FACTORS AFFECTING THE REGULATION OF LEUKOTRIENE PRODUCTION BY NEUTROPHILS

A thesis submitted to the University
of Adelaide as the requirement for the degree of
Doctor of Philosophy

by

SHAUN REUSS McCOLL, B.Sc. (HONS.)

Department of Clinical and
Experimental Pharmacology
University of Adelaide

and

The Queen Elizabeth Hospital
Rheumatology Unit

April, 1987

Awarded : September 14, 1987

This thesis is dedicated to Adriana.

DECLARATION

This thesis contains no material that has 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 written by any other person except where due reference is made in the text.

S.R. McColl

15/4/87

ACKNOWLEDGEMENTS.

This work in this thesis was carried out during the tenure of a Mary Paxton-Gibson Postgraduate Research Scholarship administered by the Arthritis Foundation of Australia.

I would like to thank Dr. L.G. Cleland for his supervision and guidance throughout the course of this work and for providing me with the oppurtunity to undertake a Ph.D. Thanks are also due to Professor F. Bochner, Dr. N.P. Hurst, Dr. J.K. French and Ms. G. Murphy for helpful advice, discussion and encouragement.

The figures and illustrations in this thesis were expertly photographed by the staff in the Clinical Photography Department of The Queen Elizabeth Hospital.

My thanks also go to my parents, my sister Kim, and my brother Damien for their love and support.

Finally, I am most grateful to Dr. W.H. Betts who acted as my unofficial supervisor during the course of this work and without whose expertise, friendship and encouragement this thesis would not have been possible.

LIST OF ABBREVIATIONS.

AA	Arachidonic acid
EPA	Eicosapentaenoic acid
LT	Leukotriene
PG	Prostaglandin
TX	Thromboxane
LO	Lipoxygenase
CO	Cyclooxygenase
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HEPE	Hydroxyeicosapentaenoic acid
PKC	Protein kinase C
PMA	Phorbol 12, 13 myristate acetate
STZ	Serum treated zymosan
IgG	Immunoglobulin G
FMLP	Formyl-methionyl-leucyl-phenylalanine
HPLC	High pressure liquid chromatography
IP_3	Inositol 1,4,5 trisphosphate
DAG	Diacyl glycerol
PUFA	Polyunsaturated fatty acid
PLA_2	Phospholipase A_2
PI	Phosphatidyl inositol
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PS	Phosphatidyl serine

PUBLICATIONS IN SUPPORT OF THESIS.

1. **S.R. McColl, W.H. Betts, G.A. Murphy, and L.G. Cleland.** Determination of 5-lipoxygenase activity in human polymorphonuclear leukocytes using high-performance liquid chromatography. J. Chromatogr. 378:444-449, 1986.

2. **S.R. McColl, N.P. Hurst, and L.G. Cleland.** Modulation by phorbol myristate acetate of arachidonic acid release and leukotriene synthesis by human polymorphonuclear leukocytes stimulated with A23187. Biochem. Biophys. Res. Commun. 141:399-404, 1986.

3. **S.R. McColl, L.G. Cleland, M.W. Whitehouse, and B. Vernon-Roberts.** Effect of dietary polyunsaturated fatty acid (PUFA) supplementation on adjuvant induced polyarthritis in rats. J. Rheumatol., in press.

4. **S.R. McColl, and C.B. Daniels.** A comparison between the inflammatory mediators produced by the blue-tongue lizard (Tiliqua scincoides) and human white blood cells. Aust. J. Zool., in press.

5. **J.K. French, S.R. McColl, and L.G. Cleland.** Fish oils and coronary disease I. N.Z. Med. J. 99:962-963, 1986.

6. **J.K. French, S.R. McColl, and W.H. Betts.** Fish oils and coronary disease II. N.Z. Med. J., in press.

7. **S.R. McColl, N.P. Hurst, W.H.Betts, and L.G. Cleland.** Modulation of human neutrophil LTA hydrolase activity by phorbol 12, 13 myristate acetate. Biochem. Biophys. Res. Commun. Submitted for publication.

8. **J.K. French, N.P. Hurst, S.R. McColl, and L.G. Cleland.** Effects of piroxicam on superoxide generation, phospholipid methylation and leukotriene production by human neutrophils. J. Rheumatol., in press.

SUMMARY

A rapid and sensitive high pressure liquid chromatography assay for the major neutrophil 5-lipoxygenase products was developed. The assay was well documented with respect to quality control, precision, accuracy and sensitivity. The assay enabled detection of leukotriene B_4, the all trans isomers of leukotriene B_4 and 5-hydroxyeicosatetraenoic acid. Detection of all four of these compounds provided a means of estimating the relative activities of phospholipase A_2, 5-lipoxygenase, leukotriene A synthetase and leukotriene A hydrolase.

Stimulation of human neutrophils by the calcium ionophore A23187 with or without arachidonic acid caused maximal leukotriene production after five minutes. The chemotactic peptide formyl-methionyl-leucyl -phenylalanine, added with arachidonic acid caused maximal production after two minutes but formyl-methionyl-leucyl-phenylalanine alone caused no detectable production of leukotrienes. Inactivation was seen when the 5-lipoxygenase system was stimulated by high doses of A23187 alone, A23187 with arachidonic acid, or formyl-methionyl-leucyl-phenylalanine with arachidonic acid. Leukotriene production stimulated by A23187 with or without arachidonic acid from the neutrophils of healthy volunteers was measured on five consecutive days. Intra-experimental results were reproducible but variations in absolute amounts of leukotrienes from day to day were too great to allow quantitative inter-experimental comparisons.

Experiments using the Ca^{++} chelators EDTA, EGTA and TMB-8 revealed that the 5-lipoxygenase pathway requires either extracellular or intracellular Ca^{++} for activation by A23187 whereas phospholipase A_2 requires both. The fatty acid eicosapentaenoic acid inhibited production of arachidonic acid-derived metabolites by human neutrophils. Production of leukotriene B_4 was inhibited to a greater extent than either the all-trans isomers or 5-hydroxyeicosatetraenoic acid, indicating a possible site of inhibition was leukotriene A hydrolase.

The influence of dietary eicosapentaenoic acid on three separate animal models of inflammation was investigated. Dietary eicosapentaenoic acid did not influence fluid accumulation in carrageenan-soaked sponges implanted in the dorsal flanks of rats, but did inhibit carrageenan-induced paw swelling. The effect of dietary eicosapentaenoic acid on adjuvant-induced arthritis in rats was dependent on the rat strain studied.

The tumour promoter, phorbol 12,13 myristate acetate augmented both release of ^{3}H-arachidonic acid and synthesis of leukotriene B_4, the all-trans isomers of leukotriene B_4 and 5-hydroxyeicosatetraenoic acid by human neutrophils stimulated by A23187. These data are consistent with the hypothesis that activation of protein kinase C inhibits lipomodulin and thereby enhances arachidonic acid release and metabolism in stimulated neutrophils. Phorbol 12,13 myristate acetate exhibited different effects on formyl-methionyl-leucyl-phenylalanine

and arachidonic acid stimulated leukotriene production. Production of leukotriene B_4 was enhanced while production of the all-trans isomers of leukotriene B_4 was reduced. No effect on production of 5-hydroxyeicosatetraenoic acid was observed. These observations imply that protein kinase C regulates the activity of leukotriene A hydrolase.

TABLE OF CONTENTS.

CHAPTER 1: LITERATURE REVIEW.

1.1 INTRODUCTION.

Developments in the field of eicosanoid research can be divided into two major sections. The first section, which took place from the 1930s to 1970 involved the discovery of the major prostaglandins (PGs) and the slow reacting substance of anaphylaxis (SRS-A). The second section occurred along with the development of technology such as gas chromatography-mass spectrometry in the mid-70s, and led to the discovery of the labile PGs, the structure of SRS-A and the lipoxygenase (LO) arm of the arachidonic acid (AA) cascade. During this latter period, there was an exponential growth in eicosanoid research as new compounds were discovered and their pharmacological actions investigated.

In this review, I will describe the progress made in the area of eicosanoid research since the initial discovery of PGs and SRS-A and present the evidence implicating eicosanoids in the process of inflammation. My major emphasis will be placed on the 5-lipoxygenase (5-LO) products of human peripheral neutrophils which are the subject of this thesis.

1.2 PROSTAGLANDINS AND LEUKOTRIENES.

1.2.1 Initial Research - Pre 1970.

PGs were first discovered in the 1930s (Kurzok and Lieb, 1930; von Euler, 1934; 1935) and by 1963, Bergstrom and coworkers had determined the chemical formulae of six of the primary PGs (Bergstrom et al., 1962a; 1962b). PGE_2 was later shown to be synthesised from AA in sheep seminal vesicles (Bergstrom et al., 1964; van Dorp et al., 1964). These discoveries generated considerable interest in cyclooxygenase (CO), the enzyme which catalyses the metabolism of AA to PGs. This topic has recently been reviewed (Lands and Hanel, 1983).

SRS-A is a product of immediate hypersensitivity reactions in the perfused guinea pig lung. Initially described as "contracting smooth muscle tissue more slowly than did histamine" (Feldberg and Kellaway, 1938), major problems were encountered in separating the biological action of histamine from that of SRS-A. When this was achieved it was possible to address the isolation of SRS-A (Brocklehurst, 1953). Over the next decade it was found that SRS-A was a polar lipid/s (Brocklehurst, 1962; Strandburg and Uvnas, 1971), and that it was not a PG (Strandburg and Uvnas, 1971). By the late 1960s, it was possible to generate large amounts of SRS-A using an immune reaction in the peritoneal cavity of rabbits (Orange et al., 1967; 1968; 1969). Chromatographic purification of SRS-A led to its identification as lipid/s with molecular weights of approximately 500 (Orange et al., 1973). In the mid-1970s it became possible to generate sufficient

amounts to enable detailed structural identification of the individual components (Lewis et al., 1975). GC-MS analysis showed that SRSA consisted of a mixture of several leukotrienes (LTs) (Orning et al., 1980).

1.2.2 Post 1970.

The major breakthrough in the field of PG research occurred with the discovery that aspirin and other non-steroidal anti-inflammatory drugs inhibit PG synthetase thereby preventing PG synthesis (Vane, 1971). This discovery not only provided an opportunity to examine the role of PGs in pathophysiological conditions but also led to the discovery of the labile PGs. Smith and Willis, (1971) subsequently showed that aspirin inhibited platelet aggregation, implicating PGs in this process. Curiously, they found that addition of PGE_2 or $PGF_{2\alpha}$ to platelet suspensions did not cause aggregation, implying the presence of undiscovered compound/s. Isolation by GC-MS of the PG endoperoxides (PGG_2 and PGH_2), and the discovery that these compounds possessed platelet aggregating activity followed (Hamberg and Samuelsson, 1973; Nugteren and Halzelhof, 1973). However, a more potent aggregating compound, thromboxane (TX), was subsequently discovered and shown to be the major platelet metabolite of the PG endoperoxides (Hamberg et al., 1975). A year later, prostacyclin (PGI_2), an anti-aggregatory compound was discovered when metabolism of AA by tissues other than platelets was examined (Moncada et al., 1976).

While conducting the experiments which eventually led to the discovery of TxA_2, Samuelsson and his colleagues found that platelets

produced two other major AA metabolites, hydroxy-heptadecatrienoic acid (also PG synthetase-derived), and a third compound whose production could not be inhibited by indomethacin. This latter compound was identified as 12 hydroxyeicosatetraenoic acid (12-HETE), a chemotaxin for neutrophils (Turner et al., 1975). Extension of these investigations to AA metabolism in neutrophils led to the discovery of the LTs (Borgeat et al., 1976).

The first neutrophil metabolite identified was 5-HETE (Borgeat et al., 1976), a more potent neutrophil chemotaxin than 12-HETE (Goetzl and Sun, 1979). Further study revealed that neutrophils also produced di-hydroxy eicosatetraenoic acids (diHETEs) with a conjugated triene structure (hence the term "leukotriene")(Borgeat and Samuelsson, 1979a). The diHETEs exhibited potent pharmacological activity (discussed in Section 1.2.5), although the major activity was due to only one of these compounds (Feinmark et al., 1981). The stereospecificity of the hydroxyl groups indicated that the two hydroxylations were enzymatic and through a series of ingenious studies using isotopically labelled oxygen, they determined that the alcohol at the C_5 position was due to a lipoxygenation reaction whereas the C_{12} hydroxyl group was derived from water indicating that the dihyroxy acids were formed via hydrolysis of an intermediate, leukotriene A_4 (LTA_4) (Borgeat and Samuelsson, 1979b). The active hydrolysis product was termed LTB_4 (Borgeat and Samuelsson, 1979b). Acid hydrolysis of synthetic LTA_4 showed that two diHETEs possessing three trans double bonds were formed (known as the all-trans isomers of LTB_4). However, when synthetic LTA_4 was added to neutrophil suspensions, LTB_4 was also formed (Radmark et al., 1980a). The

stereospecificity of this reaction indicated enzymatic hyrolysis and the enzyme responsible (LTA hydrolase) has been purified (Radmark et al., 1984a). Total organic synthesis revealed that the precise structure of LTB_4 was 5s 12r dihydroxy 6,14-cis 8,10 trans eicosatetraenoic acid (Corey et al., 1980).

1.2.3 The Arachidonic Acid Cascade.

Free AA, the precursor of PGs and LTs, is derived from cellular phospholipids (discussed in more detail in section 1.2.4). The metabolites formed from free AA depend on the cell type and the enzymatic profile of that cell. For example, platelets produce TxA_2 and therefore possess cyclooxygenase and Tx synthetase (Johnson et al., 1983) (Figure 1.2). In contrast, neutrophils produce LTB_4 and therefore possess 5-LO, LTA synthetase and LTA hydrolase (Johnson et al., 1983).

PGs and LTs are very potent, therefore some form of regulation to control their effects is critical. Some of the more labile PGs spontaneously hydrolyse to less active compounds. For example, PGI_2 to 6-keto$PGF_{1\alpha}$ or TxA_2 to TxB_2 (Oliw et al., 1983). However, other PGs, for example $PGF_2\alpha$, require enzymatic inactivation (Oliw et al., 1983). The organs primarily responsible for the enzymatic degradation of PGs are the lungs (Pace-Asciak, 1977a), liver (Anderson et al., 1976) and kidneys (Pace-Asciak, 1977b). Generally, the initial metabolic step is oxidation of the 15-OH group of a PG into a keto group, a process which decreases biological activity (Clyman et al., 1978). A second reaction, catalysed by a 15-keto reductase enzyme,

reduces the delta-13 double bond resulting in a 15-hydroxy 13,14 dihydro PG which is essentially inactive (Wasserman, 1975). Further metabolism prior to excretion involves one or two steps of β-oxidation (Johnson et al., 1972) or omega (w) 1 or w2-oxidation (Powell and Solomon, 1978). There are, however many other metabolic steps involved in the deactivation of PGs, a subject which is reviewed by Oliw et al., (1983).

In tissues containing LO enzymes, AA is converted to hydroperoxides (see figure 1.3). The specific hydroperoxide formed depends on the type of LO. For example: 5-LO converts AA to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) whereas 12-LO converts AA to 12-HPETE (see Figure 1.3). These hydroperoxy fatty acids are then converted by peroxidase enzymes to more stable alcohols (Needleman et al., 1986).

The 5-LO system is unique among the LO enzyme systems in that a host of biologically active products may be generated (Samuelsson and Hammarstrom, 1980) (see Figure 1.4). In this pathway, the hydroperoxy fatty acid formed from AA may also be enzymatically metabolised to LTA_4 by LTA synthetase (Panossian et al., 1982). LTA_4, an epoxide is extremely unstable and has four possible products. LTA_4 can be non-enzymatically hydrolysed to two all-trans diHETEs known as the all-trans isomers of LTB_4 (Borgeat and Samuelsson, 1979b). Alternatively, the enzyme LTA_4 hydrolase can convert LTA_4 to LTB_4, and LTB_4 can then be metabolised by w-oxidation to 20-OH LTB_4 (see Figure 1.4) and further, to 20-COOH LTB_4 (Hansson et al., 1981). These latter two compounds are significantly less active than LTB_4 (Jubiz at

al., 1982). In cells containing glutathione-S-transferase LTC_4 is formed from LTA_4 (Radmark et al., 1980b). Subsequent removal of glutamine from LTC_4 by γ-glutamyl transpeptidase, produces LTD_4 (Orning et al., 1980) which is metabolised by cysteinyl-glycinase to LTE_4 by removal of glycine (Bernstrom and Hammarstrom, 1981).

1.2.4 Generation of leukotrienes.

For an agonist to stimulate cells to produce LO metabolites, two requirements must be met. Firstly, interaction of the agonist with the cell must lead to release of AA and secondly, the 5-LO system must be activated.

Within cells, (for example platelets), AA is usually esterified in the sn-2 position of membrane phospholipids (Irvine, 1982). The esterification process is regulated by the action of an acyl CoA synthetase whose action is specific for long-chain polyunsaturated fatty acids (PUFAs) (Wilson et al., 1982). AA can also be stored in lipid bodies (Dvorak et al., 1983) or triglycerides (Irvine, 1982), however neutral lipids are generally less rich in AA than membrane phospholipids (Irvine, 1982).

With the exception of phosphatidyl inositol (PI), the major route of phospholipid metabolism is controlled by PLA_1 and PLA_2 enzymes (Irvine 1982). Direct release of AA from the sn-2 position of phospholipids leaving a lysophospholipid may be achieved by PLA_2 enzymes (Van den Bosch, 1982) of which two types exist. One type appears specific for PI and phosphatidic acid (Billah et al., 1981;

Billah and Lapetina, 1982), while the other appears specific for phosphatidyl choline and phosphatidyl ethanolamine (Billah et al., 1980). Both types are Ca^{++}-dependent.

Alternatively, phospholipase C can cleave the phosphodiester bond of inositol 4,5 bisphosphate, releasing 1,2 diacylglycerol (DAG) and inositol triphosphate (IP_3) (Downes and Michell, 1985). DAG may subsequently be cleaved by the action of a diglyceride lipase giving AA and a monoacyl glycerol (Bell et al., 1979). These two possible mechanisms by which esterified AA can be released from membrane sources are shown in Figure 1.1.

Activation of the 5-LO system also requires Ca^{++}, an observation which is apparent for several reasons. A23187, a calcium ionophore which promotes rapid exchange of divalent cations between the intra- and extracellular environment is a potent stimulator of 5-LO product formation in a variety of cell types (Bray, 1986). In the absence of extracellular Ca^{++}, the formation of 5-LO metabolites stimulated by A23187 is dramatically reduced, implying a dependence of the system for Ca^{++} (see Section 4.2.7). Addition of Ca^{++} to the medium is sufficient to stimulate homogenised RBL-1 cells to produce large quantities of LTs in the absence of A23187 (Jakschik et al., 1980; Parker and Aykent, 1982). However, the Ca^{++}-dependence of the system appears to be limited to the 5-LO enzyme. Other enzymes in the 5-LO pathway do not exhibit the same Ca^{++} requirement (Needleman et al., 1986). For example, addition of 5-HPETE to intact RBL-1 cells initiates LT synthesis without A23187 (Parker et al., 1980). Furthermore, LTC_4 and LTB_4 are synthesised from LTA_4 by homogenised

RBL-1 cells in a Ca^{++}-free medium with EDTA present (Jakschik and Kuo, 1983).

Stimulators of leukotriene production.

A23187 has proven a useful tool for investigation of LT biochemistry because of its ability to generate large quantities of LTs in many blood cell types (see Table 1.1). However, the Ca^{++} flux produced by A23187 may not be representative of a physiological or pathophysiological stimulus (Irvine, 1982). For this reason, a variety of immunologic and endogenously synthesised compounds have been tested for the ability to generate LTs (see Table 1.1). These compounds fall into three categories - those which alone stimulate production of LTs, those which require exogenous AA, or those which cannot stimulate LT production.

The cell type most extensively studied with respect to LT production is the neutrophil (see Table 1.1). Serum treated and unopsonised zymosan stimulate LT production by neutrophils, implying that LT production can be initiated by a receptor dependent mechanism (Palmer and Salmon, 1983; Williams et al., 1985). Another immunological signal which has been tested and found to stimulate LT generation by neutrophils is monosodium urate crystals (MSU) (Serhan et al., 1984). Complement component, C8 also stimulates LT formation by neutrophils (Seeger et al., 1986), probably by creating channels in the neutrophil membrane to allow Ca^{++} into the cell. In contrast, C5a (Clancy et al., 1983; 1985), aggregated IgG (Smith et al., 1986) and Staphylococcus aureus (Hendricks et al., 1986) require exogenous AA

for generation of LTs to occur.

The effect of stimuli on cells other than neutrophils has not been examined as thoroughly although all respond to A23187 (see Table 1.1). Serum treated zymosan (STZ) stimulates LT generation by human eosinophils (Bruynzeel et al., 1985) and rabbit alveolar macrophages (Hsueh et al., 1982) and unopsonised zymosan (Rouzer at al., 1980; 1982), latex (Tripp et al., 1985) and lipopolysaccharide (Luderitz et al., 1986) stimulate LT production by mouse peritoneal macrophages. LT production stimulated with various immunoglobulins by human basophils (Peters et al., 1982), human monocytes (Williams et al., 1984) and mouse peritoneal macrophages (Rouzer at al., 1982) has also been reported. Unfortunately, there are insufficient data to determine how these stimuli may act although it is possible that specific receptors linked in some way to Ca^{++} stores are being activated.

Among the compounds reported as ineffective stimuli are endogenously synthesised compounds such as platelet-activating factor (PAF) and LTB_4 and other leukocyte activators such as phorbol 12,13 myristate acetate (PMA), cytochalasin B and n-formyl-methionyl-leucyl-phenyalanine (FMLP) (Bruynzeel et al., 1985). However, PAF and FMLP enhanced the production of LTC_4 by opsonised zymosan (Bruynzeel et al., 1985). Addition of exogenous AA enhances production of LTs by several stimuli (Borgeat and Samuelsson, 1979c; Palmer and Salmon, 1983; Serhan et al., 1984; Salari et al., 1985b), but whether AA alone is a sufficient stimulus of LT synthesis is controversial and analysis of published literature suggests that

metabolism of AA depends on the form in which it is presented to cells. For example, several reports show that exogenous AA in ethanol is metabolised (Serhan et al., 1984; Borgeat and Samuelsson, 1979a) while others demonstrate that if it is presented esterified with bovine serum albumin, metabolism will only take place if another stimulus such as FMLP or C5a is present (Clancy et al., 1983; 1985).

The major source of controversy in the area of stimulation of LT generation relates to FMLP, a chemotactic peptide which stimulates a variety of leukocyte functions including oxy-radical production, chemotaxis, degranulation (the latter dependent on cytochalasin B) (Salari et al., 1985b). Whether FMLP can generate LT production without exogenous AA is controversial. Palmer and Salmon, (1983) and Clancy et al., (1983; 1985) found no evidence of LTB_4 production by human neutrophils with FMLP unless exogenous AA was present. In contrast, Salari et al., (1985b) found that FMLP without AA could stimulate PMNL to produce LTB_4, w-oxidation products and 5-HETE. Several possibilities to explain these findings exist. Firstly, it is possible that during the process of cell separation, the FMLP cell surface receptor expression was altered. In the study by Salari et al., (1985b), whole blood was sedimented by dextran, the buffy coat was centrifuged through ficoll-paque and contaminating erythrocytes were removed by NH_4Cl lysis. Interestingly, dextran was not used in either of the studies by Clancy et al., or that by Palmer and Salmon, (these two groups used metrizamide and ficoll-paque respectively followed by an NH_4Cl lysis).

Another possibility is that the result may depend on the time of

stimulation or on the products assayed or both of these factors (Salari et al., 1985b). For example, in the study by Salari et al., maximal LT production occurred 30 min after addition of FMLP and the major products were w-oxidation metabolites of LTB_4. Clancy et al., and Palmer and Salmon, measured only LTB_4 and used stimulation times of 5 and 1 min respectively. It is therefore possible that a combination of short stimulation time and not measuring the major product lead to the inability to detect synthesis.

An interesting theory regarding the origin of AA for metabolism by the 5-LO pathway in leukocytes has been put forward by Clancy et al., (1985) to explain the observation that immune activators such as FMLP and C5a cannot stimulate neutrophils to produce LTs unless exogenous AA is present. They suggested that stimuli such as FMLP and C5a activate 5-LO but are incapable of stimulating release of endogenous AA. Thus metabolism of AA is controlled at the level of the PLA_2. Other cells, such as platelets (Marcus et al., 1982), and monocytes (Humes et al., 1982) or damaged tissue, could supply unesterified AA for metabolism by the activated 5-LO. In fact, non-esterified AA has been measured at 100 uM in inflamed tissue (Hammarstrom et al., 1975).

Leukotrienes from various sources.

LO product formation can be stimulated by a variety of agonists in different cell types (see Table 1.1), tissues and effusions (see Table 1.2). The discovery that the calcium ionophore A23187 stimulated basophilic leukaemia cells to produce large amounts of SRS-A was an important observation (Lewis et al., 1975) in this respect as work using A23187 has now been performed on a host of different cell types and tissues, investigating their LT generating potential (see Tables 1.1 and 1.2).

Cell types examined so far include all circulating blood cells and macrophages from humans and a variety of animal sources (see Table 1.1). In general, it appears that not all cells are capable of synthesizing all products of the AA cascade. Which monoHETEs are produced is a source of controversy, particularly with respect to neutrophils. For example, most of the recent literature (Sun and McGuire, 1984; Williams et al., 1985) report that human peripheral neutrophils exposed to A23187 or zymosan synthesise 5-HETE, LTB_4 and the isomers and w-oxidation products of LTB_4. Earlier reports claim that these cells can also synthesise 15-HETE (Borgeat and Samuelsson, 1979c), 8-,9-,11-,12- and 15-HETE (Goetzl and Sun, 1979) and 12-HETE (Ford-Hutchinson et al., 1980) and therefore in addition to 5-LO, possess OTHER LO enzymes. There are several possible explanations for this. Firstly, many of the cell separation techniques used would not entirely remove platelets, and platelet contamination could account for 12-HETE production (McColl, unpublished observations). Furthermore, in the latter three studies, exogenous AA was added

either in large concentrations to amplify production or as ^{3}H-AA to increase assay sensitivity. AA is an unstable polyunsaturated fatty acid, and unless stored properly, it will spontaneously oxidise and form several monoHETES, which may then be added to cell suspensions (Johnson et al., 1983).

A further possibility arises when examining the work performed by Goetzl and Sun, (1979). The cell system used in these experiments was an homogenised neutrophil system. It is therefore possible that when neutrophils are damaged, other LO enzyme activities are expressed and synthesis of many other monoHETEs occurs. This theory is supported by observations that several NSAIDs can damage neutrophils and unmask 15-LO activity (McGuire et al., 1985).

Whether neutrophils synthesise CO products is also controversial. Some reports indicate that neutrophils can make PGE_2 (Higgs et al., 1975) and TXB_2 (Higgs and Youlten, 1972; Palmer and Salmon, 1983) implying the presence of a CO pathway. However, the amounts of these products reported are small and could be accounted for by platelet contamination.

An overview of the literature indicates that human neutrophils possess a 5-LO enzyme which upon stimulation generates LTB_4, its isomers and oxidation products but not the peptido-LTs. Similar findings have been reported for peritoneal neutrophils from rats (Ford-Hutchinson et al., 1980; Siegel et al., 1981), rabbits (Borgeat and Samuelsson, 1976; 1979a; 1979b) and guinea pigs (Bokoch et al., 1981). Although controversy regarding monoHETE production in

eosinophils also exists, it is reported that in addition to LTB_4, they produce LTC_4 (Borgeat et al., 1984; Bruynzeel et al., 1985). Similar findings have been reported for human lung mast cells (Peters et al., 1984) and human peripheral blood monocytes (Goldyne et al., 1984). In contrast, it appears that murine peritoneal macrophages can synthesise only LTC_4 (Rouzer et al., 1980) whereas rat peritoneal macrophages (Doig and Ford-Hutchinson, 1980) and rabbit alveolar macrophages (Hsueh et al., 1982; 1985) predominantly produce LTB_4. Lymphocytes also possess a 5-LO system and can produce LTB_4 (Goetzl, 1981a).

LTC_4 and LTD_4 and sometimes LTB_4 production, stimulated by several agonists has also been reported in a variety of tissues (see Table 1.2). For example, guinea-pig lung tracheal, colonic and aortic tissue, rat brain tissue, mouse ear tissue and several other tissues can synthesise the peptido-LTs (see Bray, 1986 for review). Human lung parenchymal tissue and bronchial fragments also produce LTB_4, LTC_4, LTD_4 and LTE_4 (Salari et al., 1985a). Detection of LTs in several experimental effusions has also been reported (see Table 1.2). LTB_4 and or LTC_4 and LTD_4 have also been assayed in rat peritoneal, pleural or granulomatous sponge effusions and rat bile samples (Bray, 1986).

1.2.5 Pharmacological actions of Prostaglandins and Leukotrienes.

Prostaglandins.

Some of the reported pharmacological actions of PGs and LTs C_4, D_4 and E_4 are shown in Table 1.3. Briefly, the major physiological

actions of the PGs are as either smooth muscle contractors or relaxants and include effects on blood pressure (PGE_2, $PGF_{2\alpha}$) and regulation of haemostasis (PGI_2, TxA_2). Most of the effects of LTs C_4,D_4 and E_4 are similar to the PGs in that they cause smooth muscle contraction and vasoconstriction (Table 1.3).

CO products can have direct pro-inflammatory action on tissues. For example, PGE_2 is a pyrogen and causes vasodilation and erythema at sites of injection, while PGD_2, PGI_2 and PGE_2 enhance microvascular permeability and plasma exudation caused by other mediators such as bradykinin and histamine (Ferreira, 1972). PGE_2 and PGI_2 also enhance pain evoked by other stimuli (Ferreira, 1972), and there is evidence that PGs directly effect leukocytes. For example, PGD_2, PGI_2 and PGE_2 stimulate neutrophil and eosinophil chemokinesis (Goetzl et al., 1979), and PGI_2 inhibits adherence of these cells (Boxer at al., 1980). PGE_2 and PGD_2 also inhibit lymphokine generation by T lymphocytes (Gordon et al., 1976).

MonoHETEs.

Since the discovery that 12-HETE was a chemotaxin for neutrophils, there has been great interest in the various effects of monoHETEs. Most studies have used one or two monoHETEs although several have included most of the monoHETEs (see Table 1.4). The pharmacological effects of the monoHETEs include stimulation of chemotaxis and chemokinesis by human neutrophils and eosinophils in addition to stimulation of neutrophil degranulation. An order of chemotactic potency in the concentration range of 10^{-5} -10^{-7} M was

presented by Goetzl et al., (1980a), indicating that 5-HETE >> 8-HETE = 9HETE > 11-HETE = 12-HETE >>15-HETE. MonoHETEs can also modify several other granulocyte functions. For example, increased C3b receptor expression on human neutrophils and eosinophils by 5-HETE has been reported (Goetzl et al., 1980b), and Chang et al., (1985a) showed that 5-,12- and 15-HETE inhibit neutrophil 5-LO thereby inhibiting the production of LTB_4 and 5-HETE. It appears therefore that a form of negative feedback inhibition on 5-LO exists.

MonoHETEs also affect other cells. For example, 15-HETE inhibits the 5-LO of T Lymphocytes and 5-, 12- and 15-HETE inhibit platelet PLA_2 (Chang et al., 1985b). 12-HPETE inhibits collagen-induced platelet aggregation and platelet CO but stimulates 12-LO (Siegel et al., 1981). The mechanism for inhibition of aggregation was via inhibition of CO (and therefore TXA_2 production) (Siegel et al., 1981). 5-HPETE and 5-HETE inhibit antigen-induced histamine release by basophils (Peters et al., 1982) and therefore can modify hypersensitivity reactions. 12- and 15-HETE stimulate mucus secretion by airway tissue (Marom et al., 1983; Johnson et al., 1985) and inhibit glucose-induced insulin secretion by pancreatic islets (Yamamoto et al., 1983).

Although monoHETEs have diverse actions, the doses required to demonstrate these actions are high compared to the levels produced by cells. Furthermore, compared to LTB_4, the monoHETEs appear relatively inert as chemotaxins because LTB_4 is 2-4 orders of magnitude more potent (Dahinden et al., 1984).

Leukotriene B_4.

Initially, LTB_4 attracted a great deal of interest as a stereospecific neutrophil chemotaxin (Malmsten et al., 1980). LTB_4 is the most potent endogenous granulocyte chemotaxin yet discovered acting in the concentration range of 10^{-6} -10^{-9} M (Dahinden et al., 1984; Palmblad et al., 1981). Its activity in this respect has been reported by a large number of groups (see Table 1.5). LTB_4 also stimulates several other important neutrophil functions all of which reflect cell activation. For example, it stimulates chemokinesis and aggregation (Ford-Hutchinson et al., 1980), cytochalsin B-dependent degranulation (Feinmark et al., 1981; Hafstrom et al., 1981; Showell et al., 1982a), and the hexose monophosphate shunt. Although neither LTB_4 nor 5-HETE can directly stimulate oxy-radical production (Gay et al., 1984), they enhance FMLP-induced O_2^- production. LTB_4 also enhances C3b receptor expression (Nagy et al., 1982) and stimulates release or production of second messengers thus potentially activating many other neutrophil functions (Naccache et al., 1981; Lew et al., 1984; Goetzl 1981b).

LTB_4 stimulates similar functions in eosinophils (see Table 1.5) and IL-1 production by monocytes (Rola-Pleszcynski and Lemaire, 1985) and therefore can modify the activity of a wide range of cell types. Investigation of effects of LTB_4 on lymphocytes show that it activates cytotoxic cells and natural killer cells, and inhibits T cell proliferation and the production of lymphocyte inhibitory factor by T lymphocytes (Rola-Pleszcynski et al., 1982; 1983; Payan and Goetzl, 1982).

Actions of LTB_4 on other tissue systems include contraction of lung parenchyma and perfused lung (Sirois et al., 1980) and enhancement of bradykinin-induced plasma exudation in the skin of rats, rabbits and guinea-pigs (Bray et al., 1981).

Interactions between prostaglandins and leukotrienes.

PGs and LTs can interact. For instance, LTB_4 is a relatively weak stimulator of plasma exudation, however the addition of small amounts of PGE_2 greatly enhances its action (Bray et al., 1981). LTC_4 and LTD_4 inhibit the chemotactic response of human neutrophils and eosinophils to LTB_4 (Payan et al., 1984) and LTC_4. To a lesser extent LTB_4 can enhance adherence of these cells via production of TXA_2 (Goetzl, 1981b). PGE_2 and PGD_2 not only directly stimulate leukocyte chemokinesis, but also enhance the chemotaxis induced by LTB_4 (Goetzl et al., 1979).

Leukotrienes in inflammation.

In vivo studies also provide evidence that LO products are pro-inflammatory. For example, injection of 12-HETE, 5-HETE and LTB_4 leads to a rapid accumulation firstly of eosinophils and later neutrophils in the peritoneal cavity of rats (Smith et al., 1980). Furthermore, LTB_4 causes an accumulation of PMNL in the eyes (Bhattacherjee et al., 1981) and skin (Carr et al., 1981) of rabbits. The use of inhibitory drugs in some animal models of inflammation provide further evidence of the involvement of LO products. For

example, the dual CO and LO inhibitor BW755C prevents infiltration of PMNL into rat paws after injection of carrageenan while a specific CO inhibitor fails to suppress the influx of cells (Higgs et al., 1980).

Leukocytes such as activated rabbit neutrophils (Traynor et al., 1981) and rabbit (Vadas et al., 1981) and mouse peritoneal macrophages (Wightman et al., 1981) liberate PLA_2 into the extracellular milieu. Release is evoked by several stimuli, including lipopolysaccharide (Vadas et al., 1983), calcium and opsonised zymosan (Traynor et al., 1981). Increased levels of extracellular PLA_2 have been found in inflammatory fluids, for example, in the ascitic fluid of rabbits given an intraperitoneal injection of glycogen (Franson et al., 1973) and pancreatic tissue and peripheral blood of dogs and humans with acute pancreatitis (Nevalainen et al., 1980). Vadas et al., (1984), compared the level of PLA_2 in the sera and synovial fluid of patients with classical rheumatoid arthritis and found approximately six times more PLA_2 in synovial fluid. It is possible that elevated levels of PLA_2 in inflammatory fluid may not only reflect the increased activation level of local or infiltrating cells, but may also provide a mechanism for increased release and therefore metabolism of AA by cells within an inflammatory focus.

Increased levels of PGs and LTs have been found at sites of inflammation in animal models or human inflammatory disorders and at sites of hypersensitivity reactions. Significantly increased amounts of PGE_2, TXA_2, AA and HETEs have been identified in patients with psoriasis when compared with normal skin areas from the same patients (Hammarstrom et al., 1975). Synovial fluid from patients with

rheumatoid arthritis had greater amounts of CO metabolites than synovial fluid from patients with non-inflammatory arthropathies (Robinson et al., 1975; 1979). Asthmatic attack leads to generation of LTs C_4 and D_4 which can be detected in sputum and plasma (Bray et al., 1986). Allergen challenge leads to detection of LTB_4, C_4, D_4 and E_4 in nasal washouts from patients with allergic rhinitis (Creticos et al., 1984), and LTC_4 and LTD_4 in skin blister fluid (Bisgaard et al., 1985). LTB_4 has also been detected in chronic inflammatory effusions from patients with cystic fibrosis, psoriasis, gout, uticaria and allergic or irritant contact dermatitis (Bray et al., 1986). LTB_4 has been measured in the synovial fluid of patients with rheumatoid arthritis although there is some controversy regarding the amount. Using HPLC, Klickstein et al., (1980), found 141 $\pm$ 34 ng/ml of LTB_4 in the synovial fluid of 18 patients with rheumatoid arthritis with individual levels as high as 800 ng/ml. Such high levels are improbable, since Davidson et al., (1982) could not detect LTB_4 in the synovial fluid from 12 patients using HPLC, however by RIA, they measured levels of 0.34 $\pm$ 0.14 ng/ml. Furthermore, Davidson et al., (1982) have questioned the accuracy of measuring LTB_4 in synovial fluid by HPLC.

A tentative model of the possible roles of AA-metabolites in an inflammatory disorder such as rheumatoid arthritis (RA) has been put forward (Goetzl, 1981b). The model, depicting RA of a knee joint is shown in Figure 1.5. The nature of the initial stimulus in a such a lesion is unknown but may, for example, be a persistent bacterium. The elevated levels of CO and LO metabolites could be caused by the influx of macrophages and neutrophils into the synovium. LTB_4 (and

possibly mono-HETEs) together with molecules such as C5a exacerbate the condition by promoting the local influx of more leukocytes. PGE_2 and LTB_4 possibly synergise to increase permeability of local vascular beds thus enhancing the influx of leukocytes and promoting swelling. Once activated, leukocytes can further damage the local tissue by release of a host of cytokines and chemically active molecules for example, oxy-radicals, collagenase, lysosomal enzymes. PGE_2 can stimulate resorption of bone by synoviocytes and inhibit the production of proteoglycans by synoviocytes and articular chondrocytes (Fahey et al., 1977; Lippiello et al., 1978).

1.2.6 Leukotriene inhibition.

The two main points of attack when inhibiting endogenously synthesised compounds such as LTs are inhibition of product action (eg: receptor antagonism) or product formation. In the case of LTs, there are two approaches to inhibition of product formation. They are, (a) to inhibit release of AA or (b) to inhibit subsequent metabolism of AA. A summary of the reports on LT inhibition is shown in Table 1.6.

Inhibition of leukotriene action - receptor antagonism.

The stereospecificity of LTB_4 action on neutrophils indicated the presence of specific receptors. For example, the all-trans isomers and the w-oxidation products of LTB_4 while being chemically very similar to LTB_4 are orders of magnitude less potent (Feinmark et al., 1981). High affinity LTB_4 receptors are present on neutrophils

(Goldman and Goetzl, 1982). 5-HETE and the all-trans isomers of LTB_4 bind to this receptor with approximately 100-fold less affinity than does LTB_4, reflecting the difference in chemotactic potency (Goldman and Goetzl, 1982). There are 26,000-40,000 receptors per neutrophil and LTB_4 binds to these receptors with a Kd of 10.8 - 13.9 nM (Kriesle and Parker, 1983). C5a and FMLP do not inhibit ^3H-LTB_4 binding to neutrophils, implying that the receptor which binds LTB_4 is distinct from the receptors for either of these chemotaxins.

