SYSTEMIC OXIDANT STRESS AND ITS EFFECTS ON HEPATOTOXICITY

A thesis submitted for
the degree of

DOCTOR OF PHILOSOPHY

in

The Department of Clinical & Experimental Pharmacology
The University of Adelaide, South Australia.

by

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August 1988

DECLARATION

I declare that this thesis contains no material which has previously been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge contains no material previously published by any person, except where due reference is made in the text.

Results of this thesis have been presented to:

(a) Meetings of the Australasian Society of Clinical and Experimental Pharmacologists (ASCEP), in Brisbane (1985), Sydney (1986) and Adelaide (1988, abstract submitted);

(b) X^{th} International Congress of Pharmacology, Sydney (1987, 2 papers);

(c) Australian Society of Medical Research, Adelaide (1988);

(d) Australasian Society for Free Radical Research, Adelaide (1988).

A manuscript titled "Hydroxylation of salicylate by activated human neutrophils: evidence of a role for superoxide and myeloperoxidase" has been submitted to *Biochem. Pharm.*

Paul F. A. Wright

ACKNOWLEDGEMENTS

I would like to thank:

Dr. Brian Priestly for his excellent supervision and critical evaluation of the experimental work and writing of this thesis.

Prof. Felix Bochner for the courtesy of allowing me to undertake this study in the Department of Clinical and Experimental Pharmacology at the University of Adelaide.

Dr. John K. French for his collaboration with the experiments in chapter 3; and Dr. W. Henry Betts for allowing the performance of this research in his laboratory, at the Queen Elizabeth Hospital Rheumatology Unit.

Dr. Michael W. Whitehouse for his advice and supervisory assistance with Chapters 4 and 5. David Haynes for his collaboration with experiments in chapter 5, which were performed in the Dept. of Pathology, at the University of Adelaide.

Fellow colleagues - Sam Bruschi, Anthony Simula, John Edwards, Trevor White, John Dally and Laura DeCasto, for a stimulating and enjoyable work environment; Debbie Siebert, Rod Irvine and Gordon Crabb for invaluable technical advice; and to all members of the Pharmacology Dept.

Nicola Guerin for the support and motivation needed to complete this thesis.

My mother for her encouragement and assistance throughout my secondary and tertiary studies.

The experimental work described in this thesis was performed during 1984 - 1988 in the Dept. of Clinical and Experimental Pharmacology, University of Adelaide. The author held a Commonwealth Postgraduate Research Award during this period.

I wish to dedicate this thesis to my two children,

Tonja Lee and Stefaan Ramsay.

ABSTRACT

Experimental work which concerned certain aspects of oxyradical formation *in vitro* by a cell type involved in systemic oxidant stress, and the effect of inflammation-induced systemic oxidant stress on hepatotoxicity *in vivo*, produced the results detailed below.

1. A sensitive and specific HPLC-amperometric assay for hydroxylated salicylate was developed for the detection of hydroxyl radicals (OH·). The sensitivity of the assay was attributable to the optimisation of extraction and storage procedures, and the use of electrochemical detection of the reaction products. Both specificity and sensitivity compared favourably with the [^{14}C]-benzoate decarboxylation assay in the ferrous-EDTA autoxidation and xanthine oxidase-hypoxanthine-ferric-EDTA systems. This assay was sufficiently sensitive to measure OH· formation in isolated cellular systems.

2. Human neutrophils (PMN) activated by either serum treated zymosan (STZ), phorbol myristate acetate (PMA), formyl-methionyl-leucyl-phenylalanine (with cytochalasin B) or A23187, produce a partially reduced oxygen species capable of hydroxylating salicylate to form dihydroxybenzoates (DHB), as determined by HPLC/amperometric detection. Irrespective of the stimulus, the hydroxylation was markedly inhibited by superoxide dismutase but not catalase. Azide selectively inhibited DHB formation in PMN

activated with STZ (a potent degranulating agent which stimulates myeloperoxidase release), but had less effect on PMA-stimulated hydroxylation. Desferrioxamine failed to inhibit DHB production suggesting that it is independent of the Fenton reaction. Taken together with the lack of inhibitory effect of chelating agents, the data suggest that salicylate is hydroxylated by PMN *in vitro* by a highly reactive species, probably the OH·. This hydroxylating species is superoxide- and myeloperoxidase-dependent and is formed by a Fenton reaction-independent mechanism. Therefore two phagocytic functions - the respiratory burst and degranulation, may interact to produce a toxic species in addition to the hypochlorite ion.

3. Systemic oxidant stress is a condition in which an excess of oxyradical production can lead to altered cellular antioxidant levels and other responses. It was induced in LACA Swiss mice, albino Porton and hooded Wistar rats, by the tail-base injection of the acute inflammogen, oleyl alcohol (OA), or the chronic inflammogen, adjuvant (ADJ, *Mycobacterium tuberculosis* in squalane). The resultant effects included the inhibition of the hepatic microsomal mixed function oxidase (MMFO) profiles in a temporally phasic manner. Hepatic MMFO activity was assessed *in vivo* by pentobarbital sleeping times (PST) and zoxazolamine paralysis times; and *in vitro* by ethoxycoumarin O-deethylation and diphenyloxazole hydroxylation activities.

The injection of inflammogens also resulted in decreased in body weight and fluctuations in hepatic glutathione levels.

Paracetamol-induced hepatotoxicity was ameliorated by the presence of inflammation, however hepatotoxicity induced by bromobenzene was unaffected (assessed by serum alanine aminotransferase). The effects induced by the arthritogenic ADJ treatment were more prolonged than those caused by the short-term inflammogen OA (particularly in the hooded rat). The differential effect of inflammation on two oxidative-microsomally activated hepatotoxins precludes the assumption that all MMFO-activated compounds would be affected in a similar manner by the presence of systemic oxidant stress.

4. Concomitant piroxicam administration repeatedly and specifically attenuated OA-mediated effects on the liver - daily dosage reduced OA-induced PST prolongation (Swiss mice and hooded rats) and OA-mediated ceruloplasmin synthesis (hooded rats); and restored PC-induced hepatotoxicity in OA-inflamed Swiss mice. Clozic administration reduced the inflammation-mediated effects on PC-induced hepatotoxicity in both OA and ADJ treated Swiss mice. The immunological status of Swiss mice and C3H/HeJ mice during OA and/or piroxicam treatment, which was assessed by *ex vivo* lymphocyte proliferation, suggested the presence of high levels of PGE_2 and functionally equivalent levels of IL-1; whereas ADJ treatment caused the production of mainly IL-1.

From these results it may be concluded that: (a) inflammation induced by OA is accompanied by large increases in PGE_2 and IL-1 levels which stimulates the liver (via another cytokine, IL-6) to produce Acute Phase Reactants, turn down certain MMFO systems, and cause fluctuations in hepatic glutathione levels; (b) ADJ-induced inflammation is accompanied by increases in IL-1 predominantly which promotes the same hepatic changes seen with OA treatment (also via IL-6), but in a piroxicam-insensitive (and therefore PGE_2-insensitive) manner. (c) Clozic interacts with presently unknown cytokines common to both pathways. (d) Piroxicam exhibits immunostimulatory action by inducing lymphocyte proliferation in OA-treated mice.

AIMS OF THIS STUDY

1. To develop a sensitive and specific assay for the highly reactive oxygen species, the hydroxyl radical, for use in isolated cell systems.

2. To examine the nature and mechanism of hydroxyl radical production by activated human neutrophils *in vitro* - an important cell type present in systemic oxidant stress.

3. To investigate the effects of *in vivo* animal models for systemic oxidant stress on hepatic microsomal metabolism and the hepatotoxic responses to paracetamol and bromobenzene - two oxidatively activated hepatotoxins.

4. To manipulate the systemic oxidant stress with different classes of anti-inflammatory and anti-arthritic drugs in order to modify the inflammation-induced effects on hepatotoxicity, and thereby elucidate immunological pathways which mediate these effects, *in vivo*.

ABBREVIATIONS

ADJ	Adjuvant
ALAT	Alanine aminotransferase
APR	Acute phase reactants
BB	Bromobenzene
BPS	Bathophenanthroline sulphonate
CAT	Catalase
CB	Cytochalasin B
COMT	Catechol O-methyl transferase
CONT	Control
CSF	Colony stimulating factor
CV	coefficient of variation
DAG	Diacyl glycerol
DETAPAC	Pentetic acid
DFO	Desferrioximine
DHB	Dihydroxybenzoic acid
DMPO	5,5'-dimethyl-1-pyrroline-1-oxide
DMSO	Dimethylsulphoxide
DPI	Diphenylene iodonium
DTNB	5,5'-dithiobis-[2-nitrobenzoic acid], also Ellman's reagent
ECD	Electrochemical detector
7-EC	7-Ethoxycoumarin
7-ECODase	7-Ethoxycoumarin O-deethylase
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FID	Flame ionisation detector
FMLP	Formyl-methionyl-leucyl-phenylalanine
G	Gentisic acid (also 2,5-DHB)
GC	Gas chromatography
GQ	Gentisoquinone (2-carboxy-1,4-benzoquinone)
GSH	Glutathione
HBSS	Hank's balanced salt solution
HG	Homogentisic acid
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
HX	Hypoxanthine
IC	Inhibitor concentration
IFN	Interferon
IL	Interleukin
IP_3	1,4,5-inositol triphosphate
IS	Internal standard
IU	International units
LAF	Lymphocyte activating factor (IL-1)
LPS	Lipopolysaccharide
MMFO	Microsomal mixed function oxidases
MPO	Myeloperoxidase
MT	Metallothionein
NAD(H)	β-Nicotinamide adenine dinucleotide (reduced)

NADP(H)	β-Nicotinamide adenine dinucleotide phosphate (reduced)
NSAID	Nonsteroidal anti-inflammatory drugs
O_2^-	Superoxide
OA	Oleyl alcohol
ODH	O-Dianisidine hydrochloride
OH·	Hydroxyl radical
PAH	Polycyclic aromatic hydrocarbons
PC	Paracetamol (acetaminophen)
PCN	Pregnenolone 16-α-carbonitrile
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2 (prostacyclin)
PHA	Phytohaemagglutinin A
PIC	Phosphoinositidase C
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PMA	4β-Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocytes
PPO	Diphenyloxazole
PPO-OHase	Diphenyloxazole hydroxylase
PST	Pentobarbital sleeping time
SA	Salicylate
SE	Standard error
SOD	Superoxide dismutase
STZ	Serum treated zymosan
TCA	Trichloroacetic acid
THB	Trihydroxybenzoic acid
TNF	Tumour necrosis factor
XO	Xanthine oxidase
ZPT	Zoxazolamine paralysis time

CONTENTS

Chapter 1

GENERAL INTRODUCTION

This thesis concerns certain aspects of systemic oxidant stress, and the modifying effects it exerts upon hepatotoxicity. It is therefore appropriate to first consider the concepts of reactive oxygen species and oxidant stress.

1.1 Reactive Oxygen Species.

All aerobic animals and plants require oxygen (O_2) for the efficient production of energy during respiration. However oxygen is itself a 'free radical' as it has two unpaired electrons, each located in separate π^* antibonding orbitals, and is a major promoter of radical reactions in living cells (Figure 1.1a) [Halliwell and Gutteridge, 1985].

The progressive one-electron reduction of O_2 to water results in the formation of reactive oxygen species - i.e. superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\cdot$). These reactive species may be formed (a) as by-products from normal metabolic processes, i.e. during respiration where electrons may leak from the electron transport chains; (b) via the redox-cycling of both endogenous and xenobiotic compounds, e.g. flavoproteins and quinones such as menadione; (c) or intentionally via enzymatic production, as in the case of O_2^- production from NADPH-oxidase in activated neutrophils during inflammation and infection (table 1.1). Singlet oxygen formation has

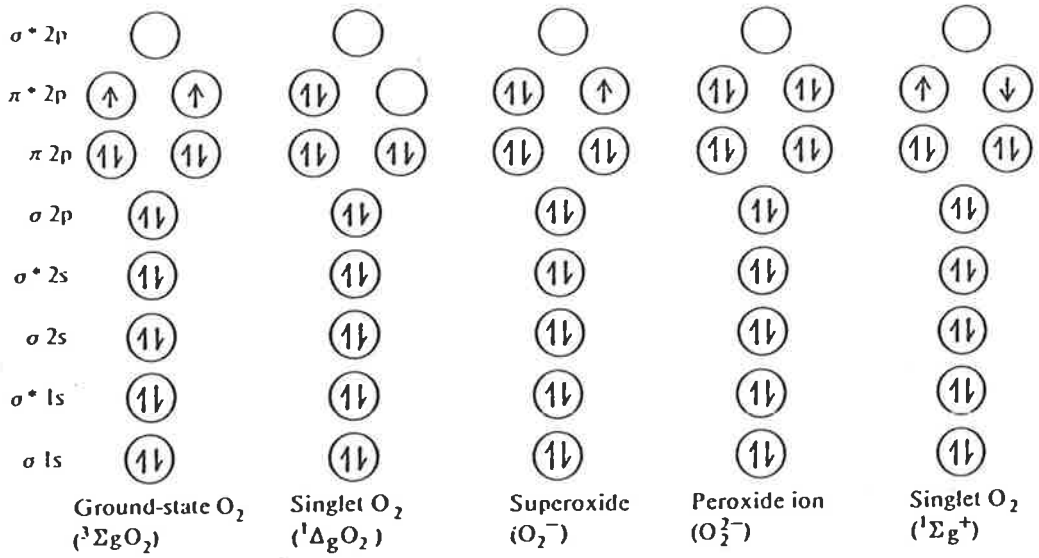

Figure 1.1 (a): The bonding orbitals in the diatomic oxygen molecule. [Halliwell and Gutteridge, 1985].

$$Fe^{3+}\text{-salt} + O_2^- \longrightarrow O_2 + Fe^{2+}\text{-salt} \qquad (1)$$

$$Fe^{2+}\text{-salt} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + Fe^{3+}\text{-salt} \qquad (2)$$

$$O_2^- + H_2O_2 \xrightarrow{Fe} OH^{\cdot} + O_2 + OH^- \qquad (3)$$

Figure 1.1 (b): The Fenton reaction. Catalysis of the Haber-Weiss reaction by transition metal ions, predominantly iron and copper [Halliwell, 1982].

Table 1.1: Sources of free radicals within cells. [Halliwell, 1987].

Endogenous sources:

- Mitochondrial electron transport chain
- Microsomal electron transport chain
- Chloroplast electron transport chain
- Oxidant enzymes
  - Xanthine oxidase
  - Indolamine dioxygenase
  - Tryptophan dioxygenase
  - Galactose oxidase
  - Cyclooxygenase
  - Lipoxygenase
  - Monoamine oxidase
- Phagocytic cells
  - Neutrophils
  - Monocytes and macrophages
  - Eosinophils
  - Endothelial cells
- Autoxidation reactions (e.g. Fe^{2+}, adrenaline)

Exogenous sources

- Redox-cycling substances (e.g. paraquat, diquat, alloxan, doxorubicin)
- Drug oxidations (e.g. paracetamol, CCl_4)
- Cigarette smoke
- Ionising radiation
- Sunlight
- Heat shock
- Substances that oxidise glutathione

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been confirmed in plants (via the photosynthetic complexes), however its role in mammalian processes is debatable.

The conversion of O_2^- to H_2O_2 and $OH^\cdot$ may also be catalysed by the free ions (or certain complexes such as EDTA) of iron and copper, in a process described as Fenton chemistry (Figure 1.1b) [Halliwell, 1982].

1.2 Protective mechanisms against oxyradicals.

The extent in which the Fenton reaction occurs in the normal biological situation may be limited as these metals are rarely present in forms that can easily promote the Fenton reaction *in vivo*. This is due to the extraordinary efforts that eukaryotes have taken in developing multiple mechanisms which limit the presence of free Fe^{2+} and Cu^+ (thus inhibiting oxyradical formation) and to quench reactive oxygen species and radicals once these toxic compounds had been formed.

For example, iron can bind to transferrin, lactoferrin, ferritin, haptoglobin (Fe in haem form), urate and albumin (weakly); and Fe^{2+} may be oxidised by ceruloplasmin. Ceruloplasmin contains Cu, and albumin can also bind copper tightly, thus preventing the presence of free Cu^+ in the plasma. The damage due to uncontrolled Fenton reactions *in vivo* can be seen in cases of iron overload, α- and β-thalassemia and Wilson's disease (low ceruloplasmin) [Halliwell, 1987].

Other protective mechanisms include: superoxide dismutase - which catalyses the breakdown of O_2^- to H_2O_2; catalase - which converts H_2O_2 safely to H_2O in a two-electron reduction; glutathione peroxidase - which also breaks down H_2O_2 but requires glutathione (a cellular tripeptide-thiol, gly-cys-lys, in the reduced form) as a cofactor; and glutathione (GSH) is itself an antioxidant and may quench radicals, as do other thiols (proteins such as metallothionein), ascorbate, glucose, urate, and a-tocopherol [Orrenius, 1985].

1.3 Oxidant stress.

Excessive formation of reactive oxygen species results in 'oxidant stress' - depletion of cellular antioxidants and the metabolic reducing agents, such as NADH and NADPH, are also depleted through antioxidant reactivation pathways (GSH-reductase) and the direct damage by oxyradicals on cellular components involved in the regeneration of reducing agents (i.e. proteins, lipids, carbohydrates and DNA) [Halliwell, 1987] (Figure 1.2).

There is extensive evidence which implicates that oxygen free radicals play some role in the development of many diseases, including drug-induced diseases (Table 1.2). Much of this evidence is based on experimental data indicating increased lipid peroxidation in diseased tissues and/or the ameliorating effect of antioxidants. Although lipid peroxidation can be a consequence of tissue damage and

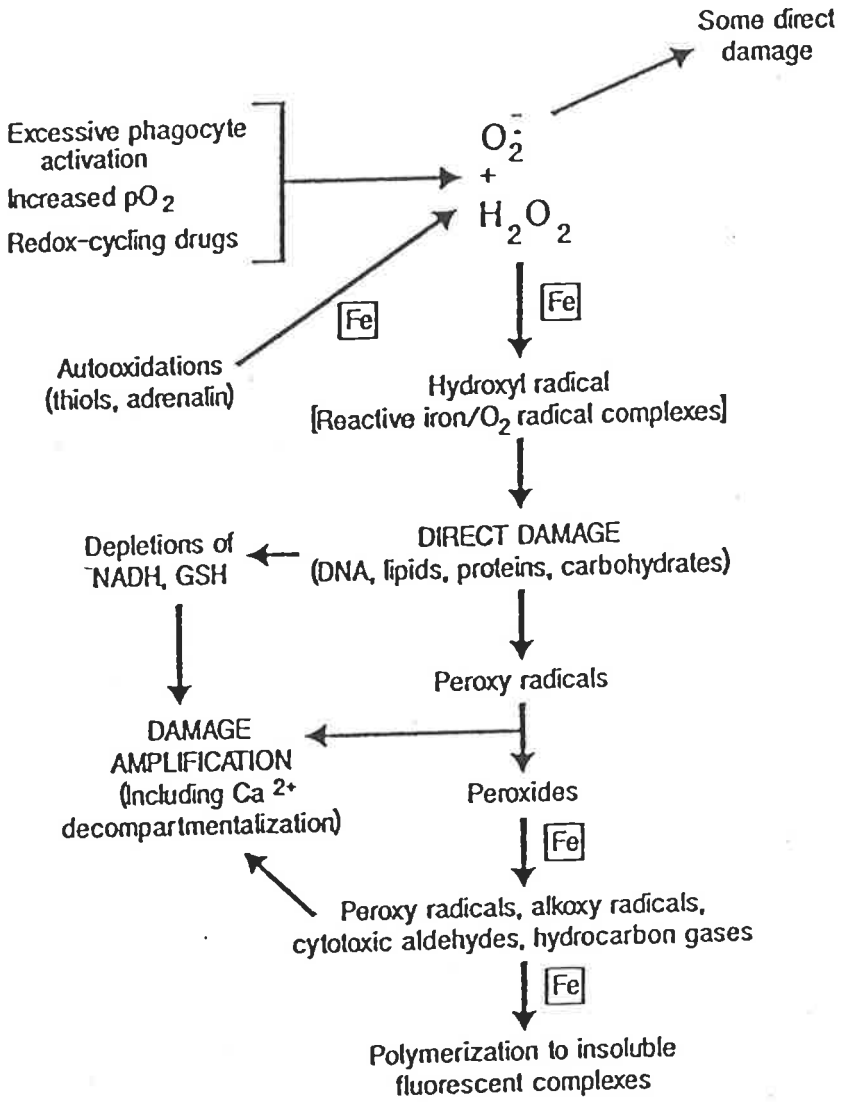

Figure 1.2: Mechanisms of cellular damage in oxidant stress. Superoxide = O_2^-; hydrogen peroxide = H_2O_2; reduced nicotinamide-adenine dinucleotide = NADH (NAD-phosphate = NADPH); reduced glutathione = GSH [Halliwell, 1987].

Table 1.2: Clinical conditions in which oxygen radicals are thought to be involved. [Cross CE, 1987].

Multiorgan involvement:

Inflammatory-immune injury: Glomerulonephritis (idiopathic, membranous), vasculitis (hepatitis B virus, drugs), Autoimmune disease.

Ischemia-reflow states

Drug and toxin-induced reactions

Iron overload: Idiopathic haemochromatosis, dietary iron overload, thalassemia and other chronic anemias.

Nutritional deficiencies: Kwashiorkor, Vit E deficiency

Alcohol

Radiation injury

Aging: Disorders of "premature aging", immune deficiency of age.

Cancer

Amyloid diseases

Primary single organ involvement:

Erythrocytes: Phenylhydrazine, primaquine, lead-poisoning, protoporphyrin photo-oxidation, malaria, sickle cell anemia, favism, Fanconi anemia.

Lung: Cigarette-smoke effects, emphysema, hyperoxia, bronchopulmonary dysplasia, oxidant pollutants, acute respiratory distress syndrome, mineral dust pneumoconiosis, bleomycin toxicity, paraquat toxicity.

Heart and cardiovascular system: Alcohol cardiomyopathy, Keshan disease (selenium deficiency), atherosclerosis, doxorubicin toxicity.

Kidney: Nephrotic antiglomerular basement membrane disease, aminoglycoside nephrotoxicity, heavy metal nephrotoxicity, renal graft rejection.

Gastrointestinal tract: Endotoxin and CCl_4 liver injury, diabetogenic action of alloxan, free-fatty-acid-induced pancreatitis, non-steroidal-antiinflammatory-drug-induced lesions.

Joint abnormalities: Rheumatoid arthritis.

Brain: Hyperbaric oxygen, neurotoxins, senile dementia, Parkinson disease-MPTP, hypersensitive cerebrovascular injury-cerebral trauma, neuronal ceroid lipofuscinoses, allergic encephalomyelitis and other demyelinating diseases, ataxia-telangiectasia syndrome, potentiation of traumatic injury, aluminium overload, A-β-lipoproteinemia.

Eye: Caractogenesis, ocular hemorrhage, degenerative retinal damage, retinopathy of prematurity, photic retinopathy.

Skin: solar radiation, thermal injury, porphyria, contact dermatitis, photosensitive dyes, Bloom syndrome.

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need not be related to the primary mechanism of tissue injury, it may result in amplification of the original injury - through disruptions of cellular metabolism (i.e. GSH depletion), or through the generation of toxic aldehydes capable of causing cell damage at other sites [Cross CE, 1987].

1.4 Inflammation-induced oxidant stress.

Inflammatory-immune injury results from the stimulation of phagocytes in particular polymorphonuclear leukocytes (PMN), by immune complexes and activated complement. In response to these stimuli PMN produce O_2^- and subsequently H_2O_2 - which may be converted to hypochlorous acid (HOCl, another potent oxidant) by myeloperoxidase, an enzyme released during the simultaneous degranulation that occurs following activation by most stimuli. HOCl can form chloramines which are relatively stable oxidants that can migrate and exert more widespread effects than the more reactive oxyradicals. The role of $OH^{\cdot}$ in this system remains contentious, as there is dispute over the presence of free iron and whether the hydroxylating activity of PMN on aromatic compounds is in fact mediated by free $OH^{\cdot}$ or ferryl ion [Winterbourn, 1988].

Inflammation is mediated and potentiated by the pharmacological actions of the endogenous transmitters of immune system. These include prostaglandins, such as PGE_2 and PGI_2 which are synthesised via the arachidonate-

cyclooxygenase pathway, and cytokines - a group of polypeptide messengers with a wide variety of structures and forms, but many have very similar properties, i.e. interferons (IFN), interleukins (IL), colony-stimulating factors (CSF) and tumour necrosis factor (TNF) [Old, 1988]. Cell culture experiments have revealed that these endogenous agents may act similarly (i.e. TNF and IL-1, in promoting inflammation), and in combination can result in either synergism (TNF and IFN, in killing tumour cells) or antagonism (PGE_2 and PGI_2 on cardiovascular tone; PGE_2 and IL-1, PG inhibits IL synthesis) depending on cell type and the particular function studied.

1.5 Hepatic changes during inflammation.

One of the interleukins (IL-6) has been shown to affect the liver and stimulates hepatocytes to produce acute phase reactants (APR) [Gauldie et al, 1987]. These serum glycoproteins include the thiol-rich metallothionein, Cu-ceruloplasmin and haptoglobin, all of which possess attributes that assist the body in overcoming oxidant stress.

In vivo animal models for acute inflammation generally involve the tail-base or foot-pad injection of irritants, such as oleyl alcohol and turpentine oil, which usually results in a marked inflammation that subsides after a few days. Arthritis can be induced by the injection of an adjuvant, such as *Mycobacterium tuberculosis* in mineral oil,

which results in an acute phase of inflammation, followed by a more prolonged chronic arthritis lasting for a few weeks [Whitehouse, 1986].

Evidence over the past two decades has revealed certain hepatic effects that accompany APR synthesis resulting from chemical-induced inflammation, such as decreased albumin synthesis and reduced metabolic function. Sleeping times of barbiturates were prolonged by adjuvant-induced arthritis but were not altered with two other immunologically mediated 'inflammatory diseases' (graft vs. host disease and allergic encephalomyelitis) [Kato, 1977]. There are conflicting reports concerning the effects of arthritis on human drug metabolism [Chalmers et al, 1969; Furst and Paulus, 1975; Selley et al, 1975], however these may be due to other confounding factors.

1.6 Metabolism of xenobiotics in the liver.

Both xenobiotics and endogenous waste compounds undergo hepatic metabolism known as Phase I and Phase II biotransformation. This process increases the polarity and water solubility of the compounds prior to transport to the kidney and subsequent excretion.

Many toxic xenobiotics cannot directly initiate toxicity but require metabolic activation to form reactive intermediates, and it is these short-lived radicals and electrophiles which are responsible for the ensuing toxicity

[Mason and Chignell, 1982]. Xenobiotic activation is mainly performed by hepatic enzymes involved in Phase I biotransformation, which catalyse the oxidation, reduction or hydrolysis of various substrates. The major group of Phase I reactions are catalysed by the microsomal mixed function oxidases (MMFO) which are a family of haemoproteins known as cytochrome P-450 [Trush et al, 1982].

Different subsets of cytochrome P-450 isozymes have greater specificities for certain classes of substrates, and four types of isozymes are inducible by their prefered substrates - i.e. phenobarbital, polycyclic aromatic hydrocarbons (PAH, i.e. 3-methylcholanthrene), ethanol and steroids (pregnenolone-16α-carbonitrile, PCN). Hypnotic barbiturates, such as phenobarbital and pentobarbital, are predominantly metabolised by the P-450 group of cytochromes, whereas PAH's are mainly metabolised by cytochrome P-448 isozymes [Atchison and Adesnick, 1983a & 1983b; Hardwick et al, 1983].

The protective mechanisms present in hepatocytes, which counter the effects of activated metabolites, involve the quenching of radicals and the resultant oxidant stress (as previously mentioned) or the formation of GSH-adducts (either directly or catalysed by GSH transferase) - both mechanisms depend on the cellular store of reducing agents.

Therefore the extent of hepatotoxicity resulting from the metabolic activation of xenobiotics depends on both MMFO activity and the perturbations of the cellular redox state with the subsequent changes in available antioxidants.

A multi-directional approach was adopted in the experimental work in this thesis, in order to examine various aspects of systemic oxidant stress. These aspects included: the *in vitro* measurement of reactive oxygen species - in particular the $OH^{\cdot}$, which required the development of a suitably sensitive and specific assay; and the effects of systemic oxidant stress on hepatotoxicity *in vivo* - in which hepatic metabolic and glutathione profiles were measured, hepatotoxic responses of inflamed animals to paracetamol and bromobenzene were assessed, and the interaction of anti-inflammatory and anti-arthritic drugs with two animal models of inflammation was examined. To elucidate the possible pathways involved in mediating the effects of systemic oxidant stress on the liver, it was also necessary to examine the immunological status of inflamed animals.

Chapter 2

HPLC-ECD ASSAY OF HYDROXYLATED SALICYLATE FOR THE DETECTION OF HYDROXYL RADICALS

2.1 INTRODUCTION

The sequential one-electron reduction of oxygen to water results in the formation of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH·). Of these 'reactive oxygen species' (ROS), OH· is the most reactive *in vitro*, and therefore an important radical to monitor in conditions of oxygen stress.

Whether the formation of OH· occurs *in vivo* remains contentious for the following reasons. Firstly, there is no certainty that iron is present in a suitable form to participate in a Haber-Weiss reaction, i.e. the catalysis of OH· formation from O_2^- and H_2O_2 [Winterbourn et al, 1986; Thomas et al, 1986; Brittigan et al, 1986.]. Furthermore, the evidence supporting OH· production so far, has been indirect, utilizing methods such as the release of ethylene from methional [Halliwell and Grootveld, 1987], decarboxylation of benzoic acid [Sagone et al, 1980], the analysis of electron spin resonance spectra generated by spin trapping reagents, such as 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) [Samuni et al, 1986], and aromatic hydroxylation.

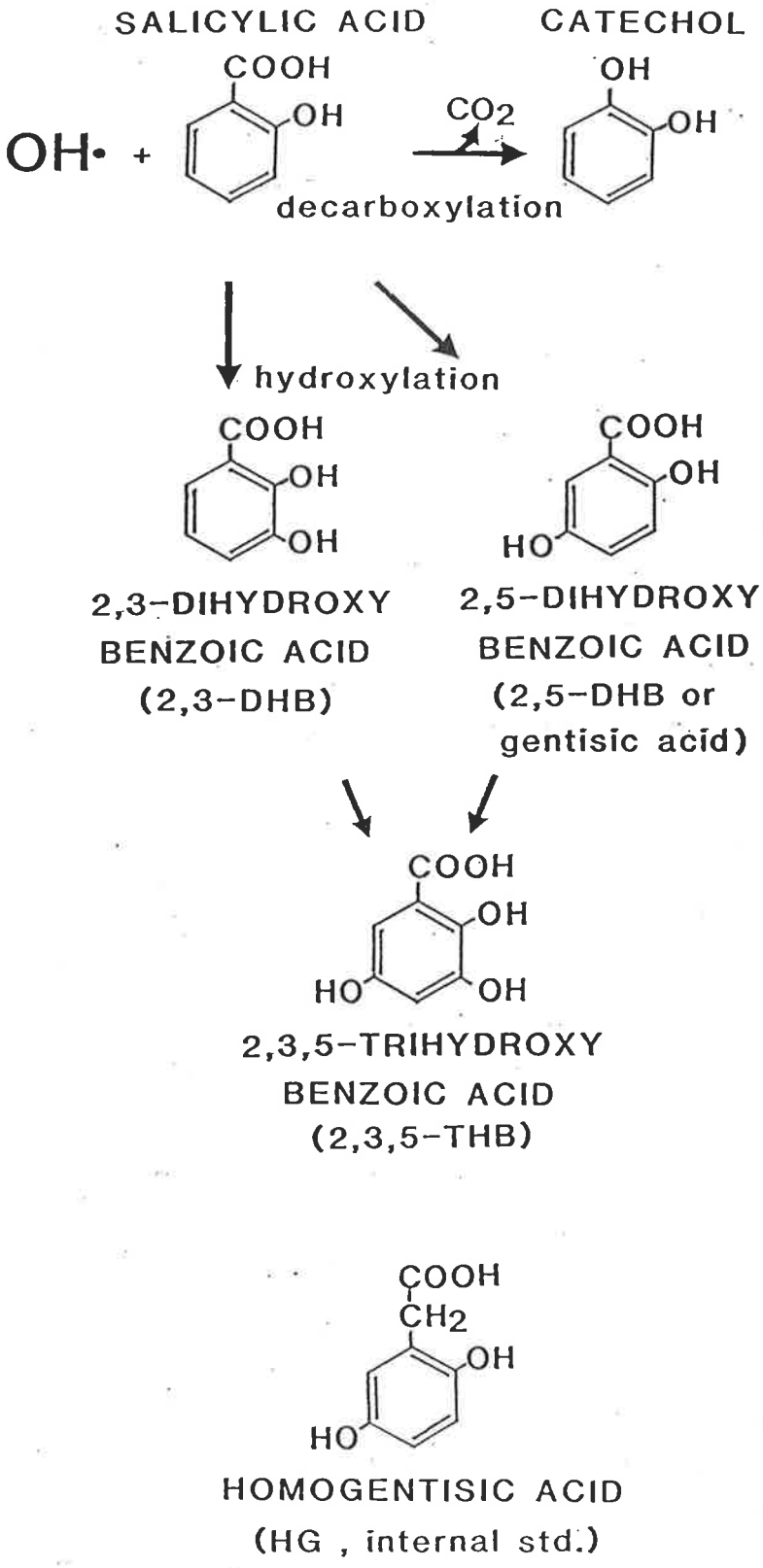
At the commencement of this research project in 1984, the most recently published methods which used aromatic hydroxylation for the detection of OH· were: (a) Richmond et al (1981), who employed gas chromatography of hydroxylated

phenols and the colourimetric determination of a tungstate-catechol complex from salicylate hydroxylation; and (b) Radzik et al (1983) who used liquid chromatography/ electrochemistry to measure the 1,2- and 1,4-hydroxylated products of aniline and phenol. Both groups used the xanthine oxidase/hypoxanthine system (XO/HX), in simple buffered aqueous media, to generate $OH^{\cdot}$.

