801; EMBL Entry X78212). This molecular mass is in close agreement with the calculated molecular mass of 85,357.6 Da reported here for the 751 amino acid enzyme subunit precursor. Since sequence analysis of the (retro)placental enzyme has identified the *N*-terminal as starting at position 20 (*q.v.* §6.2.9.1) it is likely that the 19-amino acid signal peptide is released and that the mature protein has 732 amino acids. Slight variations in calculated molecular mass have been reported for the human pregnancy-associated enzyme subunit (*vide* §1.3.4.2biii). An open reading frame of a cDNA variant form of human placental diamine oxidase codes for a 751 amino acid polypeptide enzyme precursor with a deduced molecular mass of 85,423 [758], in

close agreement with the calculation made in this study. Although originally reported as M_r 105,000 based on SDS-PAGE data [780], the calculated molecular mass of human kidney enzyme peptide subunit is 78,886 Da (M55602 is 713 amino acids and 80,871.1 Da) indicating some heterogeneity among the human amiloride-sensitive copper-containing amine oxidases.

7.2.2 Isoelectric Forms

The resolution of the human retroplacental serum polyamine oxidase into two distinct forms with different p*I*s by DEAE anion exchange chromatography is in agreement with the findings of others for the amniotic fluid [763] and placental [755,1244] enzymes (*vide* §1.3.4.4). Moreover, multiple enzyme forms from other species, notably from bovine [451] and porcine [931] plasma, have also been resolved by DEAE anion exchange chromatography. The binding of both forms of human retroplacental enzyme to the anion exchange column at pH 8.0 suggests that their p*I*s were less than 8.0 in agreement with all of the p*I* values determined for amine oxidases thus far (Table 1.6).

7.2.2.1 Analytical Isoelectric Focusing

The high degree of microheterogeneity observed for both forms I and II of the human retroplacental serum polyamine oxidase after analytical isoelectric focusing is similar to that observed for the pig plasma diamine oxidase [931] and placental 'histaminase' [752]. The microheterogeneity may be attributed to variation in the glycosylation of the enzyme protein (*qq.v.* Figure 5.11 and §6.2.9.2e). Figure 6.3 shows that there appeared to be only one major protein present in PAO II that did not focus sharply, but rather appeared as a diffuse band on the gels. In contrast, the IEF standards focused with high resolution. PAO I focused as a ladder of bands with slightly different p*I* values. The average p*I* values of the PAO II preparation appeared to be about 6.5, and for PAO I about 6.0. Similarly, some of the plant amine oxidases were shown to be heterogenous mixtures by analytical isoelectric focusing (*q.v.* §1.3.4.4). Agarose gel IEF may yield clearer results with the retroplacental polyamine oxidase because of its more open pore structure, allowing better penetration of the gel by the enzyme.

7.2.2.2 Preparative Isoelectric Focusing

Retroplacental serum polyamine oxidase was resolved into two peaks of enzymatic activity by preparative isoelectric focusing. The two forms had isoelectric points, p*I* 5.5 and p*I* 7.2. Because the proteins are in solution in their native conformation, preparative isoelectric focusing has the advantage of being a non-denaturing method, therefore the enzymes retained their catalytic activity, although this is difficult to measure because of interference by (polyamine) ampholytes.

In future studies, these ampholytes might be removed by microdialysis or gel filtration. Higher resolution data might be obtained using high performance chromatofocusing columns. The observation of two distinct isoelectric enzyme forms in retroplacental serum is in agreement with the results of anion exchange chromatography: the p*I* values obtained are also in agreement with that data. Similarly, Morel *et al.* resolved the human placental enzyme into two isoelectric forms, although with slightly different p*I* values to those found for the retroplacental enzyme [755]. In contrast, others have only reported single isoelectric forms of placental amine oxidases [744,753] (*q.v.* §1.3.4.4).

7.2.2.3 High Resolution Two-Dimensional Electrophoresis

The immunoaffinity purified human retroplacental serum polyamine oxidase showed isoelectric microheterogeneity after high resolution 2D-electrophoresis confirming other findings of this study. No previous 2D-electrophoretic studies of amine oxidases have been reported. Two dimensional electrophoresis also confirmed that the major band seen after one dimensional SDS-PAGE of immunoaffinity purified enzyme was representative of the enzyme, and contained no major contaminants of similar molecular weight obscured by the diffuse enzyme band in 1D gels. This finding was reinforced by the *N*-terminal amino acid sequencing results. The nature of the enzyme microheterogeneity may include the following: differing degrees of glycosylation (*vide supra*), or other differences in post-translational processing; different degrees of translation (i.e. slightly different polypeptide lengths); or heterogeneity in amino acid primary sequence, most likely close to the amino terminal end (*vide* §7.2.8.4). It is possible that partially degraded proteins could account for some of the microheterogeneity, although cleaved protein fragments would have different molecular weight.

7.2.3 Steady-State Kinetics

A panel of substrates, including histamine and N^1-acetylspermidine, was used here to determine steady-state kinetic parameters. This was done not only to characterize and identify the isolated retroplacental amine oxidases, but also to see if there were any catalytic differences between the two enzyme forms. The steady-state kinetics study involved the accurate determination of Michaelis constants, K_m, turnover constants, K_{cat}, and the ratio $k_{cat}/K_m = k_{eff}$, the catalytic efficiency or 'specificity constant', for the panel of substrates. Of all the kinetic constants, the catalytic efficiency, with the dimensions of an apparent second order rate constant coefficient ($M^{-1}.s^{-1}$), is the most accurate reflection of an enzyme's specificity for a substrate [1934-1936]. The substrate affinities (reflected by K_m values) and k_{cat} values for the two enzyme forms, PAO I and PAO II, appear to be almost identical. These findings are similar to those of Tufvesson who

reported that the two amine oxidase forms observed in amniotic fluid exhibited similar substrate affinities when using discontinuous radiometric assays [763]. In contrast, the two amine oxidases isolated from human placenta by Bardsley *et al.* were reported to have different K_m values [712], one of these may have been serum or mitochondrial monoamine oxidase.

The k_{cat} values are consistent with observed reaction rates; and the catalytic efficiencies, $k_{eff} = k_{cat}/K_m$ fall in the expected range for this type of enzyme [1937]. The selectivity of the enzyme based on relative k_{eff} values is:

histamine > putrescine > N^1-acetylspermine > spermidine > spermine > N^1-acetylspermidine

These findings indicate that histamine and putrescine are good substrates for the enzyme and that N^1-acetylspermine is a better substrate than free spermine. So there are similarities in substrate specificities between the retroplacental serum polyamine oxidase and the other human pregnancy-associated amine oxidases. The substrate specificity patterns of the retroplacental serum polyamine oxidase were found to be similar for amniotic fluid diamine oxidase, pregnancy serum diamine oxidase, and post-heparin serum DAO [678]. These enzymes had similar K_m values. This is of interest because it suggests that human intestinal diamine oxidase, which may be released into circulation after intravenous heparin administration [786], is similar to the human pregnancy-associated diamine oxidase enzyme. This has also been suggested by purification studies [796]. On the other hand, the enzyme released by heparin may be the vascular semicarbazide-sensitive amine oxidase (*qq.v.* §1.3.2.5g, 1.3.3, 1.4.4, 1.4.6).