The only reported specific LTB_4 receptor antagonist is LTB_4 demethylamide which appears to be a partial agonist with an ID_{50} value of between 0.001 and 0.03 uM (Showell et al., 1982b).

The presence of receptors for LTC_4 and LTD_4 has also been demonstrated (Augstein et al., 1973). Pharmacological analysis of the effect of the chromone carboxylate FPL55712, a highly selective and potent inhibitor of SRS-A-induced contraction of guinea-pig ileum, strongly suggested that it was acting as a competitive receptor antagonist (Augstein et al., 1973). Using this compound, the presence of several different populations of SRS-A receptors has been demonstrated. For instance, Schild plot analysis of experiments in guinea-pig tissue indicated that the receptors in guinea-pig ileum had a greater affinity for FPL55712 than did those in trachea or parenchyma (Fleisch et al., 1982). More recently a longer active derivative, FPL59257 has been synthesised as the action of FPL55712 was too short in vivo (O'Donnell and Welton, 1982). Unfortunately, the performance of these compounds in vivo has not lived up to expectations (Bach, 1984).

Inhibition of arachidonic acid release.

Corticosteroids have been used for many years as antiinflammatory drugs. Approximately 10 years ago, it was discovered that they inhibited PG production (Johnson et al., 1983). Initially believed to act by inhibition of CO, subsequent studies demonstrated that corticosteroids cause inhibition of AA release by inducing synthesis of PLA_2-inhibitory proteins. Two proteins with PLA_2 inhibitory activity have been isolated, one with a molecular weight of approximately 16,000 called macrocortin (Blackwell et al., 1982) and the other with a molecular weight around 40,000 called lipomodulin (Hirata et al., 1980). It now seems likely that macrocortin is a cleavage product of lipomodulin (Johnson et al., 1983).

Two other inhibitors of PLA_2, mepacrine (Burka and Flower, 1979) and p-bromophenacylbromide (Hofmann et al., 1982) have been reported. However, they are not selective in action as mepacrine also binds to nucleic acids and is a frameshift mutagen, while p-bromphenacylbromide is a potent alkylating agent which alkylates histidine (Bach, 1984).

Dual cyclooxygenase and lipoxygenase inhibition.

It is clear from the intended site of action of PLA_2 inhibitors that such inhibitors will inhibit production of both PGs and LTs. There are several other drugs which act directly on CO or LO and achieve a similar effect. These drugs, known as nonsteroidal antiinflammatory drugs (NSAIDs), range in action from predominant CO inhibition to equipotent inhibition of both types of enzyme. Examples

of the latter type are BW755c and its analogs (Randall et al., 1980; Piper and Temple, 1981; Patterson et al., 1981) and benoxaprofen (Walker et al., 1980; Dyer et al., 1982).

ETYA (5,8,11,14-eicosatetraynoic acid), a structural analog of AA in which all ethylenic double bonds have been replaced with acetylenic triple bonds, is a drug which defined as a "universal" inhibitor of PG and LT synthesis (Bach, 1984). This compound may inhibit all the major enzymes of the AA cascade as well as PLA_2 (Bach et al., 1977), however its actions are complex. For example, doses that inhibit both CO and 5-LO do not inhibit platelet 12-LO (Morris et al., 1979; Busse et al., 1982). Furthermore, doses which inhibited CO did not affect SRS-A generation (Engineer et al., 1978) and enhanced 5-HETE production (Borgeat et al., 1982).

Non-selective lipoxygenase inhibition.

Drugs more selective for the LO arm of the AA cascade may also effect several LO enzymes. An example is nordihydroguairiaretic acid (NDGA) which does not inhibit CO (Walker et al., 1980; Bokoch and Reed, 1981) and has been used as a prototype LO inhibitor. Of the coumarin derivatives, esculetin is the most potent with ID_{50} values of 4 uM and 2.5 uM for 5- and 12-LO enzymes respectively (Neichi et al., 1983). In contrast, flavonoids appear more selective for 5-LO. For example, quercetin exhibits ID_{50} values of 0.2 uM for 5-LO and 5 uM for soy-bean LO (Hope et al., 1983) and cirsiliol is more selective for the 5-LO of RBL-1 cells (ID_{50} of 0.1 uM) than the bovine 12-LO (ID_{50} of 1.0 uM) (Yoshimoto et al., 1983). Other compounds include

caffeic acid, extracted from the chinese plant Artemisa rubripes Nakai. This compound and its methyl ester, selectively inhibit 5-LO (ID_{50} of 3.7 uM and 0.48 uM respectively) in preference to 12-LO (ID_{50} of 100 uM) and CO (ID_{50} < 100 uM) (Koshihara et al., 1984). Two other compounds extracted from the same plant, eupatilin and 4' demethyeupatilin also inhibit 5-LO although their activity against 12-LO was not determined in this study (Koshihara et al., 1983).

5-lipoxygenase inhibition.

Some AA analogs with relatively specific 5-LO inhibitory activity have been synthesised, for example, acetylenic analogs such as 5,6-dehydro-AA (Corey and Munroe, 1982; Sok et al., 1982) and 5,6-methano-LTA_4 (Arai et al., 1982; Koshihara et al., 1982). These drugs respectively exhibited ID_{50} values of 10 uM and 3 uM (selectively) for 5-LO. 15-HETE inhibited the 5-LO of rabbit PMNL (Vanderhoek et al., 1980; Goetzl, 1982), although these data remain controversial as Vanderhoek et al., (1982) actually showed that 15-HETE enhanced 5-LO activity in mast cells.

Inhibition of other enzymes of the 5-LO system.

Because of the lack of pure enzymes for detailed kinetic studies, there are few reports of selective inhibitors of other enzymes in the 5-LO pathway. As a consequence intact cell systems have been used, making it difficult to assign specific sites of action. For example, in assessing the sulfasalazine metabolite, 5-aminosalicylate, Stenson and Lobos, (1982) determined that LTB_4 production was inhibited

without a reduction in 5-HETE. However, because they did not measure production of either LTC_4 or the all-trans isomers of LTB_4, they could not differentiate between inhibition of LTA synthetase or LTA hydrolase. Ebselen, an organo-selenium compound, inhibited LTB_4 synthesis with an ID_{50} value of 2.7 uM compared to 30 uM for 5-HETE (Kuhl et al., 1985). Furthermore, at a dose which inhibited production of LTB_4, formation of the all-trans isomers of LTB_4 increased implicating the LTA hydrolase as the site of action. Another drug, diethylcarbamazine with an ID_{50} value of 5 uM, inhibited the synthesis of LTB_4, LTC_4 and the all-trans isomers of LTB_4 in mastocytoma cells while enhancing 5-HETE production (Mathews and Murphy, 1982). This indicates a site of action on LTA synthetase.

Several drugs inhibit glutathione-S-tranferase (the enzyme which converts LTA_4 to LTC_4). Of note are organic anions such as bilirubin (Kaplowitz et al., 1975) and bile acids (Pattison, 1981) and steroid sulphates (Ohl and Litwack, 1977).

Fatty acid substitution - The EPA story.

Over the last 15 years, many studies examining the effect of eicosapentaenoic acid (20:5 w3, EPA) on the cardiovascular system or immune and inflammatory responses have been performed. In such experiments cells (for example, platelets or neutrophils) have either been removed from donors before EPA has been introduced, or EPA has been provided as a dietary supplement for a period of time after which cells have been removed and studied. In addition, in vivo studies investigating potential beneficial effects of EPA on the

cardiovascular or immune systems have been carried out on animals with a view for eventual emphasis on human application. Specific examples of theses studies will now be discussed.

1. EPA and the cardiovascular system.

The influence of EPA on haemostasis

As early as 1969, researchers were aware of the discrepancy between Greenland eskimos and the Danish population with respect to coronary heart disease (CHD). A report by the chief medical officer for the years 1973-1976 (Anonymous, 1978) indicated that the incidence of CHD-related mortality was 3.5 % in the eskimo population compared to greater than 40 % for Danes. Because this low rate of mortality did not apply to eskimos living off the Danish coast, it was believed that the difference was environmental and not genetic. Analysis of diet and levels of plasma lipids and lipoproteins determined that the major difference between the two populations was that eskimos ingested large quantities of w3 PUFA in their diet (Dyerberg et al., 1978) and this was reflected in their plasma lipids and lipoproteins (Bang et al., 1971; Bang and Dyerberg, 1972). It was estimated that Eskimos ingest 5-6 g EPA per day (Bang et al., 1980).

The implications of the large intake of EPA by the eskimos became clear when the labile AA-derivatives prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) were discovered (Hamberg et al., 1975; Moncada et al., 1976). TXA_2, which is produced by platelets, stimulates platelet aggregation, whereas PGI_2 which is produced by vascular

endothelial tissue has the opposite effect (Hamberg et al., 1975; Moncada et al., 1976). The pharmacological balance between these two compounds exerts a powerful influence on the control of haemostasis (Nugteren, 1978). Like its analogue AA, EPA is utilised by vascular endothelium to make a potent platelet anti-aggregatory substance (presumed at the time to be PGI_3) (Dyerberg et al., 1978). However, unlike AA, EPA does not induce platelet aggregation and furthermore, the thromboxane derivative formed from EPA (TXA_3) is non-active as a pro-aggregant (Dyerberg et al., 1978). As a consequence, it was expected that a diet rich in EPA would push the balance of haemostasis towards an anti-aggregatory state - a theory supported by the finding that the average bleeding time in eskimos was 8.1 minutes compared to 4.8 minutes in Danes (Dyerberg and Bang, 1979).

In vitro and ex vivo studies of the effect of EPA on platelets

EPA is readily incorporated into the phospholipids of human and animal platelets and it is generally agreed that there is a concommitant reduction of AA. For example, EPA is incorporated into human platelet phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) at the expense of AA (Ahmed and Holub, 1984; Galloway et al., 1985) and similar findings have been reported in rats (Nordoy et al., 1985). Analysis of the phospholipids of platelets from volunteers fed high EPA diets (0.6g EPA per day) for 4 weeks showed selective incorporation into PC and PE (Ahmed and Holub, 1984). Galloway et al., (1985) obtained similar results when six normal volunteers were given 1.8 g EPA per day for 4 weeks. Furthermore, preferential incorporation of EPA into PE (but not PC) was found in the platelets

of volunteers who had taken 25 mls of cod liver oil per day for 5 months (von Schacky et al., 1985b). A subsequent study found selective incorporation into PC and PE when volunteers were given 6 g EPA per day for 6 days (von Schacky and Weber, 1985a). Conversely, washed human platelets incubated with ^{14}C-labelled fatty acids preferentially incorporated EPA into PI (Weiner and Sprecher, 1984), a finding supported in the study by von Schacky et al., (1985a). These results indicate that in vitro incorporation of EPA into platelet phospholipids differs from that in vivo.

Platelet aggregation stimulated by a variety of substances (ADP, collagen or thrombin) is generally inhibited in the platelets of humans taking EPA supplements and there is a reduction of dienoic prostanoids with an associated production of trienoic prostanoids (Ahmed and Holub, 1984; von Schacky et al., 1985b). Ahmed and Holub, (1984) also showed that when healthy volunteers took 0.6g EPA/day for 4 weeks, bleeding times increased and that all of the above effects were reversible upon cessation of ingestion of EPA. Therefore, EPA can modify platelet function and alter eicosanoid synthesis.

In vivo studies of EPA and its effects on cardiovascular disease

In vivo studies where animals have been fed diets rich in EPA have shown beneficial effects. Ligation of the cerebral artery of cats, showed that cats fed an EPA rich diet for 18-24 days, had reduced ischaemic damage and lower mortality than those fed a normal diet (Black et al., 1979). In a similar dietary study using dogs, mortality from experimental myocardial infarction was lower in dogs

fed fish oil (Culp et al., 1980). More recently, it has been shown that development of atherosclerosis was significantly lower in pigs fed a diet rich in EPA (Weiner et al., 1986).

Hyperlipidaemia is reduced in humans after dietary treatment with EPA, although the evidence regarding cholesterol is contradictory. For example, a general decrease in total cholesterol was observed when healthy volunteers took 15 or 30 g of cod liver oil per day for four weeks (Kingsbury et al., 1961; Kinsell et al., 1961). Other studies, however have shown no change in cholesterol (van Gent et al., 1979; Saynor and Verel, 1980). Supplementation also reduced levels of plasma triglycerides in normal humans (van Gent et al., 1979; Saynor and Verel, 1980; Sanders and Younger, 1981).

Kromhout et al., (1985) demonstrated an inverse relationship between consumption of fish and mortality from coronary heart disease in a study on a Dutch village extended over a period of twenty years. They found that mortality from coronary heart disease was over 50% lower among men who consumed at least 30 g of fish daily. EPA supplementation alters platelet and vascular function in patients with atherosclerosis (Knapp et al., 1986). These workers demonstrated that a supplementation with 1 g EPA/day was not sufficient to cause these changes. However, at 10 g/day, EPA levels in platelet and erythrocyte phospholipids were raised and synthesis of TXA_2, which was elevated in the patients prior to commencement of the study, was reduced by 58% during supplementation. Furthermore, the decrease in TXA_2 production correlated with the appearance of TXA_3.

In summary, the in vitro, ex vivo and in vivo effects of EPA on the cardiovascular system of animals and humans have been examined and the findings generally support the hypothesis that increased dietary EPA has beneficial effects on coronary heart disease and reduces plasma lipid levels, further reducing coronary risk.

2. EPA and immune and inflammatory responses

Since the discovery of the LO products of AA and elucidation of their effects on the immune system, the in vitro and in vivo effects of EPA in relation to immunity and inflammation have been examined. Studies showing incorporation of dietary EPA into cells of the immune system have been performed in both animal and human tissue and in vivo experiments on the effect of EPA on animal models of inflammation have been carried out. However, very few studies on the effects of EPA-rich diets in human inflammation have been performed.

The effect of EPA on in vitro and ex vivo cellular immunity

The amount of AA present in the cellular phospholipids of mastocytoma cells from mice fed menhaden oil (MO) for 6 weeks decreased while the amount of EPA increased (Murphy et al., 1981). When these cells were stimulated by A23187, LTB_5 was produced and the amount of LTB_4 produced decreased. In contrast, the amount of LTC_4 produced was 10x the amount of LTC_5 indicating a preference of glutathione-S-transferase for LTA_4 over LTA_5.

In a similar fashion to the trienoic thromboxane produced from

EPA, some of the LO EPA products are less active than their AA-derived analogues. Human neutrophils have been used to test the potency of LTB_5 which had been biosynthesised from guinea-pig peritoneal neutrophils (Goldman et al., 1983). LTB_5 was 10 - 30-fold less potent than LTB_4 as a neutrophil chemotaxin and equipotent as a degranulating agent. Terano et al., (1984b), compared the potency of LTB_5 biosynthesised from rabbit peritoneal neutrophils with synthetic LTB_4. LTB_5 was at least 30-fold less potent in promoting chemotaxis, degranulation, aggregation and bradykinin-induced plasma exudation. LTB_4 also bound to a high affinity class of LTB receptor with a 500-fold greater affinity than LTB_5, however, the two compounds bound to the low affinity LTB receptor to the same extent (Goldman et al., 1983). A comparison of the potency of eicosanoids produced in the peritoneal cavity of Sprague-Dawley rats fed diets supplemented with either menhaden oil or beef tallow, revealed that LTB_5 was 30-60 times less potent than LTB_4 as a neutrophil chemotaxin, while LTC_4 and C_5 had equipotent actions on parenchymal strips (Leitch et al., 1984).

Studies on in vitro EPA metabolism by human cells have yielded similar results to those on animal tissue. Isolated human neutrophils incorporate and release EPA to the same extent as AA, and LTB is produced from both substrates (Prescott, 1984). Upon stimulation with A23187, neutrophils produce less LTB_4 (94 ng/10^7 cells/5 min) when 20 uM EPA is used as a substrate than when the same concentration of AA is used (401 ng/10^7 cells/5 min). LTB_5 had 10% of the potency of LTB_4 in stimulating neutrophil aggregation (Prescott et al., 1984). Similar findings have been reported by Lee et al., (1984).

Human volunteers fed 8-10 g EPA per day for 3 weeks showed an increase in the amount of EPA in neutrophil phospholipid (0%-7.4%) while the percentage of AA decreased (15.4%-12.8%) (Prescott et al., 1985). Furthermore, the amount of LTB_4 produced by stimulation of neutrophils with A23187 was reduced from 160 ng to 120 ng/10^7 cells and LTB_5 was produced (37 ng/10^7 cells). The diet had no effect on neutrophil aggregation or adherence to nylon fibers (Prescott et al., 1985). Lee et al., (1985) provided human volunteers with 18 capsules of MaxEPA/day for 6 weeks, a daily ingestion of 3.2 g EPA and 2.2 g DHA and observed a 7-fold increase in the EPA content of neutrophil and monocyte phospholipids. However, no change in AA or DHA content was observed. The A23187-induced release of ^3H-AA from neutrophils of the volunteers taking MaxEPA was reduced by 37% and the maximal stimulation of neutrophil 5-LO products by 48% compared with control values. Similar effects were observed in macrophages. Neutrophil adherence to LTB_4 pre-treated bovine endothelial cells was completely inhibited by the diet and the average chemotactic response to LTB_4 was reduced by 70% (Lee et al., 1985).

An overview of the literature on the in vitro and ex vivo effects of EPA on neutrophils reveals that EPA is readliy incorporated into cells of the immune system although there is some controversy as to whether an associated decrease in AA occurs. Also, like the effect demonstrated on CO products, some of the 5-LO metabolites of EPA have been found to be generally less potent than their AA-derived counterparts.

EPA and the immune system in vivo

The effects of dietary PUFA on autoimmune diseases can be demonstrated using essential fatty acid deficient rats (Denko, 1976). Significantly less adjuvant-induced paw swelling was observed in essential fatty acid deficient (EFAD) rats compared with rats fed a normal diet. This effect was reversed when the rats were fed a normal diet. Denko concluded that the reduction in swelling is due to inhibition of eicosanoid production. The most commonly studied auto-immune model in dietary investigations is the spontaneously developed systemic lupus erythmatosus (SLE) disease in NZB x NZW/F_1 mice where severe proteinuria associated with immune complex glomerulonephritis eventually leads to death (Prickett et al., 1981). In initial studies, onset of renal disease and hence mortality was delayed in mice fed a diet supplemented with menhaden oil compared to control rats fed beef tallow. Furthermore, levels of circulating anti-DNA antibodies were lower in the MO fed mice (Prickett et al., 1981). The protective effect is observed even if dietary treatment was not commenced until after the onset of disease (Prickett et al., 1983; Robinson et al., 1986) however in these studies the effect on anti-DNA antibodies was not observed. It therefore appears that the protective effect of menhaden oil is due to some influence other than a decrease in the levels of circulating anti-DNA antibodies (Prickett et al., 1983).

Kelley et al., (1985), using MRL-1pr mice, another strain of mouse which developes a SLE and eventually dies, reported similar results when the mice were fed a diet supplemented with MO. The

lymphoid hyperplasia and the levels of circulating retroviruses associated with the disease were decreased, and the onset of renal disease and mortality were delayed. In addition, the MO-fed mice produced trienoic eicosanoids while the levels of dienoic PGs were significantly reduced (Kelley et al., 1985).

Sprague-Dawley rats fed a diet supplemented with MO for 6 weeks and immunised with an intradermal injection of native chick type II collagen showed a higher incidence of arthritis compared with control-fed rats (Prickett et al., 1984). While no difference in joint severity was observed, the average serum titre of IgG antibodies was lower in the MO-fed rats and primary synovial explant cultures from MO-fed rats produced 21-24% of the amount of PGE_2 produced by those from the control-fed rats. There was, however, no evidence of production of trienoic PGs. In contrast dietary EPA decreased the susceptibility of mice to arthritis induced by native type II collagen from bovine nasal septa (Leslie et al., 1985) indicating a possible species difference.

Efamol, an oil rich in dihomo-gamma-linolenic acid caused a slight improvement in patients with rheumatoid arthritis (Hansen et al., 1983) and Sjogren's syndrome (Manthorpe et al., 1984). However, only two studies on EPA supplementation have been reported to date. EPA appears to prevent deterioration of IgA nephropathy in humans (Hamazaki et al., 1984). Kremer et al., (1985) conducted a dietary study on 17 patients with rheumatoid arthritis (RA). The RA patients were provided with either 1.8 g EPA per day or placed on a high saturated fat diet for 3 months. A reduction in duration of morning

stiffness and joint tenderness was found in the patients taking EPA. However, the dietary regimen in this study was not ideal since the EPA was taken with a diet which was high in PUFA - particularly w6 PUFA - and this may have reduced the possible beneficial effect of the EPA supplement. Furthermore, it is possible that a daily supplement of 1.8 g/day may not have been sufficient to cause a large change in the course of the disease (Knapp et al., 1986).

Therefore, although extensive studies on the effect of EPA on immune models have been carried out and results indicate that EPA has beneficial effects (eg: murine lupus model), remarkably few studies examining the effect of EPA on acute and chronic inflammation have been reported. Only two studies examining arthritic models in animals (Prickett et al., 1984; Leslie et al., 1985) and one on humans with rheumatoid arthritis (Kremer et al., 1985) have been reported. Some of the research reported in this thesis was performed to provide more information regarding the effect of dietary PUFA modification on inflammatory conditions.

1.3 ANIMAL MODELS OF INFLAMMATION.

1.3.1 Introduction.

In this section I will describe several of the experimental models of inflammation which have been developed to enable study into the mechanisms of inflammation and evaluation of potential treatments of inflammatory disorders. Several animal models of inflammation are available, however no single model closely mimics rheumatoid

arthritis. Each of these models yields information pertaining to specific characteristics of inflammation.

1.3.2 Irritant-induced pleurisy in rats.

This model, which involves pleural responses induced by injection of an irritant has been used to assess the cellular component of acute inflammation (Vinegar et al., 1982). It is particularly useful for monitoring the early phase of acute inflammation. In our experience, variability of cell migration within experimental groups limits the utility of this model.

1.3.3 Knee joint synovitis in rabbits.

In this model, a synovitis which histologically resembles rheumatoid arthritis can be induced by injection of an irritant such as carrageenan (Lowther and Gillard, 1976), or an antigen such as fibrin (Dumonde and Glynn, 1962) or ovalbumin (Consden et al., 1971), to which the animal has been sensitised. Quantitation of responses in this model is difficult.

1.3.4 Carrageenan paw swelling in rats.

The carrageenan paw swelling model is one of the most widely used in vivo models for evaluating anti-inflammatory treatments. It has been well characterised and three distinct phases of the response have been identified. The first phase occurs at 30 min after injection and results from the release of histamine and serotonin (Dirosa et al.,

1969). The second phase, ocurring 1-2.5 hrs after injection results from bradykinin release while the final phase, 3-4 hrs following injection, is complement-dependent and is associated with production of arachidonic acid metabolites (Velo et al., 1973).

1.3.5 Subcutaneous sponge implants in rats.

The subcutaneous sponge implant model provides information regarding the humoral and cellular aspects of inflammation. The sponges, which are impregnated with an irritant, provide a site into which fluid exudate accumulates and inflammatory cells migrate (Clarke et al., 1975).

1.3.6 Adjuvant-induced arthritis in rats.

The adjuvant-induced arthritis model is a complex model of polyarthritis which is caused by the intradermal injection of a combination of heat-killed mycobacterium emulsified in an oily vehicle (Complete Freund's Adjuvant). Assessment of various disease parameters can be made approximately 12 days after the injection (Whitehouse et al., 1974). This model is more complex than the other models desribed above because the disease affects the whole animal. The major problem with this model is that there is a great deal of variation within experimental groups and between rat strains (Swingle et al., 1969) with respect to induction and severity of disease. Therefore, although this model is more representaive of an overall "arthritic condition", its usefullness can be limited by this variability.

1.4 THE NEUTROPHIL.

1.4.1 Introduction.

Polymorphonuclear leukocytes (PMNL) are constant features of acute, subacute and sometimes chronic inflammation. They possess powerful mechanisms for host defense and are important in the elaboration and amplification of inflammatory responses. However, if they accumulate in excessive numbers, and their defense mechanisms are activated uncontrollably, loss of function and tissue damage may occur.

There are three classes of PMNL in humans - neutrophils, eosinophils and basophils all of which are characterised as such by the staining characteristics of their granules. The differential leukocyte count in human adults is approximately 40-60% neutrophils, 0-3% eosinophils and 0-1% basophils (Lentner, 1984). In general, the primary function of neutrophils and eosinophils is to ingest and destroy bacteria and parasites, whereas basophils are associated with allergic reactions. This section of the literature review will focus primarily on the neutrophil because this was the cell type studied during the course of this thesis.

Neutrophils are derived from pleuripotential stem cells in the bone marrow (Quesenberry and Levitt, 1979). Beginning as myeloblasts, in 7 days they differentiate into cells capable of ingestion and killing of microorganisms (Wade and Mandell, 1983). Two populations of granules appear in the cytoplasm during development; a) primary

(azurophillic) granules which contain mainly myeloperoxidase, acid hydrolases, cationic proteins, lysozyme and elastase, and b) secondary (specific) granules which contain predominantly lysozyme, lactoferrin and collagenase (Bainton et al., 1971). The cells remain in the bone marrow for approximately 5 days following maturation after which they migrate into the circulatory system. At any one time only about half of the cells are circulating - the other half are attached to endothelium, a process known as margination (Wade and Mandell, 1983). There are higher percentages of eosinophils and basophils in normal tissue than neutrophils which are usually found in tissue only in response to irritation or infection (Goetzl and Goldstein, 1985).

1.4.2 Recognition.

The neutrophil membrane recognises certain chemical structures on the surface of external objects, some of which exist naturally although most objects must be "opsonised" for recognition to occur (Hoffstein et al., 1982). The recognition sites on the plasma membrane are specific receptors and the human neutrophil possesses receptors for compounds such as complement fragments (C3b) or the Fc portion of immunoglobulins (Michl et al., 1979). Activation of these receptors initiates phagocytosis whereas activation of receptors for C5a and FMLP initiate chemotaxis (Hoffstein et al., 1982).

Little is understood regarding the initial events following receptor binding, however initiation of most of these functions involves alterations in Ca^{++} homeostasis (Yin and Stossel, 1982). For example, the "motor" event for chemotaxis, phagocytosis and

degranulation is thought to be mediated by interactions between actin and other proteins and this interaction is Ca^{++}-dependent (Yin and Stossel, 1982).

1.4.3 Chemotaxis.

Chemotaxis is the process of directed movement in a concentration gradient toward a chemotactic factor (Wade and Mandell, 1983). Upon stimulation by chemotactic factors, neutrophils leave the circulation by squeezing between capillary endothelial cells (Kawaoka et al., 1981), a process known as diapedesis. A prerequisite for accumulation of neutrophils at sites of irritation is the local generation of chemotactic factors (Goetzl and Goldstein, 1985). In fact, the introduction of certain microorganisms into tissues results in production of such stimuli (Snyderman and Goetzl, 1981).

1.4.4 Phagocytosis.

The ingestion of foreign or suitably opsonised particles is known as phagocytosis (Wade and Mandell, 1983). This is a key process in host defense. For phagocytosis to occur, a particle must be recognised by receptors on the surface of the cell (Griffin and Silverstein, 1974). Most organisms or particles require opsonisation with either C3b or IgG although some are recognised without opsonisation (for example, nonencapsulated pneumococci or several gram negative rods) (Wade and Mandell, 1983). Complement fixation can occur via activation of either the classical or alternative complement pathways, both of which lead to activation of an esterase which

cleaves the C3 component to C3a and C3b (Wade and Mandell, 1983). The classical pathway is antibody dependent whereas the alternative pathway is activated by cell wall components of several microorganisms (Wade and Mandell, 1983).

Although initially believed to be caused by membrane perturbation following receptor occupancy, phagocytosis is now thought to occur along the lines of a theory known as the "Zipper Theory" of phagocytosis. Griffen et al.,(1975) claimed that phagocytosis results from a sequential interaction of ligands and receptors around the surface of a particle, a process analogous to the opening and closing of a zipper. The mechanism for ingestion (and movement) is based on a meshwork of microfilaments containing actin and the contractile protein myosin (Boxer and Stossel, 1976).

1.4.5 Degranulation.

As a particle or bacterium is ingested, specific granules fuse with the developing phagosome whereupon the contents of the granule are released into the phagosome and the extracellular millieu (Wade and Mandell, 1983). This process is known as degranulation. As the phagosome closes and is drawn into the cell, metabolic activity increases and primary granules fuse to the phagosome and release their contents (Root and Cohen, 1981). Phagocytes possess two types of microbicidal chemicals; oxygen-dependent (oxy-radicals) and non-oxygen-dependant (Wade and Mandell, 1983). The non-oxygen-dependant mechanisms have been described above (lysozyme, lactoferrin, cationic proteins) (Section 1.4.1).

Activation of the oxygen-dependent mechanism is a consequence of the respiratory burst and is usually initiated by phagocytosis although it can occur in the absence of phagocytosis (Goetzl and Goldstein, 1985). The respiratory burst, (an increase in O_2 consumption), through a series of enzymatic steps results in production of the toxic oxy-radicals; superoxide (O_2^-); hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$)(Klebanoff, 1980). It has been suggested that oxy-radicals are mediators of inflammation (Goetzl and Goldstein, 1985). Oxy-radicals injure tissues (they cause lipid peroxidation of cell membranes), and irreversibly alter macromolecules such as hyaluronic acid, collagen, alpha-1-proteinase inhibitor and DNA (Goetzl and Goldstein, 1985). Secondly, superoxide dismutase and other scavengers of oxy-radicals prevent tissue injury in vitro and possess antiinflammatory effects in vivo. Furthermore, some commonly used antiinflammatory agents either scavenge or inhibit the production of oxy-radicals by phagocytes (Goetzl and Goldstein, 1985).

1.4.6 The biochemical pathways leading to superoxide production.

The key enzyme in the oxy-radical generating pathway is an NADPH oxidase which converts O_2 to O_2^-. In addition to the NADPH oxidase, the hexose monophosphate shunt is activated, thereby generating more NADPH (the substrate for NADPH oxidase). The cell possesses intracellular protective mechanisms against these powerful molecules. For example, superoxide dismutase converts O_2^- to H_2O_2 and catalase and glutathione peroxidase convert the latter to water and O_2 (Goetzl and Goldstein, 1985).

Several agonists have for years been used as stimuli for in vitro studies of O_2^- production in phagocytes. They can be divided into two main classes - receptor-dependent (eg: FMLP, IgG, STZ, C5a) or receptor-independent/soluble stimuli (eg: A23187, fluoride or PMA).

The pathways leading to activation of oxy-radical production are shown in Figure 1.6. Receptor-ligand interactions, via a guanine nucleotide binding protein, stimulate phospholipase C (PLC)-dependent breakdown of phosphatidyl inositol-4,5-bisphosphate (PIP_2), generating inositol-1,4,5-tris phosphate (IP_3) and diacyl glycerol (DAG) (Downes and Michell, 1985). IP_3 is released into the cytoplasm where it can stimulate a transient rise in intracellular Ca^{++} from the endoplasmic reticulum (Berridge and Irvine, 1984). Possible consequences of this include activation of PLA_2 and calmodulin-dependent protein phosphorylation. The other product of the PI cycle, DAG, binds to and activates protein kinase C (PKC) (Nishizuka, 1984).

PKC is a cytoplasmic protein which upon activation becomes closely bound to the cell membrane (Wolfson et al., 1985). Several factors are required for complete activation of PKC. For example, translocation and binding of PKC to the cell membrane is greatly enhanced by Ca^{++}. Furthermore, once bound to the cell membrane, phosphatidyl serine acts as a cofactor, further enhancing activation (Konig et al., 1985). A number of proteins, including transferrin (Stratford et al., 1984), interleukin-2 receptors (Shackelford and Trowbridge, 1984), lipomodulin (Hirata et al., 1984), and the NADPH oxidase (Genaro et al., 1985) are phosphorylated by the active form of

PKC.

It is interesting to note that biochemical pathways can now be assigned to most of the agonists which stimulate O_2^- production by phagocytes. The external membrane receptor-dependent stimuli have been discussed already. A23187 mimics the effect of IP_3 by raising intracellular Ca^{++} (Nishizuka, 1984), while phorbol esters such as PMA mimic DAG and directly activate PKC (Nishizuka, 1984) (Figure 1.6).

While studies on the biochemical chain of events leading to O_2^- production have recently proven fruitful, the same cannot be said of the pathways leading to LT production. Of the agonists used to stimulate O_2^- production only A23187 generates large quantities of LTs and the mechanism by which this is achieved is uncertain. FMLP is a potent stimulus of O_2^- production but appears to require exogenous AA to stimulate LT production and PMA appears totally ineffective as a LT stimulus. Several of the studies described in this thesis were designed to shed more light on some of the unanswered questions regarding pathways of LT production in human neutrophils.

1.5 OUTLINE OF STUDIES.

With the exception of the studies on rats, all of the experiments described in this thesis were performed on isolated human peripheral blood PMNL neutrophils. This cell type was chosen because it is an important inflammatory cell and produces large amounts of LTB_4.

The first experimental chapter (Chapter 3) describes the setting up of a reliable and sensitive HPLC assay for the separation and quantitation of LTB_4, the all-trans isomers of LTB_4, and 5-HETE, thus enabling assessment of the relative activities of PLA_2, 5-LO, LTA synthetase and LTA hydrolase. This assay provided the backbone of almost all of the experimental work performed during the course of study.

Experiments to characterise the 5-LO system of human neutrophils using the HPLC assay are described in Chapter 4. These experiments included dose-response curves and time courses of stimulation to A23187 and FMLP (with AA) and assessment of the day to day variation which occurs within the 5-LO system and the variation which occurs between donors. Evaluation of the dependence of the response to A23187 on Ca^{++} was also undertaken.

The next two chapters (5 and 6) describe work investigating the effect of EPA on human neutrophil 5-LO metabolism in vitro (Chapter 5) and on inflammatory models in rats (Chapter 6). Chapter 5 describes how EPA-derived 5-LO metabolites were separated and quantified. Experiments investigating the effect of EPA on both endogenous and exogenous AA metabolism by stimulated human neutrophils are also described. An extensive series of experiments evaluating the effect of modification of dietary PUFA on inflammatory models in rats is described in Chapter 6.

In the final experimental chapter, experiments examining the effect of the phorbol ester PMA on LT synthesis by human neutrophils

in response to A23187 and FMLP are described.

Abbreviations for Table 1.1.

A23187 - calcium ionophore
AA - archidonic acid
STZ - serum treated zymosan
FMLP - n-formyl-methionyl-leucyl-phenylalanine
cyto B - cytochalasin B
LPS - lipopolysaccharide
PHA - phytohaemagglutinin
L - lysis
D - dextran sedimentation

Table 1.1 Arachidonic acid lipoxygenase metabolites produced by various cell types in response to different stimuli.

Stimulus	Products	Detection	Cell Preparation	Reference
Neutrophil - Human				
A23187 + AA	5-,15-HETE, LTB_4	HPLC-GCMS	Lymphoprep	Borgeat and Samuelsson, 1979c
AA	5,8,9,11,12,15-HETE	HPLC	Ficoll-Hypaque	Goetzl and Sun, 1979
A23187	5,12-HETE,LTB_4	HPLC	"	Ford-Hutchinson et al., 1980
"	5,11,12,15-HETE	HPLC	Lymphoprep (L)	Walsh et al., 1980
A23187 ± AA STZ ± AA FMLP + AA	LTB_4,TXA_2	RIA	Ficoll-Paque (L)	Palmer and Salmon, 1983
AA (ethanol) FMLP + AA C_5a + AA	5-HETE,LTB_4	HPLC	Metrizamide	Clancy et al., 1983;1985
A23187 ± AA	LTB_4,20-OHLTB$_4$ 20-COOHLTB$_4$, LTB_4 all trans isomers 5-HETE	HPLC	Ficoll-Hypaque	Sun and McGuire, 1984
MSU ± AA AA	LTB_4,20-OHLTB$_4$ LTB_4 all trans isomers 12,15-HETE	HPLC	Lymphoprep	Serhan et al., 1984
A23187 Zymosan (U)	LTB_4,20-OHLTB$_4$ 20-COOHLTB$_4$ LTB all trans isomers 5-HETE	HPLC/RIA	Ficoll-Hypaque (L)	Williams et al., 1985

FMLP ± cyto B FMLP ± AA	LTB_4,5-HETE 20-OHLTB_4 20-COOHLTB_4	HPLC	Ficoll-Hypaque (D,L)	Salari et al., 1985b
Human serum (C_8) A23187	LTB_4	HPLC/RIA	Percoll (L)	Seeger et al., 1986
S.aureus + AA	LTB_4	HPLC	Fico-Paque (D,L)	Hendricks et al., 1986
Agg IgG + AA	LTB_4	RIA	Ficoll-Hypaque (D,L)	Smith et al., 1986
A23187 ± AA	LTB_4,5-HETE	HPLC	Percoll (D)	McColl et al., 1986

Neutrophil - Rabbit Peritoneal

AA	5-HETE	HPLC-GCMS	Glycogen	Borgeat et al., 1976
AA	LTB_4	HPLC-GCMS	Glycogen	Borgeat and Samuelsson, 1979a
AA	LTB_4,5s12sdiHETE LTB all trans isomers	HPLC-GCMS	Glycogen	Borgeat and Samuelsson, 1979d

Neutrophil - Rat Peritoneal

A23187	LTB_4	HPLC	Glycogen	Ford-Hutchinson et al., 1980
A23187 + AA	LTB_4,5-,15-HETE	HPLC-GCMS	Glycogen	Vanderhoek et al., 1980

Neutrophil - Guinea Pig Peritoneal

A23187 FMLP	TXB_2,PGE_2, 5-HETE	HPLC	Casein	Bokoch et al., 1980

Eosinophil - Human

A23187 + AA C_5a + AA FMLP + AA	LTB_4 5,9,11-HETE	HPLC	Metrizamide	Goetzl et al., 1980b
A23187	5,15-HETE,LTB_4	HPLC	Metrizamide	Turk et al., 1982
A23187 + AA	LTC_4,LTB_4, 20-OHLTB_4,5-HETE	HPLC	Ficoll-paque	Borgeat et al., 1984
A23187 STZ	LTC_4	HPLC/RIA	Ficoll-paque Percoll (L)	Bruynzeel et al., 1985

Basophil - Human

A23187 anti-IgE	PGD_2,LTB_2, LTC_4,5-HETE	HPLC	Elutriation Percoll	Peters et al., 1982

Basophil - Murine Mastocytoma

AA (ethanol)	PGD_2,LTC_4,LTD_4	HPLC	Cultured Line	Westcott and Murphy, 1983

Basophil - Rat Basophilic Leukaemia

A23187	LTC_4,LTD_4,LTE_4	HPLC	Cultured Line	Morris et al., 1980
"	"	"	"	Parker et al., 1980b

Monocyte - Human

RBC coated IgG A23187	LTC_4,LTB_4	HPLC/RIA	Ficoll-hypaque (D) Adherence	Williams et al., 1984
A23187 FMLP + Cyto B	LTC_4,LTD_4,LTB_4	HPLC/RIA	"	Williams et al., 1986
A23187	LTC_4,LTB_4,PGE_2	HPLC/RIA	Ficoll-hypaque Percoll	Ferreri et al., 1986

Macrophage - Rabbit Alveolar

A23187 STZ	LTB_4	HPLC-GCMS	Lung Lavage	Hsueh et al., 1982
A23187	LTB_4,5-HETE	HPLC	"	Hsueh et al., 1985

Macrophage - Rat Peritoneal

A23187	LTB_4,5,12,15-HETE	GCMS	Heparin	Doig and Ford-Hutchinson, 1980

Macrophage - Murine Peritoneal

Zymosan (U)	LTC_4	RIA	Peritoneal Lavage	Rouzer et al., 1980
Zymosan (U) IgG, IgE	LTC_4, PGE_2, 6-keto$PGF_{1\alpha}$	RIA	"	Rouzer et al., 1982
A23187 Zymosan (U)	"	RIA	Peritoneal Lavage	Boraschi et al., 1985
A23187, Latex, Zymosan (U)	LTC_4, PGE_2, TXB_2, 6-keto$PGF_{1\alpha}$	HPLC	"	Tripp et al., 1985
LPS	LTC_4, PGE_2, 6-keto$PGF_{1\alpha}$	HPLC (LT), RIA (PG)	"	Luderitz et al., 1986

Lymphocyte - Human

PHA + AA	5,12-HETE, TXB_2	TLC	Nylon Fibre Adherence	Parker et al., 1979
A23187	5-HETE, LTB_4, LTB_4 isomers	HPLC	Ficoll-Paque (D)	Goetzl, 1981a

Platelet - Human

AA	12-HETE	HPLC		Hamberg et al., 1974
AA	12-HETE	HPLC		Lagarde et al., 1984

Erythrocyte - Rabbit, Rat, Dog, Chicken

A23187	12-HETE	HPLC	Extensive washing, Sepaharose 4B column	Kobayashi and Levine, 1983

Table 1.2: Generation of leukotrienes in tissue and exudate fluid.