Previous problems encountered in cellular systems have included the need for the presence of extremely large concentrations of possibly noxious probe chemicals, i.e. spin trapping reagent DMPO at 10 to 100 mM [Samuni et al, 1986]. Also application of the benzoate decarboxylation assay [Sagone et al, 1980] to cellular systems has met with several difficulties, some of which were overcome only by the use of special reaction vessels with glass necks fitted to photomultiplier tubes, to enable the measurement of $[^{14}C]$-CO_2 as it was produced during the incubation (albeit with low counting efficiency).

The goal was therefore the development of a sensitive assay for $OH^{\cdot}$ production which was suitable for use in the more complex cellular systems. Thus salicylate was chosen for the aromatic probe compound as it is less toxic than phenol in biological systems [Merck Index, 1983]. The interaction of $OH^{\cdot}$ with salicylate results in hydroxylation, to form 2,3- and 2,5- dihydroxybenzoates (2,3- and 2,5-DHB), or decarboxylation to produce catechol (see figure 2.1).

Figure 2.1: Products of salicylate & OH· interaction.



Attack by OH· (generated by Fenton systems at pH 7.4) upon salicylate results in proportions of products of 49, 40 and 11% for 2,3-, 2,5-DHB and catechol, respectively [Grootveld and Halliwell, 1986].

2,5-DHB (also called gentisate) had been measured in the plasma and synovial fluid of patients on salicylate therapy, utilizing a method developed in this department for the ultraviolet detection of aspirin metabolites separated by HPLC [Cleland et al, 1985a]. However 2,3-DHB was not detected in these biological samples, even though the patients had been chronically administered with high dose aspirin. Therefore it was necessary to develop a more sensitive method for the quantitation of salicylate hydroxylation since it was anticipated that cellular preparations would better tolerate the use of low salicylate concentrations.

This chapter describes an assay which was developed to measure OH· generation *in vitro*, utilizing salicylate as a substrate, and quantitation of the 2,3- and 2,5-DHB product by HPLC/amperometric detection, with picomolar sensitivity [Wright and Priestly, 1985]. This HPLC-ECD procedure was also compared to other methodologies, e.g. luminol-dependent chemiluminescence and benzoate decarboxylation, using cell-free systems. The application of the HPLC-ECD method to a cellular system, which in this case was activated human neutrophils *in vitro*, is described in chapter 3.

2.2 INCUBATION SYSTEMS FOR OH· FLUXES

Incubations were generally performed in potassium phosphate buffer (2 ml, 0.1 M, pH 7.4). OH· fluxes were generated: (a) enzymatically by the XO/HX system - XO (0.025 IU/ml), HX (500 μM)/ ferric chloride/ EDTA (both 100 μM), at 37^{o}C for 60 minutes; (b) and chemically, by the autoxidation of ferrous sulphate (500 μM) upon the addition of EDTA (2 mM), at 20^{o}C for 5 minutes.

2.2.1 Quantitation by chemiluminescence.

The nature of these two OH· fluxes were initially investigated by the luminol chemiluminescence assay. Luminol (100 μM in K_2HPO_4 buffer, 50 mM, pH 7.4) was added to borosilicate tubes (6 x 50 mm Kimble disposables) with components of OH· flux (total volume 300 μl). After the final addition incubations were quickly vortexed without aeration, before the chemiluminescence was measured in a thermostatically controlled luminometer (Packard Pico-lite 6100) at 28^{o}C for 20 ten second intervals.

The autoxidation of ferrous sulphate (added immediately before vortexing, and placed in the luminometer within 7 secs) appeared to be almost instantaneous. Addition of ferrous ions via Hamilton syringe did not provide sufficient mixing, and as vortexing was necessary, it was not possible to measure the true maximum (figure 2.2.a). In contrast, the XO/HX generates a constant amount of chemiluminescence

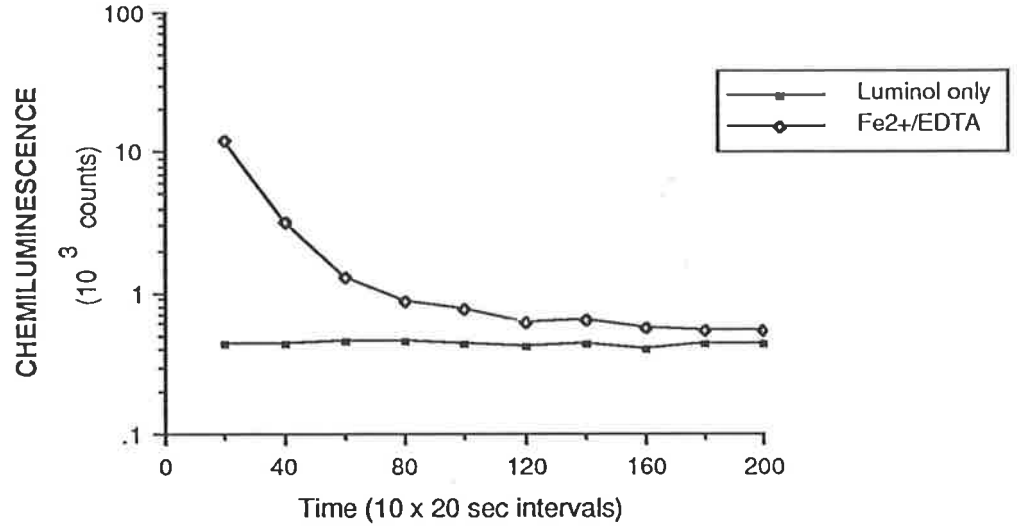
Figure 2.2: Quantitation of oxyradical fluxes generated in enzymatic and chemical systems by luminol chemiluminescence. Luminol (100 μM) in K_2HPO_4 buffer (50 mM, pH 7.4) was added to borosilicate tubes with the components of oxyradical flux, vortexed without aeration, and placed in a thermostatically controlled luminometer.

(a) chemical generation by ferrous sulphate autoxidation (500 μM, upon the addition of EDTA, 2 mM).

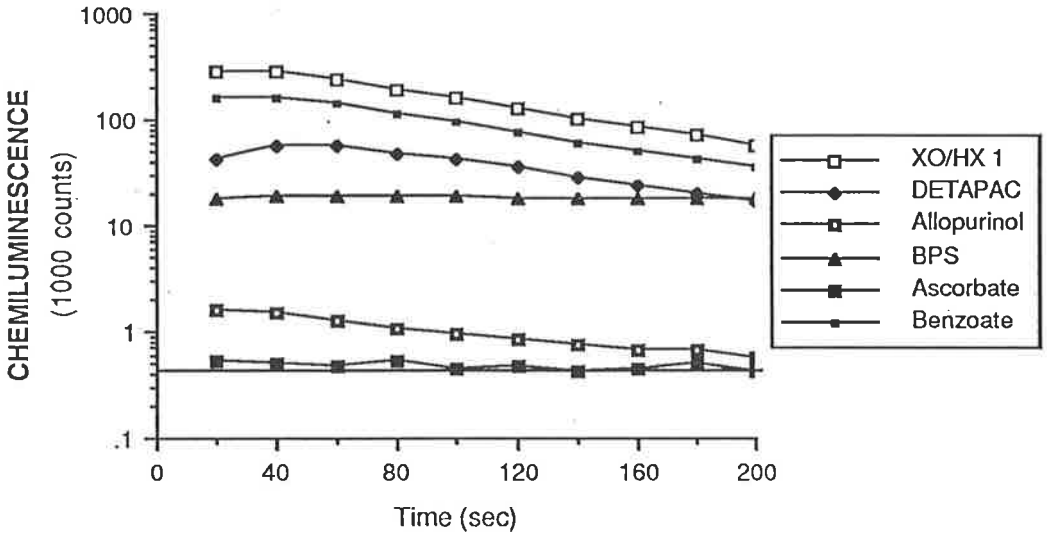
(b) enzymatic generation by the XO (0.025 IU/ml)/HX (500 μM)/ferric chloride/EDTA (both 100 μM); at 37^{o}C.

Luminol-dependent chemiluminescence is not specific for a particular reactive oxygen species, but is an indication of total oxyradical flux. The addition of Fe^{3+}/EDTA to the XO/HX system would result in the accelerated catalysis of O_2^- to H_2O_2 and the short-lived $OH^{\cdot}$, and therefore less oxyradicals would be available for reaction with luminol.

2.2 a


CHEMILUMINESCENCE
(10 3 counts)
100
10
1
.1
0
40
80
120
160
200
Time (10 x 20 sec intervals)
Luminol only
Fe2+/EDTA

2.2 b


CHEMILUMINESCENCE
(1000 counts)
1000
100
10
1
.1
0
40
80
120
160
200
Time (sec)
XO/HX 1
DETAPAC
Allopurinol
BPS
Ascorbate
Benzoate

in the presence of luminol - which is reduced by 75% by the addition of ferric chloride/EDTA (figure 2.2.b)

2.3 HPLC/ECD ASSAY METHOD

Reactions were terminated by phosphoric acid (50% v/v, 200 μl) and the internal standard homogentisic acid (HG, 10 ÌM, 50 μl) was added. DHB were back-extracted into phosphate buffer (0.1 M, pH 7.4, 75 μl) via diethyl ether (4 ml, $0^{o}C$, deperoxidated with activated alumina). Samples were acidified with phosphoric acid (5% v/v, 25 μl) and frozen until assayed. 20 μl of each sample was injected onto a reverse phase HPLC column (C18 μBondapak, 5 μm, 0.4 x 15 cm) with precolumn (C18 Sigma, 30 μm) and eluted by mobile phase (30% methanol, 70% 0.1 M phosphate/ 0.1 mM EDTA, pH 2.5) at a flow rate of 1 ml per minute. DHB were measured by electrochemical detection (LC-4A Amperometric detector, with an RE-1 glassy carbon working electrode, Bioanalytical Systems Inc.) using an oxidation potential of +0.8 volts versus the Ag/AgCl reference electrode..

2.4 ASPECTS OF ASSAY SENSITIVITY

2.4.1 Hydrodynamic voltammogram.

The optimal potential difference was determined in order to achieve maximum sensitivity. The hydrodynamic voltammogram shown in figure 2.3 was constructed by varying the oxidation potential over the range of +0.2 to +1.2 volts (vs. Ag/AgCl reference electrode) at 0.1 volt increments,

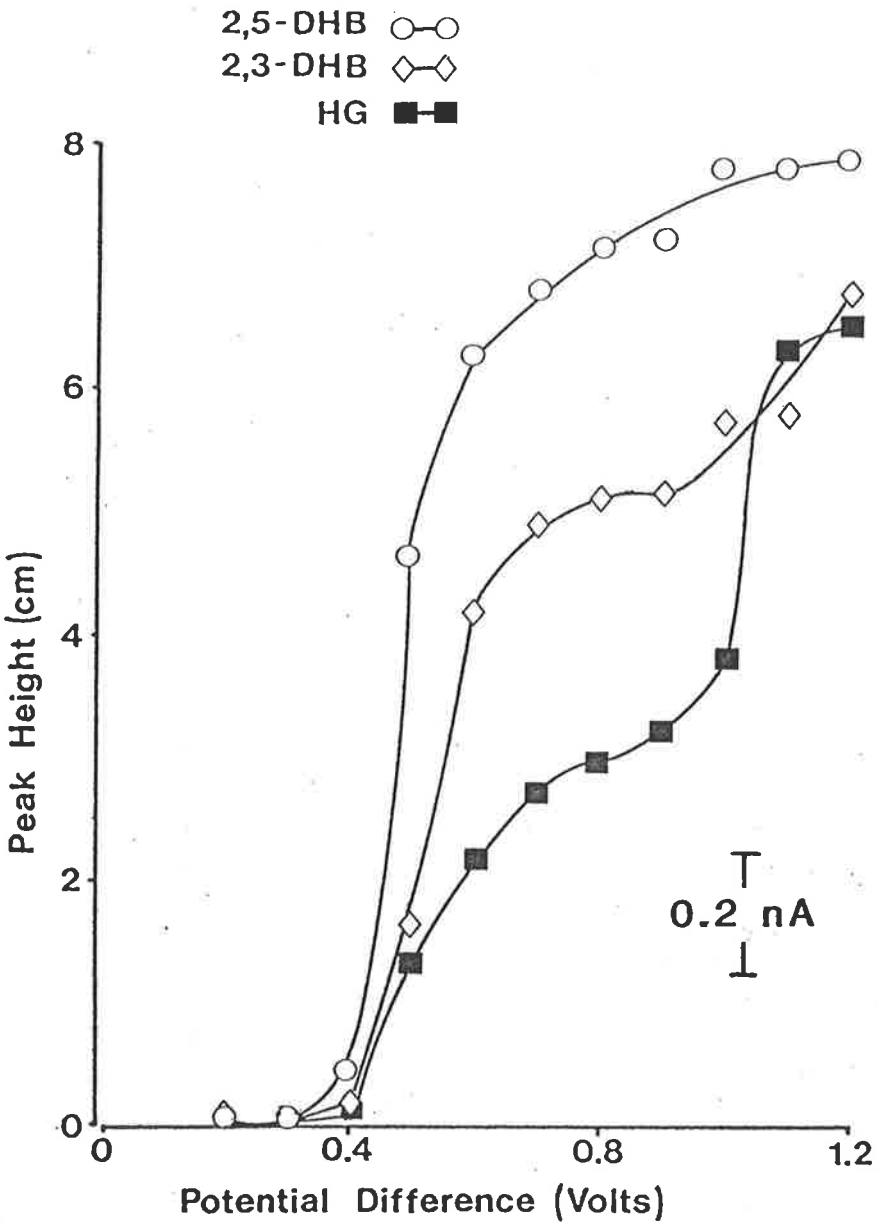


Figure 2.3: Hydrodynamic voltammograms of DHB and HG. A potential difference of 0.8 volts was chosen in the assay as this produced near maximal response with minimal noise in baseline.

for a standard sample containing HG, 2,3- and 2,5-DHB (20 pmol, injected in 20 μl, ECD range of 5 nA, 1 cm peak height = 0.2 nA).

A potential difference of 0.8 volts was chosen in the assay as this produced near maximal response (or plateau as in the case of HG) thus minimising the effects of small fluctuations in the conduction of mobile phase passing between the working and reference electrodes of the ECD. This voltage also resulted in minimal noise in baseline and no response to salicylate, at 100 nmol injected.

2.4.2 Standard Curve of DHB.

As seen in figure 2.4, a linear relationship exists between peak height and the amount of DHB standard injected, over an extremely wide range (5 to 400 pmol injected onto column), i.e. regression analysis:

2,3-DHB, $r^2 = 1.00$, $y = 0.72x + 1.51$

2,5-DHB, $r^2 = 1.00$, $y = 0.46x + 0.28$

- where dimensions for x and y are: pmoles of DHB (in 20 μl of extracted standard injected onto column); and peak height in mm, respectively (ECD range of 20 nA, 10 mm peak height = 0.8 nA).

2.4.3 Assay variability and limit of sensitivity.

Variability in the assay procedure was assessed by the analysis of a number of extracted standards which spanned the range used in the standard curves. Percentage

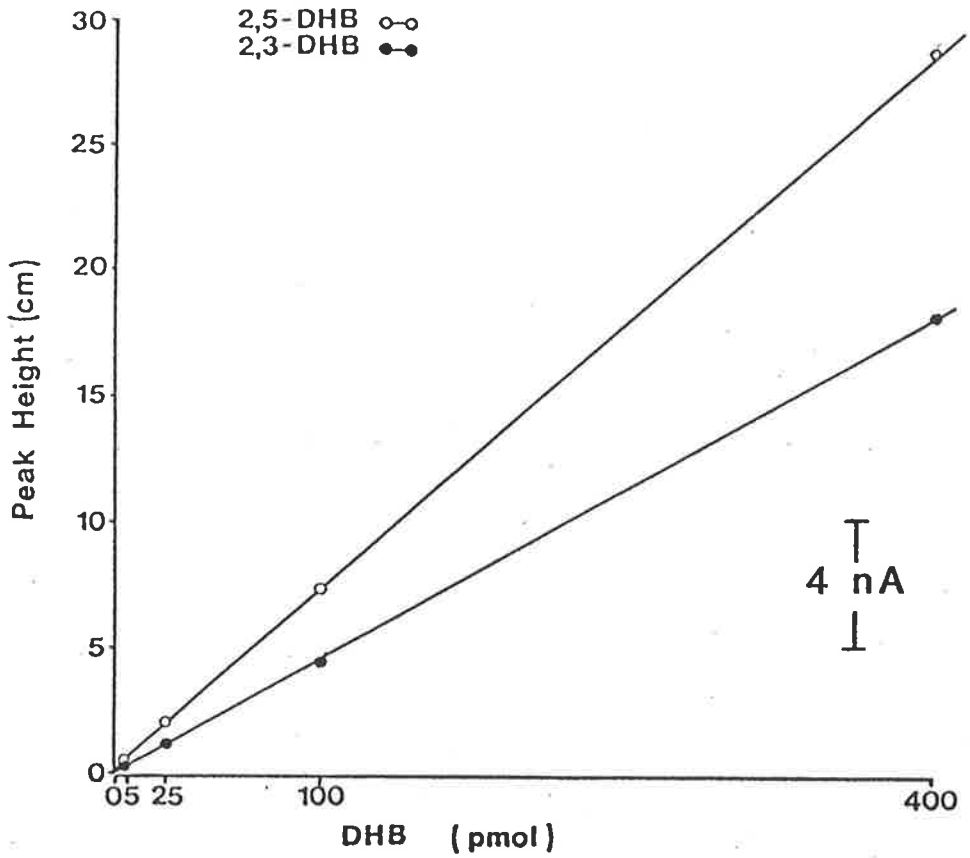


Figure 2.4: Standard curve for DHB. This graph shows the linear relationship between recorder peak height and the amount of DHB detected, over a wide range. (Scale represents pmol of DHB in the 20 μl injected onto column).

coefficient of variation (%CV) and mean coefficient of variation ($\overline{CV}$) are listed in table 2.1. Example chromatographs are shown in figure 2.5, and includes a trace from a 1 pmol extracted standard which shows an excellent signal to noise ratio (greater than 10:1).

2.4.4 Sample stability.

As DHB's oxidise easily in solution and crystalline form, standards were stored dessicated and under nitrogen, at -20^{o}C. The stability of extracted DHB standards was examined at various temperatures (-20, 5 and 25^{o}C), in light or dark, and in phosphate buffer (50 mM, pH 7.4) or perchloric acid (50 mM), over a 22 day period (figure 2.6). The final DHB concentration for storage (in triplicate) was 5 μM, and 10 μl was assayed by the HPLC/ECD method. As was expected, samples began to deteriorate at room temperature but remained stable when stored at -20 and 5^{o}C. The stabilisation of DHB with acid was effective at room temperature, however the effect was more apparent in the dark, for reasons which are unclear. All samples were acidified with phosphoric acid before freezing to reduce loss of DHB, after the back-extraction into weakly basic phosphate buffer which is necessary for the complete transfer of acidic products from the organic phase.

Ether forms strongly oxidising compounds when stored, including peroxides, which may reduce DHB recoveries during the extraction step. Therefore it was important to use

Table 2.1: Variability of DHB measurement by HPLC-ECD assay.

	pmol DHB	% CV *	N
2,5-DHB	1	4.7	9
	50	4.6	9
	500	6.1	8
		5.1 **	
2,3-DHB	1	5.6	9
	50	5.4	9
	500	7.7	8
		6.2 **	

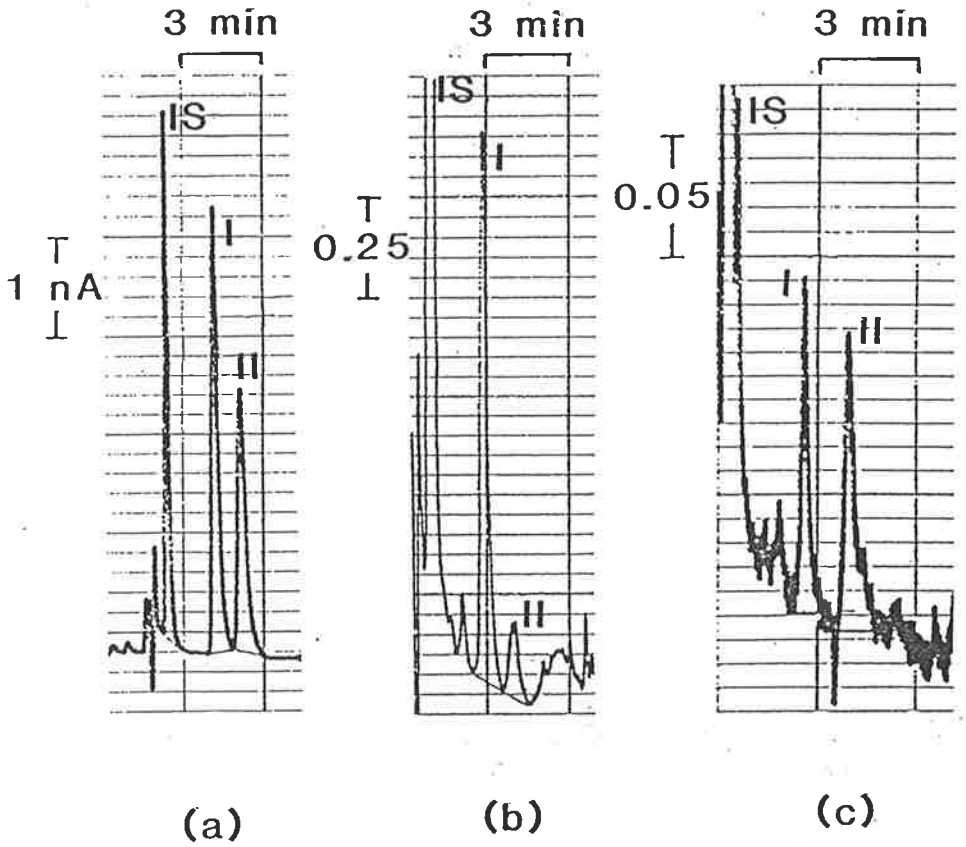
* Percentile coefficient of variation in the quantitation of extracted standards.
** Mean $\overline{CV}$ of standard curve.

Figure 2.5: Example chromatograms. Chromatography conditions as described in assay methods, except for a mobile phase flow rate of 1.5 ml/min - resulting in the retention times of 1.1, 2.8 and 3.8 minutes for HG (IS), 2,5-DHB (I) and 2,3-DHB (II), respectively (c.f. figure 3.1, flow rate 1 ml/min).

(a) Extracted DHB standards (100 pmol in 15 μl injected onto HPLC column) with internal standard.

(b) 2.9 pmol of 2,3-DHB and 18.4 pmol of 2,5-DHB was formed in a hepatic microsomal incubation (37^{o}C, 30 min., 0.1 mg/ml microsomal protein with NADPH generator, 20 μl of extracted sample was injected).

(c) 1 pmol DHB extracted standards, with a signal to noise ratio of 10 (25 μl injected).


3 min
IS
I
II
1 nA
(a)
3 min
IS
I
II
0.25
(b)
3 min
IS
I
II
0.05
(c)

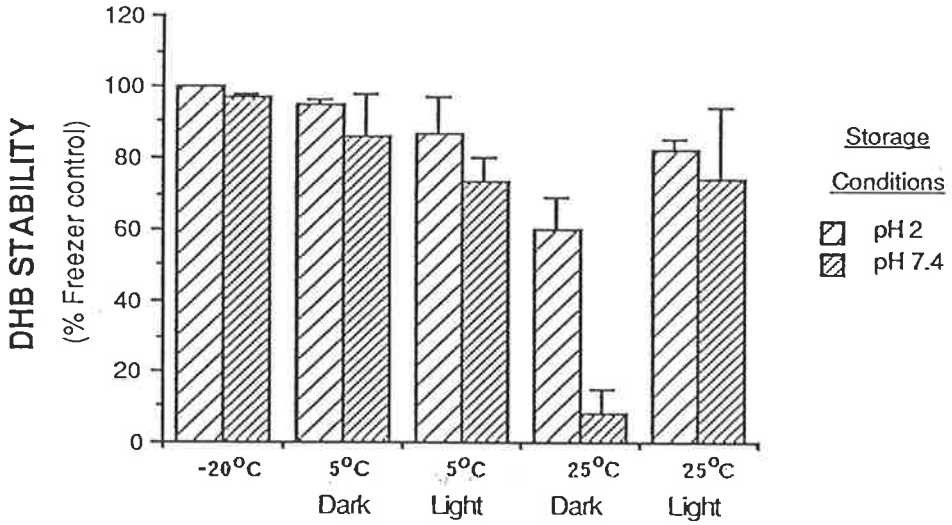


Figure 2.6: Storage stability. The stability of extracted DHB standards was examined at various temperatures (-20, 5 and 25°C), in light or dark, and in phosphate buffer (50 mM, pH 7.4) or perchloric acid (50 mM, pH 2), over a 22 day period (figure 2.6). The final DHB concentration for storage (in triplicate) was 5 μM, and 10 μl was assayed by the HPLC/ECD method.

ether which had previously been deperoxidated with aluminium oxide (anhydrous diethyl ether with basic or neutral activated Al_2O_3, 8.3 ml/g).

2.5 ASPECTS OF ASSAY SPECIFICITY

2.5.1 Specificity in the xanthine oxidase/hypoxanthine system.

The XO/HX/Fe^{3+}EDTA system described in the assay method was used to examine the specificity of the HPLC/ECD assay of hydroxylated salicylate for detecting OH·. This system relies on the conversion of O_2^- to H_2O_2 and OH· via the Fenton reaction. This enzymatically-driven reaction occurs at a constant rate for 15 min at 37°C (HX, 500 mM & XO, 0.025 IU/ml; ferric/EDTA, 100 μM), as shown in figure 2.7.

The ability of superoxide dismutase (SOD) and catalase (CAT) to inhibit salicylate hydroxylation in this system was not shared by the heat inactivated enzymes, thus implying the essential roles for both O_2^- and H_2O_2 in OH· generation (Table 2.2). These results were similar to those obtained colourimetrically by Richmond et al [1981] (CAT inhibit more than SOD). XO/HX alone resulted in 20% of the DHB production seen from the complete system of XO/HX/Fe^{3+}EDTA. This was SOD and CAT inhibitable which suggested trace metals could promote the Fenton reaction to a limited extent.

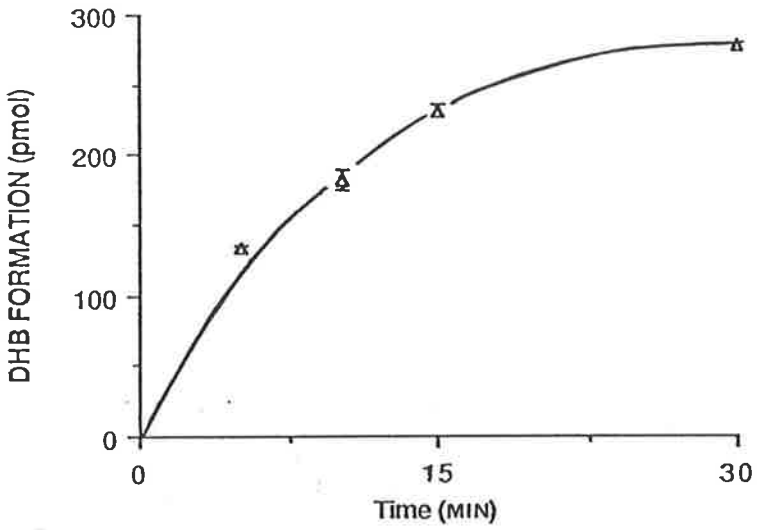


Figure 2.7: Time course of xanthine oxidase/ hypoxanthine/ ferric EDTA system. DHB formation in the XO (0.025 IU/ml)/HX (500 μM)/ferric/EDTA (both 100 μM), final incubation volume of 2 ml, at 37^{o}C.

Table 2.2: The specificity of salicylate hydroxylation assay for detecting hydroxyl radicals in the xanthine oxidase/hypoxanthine/ferric-EDTA system.

INCUBATION CONTENTS *	% CONTROL (±SE)
XO/HX + $FeCl_3$/EDTA	100 ± 3
" + SOD	34 ± 2
" + SOD-HI	87 ± 0
" + CAT	16 ± 1
" + CAT-HI	91 ± 1
XO/HX	20 ± 2
" + SOD	1 ± 0
" + CAT	1 ± 0
XO	1 ± 0
$FeCl_3$/EDTA	2 ± 0

INHIBITOR	IC_{50} **
p-Coumaric acid	0.25 mM
Dimethylurea	1.2 mM
DETAPAC	1.5 mM
Bathophenanthroline sulphonate	5.5 mM
SOD	11.5 IU/ml
CAT	9.9 IU/ml

* Total system: Incubation volume of 2 ml containing xanthine oxidase (XO, 0.025 IU/ml), hypoxanthine (HX, 500 μM), $FeCl_3$/EDTA (100 μM), incubated at 37^{o}C for 1 hr. Superoxide dismutase (SOD, 16 IU/ml) and catalase (225 IU/ml), or heat inactivated (SOD-HI and CAT-HI) by boiling for 10 minutes. The total system produced 976 ± 28 pmoles DHB.

** Inhibitor concentration required to reduce DHB production in the XO/HX + $FeCl_3$/EDTA system by 50%.

Dimethylurea and p-coumaric acid - two OH· quenchers; pentetic acid (or DETAPAC) and bathophenanthroline sulphate (BPS), - two divalent metal chelators; and SOD and CAT, were added in varying concentrations to the above system to determine the concentration of inhibitor required to reduce DHB production by 50% (IC_{50}'s listed in table 2.2). The quenching agents proved to be more efficacious inhibitors than the metal chelators in this system.

The hydroxylation efficiency of salicylate by the ferrous sulphate/ EDTA system was constant over a wide substrate range - 13% for salicylate concentrations of 0.25 to 3.75 μM (in 2ml incubation volume). This confirms the instantaneous nature of Fe^{2+} autoxidation which was seen by luminol chemiluminescence, and suggests that the proportion of the substrate hydroxylated is dependent on the density of substrate molecules present at initiation of the reaction.

2.5.2 Comparison with ^{14}C-benzoate decarboxylation assay.

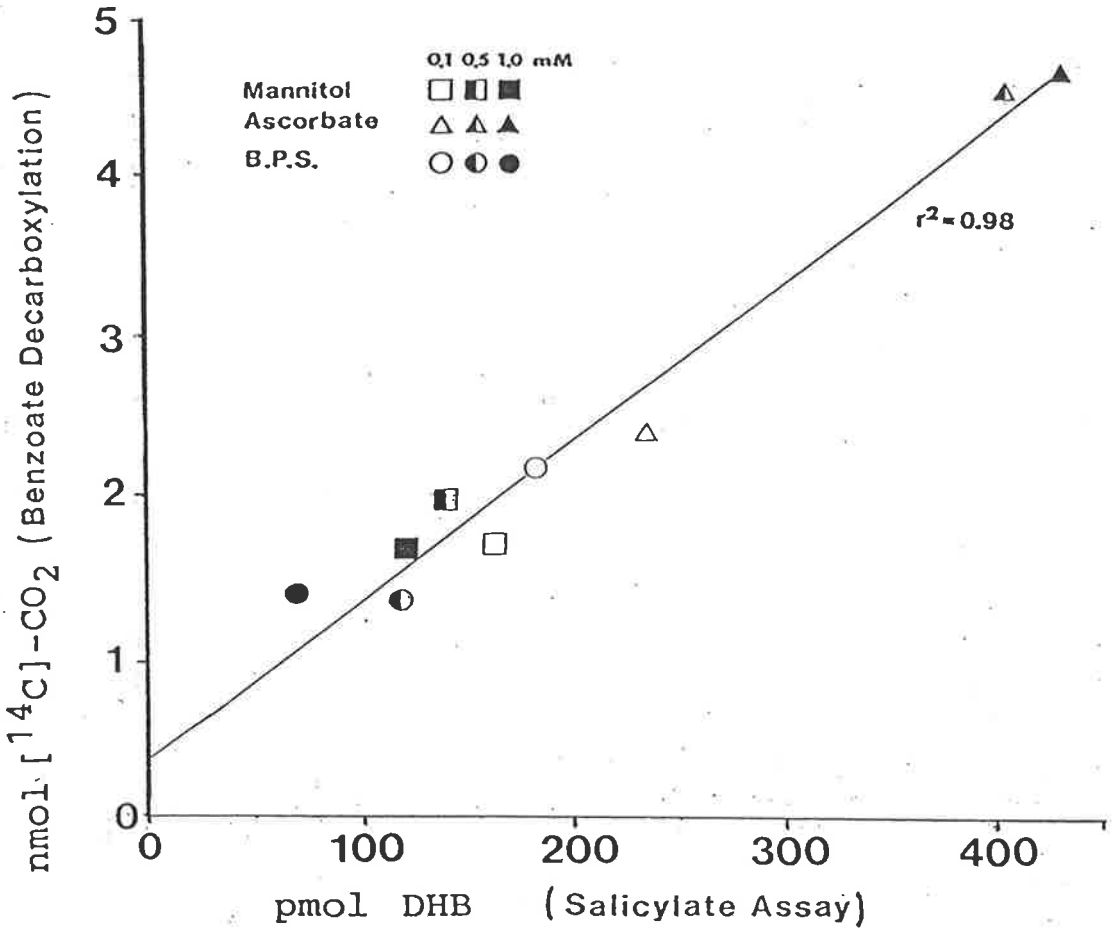
The chemical generation of OH· by the autoxidation of ferrous ions was examined by both the HPLC/ECD procedure and the radiometric benzoate decarboxylation assay. The procedure of Sagone et al (1980) was adopted with slight modifications. A reaction mixture containing [7-^{14}C]-benzoate (specific activity: 18.8 mCi/mmol, incubation concentration: 0.8 μCi/ 42.4 nmol/ml) and EDTA (2 mM) in phosphate buffer (50 mM, pH 7.4, 2.5 ml), was placed in the

outer division of a 25 ml ehrlenmeyer flask with a center well containing filter paper (3 cm^2) soaked with phenylenediamine (200 μl). The flasks were sealed with resealable rubber septae, and reactions initiated by the addition of ferrous sulphate (final concentration 500 μM, stock solution in distilled water). The reactions were terminated with trichloroacetic/hydrochloric acid (8% w/v in 0.5 M HCl, 0.5 ml) added by syringe through the septa (1 ml tuberculin syringe with 23G x 1.25 needle). The $[^{14}C]$-CO_2 was allowed over 30 minutes to absorb onto the filter paper, which was then removed and analysed by liquid scintillation (LS 3801 liquid scintillation system, Beckman Instruments Inc.).