The data presented here represent the first systematic study of human pregnancy-associated amine oxidase steady-state kinetics parameters based on the experimental considerations described below and in sections 2.3.2 and 6.2.4. Previously, k_{cat} and k_{eff} values have not been reported for amine oxidases. The K_m found for putrescine (*vide* Table 6.1) is of the same order of magnitude found by others for pregnancy-associated amine oxidases (*vide* Table 1.7). With the exception of N^1-acetylspermidine, the relative rates of polyamine oxidation by the retroplacental enzyme found by Morgan appear to be consistent with the data presented here. However, the K_m reported for spermine was two orders of magnitude larger than that found in this study [726]. Like the amniotic fluid enzymes [763] the two retroplacental enzyme forms isolated in this study were most active with putrescine and histamine. Both substrates caused substrate inhibition of the amniotic fluid and retroplacental enzymes at high concentrations.

Other investigators have simply reported the relative rates of reaction of the enzyme with various substrates at a constant concentration [712,717,726,738,741-743,1155]. The value of these studies is limited because it is not possible to determine whether a difference in reaction velocity between one substrate and another is due to a difference in the rate of reaction, or a

difference in the enzyme's affinity for its substrate; i.e. V_{max} or K_m.

Earlier kinetics studies of amine oxidases have been hampered by relatively insensitive methods; or where there had been adequate sensitivity through the use of radiolabelled substrates, by methods leading to inaccuracies. The use of radiolabelled substrates requires the use of discontinuous initial rate measurements, in which the reaction is stopped after a fixed time and reaction products are then separated from unreacted substrate for liquid scintillation counting [47,678,719,748,754,763]. These radiochemical methods are not the most suitable for accurate determination of K_m values for a number of reasons, including the difficulty of ensuring that the reaction is stopped instantaneously after a fixed incubation time; and because of errors that may be introduced by during subsequent manipulations of the samples [673,733,782,990,1154,1668, 1938]. Furthermore, there are practical limitations in the number of sample points that can be included on a reaction progress curve. In contrast, continuous methods, such as used in this study, have the advantage of following the reaction progress curve in a single assay.

The continuous indirect assay using a fluorogenic substrate (and sophisticated modern instrumentation) provided a considerable gain in sensitivity over the chromogenic substrates used in previous studies [164,712,717,734,738,741]. Furthermore, some chromogenic substrates, such as *o*-dianisidine [943] and indigo disulphonate [735], are known to interfere with the amine oxidase assay, and some have been found to inhibit amine oxidases [878]. Indigo disulphonate is capable of reacting with a variety of amines under the influence of peroxidase [765]. The fluorogenic substrate used here, homovanillic acid, has been previously demonstrated not to interfere with amine oxidase activity [1628,1629] (*q.v.* §2.3.1). The increase in sensitivity with the use of this substrate provides the advantage of accuracy when examining substrates with low K_m values. Furthermore, less enzyme was required in the more sensitive assay system thereby reducing the risk of introducing interfering impurities.

Sophisticated methods of data analysis that reduce the errors often introduced by other methods of calculation, were used here (*qq.v.* §2.3.1.5, 2.3.2.4). In the past, others have often used Lineweaver–Burk plots to calculate K_m values for placental amine oxidases. While these plots are useful for displaying steady-state kinetics data their use for calculating K_m values can lead to inaccuracies [1901,1903,1904,1907,1939,1940]. Indeed, to quote Tipton 'the double reciprocal plot is the least accurate of all those available for calculation of enzyme kinetic data' [1668] '...the worst one to use!' [1904], mainly because of its distortion of error distribution.

Because different pH values have been used in assays, is difficult to compare the results of different reports. For example, some investigators have used quite high pHs in their assays [165,410], while at other times using more physiological pH values [881]. Several investigators have reported that Michaelis constants for amine oxidases have a strong pH dependency and have

suggested that the enzyme reacts with the unprotonated form of the amino group of the substrate [638,719,900,915,924,1046,1941]. The effect of pH on the Michaelis constant and maximum velocity for the enzymatic degradation of putrescine by amniotic fluid amine oxidase was examined in detail by Tufvesson [763]. The K_m value (with respect to total putrescine concentration) was found to decrease logarithmically with increasing pH value. However, when defined with respect to putrescine with only one amino group protonated, the K_m was smallest at pH 7.4 and varied only moderately with the pH range 6.0–9.5. The radiometric assay (*q.v.* §2.4.1) indicated a pH optimum for the retroplacental serum enzyme with spermine to be 7.4, which is in agreement with the value found for putrescine (pH 7.4–7.5) using a fluorometric assay; and by others for pregnancy-associated enzymes (pH 7.3, 7.2–7.4) using a radiometric assay for putrescine [678], and a direct spectrophotometric assay with a substrate analogue [1154]. Our kinetics measurements were therefore made at pH 7.4. The $k_{eff} = k_{cat}/K_m$ optima for a range of pHs remain to be determined. In contrast, Hölttä [164], and Seiler [165] have conducted their assays at pH 9.5 (20 °C) and pH 9.0 (37 °C) respectively, which is close to the pKa_1 (= 9.71) for putrescine with one group protonated, viz.

$$H\text{–}{}^{+}NH_2\text{-putrescine-}N^{+}H_2\text{–}H \Leftrightarrow \text{putrescine}^{+}\text{–}H + H^{+} \qquad pKa_1$$

Could this be the reason for the greater apparent affinity of the acetylated polyamines for the catalytic site at higher pH conditions? Further studies in this area are required to clearly establish the variation of steady-state parameters with changes in pH. More detailed studies need to be made of the effects of various buffers on the activity of the enzyme, for example phosphate ion is apparently a competitive inhibitor of the bovine plasma amine oxidase [1651] (cf. §2.3.1.2). Furthermore, three dimensional surfaces indicating the affect of variation of the second enzyme substrate, oxygen, on the oxidation of *naturally occurring* amine substrates have yet to be made (cf. reference [716]). The characterization of the human retroplacental serum polyamine oxidase as a copper-containing amine oxidase indicates a broad substrate specificity. Surveys of enzymatic activity with a wider range of potential substrates including dopamine, 5-hydroxytryptamine (serotonin), tyramine, octopamine, phenylethylamine, benzylamine, short- and long-chain diamines, methylamine and other monoamines, bi-acetylated polyamines, adrenaline, noradrenaline, mescaline, alkaloids, amphetamine and its derivatives should be made.

7.2.4 Enzymatic Reaction Products

The results presented in section 6.2.5 show a decrease in spermine concentration during its incubation with retroplacental serum polyamine oxidase. There was a transient accumulation of spermidine, which was in turn oxidized to putrescine. Putrescine was then metabolized to γ-

aminobutyraldehyde (which spontaneously cyclizes to Δ^1-pyrroline) (Figure 7.1). These results confirm the proposed site of polyamine cleavage by the retroplacental serum enzyme [726]and indicate a mode of action similar to that of the rat liver enzyme [164] and more recently demonstrated with the bovine serum enzyme [223]. The bovine serum enzyme was previously thought to cleave polyamines at their primary amino groups only (*vide* §1.2.3.4, 1.2.5, 1.3.1). The radiolabelling of [^{14}C]spermine at the internal putrescine moiety (*N,N'*-bis(3-aminopropyl)-[1,4-^{14}C]tetramethylene-1,4-diamine) unambiguously demonstrates that the human retroplacental polyamine oxidase cleaves polyamines at their secondary amino groups, thus acting as an EC 1.5.3 type enzyme.

The progressive formation of spermidine and then putrescine suggests a feed-forward competitive inhibition of putrescine oxidation by spermidine as previously associated with extracellular adenine nucleotide metabolism by endothelial cells where ADP and/or ATP exert a feed-forward inhibition on AMP hydrolysis [1942].