Source	Species	Stimulus	Products
A. Tissue			
Brain tissue	Rat	A23187	LTC_4
Forebrain tissue	Gerbil	Ischaemia	LTC_4, D_4
Colonic tissue	Guinea-Pig	Ovalbumin	"SRS-A"
Aortic tissue	Guinea-Pig	Ovalbumin	"SRS-A"
Ear tissue	Mouse	Topical AA	LTC_4, D_4
Lung tissue	Guinea-Pig	C_5a, C_5a des arg	"SRS-A"
		A23187	LTB_4, C_4, D_4
Trachea	Guinea-Pig	A23187	LTC_4
Mastocytoma	Dog	A23187, AA, antigen, Ovalbumin	LTB_4, C_4, D_4
Liver microsomes	Rat	LTA_4	LTB_4, C_4, D_4
Perfused paw	Cat	48/80	LTD_4, E_4
B. Effusions			
Peritoneal	Rat	Antigen	LTB_4
Pleural	Rat	Carrageenan	LTB_4, C_4, D_4
Sponge exudate	Rat	A23187, FMLP, Zymosan, Uric acid, Carrageenan	LTB_4, C_4
Bile samples	Rat	Endotoxin	LTC_4, D_4

Table reproduced from Bray (1986)

Table 1.3 Some of the reported pharmacological effects of prostaglandins and LTC_4, D_4 and E_4.

Eicosanoid	Effect	Reference
PGE_2	relaxation of smooth muscle	Oliw et al., 1983
	inhibition of gastric acid release	Robert et al., 1976
	vasodilation	Nakano et al., 1973
	reduction of blood pressure	Anggard and Bergstorm, 1963
	pyrogen	Feldberg and Milton, 1978
	plasma exudation (with LTB_4)	Wedmore and Williams, 1981
	chemokinesis	Goetzl et al., 1979
	inhibition of lymphokine generation	Gordon et al., 1976
	increase collagenase secretion	Lippiello et al., 1978
	decrease proteoglycan secretion	"
$PGF_{2\alpha}$	contraction of smooth muscle	Goldberg and Ramwell, 1975
		Mathe et al., 1972
	vasoconstriction	Chapnick et al., 1978
	increase blood pressure	"
PGI_2	inhibit platelet aggregation	Moncada et al., 1976
	inhibit neutrophil adherence	Boxer et al., 1980
	chemokinesis	Goetzl et al., 1979
TXA_2	platelet aggregation	Hamberg et al., 1975
	neutrophil adherence	Goetzl et al., 1979
PGG_2/H_2	vasodilation	Feigen et al., 1978
	platelet aggregation	"
PGD_2	renal vasodilation	Feigen et al., 1977
	mesenteric vasoconstriction	"
	chemokinesis	Goetzl et al., 1979
	inhibition of lymphokine generation	Gordon et al., 1976
LTC_4, D_4, E_4	bronchoconstriction	Hedquist, 1982
	vascular permeability	"
	vasoconstriction	Michelassi et al., 1983
	reduction of coronary blood flow	"

Table 1.4 Some of the reported pharmacological effects of the monoHETEs.

Cell Type	Effect	monoHETEs	Reference
Neutrophil	chemotaxis	5-,8-,9-,11-,12- & 15-HETE	Goetzl et al., 1980a
		5-,11- & 12-HETE	Palmer et al., 1980
		5-,11- & 12-HETE	Goetzl and Pickett, 1980
		5- & 12-HETE	Stenson and Parker, 1980
	chemokinesis	5-,8-,9-,11-,12- & 15-HETE	Goetzl et al., 1980a
	degranulation	5- & 12-HETE	Stenson and Parker, 1980
	inhibit 5-LO	5-,12- & 15-HETE	Chang et al., 1985a
	FMLP-induced O_2^- production	5-,12- & 15-HETE	Beckman et al., 1985
	hexose uptake	5-HETE	Bass et al., 1981
	Ca^{++} homeostasis	5-HETE	Naccache et al., 1981
	C3b receptor expression	5-HETE	Goetzl et al., 1980b
Eosinophil	chemotaxis	5-,8-,9-,11-,12- & 15-HETE	Goetzl et al., 1980a
	chemokinesis	"	"
	hexose uptake	5-HETE	Bass et al., 1981
	C3b receptor expression	5-HETE	Goetzl et al., 1980b
Lymphocyte	inhibits 5-LO	15-HETE	Goetzl, 1981a
Platelet	12-LO activity	12-HPETE	Siegel et al., 1979
	inhibit CO activity	"	"
	inhibit platelet aggregation	"	"
	inhibit PLA_2	5,-12- & 15-HETE	Chang et al., 1985b
Basophil	inhibit histamine release	5-HPETE & 5-HETE	Peters et al., 1982
Other Tissue	mucus secretion	15-HETE	Johnson et al., 1985
		12- & 15-HETE	Marom et al., 1983
	inhibit insulin release	12- & 15-HETE	Yamamoto et al., 1983

Table 1.5 Some of the reported pharmacological effects of LTB_4.

Cell Type	Effect	Reference
Neutrophil	chemotaxis	Malmsten et al., 1980 Palmblad et al., 1981 Rollins et al., 1983 Dahinden et al., 1984
	chemokinesis	Ford-Hutchinson et al., 1980
	aggregation	Ford-Hutchinson et al., 1980
	adherence	Palmblad et al., 1981 Dahlen et al., 1981
	degranulation (cytochalasin B dep)	Feinmark et al., 1981 Hafstrom et al., 1981 Showell et al., 1982a Rollins et al., 1983 Lew et al., 1984
	Ca^{++} homeostasis	Naccache et al., 1981 Lew et al., 1984
	FMLP-induced O_2^- production	Gay et al., 1984
	C3b receptor expression	Nagy et al., 1982
	hexose uptake	Bass et al., 1981
Eosinophil	chemotaxis	Uden et al., 1983
	C3b receptor expression	Nagy et al., 1982
Monocyte	IL-1 production	Rola-Pleszcynski and Lemaire 1985
Lymphocyte	natural cytotoxic cell activity	Rola-Pleszcynski et al., 1983
	inhibits LIF production	Payan and Goetzl, 1983
	inhibits T cell proliferation	Rola-Pleszcynski et al., 1982
Other tissue	contracts lung parenchyma	Sirois et al., 1980
	contracts perfused lung	Sirois et al., 1980
	bradykinin-induced plasma exudate	Bray et al., 1981

Table 1.6 **Some of the reported inhibitors of leukotriene synthesis and action.** Sites of action; 1, phospholipase A_2; 2, 5-lipoxygenase; 3, cyclooxygenase; 4, other lipoxygenases; 5, LTA synthetase; 6, LTA hydrolase; 7, glutathione-S-transferase; *, receptor antagonist.

Drug	Site of action	ID_{50}	Reference
Dexamethasone	1	0.003 uM	Robinson, 1985
Hydrocortisone	1	0.003 uM	Robinson, 1985
BW775C	2,3,4	5 uM	Higgs et al., 1981; Randall et al., 1980
Benoxaprofen	2,3	"	
ETYA	1,2,3,4,5,6	2 uM	Bach et al., 1977; Engineer et al., 1978
NDGA	2,4	3 uM	Walker et al., 1980; Bokoch and Reed, 1981
Esculetin	2,3,4	4 uM	Neichi et al., 1983; Sekiya et al., 1982
Quercetin	2,4	0.2 uM (2), 5 uM (4)	Hope et al., 1983
Cirsiliol	2,4	0.1 uM (2), 1 uM (4)	Yoshimoto et al., 1983
Caffeic acid	2	3.7 uM	Koshihara et al., 1983; 1984
Caffeic acid (methyl ester)	2	0.48 uM	Koshihara et al., 1984
Sulfasalazine	2	1 mM	Stenson and Lobos, 1982
Piriprost	2	5 uM	Bach and Brasher, 1986
5,6 dehydro-AA	2	10 uM	Corey and Munroe, 1982; Sok et al., 1982
5,6 methano-LTA_4	2	3 uM	Koshihara et al., 1982; Arai et al., 1983
15-HETE	2 (4?)	6 uM	Vanderhoek et al., 1980; Goetzl 1981a
Ebselen	6	2.7 uM	Kuhl et al., 1985
Diethylcarbamazine	5	5 uM	Mathews and Murphy, 1982
5 aminosalicylate	5,6 ?	1 mM	Stenson and Lobos, 1982
Bilirubin	7	1 uM	Kaplowitz et al., 1975
FPL55712	*	1 uM	Augstein et al., 1973
FPL59257	*	5 uM	Sheard, 1981; Sheard et al., 1982
LTB_4 dimethylamide	*	0.003-0.01 uM	Showell et al., 1982b

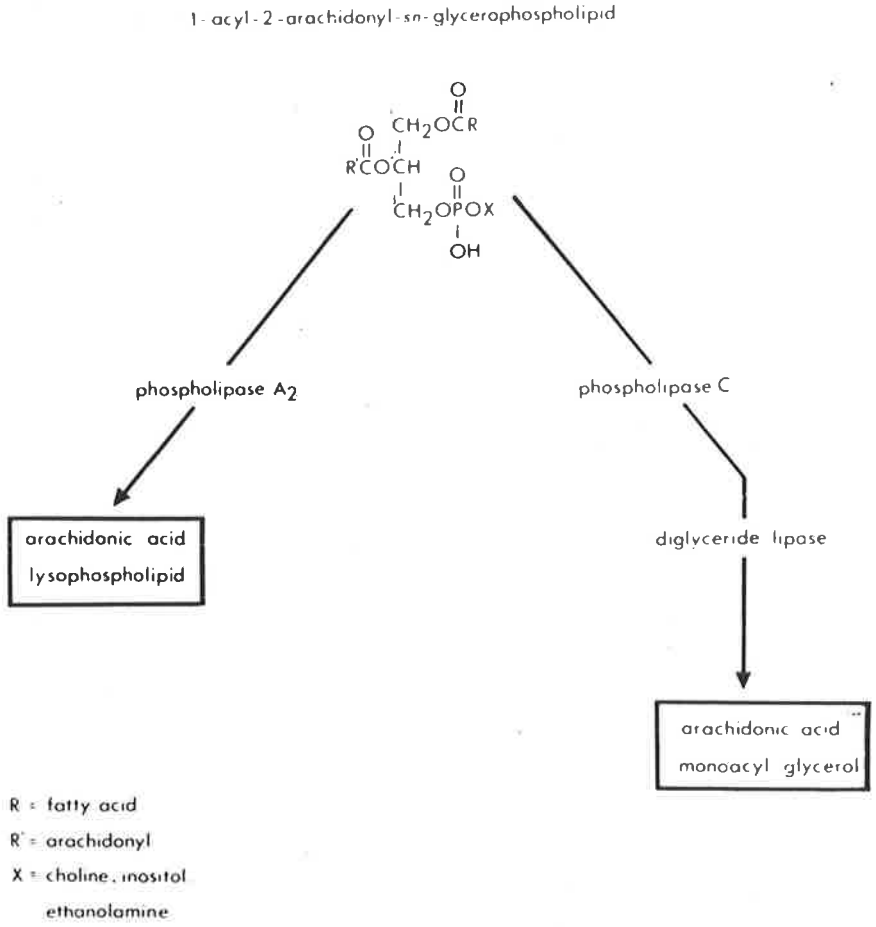


Figure 1.1: Two possible mechanisms for arachidonic acid release within leukocytes. (Adapted from Johnson et al 1983).

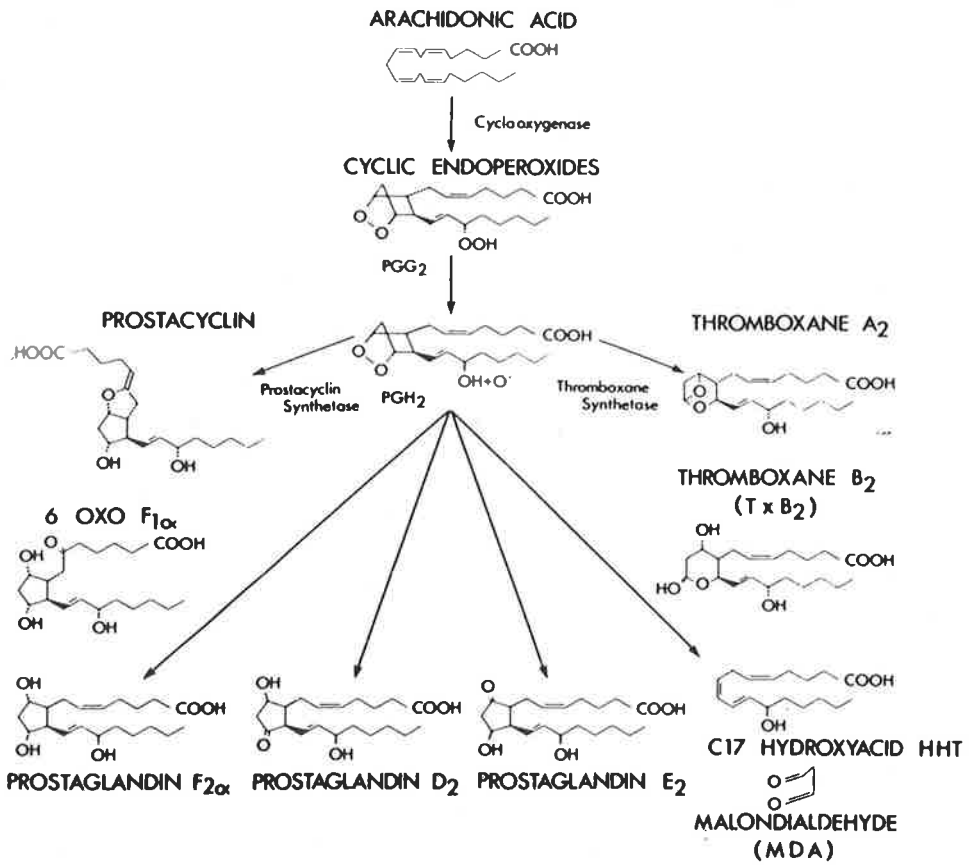


Figure 1.2: The prostaglandin pathway of arachidonic acid metabolism. (From Bhattacherjee and Eakins 1984).

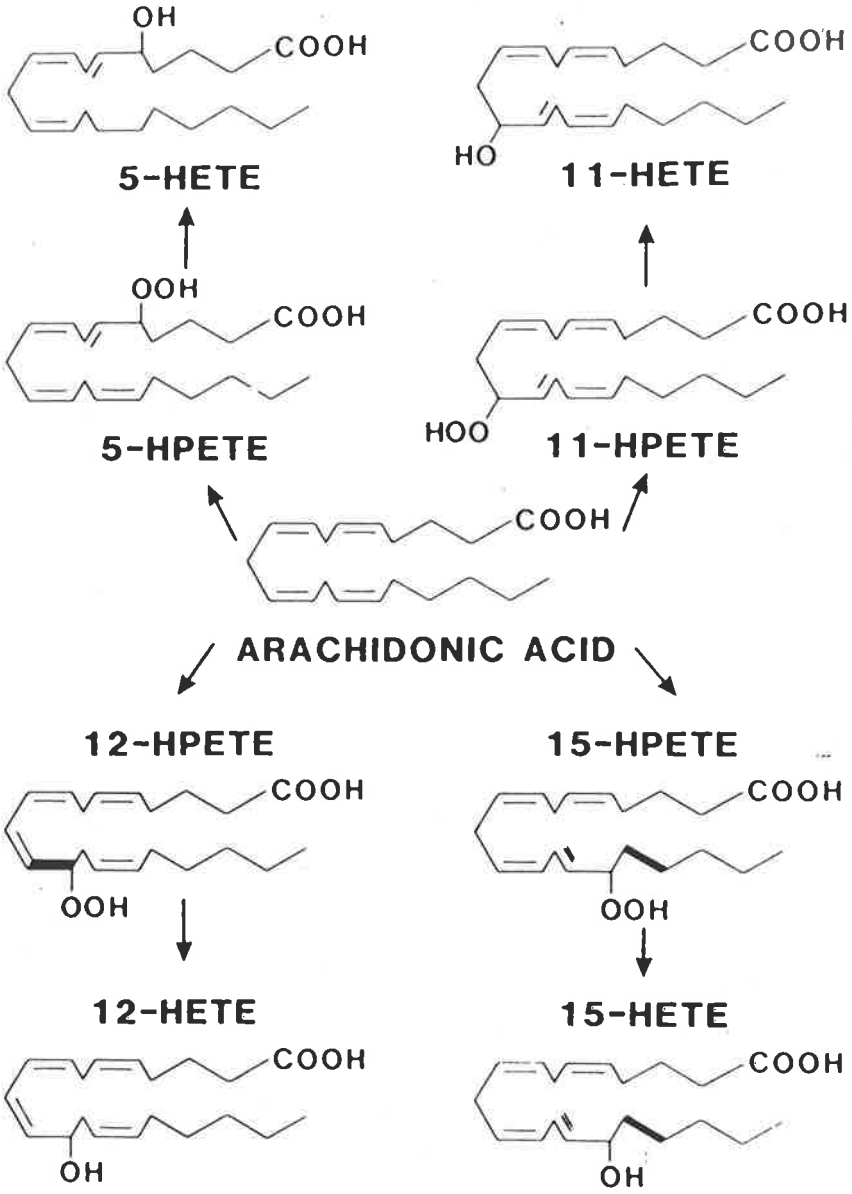


Figure 1.3: The generation of different monoHETEs from arachidonic acid. Production of specific monoHETEs is controlled by specific lipoxygenases. (From Bhattacherjee and Eakins 1984).

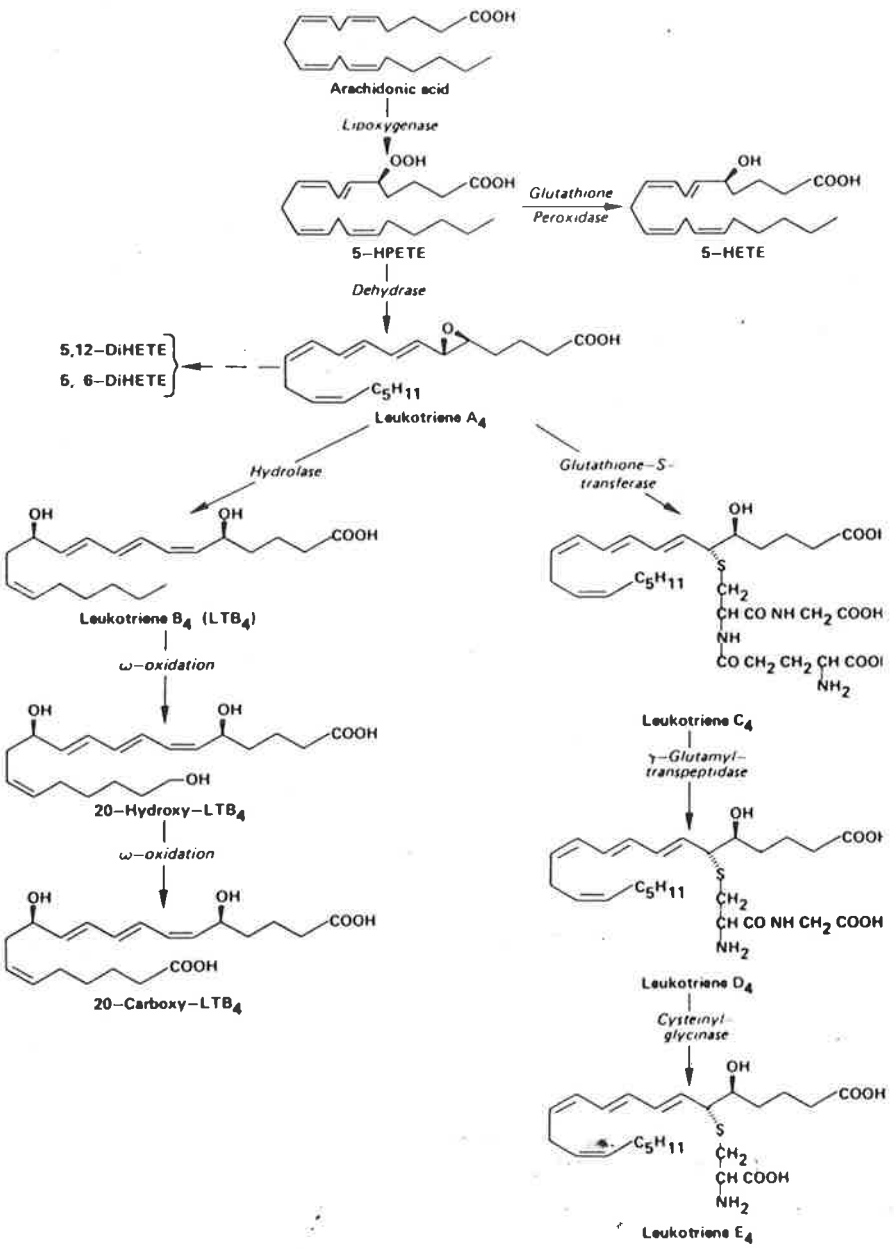


Figure 1.4: The 5-lipoxygenase pathway of arachidonic acid metabolism. (From Bhattacherjee and Eakins 1984).

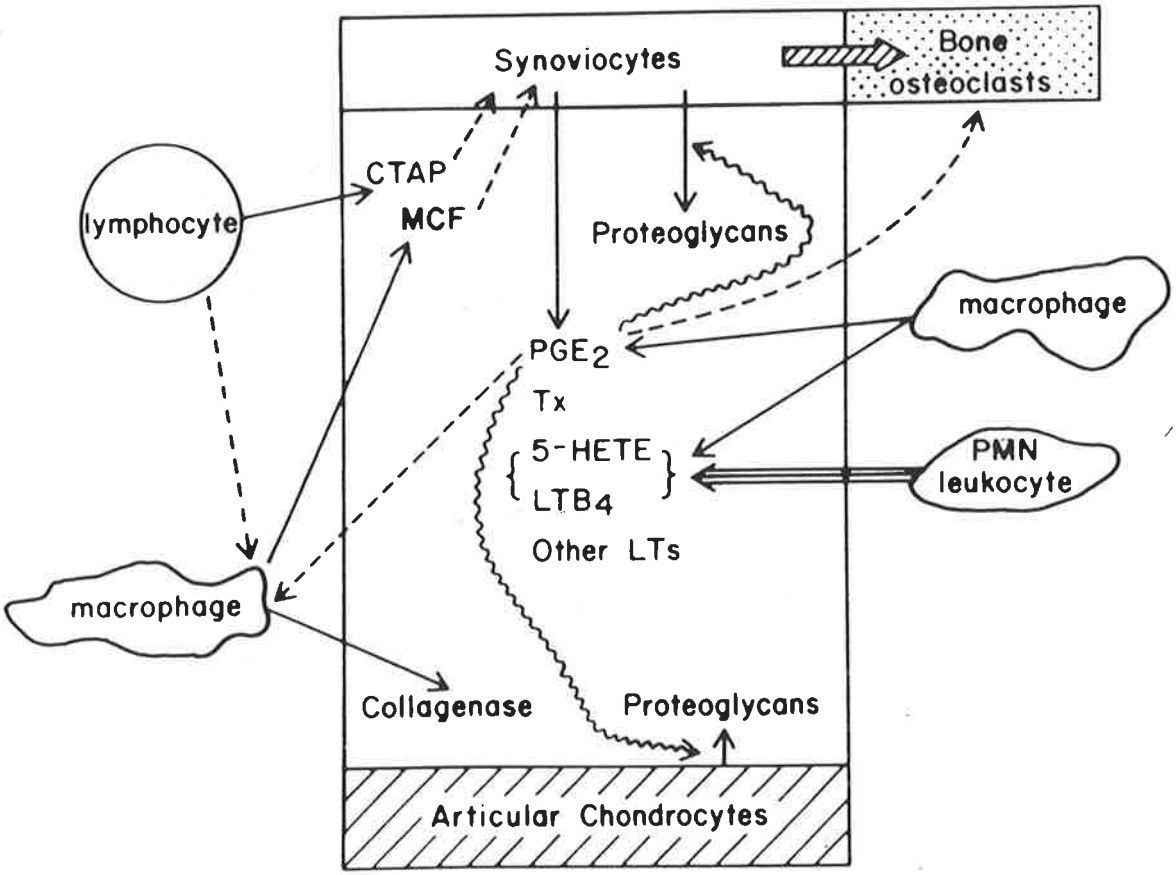


Figure 1.5: Possible role of arachidonic acid metabolites in rheumatoid arthritis. CTAP, connective tissue activating peptide; MCF, mononuclear cell factor; ~~~> erosion of bone; ⟹ chemotactic response. See text for details. (From Goetzl 1981).

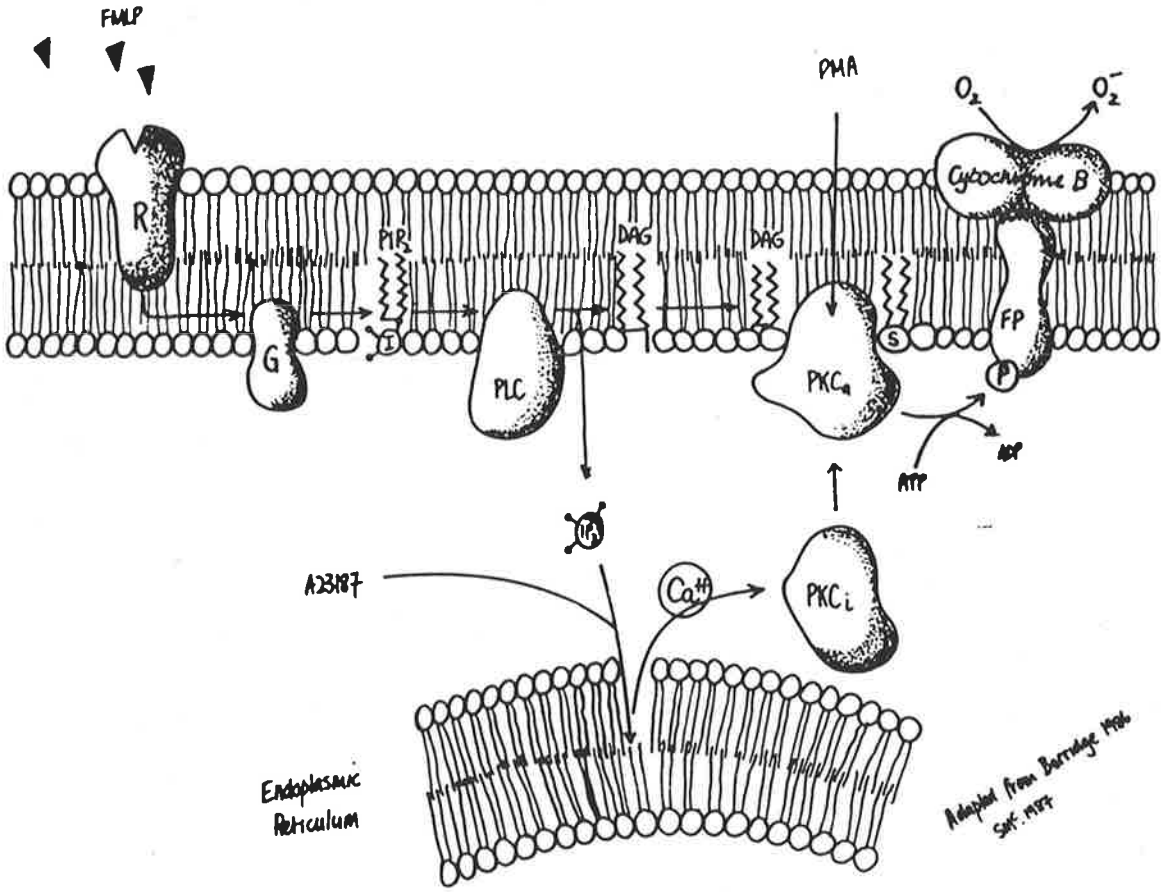


Figure 1.6: Putative pathways leading to superoxide production. FMLP, n-formyl-methionyl-leucyl-phenylalanine; R, receptor; G, guanine nucleotide binding protein; PIP_2, inositol 4,5 bisphosphate; PLC, phospholipase C; DAG, diacylglycerol; IP_3, inositol 1,4,5 trisphosphate; FP, flavoprotein; S, phosphatidyl serine; PKC, protein kinase C; O_2^-, superoxide; PMA, phorbol myristate acetate.

CHAPTER 2: MATERIALS & METHODS.

2.1 MATERIALS.

2.1.1 Chemicals. (* brackets indicate common abbreviations.)

1. Calcium Ionophore (A23187)
   -Sigma Chemical Company, St. Louis, Missouri, USA

2. N-Formyl-l-Methionyl-l-Leucyl-l-Phenylalanine (FMLP)
   -Sigma Chemical Company, St. Louis, Missouri, USA

3. Phorbol 12-Myristate 13-Acetate (PMA)
   -Sigma Chemical Company, St. Louis, Missouri, USA

4. 15-Lipoxygenase (Linoleate: Oxygen Oxidoreductase)
   -Sigma Chemical Company, St. Louis, Missouri, USA

5. Methanol - Waters Millipore, HPLC grade
   -Waters Associates, Milford, Massachusetts, USA

6. Chloroform - Waters Millipore, HPLC grade
   -Waters Associates, Milford, Massachusetts, USA.
   Redistilled prior to use.

7. Ethyl Acetate - Waters Millipore, HPLC grade
   -Waters Associates, Milford, Massachusetts, USA.
   Redistilled prior to use.

8. All water double distilled at TQEH Pharmacy and further deionised and filtered using a 4-Bowl Milli-Q System - Waters Associates, Milford, Massachusetts, USA

9. 5,8,11,14-Eicosatetraenoic acid (AA)
-Sigma Chemical Company, St. Louis, Missouri, USA.

^{3}H-AA [5,6,8,9,11,12,14,15-^{3}H(N)], 60-100 Ci/mmol
-Du Pont - NEN Research Products, North Sydney, Australia.

10. 5,8,11,14,17-Eicosapentaenoic acid (EPA)
-Sigma Chemical Company, St. Louis, Missouri, USA

[1^{14}C]Eicosa-5,8,11,14,17-pentaenoic acid, 50-60 Ci/mmol
-Amersham Australia Pty Limited, North Ryde, Sydney.

11. Butylated hydroxy toluene (BHT)
-Sigma Chemical Company, St. Louis, Missouri, USA

12. Acetic acid (analytical grade)
-Ajax Chemicals Pty. Ltd., Auburn, Australia

13. Ammonium hydroxide (analytical grade)
-BDH Chemicals, Port Fairy, Australia

14. Citric acid
-Sigma Chemical Company, St. Louis, Missouri, USA

15. Quercetin (3,3',4',5,7-Pentahydroxyflavone)
-Sigma Chemical Company, St. Louis, Missouri, USA

16. Nordihydroguaiaretic acid (4,4'-[2,3Dimethyl
1,4-butanediyl]-bis[1,2-benzenediol] (NDGA)
-Sigma Chemical Company, St. Louis, Missouri, USA

17. 5S,12R dihydroxy-eicosatetraenioc acid (LTB_4)
-Merck, Sharpe & Frosst, Canada.
-Upjohn Diagnostics, Kalamazoo, USA.

18. 5-Hydroxyeicosatetraenoic acid (5-HETE)
    -Merck, Sharpe & Frosst, Canada.
    -Organic synthesis (see methods).

19. 15-Hydroxyeicosatetraenoic acid (15-HETE)
    -Biosynthesis (see methods).

20. Ethylenediaminetetraacetic acid (EDTA)
    -Sigma Chemical Company, St. Louis, Missouri, USA

21. Ethylene glycol-bis(b-aminoethyl ether)
    N,N,N',N'-tetraacetic acid (EGTA)
    -Sigma Chemical Company, St. Louis, Missouri, USA

22. 3,4,5-Trimethoxy-benzoic acid 8-(diethylamino)-octyl ester
    (TMB-8)
    -Sigma Chemical Company, St Louis, Missouri, USA

23. Squalane (C_{30})
    -Fluka A.G., Buchs, Switzerland

24. Triolein
    -B.D.H. Chemicals, Sydney, Australia

25. Percoll
    -Pharmacia Laboratory Separation Division, Uppsala, Sweden

26. MaxEPA
    -R.P. Scherer Pty. Ltd., Oakleigh, Victoria, Australia

27. Sunflower Oil
    -Nuttlelex Food Products Pty. Ltd., Richmond, Australia.

28. Linseed Oil
    -Diggers Trading Company, Adelaide, Australia

29. Cod Liver Oil
    -Faulding Pharmaceuticals Pty. Ltd., Adelaide, Australia

30. Carrageenan
    -Sigma Chemical Company, St. Louis, Missouri, USA

31. Prostaglandin B_2 (PGB_2)
    -Sigma Chemical Company, St. Louis, Missouri, USA

32. Phosphatidyl Choline (PC)
    -Sigma Chemical Company, St. Louis, Missouri, USA

33. Phosphatidyl Inositol (PI)
    -Sigma Chemical Company, St. Louis, Missouri, USA

34. Phosphatidyl Ethanolamine (PE)
    -Sigma Chemical Company, St. Louis, Missouri, USA

35. Phosphatidyl Serine (PS)
    -Sigma Chemical Company, St. Louis, Missouri, USA

36. Adenosine Tri-Phosphate (ATP)
    -Sigma Chemical Company, St. Louis, Missouri, USA

37. Di-isopropylfluorophosphate (DFP)
    -Sigma Chemical Company, St. Louis, Missouri, USA

38. Deoxyribonuclease (DNase)
    -Promega Biotec, Madison, Wisconsin, USA

39. Acrylamide
    -Biorad Laboratories, Richmond, California, USA

40. N,N'-bis-methyleneacrylamide
    -Biorad Laboratories, Richmond, California, USA

41. Tetramethylethylenediamine
-Biorad Laboratories, Richmond, California, USA

42. Sodium dodecylsulphate (SDS)
-BDH, Chemicals, Sydney, Australia

43. Ammonium persulphate
-Biorad Laboratories, Richmond, California, USA

44. Bromophenol blue
-Biorad Laboratories, Richmond, California, USA

45. 2-mercaptoethanol
-Sigma Chemical Company, St. Louis, Missouri, USA

46. Coomassie blue
-Biorad Laboratories, Richmond, California, USA

2.1.2 Buffers

Two buffers were used throughout the course of this thesis:
- Dulbecco's Ca^{++} Mg^{++}-free Phosphate Buffered Saline (DPBS)
- Hank's Balanced Salt Solution (HBSS)

Ingredient	HBBS (g/L)	DPBS (g/L)
NaCl	8.0	8.0
KCl	0.4	0.2
$CaCl_2$	0.14	-
$MgCl_2.6H_2O$	0.2	-
$Na_2HPO_4.2H_2O$	0.06	1.15
KH_2PO_4	0.06	0.2
Glucose	1.0	-
dH_2O		

2.2 METHODS.

A. Human Studies.

2.2.1 Collection of whole human blood and purification of neutrophils.

Whole blood was collected by venepuncture and mixed with 4.5% EDTA (5:1, v:v). Dextran (6% dextran 500) was added (5:1, v:v) and the erythrocytes were sedimented at 37°C for 30 min. The white blood cell rich supernatant was collected and carefully layered onto the percoll separation medium.

Percoll was prepared as follows: 90 ml of Percoll was mixed with 10 ml 10x saline and adjusted to pH 7.0 with concentrated HCl. To 76.5 ml of diluted Percoll, 23.5 ml DPBS was added. The density of this medium was 1.092 g/ml at 25°C. To 58.5 ml of diluted Percoll, 41.5 ml of DPBS was added. The density of this medium at 25°C was 1.070 g/ml. 5 mls of the 1.092 g/ml solution was placed at the bottom of a 25 ml universal test tube (Filtrona cat. no. 128A) and 5 ml of the 1.07 g/ml solution was carefully layered above the 1.092 g/ml solution through a syringe barrel and a 21 gauge needle. This ensured an intact interface between the two solutions. 8 mls of the white blood cell rich supernatant was then layered above the top percoll layer through the syringe and needle. These test tubes were spun at 450 x g for 25 min after which the neutrophils were collected at the interface between the two percoll layers (see Figure 2.1). The

cells were then washed twice in DPBS in 50 ml polycarbonate test tubes. Prior to the second wash a lysis of contaminating erythrocytes was performed. This was achieved by exposing the cell pellet to 10 mls of 2 g/ml NaCl for 30 sec followed by 10 ml of 16 g/ml NaCl to restore isotonicity. The test tube was topped up with DPBS and spun at 500 x g for 7 min. After the two washes, the neutrophils were resuspended in HBSS at the concentration required for the experiment. Cell concentration was determined using a model ZF Coulter counter (Loos et al., 1976).

2.2.2 Cell stimulation.

PMA was dissolved in DMSO at a concentration of 1 mg/ml and stored at -10^{o}C. FMLP was dissolved in DMSO and stored at 4^{o}C at 20 uM. These stock solutions were diluted in DPBS to appropriate concentrations for each experiment. A23187, AA or EPA were dissolved in methanol at required concentrations and added directly to cell suspensions. A23187 was stored at 4^{o}C and AA and EPA were stored under nitrogen at -70^{o}C. The final concentrations of DMSO or methanol did not exceed 0.1 or 0.5% respectively and had no effect on release or detection of leukotrienes.

All cells were incubated at 37^{o}C for 15 mins prior to addition of stimuli. After stimulation for the desired period of time, 25 ul of 100 mM citric acid was used to stop cellular reactions.

2.2.3 Extraction of leukotrienes from cells.

After stimulation was terminated, 50 ng of PGB_2 and/or 170 ng of 15-HETE were added to each sample and LTB_4 and 5-HETE were added to tubes for standard curves. All tubes were vortexed briefly after which 2.5 mls of chloroform/methanol (7/3 v/v) was added. All tubes were then vortexed for 30 sec, centrifuged at 1000 x g for 5 min and the chloroform layer was removed. The chloroform was evaporated under vacuum in a Savant centrifugal evaporator, the samples were reconstituted in 100 ul of methanol and 25 ul was injected onto the HPLC.

2.2.4 Incorporation into and release of ^{3}H-AA from neutrophils.

Neutrophils were incubated with 0.1 uCi of ^{3}H-AA at 37°C for 1 hr. At 15 min prior to stimulation 10 uM NDGA was added to inhibit conversion of AA to leukotrienes. The cells were washed twice in DPBS resuspended in HBSS and incubated at 37°C for 15 min after which stimuli were added. After 5 min, reactions were stopped by addition of citric acid. The samples were centrifuged at 1000 x g for 5 min and the amount of ^{3}H-AA in 100 ul of supernatant was measured by liquid scintillation spectrometry.

2.2.5 Radiochromatography of ^{3}H-AA metabolites.

For experiments examining the metabolism of ^{3}H-AA by neutrophils, samples were treated as described in Sections 2.2.2

& 2.2.3. HPLC elution fractions were collected every 20 sec and the amount of ^{3}H present in each fraction determined by liquid scintillation counting.