The ferrous/EDTA test-system DHB formation was 83 pmol of 2,5-DHB and 74 pmol of 2,3-DHB (157 pmol total DHB production from 2.5 nmol salicylate/incubation, i.e. 6.3% conversion), and control $[^{14}C]$-CO_2 production was 1.92 nmol (or 81.75 x 10^3 DPM, from 106 nmol benzoate/incubation, i.e. 1.8% conversion).

There was good correlation between the salicylate hydroxylation assay and the benzoate decarboxylation assay (regression analysis r^2 = 0.98, y = 0.77x + 0.18) for the Fe^{2+}EDTA system plus inhibitors, mannitol and BPS, and ascorbate, which facilitates the Fenton reaction (figure 2.8).

Figure 2.8: Regression analysis comparing results obtained from the salicylate hydroxylation assay with those of ^{14}C-benzoate decarboxylation assay. Test system: $FeCl_2$ (500 μM)/ EDTA (2mM) with mannitol, ascorbate, or bathophenanthroline sulphonate at 0.1, 0.5 and 1.0 mM, in a total incubation volume of 2.5 ml. The control test-system DHB formation was 83 pmol of 2,5-DHB and 74 pmol of 2,3-DHB (157 pmol total DHB production from 2.5 nmol salicylate/incubation, i.e. 6.3% conversion), and control [^{14}C]-CO_2 production was 1.92 nmol (or 81.75 x 10^3 DPM, from 106 nmol benzoate/incubation, i.e. 1.8% conversion).


0,1 0,5 1,0 mM
Mannitol
Ascorbate
B.P.S.
r^2 = 0.98
nmol [^{14}C]-CO_2 (Benzoate Decarboxylation)
pmol DHB (Salicylate Assay)
0
1
2
3
4
5
0
100
200
300
400

2.5.3 Other products of OH. fluxes.

The amounts of DHB formed by the radical fluxes which had been investigated were easily detected and reproducible. However, some years earlier, there had been a suggestion that further products could be derived oxidatively from 2,3- and 2,5-DHB (Ledvina, 1969). These included: the quinones produced by oxidation of the hydroquinones (DHB), i.e. 2-carboxy-1,4-benzoquinone from 2,5-DHB, and 6-carboxy-1,2-benzoquinone from 2,3-DHB; and a hydroxylation product, 2,3,5-trihydroxybenzoate (THB).

In conditions of high $OH^{\cdot}$ flux, it is conceivable that the DHB produced from the hydroxylation of salicylate could also be subjected to $OH^{\cdot}$ attack. The meta-directing (deactivating) properties of the carboxyl group, and the ortho/para-directing (activating) properties of the hydroxyl groups in DHB would favour the addition of a nucleophile in the 5 position for 2,3-DHB, or the 3 position for 2,5-DHB - both hydroxylations resulting the formation of 2,3,5-THB (figure 2.1). Thus it was of interest to determine the presence of THB and investigate the possibility of utilizing THB as an additional indicator of $OH^{\cdot}$ fluxes.

In order to pursue this avenue, I attempted to obtain THB in a sufficiently purified form suitable for use as an HPLC standard. As THB was not available commercially, the synthetic procedure of Schock and Tabern (1954) was employed. This simple method relied on the persulphate

oxidation of salicylate (or alternatively 3,5-DHB) at controlled temperature and rate. Although this procedure was strictly followed, the reaction failed in three separate attempts using either salicylate or 3,5-DHB, as predominantly 2,5-DHB and some 2,3-DHB were produced (figure 2.9). The non specific nature of the persulphate oxidation, which results in many side reactions, had previously been acknowledged by Kreuchunas (1956). Criticisms involving the irreproducibility of product yield (Expt. 1, THB : 2,5-DHB = 80:20. Expt. 2, THB : 2,5-DHB = 15:85, from Schock and Tabern, 1951), and the extreme difficulty in separating the various closely-related acidic compounds in the reaction mixture, were well founded. However the assumption that conversion of a large amount of starting material would produce enough THB for a chromatography standard was unfortunately incorrect, and sufficient THB was not obtained. The more specific method of Kreuchunas employed a complex and labour-intensive five-part synthesis which involved the conversion of vanillin through 5-bromovanillin, 6-bromo-2-hydroxy-1,4-quinone, 2,3,5-trimethoxy-1-bromobenzene and 2,3,5-trimethoxy-benzoic acid to THB.

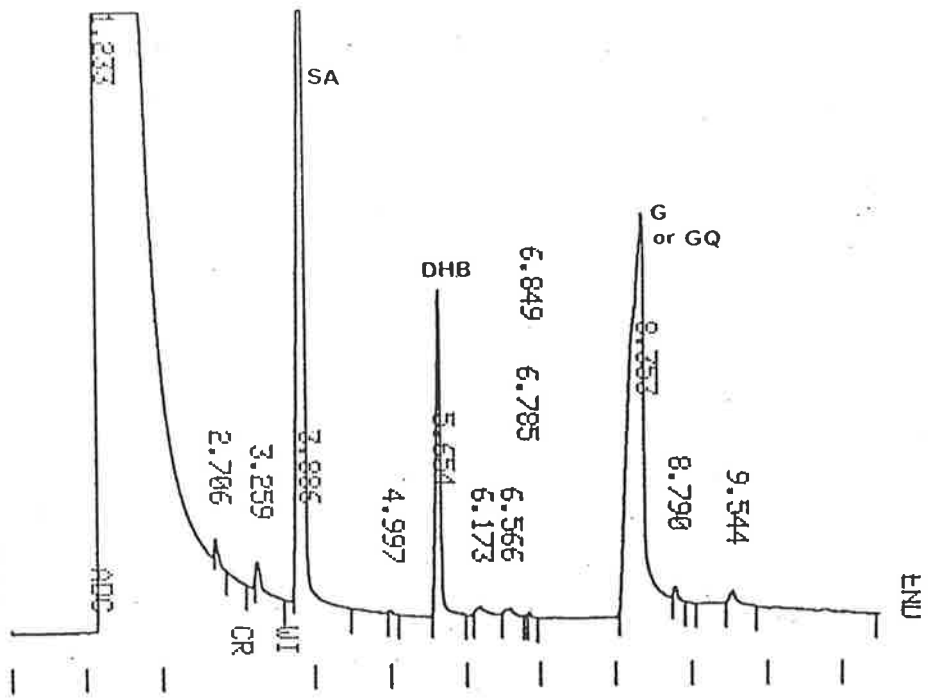
2.6 DISCUSSION

This chapter concerns a sensitive and specific HPLC-electrochemical assay for hydroxylated salicylate, in the detection of hydroxyl radicals. The sensitivity of the assay is due to the extraction and storage procedures, and

Figure 2.9: (a) Gas chromatography of THB-synthesis reaction mixture. The synthesis of THB by the method of Schock and Tabern (1954) involved the persulphate oxidation of salicylate (or alternatively 3,5-DHB) at 15°C. (1) An example of a gas chromatograph of reaction mixture solid (after solvent cleanup) in methanol (0.01 mg/ml, 3 μl with 1:30 split injection), (2) and after reaction with diazomethane (from diazald in ether, also 3 μl split injection). SA - salicylic acid, DHB - 2,3-DHB, G - gentisic acid, GQ - gentisoquinone (also called 2-carboxy-1,4-benzoquinone), MeSA - methylated SA, MeG - methylated gentisic acid, MeGQ - methylated gentisoquinone, and Diazo - an artefact due to the Diazald system.

Gas chromatography was performed with a Varian Model 3400 GC with IBDH integrator, split/splitless injector and flame ionisation detector (FID). GC conditions: Capillary column - 25m QC2/BP1 nonpolar methylsilane liquid phase, I.D 0.22 mm, 0.25 μm film thickness (S.G.E.); Initial column temp. 140°C (3 min hold time), 8°C temp. ramp to 200°C final temp. (1 min hold time), run time 11.5 min.; Injector temp. 250°C, detector temp. 280°C; FID attenuation 8, range 11; Plot speed 1 cm/min; Helium carrier gas, nitrogen make-up gas, hydrogen and air for FID.

1


FID A 8X11 1.0 CM/M 10%
INJECT
ADC
SA
DHB
G
or GQ
2.706
3.259
3.886
4.997
5.654
6.173
6.566
6.785
6.849
8.753
8.790
9.544
WI
CR
END

2

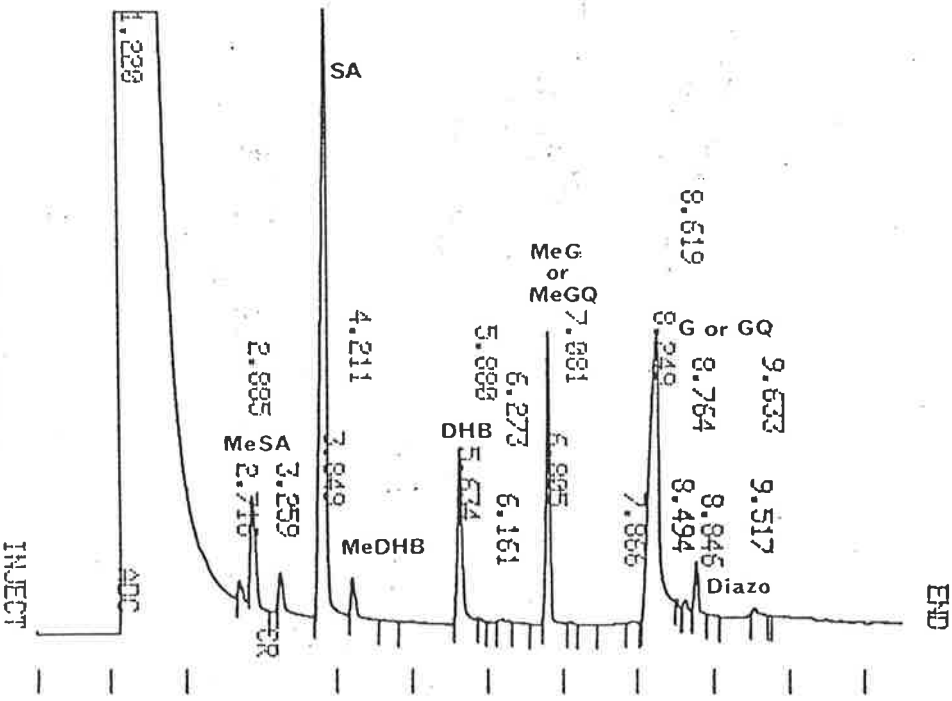

FID A 8X11 1.0 CM/M 10%
INJECT
ADC
SA
MeSA
MeDHB
DHB
MeG
or
MeGQ
G or GQ
Diazo
2.895
3.259
4.211
5.654
5.888
6.161
6.273
7.881
8.249
8.494
8.619
8.764
8.845
9.517
9.633
CR
END

Figure 2.9: (b) Assessment of THB-synthesis reaction mixture by gas chromatography-ion trap detection (ITD). (i) The mass chromatogram of diazald treated THB-synthesis reaction mixture - which is a chromatogram of the number of total particles measured during each scan (1 scan per second), and was performed with an ITD system: Perkin-Elmer 8000 Series GC-Ion trap detector-IBM PC/XT-Epson FX-85 Printer. The retention times are halved (compared to the Varian 3400-FID) due to the suction created at the interface between the capillary column and the vacuum of the mass spectrometer.

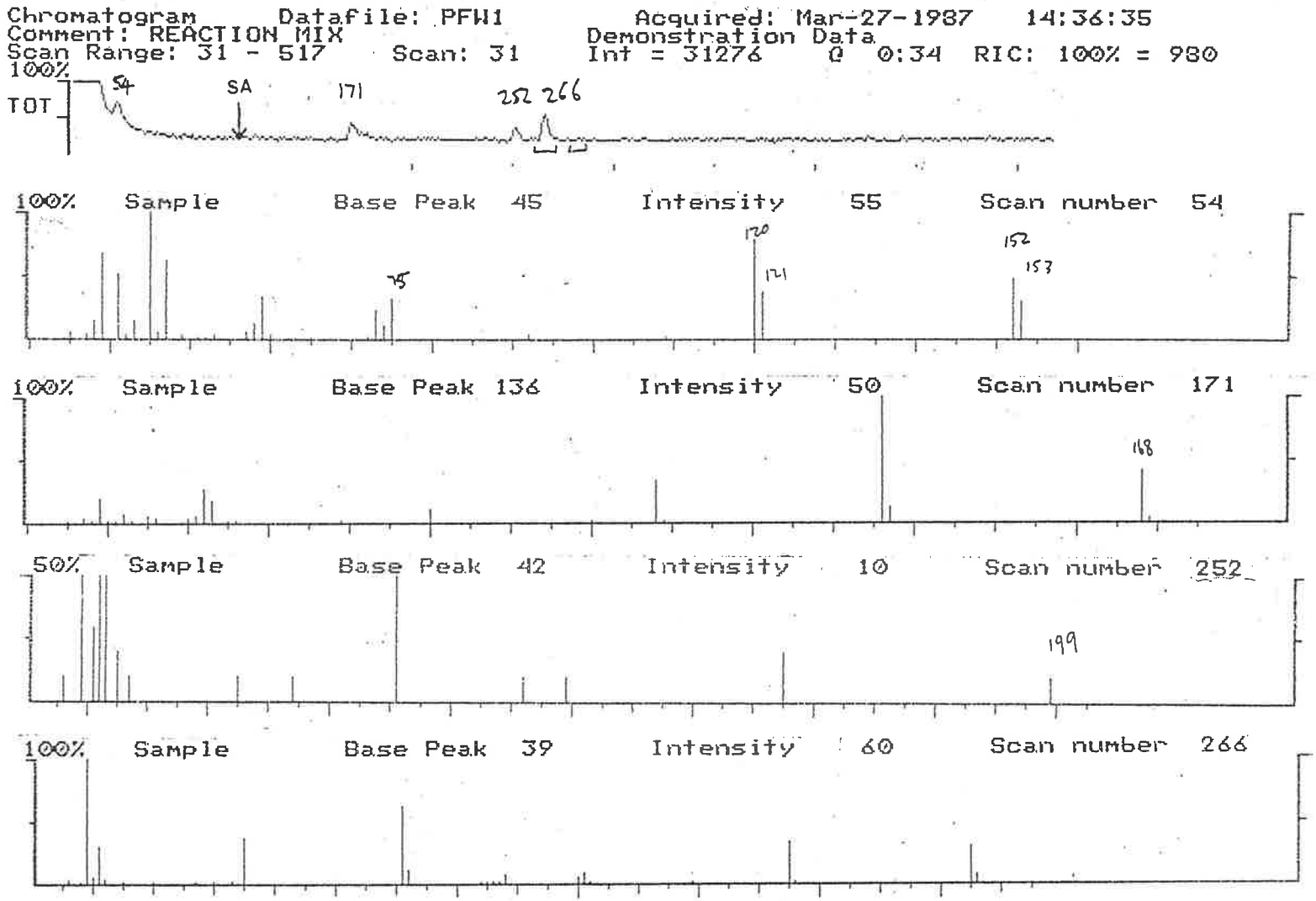
(ii) The peak at scan number 54 (0.9 min. Rt) has a molecular ion (M^+ ion 152) with the characteristic loss of *m/z* 31 ($-OCH_3$) to form a particle of ϕ-CO_2H (*m/z* 120), with the subsequent loss of *m/z* 45 ($-CO_2H$) to form ϕ (minus 2H, *m/z* 75) - this is indicative of the phenolic methyl ether of salicylate.

(iii) The peak at scan no. 171 (2.9 min. Rt) has a molecular ion (M^+ ion 168) with the characteristic loss of *m/z* 31 ($-OCH_3$) to form a particle of 2,3-diOH-ϕ-CO (*m/z* 137), with the subsequent loss of *m/z* 28 (-CO) to form diOH-ϕ (*m/z* 109) - this is indicative of the methyl ester of 2,3-DHB.

(iv) The peak at scan no. 252 (4.2 min. Rt) has a M^+ of 199 and may possibly be the trimethylated derivative of gentisoquinone (Mr 198).

(v) The peak at scan no. 266 (4.4 min. Rt) corresponds with the artefact due to the diazald preparation of diazomethane, which was used to methylate alcohols and carboxylic acids to produce more volatile ethers and esters for measurement on GC.

2.9 i


Chromatogram Datafile: PFW1 Acquired: Mar-27-1987 14:36:35
Comment: REACTION MIX Demonstration Data
Scan Range: 31 - 517 Scan: 31 Int = 31276 @ 0:34 RIC: 100% = 980
100%
TOT
54
SA
171
252 266
ii
100% Sample Base Peak 45 Intensity 55 Scan number 54
120
152
153
121
iii
100% Sample Base Peak 136 Intensity 50 Scan number 171
168
iv
50% Sample Base Peak 42 Intensity 10 Scan number 252
199
v
100% Sample Base Peak 39 Intensity 60 Scan number 266

the use of ECD to detect the easily oxidised products of salicylate hydroxylation. The extraction step reduces interference in detection of DHB in samples obtained from biological systems, and also results in a twenty-fold concentration of the sample. Deperoxidation of the extracting ether, and the storage of acidified samples at 20^{o}C, increases the stability of the dihydroquinones.

The specificity of the hydroxylated salicylate assay for OH$^{\cdot}$ was confirmed by the [^{14}C]-benzoate decarboxylation assay, using two distinct systems: the enzymatic xanthine oxidase/hypoxanthine/ferric chloride/EDTA system; and the chemical autoxidation system of ferrous sulphate/EDTA. The time-courses of the chemiluminescence produced in these two systems by the addition of luminol, showed ferrous ion autoxidation was instantaneous, and the enzymatic system was a constant radical generator. The ratio of 2,3- and 2,5-DHB products in the enzymatic and chemical system were 1:0.8 and 1:1.5, respectively.

Current methods in the literature for detecting the products of aromatic hydroxylation have included: the formation of a coloured tungstate-catechol complex [Richmond et al, 1981] or fluorescent hydroxybenzoate products [Baker and Gebicki, 1986]; the measurement of hydroxylated phenol by gas chromatography [Richmond et al, 1981] or liquid chromatography/electrochemistry [Radzik et al, 1983]; and quantitation of hydroxybenzoates with ultraviolet [Alexander

et al, 1986] and electrochemical detection [Floyd et al, 1984] following separation by HPLC.

These methods have proved to be sufficiently sensitive to detect OH· produced by the xanthine oxidase/hypoxanthine system and pulse radiolysis [Baker and Gebicki, 1986; Floyd et al, 1984], and hydroxylated salicylate in plasma and synovial fluid of patients receiving salicylate therapy for rheumatoid arthritis [Cleland et al, 1985a; Grootveld and Halliwell, 1986].

The procedure of Floyd et al [published late 1984] employed an oxidation potential of +1.1 volts to detect 2,4- and 2,6-DHB (which are not formed from the hydroxylation of salicylate by OH·) as well as 2,3- and 2,5-DHB. However additional interfering peaks from the decreased specificity (and the increased background noise) of this high voltage; the lack of sufficient separation of 2,3- and 2,5-DHB peaks; and the injection of unextracted samples onto the HPLC column, would make this method unsuitable for the quantitation of DHB in complex biological systems. The +1.1 voltage is also capable of detecting salicylate, thus producing a run time of 30 min. (as compared to the 7 min. run time seen in the Wright and Priestly [1985] procedure). Floyd's detection limit of 2 pmol of DHB standard, as determined by a signal to noise ratio of 3, also compares unfavourably to the detection of 1 pmol by this author's

assay (signal:noise = 10, figure 2.5(c); %CV of 2,3- and 2,5-DHB = 5.6 and 4.7 %, respectively).

The assay described in this chapter was considered to possess sufficient sensitivity to measure OH· production in complex cellular systems. Chapter 3 concerns the use of this assay in the activated human neutrophil system, *in vitro*.

Chapter 3

HYDROXYLATION OF SALICYLATE BY ACTIVATED HUMAN NEUTROPHILS: EVIDENCE OF A ROLE FOR SUPEROXIDE AND MYELOPEROXIDASE

3.1 INTRODUCTION

Upon activation by various stimuli, human polymorphonuclear leukocytes (PMN) exhibit a marked increase in non-mitochondrial oxygen consumption, called a "respiratory burst". Oxygen reduction is catalysed by a membrane-bound NADPH oxidase, to form O_2^- [Babior, 1987; Rossi, 1986; Hurst, 1987.] (figure 3.1). It is probable that most, if not all H_2O_2 produced, is formed indirectly via O_2^- [Makino et al, 1986; Green and Wu, 1986; Green and Pratt, 1987.]. These partially reduced oxygen species are considered important for killing microorganisms [Malech and Gallin, 1987] and may play a role in inflammation [Cross CE et al, 1987].

Both H_2O_2 and O_2^- form complexes with peroxidases such as myeloperoxidase (MPO), which is released during neutrophil degranulation, to form compounds I and III respectively [Winterbourn et al, 1985]. Compound I interacts with chloride to generate the hypochlorite ion. The species derived from compound III remain to be elucidated.

The interaction of O_2^- and H_2O_2 *in vitro* can generate hydroxyl radicals ($OH^{\cdot}$) via the Haber-Weiss reaction, with trace metal ions acting as catalysts. Whether the more reactive species, such as $OH^{\cdot}$ and singlet oxygen, are also

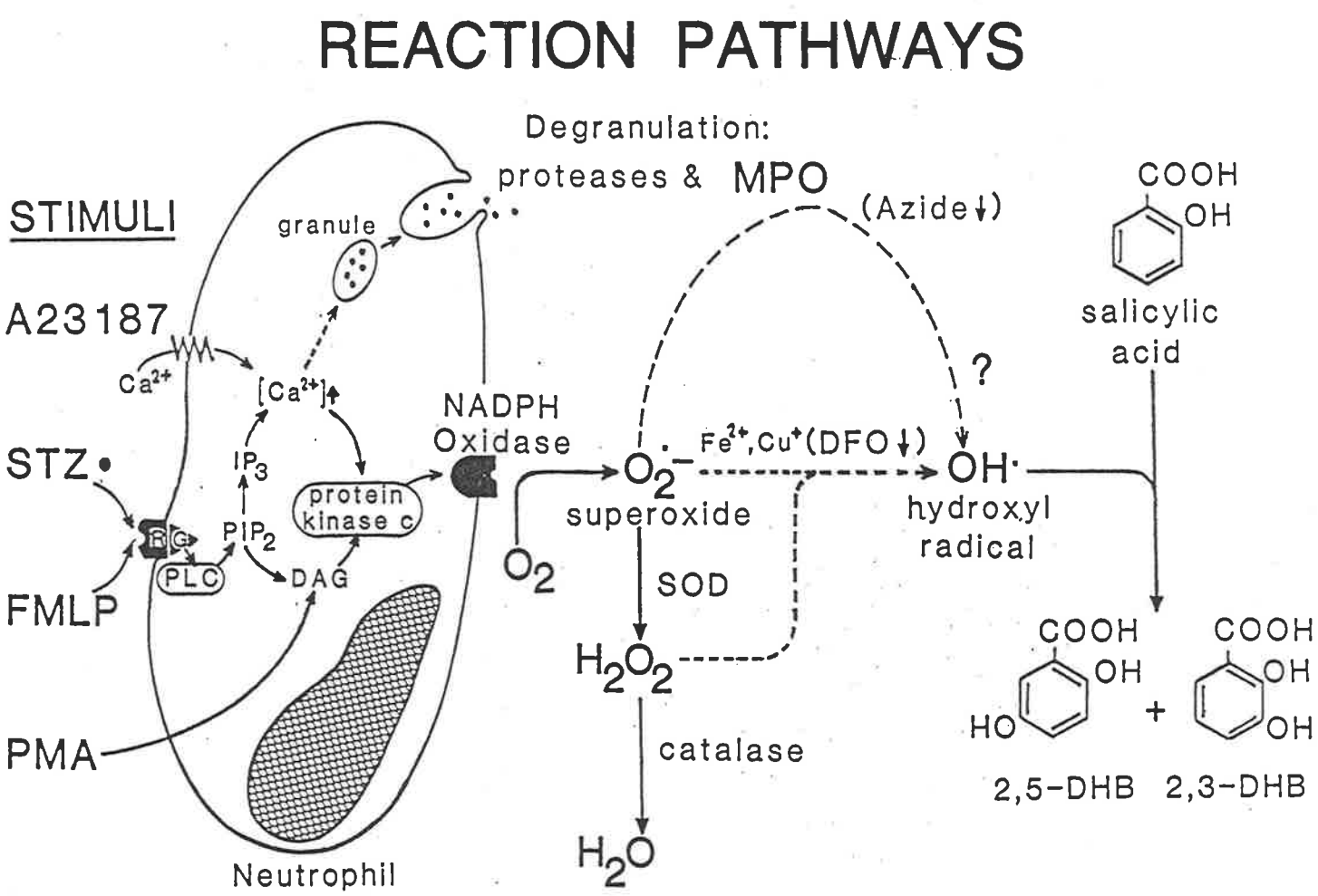


Figure 3.1: Schematic of reaction pathways in stimulated neutrophils which result in the production of oxyradicals, via cellular activation.

formed *in vivo* remains contentious for reasons previously mentioned in the introduction of chapter 2.

The HPLC/ECD method for the detection of $OH^{\cdot}$, described in chapter 2, was employed to investigate the nature and source of the hydroxylating species generated by human PMN *in vitro*, which were activated by various stimuli with different modes of action, and using agents which selectively modify components of the oxyradical flux.

The stimuli included serum treated zymosan (STZ), formyl-methionyl-leucyl-phenylalanine (FMLP with or without cytochalasin B), A23187 (the Ca^{2+} ionophore), and phorbol myristate acetate (PMA). Both STZ, which consists of yeast particles coated in C3b complement, and FMLP (the chemotactic peptide), stimulate O_2^- production via specific cell membrane receptors linked to a G-protein, which activates phosphoinositidase C (PIC), which in turn catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers - 1,4,5-inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 elevates cytosolic Ca^{2+}, and DAG is the endogenous activator of a Ca^{2+}/phospholipid dependent kinase, called protein kinase C, which phosphorylates the flavoprotein of NADPH oxidase, thus activating the enzyme catalysed formation of O_2^-. Increased cytosolic Ca^{2+} is thought to promote the degranulation process which involves the release

of myeloperoxidase and proteases from the exocytosis of granules (Figure 3.1) [French, 1988].

PMA, a phorbol ester, is an analogue of DAG and penetrates the membrane to cause the generation of large quantities of O_2^-, with very little concomitant degranulation (except at high doses). A23187 can raise the cytosolic Ca^{2+} levels via its ionophoric activity which results in both degranulation and NADPH oxidase activation (Figure 3.1).

Agents used to modify the oxyradical flux included: superoxide dismutase (SOD) - O_2^- catabolism to H_2O_2; catalase (H_2O_2 catabolism to water); desferrioximine (DFO) - a metal ion chelator which inhibits the Fenton reaction; EGTA - metal ion chelator which promotes the Fenton reaction; ascorbate - which is a reducing agent and can act as an alternative substrate to drive the Fenton reaction; diphenylene iodonium (DPI) - a specific NADPH oxidase inhibitor; and sodium azide - a less specific inhibitor of haemoproteins, but can be used at concentrations with relatively specific effects on the more sensitive myeloperoxidase enzyme (Figure 3.1).

3.2 METHODS

3.2.1 Preparation of neutrophils.

Human peripheral blood was obtained by venepuncture from healthy volunteers and anticoagulated with 4.5% (w/v)

EDTA (pH 7.4, 2 ml per 10 ml blood), or from units of whole blood (anticoagulated with citrate). The red cells were sedimented with 5% (w/v) dextran (also 2 ml per 10 ml blood). PMN were separated on a Ficoll-hypaque gradients [Sagone et al, 1984]. Following hypotonic lysis and washing with Dulbecco's phosphate buffered saline, PMN were resuspended in indicator-free Hanks balanced salt solution (HBSS), pH 7.4, at 4 to 8 x 10^6 per ml. The iron content of HBSS was less than 0.3 μM as detected by flame photometry. The average yield was 3 x 10^6 cells per ml of whole blood (95% - 99% granulocytes, and less than 1% mononuclear cells).

3.2.2 Preparation of stimuli and inhibitors.

Serum treated zymosan (20 mg/ml in HBSS) was prepared by the procedure of Hurst et al, [1986], and stored at -10^{o}C until use. PMA (1 mM) and FMLP (25 mM) were dissolved in dimethyl sulphoxide and stored at -10 and 4^{o}C, respectively. A23187 (1 mM) and cytochalasin B (1 mg/ml) were dissolved in methanol and stored at 4^{o}C. All stimuli, except A23187, were diluted in HBSS immediately prior to use, into working stock solutions which were ten or twenty-times the final concentrations.

SOD, DFO, sodium ascorbate and EGTA were dissolved in HBSS, and sodium azide in distilled water, at forty-times the final concentration, immediately prior to use. Catalase (130,000 units/ml) was dissolved in buffer and stored at 4^{o}C

and diluted with HBSS prior to use. DPI (2mM) was stored in 50% ethanol at $4^{o}C$ and used undiluted. The incubation concentrations were: STZ (2 mg/ml), PMA (500 nM), FMLP (1 μM) with cytochalasin B (5 μg/ml, added during pre-incubation), A23187 (5 μM), SOD (300 units/ml), catalase (1000 units/ml), sodium azide (0.5 mM), DPI (20 μM), sodium ascorbate (2 mM), DFO (50 μM) and EGTA (2mM).

3.2.3 *In vitro* hydroxylation of salicylate.

PMN (0.8 to 1.5 x 10^{7} in 2 mls) were preincubated with 1 mM salicylate at $37^{o}C$ for 15 minutes, and then stimulated for 20 minutes at $37^{o}C$. The reaction was terminated by phosphoric acid and samples were processed as described in HPLC/ECD assay method.

3.2.4 Measurement of superoxide production by PMN.

O_2^- production was measured as the SOD-inhibitable reduction of ferricytochrome c, using an extinction coefficient (550 minus 540 nm) of 21.1 nM^{-1} cm^{-1}. PMN (0.6 to 1 x 10^{6}/ml) in HBSS containing cytochrome c (100 μM) were equilibrated at $37^{o}C$ and then stimulated with STZ, PMA, A23187, and FMLP with or without cytochalasin B, in triplicate for 20 minutes, according to the method of Weening et al [1975]. Changes in absorbance were measured on a Varian DM-100S double-beam spectrophotometer.

DHB data presentation: all results presented are measurable DHB production by 10^{7} PMN per incubation (±SE,

standard error of the mean) from duplicate values which were normalised within each experiment, and the resultant percentage control values were combined from two or three similar experiments. Since the results were not corrected for DHB recovery they underestimate the DHB production. The results in figures 3.5, 3.6, 3.7, 3.8, and tables 3.4 and 3.5 are expressed as a percentage of their respective stimuli controls after the subtraction of DHB production by unstimulated PMN.

3.3 RESULTS

3.3.1 Formation of dihydroxybenzoates by PMN.

PMN stimulated with PMA, STZ, A23187 and FMLP (with or without cytochalasin B) hydroxylate salicylate to 2,3- and 2,5-DHB (Sample chromatogram in figure 3.2). Each of the stimuli tested, resulted in hydroxylation which was readily detectable above unstimulated levels at salicylate concentrations as low as 50 μM (Table 3.1).

The lability of the products of salicylate hydroxylation (DHB's) was examined by determination of the recovery of exogenously added DHB (in the absence of salicylate) which had been incubated with stimulated neutrophils. DHB lability was concentration-, stimulus- and inhibitor-dependent (Table 3.2 and 3.3).

The total amount of O_2^- production and $OH^\cdot$ formation (measured by HPLC/ECD determination of DHB production) were

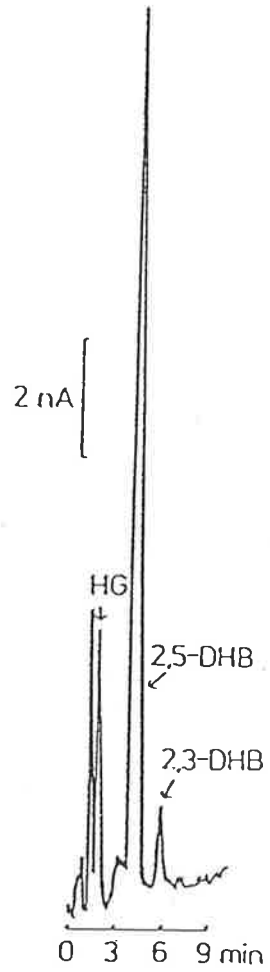


Figure 3.2: A chromatograph of dihydroxybenzoates. A typical chromatograph of DHB formed by STZ-stimulated PMN incubated with salicylate is shown. Similar chromatograms were obtained with other stimuli. The respective retention times of HG (internal standard), 2,5-DHB (or gentisic acid) and 2,3-DHB were 2.1, 4.2 and 5.7 minutes. At the potential difference of 0.8 volts, salicylic acid produces a small broad peak at 14.7 mins. The 2,3- and 2,5-DHB peaks correspond to 198 pmol and 1.01 nmol, respectively. There was no interference from endogenous peaks generated by the cellular system.

Table 3.1: Dihydroxybenzoate formation by activated neutrophils incubated with various salicylate concentrations.