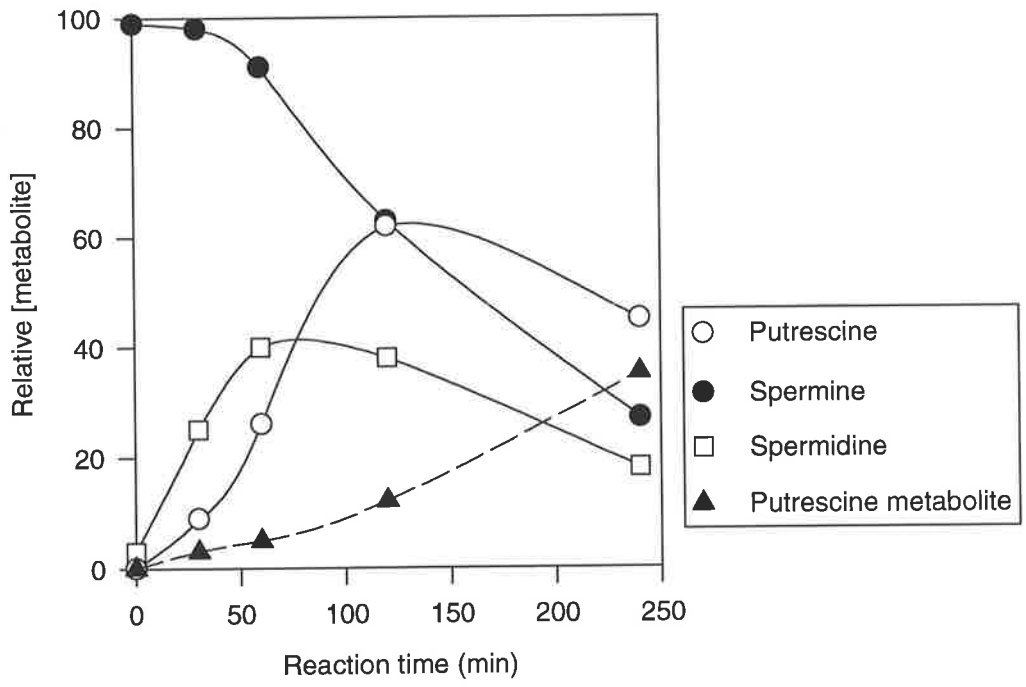


FIGURE 7.1. Polyamine Oxidase Reaction Progress Expressed as Relative Concentrations of Polyamines and their Metabolites

7.2.5 UV–Visible Spectrum

The characteristic pink-orange colour of concentrated human retroplacental polyamine oxidase solution suggests that the enzyme is likely to be TPQ- and copper- containing (*vide* §1.3.4). All amine oxidases of this class have a characteristic peach-pink colour as a result of the TPQ

cofactor (Table 1.4). When measured, only weak absorption was seen in the 450–500 region: this was because the particular enzyme solution used was too dilute to provide an adequate spectrum.

The human retroplacental polyamine oxidase spectrum lacked the intense absorbance bands at 370–360 nm and 460–470 nm that are characteristic of flavin-containing amine oxidases [526]. Furthermore, unlike those of flavoproteins, concentrated enzyme solutions were not yellow. Similarly, the absorption spectrum of the purified rat liver polyamine oxidase, although thought to contain flavin–adenine dinucleotide as a cofactor, did not show the typical three-banded spectrum for flavoproteins. Only a very small peak at 456 nm in addition to the major absorption peak at 275 nm was observed for the native enzyme [164] suggesting that its cofactor may actually be similar to that of the human retroplacental serum polyamine oxidase.

It was significant that concentrated solutions of purified enzyme were not blue, i.e. that there was no strong absorption band in the 600 nm region. This provides evidence against the enzyme being ceruloplasmin or a multi-copper proteins (*q.v.* §1.3.4.1).

7.2.6 Copper Content

Like the other copper-containing amine oxidases, the human retroplacental polyamine oxidase was found to contain one copper atom per subunit (*vide* Table 1.3). The copper content of the enzyme was supported by the spectral data discussed above. Furthermore, primary sequencing studies indicated that the retroplacental enzyme had histidine containing copper consensus sites (*q.v.* §7.2.8.4). The presence of copper (Cu^{2+}) in the human pregnancy-associated enzyme had been suggested in earlier studies. Crabbe *et al.* identified copper by EPR spectroscopy in a partially purified placental diamine oxidase preparation (77% by scanned SDS-PAGE). Copper stoichiometry was suggested to be 0.7 g-atom per M_r 70,000 unit by EPR and was found to be 1.0 g-atom per 70,000 m.wt. unit by atomic absorption spectroscopy [744]. Morgan examined a preparation of retroplacental serum amine oxidase by electrothermal atomic absorption spectroscopy and found 0.09 g-atoms of copper per 67,000 molecular weight. However, as discussed in section 1.3.4.2 this preparation of amine oxidase was probably less than 10% pure [726]. Like the other copper-containing amine oxidases of the EC 1.4.3.6biii class, the copper in the human retroplacental serum enzyme is probably associated with the TPQ cofactor at the active site (*q.v.* §1.3.4.1). Since other metal ions such as Mn^{2+} and Fe^{2+} are reported to be of possible importance in amine oxidases (*q.v.* §1.3.4.1e), it would be of interest to examine the retroplacental serum amine oxidase for the presence of these ions, these studies may be conducted by atomic absorption analysis, EPR and X-ray crystallography.

7.2.7 Inhibition

While there is a need to exercise caution in interpreting data derived from the use of inhibitors, these types of experiments have proven useful in the characterization of amine oxidases [2,170,439,1244].

The inhibition of coupled reactions are difficult to interpret without first demonstrating that the inhibitors do not affect the coupling enzyme. Therefore, a radiometric amine oxidase assay was used in this study. Further detailed studies, using coupled reactions may be possible where the inhibitor being studied has been shown not to interfer with the coupling enzyme. The enzyme inhibition data presented here indicates that the human retroplacental polyamine oxidase is inhibited in a similar way to the rat liver enzyme; but also has similarities to the bovine serum amine oxidase. Inhibition of the placental enzyme by quinacrine, a known inhibitor of flavoproteins, was greater than 90% at 10^{-5} M whereas inhibition of the bovine enzyme was less than 10% at this concentration [439]. The placental amine oxidase was only weakly inhibited by the 'pyridoxal phosphate enzyme inhibitor' isoniazid. In contrast, the bovine serum enzyme activity was inhibited more than 60% by 10^{-4} M of this inhibitor [439]. That the bovine serum enzyme is now known to contain TPQ as a prosthetic group and not pyridoxal phosphate suggests that the inhibitor does not have rigorous specificity. Like the rat liver polyamine oxidase, the retroplacental enzyme was strongly inhibited by the carbonyl reagents, aminoguanidine and semicarbazide and therefore belongs to that class of enzymes known as semicarbazide-sensitive amine oxidases. The carbonyl group reagents also act on amine oxidases of the porcine kidney and bovine serum types, so they are not particularly discriminating in that they probably act on all enzymes of both the TPQ- and flavin-containing types. The enzyme was not inhibited by the monoamine oxidase inhibitors pargyline or clorgyline.