2.2.6 Thin Layer Chromatography of phospholipids.

Thin layer chromatography of phospholipids was performed according to the method of von Schacky et al., (1985b). Phospholipids were extracted from the cells by the addition of 2.5 mls of chloroform/methanol (7/3). The samples were vortexed for 15 sec and centrifuged, and the chloroform layer was removed for concentration in a centrifugal evaporator (Savant). 10 ug of pure standards of PC, PI, PE and PS in chloroform were used as standards. The standards (approximately 40 ul) were loaded onto a Silica G60 TLC plate (Merck). The plates were run in a solvent system comprising chloroform/methanol/acetic acid/water (75/45/12/3, v/v/v/v) until the solvent front was approximately 1 cm from the top of the plate. The plates were removed from the tank and allowed to dry at room temperature after which they were placed in an iodine tank for lipid staining. The relevant (i.e. PI, PC, PE and PS) areas on the plates were removed and dissolved in scintillation fluid (Beckman EP) and the amount of ^{3}H-AA present was determined by liquid scintillation counting.

2.2.7 HPLC analysis of leukotrienes.

Biosynthesis of internal standard for HPLC of leukotrienes.

15-HETE was biosynthesised as follows: 15-lipoxygenase (10 ul, 7350 units) was added to 1 ml of sodium tetraborate buffer (200 mM at pH 9.0) at 25°C followed by 10 ul of 1 mM AA. The reaction mixture was stirred for 20 min prior to addition of 10 ul of 100 mg/ml sodium borhydride (to reduce 15-hydroperoxyeicosatetraenoic acid to 15-HETE). The reaction mixture was then stirred continuously overnight. 15-HETE was extracted into ethyl acetate or chloroform/methanol (7:3), evaporated to dryness and reconstituted in benzene (for storage) at a concentration of 0.19 mM. Aliquots were dried and reconstituted in methanol for use as internal standard. Quantitation was performed in methanol using a Perkin-Elmer spectrophotometer (molar extinction coefficient of E = 30,500 at 234 nm UV).

Organic synthesis of 5-HETE.

5-HETE was organically synthesised according to the method of Corey et al., (1980). An iodolactone was obtained from AA by reaction with KI, I_2, $KHCO_3$ (8/15/5 v/v/v) in 1:2 aqueous THF at 0°C for 18 hrs. Reaction of the iodo lactone with 2.5 equivalents of 1,5-diazabi-cyclo[5.4.0]undec-5-ene in benzene at 23°C for 7 hrs produced an unsaturated lactone which was transformed into the methyl ester of (±)-5-HETE by treatment with

6 equivalents of triethylamine in methanol at 23°C for 30 min. Saponification of the methyl ester in lithium hydroxide in dimethoxyethane-water yielded (±)-5-HETE.

Equipment.

Two HPLC systems were used throughout the study. All chromatographic equipment was from Waters Associates (Milford, MA, USA). Assay system 1 comprised a model 501 single piston pump, a model 281 variable wavelength UV spectrophotometric detector, a 710B WISP autoinjector and a BBC Goerz Metrawatt SE 120 dual pen chart recorder. Assay system 2 comprised a model 510 dual piston pump, a model 490 programmable multiwavelength UV spectrophotometric detector, a model 680 automated gradient controller, a 710B WISP autoinjector and a model 750 data module. The columns used for both systems were Nova-Pak C_{18} (15 cm x 3.9 mm, 4 um average particle size). A 1 cm C_{18} RCCS Waters guard column was fitted at all times. (10 um particle size insert). Chromatography was carried out at room temperature and mobile phases were constantly purged with nitrogen to remove dissolved gases.

Solvent Conditions.

Chromatography of LTB_4 and PGB_2 was performed using assay system 1 A (see Table 3.3 A). The solvent conditions used were methanol/water/acetic acid (70/30/0.08) at a pH of 6.2 (adjusted with NH_4OH). Assay system 1 B was used for chromatography of 5-

and 15-HETE (see Table 3.3 B). The solvent conditions used were methanol/water/acetic acid (80/20/0.08) at a pH of 6.2 (adjusted with NH_4OH).

Assay system 2 which enabled chromatographic separation of PGB_2, LTB_4, 15-HETE and 5-HETE used solvent conditions of methanol/water/acetic acid (70/30/0.08) at pH 6.2 (adjusted with NH_4OH) (see Table 3.4).

Standard curves.

Standard curves consisting of five points in the range of 0-50 ng for LTB_4 and 0-250 ng for 5-HETE were performed for each experiment. Standards were pipetted into 1 ml of cell suspension which had been inactivated by the addition of 10 ul of 100 mM citric acid. Internal standards were added. These standards were then extracted by the same procedure as the experimental tubes. Lines of best fit as calculated using a linear regression programme were drawn for each standard curve and the peak height ratio values were transformed into ng/10^6 neutrophils after taking the appropriate cell concentration into account.

2.2.8 Phosphorylation of neutrophils.

Phosphorylation of neutrophil proteins was carried out according to the method of Changelian and Fearon, (1986). One x 10^7 cells/ml were initially depleted of intracellular phosphate by incubation with buffer lacking PO_4^{-3} (30 mM HEPES, pH 7.4, 110

mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM L-glutamine and 2 mg/ml BSA) for 60 min at 37°C. Cells were resuspended to 5 x 10^7 cells/ml in buffer with ^{32}P-labelled phosphoric acid (orthophosphoric acid, 50 mCi/ml in 0.02 N HCl; New England Nuclear, Boston, USA) at 1 mCi/ml for 90 min at 37°C. After labelling, the cells were washed twice, resuspended in buffer and incubated with stimuli.

Phosphorylation reactions were stopped and the cells were lysed by the addition of an equal volume of ice-cold 2 x RIPA buffer (2% NP-40. 0.2% SDS, 0.15 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 0.68 M sucrose, 5 mM EDTA, 10 mM NaF, 5mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM ATP, 10 mM DFP, 100 ug/ml DNase, 5% FCS, pH 7.2). This lysis buffer was designed to maximally inhibit phosphatase and protease activities. Cell lysates were incubated on ice for 30 min, and insoluble material was removed by centrifugation at 25,000 x g for 20 min at 4°C. The proteins were separated by SDS-polyacrylamide electrophoresis (SDS-PAGE).

2.2.9 SDS-PAGE.

Gels containing 10% acrylamide were prepared from a stock solution of 30% acrylamide and 0.8% N,N'-bis-methyleneacrylamide. The final buffer concentrations in the separation gel were as follows: 0.375 M Tris-HCl (pH 8.8) and 0.1% sodium dodecyl sulphate (SDS). The gels were polymerized chemically by the addition of 0.025% by volume of tetramethylethylenediamine and

ammonium persulphate. Ten cm gels were prepared in glass tubes of a total length of 15 cm and an inside diameter of 6 mm. The electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine and 0.01% SDS (pH 8.3). 200 ul of the cell lysate contained the final concentrations: 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue dye. The proteins were completely dissociated by immersing the samples in boiling water for 1.5 min. Electrophoresis was carried out with a 30 mA current per gel until the bromophenol blue marker reached the bottom of the gel (about 4 hrs). The proteins were stained for 2 hr at room temperature with a 0.25% Coomassie brilliant blue indicator made up in a solution of methanol/water/acetic acid (50/50/0.46; v/v/v). The gels were diffusion destained by repeated washing in a solution of water/ethanol/acetic acid (60/30/10; v/v/v) until the protein bands were clearly visible (Laemmli, 1970). Autoradiographs were prepared by exposing dried gels to XAR X-Omat film (Kodak) for 2 days at -70^{o}C.

2.2.10 Experimental design.

All experiments on human neutrophils were performed in quadruplicate and repeated on at least three occasions. Unless otherwise stated, results are expressed as mean $\pm$ standard deviation.

B. Rat Studies.

2.2.11 Rats.

Male weanling rats (four weeks old) from the South Australian Department of Agriculture, Gilles Plains animal house, were used for all experiments. Dark Agouti (DA) rats are an inbred strain (greater than 35 generations) whereas Wistar-Hooded (WH) rats are outbred. Experimental groups comprised 8-12 rats. Upon arrival at the animal housing facilities (Animal House, Institute of Medical & Veterinary Science, Adelaide) where the experiments were performed, the rats were weighed and randomised into groups of similar number and average weight, prior to commencement of dietary treatment. Each group was fed exclusively one of four diets (three diets in early experiments; outlined below) from 4 weeks of age until termination of the experiment. The effect of the dietary manipulation on the models of inflammation was examined during the fifth and sixth weeks of feeding. Rats were maintained in large cages, up to ten to a cage and fed diet and water "ad libitum". The diurnal cycle was 12 hr light/12 hr dark.

2.2.12 Diets.

A commercially available rat chow (Milling Industries Australia Pty. Ltd.), containing 4% fat w/w, was crushed, repelleted without supplementation and used as a baseline diet. This chow was also used as a basis for diets supplemented with

the following oils (12% w/w): sunflower-seed oil; MaxEPA (except in experiments 1 & 2 where cod liver oil was used); and linseed oil. The oils were added to crushed chow, mixed, repelleted and stored at -20°C prior to use in animal experiments. Sunflower-seed oil was obtained from Nuttelex Food Products Pty. Ltd. Australia, MaxEPA was a gift from R.P. Scherer Pty. Ltd., cod liver oil was a gift from Faulding, Australia Pty. Ltd. and unboiled linseed oil was obtained from Diggers Trading, Adelaide, Australia. Upon delivery, 0.05% (w/v) butylated hydroxy-toluene was added to oils as antioxidant.

2.2.13 GLC analysis of PUFA.

Lipid samples were converted to their constituent fatty acid methyl esters by heating in a solution of 1% H_2SO_4 in methanol in a sealed tube under nitrogen at 70°C for 3 hr. The esters were extracted with petroleum spirit (bp 40-60°C). Analysis was performed by gas liquid chromatography on 1.5 m columns (2 mm i.d.) packed with 5% SP2310 (Supelco Inc., Bellefonte, PA). The chromatographic conditions were as follows; injection port temperature 200°C, flame ionization detector temperature 300°C, initial oven temperature 125°C rising to 225°C at 4°C/min and holding for 20 min. The carrier gas used was nitrogen at a flow rate of 16-20 ml/min (Gibson, 1983).

2.2.14 Models of inflammation.

Carrageenan-induced Paw odoema

After four weeks of feeding, rats were injected with 100 ul of carrageenan (1%) in saline in the left rear paw. Paw volume was determined by the weight of water displaced from a 12 x 75 mm test tube by immersion of the paw up to the anatomical hairline. Paw volumes were measured before and 4 hours after injection of carrageenan.

Granulomatous sponge model

Polyurethane sponges (Dunlopilo Ltd., Adelaide, Australia) weighing 40 mg were washed twice in distilled water and then immersed in a suspension of heat killed M. Tuberculosis, 0.5 mg/ml, in distilled water as follows: 80 mg of heat killed, lyophilized M. Tuberculosis (a mixture of strains C, DT and PN, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey, UK) were ground to a paste in 0.5 ml of distilled water with a mortar and pestle, diluted to the final concentration and sonicated for 2 min using a 60 W Mullard tissue sonicator with a 0.25 inch probe, to assure an even suspension. These sponges were dried overnight at 37^{o}C to constant weight and sterilized in ethylene oxide. Each sponge contained approximately 1.5 mg dry M. Tuberculosis. After four weeks of dietary treatment, one sponge was implanted into a flank of each rat through a dorsal midline incision under ether

anaesthesia. The wound was closed with staples. The sponges were removed on day 4 when the rats were sacrificed. Adherent tissue overlapping the sponges was carefully cut away and care was taken not to squeeze out any of the fluid contained in the sponges. The sponges were dried overnight at 37°C (to constant weight) and the difference between initial and final dry weight was recorded as the dry weight gain.

Adjuvant-induced arthritis.

After 4 weeks of feeding, a complete Freund's adjuvant containing heat-killed Mycobacterium Tuberculosis (M.Tb., Weybridge Laboratories, Ministry of Agriculture, U.K.) dispersed in squalane or triolein at a final concentration of 10 mg/ml was injected intradermally into the tail of the rats. Unless otherwise stated, assessment of inflammation was made 12 days after the injection. For ethical reasons, experimentation was not allowed to proceed beyond this time. Four parameters were used to quantify the severity of the disease: (1) rear paw swelling between the dorsum and the sole of the foot measured at the mid-point between the posterior border of the the calcaneum and the fifth metatarso-phalangeal joint (using digital calipers); (2) widest diameter of swelling of the tail in the region of injection (using digital calipers); (3) weight change and (4) disease activity score (total of 0-14 points) determined as follows: for each rear paw (0-4 points) where 0 = no localised articular lesion or swelling, 1 = localised articular lesions, or ankle swelling, 2 = localised articular lesions and ankle

swelling, 3 = moderate generalised ankle and foot swelling, and 4 = gross generalised ankle and foot swelling: for each forepaw (0-3 points) where 0 = no localised articular lesion or swelling, 1 = localised articular lesions or wrist swelling, 2 = localised articular lesions and wrist swelling and 3 = gross generalised paw and wrist swelling.

2.2.15 Assessment of homogeneity of rat groups.

Each rat was weighed twice per week throughout the experiment. Growth patterns were observed for the first four weeks of feeding. The rats did not differ significantly from each other prior to application of the experimental model of inflammation (one-way analysis of variance, $p < 0.05$).

2.2.16 Statistical analysis.

One way analysis of variance was performed for statistical analysis of parametric variables. When significant variance was found by this method (at $p < 0.05$), a students t-test was performed to identify which groups were the source of variance. Disease scores were assessed non-parametrically using a Kruskal-Wallis one way analysis of variance. If significant variance was found using this method, a Mann-Whitney U-test was used to determine which groups were the source of variance (Daniel, 1983).

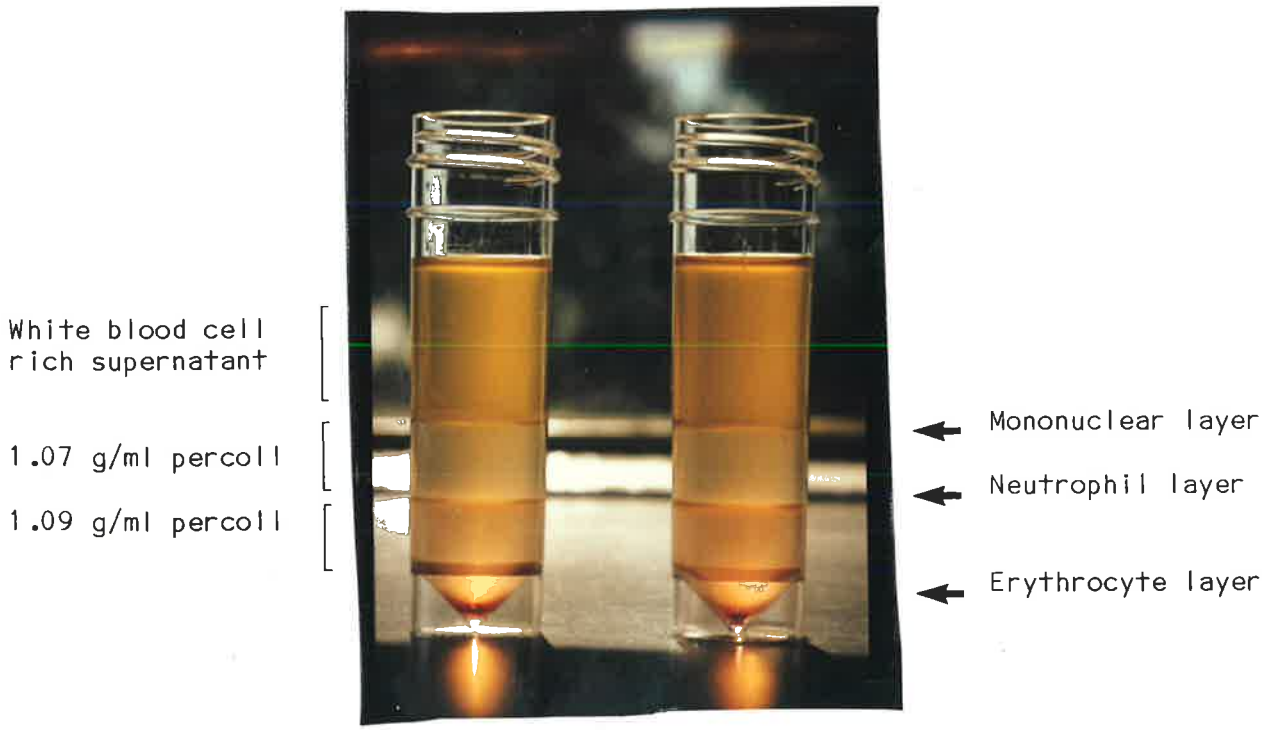


Figure 2.1 A typical Percoll cell separation showing the location of the neutrophil layer.

CHAPTER 3: HPLC OF THE LIPOXYGENASE PRODUCTS OF HUMAN NEUTROPHILS.

3.1 INTRODUCTION

Many techniques have been developed to assay the LO products of AA since their initial discovery by Borgeat and Samuelsson (1979a). The procedures range from bioassay, radioimmunoassays, thin layer chromatography, gas chromatography-mass spectrometry and high pressure liquid chromatography (HPLC). All these methods have advantages and disadvantages (Table 3.1)

HPLC is a technique which is being used with increasing frequency. It is versatile and assays can be developed to separarate and measure structures ranging from small compounds such as leukotrienes to large compounds such as enzymes. It is rapid, reproducible and can be automated. These characteristics, combined with high sensitivity make it ideal for accurate measurement of closely related compounds.

Several leukotriene HPLC assays are available, most of which use a c_{18} stationary phase (Table 3.2). The mobile phases usually consist of a mixture of methanol/water/acetic acid, although there are some reports of assays using mobile phases containing acetonitrile, tetrahydrofuran or benzene (see Table 3.2). The ratio of methanol/water/acetic acid used depends on which LTs are measured. For example, the major products measured when studying mast cell are LTC_4, LTD_4 and LTE_4 (Yecies et al., 1979), or when studying production by isolated peripheral blood mononuclear cells, LTC_4, LTB_4 and

monoHETEs (Goldyne et al., 1984). The aim of the experiments described in this chapter was to develop and document a rapid, sensitive and reproducible HPLC assay for the LO products of human neutrophils.

3.2 RESULTS

3.2.1 Chromatography of standards.

Figure 3.1 shows a typical chromatogram of PGB_2 and LTB_4 standards using assay system 1 A (Table 3.3 A). Under these conditions, PGB_2 and LTB_4 elute with k'^s of 1.7 min and 3.7 min respectively. Using assay system 1 B (Table 3.3 B), 15- and 5-HETE have k'^s of 3.3 & 4.5 min respectively (see Figure 3.2).

A typical chromatogram of all four standards run on assay system 2 (Table 3.4) is shown in Figure 3.3. Under these conditions, PGB_2 and LTB_4 elute with the same k' as assay system 1 A, whereas elution of 15- and 5-HETE takes longer (k' of 12.9 min and 19.5 min respectively).

Once the chromatographic conditions for separation of these compounds had been established, I turned my attention to development of the extraction procedures required to remove eicosanoids from an aqueous environment.

3.2.2 Extraction procedure.

Choice of solvent for extraction.

Two methods of extraction were compared; a) ethyl acetate and b) chloroform/methanol (7:3 v/v). To assess the extraction efficiencies, all four standards were added to a suspension of cells which had been pretreated with citric acid (final pH $<$ 5) and either extracted into ethyl acetate or the mixture of chloroform/methanol. Internal standards (PGB_2 and 15-HETE) were added to all tubes, whereas 10 ng of LTB_4 and 50 ng of 5-HETE were added to one set of tubes while 50 ng of LTB_4 and 250 ng of 5-HETE were added to the remainder. There was no difference in the extraction efficiencies using the two solvent systems (Table 3.5). LTB_4 and PGB_2 are extracted by both methods with approximately 50% efficiency, whereas the monohydroxy acids 5- & 15-HETE are extracted with an efficiency of approximately 85%. Chloroform/methanol was used as the organic phase for all subsequent experiments.

Effect of pH on extraction.

The effect of pH on extraction was tested by adding different amounts of citric acid to decrease the pH of HBSS containing neutrophils. This provided samples in the pH range of 2-7, to which 50 ng of LTB_4, 50 ng of PGB_2, 200 ng of 15-HETE and 125 ng of 5-HETE were added. The samples were then extracted in chloroform/methanol and HPLC was performed. The optimal pH for extraction of all four compounds from an aqueous medium containing neutrophils was between 4

and 5 (see Figures 3.4 & 3.5). This was achieved by adding 10 ul of 100mM citric acid to 1 ml of cell suspension in HBSS.

3.2.3 Internal standards.

PGB_2 is commonly used as internal standard for HPLC of LTs because it is not endogenously synthesised and unlike other prostaglandins has an absorption maximum of approximately 270 nm (see figure 3.6). However, with automation of the HPLC assay, the need for an internal standard which could be detected at 234 nm arose. 15-HETE appeared suitable, as it could be readily biosynthesised from 15-LO (Lipoxidase, Sigma Chemical Company) and was not produced by neutrophils (Lands, personal communication). This latter point was confirmed by stimulating cell suspensions with A23187 alone and in the presence of exogenous AA. A23187 alone (0.05 uM - 10 uM) or with up to 50 uM AA did not stimulate production of 15-HETE. To increase sensitivity, ^{3}H-AA was added with A23187 and after extraction, HPLC fractions were collected and the ^{3}H-AA metabolites produced were measured by liquid scintillation counting. No ^{3}H-15-HETE was detected under these conditions (see Figures 3.7 & 3.8).

3.2.4 Production and quantification of leukotrienes from neutrophils.

The chromatogram depicted in Figure 3.9 shows the HPLC elution profile of the compounds extracted from 10^6 neutrophils/ml stimulated for 5 mins with 5 uM A23187, run on assay system 2. The twin peak which elutes between PGB_2 and LTB_4 is made up of all trans-isomers of

LTB_4 (Forrest, personal communication). These can be separated by lowering the percentage of methanol in the mobile phase to 67%. Under the solvent conditions in assay system 2 (and therefore assay system 1 B), the w-oxidation metabolites of LTB_4 (20-OH-LTB_4 and 20-COOH-LTB_4) elute before PGB_2 and are too close to the solvent front to be quantified.

Ratios of peak heights of LTB_4/PGB_2, LTB_4 isomers/PGB_2 and 5-HETE/15-HETE were calculated from measurements from chromatograms and the amount of LTB_4, LTB_4 isomers and 5-HETE that these ratios corresponded to were determined by use of appropriate standard curves. Quantitation of the LTB_4 isomers was achieved using the standard curve for LTB_4 since these compounds all have the same molar extinction coefficient (Borgeat and Samuelsson, 1979c).

3.2.5 Sensitivity.

Using the criterion signal to noise ratio of 2:1, the lower level of detection for both LTB_4 and 5-HETE for the assay was determined as approximately 500 pg.

3.2.6 Precision of assay.

Coefficients of variation for both intra- and inter-assay variability were calculated for both LTB_4 and 5-HETE. Intra-assay variabilty was determined by assay of replicate samples (sextuplicate) from one assay. The coefficients of variation for LTB_4 and 5-HETE were 9.3% and 7.1% respectively (Table 3.6). For inter-assay

variability, coefficients of variation were calculated for two points on standard curves performed on 11 separate experimental days. The values were 15.3% for LTB_4 and 9.5% for 5-HETE (Table 3.6). All coefficients of variation were less than 16% indicating acceptably low variability. As expected intra-assay variability is smaller than inter-assay variability.

3.2.7 Quality control.

Quality control was performed to assess the acceptability of standard curve values in an assay on any one day. Aliquots of LTB_4 and 5-HETE in methanol were kept as quality control (QC) standards and 25 ul was added to acidified cell suspensions for each assay. These volumes represented 25 ng of LTB_4 and 125 ng of 5-HETE. Figures 3.10 and 3.11 show the sequential values of these QC standards as determined from the standard curves performed on the same day. As seen from the figures, variation of the values of the QC standards from their absolute value was bi-directional, indicating that no directional deterioration of the assay occurred. The precision of the HPLC assay was further confirmed by establishing coefficients of variation for the QC values. Over a period of six months the coefficient of variation were 3.3% for 5-HETE and 9.4% for LTB_4.

3.3 DISCUSSION.

The assay described in this chapter formed the backbone of all work performed during the course of this thesis. Many other HPLC assays for separation and quantitation of LTs and monoHETEs have been

described in the past five years. These assays vary in simplicity and sensitivity. Several enable measurement of a large number of different eicosanoids in one chromatographic run (Van Rollins et al., 1980; Cockrell and Ellis, 1984; Eling et al., 1980) although these assays require complex gradient chromatography. The LTB_4 and 5-HETE assay described in this chapter was specifically designed to separate and quantify the LO products of human neutrophils, a pre-requisite which greatly simplified the assay conditions. The extraction and sample cleanup procedures are both rapid and simple while sensitivity and reproducibility are high.

Review of the literature shows several methods used for extraction and purification of PGs and LTs released from stimulated cells prior to HPLC. Some methods involve adsorption onto amberlite resins (Mathews et al., 1981), purification either by silicic acid (Borgeat et al., 1976) or using sep-pak cartridges (Harvey and Osborne, 1983) or direct injection of supernatants onto HPLC (Lee et al., 1984; 1985). The simplest procedure involves acidification of the cell suspension after stimulation, followed by addition of nonpolar organic solvent/s such as ethyl acetate (Elings et al., 1980; Van Rollins et al., 1980), or chloroform/methanol (Clancy and Hugli, 1983). Acidification stops cellular reactions and therefore provides a convenient end point of stimulation. It also facilitates partition of LTs into the organic phase. Using ethyl acetate or chloroform/methanol, I achieved 45-55% extraction of LTB_4 from HBSS. This compares with 97% using chloroform/methanol at an unspecified acidic pH (Clancy and Hugli, 1983) and 80% using ethyl acetate at pH 3 (Cockrell and Ellis, 1984). The results from my experiments examining

the effect of pH on the extraction of LTB_4 from HBSS using chloroform/methanol (Figure 3.4) do not shed any light on why this discrepancy exists as they indicate that maximal extraction occurs between pH 4.0 and 5.0. Interestingly, the extraction efficiency I achieved with 5-HETE was similar to those of Clancy and Hugli, (1983) and Cockrell and Ellis, (1984).

During the course of this thesis, two automated chromatographic systems were used to separate and quantify LO products. The first system involved detection and quantification of LTB_4 , the LTB_4 isomers and 5-HETE on two separate chromatographic assays (designated assay system 1; A and B respectively). This was necessary because these compounds have UV maximum absorbances at two different wavelengths and when the system became automated, it proved unpractical to manually alter wavelength during each chromatographic run. Therefore, the need for a separate internal standard for each assay arose. The choice of PGB_2 as internal standard for LTB_4 was simple as this compound is widely used as such (Osborne et al., 1983; Harvey and Osborne, 1983; Muller and Sorrell, 1985; Sun and McGuire, 1984). However, the choice of internal standard for 5-HETE was more difficult. Decanophenone has been used as internal standard for mono-HETEs (Sun and McGuire, 1984), but I found this compound unsuitable for two reasons. It did not elute until well after 5-HETE (using assay system 1 B, it had a k' of 19.9 min compared with 3.7 min for 5-HETE). More importantly, the compound proved unstable under the evaporation conditions employed and was therefore unreliable. I evaluated other monoHETES such as 12- or 15-HETE and eventually chose 15-HETE as internal standard. 12-HETE was unsuitable because the

presence of platelets in cell preparations could lead to 12-HETE contamination. 15-HETE was suitable as I established that it is not synthesised by intact neutrophils. There have been several reports indicating that neutrophils can synthesise 15-HETE (Goetzl et al., 1979; McGuire et al., 1985) but these studies were not performed using an intact cell system. Problems can still arise in an intact cell system if exogenous AA is used in experiments. Care must be taken to ensure that the AA has not oxidised otherwise detectable amounts of 15-HETE (as well as 5-HETE) can be added to the sample (Johnson et al., 1983).

Assay system 1 was eventually superceded by a second system comprising more advanced equipment (termed "assay system 2"). The detection apparatus for assay system 2 was the Waters model 490 programmable multiwavelength detector which enabled detection of LTB_4, LTB_4 isomers and 5-HETE during the same chromatographic run because it could be programmed to change detection wavelength at any time through the run. This system was developed to enable HPLC analysis of EPA-metabolites of 5-LO, conditions under which PGB_2 is unsuitable as internal standard because it interferes with the measurement of LTB_5. When analysis of EPA-metabolites was performed, 15-HETE alone was used as internal standard.

Neither the w-oxidation products of LTB_4 nor the peptido-LTs are resolved using the conditions described in this chapter. The w-oxidation products of LTB_4 are not separated from the solvent front unless the mobile phase consists of approximately 60% methanol and 40% water. Special extraction, solvent and column conditions are required

for separaton and measurement of the peptido-LTs as they are highly labile (Clancy and Hugli, 1983; Muller and Sorell, 1985).

TABLE 3.1

TECHNIQUES FOR THE MEASUREMENT OF EICOSANOIDS

TECHNIQUE	ADVANTAGES	DISADVANTAGES
Bioassay	Sensitive technique. Specificity enhanced by differential bioassay. Measures labile metabolites. Endogenous and exogenous AA can be used as precursor. High throughput.	Problems with mixtures of compounds containing substances with opposing biological actions. Biological fluids may require prior purification. Quantitation difficult.
Radioimmunoassay	Specific and sensitive. Endogenous and exogenous AA can be used as precursor. High throughput.	Measures only stable metabolites. Specificity depends on antibody. Only one eicosanoid can be assayed.
Thin Layer Chromatography	Can assay several metabolites at once.	Measures only stable metabolites. Sample extraction required. Can use exogenous substrate only. Requires use of radiolabelled precursors. Accurate quantitation difficult.
Gas Chromatography Mass Spectrometry	Provides sensitive assay and structural information.	High cost, low throughput. Requires deuterated samples. Requires sample purification.
High Pressure Liquid Chromatography	Sensitive and accurate quantitation. Can use endogenous or exogenous AA. High resolution. Assays several eicosanoids at once. Assay can be automated.	Medium throughput. Measures only stable metabolites. Sample purification required.

Table 3.2: Details of published HPLC assays.

Column	Solvent conditions	Metabolites assayed	Extraction	Reference
Radial Pak-A	Gradient 40' run Methanol/Water	HHT, TXB_2, monoHETEs used ^{3}H-AA	Ethyl Acetate	Eling et al., 1980
Ultrasphere ODS Spherisorb ODS	Gradient 150' run Acetonitrile/Phosphate	HHT, TXB_2, PGE_2, 6keto$PGF_{1\alpha}$, PGD_2, $PGF_{2\alpha}$ 12-HETE, AA used ^{14}C-AA	Ethyl Acetate	Van Rollins et al., 1980
Nucleosil C_{18}	2 systems at different pH Methanol/Water/Ac 16-70' run	LTB_4, LTC_4, LTD_4	Amberlite XAD-7	Mathews et al., 1981
Nucleosil C_{18}	Methanol/Water/Ac pH 4.6 20' run	HHT, LTB_4, 5-HETE, TXB_2 PGE_2, $PGF_{2\alpha}$ used ^{14}C-AA	C_{18} Seppak	Harvey et al., 1983
Spherisorb-2	Gradient 70' run Methanol/Water/Ac 2.5 mM Pentanesulfonic acid	LTB_4, LTC_4, LTD_4 Trans isomers	Amberlite XAD-7	Ziltener et al., 1983
Nucleosil C_{18}	Methanol/Water/Ac 30' run pH 4.2	LTC_4, LTD_4, LTE_4	Silicic acid	Clancy et al., 1983

Several Columns	Methanol/Water/Ac	PGB_2,LTB_4, 5s12sdiHETE, LTC_4,LTD_4	C_{18} Seppak	Osborne et al., 1983
uBondapak C_{18}	Gradient 100' run Methanol/Acetonitrile /Benzene/Ac to Methanol/Water/Ac	6ketoPGF$_{1\alpha}$,TXB_2 $PGF_{2\alpha}$,PGD_2 LTB_4,LTC_4,LTD_4 5,12,15-HETE used ^{3}H-AA	Ethyl acetate	Cockrell et al., 1984
Nucleosil C_{18}	THF/Methanol/Water/Ac 40' run pH 5.5	LTB_4, isomers PGB_2,LTC_4	C_{18} Seppak	Verhagen et al., 1984
Nucleosil C_{18}	Gradient 60' run Methanol/Water/Ac	LTB_4,LTB_5 5-HETE,5-HEPE	direct injection	Lee et al., 1984
Ultrsphere ODS	Methanol/Water/Ac pH 5.6 40' run	PGB_2,LTB_4 LTC_4,LTD_4,LTE_4	petroleum spirit chloroform	Muller et al., 1985
Ultrasil ODS	Gradient 60' run Methanol/Water/Ac	LTB_4,LTB_5 5-HETE,5-HEPE	direct injection	Lee et al., 1985

Table 3.3 A: Details of conditions for assay system 1 A.

Compounds measured	PGB_2, k' 1.7 min LTB_4, k' 3.7 min
Solvent conditions	70:30:0.08 methanol/water/acetic acid, pH 6.2 with ammonium hydroxide, 1 ml/minute
Equipment	Waters 501 pump, 710B WISP, 481 UV spectrophotometer, BBC Goerz metrawatt SE 120 recorder, Nova-PAK 5 um C_{18} column.
Detection	Waters variable UV detector, 270 nm UV at 0.005 aufs
Run time	10 minutes.

Table 3.3 B: Details of conditions for assay system 1 B.

Compounds measured	15-HETE, k' 3.3 min 5-HETE, k' 4.5 min
Solvent conditions	80:20:0.08 methanol/water/acetic acid, pH 6.2 with ammonium hydroxide, 1 ml/minute
Equipment	Waters 501 pump, 710B WISP, 481 UV spectrophotometer, BBC Goerz metrawatt SE 120 recorder, Nova-PAK 5 um C_{18} column.
Detection	Waters variable UV detector, 234 nm UV at 0.02 aufs
Run time	10 minutes.

Table 3.4: Details of conditions for assay system 2.

Compounds measured	PGB_2, k' 1.7 min LTB_4, k' 3.7 min 15-HETE, k' 12.9 min 5-HETE, k' 19.5 min
Solvent conditions	70:30:0.08 methanol/water/acetic acid. pH 6.2 with ammonium hydroxide. 1 ml/min
Equipment	Waters 510 pump, 710B WISP, 490 UV spectrophotometer, 730 data module, gradient controller, Nova-Pak 5 um C_{18} column
Detection	Waters programmable multiple wavelength UV detector, 0 mins 270 nm UV, 15 mins 234 nm UV (all at 0.005 aufs).
Run time	30 minutes.

Table 3.5: Extraction efficiencies for eicosanoids using either ethyl acetate or chloroform/methanol.

Eicosanoid	ethyl acetate	chloroform/methanol
PGB_2	62.4%	56.0%
LTB_4	56.1%	45.0%
5-HETE	86.9%	91.5%
15-HETE	73.7%	83.4%

Table 3.6: Coefficients of variation for both intra- and inter-assay variability.

Eicosanoid	Amount (ng)	Intra-assay CV n=6	Inter-assay CV n=11
LTB_4	10	9.3	15.3
	50	8.9	13.2
5-HETE	50	7.0	9.5
	250	7.1	7.8

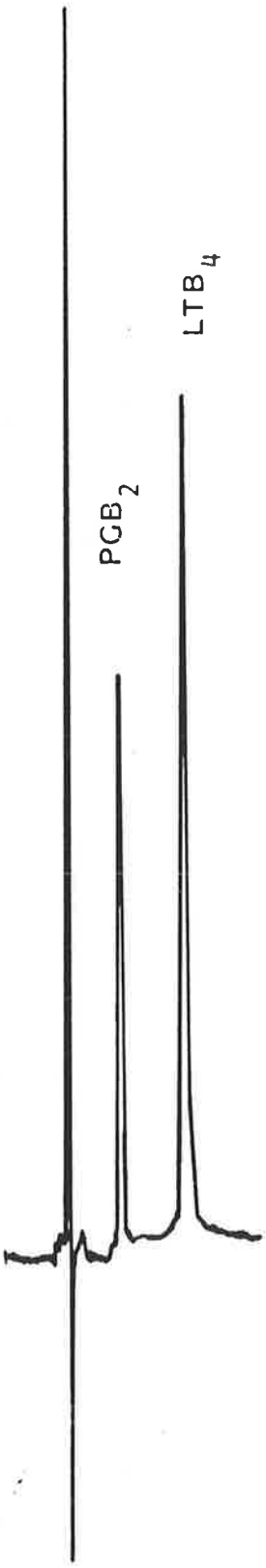


Figure 3.1: Chromatogram of 5 ng of PGB_2 and LTB_4 standards using assay sytem 1A. HPLC conditions were methanol/water/acetic acid (70/30/0.08) pH 6.2 using a Waters Nova-Pak C_{18} column and a flow rate of 1 ml/min. Detection was at 270 nm and 0.005 aufs.

Figure 3.2: Chromatogram of 5 ng of 15-HETE and 5-HETE standards, using assay sytem 1B. HPLC conditions were methanol/water/acetic acid (80/20/0.08) pH 6.2 using a Waters Nova-Pak C_{18} column and a flow rate of 1 ml/min. Detection was at 234 nm and 0.02 aufs.

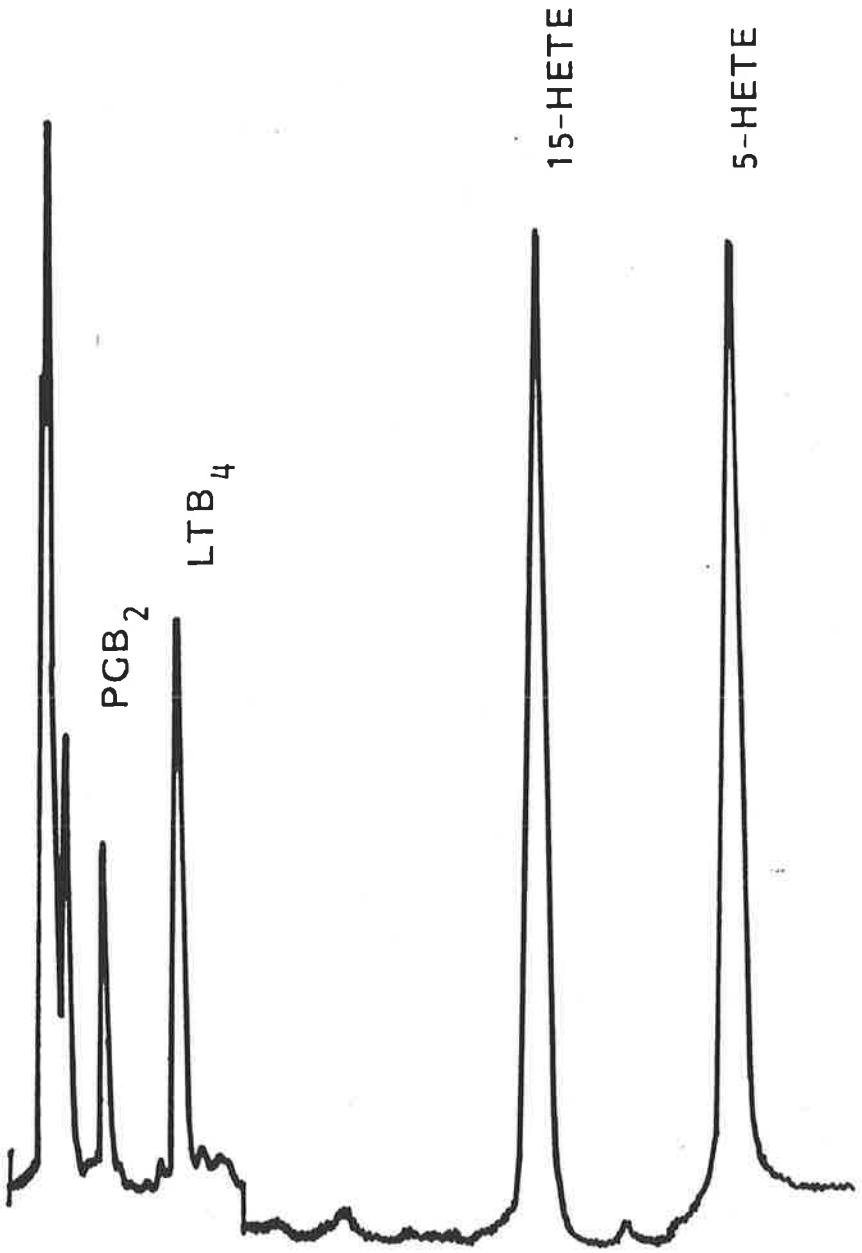


Figure 3.3: Chromatogram of 5 ng of PGB_2, LTB_4, 15-HETE and 5-HETE, using assay system 2. HPLC conditions were methanol/water/acetic acid (70/30/0.08) pH 6.2 using a Waters Nova-Pak C_{18} column and a flow rate of 1 ml/min. Detection was at 270 nm for PGB_2 and LTB_4 (0.005 aufs) and at 234 nm for 15-HETE and 5-HETE (0.02 aufs).