Stimuli *	DHB FORMATION (pmol DHB / 10^7 PMN)		
	Salicylate concentration (mM)		
	0.05	0.25	1.0
STZ	9 ± 4	47 ± 10	576 ± 82
PMA	21 ± 2	107 ± 5	270 ± 42
A23187	9 ± 5	87 ± 3	318 ± 27
FMLP	2 ± 1	13 ± 1	86 ± 2

* PMN were incubated with FMLP (1 μM, for 5 min), A23187 (5 μM, for 10 min), STZ (2 mg/ml, for 20 min) and PMA (500 nM, for 15 min).

Table 3.2: Dihydroxybenzoate recoveries from incubations with activated neutrophils at various DHB concentrations.

Stimuli **	% DHB RECOVERY*			
	DHB concentration (μM)			
	0.05	0.2	1.0	5.0
STZ	-	3 ± 1	8 ± 3	53 ± 5
PMA	-	6 ± 1	16 ± 1	66 ± 1
A23187	71 ± 2	94 ± 5	94 ± 3	-
FMLP	26 ± 4	59 ± 1	82 ± 6	-
FMLP + CB	8 ± 1	8 ± 1	19 ± 1	-
Unstimulated	82 ± 7	93 ± 3	100 ± 6	124 ± 6

* Results are expressed as percentage of DHB recovery from HBSS, with standard errors. n.d. = not determined.

** PMN were incubated with DHB at 0.05, 0.2, 1.0 and 5.0 μM in 2 ml HBSS, and then stimulated as previously described in methods.

Table 3.3: Effects of inhibitors on DHB recovery.

	% DHB RECOVERY*					
Additions:	**None**	**SOD**	**CAT**	**SOD+CAT**	**DFO**	**AZIDE**
Stimuli:						
STZ	12±0	46±4	18±4	60±2	26±1	39±2
PMA	12±0	62±1	58±2	79±1	33±0	60±1
A23187	82±5	85±1	92±1	95±4	73±1	57±1
FMLP	66±4	75±1	85±0	89±5	69±2	79±1
FMLPbCB	36±1	62±0	65±1	76±1	50±1	58±2

* Results expressed as %DHB recovery in unstimulated PMN (97 ± 1%) with standard errors. PMN were incubated with DHB at 0.4 μM in 2 ml, and stimulated as before.

stimulus dependent. The most potent stimulus for DHB production was STZ (Figure 3.3), whereas the most potent O_2^- stimulus was PMA (Figure 3.4). Salicylate itself did not alter O_2^- fluxes stimulated by STZ, PMA and A23187 (but did potentiate FMLP stimulated O_2^- flux).

Therefore, salicylate hydroxylation generated by these stimuli was not directly proportional to the O_2^- flux. As can be seen from Table 3.2 the stimulus-dependent differences in measurable DHB formation cannot be accounted for by differences in lability. For example, added DHB were most labile in the presence STZ-stimulated PMN, but also yielded the highest rate of DHB formation from salicylate.

3.3.2 Effects of catalase, superoxide dismutase and diphenylene iodonium on DHB formation by PMN.

SOD markedly inhibited DHB formation mediated by all stimuli (figure 3.5), whereas catalase had little inhibitory effect. This inhibitory effect of SOD was unaltered by the presence of catalase. All incubations containing stimuli plus SOD, with or without catalase, were significantly lower than those containing stimuli plus catalase or stimuli alone. Diphenylene iodonium, an inhibitor of NADPH oxidase, completely abolished salicylate hydroxylation by STZ- and PMA-activated PMN, and DHB production was reduced to 62 ± 4% and 38% in unstimulated and FMLP/CB-activated PMN, respectively.

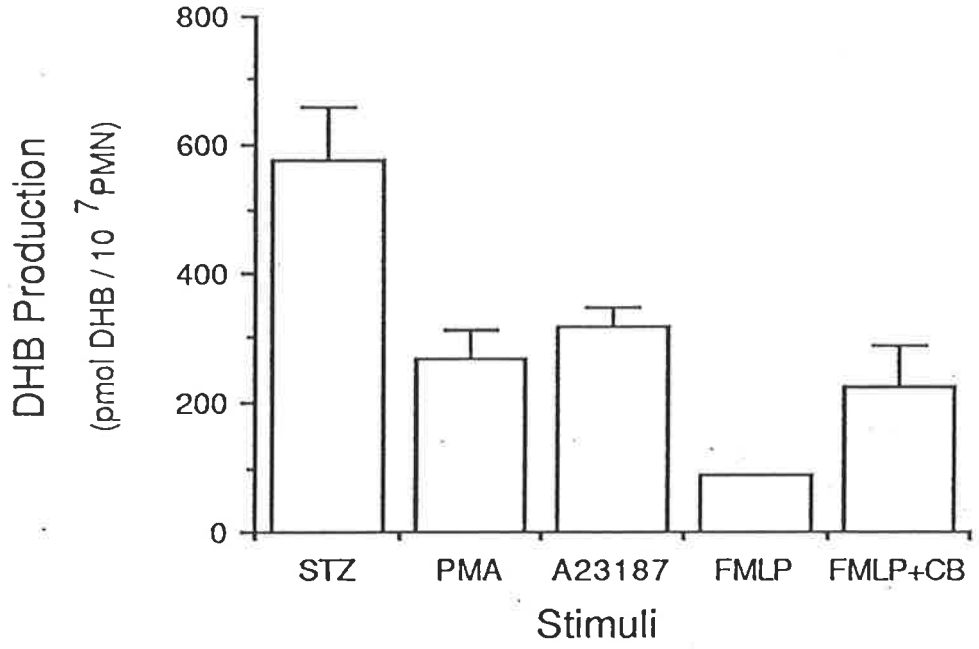


Figure 3.3: Production of DHB by activated PMN. DHB formation was measured from PMN stimulated by STZ, PMA, FMLP (with or without cytochalasin B) and calcium ionophore A23187. Unstimulated PMN mediated DHB production was 38 ± 5 pmoles DHB/10^7 PMN. There was no statistically significant difference between DHB levels obtained from PMA, A23187, and FMLP with cytochalasin B.

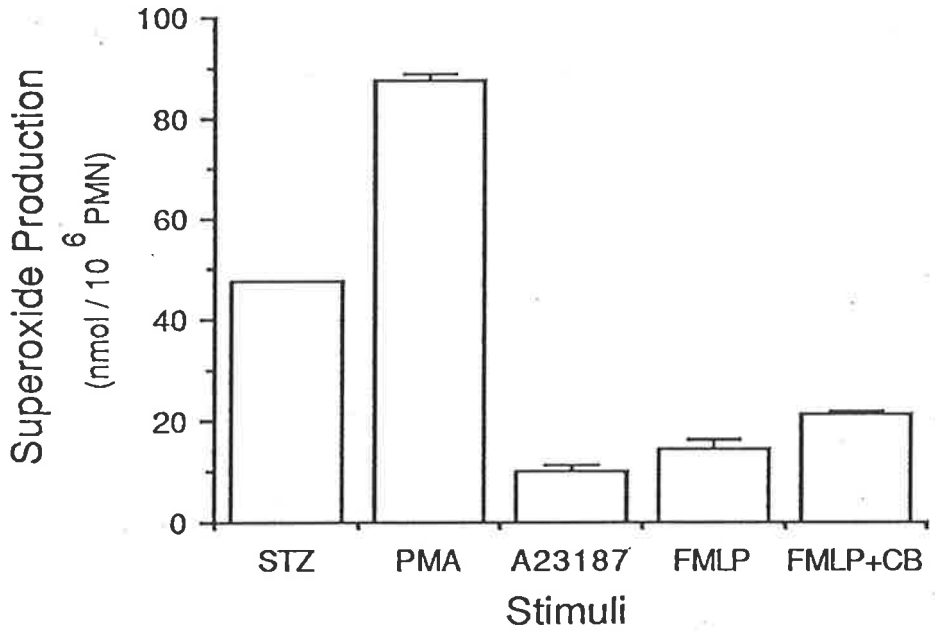


Figure 3.4: Production of O_2^- by activated PMN. O_2^- production was measured from PMN which were stimulated in the same manner as in figure 3.3.

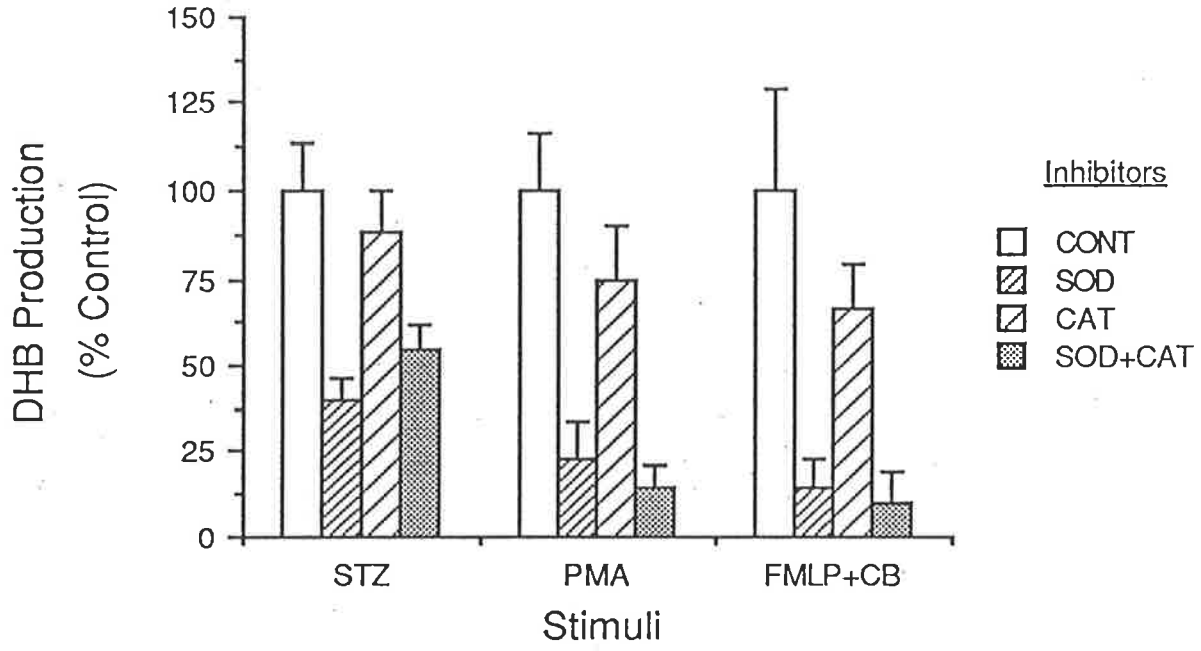


Figure 3.5: Effect of superoxide dismutase and catalase on DHB production. DHB formation was measured in PMN stimulated by STZ, PMA FMLP with cytochalasin B in the presence of SOD and/or catalase (from three separate experiments). DHB production by stimuli controls are shown in figure 3.3.

The data showing (a) the inhibition of DHB formation by the NADPH oxidase inhibitor - DPI, and the O_2^- catabolic enzyme - SOD, and (b) the lack of effect of the H_2O_2 catabolic enzyme - catalase; imply that O_2^-, and not H_2O_2, was the major contributor to the hydroxylation reaction.

3.3.3 Effects of azide.

Sodium azide, at 0.5 mM, caused only a slight reduction (10%) in O_2^- production by STZ- and PMA- stimulated PMN (figure 3.6), but markedly inhibited DHB production by PMN activated with STZ (figure 3.7). Azide was a less effective inhibitor of DHB production in PMA-stimulated cells, had no effect on unstimulated cells, and appeared to increase DHB production in FMLP-activated cells. Neither SOD nor catalase, by themselves, significantly modified the azide effect, except in PMA-treated cells where catalase restored activity to stimulated control levels. The combined effect of catalase, SOD and azide was sufficient to abolish stimulus-induced DHB production in STZ- and PMA-treated cells, but not in FMLP/cytochalasin B-stimulated PMN, where the effect was minimal.

3.3.4 Effects of iron chelators and ascorbate.

To further investigate the possible contribution of a Fenton reaction to salicylate hydroxylation by activated PMN, the effects of two iron chelators, DFO (Figure 3.8) and EGTA (Table 3.4), and the reducing agent, ascorbate were

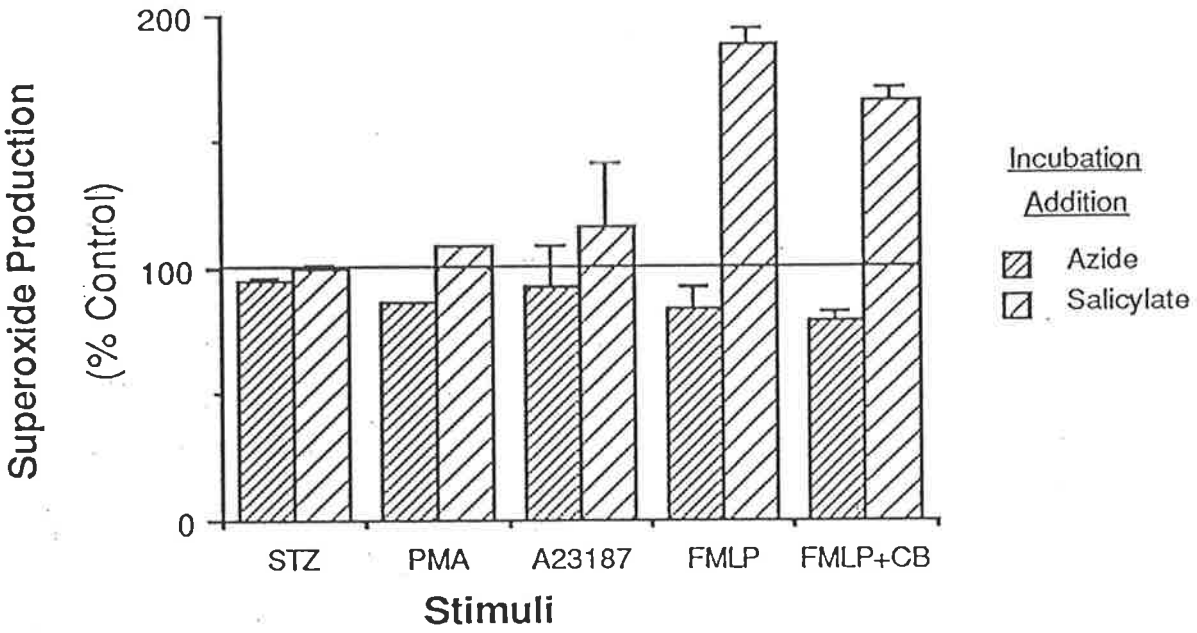


Figure 3.6: Effect of salicylate and azide on O_2^- production by activated PMN. The addition of azide (0.5 mM) resulted in a slight reduction of O_2^- formation. Salicylate (1 mM) had little effect on STZ, PMA and A23187 mediated O_2^- formation, but exhibited a marked potentiating effect on both forms of FMLP stimulation.

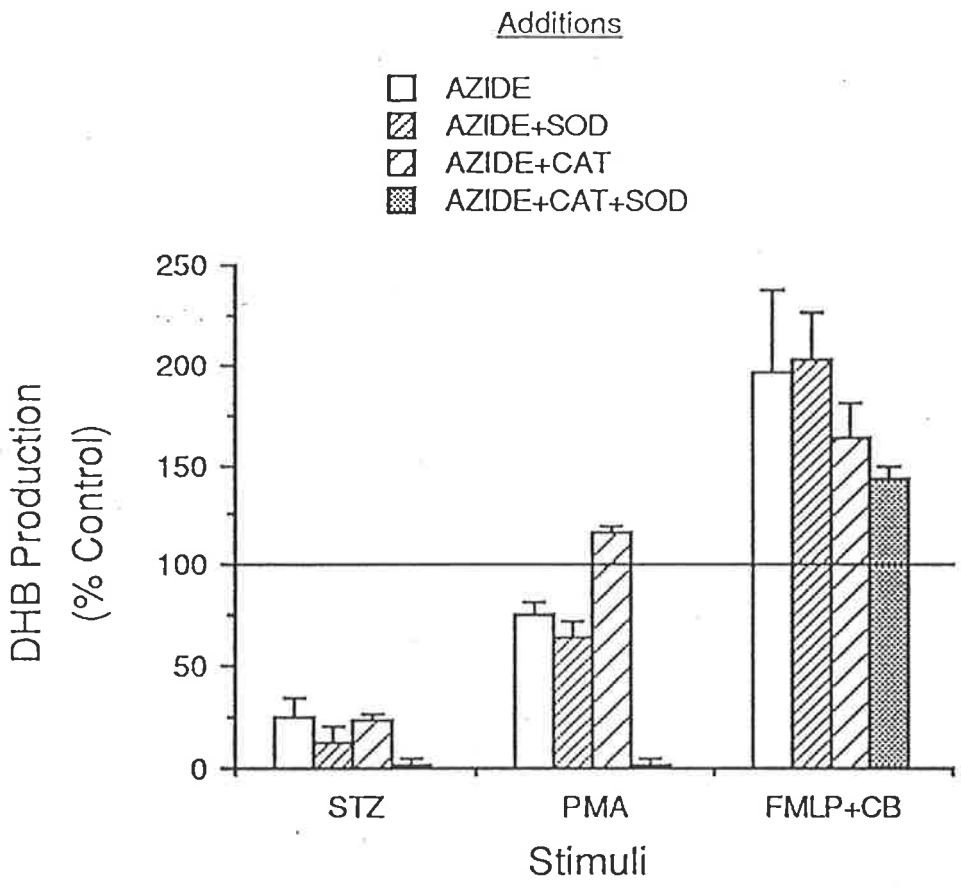


Figure 3.7: Effect of azide on DHB production. DHB formation was measured in PMN stimulated as described in methods, in the presence of azide. Unstimulated DHB levels were not significantly altered following the addition of azide (103 ± 13%). Values were derived from two separate experiments.

examined (Table 3.5). These agents increased unstimulated salicylate hydroxylation. In activated PMN, irrespective of the stimulus, DFO enhanced DHB formation. However EGTA and ascorbate were more selective in their effects. EGTA increased PMA- and FMLP/cytochalasin B-mediated hydroxylation, and ascorbate potentiated PMA-mediated DHB formation, but not FMLP-mediated hydroxylation. Neither ascorbate nor EGTA altered the hydroxylating ability of STZ-activated PMN.

Catalase and SOD had different effects on DHB formation in the presence (Figure 3.8) and absence (Figure 3.5) of DFO. For example, the effect of DFO on DHB formation by FMLP-stimulated cells was enhanced by SOD, for reasons that are unclear.

3.4 DISCUSSION

It has been determined that PMN activated by either STZ, PMA, FMLP (with cytochalasin B) or A23187, produce a partially reduced oxygen species capable of hydroxylating salicylate to form dihydroxybenzoates as determined by HPLC/amperometric detection. This hydroxylation was markedly inhibited by SOD (an enzyme which removes O_2^-) but not by catalase (which removes H_2O_2), irrespective of the stimulus. DHB production was completely abolished by DPI, an inhibitor of NADPH oxidase - the enzyme that produces O_2^- when activated. Azide selectively inhibited STZ-mediated DHB formation compared to PMA-stimulated hydroxylation.

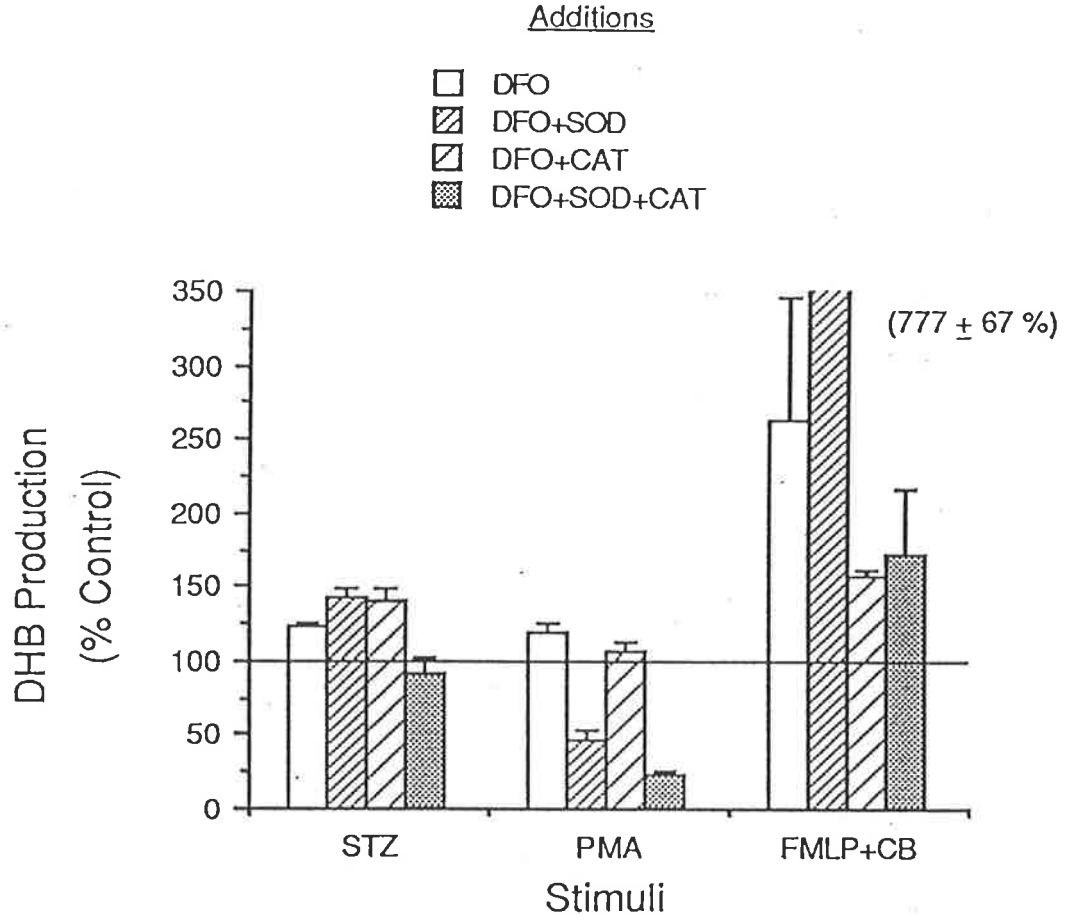


Figure 3.8: Effect of desferrioxamine on salicylate hydroxylation. DHB formation was measured in PMN stimulated as described in methods, in the presence of DFO. DFO significantly increased unstimulated levels (187 ± 19%). Values were derived from two separate experiments.

Table 3.4: Effect of EGTA and ascorbate on DHB formation.

STIMULI	% STIMULI CONTROL * EGTA
STZ	99 ± 1
PMA	476 ± 74 **
FMLP/cytochalasin B	424 ± 174
Unstimulated	131 ± 30

* Control DHB formation was 772 ± 371, 185 ± 49, 318 ± 87 and 46 ± 7 pmol DHB/10^7 PMN for STZ, PMA, FMLP/cytochalasin B and unstimulated DHB production, respectively.

** Significantly different from respective stimulus control.

Table 3.5: Effect of ascorbate on DHB formation.

STIMULI	DHB FORMATION (pmol)	% STIMULI CONTROL *
STZ	635 ± 364	82 ± 47
PMA	311 ± 42	168 ± 28
FMLP/cytochalasin B	332 ± 20	104 ± 6
Unstimulated	310 ± 46	791 ± 88 **

* Control DHB formation is listed in the legend to table 3.4.

** Significantly different from respective stimulus control.

Table 3.6: The effects of agents on unstimulated PMN mediated DHB formation.

AGENT ADDED	% UNSTIM.CONTROL
DFO	187 ± 19
EGTA	131 ± 30
Ascorbate	791 ± 88
Azide	103 ± 13
DPI	62 ± 4

DFO, a chelator of metal ions critical for the Fenton reaction, failed to inhibit DHB production in activated PMN.

Since DHB were labile in this system, the actual productions of DHB were underestimated. As the presence of SOD and azide enhanced the stability of DHB, their inhibitory effects on DHB formation were further underestimated. This does not compromise the interpretation of the effects of the inhibitors or the significance of the pathways they influence, but it does limit the quantitative usefulness of the assay in this system. It is unfortunately impossible to determine whether other researchers evaluated the effects of product lability during salicylate hydroxylation (or any aromatic hydroxylation) by oxyradical fluxes, on the data interpretation, as it has not been critically assessed in the published reports.

As the proportion of 2,3- and 2,5-DHB formation is 1:1 in the noncellular system, it was of interest to note that 2,5-DHB was by far the major product in the activated PMN system. Catechol and 3,4-DHB (2 μM) were incubated with PMA-activated and unstimulated PMN, in order to determine whether 2,3-DHB was being selectively removed from the system by catechol specific mechanisms, such as catechol O-methyl transferase (COMT). However this was not the case as neither catechol or 3,4-DHB altered the proportion or yield of DHB in this system, i.e. 98 ± 25% and 103 ± 9% of PMA-activated DHB production, and 93 ± 16% and 76 ± 12% of

unstimulated DHB formation, for catechol and 3,4-DHB additions, respectively.

The inhibition of DHB formation by SOD and not catalase would suggest that the generation of O_2^- is of greater importance than H_2O_2 in OH· production. DPI totally abolished DHB formation by stimulated PMN, implying that the activity of NADPH oxidase is a critical source of O_2^-. Although DPI selectively inhibits phagocyte superoxide release [Cross AR, 1987; Hancock and Jones, 1987], it also inhibits xanthine oxidase activity [French, 1988] thus raising the possibility that it may also inhibit other haemoproteins, such as MPO.

Azide, at a concentration which did not markedly affect O_2^- production (Figure 3.6) and therefore preserved NADPH oxidase activity [Sagone et al, 1984], selectively inhibited STZ-mediated DHB formation compared with PMA-stimulated hydroxylation. STZ is a potent degranulating agent and more efficient in stimulating MPO release than the weak degranulating stimulus, PMA [Tanekawa et al, 1985]. Therefore the differential inhibition of azide strengthens the hypothesis that MPO may also play a role in the production of OH·.

These findings are supported by recent studies of Sagone and coworkers [Alexander at al, 1986], in which SOD and azide (but not catalase) inhibited benzoate hydroxylation and [^{14}C]-salicylate decarboxylation [Sagone

and Husney, 1987] by zymosan-stimulated PMN. They concluded that there was a necessity for O_2^- production and an unidentified azide-sensitive, haem-dependent enzyme.

There is evidence [Winterbourn et al, 1985] that O_2^- may bind MPO to form a superoxo-ferric complex called "compound III", which is spectrally distinct from the peroxide-MPO complexes of "compound I (peroxy-ferrous) and compound II (peroxy-ferric)". Compound III may interact with compound II, but whether OH· is evolved from compound III has not been determined [Winterbourn et al, 1985]. Inactivation of MPO has been previously shown to enhance O_2^- release by FMLP-stimulated PMN, and MPO-deficient individuals have enhanced O_2^- production [Edwards and Swan, 1986] which implies a role for MPO in the metabolism of O_2^-. It has recently been reported that MPO possessed an inhibitory effect on OH· production [Winterbourn, 1986], but this was under different incubation conditions which included exogenous iron and chelators, and OH· was quantitated by a different method (deoxyribose oxidation). The most recent scheme of the oxyradical interactions with MPO, from Kettle and Winterbourn [1988], is shown in figure 3.9.

The lack of effect of catalase on DHB formation would suggest that the Haber-Weiss reaction is unlikely to be a major source for the hydroxylating species, and that trace amounts of free iron in our buffers (<0.3 μM) were not

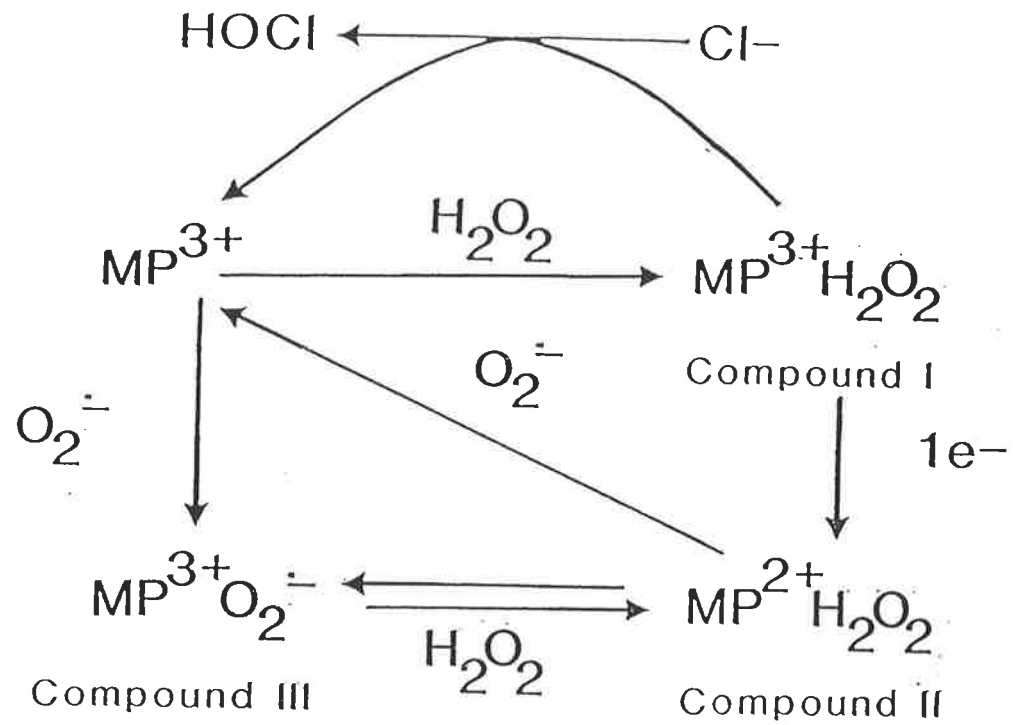


Figure 3.9: Oxyradical interactions with myeloperoxidase [Kettle and Winterbourn, 1988]. 30% of O_2^- production is involved in HOCl formation. SOD and catalase inhibit HOCl production by 80%, but there was no inhibition by DFO, pentetic acid or mannitol. The rate limiting step is the conversion of Compound II to MP^{3+}, which is catalysed by O_2^-, and thus potentiating the oxidant damage at sites of inflammation by optimising the MPO-dependent production of HOCl.

significant in this regard. This concept was supported by the observations that DFO, a potent iron chelator which decreases OH· production by Fenton chemistry *in vitro* [Graf et al, 1984], failed to inhibit the observed DHB formation by PMA- and STZ-stimulated PMN. However, since DFO enhanced the recovery of DHB, a small decrease in hydroxylation activity may have been masked.

Furthermore, DHB formation by unstimulated PMN in the presence of ascorbate, which can substitute for H_2O_2 in Fenton reactions [Levine, 1986], indicates the extent to which salicylate hydroxylation may occur under conditions which are suitable for the Fenton reaction. Ascorbic acid potentiates DHB production by unstimulated PMN to the same levels seen with PMA- and FMLP-stimulation (table 3.5). Amoung the implications of this finding is that there may be an general amplification of an oxyradical flux from previously resting PMN, an observation which is relevant in chronic ascorbate therapy.

The effects of various agents on DHB production by unstimulated PMN are summarised in table 3.6. These data highlight the superior sensitivity of this HPLC-ECD assay when compared to the methods of Alexander et al [1986] and Sagone and Husney [1987], as these groups reported that unstimulated PMN had no detectable hydroxylation or decarboxylation activity.

The presence of salicylate in all incubations had very little inhibitory effect on STZ-, PMA- and A23187-stimulated O_2^- production (but did potentiate FMLP-stimulated O_2^- formation), may suggest that salicylate is a weak cyclooxygenase inhibitor. However other mechanisms may contribute to the anti-inflammatory effects of salicylate. The hydroxylation of salicylate *in vitro*, at concentrations similar to plasma levels achieved during chronic anti-inflammatory treatment, may have dual effects. The quenching of hydroxyl radicals by salicylate may inhibit tissue damage. The resultant hydroxylation products, including 2,3- and 2,5-DHB, catechol and 2,3,5,-trihydroxybenzoate, which also possess antioxidant properties, may contribute further to oxyradical quenching [Betts et al, 1985]. A further more novel effect of 2,5-DHB (gentisate) is the inhibition of interleukin-1 induced lymphocyte proliferation [Haynes et al, 1988c]. Thus the anti-inflammatory effects of gentisate may involve both local antioxidant effects [Cleland et al, 1985b], and systemic effects acting via a reduction in circulating levels of interleukins. This systemic effect may also be responsible for the changes in hepatic function, notably the synthesis of acute phase reactants [Dinarello, 1985], and the subsequent reduction in the ability of hepatic microsomal mixed-function oxidases to metabolise xenobiotics to reactive metabolites [Wright and Priestly, 1986] (further discussed in chapter 4).

The present findings lead to the conclusion that a highly reactive species, probably the $OH^{\cdot}$, is produced by activated neutrophils via a superoxide- and MPO-dependent mechanism. A more complete understanding of the interaction and dissociation of the superoxide-MPO complex (compound III) is necessary to conclusively identify that the reactive species in question is the $OH^{\cdot}$.

This study suggests a mechanism by which two phagocyte functions, i.e. the respiratory burst and degranulation, may interact to produce a toxic species in addition to the hypochlorite ion (refer fig. 3.9). The relative extent in which these two processes contribute to phagocytic functions, such as microbial killing and tissue damage in inflammation, remains to be determined.

Chapter 4

MODIFICATION OF HEPATOTOXICITY BY INFLAMMATION-INDUCED SYSTEMIC OXIDANT STRESS

4.1 INTRODUCTION

The concept that sickness may impair drug and hormonal metabolism has been the subject of extensive investigation by many researchers over the last two decades. Reduced hepatic drug metabolism has been demonstrated in rats following surgical trauma [Griffeth et al, 1983] or the establishment of chronic inflammation in the polyarthritic syndrome of adjuvant disease [Mathur et al, 1977; Beck and Whitehouse, 1974]. The processes involved in the inflammatory response are described in chapter 5.