Substrate inhibition was shown for the retroplacental serum polyamine oxidase at high substrate concentrations, with putrescine and histamine the most potent inhibitors. This finding is similar to that for the human amniotic fluid enzyme [763]. The pronounced substrate inhibition by histamine was also observed for amine oxidases from other sources [900,1053,1164].

Because the retroplacental polyamine oxidase studied here was quite sensitive to carbonyl group reagents, is therefore not excluded from having a TPQ active site cofactor. Although the enzyme is sensitive to quinacrine suggesting that it is a flavoprotein, this inhibitor is not specific and shows inhibitory activity toward diamine oxidases demonstrated not to be flavoproteins [1168]. Future studies could include using MDL 72521 and 72527 to determine whether these inhibitors are truly specific for the FAD-containing rat liver type polyamine oxidase.

7.2.8 Primary Sequence Data

7.2.8.1 Similarity with Amiloride Binding Protein

In July 1992 when the *N*-terminal sequence of the human retroplacental serum polyamine oxidase was determined for the first time there were at least two immediate interpretations of the sequencing data. Namely, that:

(1) amiloride-binding protein is the polyamine oxidizing enzyme, or;

(2) amiloride-binding protein represented a major contaminant in the polyamine oxidase preparations.

On the other hand, perhaps the amiloride-binding protein and polyamine oxidase have a very similar molecular weight and the enzyme normally possesses a modified *N*-terminus precluding *N*-terminal sequencing using standard methods. However, both proteins would also have to behave very similarly in the highly specific purification scheme if this were the case. Further evidence aginst this scenario was that the initial yields of PTH amino acids were as expected for the protein preparations with typical transfer losses.

It was the primary structure of human amiloride-binding protein, deduced from the DNA sequence of cloned amiloride-binding protein from a human kidney cDNA library using synthetic oligonucleotide probes derived from partial sequences of the purified M_r 105,000 subunit from pig kidney, that showed strong identity with the NH_2-terminal sequence of the human retroplacental serum polyamine oxidase in a search of Swiss-Prot release no. 22 (*q.v.* §6.2.9). Barbry *et al.* had previously reported the primary structure of human amiloride-binding protein and deposited the sequences in the GenBank and EMBL data bases (accession no. 36335; EMBL entry M55602) [781].

This situation persisted in 1993 when the NH_2-terminal sequences of two multiple forms and that of the retroplacental enzyme dimer were determined. It is now known that the amiloride-binding protein is an amine oxidizing enzyme [47]. Yet even before the publication of that definitive work there was evidence that the 'amiloride binding protein' that had been isolated and cloned was *not* one of the components of the amiloride-sensitive sodium channel, and that by structural analogy was likely to have identity with the retroplacental serum polyamine oxidase described in this work.

Studies of amiloride binding proteins by other groups of investigators had shown quite different results to those of Barbry's group. Benos *et al.* had previously purified an epithelial sodium channel from bovine kidney using methylbromoamiloride. The native channel was a M_r 730,000 complex of six major nonidentical subunit polypeptides held together by disulphide bonds with apparent molecular weights of 315k, 150k, 95k, 70k, 55k, and 40k [1943,1944]. Using

another amiloride analogue, bromobenzamil, Kleyman *et al.* affinity labelled a series of proteins in the bovine kidney with apparent M_rs 176k, 77k and 47k. Then, using an anti-idiotypic antibody, this group immunoprecipitated a M_r 725,000 protein under nonreducing conditions [1945]. Under reducing conditions major non-identical polypeptides with apparent M_rs 260k–230k, 180k, 140k–110k, 70k, and 50k were observed [1057] in agreement with the biochemical characterization by Benos *et al.* They reported that methylbromoamiloride binds predominantly to the M_r 150,000 subunit of the purified channel [1058,1944]. However, the apparent molecular weight of the subunit varied between M_r 130k and 180k for reasons possibly related to its glycosylation. They also noted the specific labeling of a second lower molecular mass component at M_r 55k – 60k. Similarly, Kleyman *et al.* found an amiloride binding site on a M_r 170k subunit [1945] and subsequently confirmed that the M_r 150k – 130k polypeptide binds amiloride using the amiloride analogue NMBA (9,2′-methoxy-5′-nitrobenzamil).

Although eukaryotic cells transfected with amiloride-binding protein gene showed amiloride binding in the cell membrane fraction, no amiloride-sensitive Na^+ channel activity was detected. The epithelial Na^+ channels isolated by both Benos *et al.* and Kleyman *et al.* are both described as H-type ('H' denoting high amiloride affinity) [1057] and have a high sensitivity to amiloride (K_i < 1 μM at physiological Na^+). In contrast, the phenamil (amiloride) binding protein isolated by Barbry *et al.* appeared to be an L-type (or low affinity) type receptor (k_d = 4.2 μM) [1946]. The phenamil binding protein they purified was not part of a large molecular weight complex as found by the Benos and Kleyman groups. Cell membranes derived from eukaryotic cells transfected with Barbry's amiloride binding protein showed high amiloride binding affinity (k_d = 0.1 μM) suggesting that the cloned cDNA coded for an amiloride binding protein that might be part of an H-type channel. However, Barbry *et al.* failed to detect amiloride-sensitive Na^+ channel activity in transfected mammalian cells or in a *Xenopus* oocyte expression system [781].

The primary structure of the human amiloride binding protein had no sequence similarity with previously cloned receptors and ionic channels [781]. The phenamil/amiloride binding protein was found to be glycosylated, its hydropathy plot (Figure 6.8) did not show any membrane spanning segments. The hydropathy plot shows more hydrophilic than hydrophobic regions in contrast to those of membrane spanning proteins, which usually display numerous hydrophobic helices. The amiloride binding subunit of the classic H-type Na^+ channel is likely to be an integral membrane protein [1057], so the protein with high amiloride binding affinity isolated by Barbry and his collaborators does not seem to have any structural homology with the H-type Na^+ channel. Furthermore, polyclonal antibodies raised to the amiloride binding protein isolated by the Barbry group do not react on Western blots with the classic H-type Na^+ channel from bovine kidney [1057].

Barbry *et al.* suggested that the amiloride binding subunit and the channel subunit might be distinct and that the observation of electrogenic sodium transport in reconstituted purified amiloride binding protein might be due to contaminating channel subunits; or that alternative splicing in transfected cells might remove a protein segment that is short, but essential for channel activity [1057]. The latter suggestion appears unlikely because the cloned protein is not a membrane spanning protein.

Previously, when Barbry's group had reincorporated a purified phenamil receptor preparation into large unilamellar vesicles, they found that the purified and reconstituted material had relatively low transport capacity and suggested that only a few of the reconstituted M_r 105,000 proteins were in active form *or* that transport activity necessitated another protein other than the M_r 185/105k protein which would have been present in relatively minor abundance in their preparation [909].

When the structural and biochemical features of the phenamil/amiloride binding protein and diamine oxidase are examined, a number of similarities are seen. Similar to the human phenamil/amiloride binding protein, the subunit molecular weight of the diamine oxidase isolated by Suzuki and Matsumoto from human kidney was M_r 105,000 by SDS-PAGE under reducing conditions and presents as a diffuse band characteristic of glycoproteins [780]. The enzyme bound to con A-Sepharose contributing further evidence of its glycoprotein nature. The human retroplacental serum polyamine oxidase described here is comprised of M_r 108,000 subunits. Deglycosylation of the subunits with peptide:N-glycosidase F (EC 3.5.1.52) resulted in a reduction of the apparent M_r of the subunit to 86,000 with a concomitant sharpening of the protein band. A M_r 218,000 band that vanished after deglycosylation and remained under nonreducing conditions is most likely an unreduced homodimer of the M_r 108,000 subunit, the polypeptides in this band had an NH_2-terminal primary sequence identical to that of the subunit for at least 10 cycles of Edman degradation. The phenamil/amiloride binding protein isolated from pig kidneys [906,909] also had a number of structural similarities with the human retroplacental serum polyamine oxidase. The purified phenamil/amiloride binding protein consisted of a homodimer of M_r 88,000 subunits that migrate anomalously in SDS-PAGE to give an apparent M_r 105,000 under reducing conditions.