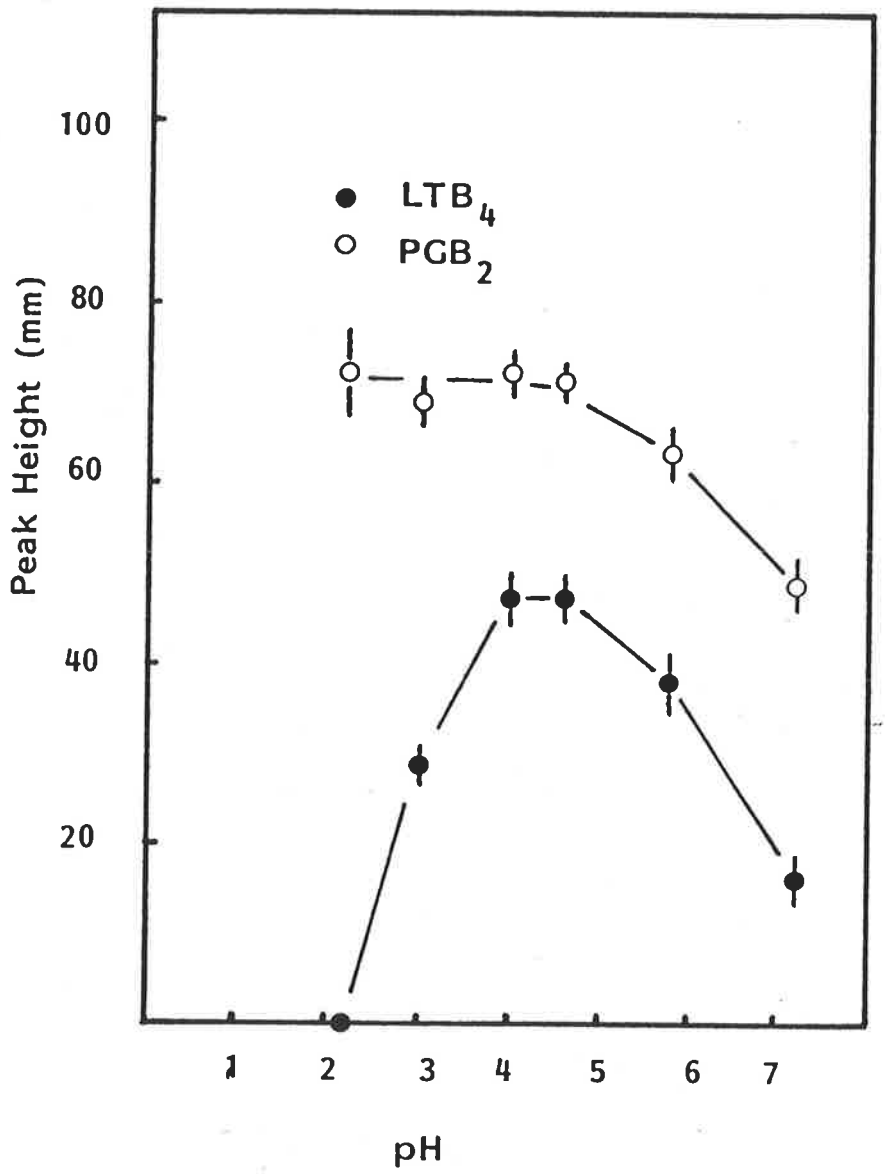


Figure 3.4: The effect of pH on extraction of PGB_2 and LTB_4 from HBSS containing neutrophils. Citric acid was added to the cell suspension to achieve the pH levels indicated after which PGB_2 and LTB_4 standards were added. The leukotrienes were then extracted from the samples and HPLC analysis was performed (see Chapter 2 for details).

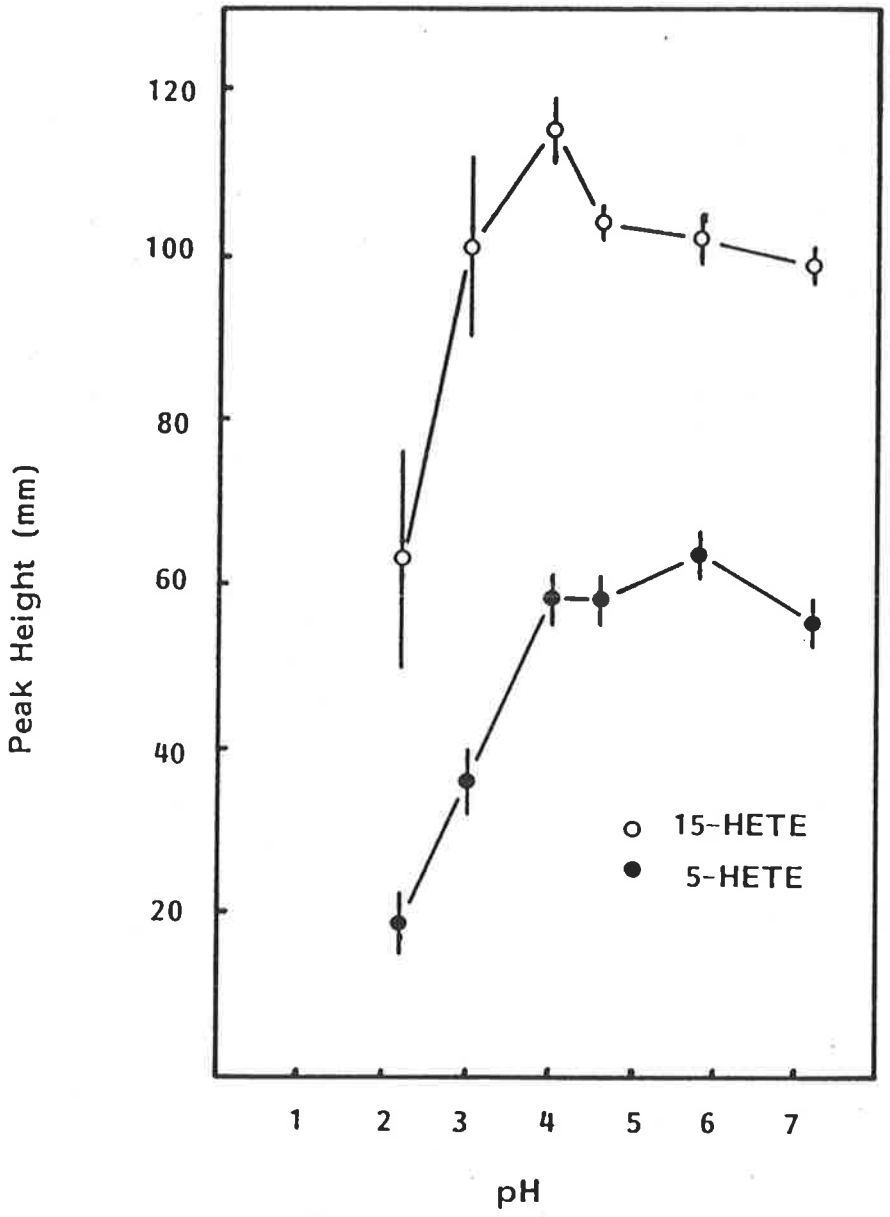


Figure 3.5: The effect of pH on the extraction of 15-HETE and 5-HETE from HBSS containing neutrophils. Citric acid was added to the cell suspensions to achieve the pH levels indicated after which 15-HETE and 5-HETE standards were added. The leukotrienes were then extracted and HPLC analysis was performed (see Chapter 2 for details).

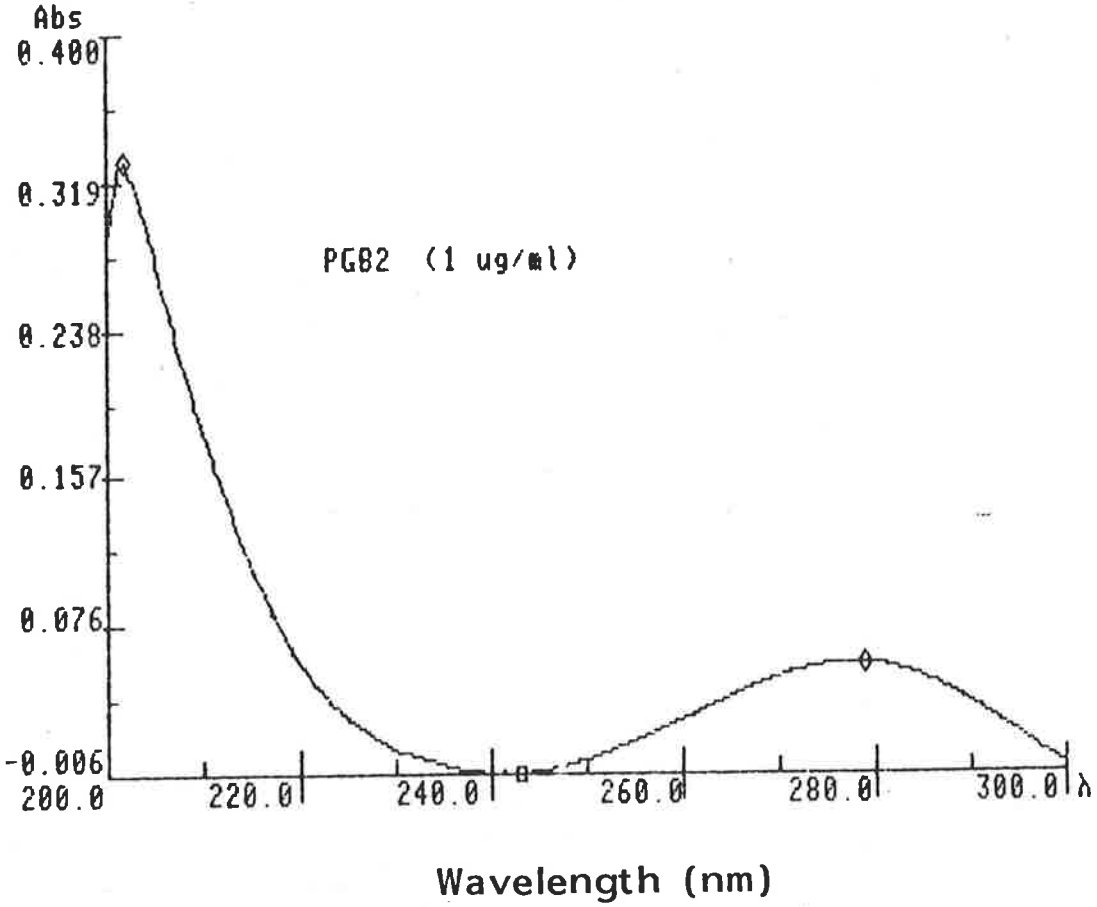


Figure 3.6: The UV absorption spectrum of 1 ug of PGB_2. The wavelength scan was performed on a Varian model DMS 100S spectrophotometer.

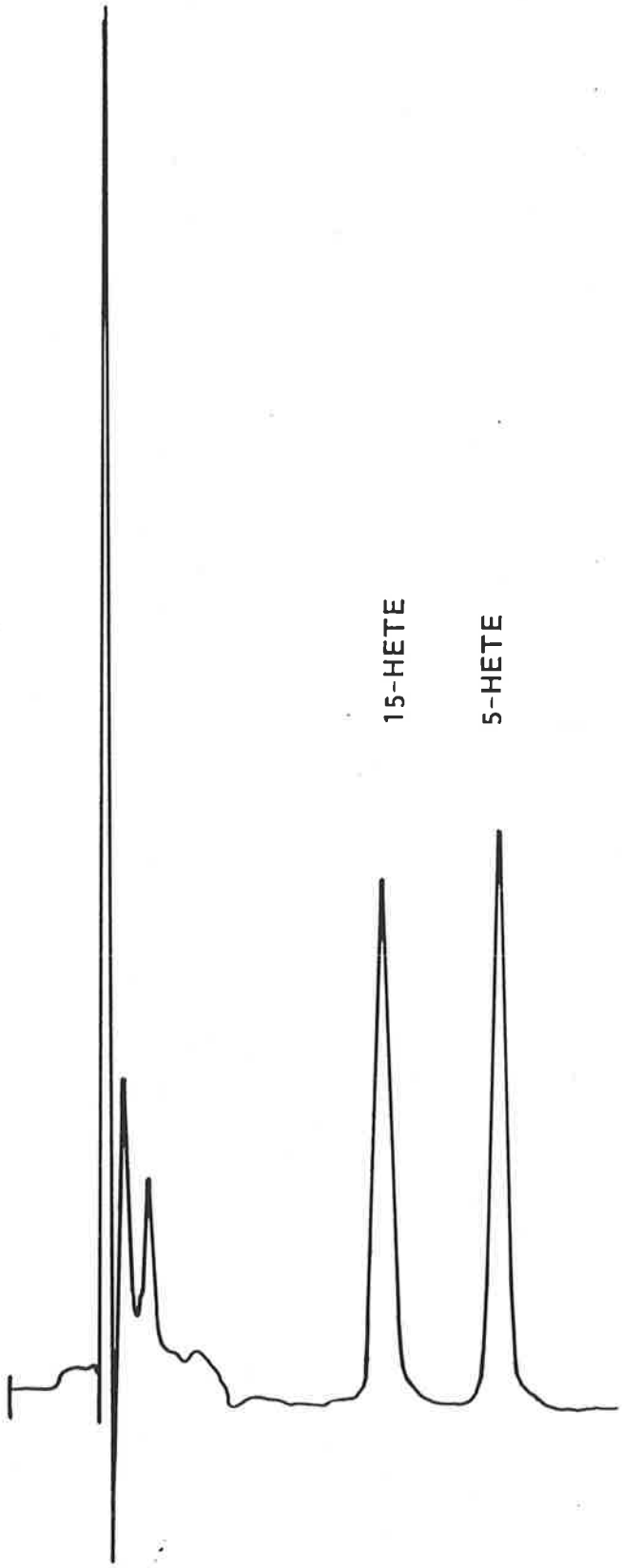


Figure 3.7: Chromatogram of an extract of a neutrophil preparation stimulated by 5 uM A23187 for 5 min. 0.1 uCi of ^{3}H-AA was added simultaneously with A23187 (see Figure 3.8 for radiochromatogram).

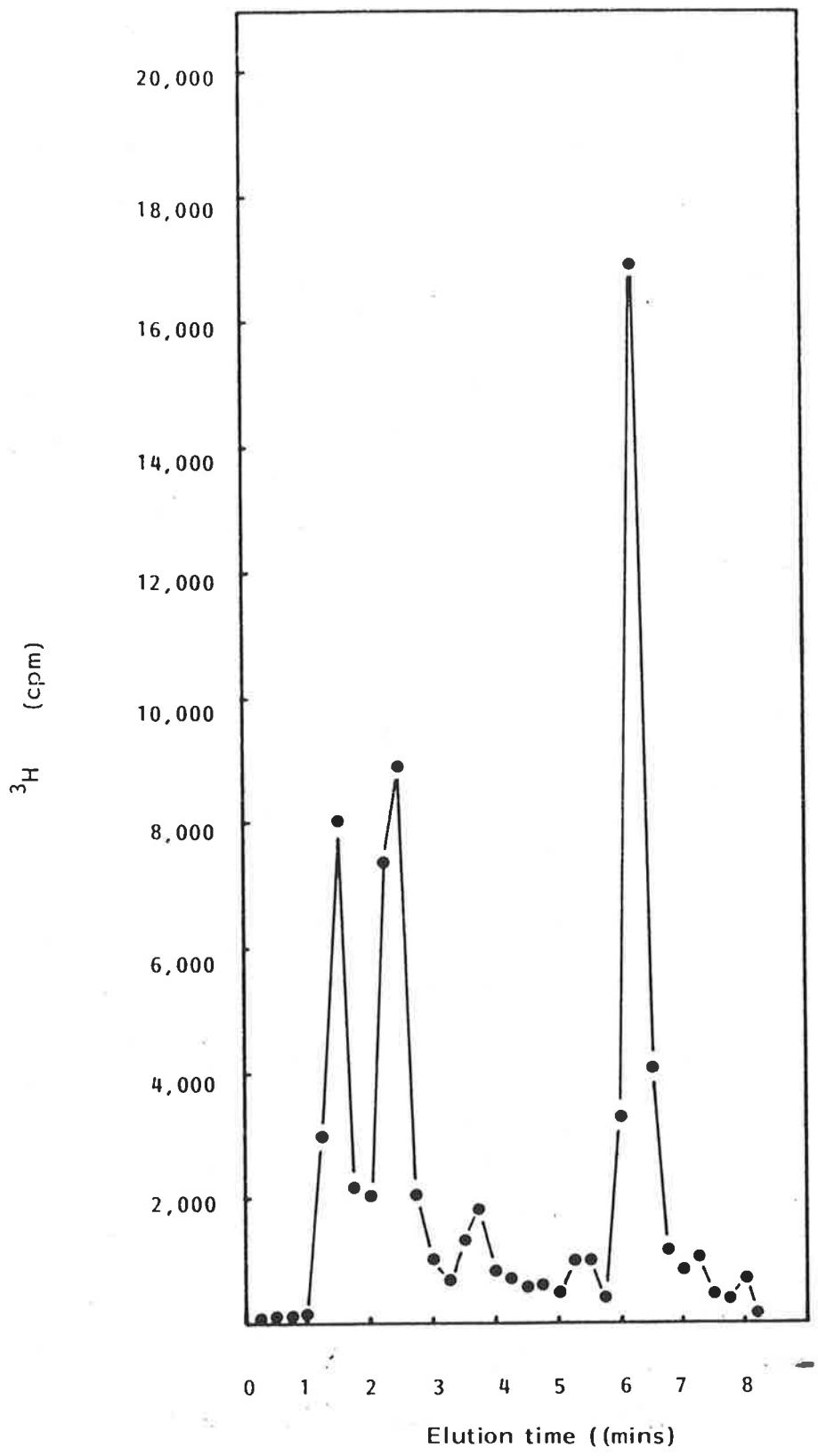


Figure 3.8: Radio-chromatogram of neutrophil preparation to which 0.1 uCi ^{3}H-AA has been added prior to stimulation with 5 uM A23187.

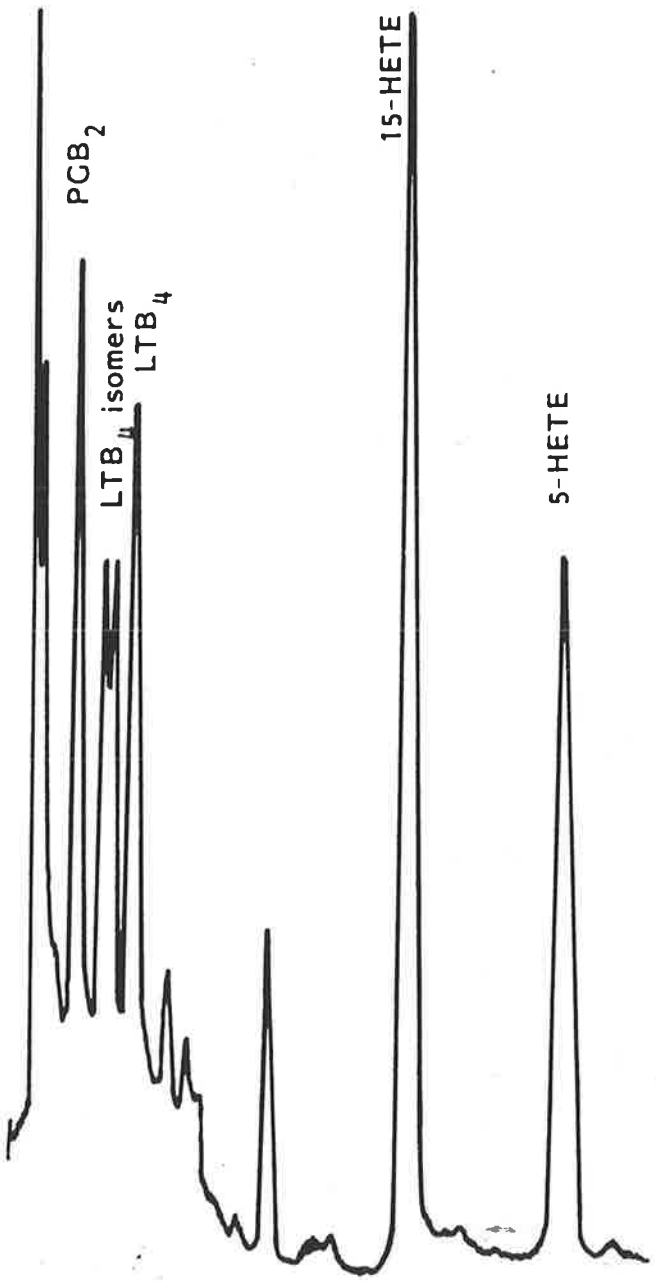


Figure 3.9: Chromatogram of stimulated neutrophil extract using assay system 2. Neutrophils were stimulated with 5 uM A23187 for 5 min.

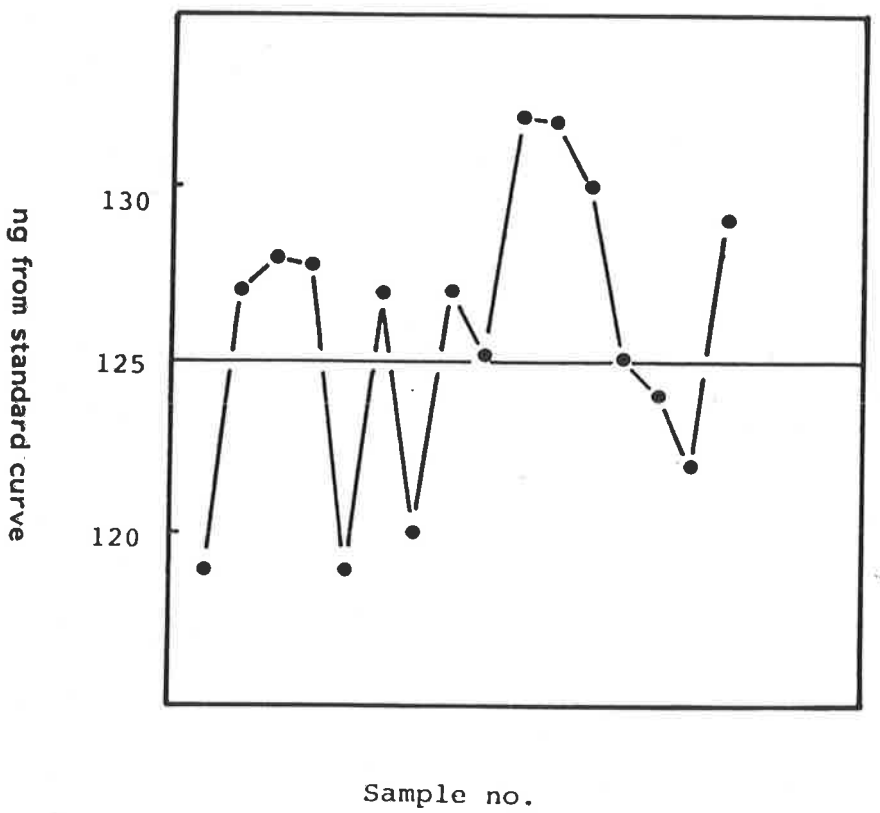


Figure 3.10: Absolute values of Q.C. standards of 5-HETE determin over a period of six months.

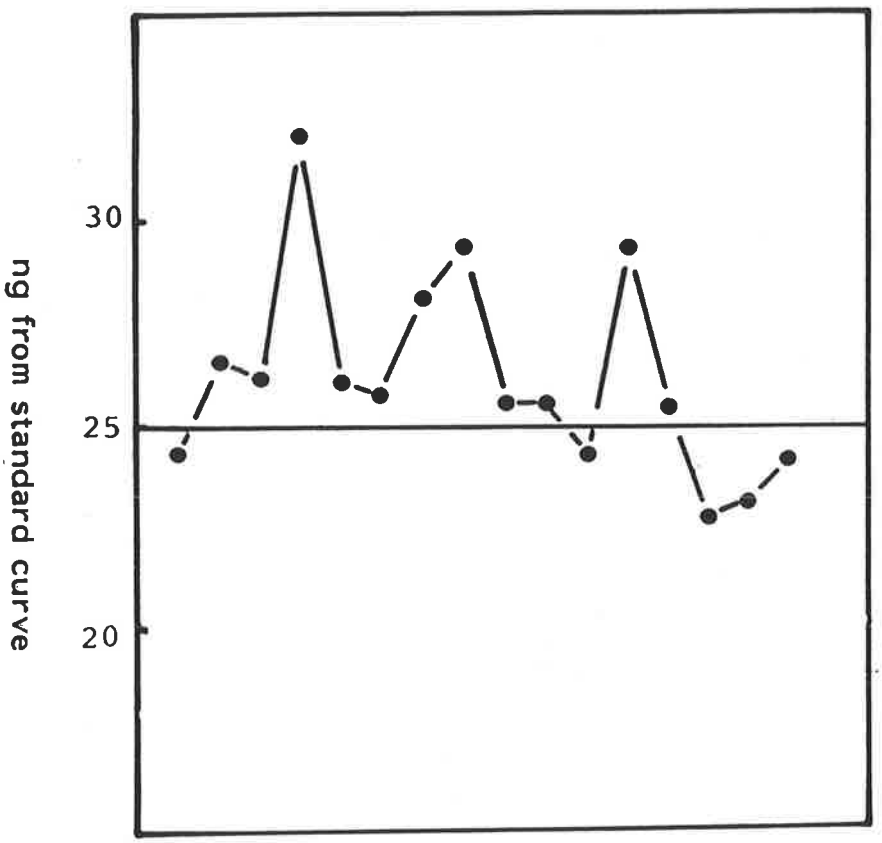


Figure 3.11: Absolute values for Q.C. standards of LTB_4 determined over a period of six months.

CHAPTER 4: CHARACTERISTICS OF THE 5-LIPOXYGENASE ENZYME (5-LO) SYSTEM OF HUMAN NEUTROPHILS.

4.1 INTRODUCTION.

Refinements in the understanding of a biological system are likely to lead to refinements in the experimental design of further studies into that system. Criteria such as dose of stimulus, stimulation time, variability between donors or day to day variation on cell preparations are likely to be factors determining the variation in the results of leukotriene studies to date. For example, the values reported for LTB_4 vary from 12 ng/10^6 neutrophils using 10 uM A23187 for 5 min as stimulus to 51 ng/10^6 neutrophils using 1.5 uM A23187 for the same period of time (Table 4.1).

The experiments described in this chapter were performed to better characterise the 5-LO system of human neutrophils with respect to (a) dose response to A23187 or FMLP, and (b) time course of leukotriene production in response to A23187 or FMLP; c) day to day variability in production of leukotrienes by cells of the same donor; d) variability in leukotriene production by cells of several donors and e) characteristics of the dependence of the 5-LO system on Ca^{++}.

4.2 RESULTS.

4.2.1 Relationship between cell concentration and generation of leukotrienes*.

To ascertain whether cell concentration influenced generation of leukotrienes in a linear fashion, and if so, what range would be suitable for subsequent experimentation, neutrophils were collected, purified as described and serially diluted to give five cell concentrations ranging from 0.35 - 5.0 x 10^6/ml. These samples were stimulated by 5 uM A23187 for 5 min. The amount of 5-HETE and LTB_4 generated increases in proportion with cell concentration in the range of 0.35 - 2.75 x 10^6 cell/ml (Figures 4.1 & 4.2). The linear regression coefficients in this range were 0.997 and 0.998 for 5-HETE and LTB_4 respectively. The linear relationship was not maintained at high concentrations (greater than 2.5 x 10^6). All subsequent experiments were performed using cell concentrations which fell between 0.75 and 2 x 10^6 cells/ml.

(* Although 5-HETE is not a leukotriene, for convenience the term "leukotrienes" will refer to LTB_4, the all-trans isomers of LTB_4 and 5-HETE.)

4.2.2 Time course of leukotriene production by A23187 with and without exogenous AA.

The next series of experiments were designed to ascertain the stimulation time which generates maximal amounts of leukotrienes. The

time course of leukotriene production by neutrophils stimulated with 0.5 uM A23187 is shown in Figures 4.3 and 4.4. Maximal levels of LTB_4, the LTB_4 isomers and 5-HETE were consistently achieved at 5 min. In most experiments, the amount of LTB_4 isomers detected decreased after 5 min.

Simultaneous addition of 2.5 uM AA to cell suspensions stimulated with A23187 enhanced leukotriene production (Figures 4.5 and 4.6), but gave the same time course as A23187 alone. Maximal amounts were detected by 5 min. of stimulation, after which the levels detected remained stable.

4.2.3 Dose response curve to A23187 with and without exogenous AA.

Dose response curves to A23187 in the range of 0.01-10.0 uM were obtained using neutrophils from seven healthy volunteers (Figures 4.7-4.12). A stimulation time of 5 min was chosen because maximal levels of the leukotrienes had been observed at this time point (Section 4.2.2). 0.01 uM A23187 did not stimulate neutrophils to produce detectable amounts of leukotrienes in any of the experiments. Production occurred at 0.05 uM A23187 in all experiments, reached a maximum at 0.1 uM and above this concentration, the amounts measured decreased. (The minimum concentration of A23187 which stimulated detectable amounts of these compounds was 0.03 uM (see Section 4.2.7)).

It was observed that the ratio of production of LTB_4:LTB_4 isomers sometimes changed. Generally, the ratio was approximately 1:1,

however the ratio varied between 2:1 and 1:2. These three possible outcomes are illustrated in the dose response curves of the three individuals shown here (see Figures 4.8, 4.10 and 4.12). It is possible that this phenomenon reflects different activation levels of LTA hydrolase, the enzyme which catalyses the conversion of LTA_4 to LTB_4. When this enzyme has a high activity level, more LTB_4 is produced while the amount of isomers is decreased and vice versa. While the factors involved in the regulation of LTA hydrolase are largely unknown, the activity of this enzyme is possibly modulated by protein kinase C (Chapter 7).

Addition of exogenous AA to neutrophils in combination with A23187 amplified the production of leukotrienes as previously described (Section 4.2.2). The amplification was approximately 2-fold and was dependent on the dose of exogenous AA supplied (Figures 4.13, 4.14 and 4.15). Maximal levels were measured using 0.5 uM A23187 with 2.5 uM AA. When AA was added with A23187, the amount of LTB_4 isomers detected was always larger than LTB_4 (compare Figures 4.14 and 4.15). A likely explanation for this is that when exogenous substrate is added with A23187, the amount of LTA_4 generated is too great for LTA hydrolase to metabolise to LTB_4 so a larger proportion is non-enzymatically converted to the all-trans isomers.

4.2.4 Effect of A23187 and FMLP on ^{3}H-AA release and metabolism by neutrophils.

These experiments were performed to determine whether FMLP alone could stimulate release and metabolism of endogenous AA in

neutrophils. FMLP alone did not stimulate detectable production of leukotrienes (data not shown). To increase sensitivity, neutrophils were preincubated with ^{3}H-AA (0.1 uCi, 1.0-1.7 x 10^{-15} moles) for 60 min at 37^{o}C prior to stimulation. FMLP did not stimulate production of ^{3}H-AA metabolites (Figure 4.16), whereas A23187 did (Figure 4.17). When FMLP and ^{3}H-AA, or A23187 and ^{3}H-AA, were added simultaneously to cells which had been preincubated with buffer for 1 hr, ^{3}H-AA metabolites were detected (Figures 4.18 & 4.19). ^{3}H-AA alone was not metabolised (data not shown).

4.2.5 Dose response curve to FMLP and exogenous AA.

To determine the concentration of FMLP which maximally stimulates maximal levels of leukotrienes, neutrophils were incubated with increasing doses of FMLP in the presence of 2.5 uM AA for 5 min. The dose response curves to FMLP and AA (Figures 4.20 and 4.21) were similar to those produced by stimulation with A23187 (Figures 4.7-4.12). Increasing the dose of stimulus increased leukotriene levels until a maximum was reached. Amounts measured decreased thereafter. Maximal amounts of all products (LTB_4, LTB_4 isomers and 5-HETE) occurred at 1 uM FMLP. This dose was used in all subsequent experiments involving FMLP.

The effect of stimulation with increasing doses of AA and a fixed dose of FMLP (1 uM) for 5 min is shown in Figure 4.22 and 4.23. Maximal levels were detected with 2.5 uM AA after which the response rapidly decreased.

4.2.6 Time course of production of leukotrienes by FMLP and exogenous AA.

These experiments were performed to determine the time at which maximum levels of leukotrienes are produced by neutrophils stimulated with FMLP. Doses of 1 uM FMLP and 2.5 uM AA were chosen as stimuli in these time course experiments. In contrast to A23187, maximal production of leukotrienes occurred after 2 min of stimulation with FMLP and AA, after which the level of all compounds rapidly decreased (see Figures 4.24 and 4.25).

4.2.7 Variability in leukotriene production.

These experiments were designed to investigate intra-subject and inter-subject variation in neutrophil leukotriene synthesis. In the first set of experiments, blood was collected from two donors every day for 1 week (5 days). Neutrophils were isolated and stimulated at 37^{o}C for 5 min with either 5 uM A23187 or 5 uM A23187 with 10 uM AA. The coefficients of variation were large irrespective of whether A23187 was used alone or in conjunction with AA indicating a high degree of day to day variation (Table 4.2).

In the second set of experiments, which took place over a period of 6 weeks, blood was taken from 31 separate donors (age range 20-65 years). Their neutrophils were stimulated for 5 min under three different conditions; 5 uM A23187, 5 uM A23187 + 10 uM AA and 1 uM FMLP + 10 uM AA. The coefficients of variation observed in this experiment were similar to those found when measuring the variability

in LTB_4 and 5-HETE generation by neutrophils from the same donor over a period of 5 days (Table 4.3).

4.2.8 Effect of calcium on generation of leukotrienes by A23187 with and without AA.

The 5-LO pathway and PLA_2 are Ca^{++}-dependent (Section 1.2.4) and both are believed to be stimulated by A23187. However, the Ca^{++} requirements for either enzyme may differ. This question is addressed in this section.

The first set of experiments were performed to determine (a) the minimum concentration of A23187 required to stimulate production of detectable quantities of leukotrienes and (b) whether addition of exogenous AA imparts responsiveness to sub-stimulatory doses of A23187. Neutrophils responded to A23187 alone at doses of 0.03, 0.04 and 0.05 uM by producing LTB_4, LTB_4 isomers and 5-HETE in a dose-dependent manner, however, these compounds were not detected using 0.01 or 0.02 uM A23187 (Figures 4.26 & 4.27). In contrast, when 2.5 uM AA (which alone did not stimulate leukotriene production) was added with 0.01 and 0.02 uM A23187, detectable amounts of leukotrienes were observed (Figures 4.26 &4.27). This suggests that the 5-LO pathway may be less dependent on Ca^{++} than PLA_2. When 2.5 uM AA was added, enhanced production of leukotrienes was observed at 0.03, 0.04 and 0.05 uM A23187.

The next set of experiments were designed to investigate the effect of decreasing the concentration of extracellular Ca^{++} on the

ability of A23187 (alone or in the presence of 2.5 uM AA) to stimulate leukotriene production. For these experiments, neutrophils were resuspended in a modified DPBS (containing glucose and Mg^{++}) to which increasing concentrations of $CaCl_2$ were added. Stimulation for 5 min with 0.5 uM A23187 did not produce detectable levels of leukotrienes in the absence of external Ca^{++} (Figures 4.28 and 4.29). In contrast, when 2.5 uM AA was added with 0.5 uM A23187, production of all three compounds occurred even in the absence of external Ca^{++}. Furthermore, maximal production of these compounds using A23187 and exogenous AA was reached with 0.1 mM extracellular Ca^{++} whereas maximal production stimulated by A23187 alone was not achieved until 0.8 mM Ca^{++} (Figures 4.28 & 4.29). These data suggest that PLA_2 has a greater requirement for extracellular Ca^{++} than does the 5-LO pathway .

The final series of experiments involved the use of three modifiers of free Ca^{++}: EDTA, EGTA and TMB-8. These antagonists were used to determine whether the influx of extracellular Ca^{++} or mobilization of intracellular Ca^{++} is more important in activation of PLA_2 or the 5-LO pathway. EDTA is believed to chelate both extra- and intracellular Ca^{++} (Williams and Cole, 1981), however, some caution must be taken when assessing results using EDTA because chelating effects on Mg^{++} cannot be excluded. EGTA was chosen as an extracellular Ca^{++} chelator (Hallett and Campbell, 1983; Smolen and Boxer, 1983) because it does not cross the cell membrane and exerts little effect on intracellular Ca^{++} unless cells are exposed to it for long periods of time (Dyett et al., 1986; Hallett and Campbell 1983; Di Virgillio et al., 1984). TMB-8 inhibits intracellular Ca^{++} mobilization although the mechanism by which this is achieved is

uncertain (Lad et al., 1985; Smolen et al., 1981; Smith and Iden, 1979).

In this series of experiments, purified human neutrophils were resuspended in HBSS at 37°C. Half the samples were incubated with 20 ul of inhibitor while the other half received the same volume of HBSS for 10 min before stimulation. The cells were then stimulated for 5 min with A23187 alone (0.05, 0.5 or 5.0 uM) or with 2.5 uM AA. The final concentrations of inhibitors were 2 mM EDTA, 2 mM EGTA or 65 uM TMB-8.

Addition of A23187, or A23187 and AA without EDTA, resulted in production of leukotrienes at all three doses of A23187. Production at each dose was enhanced by the addition of exogenous AA (Figures 4.30, 4.31 & 4.32). When the cells were preincubated for 10 min with EDTA, production of all three compounds was completely inhibited regardless of whether exogenous AA was added, implying that both the 5-LO pathway and PLA_2 activation are inhibited by this concentration of EDTA.

EGTA completely inhibited the production of leukotrienes when A23187 alone was used as stimulus (Figures 4.33, 4.34 & 4.35). However, in contrast to the effect of EDTA, production of the leukotrienes was only partially reduced in the presence of EGTA when A23187 and AA were used as stimulus, suggesting that EGTA inhibits PLA_2 activation and reduces 5-LO pathway activation by A23187.

When TMB-8 was used, 5-HETE production could not be measured

because TMB-8 interfered with the chromatography of 5-HETE. Production of LTB_4 and LTB_4 isomers was observed at all doses of A23187 and enhanced by the addition of exogenous AA (Figures 4.36 and 4.37). The effect of TMB-8 was similar to that of EGTA. Production of both LTB_4 and its isomers stimulated by A23187 alone was completely inhibited whereas when AA was added with A23187, production was only partially inhibited. These results again imply that a difference in sensitivity to Ca^{++} exists between PLA_2 and the 5-LO pathway, with the 5-LO pathway being less dependant on Ca^{++}.