A deficiency of natural anti-oxidants, particularly soluble thiols, has been observed in the sera and livers of arthritic rats [Yoshikawa et al, 1985; Ishizuki and Fujihara, 1984]. This suggests that inflammatory disease may bring about a state of systemic 'oxidant stress', in which the protective mechanisms of the body as a whole may be compromised in its ability to handle perturbations in its oxidant status.

One consequence is that membranes of cells outside the inflammatory locus may be peroxidised, especially in the case of highly metabolic cells, such as hepatocytes [Ishizuki and Fujihira, 1984]. This may result in the loss of associated enzyme functions, such as MMFO in the endoplasmic reticulum, and the activation of enzyme

catabolic processes, i.e. the conversion of cytochrome P450 to P420 [Mathur et al, 1977].

Extrahepatic trauma/inflammation activates the liver to produce more serum glycoproteins, the so-called 'Acute Phase Reactants' (APR), at the expense of normal albumin synthesis. The production of these natural scavengers is stimulated by interleukins released from activated mononuclear phagocytes [refer chapter 5]. Interleukin-1 (IL-1) can also depress cytochrome P450 activity in isolated hepatocytes [Ghezzi et al, 1987].

This direct depression of MMFO activity, compounded with alterations in hepatic membrane integrity associated with thiol depletion, may have a number of consequences where drug exposure also occurs. The reduction in MMFO activity could enhance drug effects by reducing metabolic clearance, for example, prolongation of hexobarbital sleeping time in rats with adjuvant disease [Mathur et al, 1977]. The MMFO system also plays an activating role for many types of hepatotoxic chemicals [Nelson, 1982] and therefore it is possible that a reduction in MMFO activity associated with inflammatory disease may actually reduce sensitivity to such hepatotoxins. However the accompanying oxidant stress of inflammation may be a confounding factor, as drug-induced cytotoxicity is markedly influenced by the efficiency with which cells can inactivate toxic metabolites produced by the MMFO system [Trush et al, 1982].

The inactivating pathways include: glutathione (GSH) conjugation; dismutation of O_2^- produced by redox cycling (followed by H_2O_2 breakdown); and scavenging of radicals by endogenous anti-oxidants, e.g. ascorbate, α-tocopherol and sulphur-rich proteins. These systems are likely to be disrupted during inflammatory stress, leading to possible potentiation of hepatotoxicity.

This chapter focuses on the question of whether acute inflammation (produced by oleyl alcohol, OA) or chronic inflammation (induced with adjuvant, ADJ) is able to modify the following parameters:

(a) the hepatic MMFO metabolic profiles of pentobarbital and zoxazolamine, *in vivo*; and diphenyloxazole hydroxylation and 7-ethoxycoumarin O-deethylation, *in vitro*;

(b) the hepatic glutathione profile;

(c) the hepatotoxic responses to two hepatotoxins which are activated by microsomal oxidation - paracetamol and bromobenzene, as determined by serum alanine aminotransaminase activity.

The experiments were performed in two species commonly used in hepatotoxicity studies - LACA Swiss mice and Porton albino rats; and also in Hooded Wistar rats, as this strain is this strain tends to be most resposive to adjuvant-induced polyarthritis and may represent a more suitable model in which to study chronic inflammation. In these well-established models, the administration of ADJ results

in an acute inflammation which peaks 4 days after the tail-base injection, and is followed by a longer term progressive arthritis lasting for weeks - whereas the shorter acting OA produces only the acute inflammation [Whitehouse, 1986].

4.2 METHODS

4.2.1 Induction of acute and chronic inflammation.

OA and ADJ were administered, via a tuberculin syringe and 26 gauge x 0.5 inch Terumo needle, as subcutaneous injections in the tail approximately 1 to 2 cm from the base of the tail. OA was administered neat, whereas the ADJ contained *Mycobacterium tuberculosis* in a squalane solvent (injection volumes are listed in table 4.1).

4.2.2 *In vivo* assessment of hepatic microsomal mixed function oxidases (MMFO).

Animals were administered with either intraperitoneal sodium pentobarbital or zoxazolamine (also i.p.) on alternate days, following tail injection with OA or ADJ, as described in the administration table (table 4.1). An untreated group was carried through the procedure to control for changes in enzyme activities that may result from repeated administrations of agents with MMFO inducing potential.

The period of loss of righting reflex was used as the index of pentobarbital sleeping time (PST), and the duration

Table 4.1: Drug administration procedure.

Species:	LACA Swiss Mice	Albino Portons	Hooded Wistars
Inflammogens: (μl)			
Oleyl Alcohol	50	100	100
Adjuvant	50	50	50
Drugs: (mg/kg)			
Pentobarbital	50	25	25
Zoxazolamine	200	60	60
[Period studied, days]	14	16	18
Hepatotoxins: (mg/kg)			
Bromobenzene	250	1000	1000
Paracetamol	250	[day 6] 1000 [day 13] 500	1000
[Days studied]	2, 6, 12	6, 13	4, 12

of immobility an index of zoxazolamine paralysis time (ZPT). Extremely large variations in ZPT within dose groups were observed in pilot studies which used injections of peanut oil suspensions. This variation was markedly reduced by the administration of zoxazolamine in a 50% Tween-80 solution, and also resulted in an onset time of 1 to 2 minutes (c.f. 5 minutes or more in peanut oil).

4.2.3 *In vitro* assessments of MMFO activities.

7-Ethoxycoumarin O-deethylase (7-ECODase) and diphenyloxazole hydroxylase (PPO-OHase) activities were determined in the cofactor-fortified supernatant fractions (i.e. Mg^{2+} and NADPH generating system) from homogenised liver (Ultraturrax homogeniser) which had been centrifuged at 9000 g for 15 minutes at 2^{o}C (Beckman J2-21 centrifuge). The hydroxylation of diphenyloxazole (PPO) to the flourescent PPO-OH, and the deethylation of 7-ethoxycoumarin (7-EC) to umbelliferone is indicative of cytochrome P-448 and P-450 (less specifically) activities, respectively.

PPO (22 μg/ml) was incubated with S9 fraction (4 mg liver/ml) and NADPH generating system ($MgCl_2$, 15 mM; glucose-6-phoshate, 5 mM; NADP, 0.5 mM; glucose-6-phosphate dehydrogenase, 2 IU/ml) in phosphate buffer (0.1 M, pH 7.4), at total incubation volume of 1 ml, at 37^{o}C. Reactions were quenched after 30 minutes with cold acetone (1 ml), and

samples were back-extracted into NaOH (1 N, 2.5 ml) via n-hexane (3 ml).

7-EC (0.1 mg/ml) was incubated with S9 fraction (2 mg liver/ml) and NADPH generating system (as above). The reaction was quenched after 10 minutes with trichloroacetic acid (20 % w/v, 125 μl), and samples were back-extracted into NaOH/NaCl (0.01 N / 1 M, 2.5 ml) via chloroform (1 ml). The flourescent products of PPO and 7-EC were measured according to the procedures of Phillipides et al (1982, at Em 505 nm / Ex 355 nm) and Greenlee and Poland (1978, at Em 456 nm / Ex 368 nm) respectively, using a Perkin-Elmer Flourimetric Spectrophotometer, model 203.

4.2.4 Determination of hepatic non-protein sulphydryls.

The protein was precipitated from aliquots of freshly homogenised liver with sulphosalicylic acid (1.82 % w/v, final concentration, pH 8). Following the removal of precipitate by centrifugation, a portion of the supernatant was treated with Ellman's reagent (DTNB or 5,5'-dithiobis-[2-nitrobenzoic acid] at 0.12 mM final concentration in 0.1 M PO_4/ 1 mM EDTA/ 0.12 % sodium citrate, pH 7.4) and the absorption of the sample at 412 nm was measured using a Pye Unicam SP1800 Ultraviolet Spectophotometer. Standard curves were constructed each time samples were assayed using a freshly prepared solution of GSH.

4.2.5 Administration of hepatotoxic probes.

Animals were challenged with paracetamol (PC, in saline) and bromobenzene (BB, 5% Tween-80 in saline) intraperitoneally, These two times represented points of maximal MMFO depression as observed in the PST profiles (Figure 4.1). An additional group of mice were similarly treated with hepatotoxins at a time point which corresponded with a return towards approximately normal MMFO activity (i.e. day 6 post tail-base injection). An initial comparison between preparations of bromobenzene was performed in mice (table 4.2, Tween 80 - 250, 500 and 1000 mg BB/kg; and isopropyl myristate - 1000 mg BB/kg), however all other doses of bromobenzene were administered in Tween 80 (5% v/v).

4.2.6 Determination of serum alanine aminotransferase activity.

Elevation of serum alanine aminotransferase (ALAT) was used as an index of hepatotoxicity. Blood was sampled 18 hours after hepatotoxin administration from rats (abdominal aortia, via syringe, and during ether anaesthesia) or by cardiac puncture in mice. The serum was frozen and ALAT was determined by the formation of a brown pyruvate hydrazone, quantitated colourimetrically by the method of Reitman and Frankel [1957]. Samples were assayed within two days of collection as ALAT activity is unstable with prolonged storage [Stacey et al, 1978].

4.3 RESULTS

4.3.1 The effect of inflammation on MMFO activity *in vivo*.

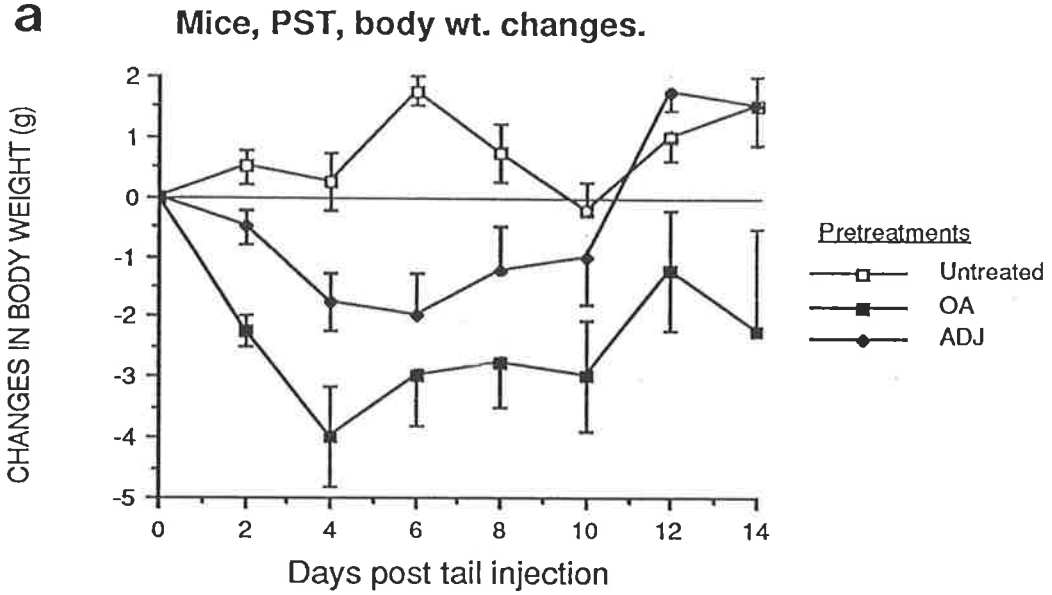
Immediate reddening and swelling was evident following the subcutaneous tail injection of OA and ADJ. A decline in the rate of body weight gains of inflammogen-treated animals were apparent when they were weighed on alternate days prior to the administration of pentobarbital or zoxazolamine (figures 4.1 - mice, 4.2 - porton rats, 4.3 - hooded rats). This effect correlated with the inflamed appearance of the animals' tails, and was most clearly demonstrated during the pentobarbital sleeping profiles, as repeated zoxazolamine administration alone was shown to inhibit weight gain.

A single tail injection of either OA and ADJ prolonged both PST and ZPT, which are *in vivo* correlates of cytochrome P450 and P448, in a mainly biphasic manner for all animal types (Figures 4.4 - mice, 4.5 - porton rats, 4.6 - hooded rats). The maxima were at days 2 to 4 and 10 to 12 in mice, while a more delayed profile was evident in the rats. Both rat strains showed marked increases in PST at 14 to 16 days after the tail injection of ADJ, whereas the shorter acting OA treatment had little effect on MMFO at this stage.

The profiles of ZPT and PST were comparable and the marked inhibitory effect on pentobarbital and zoxazolamine metabolism seen in mice was probably due to the relatively

Figure 4.1: Effect of inflammation on body weight in mice. Mean body wt. (± SEM) was measured after tail-base injection of the inflammogens, OA and ADJ, on days in which (a) pentobarbital sleeping times and (b) zoxazolamine paralysis times, were determined (n = 4).

a


Mice, PST, body wt. changes.
CHANGES IN BODY WEIGHT (g)
2
1
0
-1
-2
-3
-4
-5
0
2
4
6
8
10
12
14
Days post tail injection
Pretreatments
Untreated
OA
ADJ

b

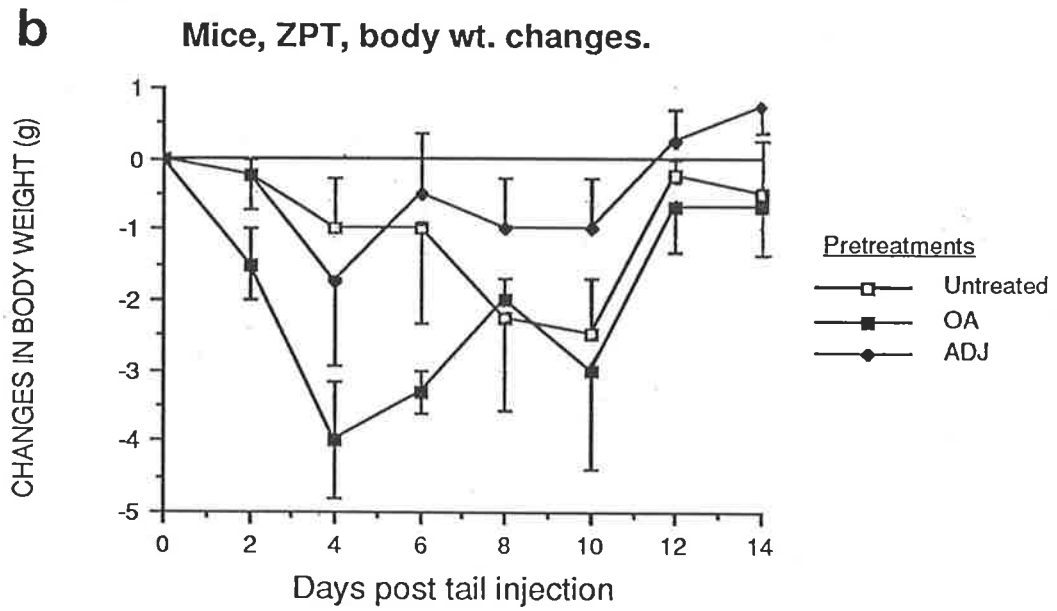
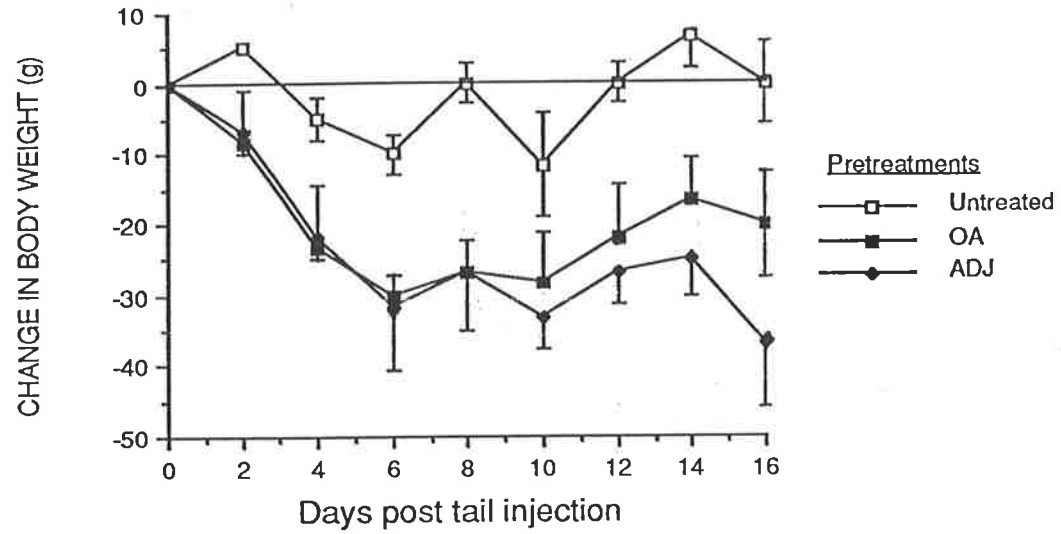

Mice, ZPT, body wt. changes.
CHANGES IN BODY WEIGHT (g)
1
0
-1
-2
-3
-4
-5
0
2
4
6
8
10
12
14
Days post tail injection
Pretreatments
Untreated
OA
ADJ

Figure 4.2: Effect of inflammation on body weight in porton rats. Mean body wt. (± SEM) was measured after tail-base injection of the inflammogens, OA and ADJ, on days in which (a) pentobarbital sleeping times and (b) zoxazolamine paralysis times, were determined (n = 3).

a **Portons, PST, body wt. changes.**



b **Portons, ZPT, body wt. changes**

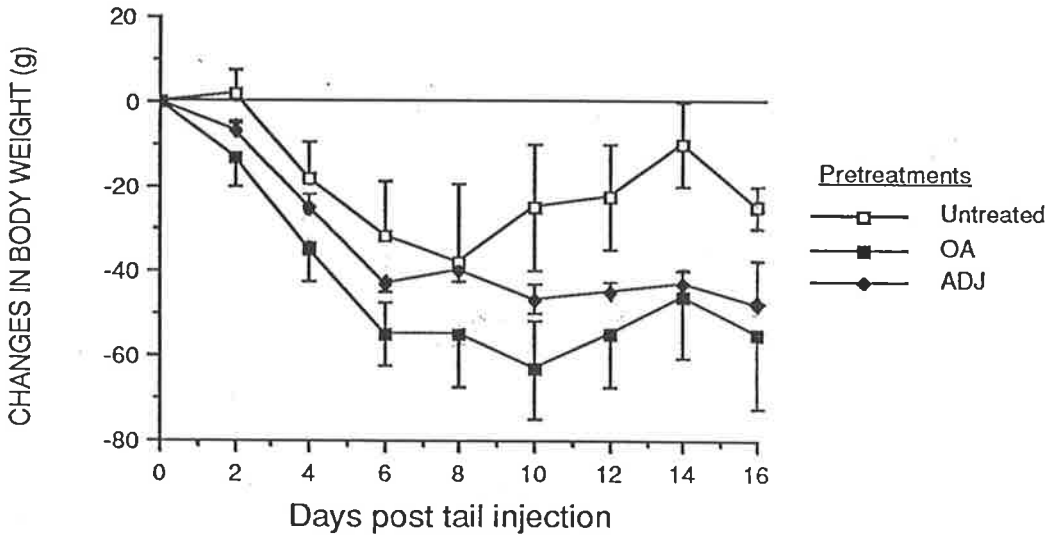
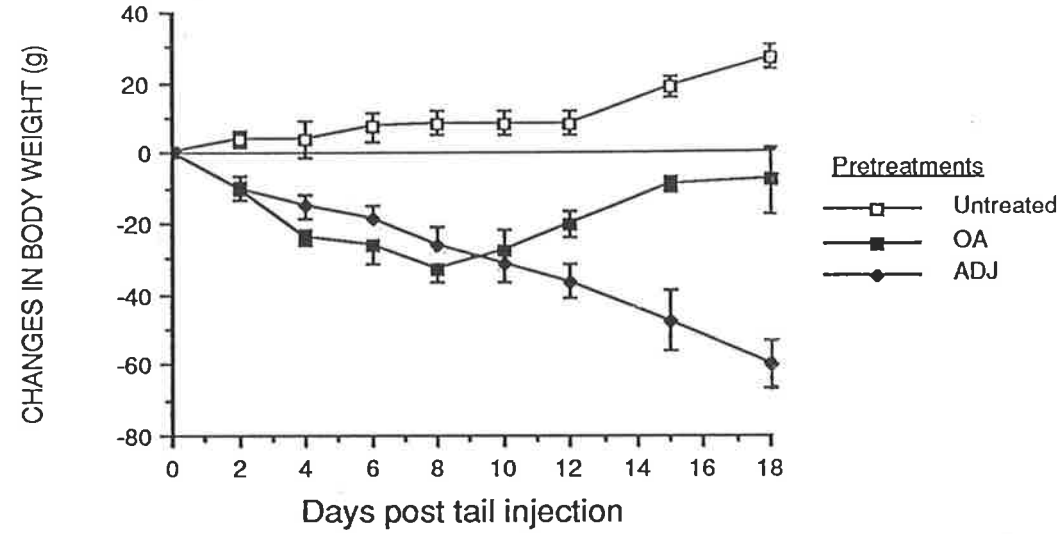


Figure 4.3: Effect of inflammation on body weight in hooded rats. Mean body wt. (± SEM) was measured after tail-base injection of the inflammogens, OA and ADJ, on days in which (a) pentobarbital sleeping times and (b) zoxazolamine paralysis times, were determined (n = 4).

a **Hooded, PST, body wt. changes.**



b **Hooded, ZPT, body wt. changes.**

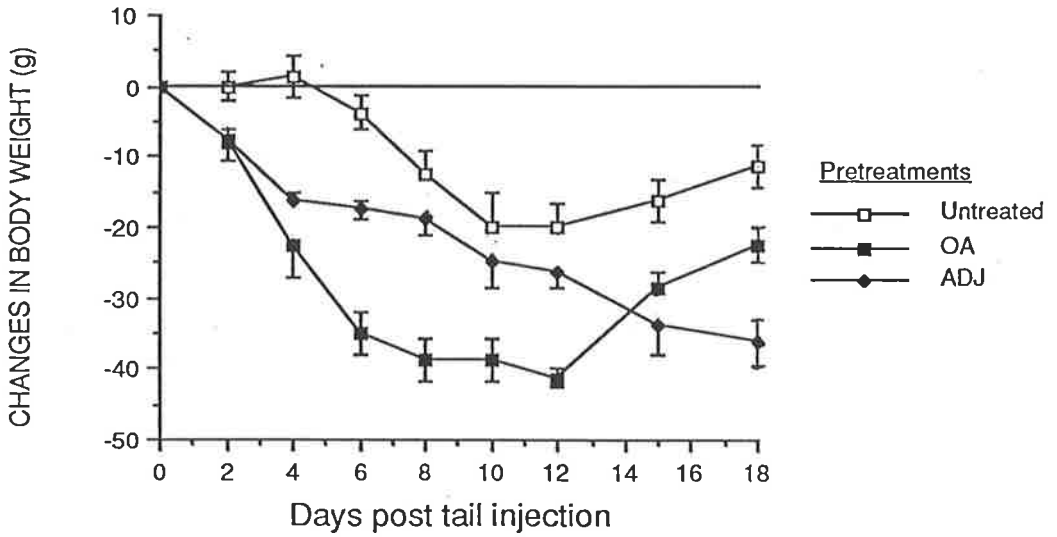
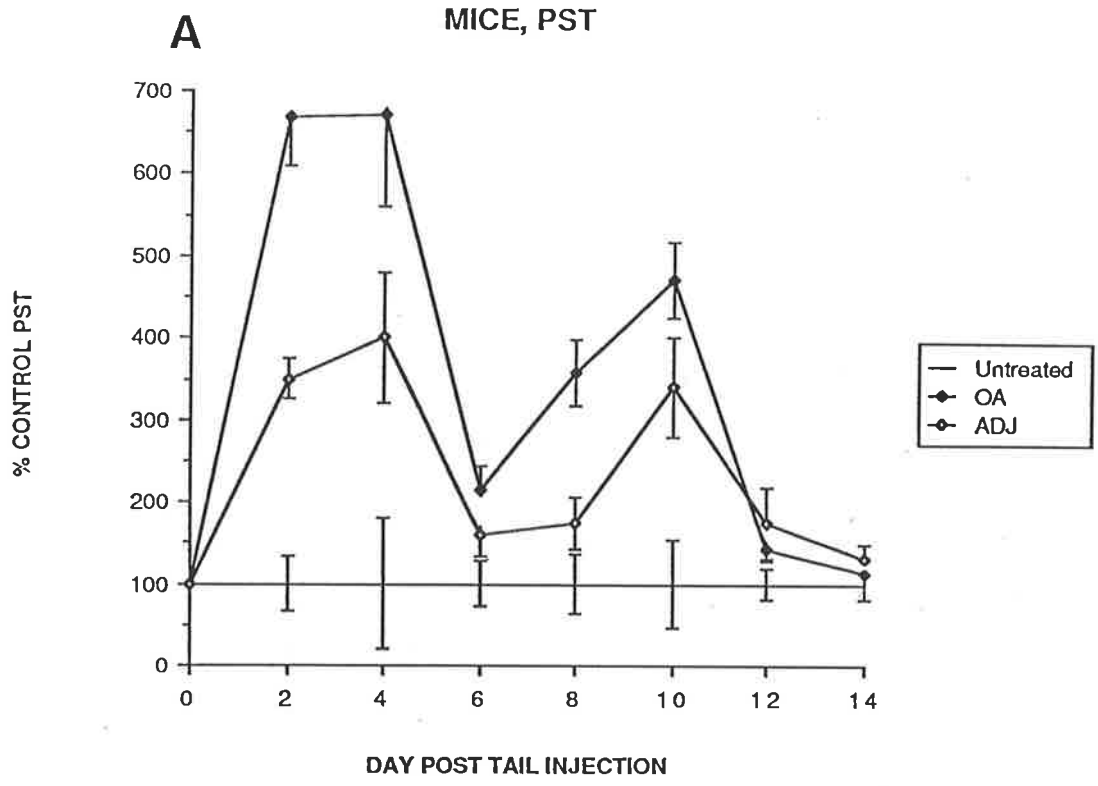


Figure 4.4: Effect of inflammation on MMFO activity *in vivo* in mice. (a) Pentobarbital sleeping times and (b) zoxazolamine paralysis times were assessed on alternate days after tail-base injection with inflammogens. Control PST on day 0, 50 mg/kg = 35 ± 13 minutes; and control ZPT on day 0, 200 mg/kg = 51 ± 11 minutes (n = 4).


A
MICE, PST
% CONTROL PST
700
600
500
400
300
200
100
0
0
2
4
6
8
10
12
14
DAY POST TAIL INJECTION
Untreated
OA
ADJ

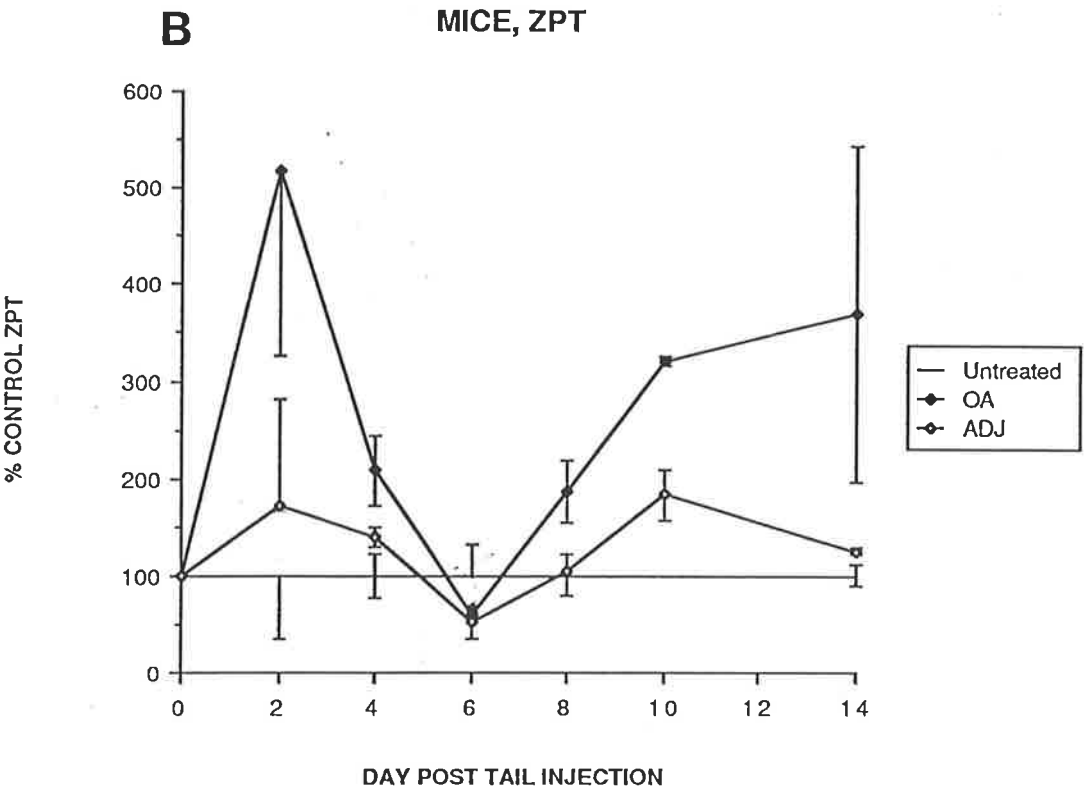
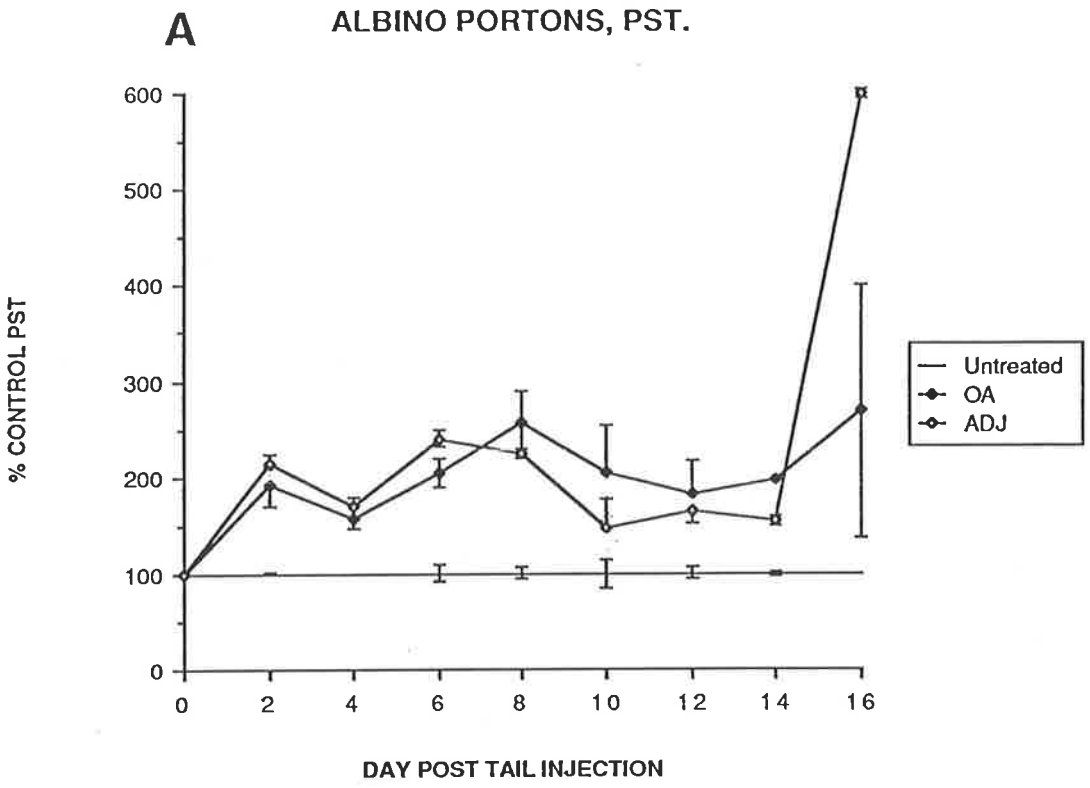

B
MICE, ZPT
% CONTROL ZPT
600
500
400
300
200
100
0
0
2
4
6
8
10
12
14
DAY POST TAIL INJECTION
Untreated
OA
ADJ

Figure 4.5: Effect of inflammation on MMFO activity *in vivo* in porton rats. (a) Pentobarbital sleeping times and (b) zoxazolamine paralysis times were assessed on alternate days after tail injection with inflammogens. Control PST on day 0, 25 mg/kg = 33 ± 3 minutes; and control ZPT on day 0, 60 mg/kg = 93 ± 1 minutes (n = 3).