The molecular mass of the pig kidney amiloride binding protein subunit was initially determined using a radiation-inactivation technique [906]. The data presented indicated that the membrane bound phenamil receptor had a functional molecular mass of 90k ± 10k. The discrepancy between the apparent molecular mass obtained by radiation inactivation and that of the purified material after reduction with 2-mercaptoethanol (M_r 105,000) was attributed to the tendency of reduced glycoproteins to migrate anomalously in SDS-PAGE. It was later shown

[909] by Ferguson analysis that the apparent M_r 105,000 phenamil receptor subunit had a true M_r closer to 88,000. Deglycosylation of the purified phenamil receptor with neuraminidase and glycopeptidase F or endoglycosidase F under reducing conditions caused a shift in apparent M_r from 105,000 to 102,000. Under nonreducing conditions the major polypeptide was at M_r 185,000 and upon storage, the purified Na^+ channel tended to aggregate into larger molecular mass forms as also observed for the human retroplacental serum polyamine oxidase.

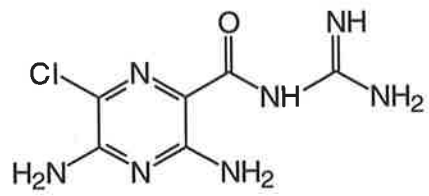
The phenamil binding protein and amine oxidases showed similar binding to chromatographic media. The phenamil binding protein bound to anion exchange (QAE) Sephadex and both retroplacental serum polyamine oxidase and kidney diamine oxidase bound to DEAE ion exchange media. The amiloride binding protein also bound to a lectin column indicating that it was likely to be a glycoprotein [909]. The kidney diamine oxidase also bound to a con A lectin column. The glycoprotein nature of the human retroplacental polyamine oxidase was demonstrated in deglycosylation experiments (*vide* Figure 5.11). Like the phenamil/amiloride binding protein, human kidney diamine oxidase also bound to hydroxyapatite [780].

Of particular interest is the structural similarity of amiloride and its analogues to the endogenous substrates of amine oxidases. The benzene ring of phenamil increases its hydrophobicity, which would facilitate its binding to the hydrophobic region of the substrate binding site of amine oxidases (*q.v.* Figure 1.12). Pig kidney diamine oxidase has the capacity to bind [^{3}H]phenamil, a tritiated amiloride derivative. Indeed, amiloride and its analogues appear structurally similar to the polyamines and histamine as shown in Figure 7.2.

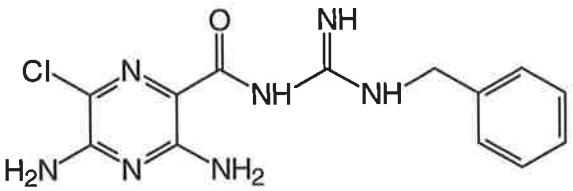
7.2.8.2 Amiloride Binding Protein has Diamine Oxidase Activity

Klinman and her colleagues had found as early as November 1992 that tryptic peptides derived from the active site of bovine serum amine oxidase which contained the TPQ consensus sequence showed a high degree of sequence similarity to human kidney amiloride binding protein (Prof Judith Klinman, Department of Chemistry, University of California, Berkeley; *personal communication*). Furthermore, a tryptic peptide from the porcine kidney diamine oxidase active site was identical to a segment of human kidney amiloride-binding protein primary structure. Moreover, they found that amiloride was a competitive inhibitor for the enzymes and concluded (correctly) that the cloned human kidney amiloride-binding protein was actually a human kidney diamine oxidase.

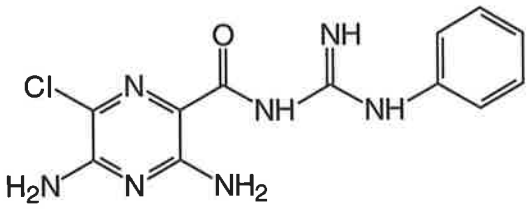
Subsequently, in 1994 a pair of papers [47,411] were published and reported that human kidney amiloride-binding protein was an amiloride-sensitive amine oxidase and elaborated on the Klinman group's evidence. The cDNA of a second amiloride-sensitive sodium channel has been isolated from the epithelial cells of rat distal colon [1945,1947]. The deduced protein



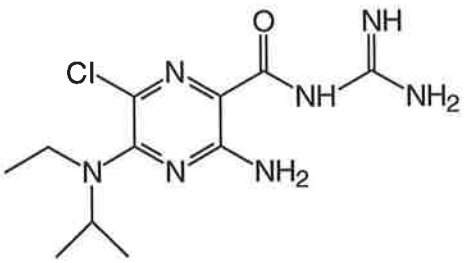
amiloride



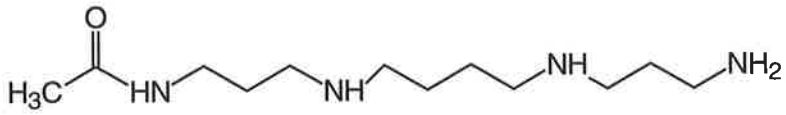
benzamil



phenamil

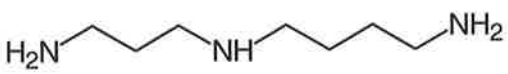


N-5-ethylisopropylamiloride

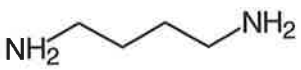


N^1-acetylspermine

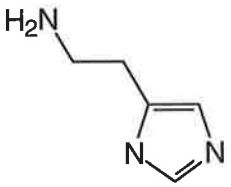
spermine



spermidine



putrescine



histamine

FIGURE 7.2. Structures of Amiloride and its Analogues Compared with those of Polyamines and Histamine

sequence showed two putative membrane spanning segments lacking any significant sequence homology to the human kidney amiloride binding protein isolated by Barbry's group. Furthermore, protein expression in *Xenopus* oocyte reconstituted the activity of the highly selective amiloride-blockable sodium channel (not found by Barbry's group).

Novotny *et al.* demonstrated that human placental diamine oxidase was identical to the human kidney amiloride binding protein [47].

(1)They found that 20 residues of the NH_2-terminal sequence of human placental diamine oxidase (purification data not shown) differed by only 1 amino acid from that of the human kidney amiloride-binding protein sequence. This amino acid (Arginine) was consistently found to be the same in the sequence of both of the retroplacental serum polyamine oxidase forms.

(2)Monoclonal antibodies raised against the pig-kidney amiloride-binding protein immunoprecipitated a protein with the same molecular weight (105,000) as that of the pig-kidney diamine oxidase. This protein had both diamine oxidase activity and bound phenamil.

(3)Cells transfected with human kidney amiloride binding protein cDNA expressed a high diamine oxidase activity. In transfected cells, as well as with purified enzyme, the diamine oxidase activity was inhibited by amiloride and some of its analogues, such as phenamil and ethylpropylamiloride. The amiloride inhibition appears to be due to competitive drug binding at the active site of the enzyme [47,411].