4.1 DISCUSSION.

In the experiments described in this Chapter, FMLP alone failed to stimulate metabolism of endogenous AA to leukotrienes (see also Clancy et al., 1983; 1985; Palmer and Salmon, 1983). The lack of detection was not caused by lack of sensitivity because FMLP also failed to stimulate ^3H-LTB_4 production by neutrophils which had been preincubated with ^3H-AA for 1 hr. Furthermore, the lack of response was not due to the preincubation time because cells preincubated with buffer for the same period of time responded to stimulation with FMLP and ^3H-AA. Production of leukotrienes did occur if exogenous AA was added with FMLP (see also Clancy et al., 1983; 1985), implying that stimulation of neutrophils with FMLP is sufficient to activate the 5-LO pathway but not to stimulate release of endogenous AA. It is possible that the Ca^{++} influx caused by FMLP is insufficient to stimulate PLA_2.

Maximal levels of LTB_4, LTB_4 isomers and 5-HETE were reached

after stimulation with A23187 for 5 minutes (see also Clancy et al., 1985; Sun and McGuire, 1984; Bonser et al., 1981). Co-addition of AA did not alter this characteristic. In contrast, when FMLP and AA were used as a stimulus, maximal levels were reached after two minutes of stimulation (see also Bonser et al., 1981; Clancy et al., 1983). It is likely that the difference in response to A23187, or FMLP with AA reflects the different Ca^{++} fluxes they generate. A23187 generates a large, sustained influx of Ca^{++} (French, personal communication) whereas FMLP generates a smaller, more transient flux (Lazzari et al., 1986).

It was observed in the time course experiments using FMLP and AA as stimulus, that after maximal levels of the leukotrienes were reached, the amounts detected rapidly decreased. The disappearance of 5-HETE from the supernatant is thought to be due to its re-esterification into membrane phospholipids, which has been demonstrated, using FMLP as a stimulus, in macrophages (Pawlowski et al., 1982; Stenson et al., 1983), HL-60 granulocytes (Bonser at al., 1981) and neutrophils (Clancy et al., 1985). The disappearance of LTB_4 is believed to occur because of w-oxidation. This hypothesis is supported by work showing increased levels initially of 20-OH LTB_4 and later 20-COOH LTB_4 after stimulation times greater than 5 min (Salari et al., 1985b). Similar findings have been reported using A23187 as a stimulus (Sun and McGuire, 1984), an effect I have been unable to reproduce. At least part of the decrease in measurement of metabolites is likely to be due to enzyme inactivation since upon stimulation, several types of leukocyte characteristically produce a rapid burst of 5-LO activity followed by cessation of activity

(Parker, 1984) and the 5-LO enzyme system in the 10,000 x g homogenate from RBL-1 cells inactivates after 10-15 minutes (Jakschik and Kuo, 1983). It is believed that generation of a peroxide product causes the inactivation (Jakschik and Kuo, 1984).

Published data showing complete dose response curves to A23187 or FMLP with AA are rare. Using FMLP in the presence of cytochalasin B, Williams et al., (1986) were able to demonstrate that 1 uM FMLP stimulated maximal release of LTB_4 by human monocytes, a result in agreement with my findings using neutrophils stimulated with FMLP and AA. Jorg et al., (1982) stimulated equine eosinophils with increasing doses of A23187 and found that 10 ug/ml (approximately 20 uM) stimulated maximal release of LTC_4. In contrast, Lee et al., (1985) reported maximal leukotriene release (LTB_4, LTB_4 isomers and 5-HETE) by human neutrophils and monocytes using between 10 and 20 uM A23187. I have found that maximal leukotriene release was stimulated by 0.1 uM A23187. It is possible that there is greater bioavailability of A23187 when added in methanol compared to aqueous solutions. Both Jorg et al., (1982) and Lee et al., (1985) added A23187 in an aqueous solution. A further explanation may be that the cell concentration used in the study by Jorg et al., (1982) was 5×10^6/ml and I have demonstrated that at this cell concentration, the relationship between leukotriene production and concentration of A23187 is not linear (Section 4.2.1). Therefore using 5×10^6 cell/ml or greater may shift the dose response curve to the right. It is also possible that differences in cell separation techniques may account for such variation (McColl, unpublished observations).

The dose response curves I performed indicated that doses of the stimulus higher than required for maximum levels of leukotriene detection resulted in progressive inhibition. More specifically, doses above 0.1 uM A23187, or 1 uM FMLP with AA begin to inhibit LT production and as the dose of stimulus is further increased, greater inhibition occurs (see also Jorg et al., 1982). Furthermore, once stimulated, the cells respond less readily to subsequent stimulation (Jakschik and Kuo, 1984; Sun and McGuire, 1984; McColl, unpublished observations). It is possible that the higher doses of agonist stimulate re-esterification of 5-HETE and w-oxidation of LTB_4, however another explanation is that higher doses of stimulus progressively inhibit 5-LO. Prostaglandin H Synthetase catalyses its own inactivation, a phenomenon which is related to enzyme turnover and is therefore proportional to product formation (Lands and Hanel, 1983). It is possible that 5-LO also possesses this characteristic, however, from the data gathered here it appears that the mechanism of inactivation of 5-LO is much slower than that of prostaglandin H synthetase.

The experiments examining variability of leukotriene production within and between donors indicated that variation in absolute amounts of leukotrienes generated from day to day were too great to allow quantitative inter-experimental analyses. Why such variability occurs may be related to day to day differences in the level of lipomodulin within cells.

The experiments investigating the dependence of the system on Ca^{++} demonstrates that in neutrophils, metabolism of AA to

leukotrienes is less sensitive to Ca^{++} than the release of esterified AA. Doses of A23187 which alone failed to stimulate production of detectable levels of LTs, stimulated production of LTs when exogenous AA was added. AA alone did not stimulate LT generation. Furthermore, when neutrophils were incubated in buffer without extracellular Ca^{++}, the combination of A23187 and AA stimulated production of LTs whereas A23187 alone did not. Maximal production of LTs occurred at 0.05 mM extracellular Ca^{++}. In contrast, maximal production using A23187 alone required 0.4-0.8 mM extracellular Ca^{++}.

Further supportive evidence was obtained from the studies using the Ca^{++} chelators EDTA, EGTA and TMB-8. Using the latter two compounds, it was possible to completely inhibit LT production stimulated by A23187 alone whereas production stimulated by A23187 and AA was reduced but not completely inhibited. On the other hand, EDTA, which chelates both intracellular and extracellular Ca^{++}(Williams and Cole, 1981), completely inhibited leukotriene production regardless of whether A23187 was used alone or with AA. This implies that both PLA_2 and the 5-LO pathway have a requirement for Ca^{++}. However, EGTA, an extracellular Ca^{++} chelator (Smolen and Boxer, 1983) only partially inhibited the response to A23187 with AA, indicating that the intracellular Ca^{++} flux induced by A23187 is sufficient to partially activate the 5-LO pathway. The same result was observed using TMB-8, which inhibits intracellular Ca^{++} release (Smith and Iden 1979). Taken together, these results imply that the 5-LO pathway requires either influx of extracellular Ca^{++} or release of intracellular Ca^{++}, whereas activation of PLA_2 requires both.

Table 4.1 Some of the reported amounts of 5-lipoxygenase products generated in response to A23187. Amounts expressed as ng/10^6 cells

Conditions	Cell Type	Product	Amount	Reference
1.5 uM for 5'	PMNL	LTB_4	51	Borgeat et al., 1984
		5-HETE	107	
		20-OHLTB_4	8	
2.5 uM for 10'	PMNL	LTB_4	25	Williams et al., 1985
10 uM for 2'	PMNL	LTB_4	12	Sun and McGuire, 1984
		5-HETE	26	
2 uM for 5'	PMNL	LTB_4	5-10	Palmer and Salmon, 1983
10 uM for 5'	Leukocytes	LTB_4	12	Clancy et al., 1985
		20-OHLTB_4	4	
		20-COOHLTB_4	1	
5 uM for 5' (+ 150 uM AA)	Leukocytes	LTB_4	3	Claesson et al., 1985
1 uM for 5'	MNC	LTB_4	48	Goldyne et al., 1984
		20-OHLTB_4	16	
		5-HETE	59	

Table 4.2 Variability of a) LTB_4 and b) 5-HETE production within donors. Results are expressed as mean ± sem.

A) LTB_4

Condition	Donor 1	CV (n=5)	Donor 2	CV (n=5)
5 uM A23187	11.2±5.5	48.7%	8.9±7.8	87.6%
5 uM A23187 + 10 uM AA	18.6±6.3	33.7%	15.8±4.9	31.0%

B) 5-HETE

5 uM A23187	27.2±10.4	38.2%	24.5± 9.0	36.7%
5 uM A23187 + 10 uM AA	155.2±55.0	35.4%	196.3±68.2	34.8%

Table 4.3 Variability of a) LTB_4 and b) 5-HETE production between donors. Results are expressed as mean ± sem.

A) LTB_4

Condition	Male (n=15)	Female (n=16)	Total (n=31)	CV (n=31)
5 uM A23187	11.1±0.8	9.3±0.9	10.1±0.6	32.7%
5 uM A23187 + 10 uM AA	15.9±0.7	16.0±0.8	15.9±0.6	20.8%
1 uM FMLP + 10 uM AA	14.1±0.9	13.8±0.8	14.0±0.7	27.9%

B) 5-HETE

Condition	Male (n=15)	Female (n=15)	Total (n=30)	CV (n=30)
5 uM A23187	54.1± 4.1	49.9± 7.0	52.0± 4.0	42.1%
5 uM A23187 + 10 uM AA	151.1±10.6	174.3±16.1	162.7± 9.7	32.6%
1 uM FMLP + 10 uM AA	139.0±10.1	151.1±11.7	141.4± 7.9	30.6%

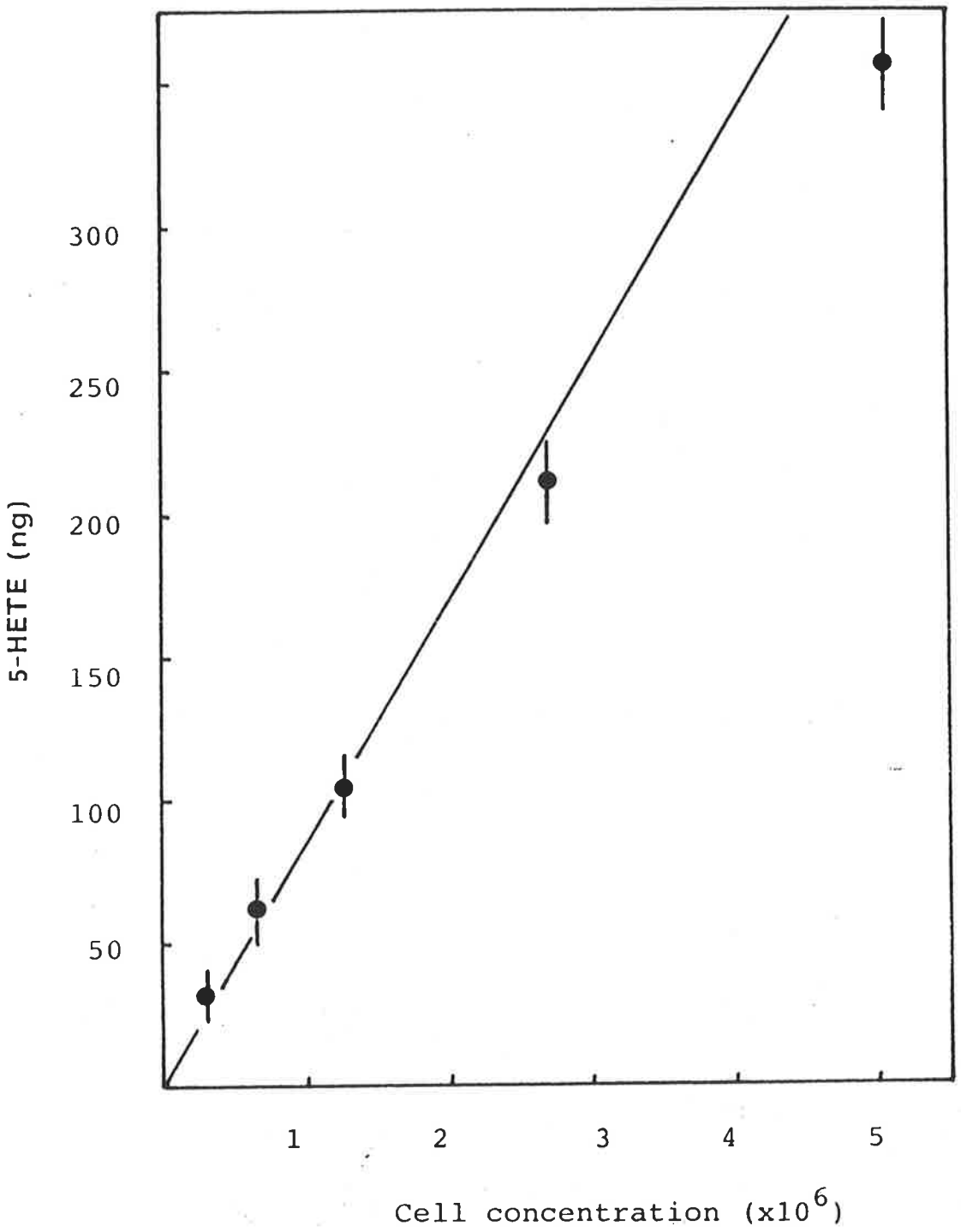


Figure 4.1: The effect of increasing cell concentration on the amount of 5-HETE generated by neutrophils. Each cell sample was stimulated with 5 uM A23187 for 5 min after which time the reaction was stopped using citric acid. 170 ng of 15-HETE was added as internal standard, the leukotrienes were extracted from the samples and HPLC analysis was performed (see Chapter 2 for details).

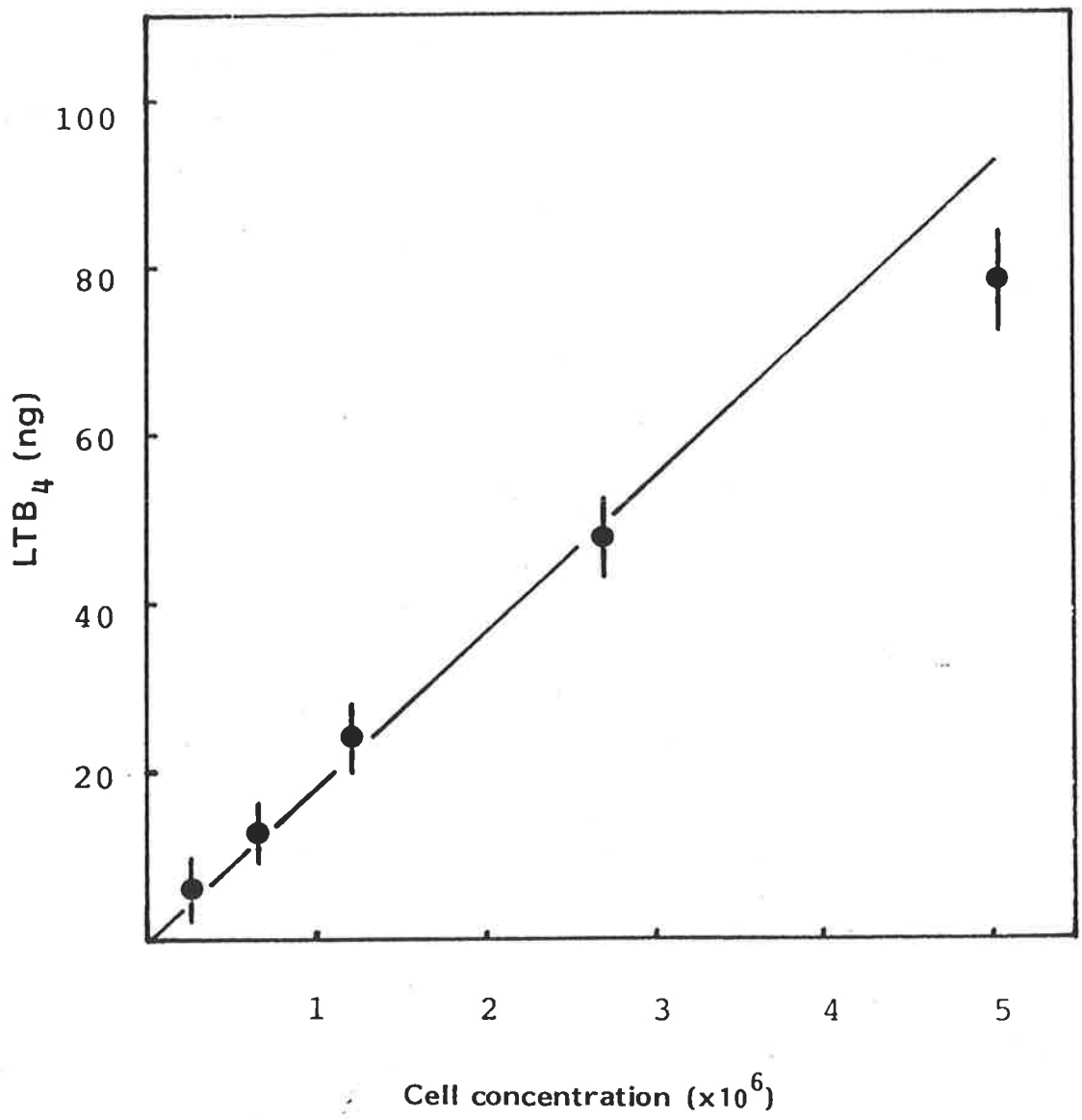


Figure 4.2: The effect of increasing cell concentration on the amount of LTB_4 generated by neutrophils. Each sample was stimulated with 5 uM A23187 for 5 min after which time the reaction was stopped using citric acid. 50 ng of PGB_2 was added as internal standard, the leukotrienes were extracted from the samples and HPLC analysis was performed (see Chapter 2 for details).

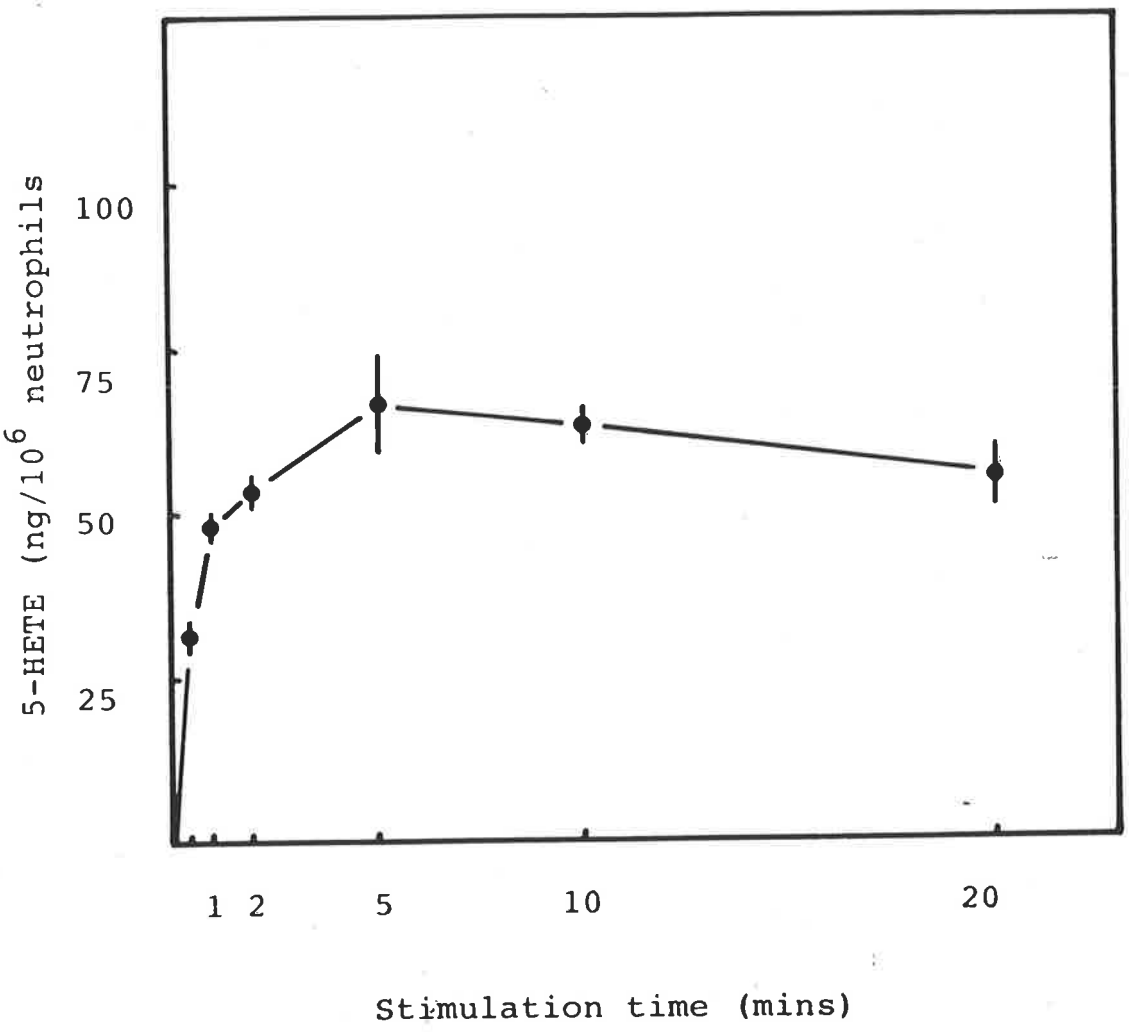


Figure 4.3: Time course of production of 5-HETE by neutrophils. Neutrophils were stimulated with 0.5 uM A23187 for increasing periods of time.

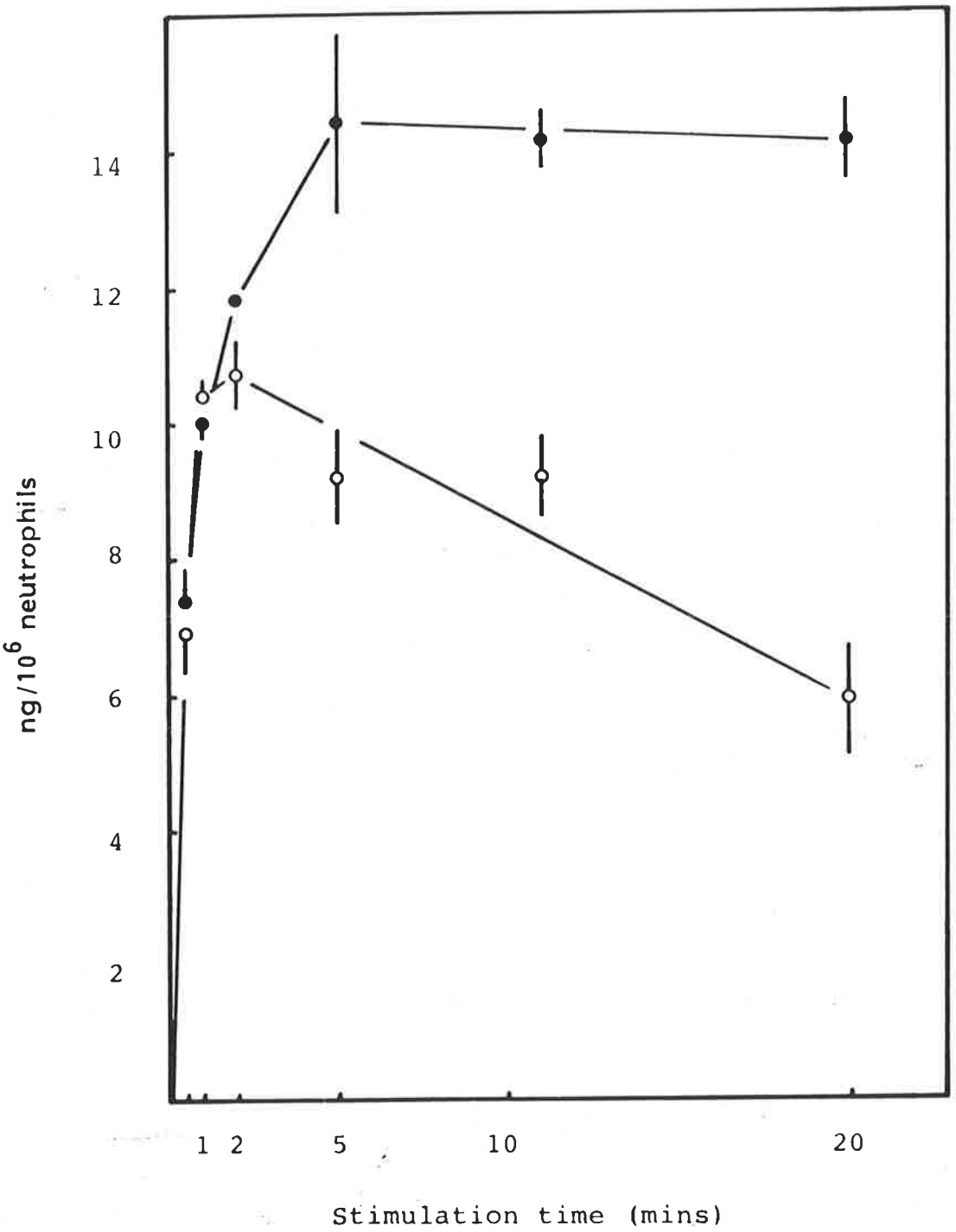


Figure 4.4: Time course of production of LTB_4 (●) and LTB_4 isomers (o) by neutrophils. The cells were stimulated with 0.5 uM A23187 for increasing periods of time.

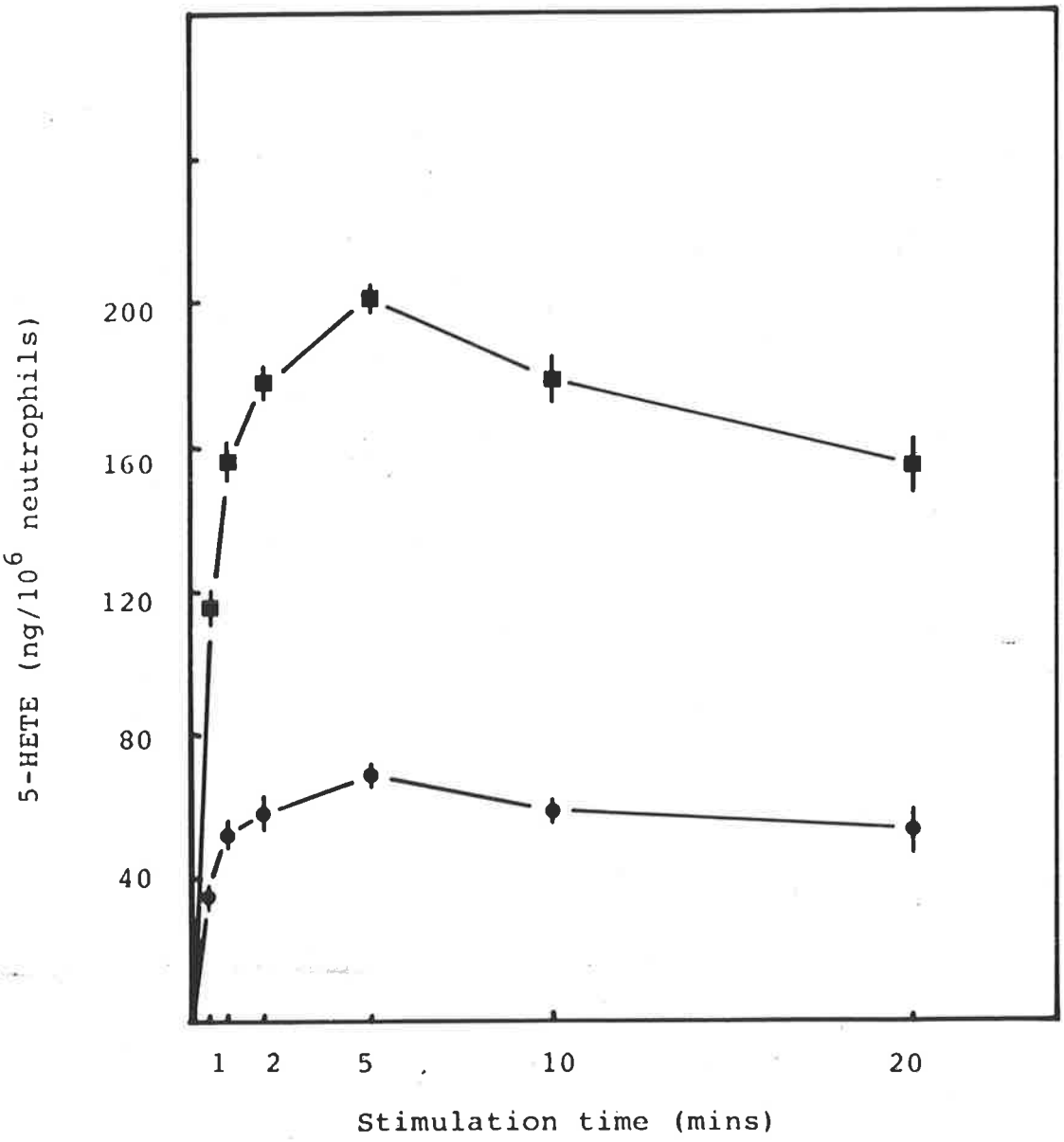


Figure 4.5 Time course of production of 5-HETE by neutrophils. Cells were stimulated with 0.5 uM A23187 (●) or 0.5 uM A23187 and 2.5 uM AA (■) for increasing periods of time.

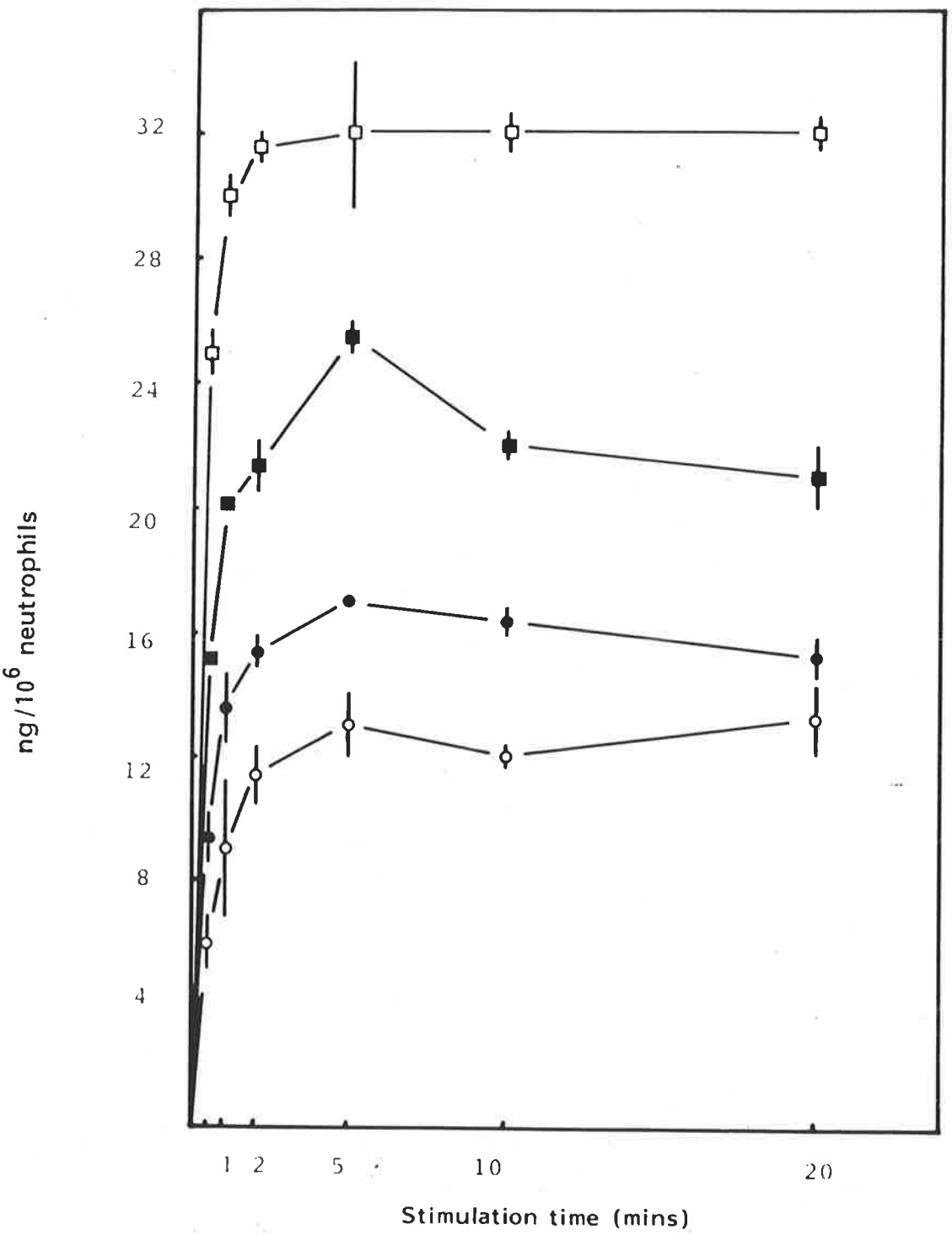


Figure 4.6 Time course of production of LTB_4 and LTB_4 isomers by neutrophils. Cells were stimulated with 0.5 uM A23187 (● , LTB_4; o , LTB_4 isomers) or 0.5 uM A23187 and 2.5 uM AA (■ , LTB_4; □ , LTB_4 isomers) for increasing periods of time.

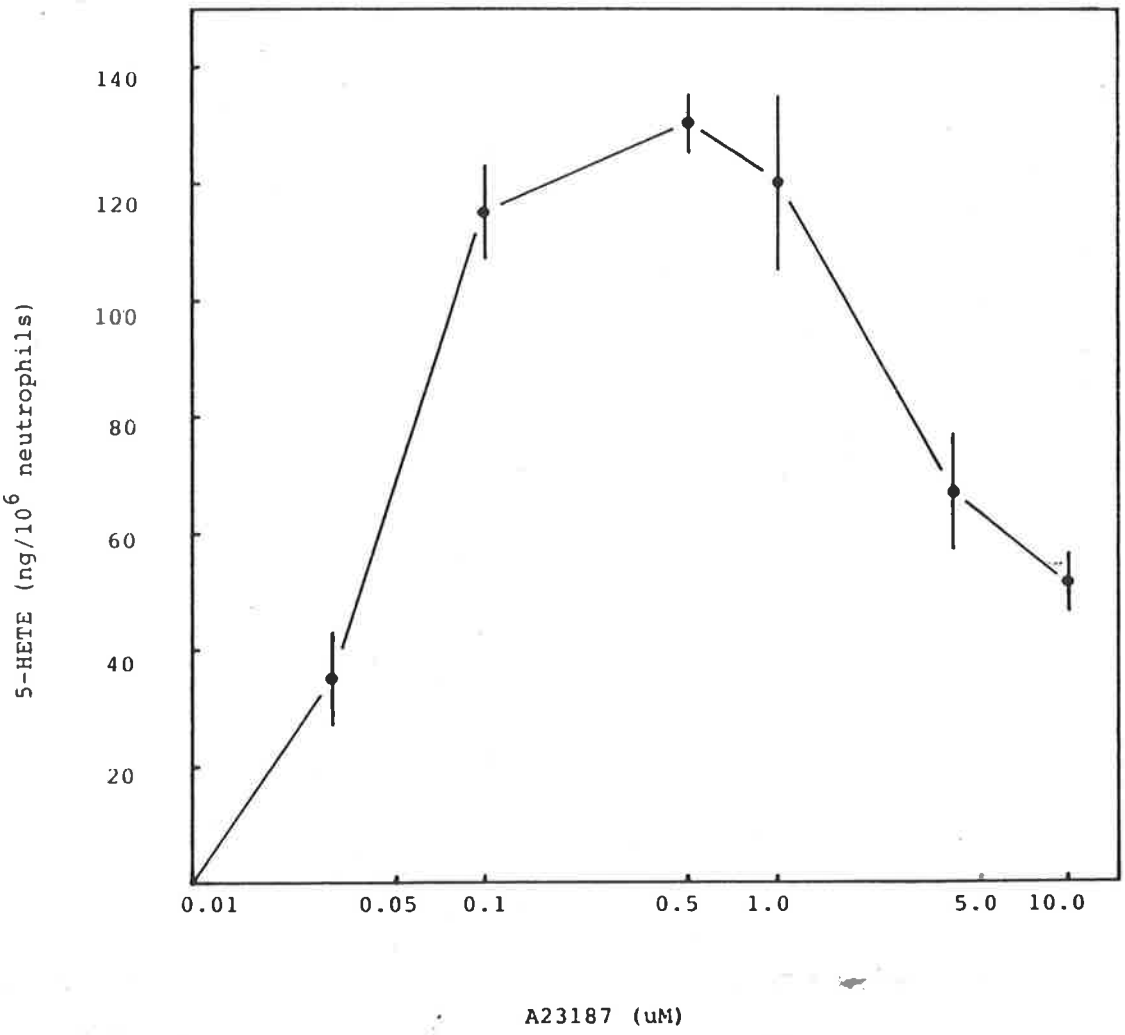


Figure 4.7 Dose reponse curve of production of 5-HETE by neutrophils stimulated by A23187 (volunteer A). Neutrophils were stimulated with increasing doses of A23187 for 5 min.

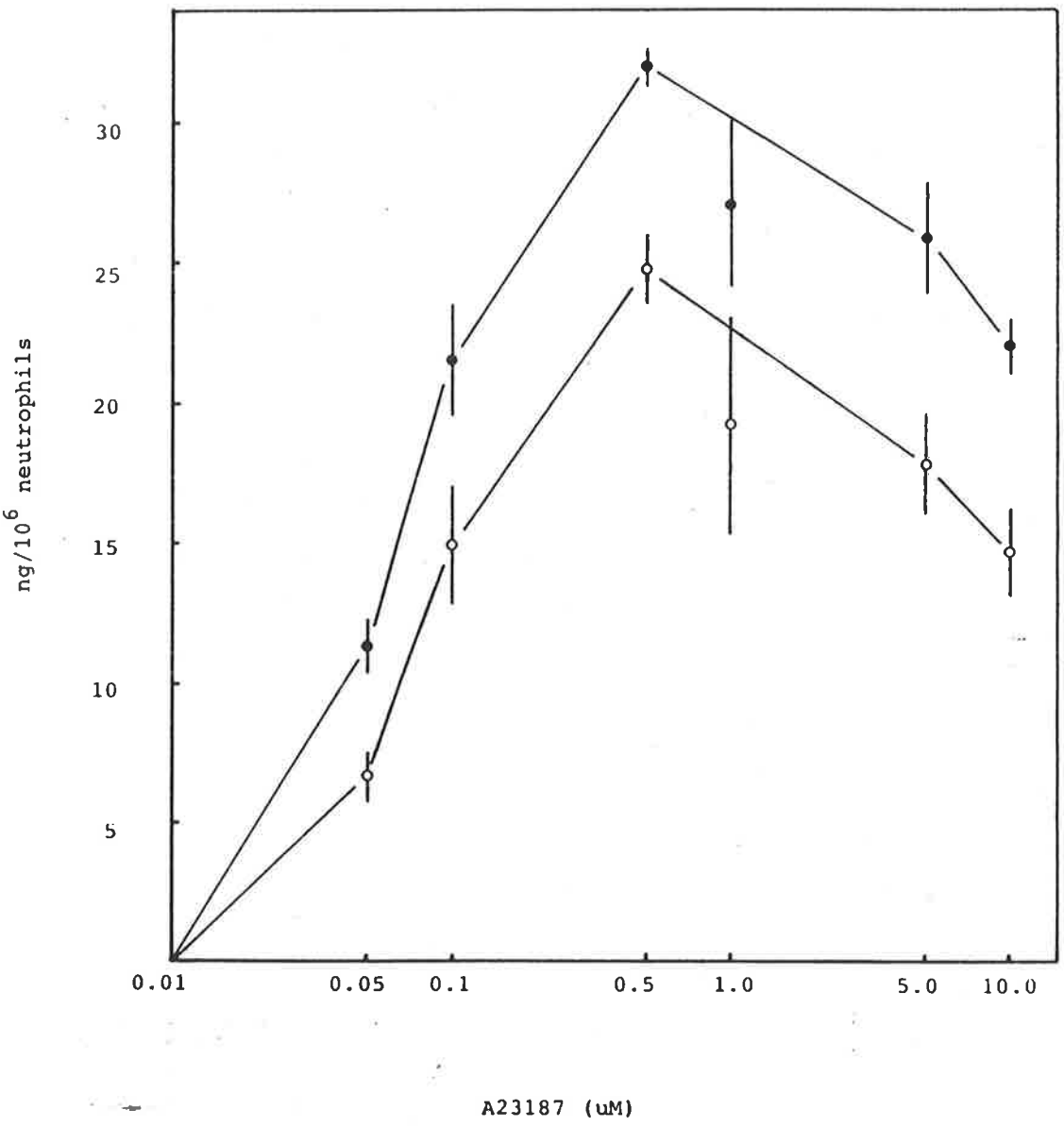


Figure 4.8 Dose response curves of production of LTB_4 (●) and LTB_4 isomers (○) by neutrophils stimulated by A23187 (volunteer A). Neutrophils were stimulated with increasing doses of A23187 for 5 min.