A
ALBINO PORTONS, PST.
% CONTROL PST
600
500
400
300
200
100
0
0
2
4
6
8
10
12
14
16
DAY POST TAIL INJECTION
Untreated
OA
ADJ

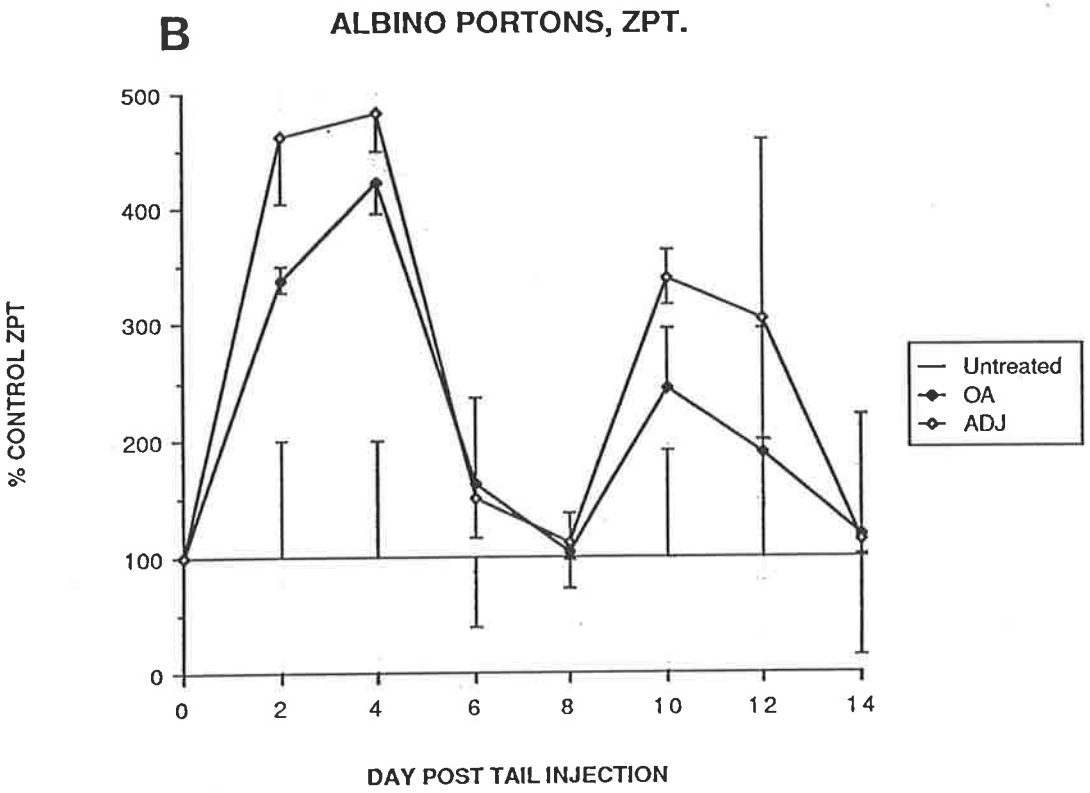
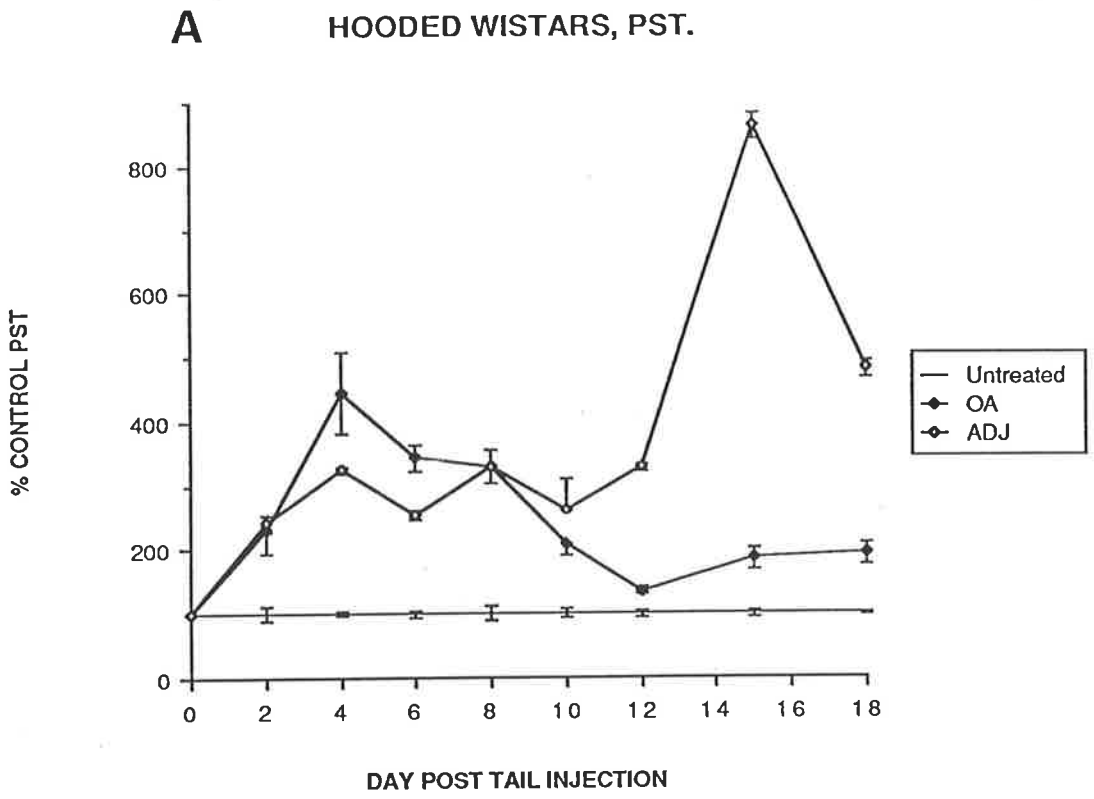
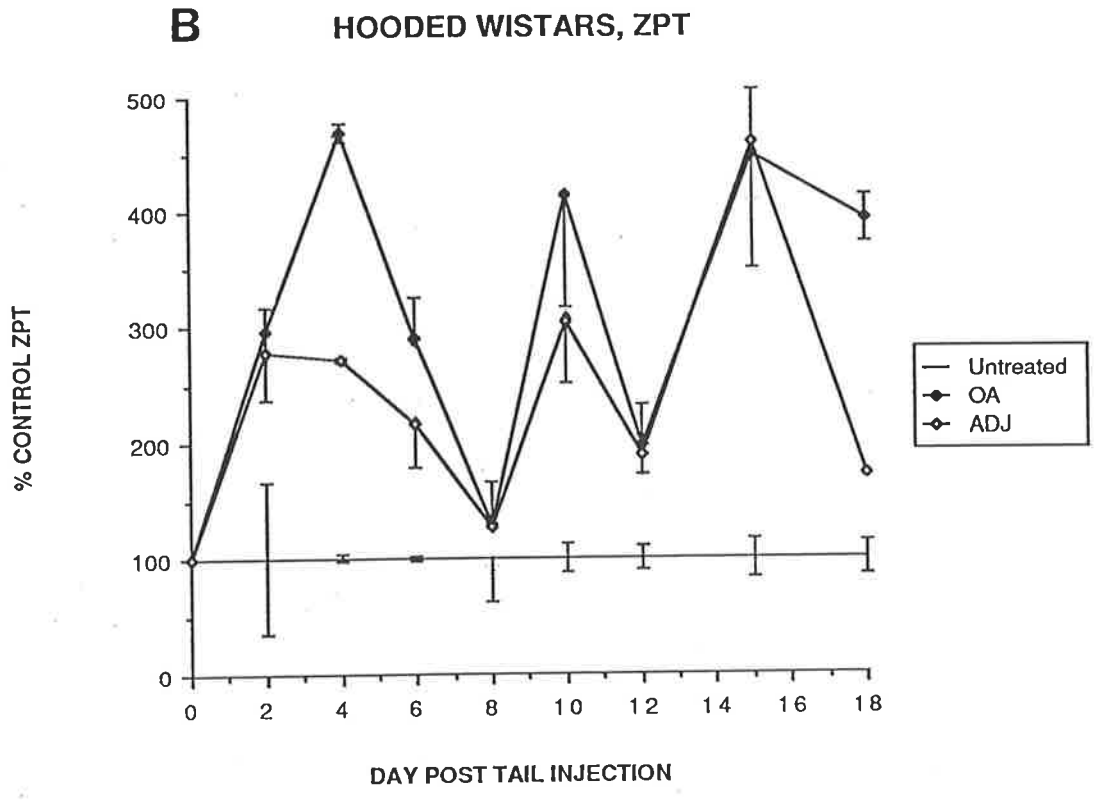

B
ALBINO PORTONS, ZPT.
% CONTROL ZPT
500
400
300
200
100
0
0
2
4
6
8
10
12
14
DAY POST TAIL INJECTION
Untreated
OA
ADJ

Figure 4.6: Effect of inflammation on MMFO activity *in vivo* in hooded rats. (a) Pentobarbital sleeping times and (b) zoxazolamine paralysis times were assessed on alternate days after tail injection with inflammogens. Control PST on day 0, 25 mg/kg = 26 ± 3 minutes; and control ZPT on day 0, 60 mg/kg = 44 ± 2 minutes (n = 4).


A
HOODED WISTARS, PST.
% CONTROL PST
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
DAY POST TAIL INJECTION
Untreated
OA
ADJ


B
HOODED WISTARS, ZPT
% CONTROL ZPT
500
400
300
200
100
0
0
2
4
6
8
10
12
14
16
18
DAY POST TAIL INJECTION
Untreated
OA
ADJ

large dose per body weight of arthritogens (50 μl/ 30 g), compared to rats (50 and 100 μl/ 300 g).

4.3.2 The effect of inflammation on MMFO activity *in vitro*.

A comprehensive time-course of MMFO activities *in vitro* was assessed in hooded rats only (Figure 4.7). ECODase and PPO-OHase activities, which are indicative of cytochrome P450 and P448 activities respectively, were depressed in a biphasic manner similar to the PST and ZPT profiles. Maximal effects were observed on days 2 to 4 and 12, with ADJ treatment reducing PPO-OHase and ECODase activities in a sustained manner from day 10 onwards.

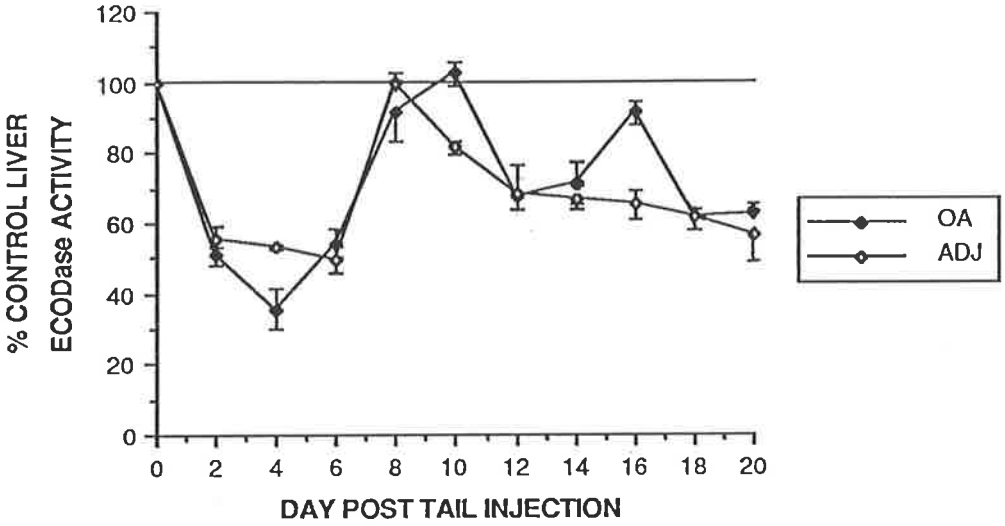
4.3.3 The effect of inflammation on hepatic non-protein sulphydryl levels.

A detailed profile of the time course of hepatic non-protein sulphydryl (predominantly GSH) content, following tail injections of inflammogens, was performed only in hooded rats (Figure 4.8). Initial hepatic GSH levels were slightly raised 2 days after the OA tail injection, but became depressed at days 4 to 8. ADJ administration resulted in lower hepatic GSH than with OA treatment on days 16 to 18 - the extent of GSH depression was 40 % for ADJ, compared to 20 % with OA treatment.

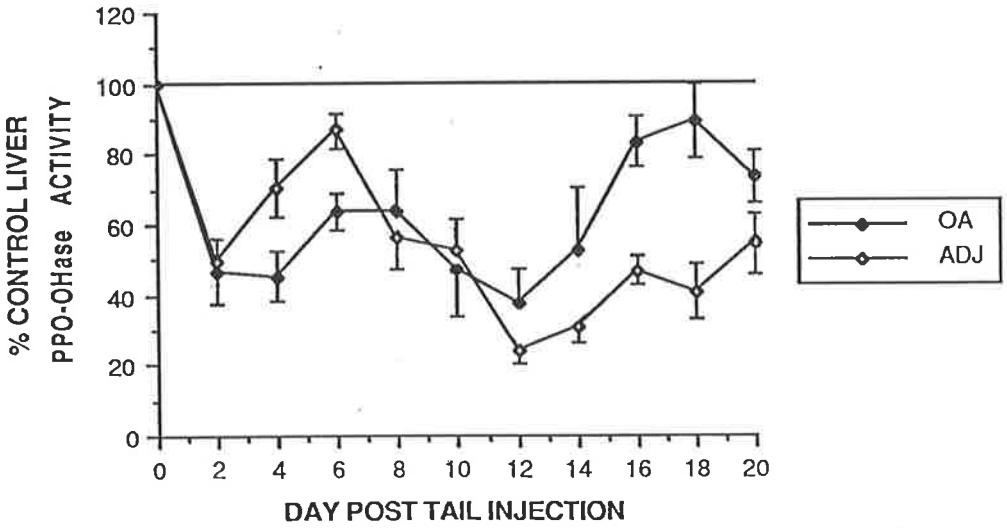
Fewer time points were assessed in mice, but the increase in hepatic GSH was more apparently greater, since

Figure 4.7: Effect of inflammation on MMFO activity *in vitro* in hooded rats. (a) ECODase activity and (b) PPO-OHase activities were assessed on alternate days after tail injection with inflammogens (n = 4).

A HOODED WISTARS, ECODase.



B HOODED WISTARS, PPO-OHase



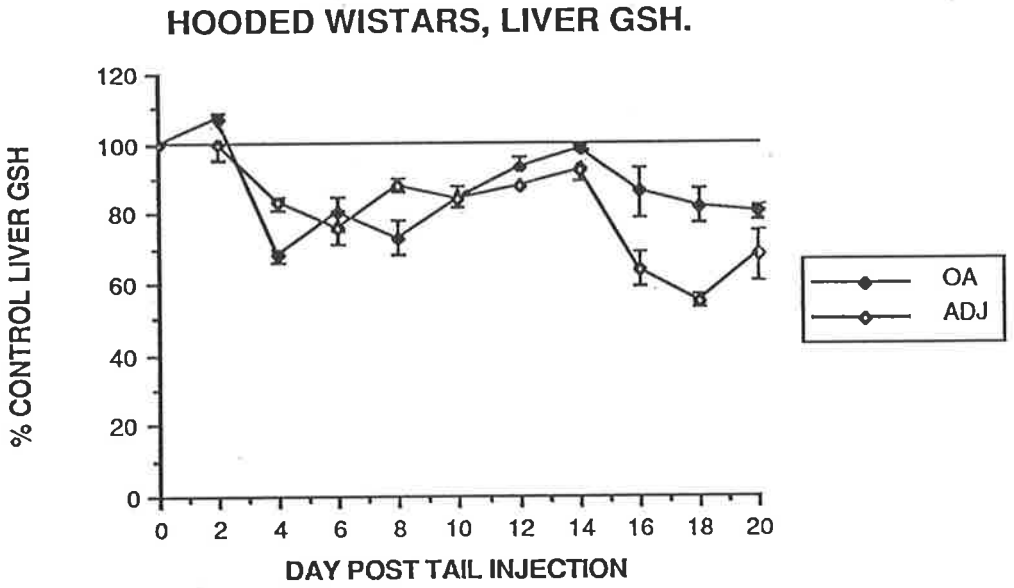


Figure 4.8: Effect of inflammation on hepatic GSH levels in hooded rats. Hepatic GSH was assessed on alternate days after tail injection with inflammogens. Control hepatic GSH, on day 0 = 9.01 ± 0.36 μmol / g liver (n = 4).

liver tissue obtained 2 days after inflammogen tail injection contained 164 ± 31 % and 122 ± 7 % of control GSH levels (4.75 ± 0.33 μmol GSH/ g liver) in OA and ADJ treated animals, respectively. The depression of hepatic GSH levels 12 days after the tail injection was also greater than that seen in hooded rats, i.e. 39 ± 12 % and 79 ± 9 % in OA and ADJ treated mice, respectively.

4.3.4 Effect of inflammation on the hepatotoxic responses to paracetamol and bromobenzene.

(a) MICE: The administration of paracetamol in mice resulted in the expected frank hepatotoxicity. This was confirmed by both the gross morphological appearance of the liver, and by the high levels of serum ALAT. In contrast, inflammogen-treated mice, which had been challenged with paracetamol at time points of greatest MMFO depression, did not exhibit hepatotoxicity, had no abnormal gross liver morphology and had essentially normal serum ALAT levels (Figure 4.9). A similar insensitivity to paracetamol-induced liver damage was seen in mice 6 days post arthritogen tail injection, despite the fact that MMFO activities had returned to near control values.

Bromobenzene is also hepatotoxic in mice, causing elevated serum ALAT activity (Figure 4.9). During pilot studies it was noted that bromobenzene was more toxic when administered in a micellar Tween 80 suspension, than when given in isopropyl myristate - a 14-carbon chain fatty acid

Figure 4.9: Effect of inflammation on the hepatotoxic responses of mice to paracetamol and bromobenzene. Mice were challenged with the hepatotoxins at 2, 6 and 12 days after tail injection with inflammogens (n for each group shown on figure).

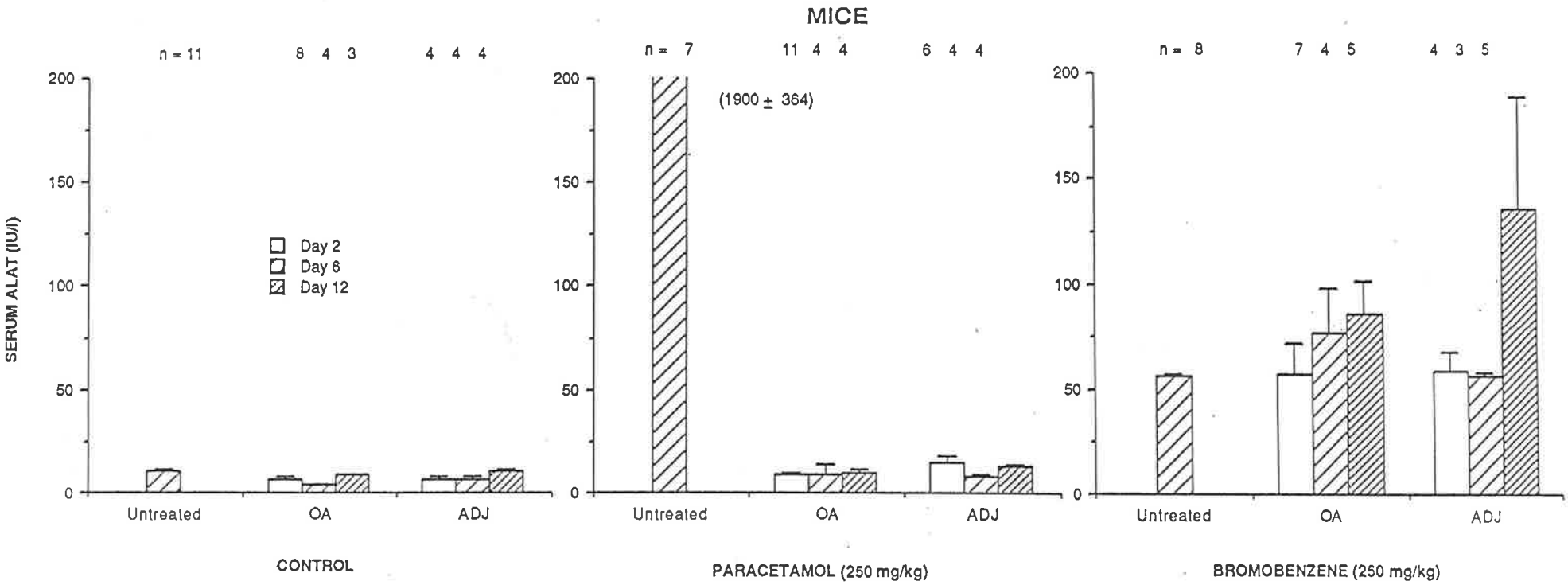

MICE
n = 11
8 4 3
4 4 4
n = 7
11 4 4
6 4 4
n = 8
7 4 5
4 3 5
(1900 ± 364)
SERUM ALAT (IU/l)
200
150
100
50
0
Day 2
Day 6
Day 12
Untreated
OA
ADJ
CONTROL
PARACETAMOL (250 mg/kg)
BROMOBENZENE (250 mg/kg)

Table 4.2: The hepatotoxicity and mortality of bromobenzene in inflamed mice.

Dose (mg/kg) -Vehicle * -Time (hr)	Mortality			Serum ALAT (IU/L)		
	CON	OA	ADJ	CON	OA	ADJ
250, Tw 80, 18 hrs	0/4	0/4	0/4	62 ± 18	45 ± 14	72 ± 4
500, Tw 80, 18 hrs	2/4	4/4	3/4	62 ± 3	#	45
24 hrs	4/4	nd	nd	#	nd	nd
1000, Tw 80, 9 hrs	4/4	4/4	4/4	#	#	#
C14:0, 18 hrs	1/4	0/4	nd	32 ± 11	59 ± 36	nd

* Tw 80, Tween 80 at 3 % v/v; C14:0, isopropyl myristate.
\# no survivors for serum ALAT determination.
n.d. not determined
NB: Bromobenzene administered 2 days after tail injections of OA and ADJ.

ester (Table 4.2). It is probable that the vehicle was influencing the bioavailability of bromobenzene.

The effects of inflammogen treatment on bromobenzene - induced hepatotoxicity were different from those seen with paracetamol. Both inflammogens increased bromobenzene-induced mortality and serum ALAT (Table 4.2 & Figure 4.9).

(b) RATS: Rats were less susceptible to liver damage induced by paracetamol and bromobenzene, although a modest elevation in serum ALAT could be detected after either hepatotoxin challenge. In Porton rats, both inflammogens were able to protect against paracetamol challenge, as shown by the failure of ALAT's to become elevated (Figure 4.10). Both arthritogens also ameliorated the mild serum ALAT elevation caused by bromobenzene, 6 days after tail injections. This effect was not present 13 days post tail injection.

In hooded Wistars, ADJ protected against paracetamol-induced hepatotoxicity, at both 4 and 16 days post tail injection, whereas OA, the shorter acting inflammogen, only reduced the paracetamol effect during the initial phase of inflammation (Figure 4.11). The effect of bromobenzene on serum ALAT was unchanged by pretreatment with arthritogens.

4.4 DISCUSSION

Systemic oxidant stress induced by the tail-base injection of oleyl alcohol or a rodent adjuvant

Figure 4.10: Effect of inflammation on the hepatotoxic responses of Porton rats to paracetamol and bromobenzene. Mice were challenged with the hepatotoxins at 6 and 13 days after tail injection with inflammogens (n = 3).

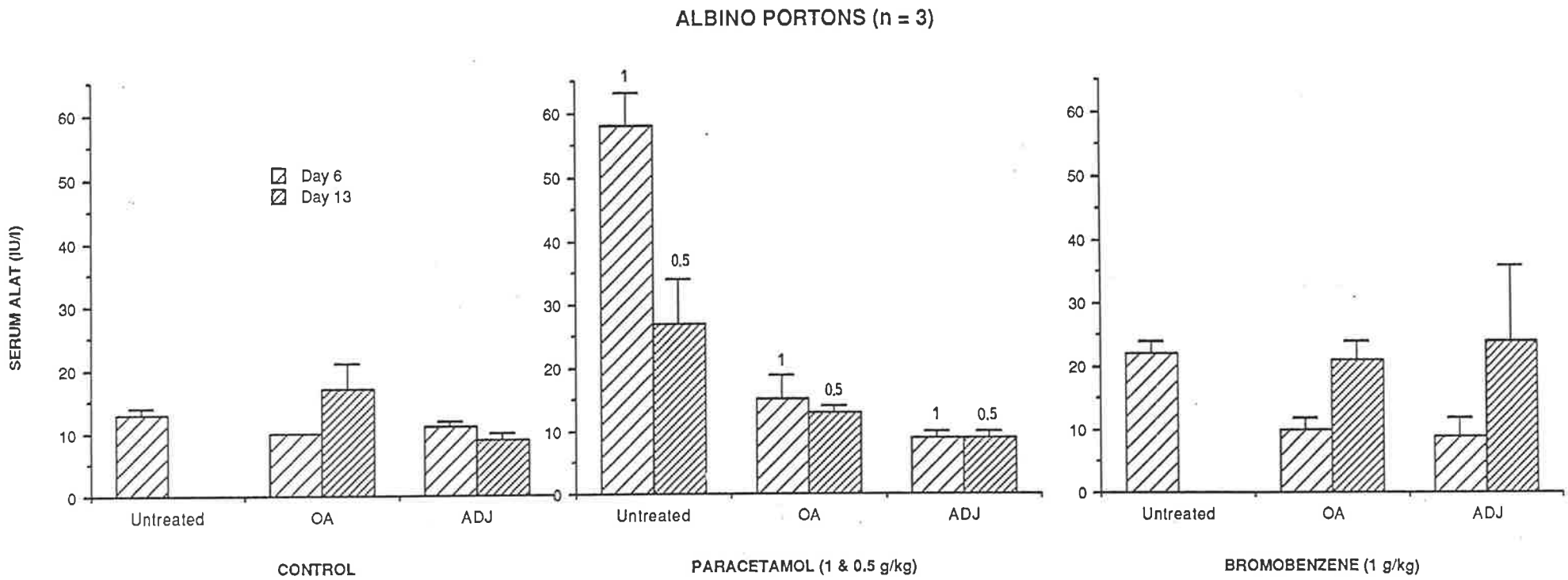
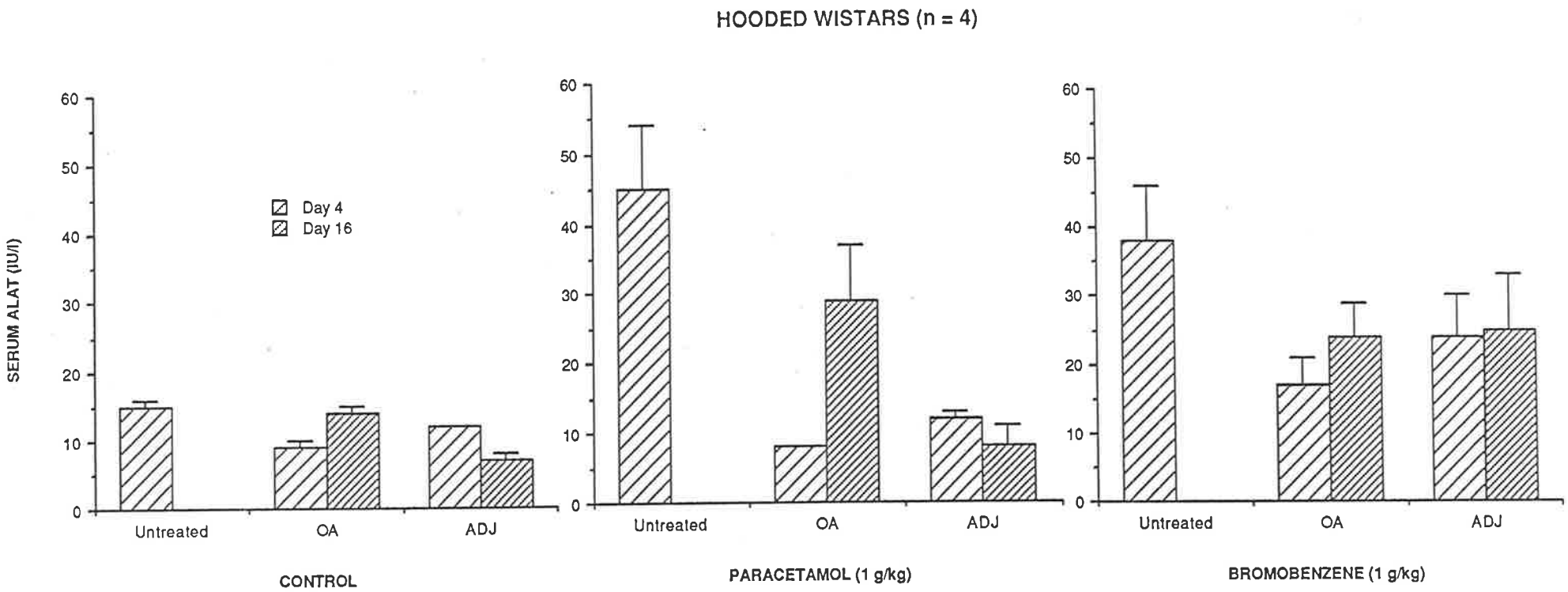

ALBINO PORTONS (n = 3)
SERUM ALAT (IU/l)
Day 6
Day 13
0
10
20
30
40
50
60
Untreated
OA
ADJ
1
0.5
CONTROL
PARACETAMOL (1 & 0.5 g/kg)
BROMOBENZENE (1 g/kg)

Figure 4.11: Effect of inflammation on the hepatotoxic responses of hooded rats to paracetamol and bromobenzene. Mice were challenged with the hepatotoxins at 4 and 16 days after tail injection with inflammogens (n = 4).


HOODED WISTARS (n = 4)
SERUM ALAT (IU/l)
Day 4
Day 16
Untreated
OA
ADJ
CONTROL
PARACETAMOL (1 g/kg)
BROMOBENZENE (1 g/kg)

(*Mycobacterium tuberculosis* in squalane) is accompanied by inhibition of hepatic MMFO activity, as assessed by the *in vivo* indices of pentobarbital sleeping and zoxazolamine paralysis times, and by the *in vitro* indices of ethoxycoumarin O-deethylation and diphenyloxazole hydroxylation.

The inhibitory profiles were temporally phasic and corresponded well in hooded rats with the acute inflammation seen with OA treatment, and with the acute and chronic phases of inflammation which result from adjuvant administration. Both agents had sustained effects on MMFO activities in mice but this may have been due to the relatively large doses of inflammogens administered to this specie. The inflammation was also characterised by a decline in body weight, and local swelling and stiffness in the tails of inflammogen-treated animals. These results correlated well with previous studies by other workers [Whitehouse and Brooks, 1986; Mathur et al, 1977].

Administration of the cytochrome P-450-activated hepatotoxins, paracetamol and bromobenzene, to animals at the peak of the inflammatory response, resulted in the abolition of PC-induced hepatotoxicity but had no effect on BB-induced liver damage, as graded by plasma ALAT elevation 18 hours after hepatotoxin administration. Although both PC and BB are oxidative-microsomally activated hepatotoxins, the resultant hepatotoxic response to the two compounds were

not uniformly affected by inflammation. This would suggest that mechanisms, other than MMFO inhibition, are involved to account for this pattern of responses.

The liver GSH status appeared elevated 2 days after inflammogen treatment, however this 'rebound' effect to the administration of oxidisable xenobiotics is common [Jollow et al, 1974]. Therefore the abolition of PC-induced hepatotoxicity is consistent with the inflammation-dependent inhibition of MMFO activity and the initial elevation of hepatic thiol status. The decline in hepatic GSH levels at the later stage of inflammation may not truly reflect the antioxidant status of the liver, as the induction of APR synthesis results in elevated metallothionein levels within 36 hours of the onset of inflammation [Gitlin, 1988].

There is growing evidence that MT is a powerful antioxidant and capable of binding the electrophilic metabolites of xenobiotics (including PC) by utilising the 20 sulphydryl groups per molecule of protein [Chengalis et al, 1986]. Previous interests in this protein have concerned the binding properties of, and ready induction by, heavy metals such as cadmium, zinc and mercury. In normal livers GSH is the predominant free-sulphydryl containing compound, while a very small portion can be attributed to MT. However, following induction with heavy metals, hepatic MT content can increase to account for three quarters of the elevated thiol level present in the liver [Thornalley and

Vasak, 1985]. Animal models of systemic and hepatic inflammation show rapid induction of metallothionein synthesis [Sobocinski and Canterbury, 1982; Maitani and Suzuki, 1981]. Induction of hepatic MT by heavy metals has been shown to reduce the lethality of γ-irradiation in mice [Matsubara et al, 1987], and diminish the rate of GSH oxidation in isolated perfused rat lungs following exposure to t-butyl hydroperoxide [Buckley and Bassett, 1987]. Further evidence for the protective role of MT was the beneficial effects of loading with exogenous zinc in two experimental models - paw oedema in adjuvant-induced polyarthritic rats [Cousins and Swerdel, 1985] and PC-induced hepatotoxicity in mice [Chengalis et al, 1986]. Although there have been reports that administration of zinc ameliorates BB-induced hepatotoxicity [Schnell et al, 1984; McMillan et al, 1985], the effects may have been confounded by the fact that the zinc dose inhibited cytochrome P-450 activity.

Applications of genetic engineering have brought MT to renewed prominence, with use of the easily inducible MT genes to 'switch on' artificially linked growth factor genes in the production of transgenic animal strains. Recombinant DNA technology has also revealed the time course and control of APR gene expression during rat adjuvant disease, especially in the cases of MT and ceruloplasmin [Gitlin, 1988]. As the oxidised MT-thiol groups are easily reduced in the presence of GSH, MT appears to provide a readily

the potential to represent a significant antioxidant process in the liver.

In order to confirm literature reports of the protective effects of MT-induction, an additional preliminary experiment was conducted, in which mice were dosed with zinc sulphate (or the better tolerated zinc lactate) or cadmium sulphate (all were injected i.p., n = 4 per group, at 2 mg/kg of zinc and 0.1 mg/kg of cadmium) 48 and 24 hours prior to challenge with PC (250 mg/kg). The serum ALAT 18 hours later revealed that cadmium and zinc sulphate were more effective than zinc lactate, i.e. serum ALAT for cadmium sulphate, zinc sulphate and zinc lactate were 53 ± 5 %, 65 ± 20 % and 83 ± 13 % of the control value (1150 ± 50 IU/L), respectively. Mice similarly pretreated before administration with BB (250 mg/kg) also showed decreased serum ALAT, i.e. cadmium sulphate, zinc sulphate and zinc lactate produced 34 ± 3 %, 22 ± 6 % and 55 ± 22 % of control serum ALAT (135 ± 53 IU/L). Therefore it would appear that Cd and Zn treatment can ameliorate chemically-induced hepatotoxicity, however cytochrome P-450 activity was not measured, and so the extent of heavy metal effect on chemical activation was not known.