Thus, the human kidney amiloride binding protein cloned by Barbry's group [EMBL entry M55602] was actually kidney diamine oxidase and is almost identical to the human retroplacental polyamine oxidase and the enzyme identified by Novotny *et al.* as human placental diamine oxidase [47].

The human gene coding for a diamine oxidase has now been cloned and sequenced [EMBL entry X78212]. The human diamine oxidase corresponds to a 751-residue polypeptide, its upstream promoter sequence has been analysed and essential motifs described [757].

Since copper-containing amine oxidases show a high degree of sequence homology even if isolated from different species, it is most likely that the human (retro)placental serum polyamine oxidase is very similar to the human kidney and human gene diamine oxidase.

7.2.8.3 Consensus Sites

a) N-glycosylation. At least three potential *N*-glycosylation sites [1948] have been identified in the sequence of human placental amine oxidase, viz. Asn^{110}, Asn^{168} and Asn^{538}. These sites appeared likely because of their location in hydrophobic regions (*q.v.* Figure 6.8) (cf. the conserved sites in the pea seedling enzyme that are located on the surface of the enzyme (*q.v.* §1.3.4.3). Glycosylation has been demonstrated for the human retroplacental enzyme (*q.v.* Figure

5.11); and for many other amine oxidases (*qq.v.* §1.3.4.3 and 7.2.8.1).

The calculated molecular mass of the mature human placental amiloride-sensitive amine oxidase subunit, 83,415.6 Da, is consistent with the observed relative molecular mass of 86,000 and the observed 15–20% glycosylation of the mature protein subunit (*q.v.* Figure 5.11).

b) N- myristylation. Several potential *N*- myristylation sites were identified in the sequence of human placental amine oxidase. These sites are fairly common among eukaryotic proteins [1949,1950]. *N*- myristylation involves acylation by the covalent addition of myristate (a C_{14}-saturated fatty acid) to the *N*-terminal residue through an amide linkage. However, internal glycines are not usually myristylated. Since the only glycine actually exposed in the mature human placental enzyme is Gly^{20} and does not form part of a myristylation consensus pattern, the enzyme is not likely to be myristylated.

c) Phosphorylation. In vivo, protein kinase C exhibits a preference for the phosphorylation of serine or threonine residues found close to a C-terminal basic residue [1951]. A number of potential sites in the amino acid sequence of human placental amine oxidase were identified by their consensus pattern (*q.v.* 6.2.9.2e). Similarly casein kinase II (CK-2) phosphorylation site consensus patterns, which are common on eukaryotic proteins [1952], were also identified in the enzyme sequence. CK-2 is a protein serine/threonine kinase whose activity is independent of cyclic nucleotides and calcium.

d) TPQ. An important element of the active site of amine oxidases is a tyrosine that has been modified to form the redox cofactor, TPQ, which was first discovered in bovine serum amine oxidase [843] (*q.v.* §1.3.4.1). Mu *et al.* [411] found that the TPQ-containing active site peptide from bovine serum amine oxidase was highly similar to a segment of the cloned amiloride-binding protein [781]. Furthermore, a second peptide from the bovine serum oxidase showed a high degree of similarity with another portion of the human amiloride-binding protein. Likewise, the active site peptide from porcine kidney diamine oxidase [613] was found to be wholly contained within the human kidney amiloride binding protein. TPQ was subsequently shown to be the cofactor in other copper-containing amine oxidases and is associated with a NYD/E consensus sequence for the TPQ modification of tyrosine found in the sequence of the placental enzyme.

e) Heparin binding site. Heparin, a negatively charged polysaccharide, binds to a variety of proteins. It is involved in the mediation of protein–protein interactions, including some of those involved in blood clotting. Protein–protein interactions that are modulated by heparin may occur at the cell surface, in the extracellular matrix of tissues, and in the serum. By analogy with the pea

seeding and *E. coli* amine oxidases, the consensus heparin-binding motif corresponds to a region on a structurally conserved solvent exposed loop at the bottom of a shallow depression near the centre of a subunit domain [650], forming a potential heparin-binding site. Amiloride binding protein was initially purified from pig kidney membranes [909]. This, and other evidence suggests its binding to endothelial cell surfaces (*qq.v.* §1.3.2.5g, 1.3.3, 1.4.4, 1.4.6). This binding is thought to be through attachment to glycosaminoglycans [1082]. It is well recognized that diamine oxidase is released into the blood stream *in vivo* by intravenous heparin [674]. Therefore, attachment of amiloride-sensitive copper-containing amine oxidase to glycosaminoglycans may be mediated by the heparin-binding consensus motif of the type starting at Arg^{569} in the human placental amine oxidase sequence.

7.2.8.4 Sequence comparison with other oxidases

A search of NCBI-GenBank Release 91.0 [current March 1996] [1953] @ iubio.bio.indiana.edu for 'amine oxidase' found 201 items. Many other copper-containing oxidases, such as glucose oxidase, are known to exist [464,1088]. However, only a few of the copper-containing amine oxidases of the type having the NYD/E consensus sequence for the TPQ modification of tyrosine group had been cloned at this time. Full length DNA sequences have been elucidated for amiloride-sensitive amine oxidases from human gene, human placental, human kidney, and rat colon/lung (amiloride-sensitive) copper-containing amine oxidase. These sequences were compared to known sequences for other amine oxidases of the same Enzyme Commission classification, those having the same cofactor, those thought to be related, and those catalysing the similar reactions. This was done to identify sequence similarities suggesting homology, and to identify conserved, and therefore probably important, residues. The results of a CLUSTAL W [1922,1923] multiple sequence alignment are presented in Appendix E.

Pairwise comparisons of human DAO [Swiss-Prot 19800; EMBL 78212] [757] with the other copper- and TPQ- containing enzymes showed degrees of similarity presented in Table 7.1. The similarity of the copper-containing amine oxidases between the various species is striking. The similarity between the human and rat enzyme sequences places the enzymes in the same subfamily [1958]. The bovine enzyme is more distantly related though almost within the same family.

The pairwise similarities between the sequences may also be represented by bi-dimensional dot matrix plots having one protein sequence along the X-axis and the other on the Y-axis as described in reference [1959]. Segment comparisons are made and longer contiguous sequences that match appear as diagonal lines on the plots and are easily interpreted by eye. ANTHEPROT software (*q.v.* Figure 6.8 caption), using the unity matrix to score each substitution in the

TABLE 7.1. Similarity of Human Amiloride-Sensitive Copper-Containing Amine Oxidase with Other Copper- and TPQ- Containing Amine Oxidases

Enzyme (reference); EMBL No.	*% identity (global alignment)*	*Comparison score (standard deviations)*
Human Kidney ABP/DAO [781]; M55602	86.1%	14.86
Rat colon/lung ABP/DAO [982]; X73911	81.2%	14.65
Bovine serum/liver copper AO [411]; S69583	39.1%	13.71
E. coli amine oxidase (maoA) [529]; L47571	23.0%	13.14
Hansenula polymorpha AO [566]; X15111	22.9%	13.26
Anthrobactr methylamine oxidase (maoxII) [516]; L12990	22.4%	13.09
Pea seedling AO [649]; L39931	22.3%	13.16
Lentil seedling AO [617]; X64201	20.0%	13.09
K. aerogenes AO [1092]; D10208	22.1%	13.19

Notes to Table 7.1.
Comparisons were made using the ALIGN [1954] and RELATE [1955] programs of the FASTA [1919,1956,1957] program package (Version 2.0x by W.R. Pearson of the University of Virginia, Charlottesville, VA. Available for IBM-PC/DOS @ <uvaarpa.virginia.edu/pub/fasta/dos/fa20u316.zip> by anonymous ftp).

compared sequence segments, was used to generate dot plots presented in Appendix F.