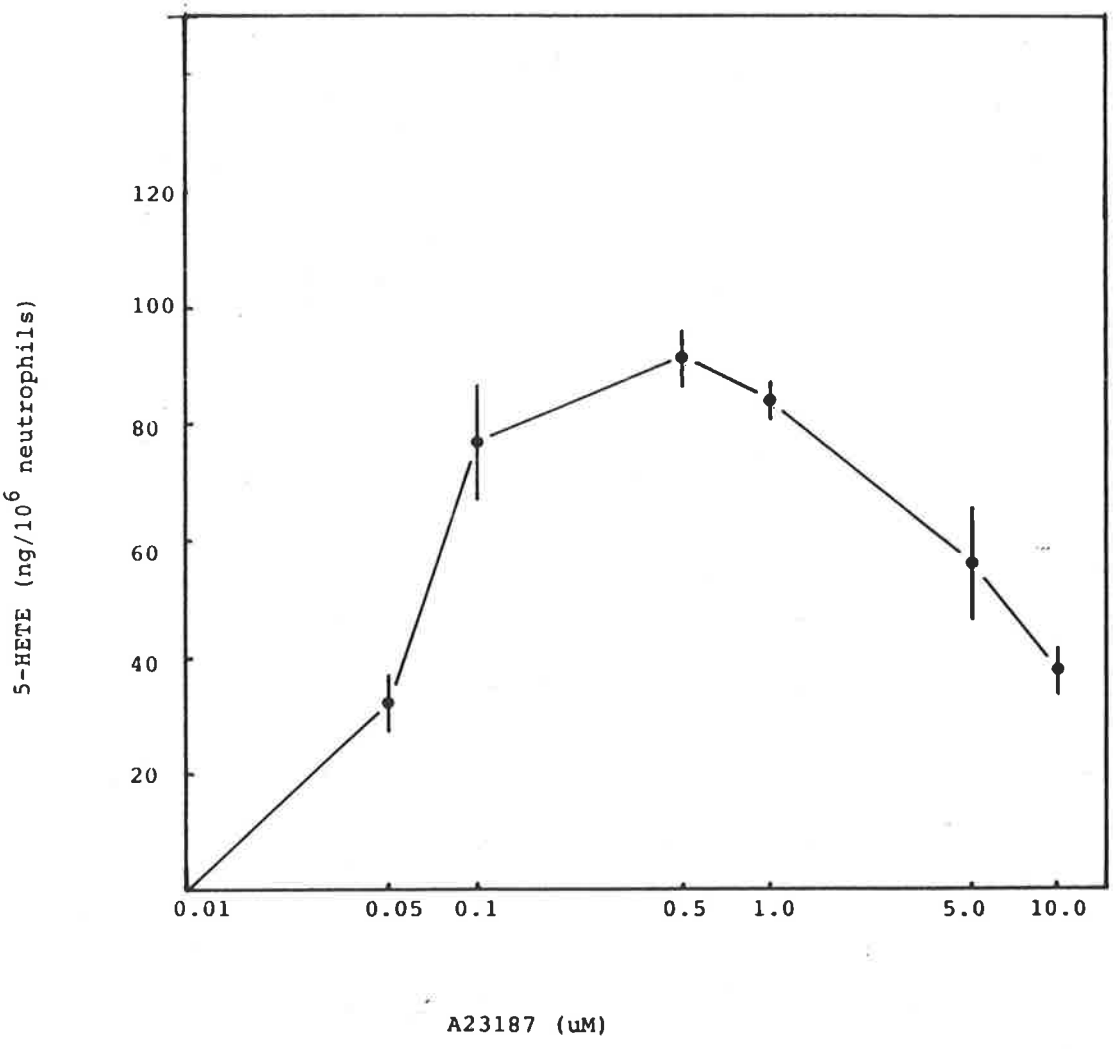


Figure 4.9 Dose response curve of production of 5-HETE by neutrophils stimulated by A23187 (volunteer B). Neutrophils were stimulated with increasing doses of A23187 for 5 min.

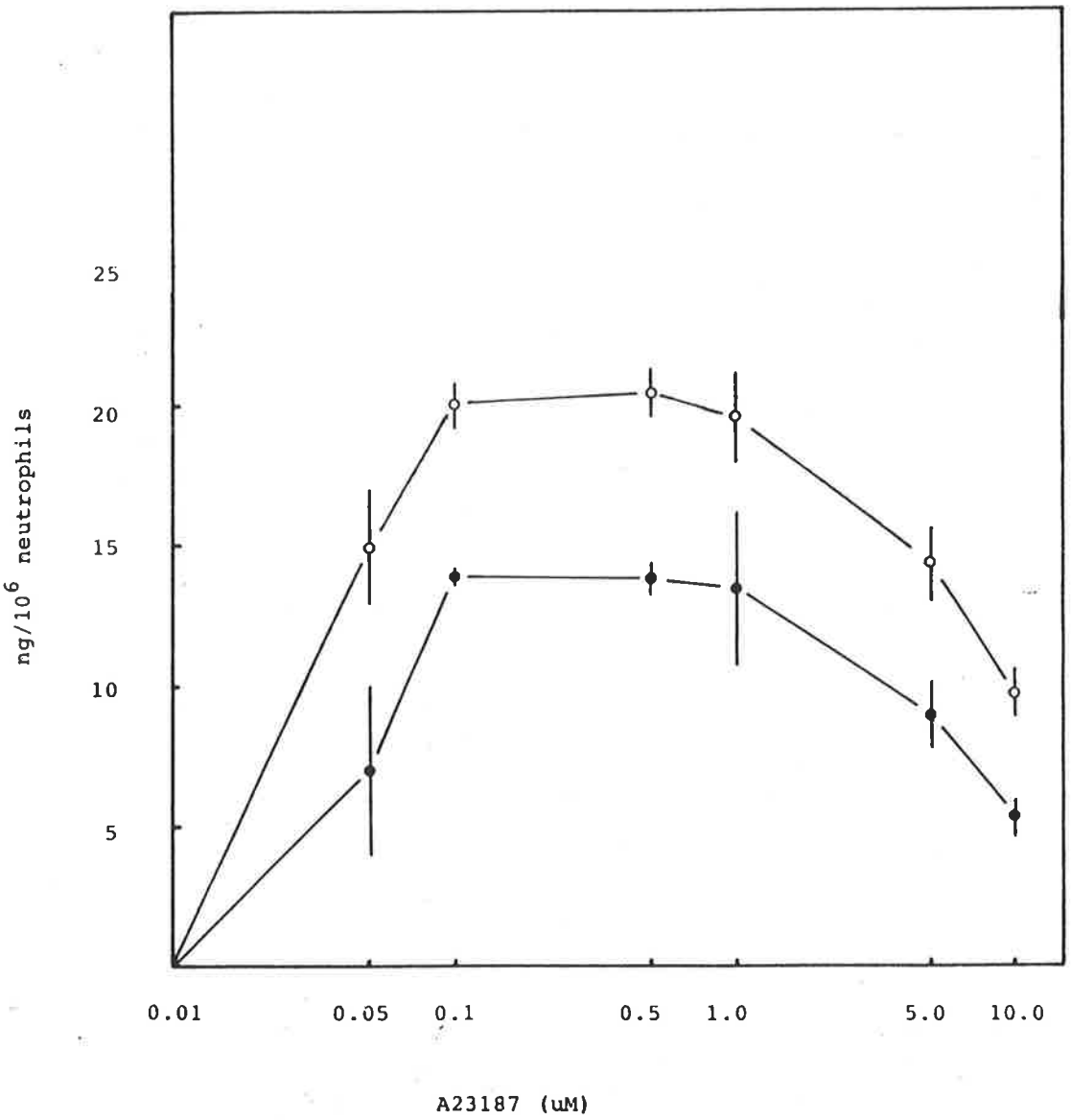


Figure 4.10 Dose response curves of production of LTB_4 (●) and LTB_4 isomers (○) by neutrophils stimulated by A23187 (volunteer B). Neutrophils were stimulated with increasing doses of A23187 for 5 mins.

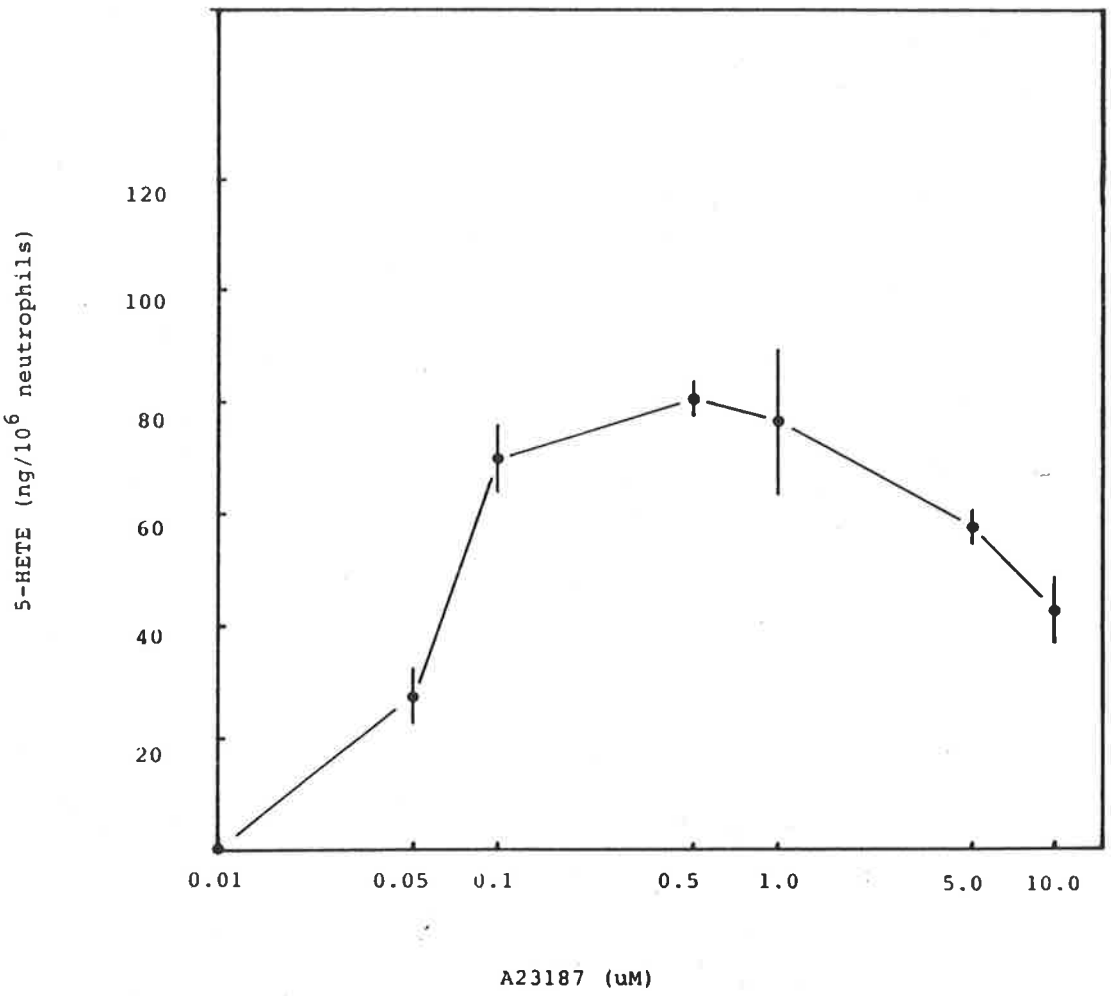


Figure 4.11 Dose response curve of production of 5-HETE by neutrophils stimulated with A23187 (volunteer C). Neutrophils were stimulated with increasing doses of A23187 for 5 min.

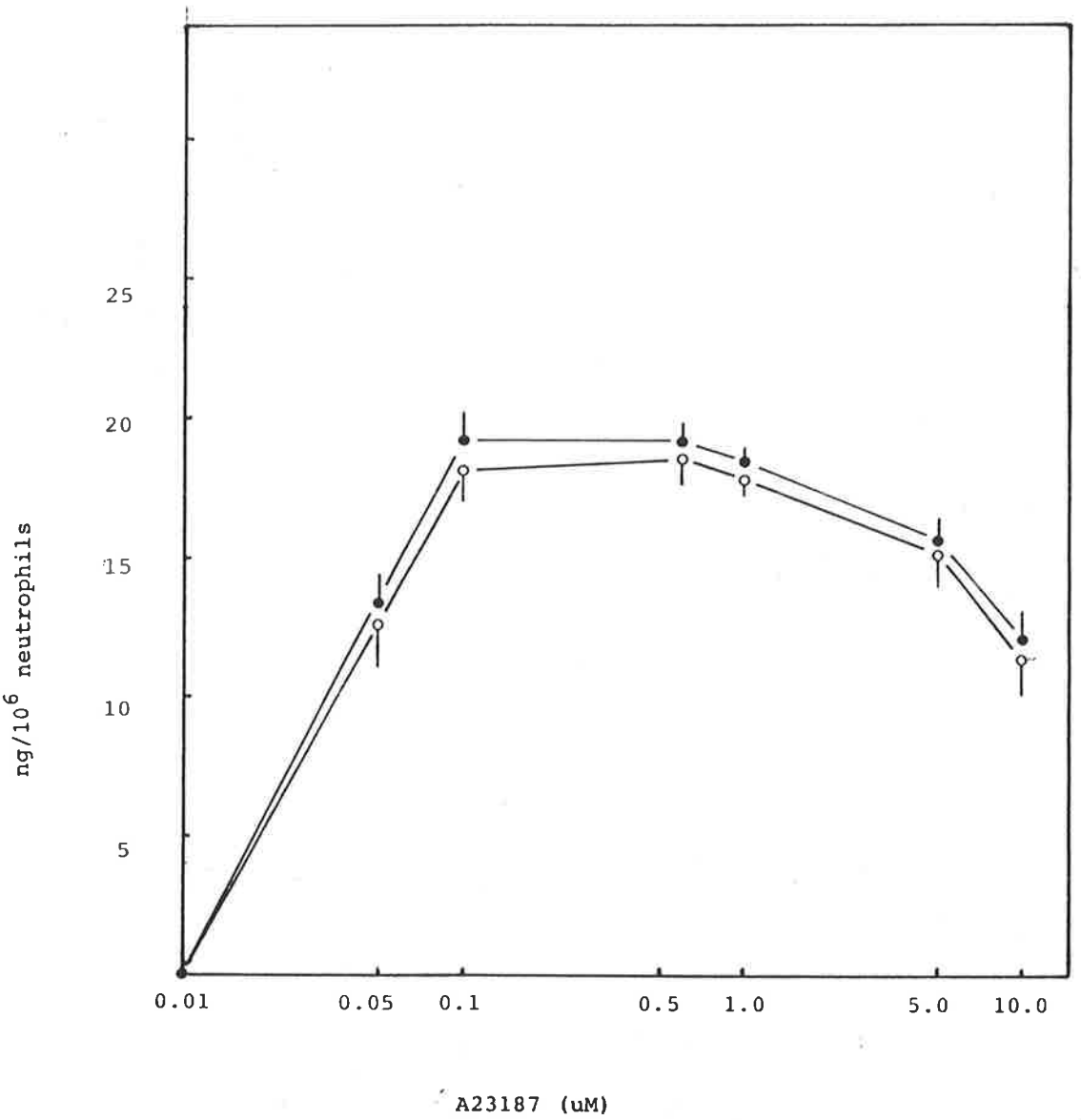


Figure 4.12 Dose response curves of production of LTB_4 (●) and LTB_4 isomers (○) by neutrophils stimulated with A23187 (volunteer C). Neutrophils were stimulated with increasing doses of A23187 for 5 min.

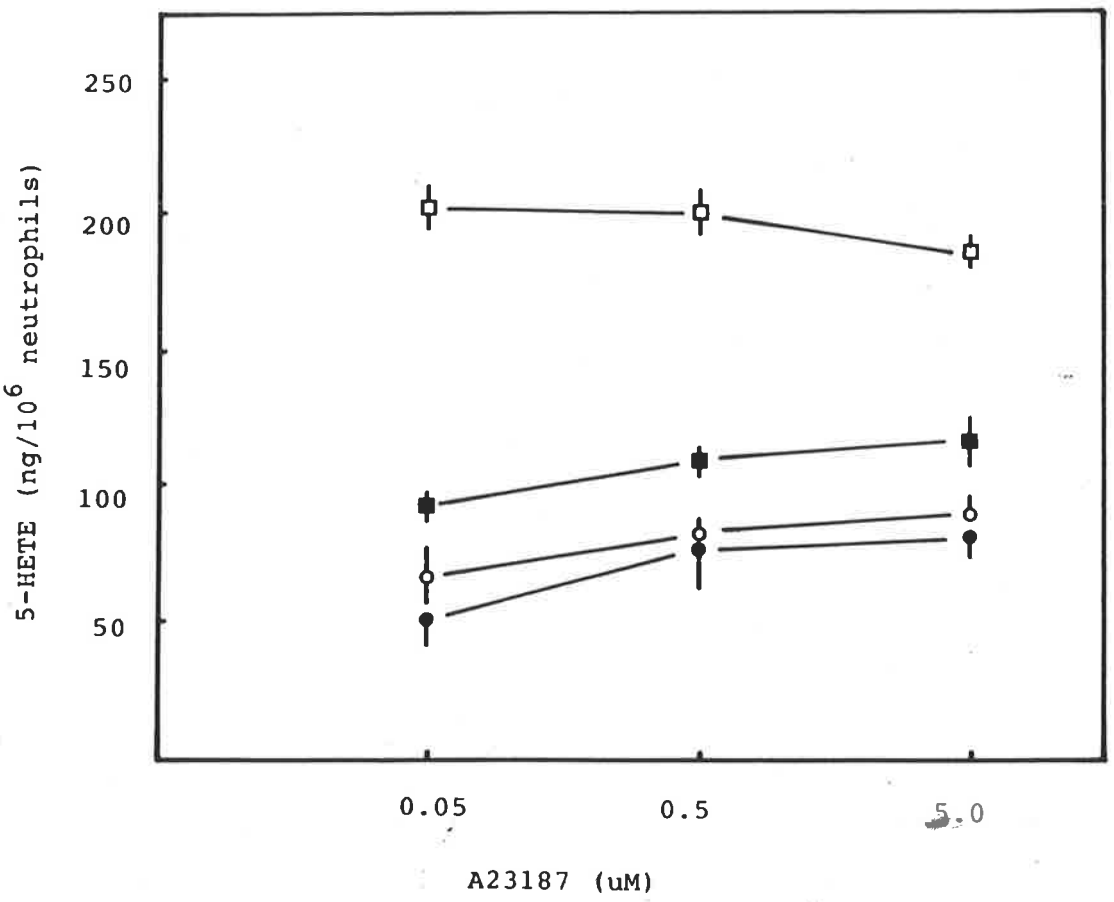


Figure 4.13 Dose response curves of production of 5-HETE by neutrophils stimulated for 5 min with three different doses of A23187 either alone (●) or with 0.63 uM AA (○), 2.5 uM AA (□) or 10 uM AA (■).

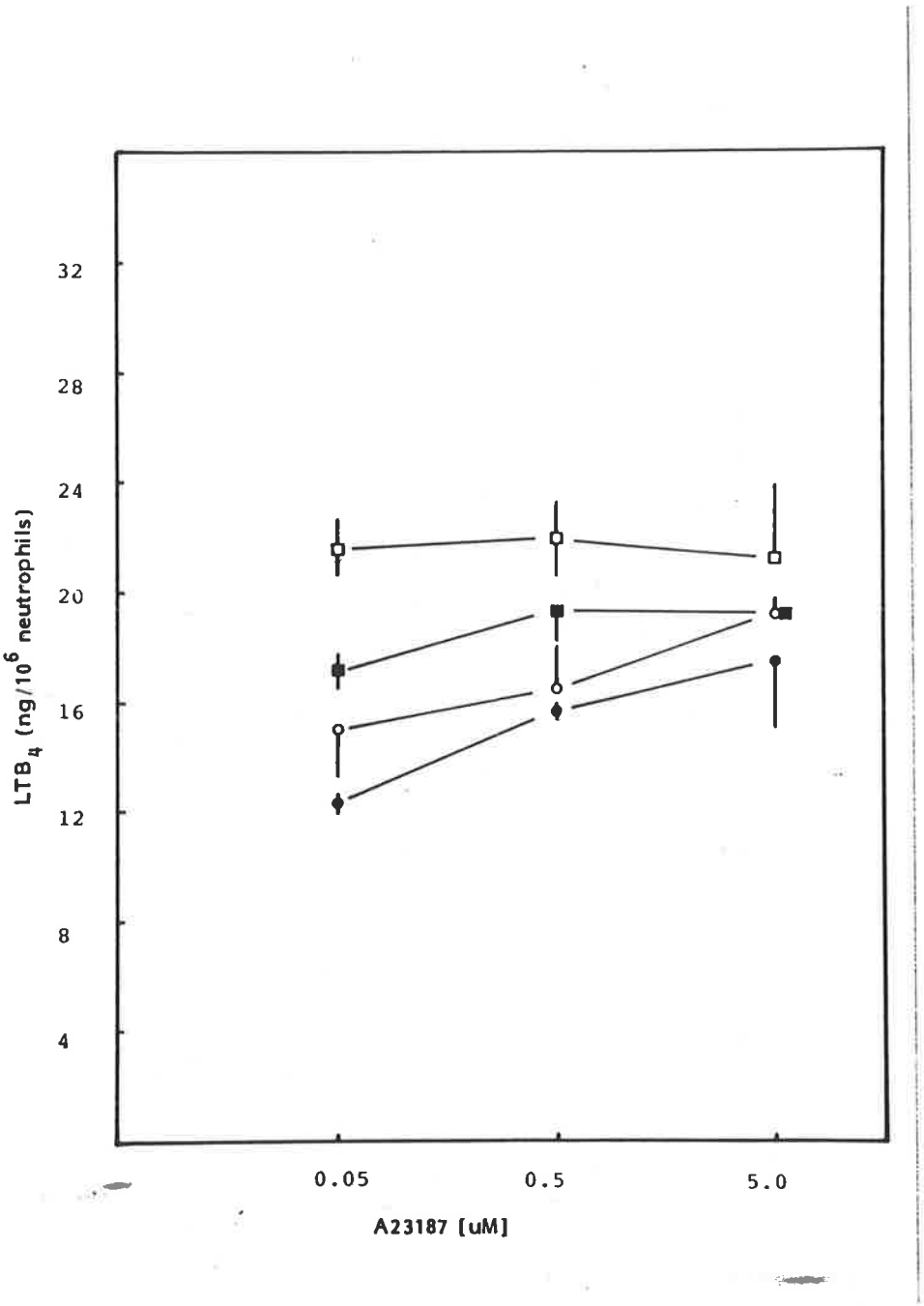


Figure 4.14 Dose response curves of production of LTB_4 by neutrophils stimulated for 5 min with three different doses of A23187 either alone (●) or with 0.63 uM AA (○), 2.5 uM AA (□), or 10 uM AA (■).

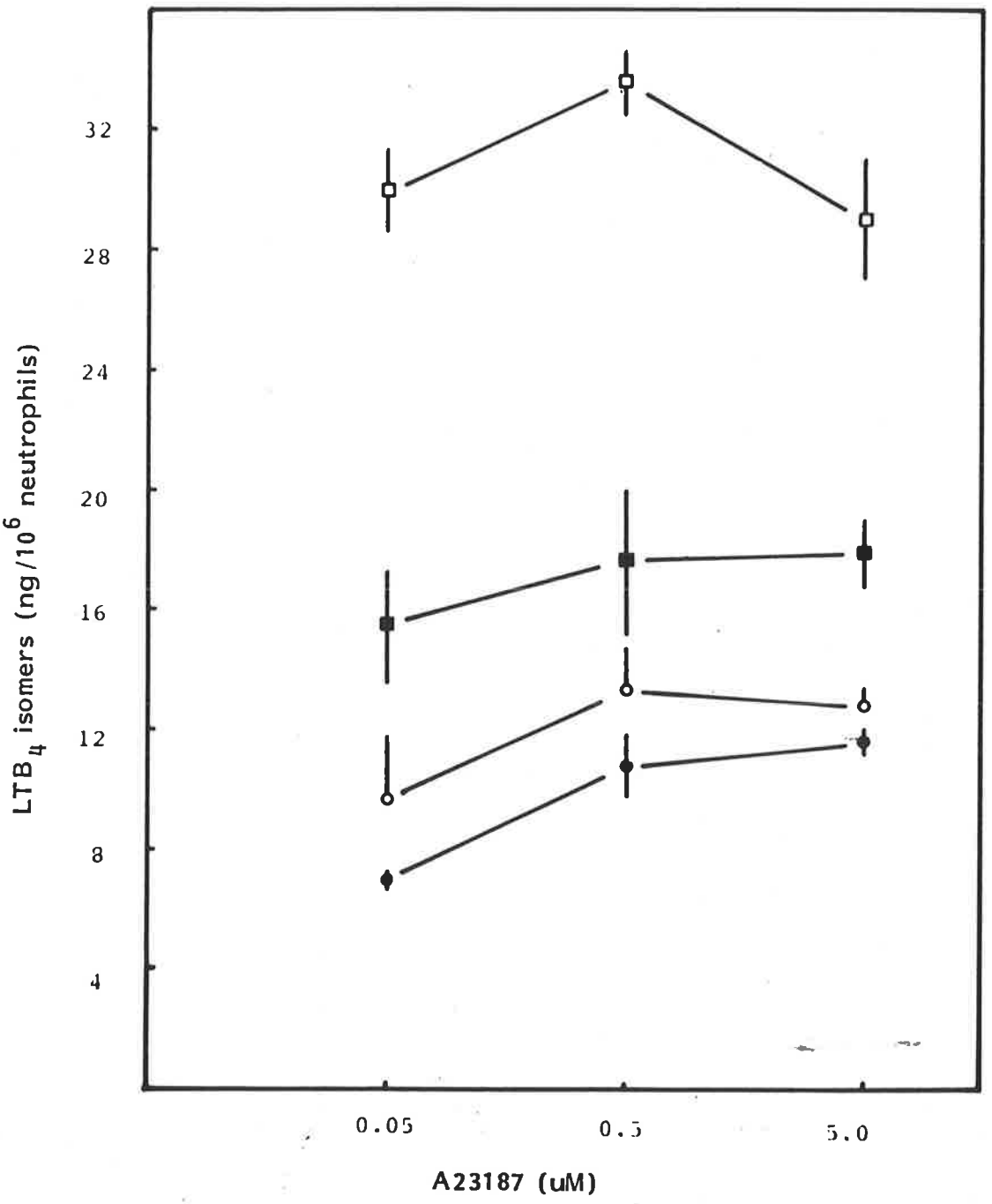


Figure 4.15 Dose response curves of production of LTB_4 isomers by neutrophils stimulated for 5 min with three different doses of A23187 either alone (●) or with 0.63 uM AA (○), 2.5 uM AA (□) or 10 uM AA (■).

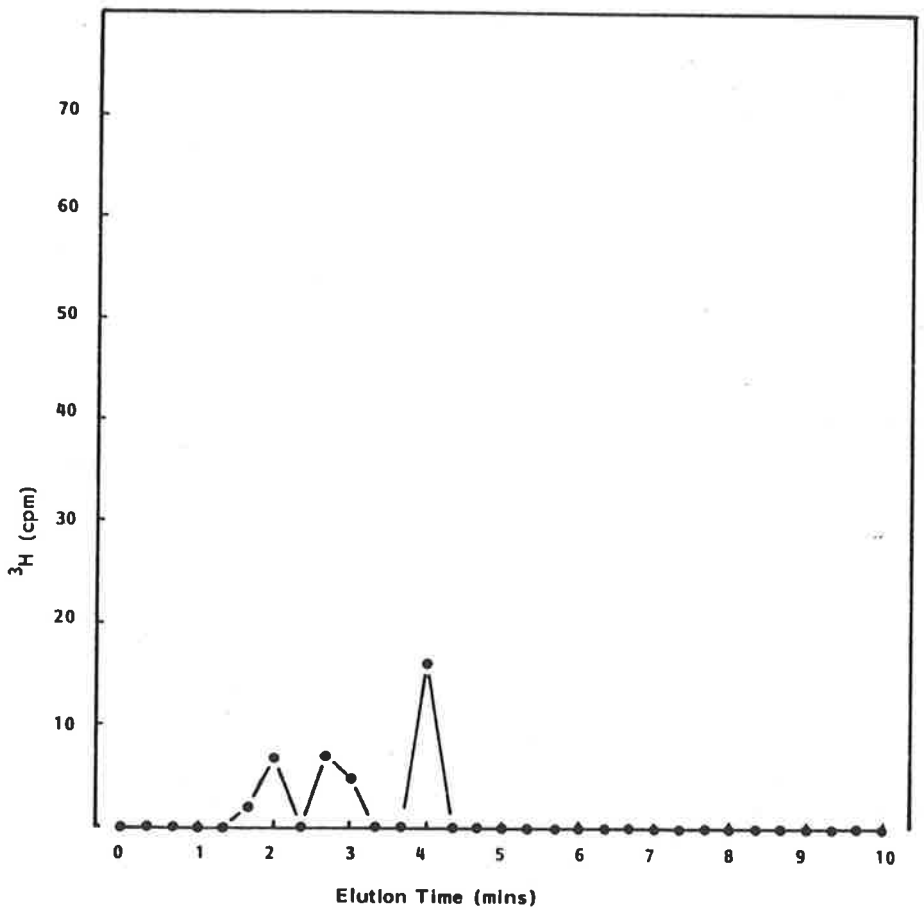


Figure 4.16 Radiochromatogram of neutrophil preparation stimulated for 5 min with 1 uM FMLP following a 1 hr preincubation with ^{3}H-AA.

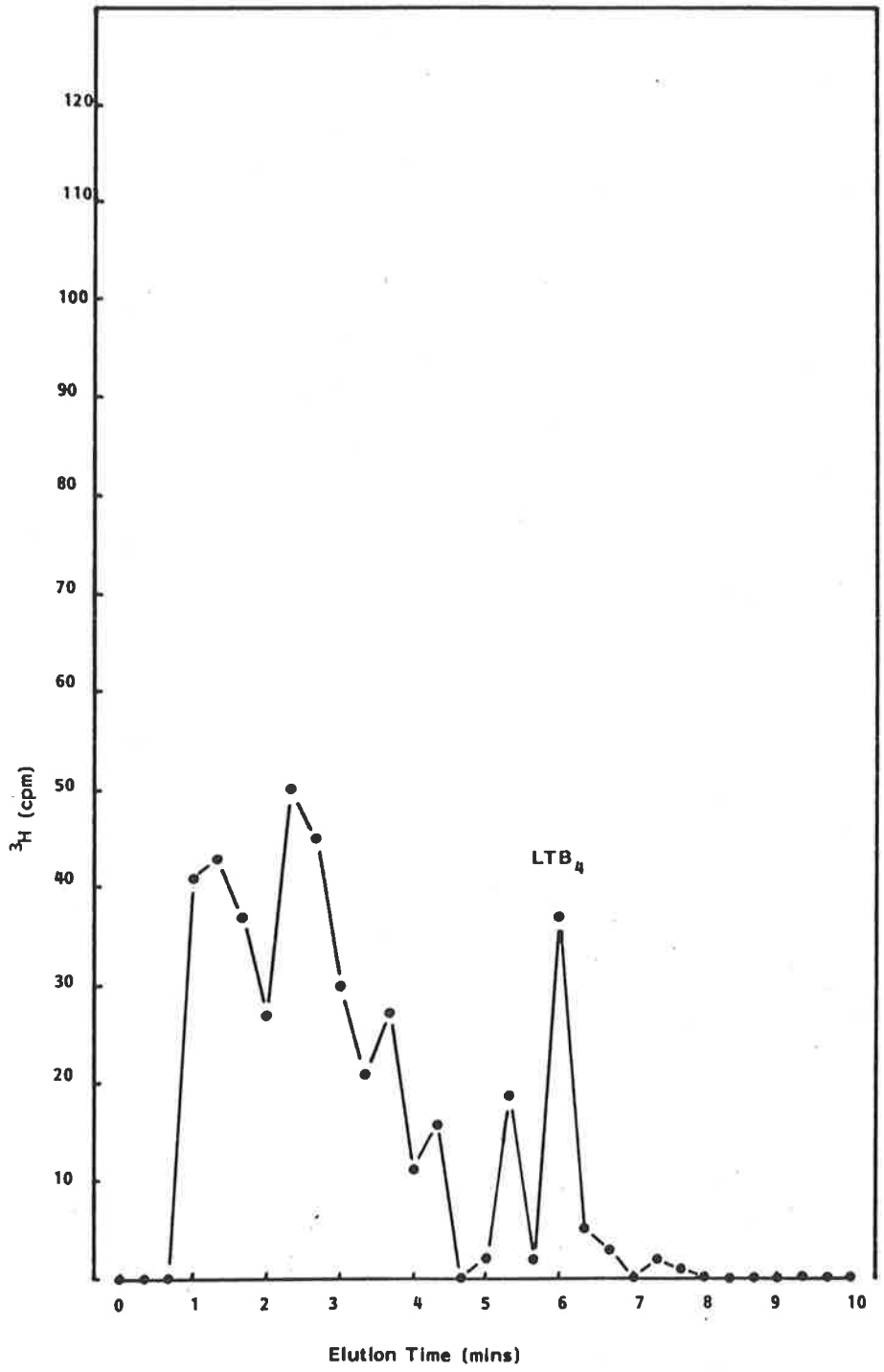


Figure 4.17 Radiochromatogram of neutrophil preparation stimulated for 5 min with 5 uM A23187 following a 1 hr preincubation with ^{3}H-AA.

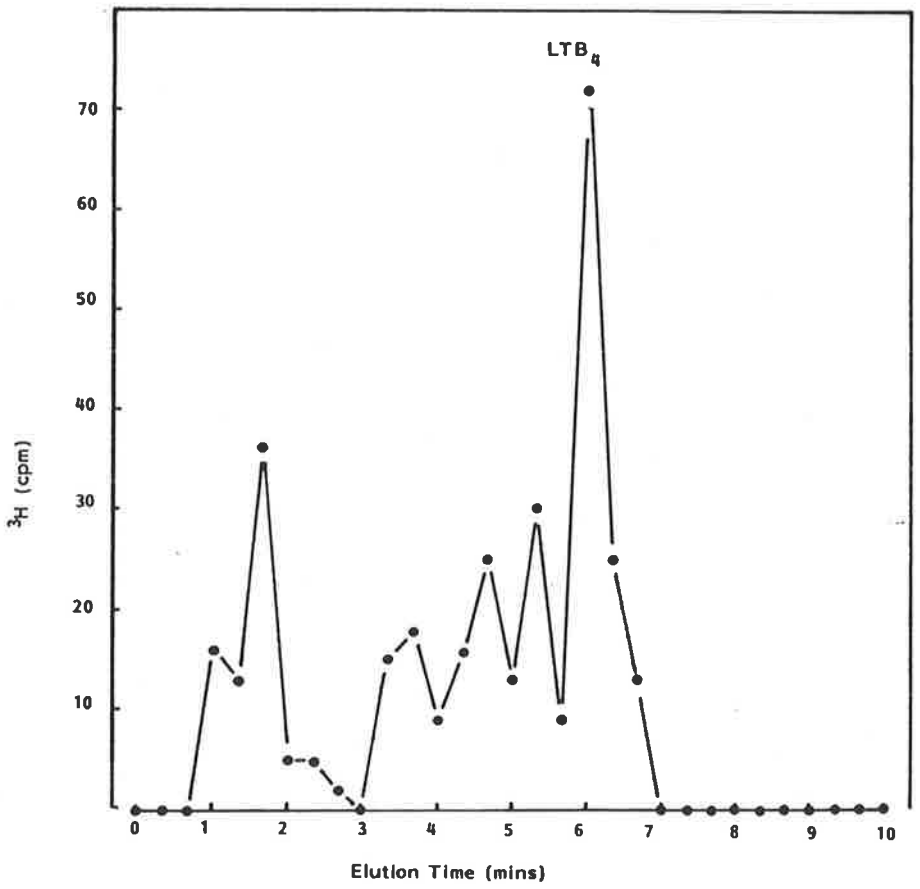


Figure 4.18 Radiochromatogram of neutrophil preparation stimulated for 5 min with 1 uM FMLP and ^{3}H-AA following a 1 hr preincubation with buffer.

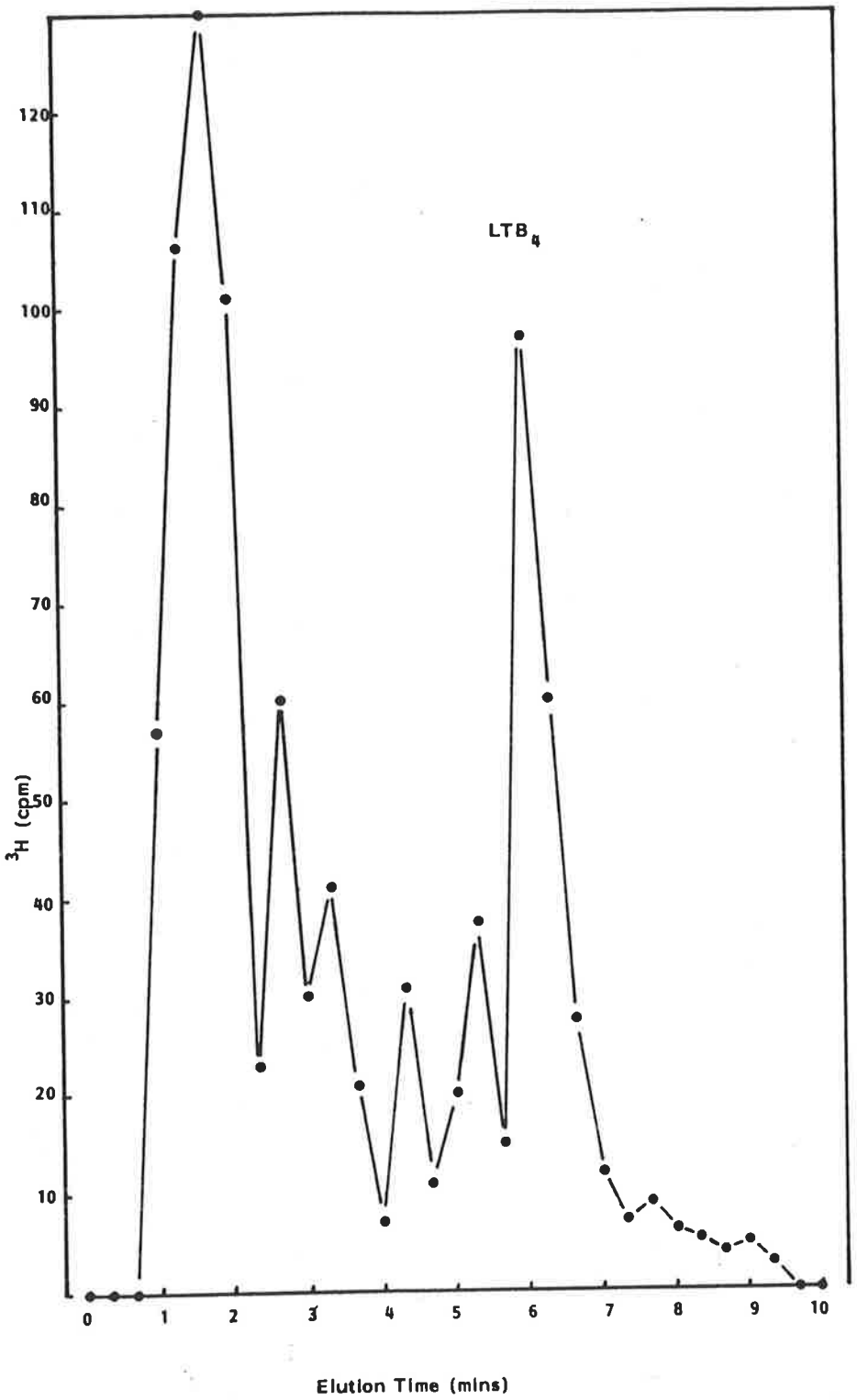


Figure 4.19 Radiochromatogram of neutrophil preparation stimulated for 5 mins with 5 uM A23187 and ^{3}H-AA following a 1 hr preincubation with buffer.