The lack of effect on BB-induced hepatotoxicity may be due to the less important role of thiol-dependent inactivation in the metabolism of BB, as the aromatic epoxide metabolite of BB may chemically rearrange to form

more stable, less reactive products [Dankovic et al, 1985; Casini et al, 1982]. The detoxification of BB is unlike that for PC metabolites, which may generate O_2^- by redox cycling, and thereby deplete GSH (via GSH peroxidase catabolism of H_2O_2) which is necessary for the inactivation of the quinoneimine metabolite [Moore et al, 1985] (Figure 4.12).

The effects of inflammation on hepatic metabolism, thiol status and paracetamol-induced hepatotoxicity are marked and prolonged. The differential effect on the hepatotoxic responses to two oxidative-microsomally activated hepatotoxins denies any assumption that the hepatotoxic responses to all cytochrome P450 activated compounds would be affected in a similar manner by the presence of systemic oxidant stress.

The following chapter describes further research into the immunological status of arthritogen-treated rats and mice, in order to isolate which immunological mediators may have influenced the hepatic changes during inflammation.

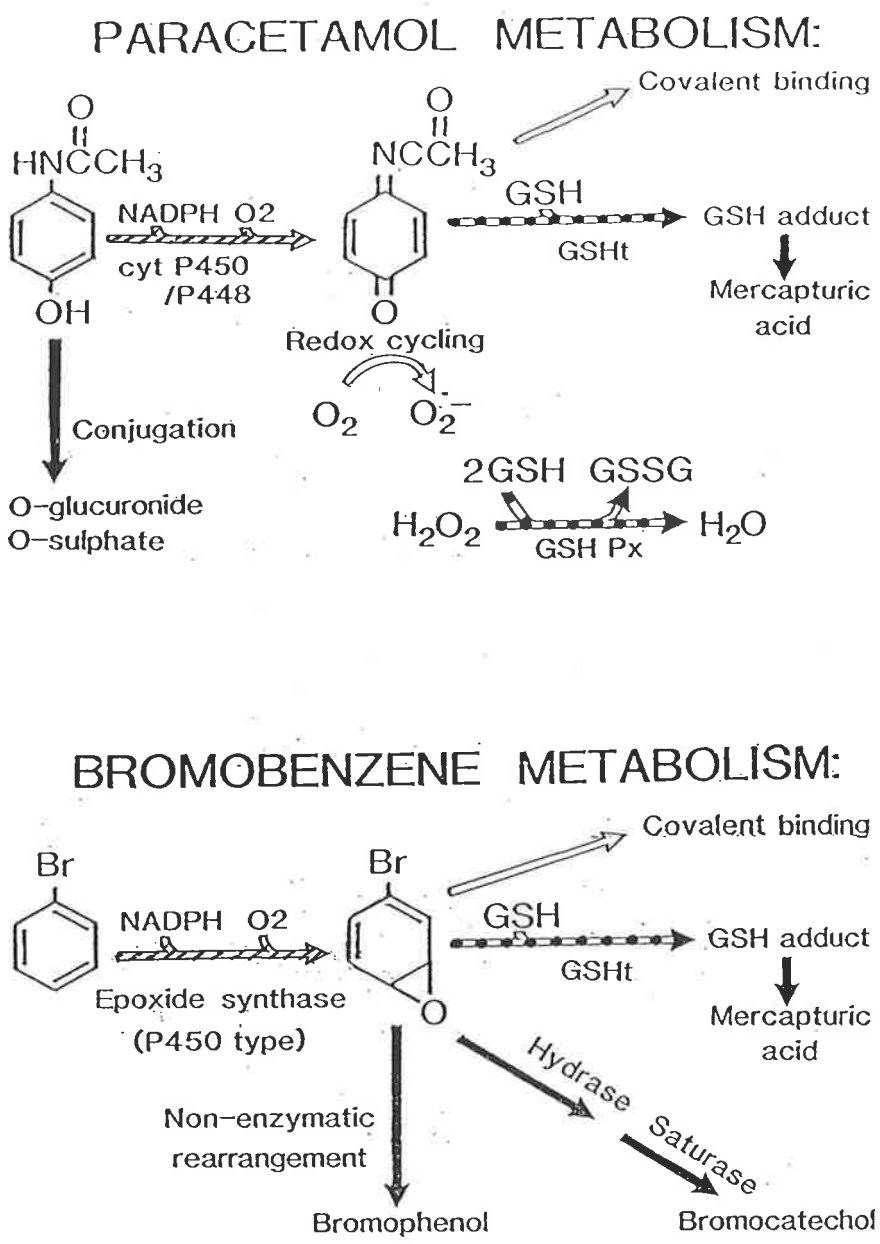


Figure 4.12: Metabolic pathways of paracetamol and bromobenzene.

Chapter 5

MODIFICATION BY ANTI-INFLAMMATORY DRUGS OF SYSTEMIC OXIDANT STRESS AND ITS EFFECTS ON HEPATOTOXIC RESPONSES

5.1 INTRODUCTION

The inflammatory response is an integral part of the host response to mechanical and chemical damage, or microbial infections. In the first phase of inflammation polymorphonuclear leukocytes leave the blood-flow and adhere to the endothelial cells which line the walls of blood vessels. The endothelial cells spread to allow activated PMN to migrate through the blood vessel walls to the site of injury, where they proceed to break down and phagocytose foreign matter by releasing reactive oxygen species, myeloperoxidase and various proteases (refer chapter 3).

Macrophages replace PMN as the predominant immune cell type a few hours after initiation of damage. Macrophages engulf damaged cells and foreign matter, particularly antibody-coated material. *T* lymphocytes (originating from the thymus) also proliferate at this point and activate other macrophages and *T* cells, as well as stimulating B lymphocytes to divide, differentiate and secrete large quantities of antibodies. Finally gross morphological damage is repaired by proliferating fibroblasts resulting in scar tissue.

Activated macrophages release a range of cytokines, including interleukins, interferons, tumour necrosis factor and colony stimulating factors, which act on a wide variety

of cell types (fig. 5.1, and present list of cytokines in table 5.1) [Old, 1988].

Interleukin-6 (IL-6, previously called interferon-β2) is produced by endothelial cells, fibroblasts and *T* cells, in response to the IL-1 secreted by macrophages. Within the first 24 hours of initiation, IL-6 (also known as hepatocyte-stimulating factor) mediates the IL-1 stimulated rapid increase in the hepatic production of a group of serum glycoproteins, known as acute phase reactants (APR).

APR are a particularly interesting group of proteins with many attributes to assist in controlling the oxidant stress induced by inflammation. They include some natural scavengers of pro-oxidant species present in tissue breakdown products, i.e. haptoglobin for certain haemoproteins, ferritin for iron (III), and ceruloplasmin to oxidise iron (II). APR can also scavenge reactive oxidant species. In particular, ceruloplasmin and the haptoglobin/haemoglobin complex have peroxidase activity; and metallothionein, a protein with no aromatic amino acids and containing 20 thiol groups which bind 7 zinc/cadmium atoms, has recently been found to possess the fastest rate reaction constant of any protein with $OH^{\cdot}$ ($k_{OH/MT}$ = 3 x 10^{12} $M^{-1}.s^{-1}$, [Thornalley and Vasak, 1985], see chapter 4).

APR can also enhance the ongoing inflammatory process, as in the case of human C-reactive protein, which can initiate the complement cascade and activated macrophages;

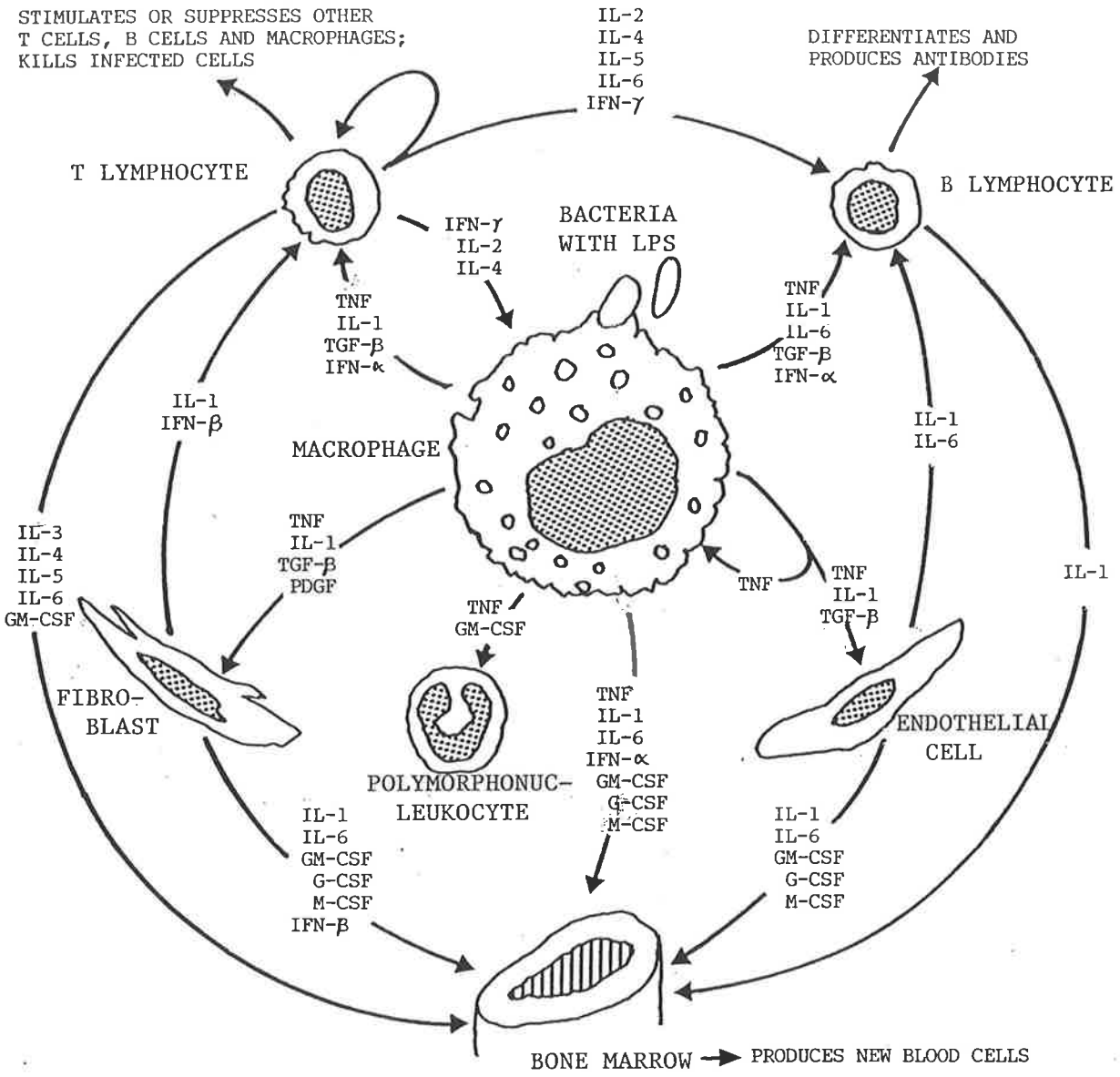


Figure 5.1: Schematic representation of cytokine secretion by various cell types. This diagram represents a simplified version of cytokine release by various immunological cell types as well as endothelial cells and fibroblasts [Old, 1988].

Table 5.1: Cytokines involved in inflammation, immunity, and cell growth and inhibition. There is considerable overlap in the activities of the factors, even though they may be structurally unrelated (e.g. IL-1 and TNF) [Old, 1988]. Some of the factors may also cause the release of other cytokines that potentiate or inhibit each other. For example, INF-γ released by *T*-cells is inhibitory on other *T*-cells, but promotes the release from macrophages of IL-1 (which stimulates *T*-cells to produce the stimulatory factor IL-2) and PGE_2 (a prostaglandin that inhibits lymphocyte proliferation).

FAMILY/MEMBERS	OTHER NAMES
Interferons (IFN)	
...IFN-α	...Leukocyte Interferon
...IFN-β	...Fibroblast Interferon
...IFN-γ	...Immune Interferon
Tumour Necrosis Factors (TNF)	
...TNF	...TNF-α, Cachetin
...Lymphotoxin	...TNF-β
Interleukins (IL)	
...IL-1α, IL-1β	...Endogenous Pyrogen, Lymphocyte-Activating Factor, Leukocyte Endogenous Mediator, Haemopoietin 1
...IL-2	...*T*-Cell Growth Factor
...IL-3	...Multipotential CSF, Mast Cell Growth Factor
...IL-4	...*B*-Cell Stimulatory Factor 1 (BSF-1)
...IL-5	...*T*-Cell Replacing Factor (TRF), Eosinophil Differentiation Factor
...IL-6	...*B*-Cell-Stimulating Factor 2 (BSF-2), Interferon-β_2, Hepatocyte Stimulating Factor (HSF)
Colony-Stimulating Factors (CSF)	
...Granulocyte Macrophage-CSF (GM-CSF)	...CSF-2
...Granulocyte-CSF	...Pluripoietin
...Macrophage-CSF	...CSF-1
...Erythropoietin	
Other Growth and Regulatory Factors (GF)	
...Epidermal Growth Factor (EGF)	
...Fibroblast Growth Factors (Acidic and Basic-FGF)	
...Insulin-like Growth Factor-1 (IGF-1)	...Somatomedin C
...IGF-2	...Somatomedin A
...Nerve Growth Factor (NGF)	
...Platelet-Derived Growth Factor (PDGF)	
...Transforming Growth Factor-α (TGF-α)	
...TGF-β	

or murine serum amyloid P component, the major mouse APR, which may enhance IL-1 production by macrophages and thus potentiate events mediated by IL-1 [Sarlo and Mortensen, 1985]. Both IL-1 and IL-6 have been shown to exert a direct effect on the transcriptional regulation of APR genes in order to enhance gene expression, in particular metallothionein [Karin et al, 1985] and ceruloplasmin [Gitlin, 1988].

Lymphocytes isolated from the joints of individuals with active rheumatoid arthritis are in a state of activation, having undergone (or about to undergo) cell division [Keystone et al, 1986]. The lymphoproliferative activity of IL-1 within the inflamed synovia depends not only on the IL-1 concentration, but also on the presence of local inhibitors of its mitogenic action.

Many nonsteroidal anti-inflammatory drugs (NSAID) control the pain and inflammation associated with rheumatic diseases. Since Vane [1971] showed that NSAID were potent inhibitors of prostaglandin (and prostacyclin) synthesis via arachidonate-cyclooxygenase, it has been widely accepted that this inhibition is primarily responsible for the beneficial effects of NSAID in rheumatoid therapy. However this simple model of a single mode of NSAID action has been challenged recently [Haynes et al, 1988a; Goodwin, 1984; Abrahamson et al, 1983].

In chapter 4, it was shown that inflammatory stress modified the response to an oxidatively-activated hepatotoxin, paracetamol. Contributing factors may have included the inhibition of MMFO activity, or changes in antioxidant thiol status. This chapter describes experiments designed to investigate which immunological mediators may be involved in modifying the hepatotoxic response.

The aim was to use anti-inflammatory and anti-arthritic drugs which selectively modify the actions of prostanoids and cytokines involved in the inflammatory response, and to observe how these drugs modified the inflammogen effects on:

(a) the hepatic MMFO metabolism of pentobarbital, *in vivo*;

(b) the hepatotoxic responses to paracetamol and bromobenzene, as determined by serum alanine aminotransaminase activity.

(c) the serum ceruloplasmin levels;

(d) the proliferative status of certain immunological cell types - splenocytes, thymocytes, peritoneal macrophages and peripheral blood lymphocytes.

The hepatic experiments were performed in LACA Swiss mice and Hooded Wistar rats. The immunological experiments were performed in Swiss and C3H/HeJ mice - the latter strain

is classed as a lipopolysaccharide non-responder strain (or slow-responders). The anti-inflammatory and anti-arthritic drugs which were used in this chapter were: sodium salicylate; piroxicam, an oxicam NSAID; naproxen, an aryl propionic acid NSAID; 6-mercaptopurine and carboplatin, both anti-arthritic/ lymphosuppressive agents; and clozic, an agent which suppresses the arthritic, but not the inflammatory, response in rodents [Billingham and Rushton, 1985].

5.2 METHODS

5.2.1 Administration of anti-inflammatory drugs.

The anti-inflammatory drugs were administered intraperitoneally two hours prior to inflammogen tail-base injection, with the same dosage maintained on a daily schedule, including the day of PST and hepatotoxicity assessments. Anti-inflammatory drugs used were: sodium salicylate (200 mg/kg), piroxicam (5 mg/kg) and carboplatin (60 mg/kg) in saline; naproxen (20 mg/kg), clozic (50 mg/kg) and 6-mercaptopurine (100 mg/kg) in saline/3 % Tween 80.

5.2.2 *In vivo* assessment of hepatic MMFO.

Swiss mice and Hooded rats were administered intraperitoneal sodium pentobarbital following a tail injection of OA or ADJ, and 2 hours after anti-inflammatory treatment (40 mg/kg, 2 days; and 25 mg/kg, 4 days post arthritogen tail injection, respectively). The

pentobarbital sleeping times were compared with PST in untreated animals.

5.2.3 Administration of hepatotoxic probes.

Swiss mice were challenged with PC and BB intraperitoneally (both 250 mg/kg), two days after the tail injection of inflammogens, and 2 hours after anti-inflammatory administration.

5.2.4 Determination of serum alanine aminotransferase activity.

ALAT activity was determined as described in chapter 4, in sera obtained 18 hours after hepatotoxin challenge.

5.2.5 Determination of serum ceruloplasmin activity.

The procedure of Henry et al [1960] employed the oxidation of p-phenylenediamine at 37^{O}C which was maintained at pH 6.0 with an acetate buffer (0.1 M). They had noted that ceruloplasmin activity was halved with 1 M acetate buffer, and was also inhibited by the presence of chloride, phosphate and sulphate ions, amoung many others. It had been reported that as a general oxidase, ceruloplasmin was capable of oxidising a wide range of compounds, such as benzidine, ascorbic acid and o-dianisidine [King, 1965]. Thus o-dianisidine HCl (ODH) was used as an oxidisable substrate for the determination of ceruloplasmin activity, in 0.1 M acetate at pH 6.0 and 37^{O}C. Serum (40 μl) was

added to ODH (400 μl of 0.8 mM, in 0.1 M acetate pH 6.0) and acetate buffer (800 μl of 0.1 M, pH 6.0) in a microcuvette, and incubated for 40 minutes at 37^{o}C. The absorption of the incubate at 440 nm was measured on the Unicam SP1800 at 10 and 40 minutes, against a blank which contained azide (5 mM final concentration) as well as the serum and ODH (0.25 mM final concentration). The results were expressed as the change in absorption units during 30 minutes (A_{440} at 40 min minus A_{440} at 10 min). Ascorbate can reduce oxidised ODH and the presence of endogenous ascorbate results in a lag phase of no greater than 10 minutes, therefore it is necessary to delay the first measurement by 10 minutes so that it is made during the linear phase of the reaction [Kerfeldt, 1957].

5.2.6 Isolation of immunological cell types.

Mice (n = 3 per dose group) were exanguinated via cardiac puncture after ether anaesthesia, and their peritoneal cavities were washed out with Hank's BSS to obtain peritoneal macrophages. Spleens were removed and homogenised in HBSS with a teflon pestle and glass mortar, and the thymus was removed and squeezed through a fine-wire mesh. Pooled blood samples, and individual spleen and thymus samples were then underlayed with Ficoll and centrifuged (1700 rpm in a benchtop centrifuge for 40 minutes), and the buffy coat at the Ficoll-Paque layer interface was washed with HBSS and RPMI medium.

Splenocytes, thymocytes, peritoneal macrophages and peripheral blood monocytes were suspended at typically 2 x 10^6 cells/ml of RPMI-1640 medium/10 % FCS. All cell types were studied in the C3H/HeJ mice, but only peripheral blood monocytes and splenocytes were studied in the Swiss mice. All assay incubations were performed in triplicate.

5.2.7 Immunological parameters.

5.2.7.1 *Ex vivo* proliferation.

Splenocytes, thymocytes and peripheral blood monocytes (typically 10^5 cells/well of a 96-well microtiter tray) were immediately incubated with [^{3}H]-thymidine (0.5 μCi/well, final volume 200 μl) for 3 hours at 37^{o}C in 5% CO_2, and the radiolabelled thymidine incorporation was used as an index of *ex vivo* cell proliferation.

5.2.7.2 IL-1 production by peritoneal macrophages.

The production of IL-1 by peritoneal macrophages, in the presence and absence of LPS, was determined in the LAF bioassay [Haynes et al, 1988b]. Thymus glands from 10 week-old C3H/HeJ mice were removed aseptically and squeezed through a fine-wire mesh to obtain a thymocyte suspension. Adherent cells were removed by incubation in a Petri dish for 20 minutes at 37^{o}C. Nonadherent cells were collected, washed and adjusted to a concentration of 5 x 10^6 cells/ml in RPMI-1640 medium. Thymocytes (2.5 x 10^5 cells/well of a 96-well microtiter tray) were cultured in the presence of

PHA (1 μg/ml), 2-mercaptoethanol (50 μM) and 50 μl aliquots of media from peritoneal macrophage 24 hour-incubations (with or without LPS), to give a final volume of 150 μl/well. After incubation for 48 hours at 37^{o}C and 5% CO_2, [^{3}H]-thymidine (0.5 μCi in 50 μl) was added and the radiolabel incorporation over 24 hours was measured by scintillation counting (Beckman, LS3801) of a trichloroacetic acid precipitate (TCA, 5% w/v) on glass filters via an automated cell harvester (Titer Teck). One unit/ml of IL-1 was defined as the amount resulting in 50 % of the maximal thymocyte proliferation in the presence of 1 μg/ml PHA. Results were expressed in IL-1 units/10^6 macrophages.

5.2.7.3 IL-2 stimulated cell proliferation.

The proliferation of splenocytes, thymocytes and peripheral blood monocytes (10^5 cells/well) incubated with IL-2 (4 U/well) over 48 hours was determined by the incorporation of a 4 hour [^{3}H]-thymidine pulse.

5.3 RESULTS

5.3.1 The effects of anti-inflammatory drugs on the *in vivo* hepatic pentobarbital metabolism by inflamed animals.

Daily administration of piroxicam (5 mg/kg) reduced the inhibitory effects of OA-induced inflammation on pentobarbital metabolism (Table 5.2, mice; Table 5.3, rats).

Table 5.2: Pentobarbital sleeping times of Swiss mice treated with inflammogens and concomitant anti-inflammatory agents. PST of Swiss mice (n = 3 per dose group) were assessed 2 days after tail injection of inflammogen. Piroxicam (5 mg/kg), Naproxen (20 mg/kg) or Clozic (50 mg/kg) was administered 2 hours prior to tail injection (and prior pentobarbital dosing), followed by a single daily injection. Control PST = 36 ± 7 minutes (pentobarbital 40 mg/kg).

	PST (% CONTROL, ±SE)		
	Untreated	OA	ADJ
Saline	100 ± 20	335 ± 45	246 ± 19
Piroxicam	131 ± 36	190 ± 25 *	184 ± 24
Naproxen	134 ± 31	264 ± 52	211 ± 27
Clozic	144 ± 6 **	337 ± 10	137 ± 0

* Significantly different from OA treatment ($P<0.05$).

** Significantly different from untreated mice ($P<0.05$).

Table 5.3: Pentobarbital sleeping times of hooded rats treated with inflammogens and concomitant piroxicam. PST of Hooded rats (n = 3 per dose group) were assessed 4 days after tail injection of inflammogen. Piroxicam (5 mg/kg) was administered 2 hours prior to tail injection (and prior pentobarbital dosing) followed by a single daily injection. Control PST = 24 ± 1 minute (pentobarbital 25 mg/kg).

	PST (% CONTROL, ±SE)	
	-Piroxicam	+Piroxicam
Untreated	100 ± 4 %	n.d.
OA	299 ± 21 %	189 ± 15 % *
ADJ	256 ± 14 %	247 ± 1 %

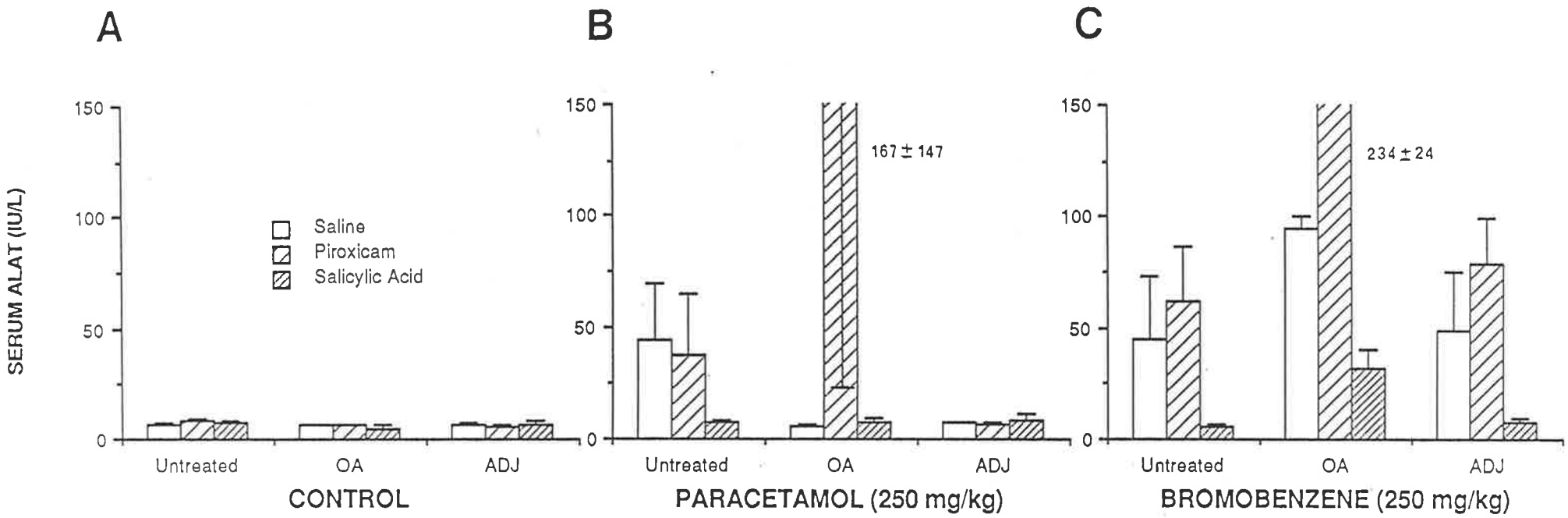
* Significantly different from OA pretreatment ($P<0.05$).

This resulted in a decrease in PST in both inflamed Swiss mice and Hooded rats. However piroxicam had very little effect on the ADJ-induced prolongation of PST. Administration of another NSAID, the propionic acid derivative naproxen (20 mg/kg), was less effective than piroxicam - this may be due to the comparatively lower efficacy of naproxen. The administration of clozic in mice, an anti-arthritic drug which does not show anti-inflammatory activity in pharmacological assays that identify cyclooxygenase inhibitors (e.g. mouse writhing and rat carrageenan paw oedema), reduced the prolonged PST caused by ADJ treatment, but did not alter the OA-dependent effects on PST (Table 5.2).

5.3.2 The effects of anti-inflammatory drugs on the hepatotoxic response of inflamed Swiss mice.

Daily administration of piroxicam (as described previously) potentiated the hepatotoxicity of PC (and BB) in OA-treated mice, but did not alter the ADJ-mediated suppression of PC hepatotoxicity (Fig 5.2). Unlike piroxicam, salicylic acid (200 mg/kg i.p.) inhibited the expression of both BB and PC-induced hepatotoxicity in untreated animals. High doses are required in order to modify inflammation with salicylate. These high doses modify the hepatotoxicity in controls, and therefore salicylate is unsuitable for the evaluation of the mechanisms involved in inflammation.

Figure 5.2: Serum ALAT levels in Swiss mice treated with inflammogens and concomitant sodium salicylate and piroxicam, prior to hepatotoxin challenge. The anti-inflammatory drugs were administered intraperitoneally (n = 4 per dose group) two hours prior to inflammogen tail injection, followed by daily dosage - including the day of hepatotoxicity assessments to PC and BB (2 days post tail injection). Doses of anti-inflammatory drugs were: Salicylate (200 mg/kg) and Piroxicam (5 mg/kg).


A
B
C
SERUM ALAT (IU/L)
150
100
50
0
Saline
Piroxicam
Salicylic Acid
167 ± 147
234 ± 24
Untreated
OA
ADJ
CONTROL
PARACETAMOL (250 mg/kg)
BROMOBENZENE (250 mg/kg)

The effects of anti-inflammatory and anti-arthritic drug administration, on the inflammogen-mediated suppression of PC-induced hepatotoxicity are shown in Figure 5.3. Piroxicam exhibited the same effects observed in Figure 5.2, however neither naproxen nor carboplatin (the less toxic analogue of cisplatin - the cytostatic anti-arthritic/ lymphosuppressive agent) altered the effects of the inflammogens on PC hepatotoxicity. 6-Mercaptopurine inhibited the hepatotoxic response in control mice, which can be attributed to the non-specific inhibition of cellular metabolic processes seen with cytostatic drugs, and had no effect on the lack of PC-response in inflammogen-treated mice.

Although clozic had an inhibitory effect on control mouse PC-induced hepatotoxicity, it reversed the inflammation-mediated inhibition of PC-induced hepatotoxicity (in both OA and ADJ treated mice). This result correlates with the reduction in ADJ-PST in mice coadministered with clozic (see summary table 5.4).

5.3.3 The effects of piroxicam and clozic administration of inflammogen treatment on serum ceruloplasmin activity.

Both OA and ADJ increased serum ceruloplasmin activity two to threefold in both Swiss mice and hooded rats. Piroxicam administration resulted in a marked reduction this effect in Hooded rats treated with OA, but had little effect in mice. Clozic (administered in hooded rats only) was less

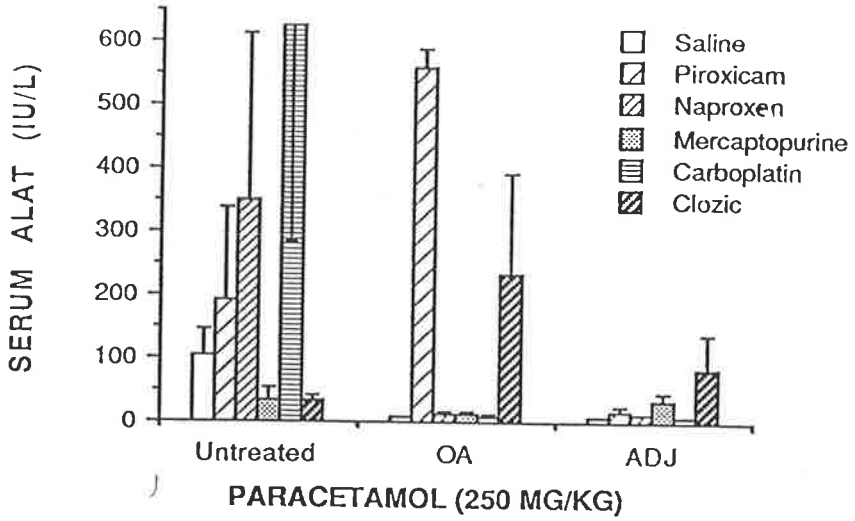


Figure 5.3: Serum ALAT levels in Swiss mice treated with inflammogens and concomitant anti-inflammatory agents, prior to paracetamol challenge. The anti-inflammatory drugs were administered intraperitoneally (n = 4 per dose group) two hours prior to inflammogen tail injection, followed by daily dosage - including the day of hepatotoxicity assessment (2 day post tail injection). Doses of anti-inflammatory drugs used were: Piroxicam (5 mg/kg) and Carboplatin (60 mg/kg) in saline; Naproxen (20 mg/kg), Clozic (50 mg/kg) and 6-Mercaptopurine (100 mg/kg) in saline/3 % Tween 80.

Table 5.4: A summary of the effects of anti-inflammatory and anti-arthritic drugs on inflammation-mediated changes in PST and paracetamol hepatotoxicity, in the Swiss mouse.

Drug	PST*			PC Hepatotoxicity**		
	CON	OA	ADJ	CON	OA	ADJ
Saline	o	↑↑↑	↑↑	o	↓↓↓	↓↓↓
Piroxicam	o	↑	↑↑	o	↑	↓↓↓
Clozic	↑	↑↑↑	o	↓	o	o
Salicylate	-	-	-	↓↓↓	↓↓↓	↓↓↓
Naproxen	o	↑↑↑	↑↑	o	↓↓↓	↓↓↓
Carboplatin	-	-	-	o	↓↓↓	↓↓↓
6-Mercaptopurine	-	-	-	↓	↓↓↓	↓↓↓

* PST changes compared to control PST (36 ± 7 minutes): o = no change; ↑ = 0 to 100% increase; ↑↑ = 100 to 200% increase; ↑↑↑ = 200 to 300% increase in PST (- = not determined).

** PC hepatotoxicity changes, as compared to the serum ALAT activity in control mice given only PC: o = no change; ↑ = an increase; ↓ = small decrease; ↓↓↓ = total abolition of PC-induced hepatotoxicity.

efficacious in ameliorating the raised serum ceruloplasmin activity, in both OA and ADJ treated rats (Table 5.5).

5.3.4 The effects of piroxicam on the *ex vivo* proliferation of lymphocytes from inflamed mice.

Similar trends were observed between the *ex vivo* proliferation of peripheral blood and splenic lymphocytes, obtained from OA treated and untreated Swiss mice (Figure 5.4: a & b). Piroxicam administration (as previously described) did not alter control levels of lymphocyte proliferation. The tail injection of OA, 2 days prior to the immunological assessment, caused a minor increase in cell division, however the concomitant dosage of piroxicam caused a marked increase in cell proliferation (a two-fold and ten-fold increase in the rate of cellular proliferation in blood and splenic lymphocytes, respectively).