Phylogenetic relationships between these homologous enzymes are illustrated using the PHYLIP DRAWTREE program with an input file (*.ph) generated by the CLUSTAL W program (*q.v.* Appendix E). The output of the PHYLIP DRAWTREE program is an uprooted phylogenetic tree (dendrogram), Figure 7.3. As can be seen in the dendrogram, the human and rat enzymes appear closely related. The bovine serum enzyme, while similar, is more distantly related. A close homology exists between the pea and lentil seedling enzymes (ALIGN and RELATE scores were 78.5% sequence similarity and 14.88 standard deviations respectively). The *E. coli*, *Hansenula* and *Anthrobactr* enzymes had RELATE scores around 13.5 and global alignment scores between 34.7% (Hans_anthr) and 24.1% (E.coli_Hans).

CLUSTAL W analysis [1923] demonstrated that potentially important residues are conserved in the copper- and TPQ- containing amine oxidases and are found in the human enzymes. All contained the NYD/E consensus sequence for TPQ modification of tyrosine. Human ceruloplasmin (Swiss-Prot entry P00450; EMBL entry M13699, EC 1.16.3.1 [1532]) and human lysyl oxidase (protein-lysine 6-oxidase; Swiss-Prot P28300; EMBL S78694; EC 1.4.3.13 [1533]), which have similar amine oxidase activity to the human (retro)placental enzyme [751,1115,1614,1857-1862] did not show these conserved sites. Only a low degree of sequence similarity was seen when these sequences were compared with the human amiloride-sensitive amine oxidase sequence in a pairwise manner using the ALIGN program [1954,1958], 17.5% and 15.3% respectively using the BLOSUM50 scoring matrix with -12/-2 gap penalties. Similarly, the putrescine oxidase from *Micrococcus rubens* [an FAD-containing enzyme; Swiss-Prot P40974; EMBL D12511; EC 1.4.3.10 [538]] showed no conserved sites and a low degree of sequence

similarity (17.8%). Nevertheless, the RELATE standard deviation comparison scores [1958,1960] for these three proteins were ceruloplasmin (12.87), lysyl oxidase (12.98) and putrescine oxidase (13.06). The probability of getting a score over 10 standard deviations by chance is less than 10^{-23} [1958,1960], suggesting these proteins originated from a common ancestral gene.

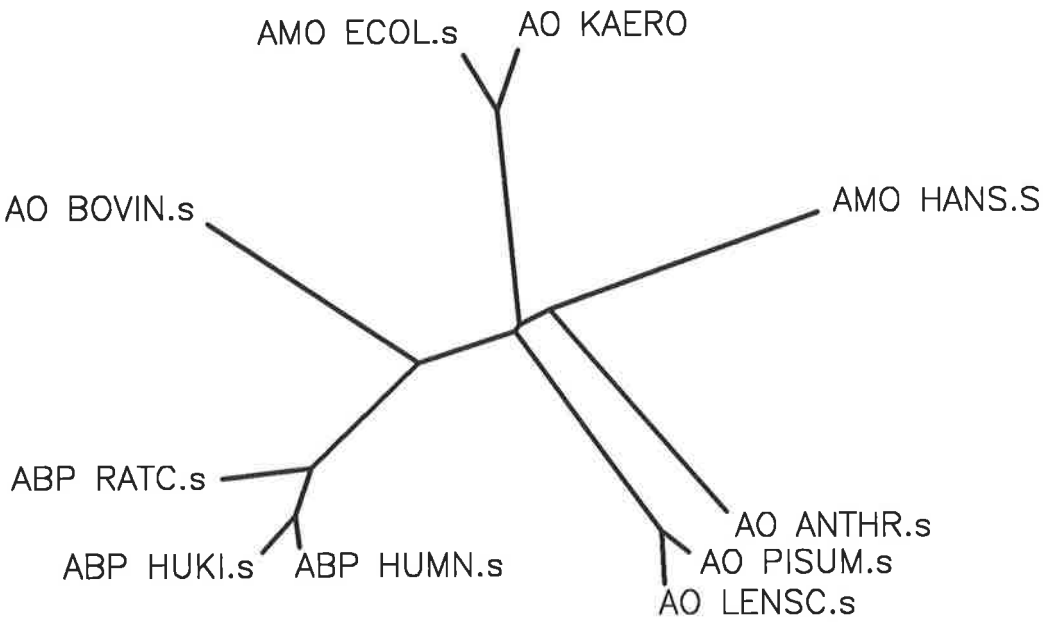


FIGURE 7.3. Theoretical Homology Between Copper-Containing Amine Oxidases. Homologies are displayed as an uprooted phylogenetic tree (dendrogram) showing ABP_HUMN.s, human amiloride binding protein/amine oxidase; ABP_HUKI.s, human kidney amiloride binding protein/amine oxidase; ABP_RATC.s, rat colon amiloride binding protein/amine oxidase; AO_BOVIN.s, bovine serum/liver amine oxidase; AMO_ECOL.s, *E. coli* amine oxidase; AO_KAERO, *K. aerogenes* amine oxidase; AMO_HANS.s, *Hansenula* amine oxidase; AO_ANTHR.s, *Anthrobactr* amine oxidase; AO_PISUM.s, Pea seedling amine oxidase; AO_LENSC.s, Lentil seedling amine oxidase. The dendrogram, illustrating phylogenetic relationships between the sequences, was constructed using the DRAWTREE program of the PHYLIP (phylogeny inference package) program suite by J. Felstenstein. PHYLIP, version 3.752 for MS-Windows, was obtained by anonymous ftp from <evolution.genetics.washington.edu/> by transferring the three 386 Windows executables, C sources and *.doc(s).

Indeed, where the probability of similarity by chance is $<10^{-6}$ (a score of more than about 5 standard deviation units), the proteins are considered to belong to the same superfamily [1958]. However, it is not until the sequences are <50% different that they are considered to belong to the same family. It is pertinent that a less stringent PROSITE search of the human (placental) amine oxidase (allowing 2 mismatches) identified multicopper oxidase [PS00079] [1961,1962] and Type 1 copper (blue) protein [PS00196][1963,1964] signatures. These signatures are found in other oxidases, ceruloplasmin and in a diverse variety of copper binding proteins. In particular, the copper ligand sites of the later group include a pattern which contains cysteine and histidine [PDOC00174]. ClustalW analysis shows that the cysteine and histidine residues, C^{391}, C^{417}, H^{510}, H^{512}, and H^{675} in the human (placental) amine oxidase sequence are highly conserved in the copper- and TPQ- containing amine oxidases that were examined, and may well participate in the copper binding site as others have suggested [411]. Furthermore, spectroscopic studies have shown that the copper ion in bovine serum amine oxidase should have three histidines coordinated [1050]. The conserved histidines possibly functioning as copper ligands are embedded in hydrophilic segments supporting the empirical observation that metal ions are ligated by a shell of hydrophilic groups [1112]. Coordination of a copper ion by the enzyme is supported by the finding of copper using atomic absorption spectroscopy (*q.v.* §6.2.7). Experiments using site directed mutagenisis, or X-ray crystallographic analysis may in future demonstrate that the three histidines are actually the copper ligands.