Similar trends were also observed between peripheral blood and splenic lymphocytes obtained from C3H/HeJ mice, but these cells did not respond in the same manner as those obtained from the Swiss mice. Whereas the proliferation of cells obtained from OA-treated mice were similar to control cells, the coadministration of piroxicam resulted in a marked potentiation of thymidine incorporation in cells from both untreated and OA-treated mice (Figure 5.5: a, b). This variation between mouse strains in the control lymphocyte responses to piroxicam treatment may be due to the special immunological C3H/HeJ phenotype - this strain is classed as

Table 5.5: Serum ceruloplasmin levels in (a) Swiss mice, and (b) Hooded rats (both n = 3 per dose group) which had been treated with inflammogens and concomitant piroxicam and clozic. Piroxicam (5 mg/kg, i.p.) or clozic (200 mg/kg) was administered 2 hours prior to the tail injection of inflammogen, and then daily for a further 2 and 4 days in mice and rats, respectively. Control ceruloplasmin activity in mice and rats were 0.031 ± 0.009 and 0.083 ± 0.009 Abs. Units per 30 minute incubation at pH 6.0 and 37°C, respectively (see methods).

Treatment	CERULOPLASMIN ACTIVITY (% CONTROL, ±SE) LACA Swiss Mice	Hooded Wistar Rats
Untreated		
+ Saline	100 ± 3	100 ± 11
+ Piroxicam	81 ± 32	n.d.
OA + Saline	215 ± 12	273 ± 11
+ Piroxicam	248 ± 32	139 ± 10 *
+ Clozic	n.d.	208 ± 33 *
ADJ + Saline	267 ± 35	186 ± 33
+ Piroxicam	238 ± 14	217 ± 10
+ Clozic	n.d.	140 ± 10 **

* Significantly different from OA treatment ($P<0.05$).

** Significantly different from ADJ treatment ($P<0.1$).

Figure 5.4: ***Ex vivo*** **proliferation of (a) peripheral blood lymphocytes and (b) spleen lymphocytes from Swiss mice treated with either inflammogen, or OA with concomitant piroxicam.** Piroxicam (5 mg/kg, i.p.) was administered 2 hours prior to the tail injection of inflammogen, followed by daily dosage - including the day of immunological assessment (2 days post tail injection). The proliferation of blood lymphocytes (2 x 10^4 cells/well) and splenocytes (5 x 10^5 cells/well) was assessed by the [^{3}H]-thymidine uptake over 3 hours.

Swiss mice: ex vivo proliferation.

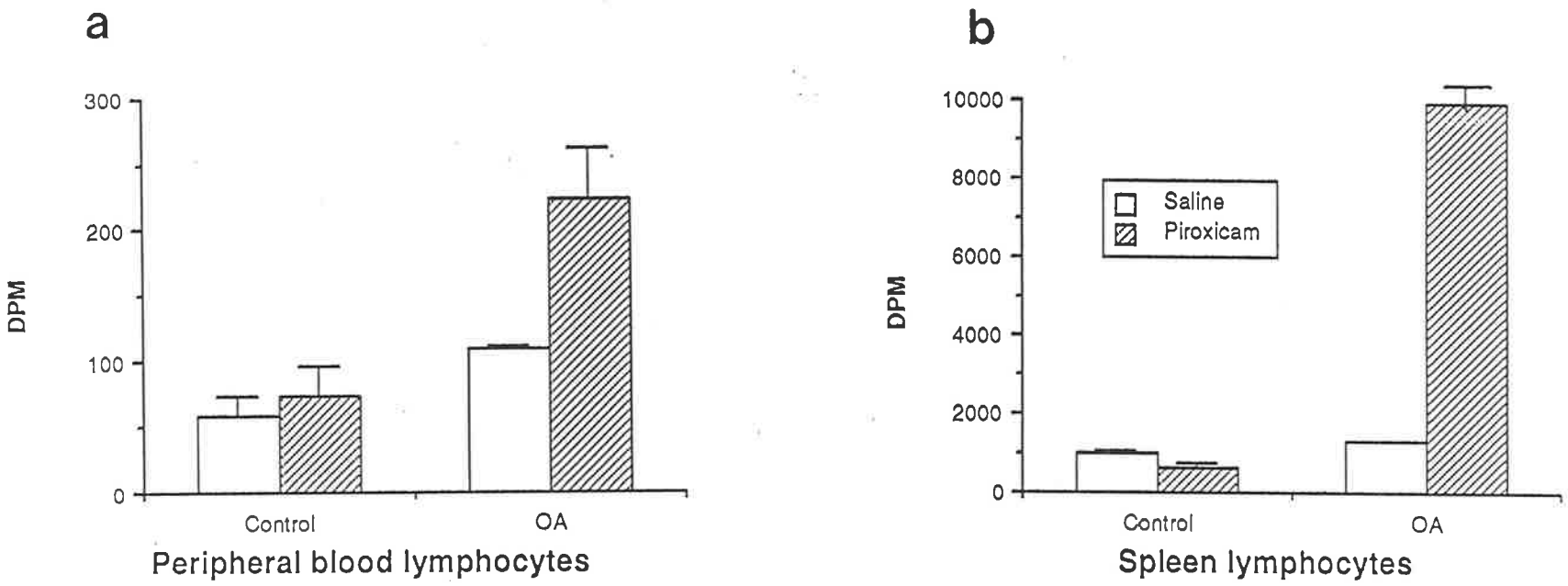
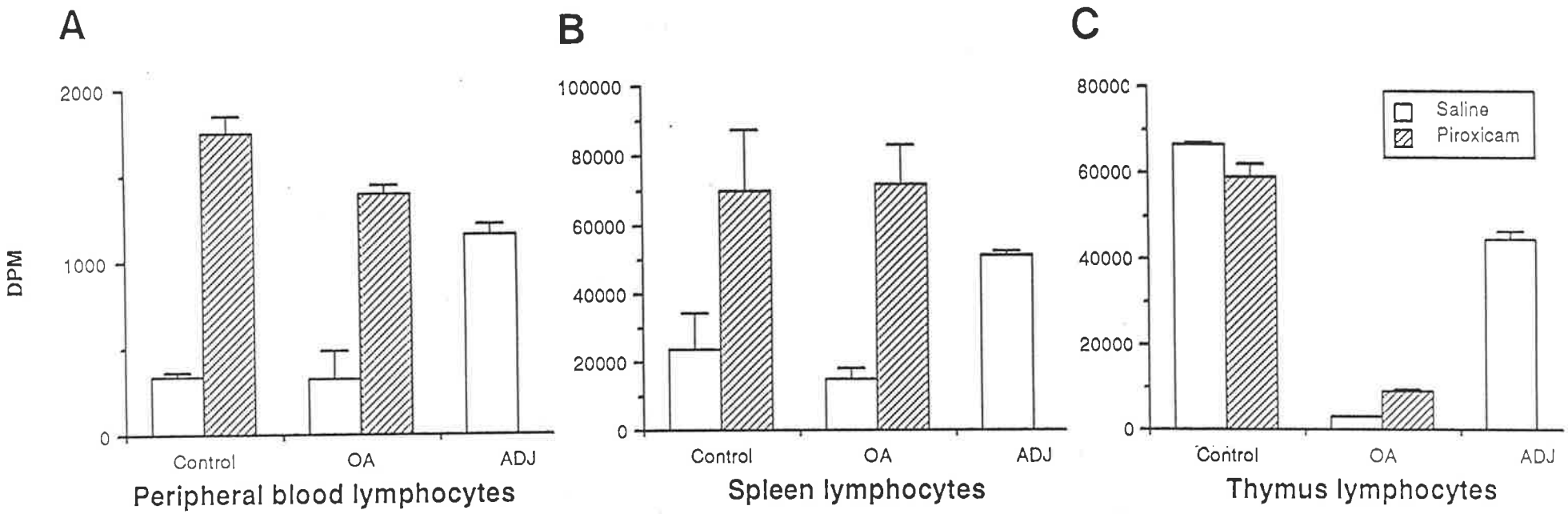


Figure 5.5: *Ex vivo* proliferation of (a) peripheral blood lymphocytes, (b) spleen lymphocytes and (c) thymus lymphocytes from mice (C3H/HeJ) treated with either inflammogen, or OA with concomitant piroxicam. Dosage as in figure 5.4. The proliferation of lymphocytes (10^5 cells/well) were assessed by the [^{3}H]-thymidine uptake over 3 hours.

C3H/HEJ mice: ex vivo proliferation.



a "poor-LPS responder", i.e. has a relatively delayed IL-1 release in response to LPS administration. Thus control C3H/HeJ mice maintain a high PGE_2 tone in comparison to Swiss mice, and the inhibition of prostaglandin synthesis by piroxicam treatment results in the full expression of circulating IL-1 (which would also exist at high resting blood levels, as part of the immunological feedback inhibition).

The thymocytes exhibited a different profile to the other lymphocytes (Figure 5.5 c). OA-treatment markedly inhibited thymidine incorporation by thymus-derived lymphocytes, and this effect was only partially reduced by piroxicam treatment. This correlated with the atrophic gross morphology seen during removal of thymus glands from OA-treated mice and can be attributed to the effects of glucocorticoids (released from the adrenal cortex in response to inflammation-induced increases in corticotrophin secretion by the anterior pituitary) circulating in a "steroid-susceptible" species.

ADJ-treatment resulted in modest increases in peripheral blood and splenic lymphocyte proliferation, suggesting that levels of IL-1 and other stimulating lymphokines are in excess (functionally) to LAF-inhibitory factors. ADJ caused only a slight reduction in thymocyte proliferation which suggests that, unlike OA, adjuvant does

not promote the release of large quantities of glucocorticoids from the adrenals.

5.3.5 Effects of inflammogens and piroxicam on IL-1 production by peritoneal macrophages.

The IL-1 production by peritoneal macrophages resulted in near similar profiles, whether in the presence (stimulated IL-1 release) or absence (basal IL-1 release) of LPS - i.e. control and OA-mediated IL-1 production were equal, but treatment with piroxicam potentiated a 10-fold increase in IL-1 production. Exceptions were (a) ADJ was potentiated 40-fold by LPS, while the other treatments were increased by a factor of between 13 and 26; (b) control IL-1 production was not detectable (<1 IL-1 unit/10^6 cells) unless LPS was present (figure 5.6 a & b).

5.3.6 Effects of inflammogens and piroxicam on IL-2 stimulated lymphocyte proliferation.

The measurement of IL-2 stimulated cell proliferation enables differentiation between *ex vivo* proliferative effects which are due to decreased IL-2 levels or to "down-regulation" of IL-2 receptors (as the expression of IL-2 and transferrin receptors, among others, are essential for the IL-1 mediated proliferative response) [Figure 5.7 a, b & c].

Splenocyte proliferation in response to a 48 hour IL-2 incubation was essentially unchanged by the treatments - the OA treated group showed a slightly increased response to the

Figure 5.6: IL-1 production by peritoneal macrophages from inflammogen treated mice (C3H/HeJ) in (a) the absence, and (b) the presence of LPS. Dosage as in figure 5.4. IL-1 production was assessed by the LAF bioassay (see method). One unit/ml of IL-1 was defined as the amount resulting in 50 % of the maximal test-thymocyte proliferation in the presence of 1 μg/ml PHA. Results are expressed in IL-1 units/10^6 macrophages.

C3H/HeJ mice: Peritoneal macrophages

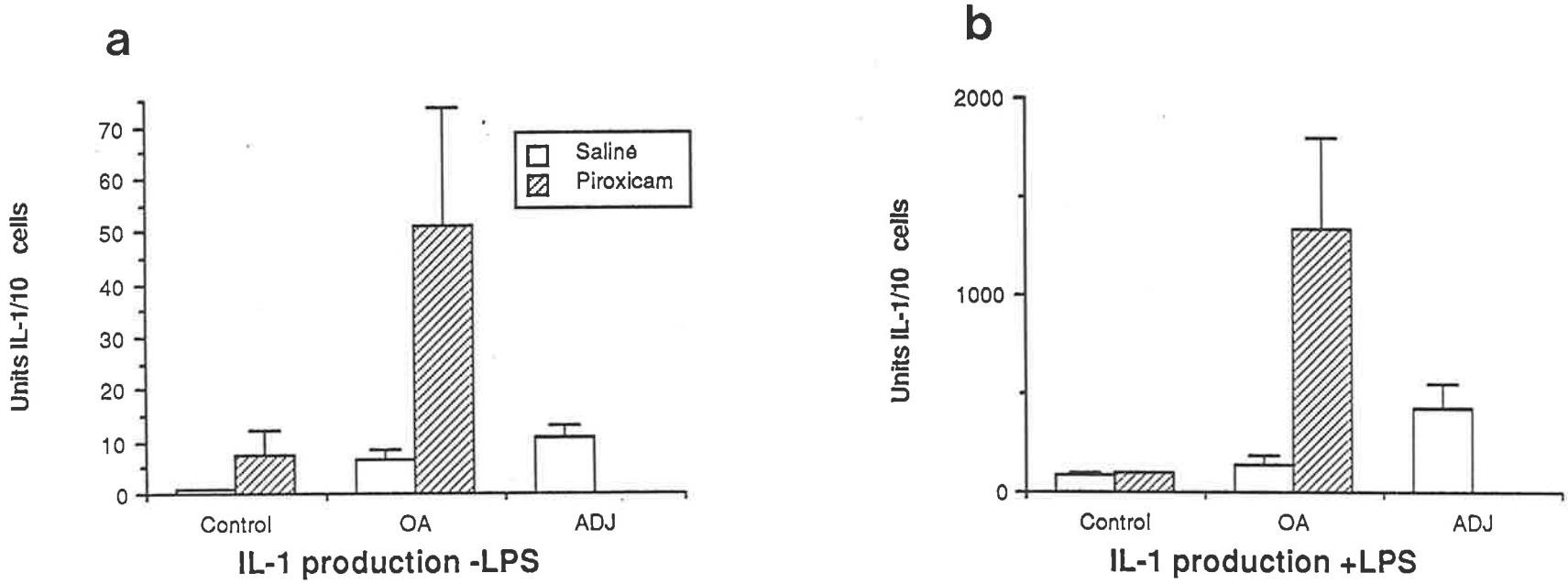
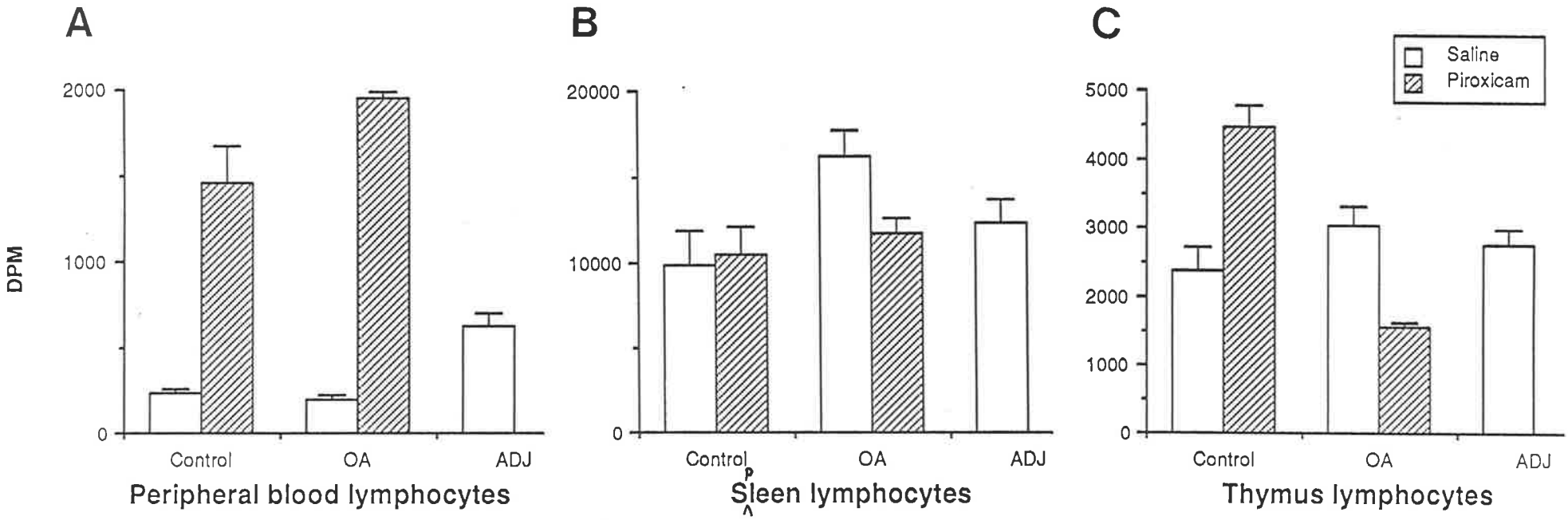


Figure 5.7: IL-2 stimulated proliferation over 48 hours in (a) peripheral blood lymphocytes, (b) spleen lymphocytes and (c) thymus lymphocytes from mice (C3H/HeJ). Dosage as in figure 5.4. The proliferation of cells (10^5 cells/well) incubated with IL-2 (Boehringer, 4 U/well) over 48 hours was determined by the incorporation of a 4 hour [^{3}H]-thymidine pulse.

C3H/HeJ mice: 48 hr IL-2 incubation.



the fixed dose of IL-2. Peripheral blood lymphocytes proliferation did not reflect the splenocyte profile, and this indicates that treatments did alter IL-2 receptor expression. Although piroxicam increased IL-2 receptor density in the membranes of OA-treated and untreated cells, it has been recognised that subpopulations of lymphocytes obtained from various sites may respond differentially to drug treatments. The reverse trend seen in thymocytes obtained from OA/piroxicam-treated mice may involve the interaction of glucocorticoids with thymus function.

5.4 DISCUSSION

This chapter investigated the effects of anti-inflammatory and anti-arthritic agents on the inflammation-mediated changes in hepatic function and toxic responses. Examination of the immunological status of identically dosed mice, assisted in elucidating possible mechanisms responsible for the modifying effects of inflammation on the liver, *in vivo*.

Piroxicam, an oxicam which can inhibit cyclooxygenase (and therefore PGE_2 synthesis), repeatedly and specifically attenuated OA-mediated effects on the liver. Daily dosage of piroxicam reduced the OA-mediated PST prolongation that resulted from the decreased cytochrome P450 activity, which is a characteristic of inflammogen treatment. This is confirmed by other studies [Whitehouse, 1986], where PST was

prolonged in male and female hooded rats treated with piroxicam for 4 days following a tail injection of OA.

Piroxicam also restored PC-induced hepatotoxicity to OA-inflamed mice. Clozic was the only other agent to affect the PST and hepatic response to PC administration without also affecting PC metabolic activation (as with salicylic acid and 6-mercaptopurine). Piroxicam and clozic did not appear to alter the inflamed and stiff appearance of tails in inflammogen-treated animals, however partial restoration of PST values were accompanied with near-untreated body weight gains.

Confirmation of the ability of piroxicam to affect the immunological status of inflamed mice, was obtained from the lymphocyte proliferation studies. Lymphocytes from OA-inflamed mice behaved like control cells, unless animals were concomitantly receiving piroxicam. This implies that OA-treatment promotes the synthesis of both PGE_2 and IL-1 in functionally equal amounts - as IL-1 is a lymphocyte activating factor and PGE_2 has an opposite inhibitory action on lymphocyte activation.

It was recently reported by Knusden et al [1986] that PGE_2 could inhibit monocyte expression of IL-1 post-transcriptionally, and that IL-1 stimulates the synthesis of PG's as part of an autoregulatory pathway. The results reported here (figure 5.5) infer that the resting levels of PGE_2 in C3H/HeJ mice are high compared to Swiss mice, since

piroxicam treatment of uninflamed mice resulted in marked lymphoproliferation.

The lymphocyte proliferation profiles for ADJ treated C3H/HeJ mice suggest that this form of inflammation promotes the release of mostly IL-1 with very little PGE_2, due to the elevated rate of thymidine incorporation in mice which had only been treated with adjuvant (and by the LAF bioassay). As C3H/HeJ mice have a high basal level of PGE_2 one may presume that, had there been sufficient time remaining in the project, the determination of *ex vivo* proliferation of lymphocytes obtained from ADJ-inflamed Swiss mice would reveal even greater rates of IL-1 production.

One of the more interesting observations concerns clozic's ability to affect both models of inflammation employed in this thesis. While clozic has not shown anti-inflammatory activity from the inhibition of cyclooxygenase, it nethertheless possesses anti-arthritic activity, but via an unknown mechanism which probably involves attenuation of the release of some IL-1-like cytokines (other than IL-1) [Billingham and Rushton, 1985].

A summary of the conclusions drawn from the studies described in this chapter are represented by the scheme in figure 5.8. The evidence gained from these studies suggest:

(a) that inflammation induced by OA is accompanied by large increases in PGE_2 and IL-1 levels, and that another

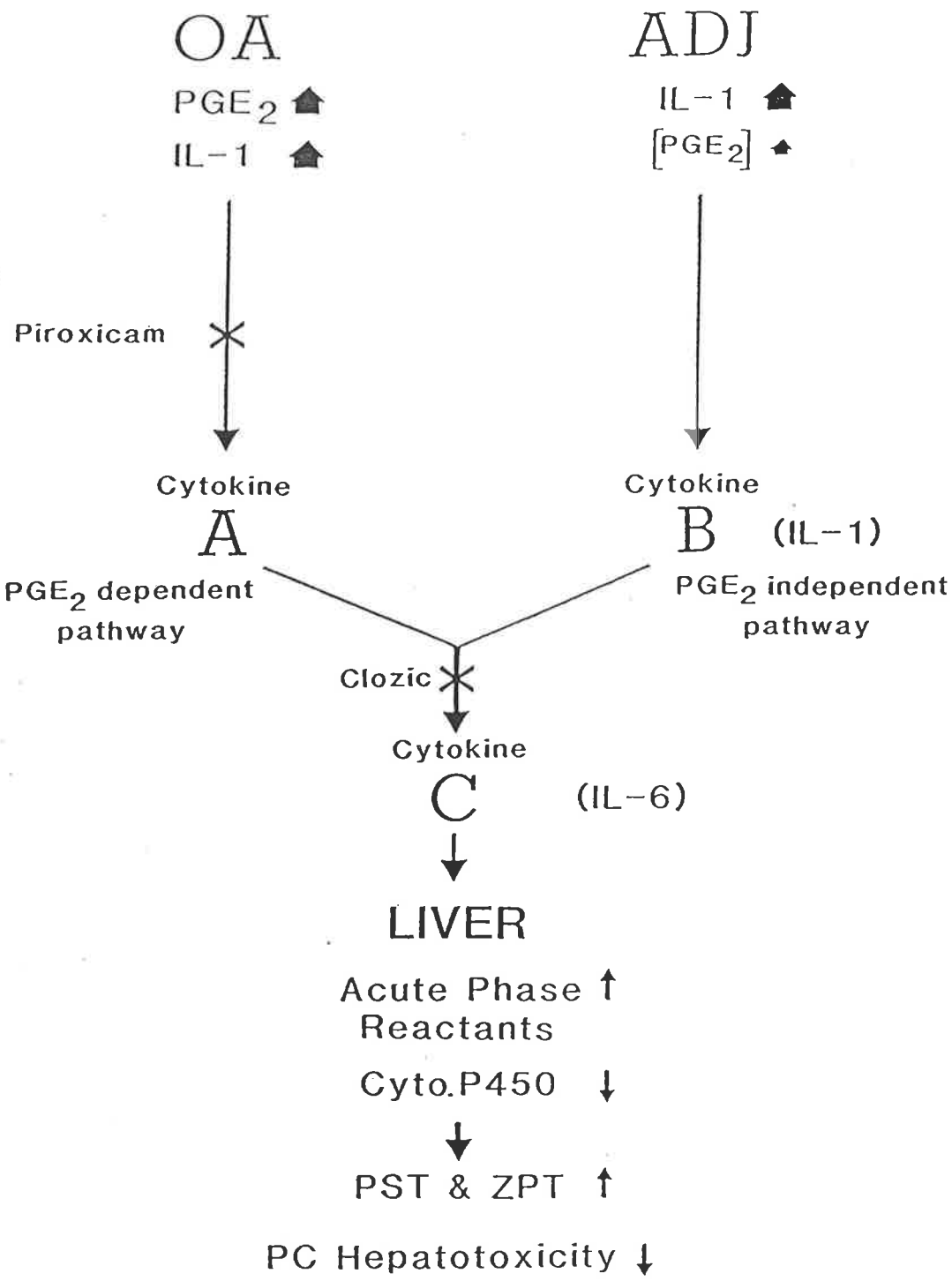


Figure 5.8: Diagram showing the immunological mediators involved in the two models of inflammation, OA and ADJ - as implied by the experimental results described in this chapter.

cytokine promotes the eventual release of IL-6 - which stimulates the liver to produce Acute Phase Reactants, turn down albumin synthesis and certain MMFO systems, and initial rise in hepatic GSH levels (followed by a longer term depletion). The release of this unknown intermediary cytokine is piroxicam-sensitive and therefore presumably PGE_2- dependent.

(b) that inflammation induced by ADJ is accompanied by large increases in predominantly IL-1 and comparatively little PGE_2, which promotes the release of IL-6 - the cytokine responsible for the same hepatic changes which arise from OA-induced inflammation. This pathway is piroxicam insensitive, and therefore primarily PGE_2-independent.

(c) that clozic interacts with cytokines as yet unknown, but common to both, pathways.

(d) that piroxicam exhibits immunostimulatory action by inducing lymphoproliferation in OA-treated mice.

Chapter 6

GENERAL CONCLUSIONS

Assessment of *in vitro* and *in vivo* oxidant stress by measuring the formation of short-lived oxygen radicals, has presented quite a problem to researchers in recent years. This point is accentuated in oxyradical assays which rely on the conversion of probe chemicals to products that are themselves unstable in the radical flux, and undergo further reactions. Despite this shortcoming, it was possible to semi-quantitatively determine the $OH^{\cdot}$ formation in an *in vitro* oxidant stress system, such as the activated human neutrophil, using the HPLC-ECD assay developed in the initial stages of this project.

The assay was extremely sensitive and compared most favourably with subsequent assay procedures which appeared in the literature [Floyd et al, 1984], e.g. measurement of changes in unstimulated-PMN salicylate hydroxylation was possible using this method, but was undetectable by other researchers [Alexander et al, 1986; Sagone and Husney, 1987].

An important characteristic of the studies described in chapter 3, is the use of more than one method of neutrophil stimulation, which highlighted the differences in the azide inhibition of DHB formation between STZ- and PMA-activated PMN. A summation of the effects from the addition of various agents - which included SOD, catalase, DPI, DFO and azide (mechanisms of action are defined in chapter 3); upon

DHB formation by activated PMN, leads one to the conclusion that a highly-reactive hydroxylating species (probably the OH·) is produced by activated neutrophils via a superoxide- and MPO-dependent mechanism. Further research is necessary to conclusively identify the reactive species as the OH·, and this may involve examination of the binding and dissociation characteristics of substrates with the MPO active site.

The neutrophil study suggests a mechanism by which two phagocyte functions (respiratory burst and degranulation), may interact to produce a toxic species in addition to HOCl.

The effects of *in vivo* oxidant stress on hepatic function was examined in both acute (OA) and chronic (ADJ) inflammatory models. Inflammation-mediated effects on hepatic metabolism, thiol status and paracetamol-induced hepatotoxicity were marked and prolonged.

Although hepatic MMFO activity was depressed at the height of inflammation in response to both inflammogens, the effects on hepatotoxic responses to two oxidatively activated hepatotoxins were not uniform. Paracetamol hepatotoxicity was ameliorated by both OA- and ADJ-induced inflammation, however bromobenzene hepatotoxicity was not attenuated. This suggests that the hepatotoxic responses to all cytochrome P-450 activated compounds would not uniformly be affected by the presence of systemic oxidant stress.

Metabolic activation of xenobiotics is only one aspect which determines the extent of hepatotoxicity. Inactivation of reactive metabolic intermediates by glutathione and other cellular antioxidants is another important aspect in the expression of hepatotoxicity. Glutathione and thiol-rich proteins (as well as ascorbate, α-tocopherol and other antioxidants) play an important role in the quenching of reactive oxygen species, and other radicals, that may be formed during the redox cycling of some metabolic intermediates. These oxyradicals may mediate the hepatotoxicity caused by the activation of certain chemicals, e.g. paracetamol [Moore et al, 1985; Cross CE, 1987]. Alterations in the balance between metabolic activation and inactivation result in modified responses to hepatotoxins. The increased synthesis of acute phase reactants during systemic inflammation results in elevated metallothionein (a thiol-rich protein) levels, which would offset any minor decline in hepatic glutathione levels. This may well have a protective effect on the liver may explain the effect of inflammation on PC-induced hepatotoxicity.

The following conclusions may be drawn from the evidence gained in the studies which concerned the modification by anti-inflammatory and anti-arthritic drugs of systemic oxidant stress and its effects on hepatotoxic responses:

(a) That inflammation induced by OA is accompanied by large increases in PGE_2 and IL-1 levels, hepatic production of acute phase reactants, reduced albumin synthesis and decreased MMFO activity and initial increases in hepatic GSH levels (followed by a longer term depletion). The effects promoted by OA-mediated inflammation are piroxicam-sensitive and therefore PGE_2-dependent. This implies that in OA-mediated inflammation, PGE_2 or a PGE_2-dependent cytokine stimulates the release of IL-6 which has been shown to mediate the major acute phase protein response in hepatocytes [Gauldie et al, 1987].

(b) That inflammation induced by ADJ is accompanied by large increases in predominantly IL-1 and comparatively little PGE_2, and the same hepatic changes that occur in OA-induced inflammation. The ADJ-mediated process is piroxicam-insensitive, and therefore primarily PGE_2-independent. This implies that in ADJ-induced inflammation high IL-1 levels cause changes in hepatic function, which correlates with reports that IL-1 can induce metallothionein synthesis [DiSilvestro and Cousins, 1984] and depress cytochrome P-450 function in hepatocytes [Ghezzi et al, 1987]. This group also found that endotoxin and TNF effects on the liver were mediated via IL-1.

This pathway is PGE_2-insensitive, and ultimately stimulates the release of IL-6 from endothelial cells,

fibroblasts, macrophages and lymphocytes in response to the circulating levels of IL-1.

(c) That the anti-arthritic agent, clozic, can alter the effects of inflammation on the liver - irrespective of whether OA or ADJ is administered. This implies that clozic interacts with cytokines as yet unknown, but common to, both OA- and ADJ-induced inflammatory processes. Although IL-6 is a cytokine common to both pathways, there is no present evidence that clozic can directly affecting IL-6 production as a mechanism of ameliorating the effects of inflammation on hepatic function.

(d) That piroxicam exhibited immunostimulatory action by inducing lymphoproliferation in OA-treated mice. This is an important observation as most research into the mechanism of anti-rheumatic drug action has been directed at their ability to suppress inflammatory and immunological mechanisms.

Little attention has been directed at a drug's ability to stimulate immune responses, even though an enhanced immune response could possibly resolve chronic inflammation by improving the elimination or neutralization of the arthritogen involved. Haynes and coworkers [1988b] have shown that an anti-arthritic drug, gold sodium thiomalate, can stimulate immune receptor expression on macrophages both *in vivo* and *in vitro*, at concentrations found in the serum of patients undergoing anti-arthritic drug therapy.

The salicylate effect of potentiating FMLP-stimulated PMN O_2^- production (chapter 3) correlates with the piroxicam effect on lymphoproliferation in OA-inflamed mice, and suggests that non-steroidal anti-inflammatory drugs, which are known to inhibit prostaglandin synthesis, can augment the lymphoproliferative action of IL-1. Thus one may conclude that some anti-rheumatic drugs act as immunostimulants, and that this may contribute to their beneficial effects in reducing chronic inflammation.

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APPENDIX

SOURCES OF DRUGS AND CHEMICALS

BDH, Ltd., Poole, England:
Al_2O_3
Ascorbic acid
Bromobenzene
Cadmium chloride
Diethyl ether
Dimethylsulphoxide
EDTA
EGTA
Ferric chloride, 60% solution
Ferrous sulphate
Hydrochloric acid
Hydrogen peroxide
K_2HPO_4 and KH_2PO_4
Paracetamol
Phosphoric acid
Toluene
Trichloroacetic acid
Triton X100
Zinc acetate
Zinc sulphate

CALBIOCHEM-BEHRING Biochemicals, HOECHST Amer. Corp.:
A23187
Ferricytochrome c
Superoxide dismutase

CIBA-GEIGY:
Desferrioximine

FARMACIA FINE CHEMICALS:
Ficoll-Hypaque

FLOW LABS:
Phytohaemagglutinin A
RPMI-1640 culture medium

MALLINCKRODT, Victoria, Australia:
Methanol, HPLC grade

MANNHEIM-BOEHRINGER:
Interleukin-1
Interleukin-2

NEW ENGLAND NUCLEAR, DUPONT:
[7-^{14}C]-Benzoic acid
[^{3}H]-Thymidine

SIGMA CHEMICAL COMPANY, St. Louis, MO, USA.:
dl-Alanine
Bathophenanthroline sulphonate

C18, 30 micron precolumn packing material
Catalase
p-Coumaric acid
Cytochalasin B
DETAPAC
O-Dianisidine
Diazald
2,3-; 2,5-; and 3,5-Dihydroxybenzoic acids
Dimethylurea
2,4-Dinitrophenylhydrazine
Diphenyloxazole
7-Ethoxycoumarin
N-formyl-methionyl-leucyl-phenylalanine
Glucose-6-phoshate
Glucose-6-phosphate dehydrogenase
Glutathione
Homogentisic acid
Hypoxanthine
α-Ketoglutaric acid
Lipopolysaccharide from E. Coli B
Luminol
Mannitol
NAD and NADP
Phenylenediamine
Phorbol 12-myristate 13-acetate
Pyruvic acid
Sodium azide
Sodium salicylate
Sulphosalicylic acid
Xanthine oxidase
Zoxazolamine
Zymosan

Thanks to Dr. Michael W. Whitehouse for supplying:
Carboplatin
Clozic (ICI Pharmaceuticals)
Freund's adjuvant (Sigma Chem Co)
6-Mercaptopurine (Sigma Chem Co)
Naproxen (Syntex USA)
Piroxicam (Pfizer)