The sequence of an extended active site peptide isolated from porcine kidney and porcine serum DAOs (full protein sequences not yet available) have been found to be identical to that of human amiloride-sensitive amine oxidase and contain the TPQ consensus site (NYD/E) [613]. In the TPQ consensus site a hydrophobic residue is always found toward the N terminus of Asn(N); and Tyr(Y) is preferentially located toward the C-terminus of Asp.

A truncated form of the rat amiloride-binding protein (DAO), pSV2 [982], corresponding to the COOH terminus part of the enzyme begins at Met^{534} and binds amiloride and its analogues, although with lower affinities than the full length form.

It is possible that a conserved negatively charged residue situated in the C-terminal part of the amiloride binding protein, such as Asp^{664}, located nearby a conserved hydrophobic sequence (LVAWV) might participate in amiloride binding as suggested by others [47,982]. However, since amiloride and its analogues behave as competitive inhibitors of diamine oxidase activity and have structures similar to the enzyme's substrates it seems likely that the active site of the enzyme is also responsible for amiloride binding corresponding to the lower affinity site as described for the porcine kidney enzyme two-site model [411,909]. There is a similarity between the K_i = 9.1 μM, obtained by porcine kidney enzyme activity inhibition experiments with amiloride and the K_d =

4.2 μM, determined with amiloride binding protein [909].

The long form of the ABP/DAO detected in rat corresponding to the cDNA cloned in rat colon, was also detected in duodenum, placenta and thymus and is similar (81%) to that identified in human kidney. The shorter form was detected in lung, colon, placenta and spleen [982]. That the short form of the rat ABP/DAO was only detected in lung suggests that different forms of the ABP/DAO may be produced by alternate splicing; differentially activated by tissue-specific nuclear factors.

Several differences between the protein sequences translated from the human kidney cDNA clone [781] and the human cDNA clone [757] are evident. Differences in the cDNA modify the protein sequence between Asp^{265} and Arg^{328} and between Gly^{493} and Leu^{511} and shift the stop codon from Ser^{707} and Val^{751} making the latter slightly larger. Human polymorphism has been reported for the gene [398,758]. It is possible that slightly different proteins sharing the highly conserved structural domain containing the active site are produced from the same gene through the operation of tissue-selective promoters [757]. It may be that there are enzyme isoforms with differences in substrate specificity due to slight differences in protein sequence similar to those of UPD-glucuronosyltransferase [1965]. Sequence alignment suggests the presence of separate protein domains with a carboxyl-terminal domain encoding the cofactor consensus sequence and putative copper binding sites, whereas the more divergent amino-terminal domain is proposed to accommodate the differing physiological functions and substrate specificities of the copper- and TPQ- containing amine oxidases [411].

7.3 Conclusions

Structural and functional analogies presented here suggest that human retroplacental serum polyamine oxidase likely to be identical to human pregnancy-associated amine oxidases found in placenta, amniotic fluid and pregnancy serum. It is therefore similar to the bovine serum enzyme and other amine oxidases currently classified as EC 1.3.4.6, semicarbazide-sensitive or, more recently, as copper- and TPQ-containing amine oxidases. Metabolic studies reported in this thesis indicate that, like the rat liver and bovine serum enzymes, the human retroplacental serum enzyme cleaves spermine and spermidine at their secondary amino groups. The enzyme thus acts as an oxidoreductase acting on the CH—NH group of donors with oxygen as an electron acceptor. This EC 1.5.3 action suggests a broader classification than the current EC 1.4.3.6 classification for many copper- and TPQ- containing semicarbazide-sensitive amine oxidases.

SDS-PAGE and SE-HPLC analyses revealed that the human retroplacental serum polyamine oxidase probably exists in its native form as a homodimer of glycoprotein subunits each with an apparent relative molecular mass of 108,000. Higher molecular weight forms of the

enzyme observed in retroplacental serum and preparations derived from RPS may be aggregates of the native enzyme dimer or perhaps tetramers of the glycoprotein subunit. Enzymatic deglycosylation of the glycoprotein subunit reduced its apparent M_r to 86,000, consistent with the molecular mass of the 732 amino acid mature enzyme subunit polypeptide moiety (83,415.6 Da) calculated from the human gene derived diamine oxidase sequence [EMBL entry no. X78212]. An atomic absorption spectroscopic study indicated one copper atom per subunit. Further studies are required to demonstrate the presence of other metal atoms, such as magnesium or iron, which may be present in the structure. *N*-terminal protein sequence similarities with other amine oxidases, UV-visible spectroscopy and inhibitor studies suggested that the copper atom lies at the active site of the enzyme along with TPQ. The enzyme lacked the intense cerulean (sky-blue) colour of Type-I copper-containing proteins and was therefore unlikely to be ceruloplasmin. Moreover, there was no sequence or molecular weight identity with that multicopper protein.

The expression of the pregnancy-associated polyamine oxidase gene has not yet been accomplished. Although profiles of the human copper- and TPQ- containing amine oxidase secondary structure, derived from analysis of the its primary protein sequence, suggest the structure of a soluble globular protein, the organization of structural domains and the atomic structure of the human enzyme have yet to be established. X-ray crystallographic studies have not yet been reported for mammalian enzymes and may have been hampered by the glycosylation of the native enzyme. These studies would reveal the active site arrangement of the enzyme which may prove to be an attractive target for the structure-based design of new enzyme inhibitors. A better understanding of disorders associated with polyamine oxidase dysfunction and the physiological role of these enzyme may revealed by future studies using the new inhibitors. And a more complete understanding of the molecular nature of catalysis by the human amine oxidases would be facilitated by the availability of atomic resolution structures for the enzyme and catalytic intermediates.

Direct comparison of the human retroplacental serum amine oxidase with other amine oxidases is required, before we can appreciate the subtle differences responsible for the substrate specificity, stereoselectivity and kinetic rate differences between members of the copper- and TPQ-containing amine oxidase family. Substrate specificities of the human RPS enzyme are reported here: the enzyme appears to have a preference for histamine, putrescine, N^1-acetylspermine, spermidine and spermine, in that order. Further studies might examine a broader range of substrates and reaction conditions. The enzyme was not found to be inhibited by pargyline and clorgyline and was therefore not a classical-type monoamine oxidase; this was supported by lack of structural and sequence similarity with the monoamine oxidases.

The monclonal antibodies generated in this study have shown promise in the development

of a sensitive ELISA for the enzyme and for immunohistochemical and ultrastructural studies (data not presented). These sorts of studies, and studies with cDNA probes may contribute to the identification of polyamine oxidases seen in other human tissues and cells. Because of its potential importance in non-specific immunity, it would be of great interest to characterize the polyamine oxidase found in human macrophages. Biochemical and immunochemical tools would be useful to investigate the involvement of the enzyme in antimicrobial, anti-inflammatory, immunosuppressive function. A investigation of the nature of the enzyme observed in synovial fluid is required for its identification and characterization. Studies reported here suggest that polyamine oxidase is responsible for the biological effects previously associated with unpurified human RPS and pregnancy serum. Enzymes of its class have been shown to have a wide range of biological effects. The preparative scale purification of the human retroplacental enzyme could potentially supply a source of exogenous enzyme for the further investigation of these biological effects.

Like many scientific studies this thesis has generated more questions than it has answered. The more knowledge that is obtained: the more it becomes evident what is not known.