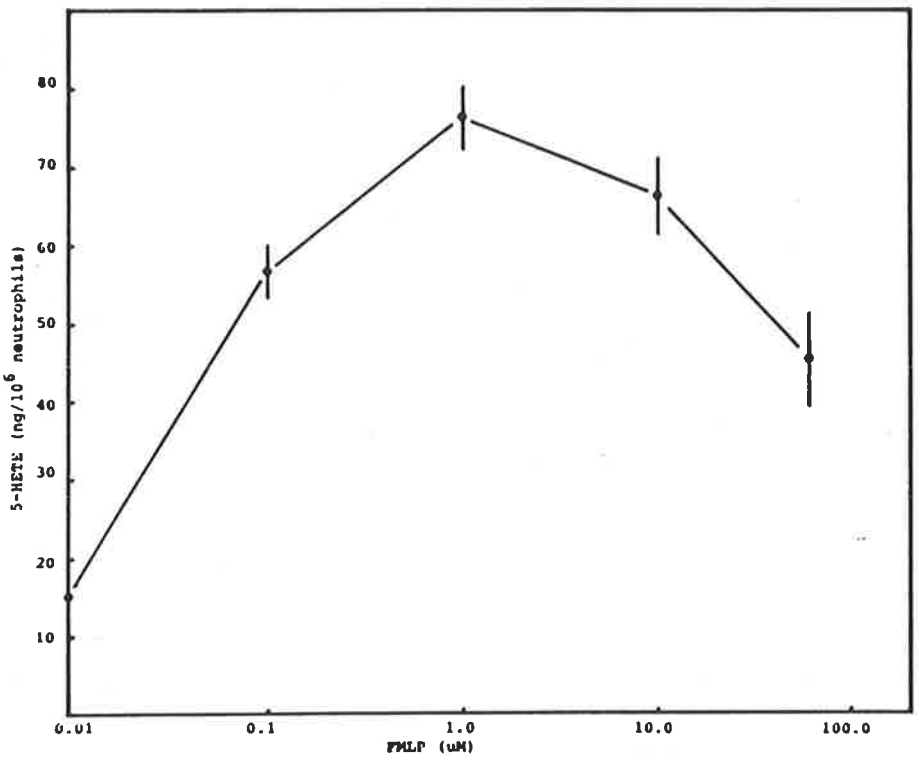


Figure 4.20 Dose response curve of production of 5-HETE by neutrophils stimulated with FMLP. Cells were stimulated for 5 min with increasing doses of FMLP added with 2.5 uM AA.

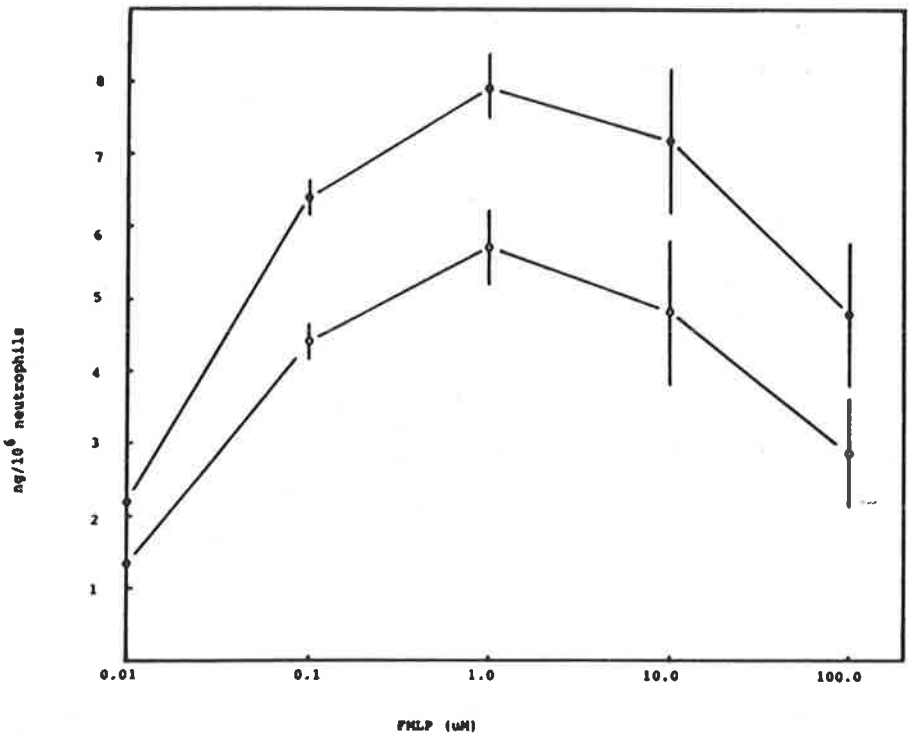


Figure 4.21 Dose response curves of production of LTB_4 (●) and LTB_4 isomers (○) by neutrophils stimulated with FMLP. Cells were stimulated for 5 min with increasing doses of FMLP added with 2.5 uM AA.

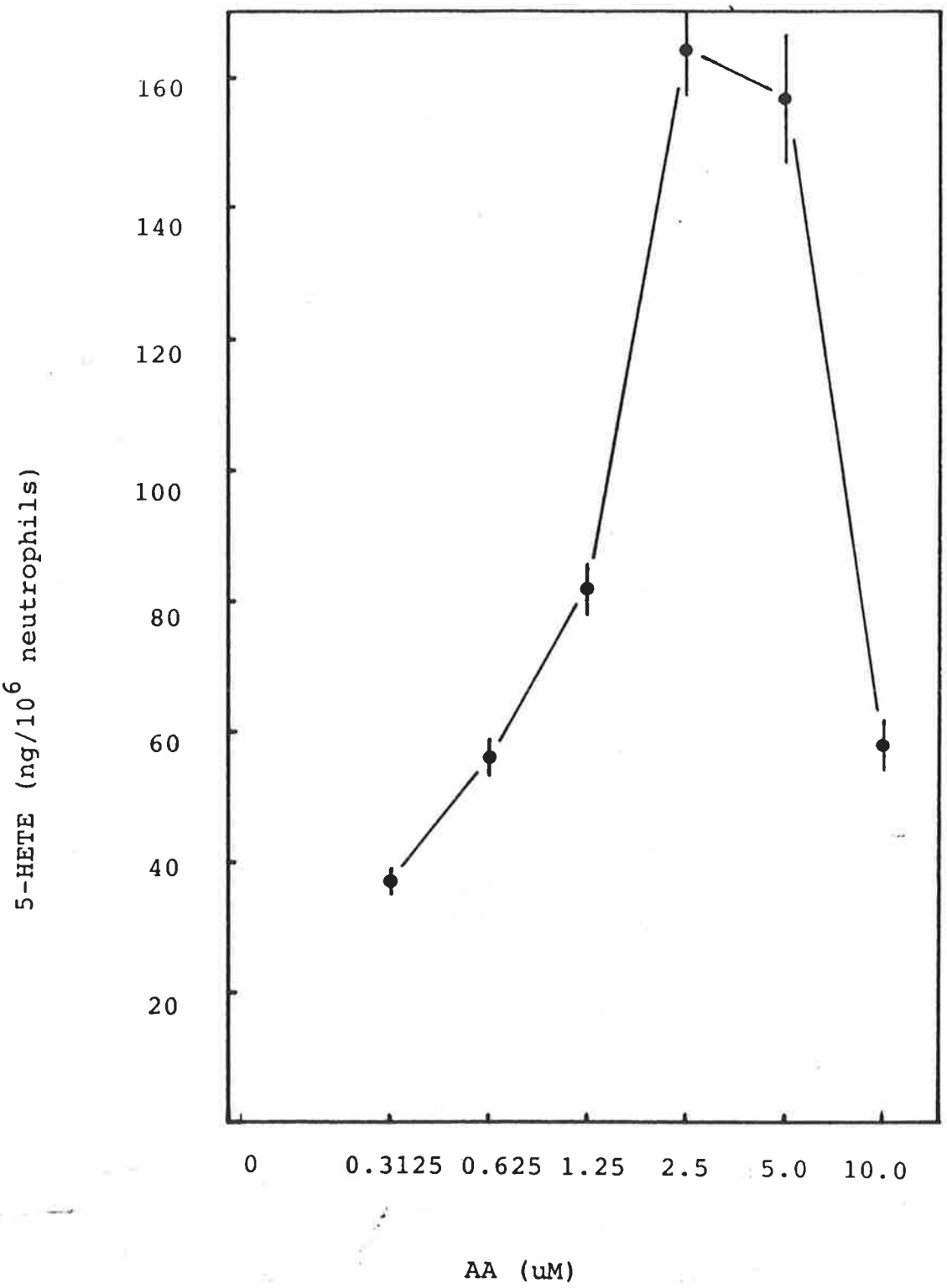


Figure 4.22 Dose response curve of production of 5-HETE by neutrophils stimulated for 5 min with 1 uM FMLP added with increasing doses of AA.

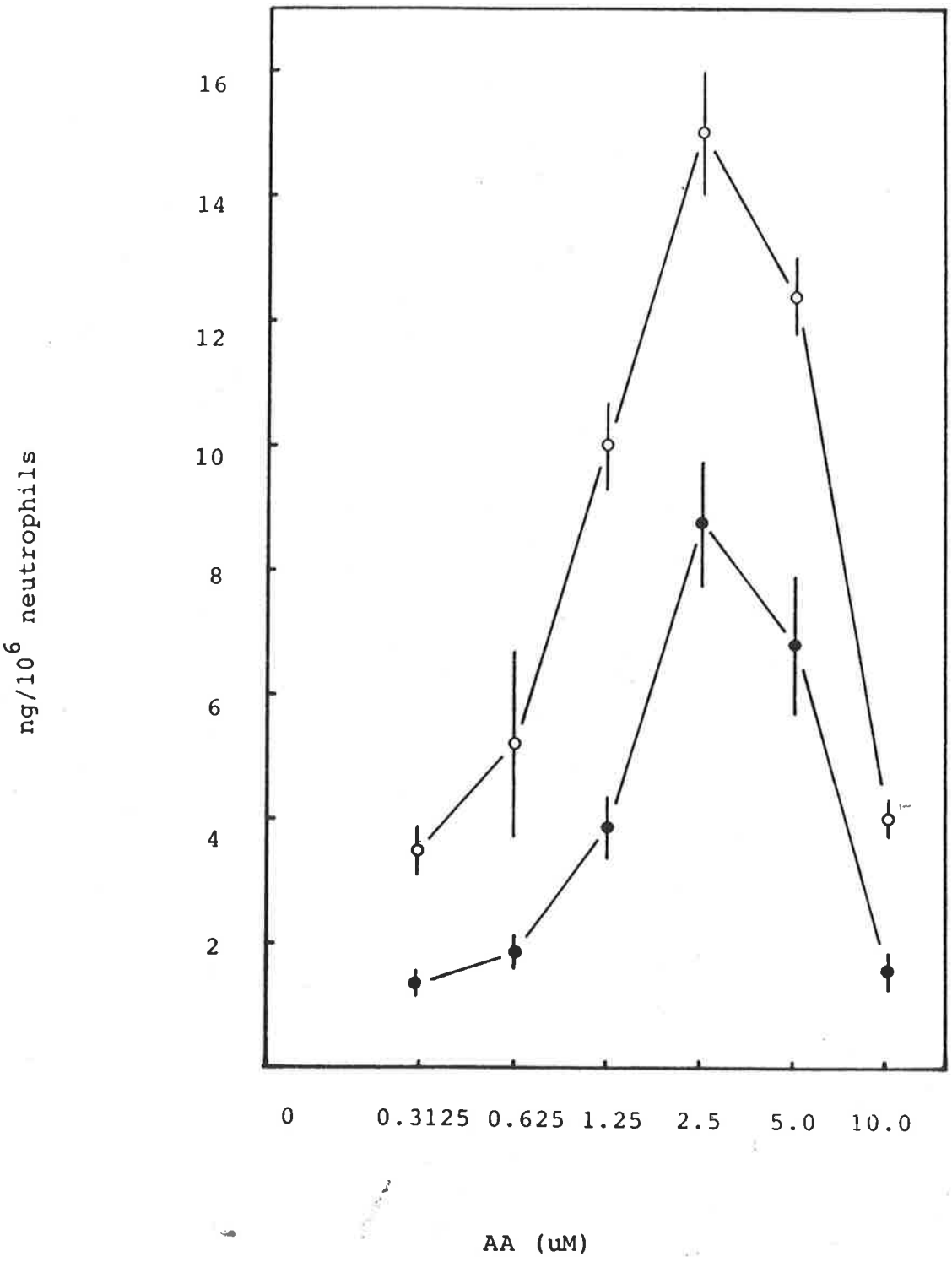


Figure 4.23 Dose responses curve of production of LTB_4 (●) and LTB_4 isomers (○) by neutrophils stimulated for 5 min with 1 uM FMLP added with increasing doses of AA.

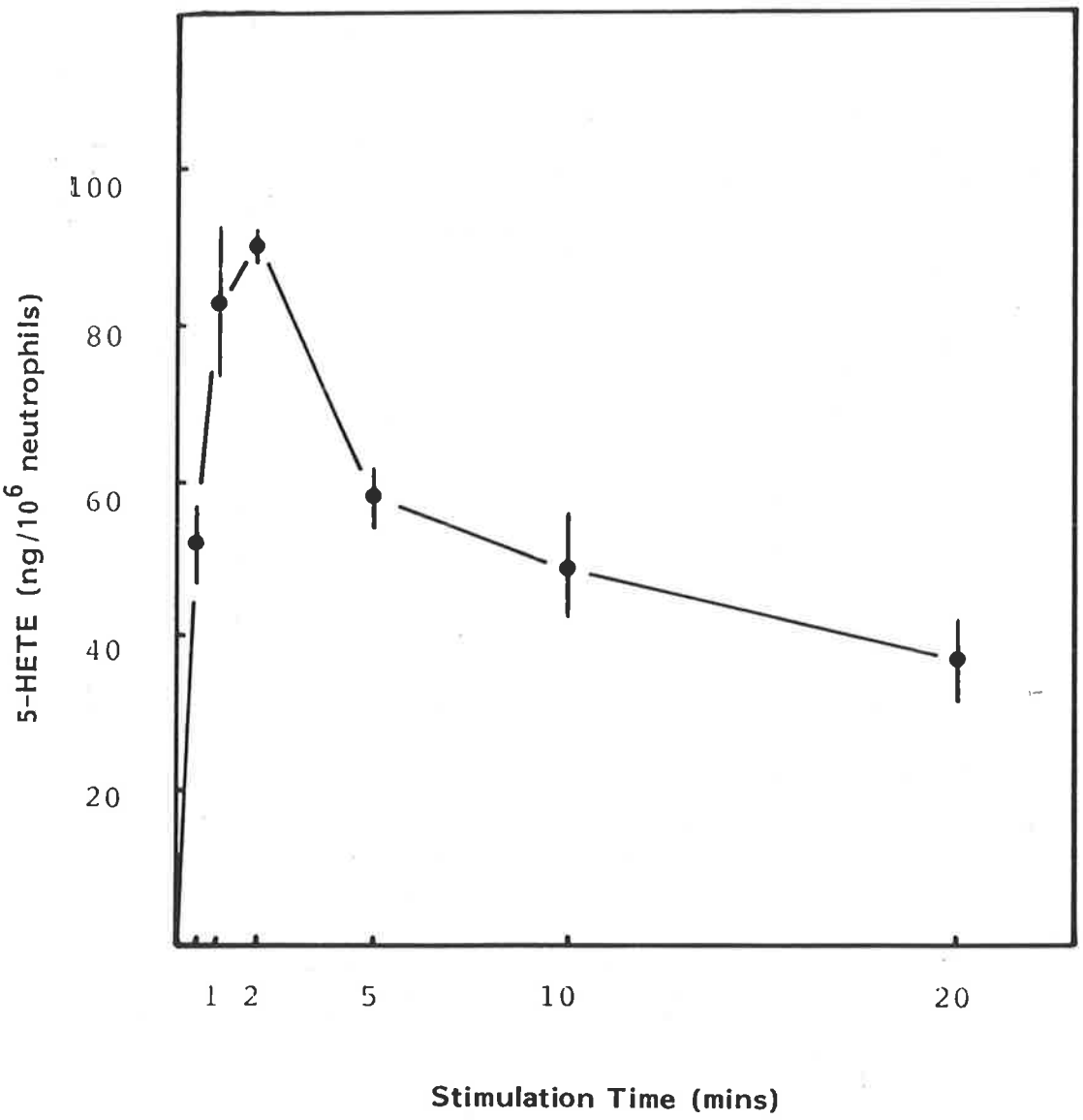


Figure 4.24 Time course of production of 5-HETE by neutrophils. Cells were stimulated for increasing periods of time with 1 uM FMLP and 2.5 uM AA.

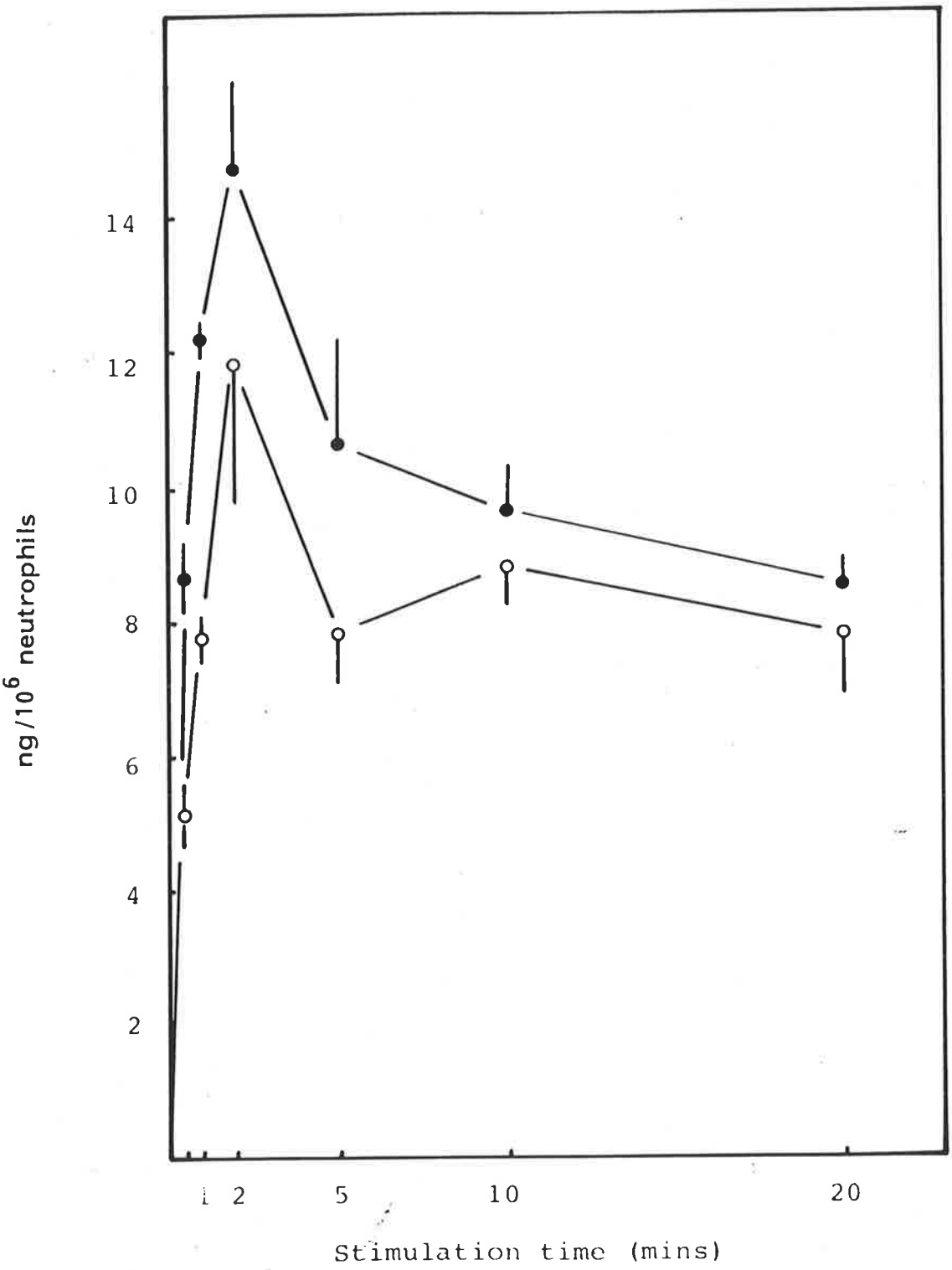


Figure 4.25 Time courses of production of LTB_4 (●) and LTB_4 isomers (○) by neutrophils. Cells were stimulated for increasing periods of time with 1 uM FMLP and 2.5 uM AA.

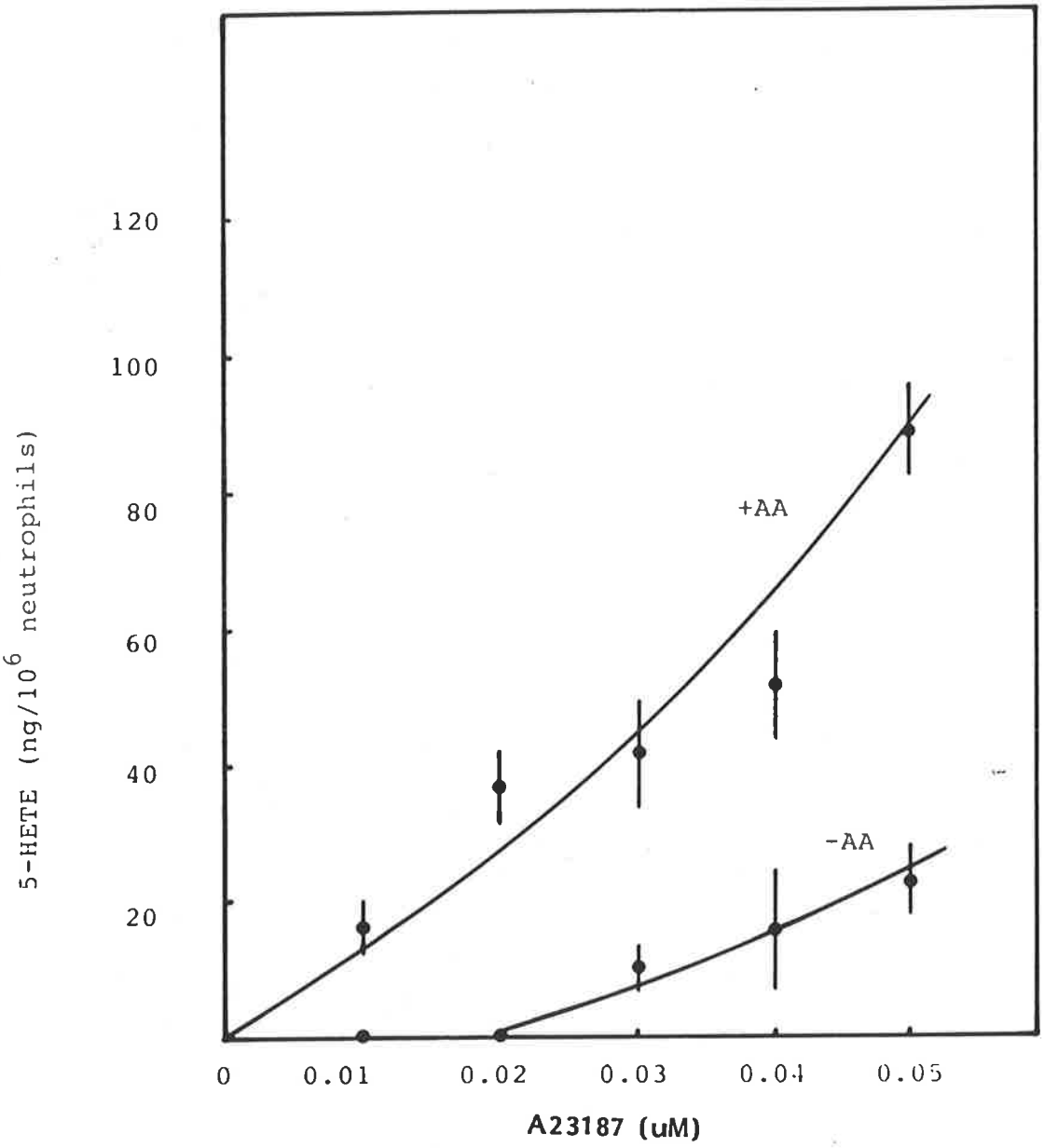


Figure 4.26 Dose response curve of production of 5-HETE by neutrophils stimulated for 5 min by increasing doses of A23187.

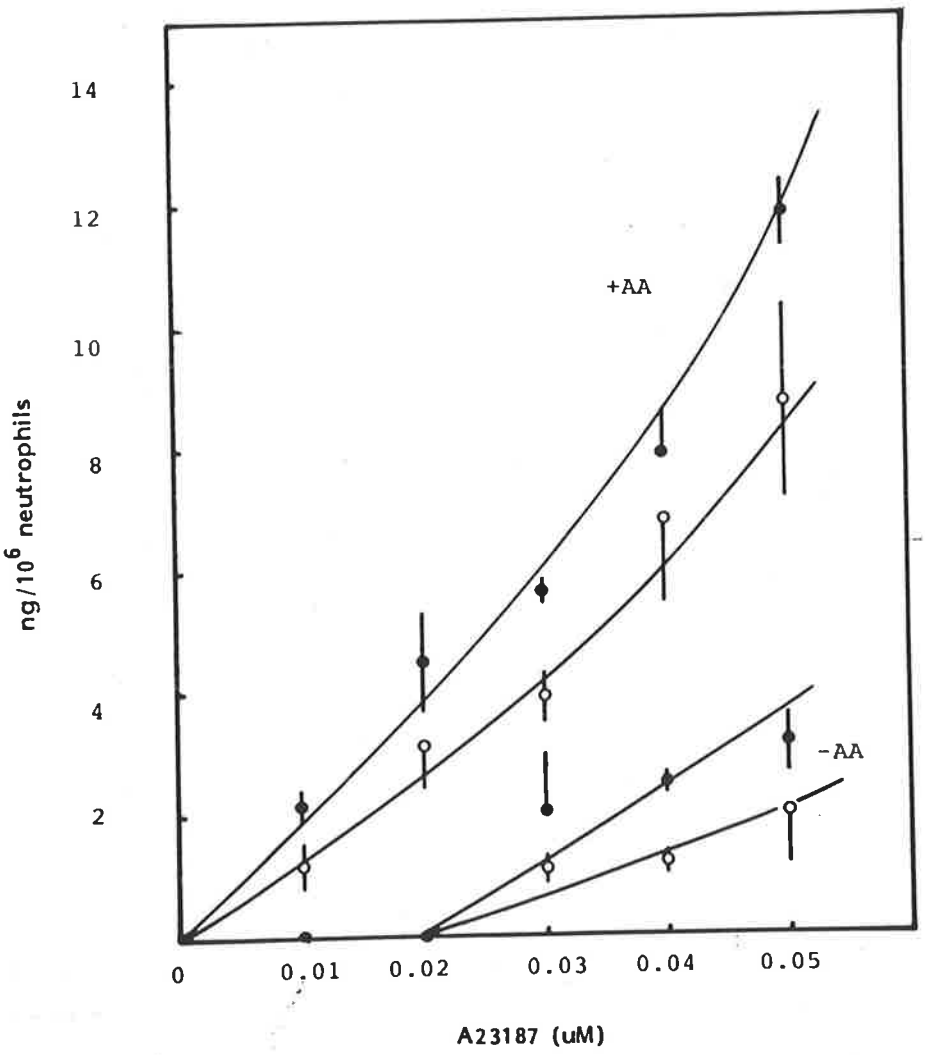


Figure 4.27 Dose response curve of production of LTB_4 (●) and LTB_4 isomers (○) by neutrophils stimulated for 5 min with increasing doses of A23187.

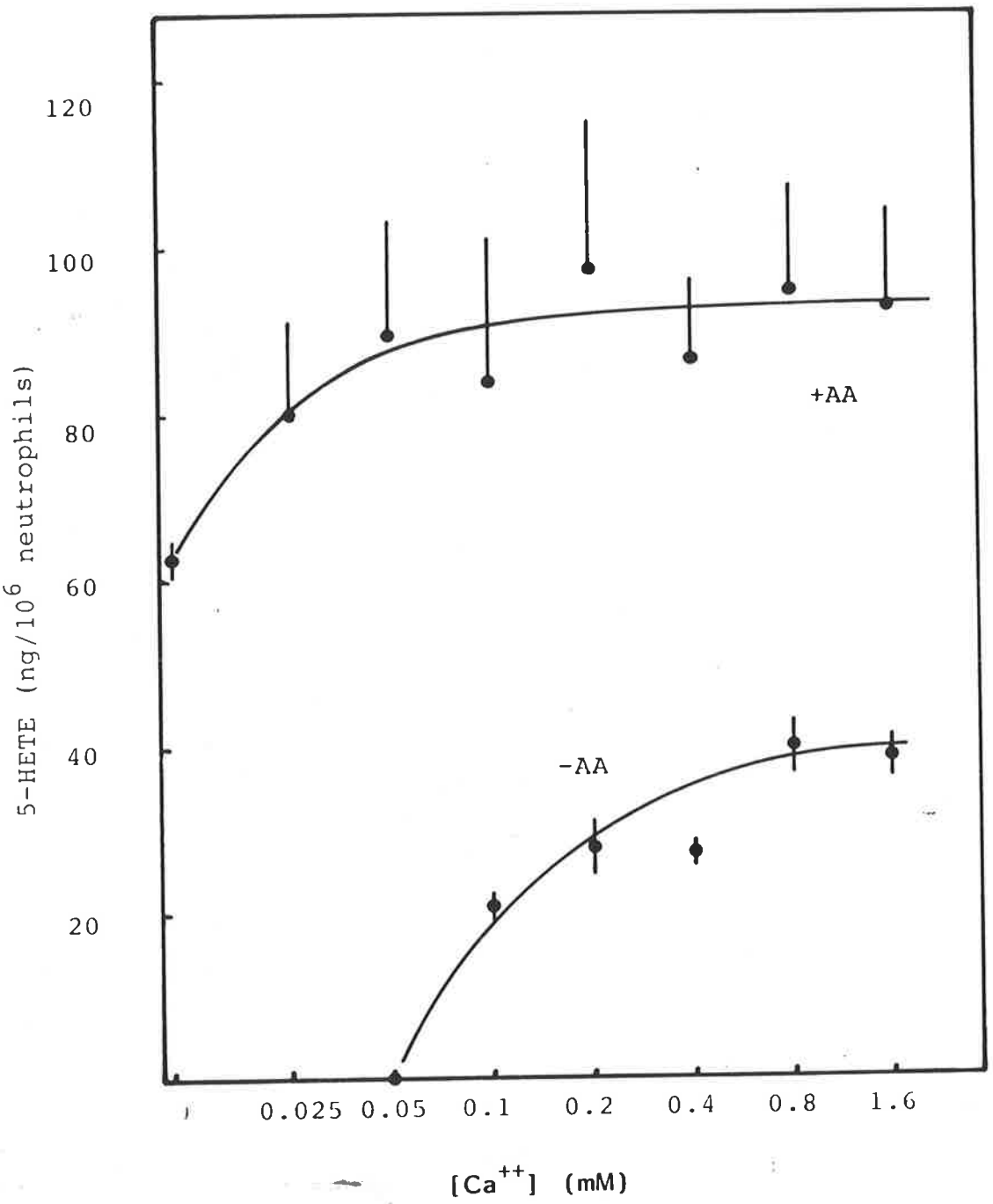


Figure 4.28 Effect of increasing $[Ca^{++}]$ in the external medium on the production of 5-HETE by neutrophils. Cells were stimulated for 5 min by either 0.5 uM A23187 alone or with 2.5 uM AA.

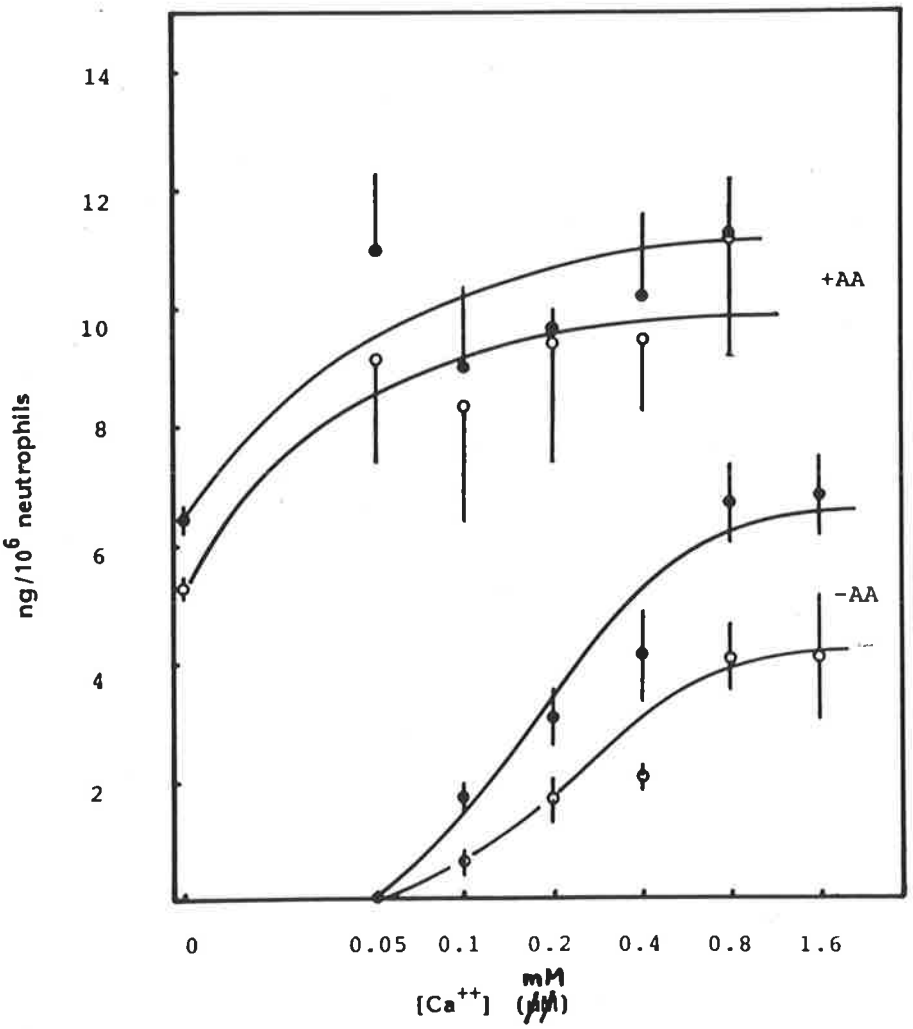


Figure 4.29 Effect of increasing $[Ca^{++}]$ in the external medium on the production of LTB_4 (●) and LTB_4 isomers (o) by neutrophils stimulated by either 0.5 uM A23187 alone or with 2.5 uM AA.

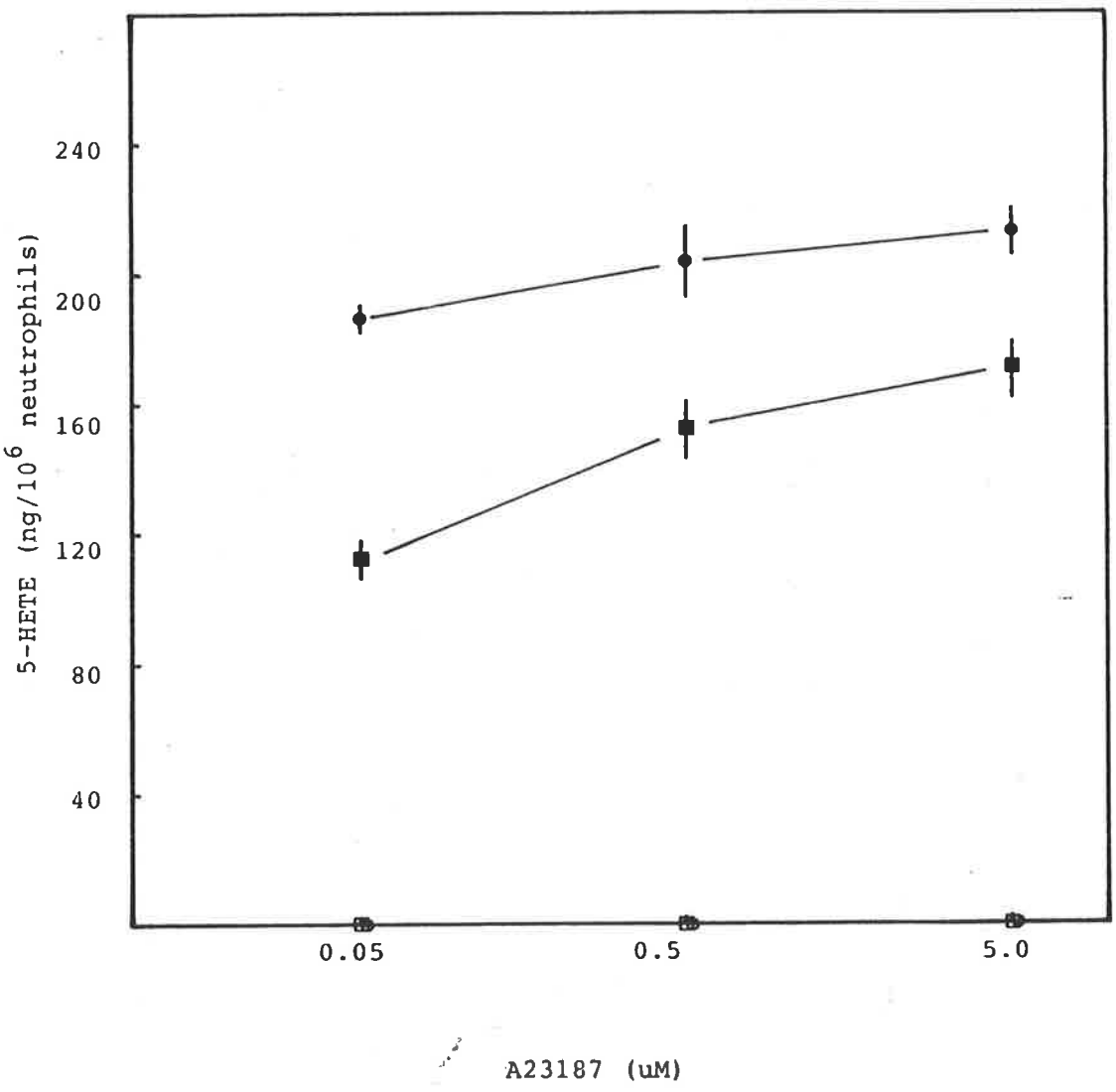


Figure 4.30 Effect of 10 min preincubation with 2 mM EDTA on the production of 5-HETE by neutrophils. Cells were stimulated for 5 min with three different doses of A23187 either alone or with 2.5 uM AA. Symbols represent A23187 alone (■), A23187 + AA (●), A23187 + EDTA (□) or A23187 + AA + EDTA (o).

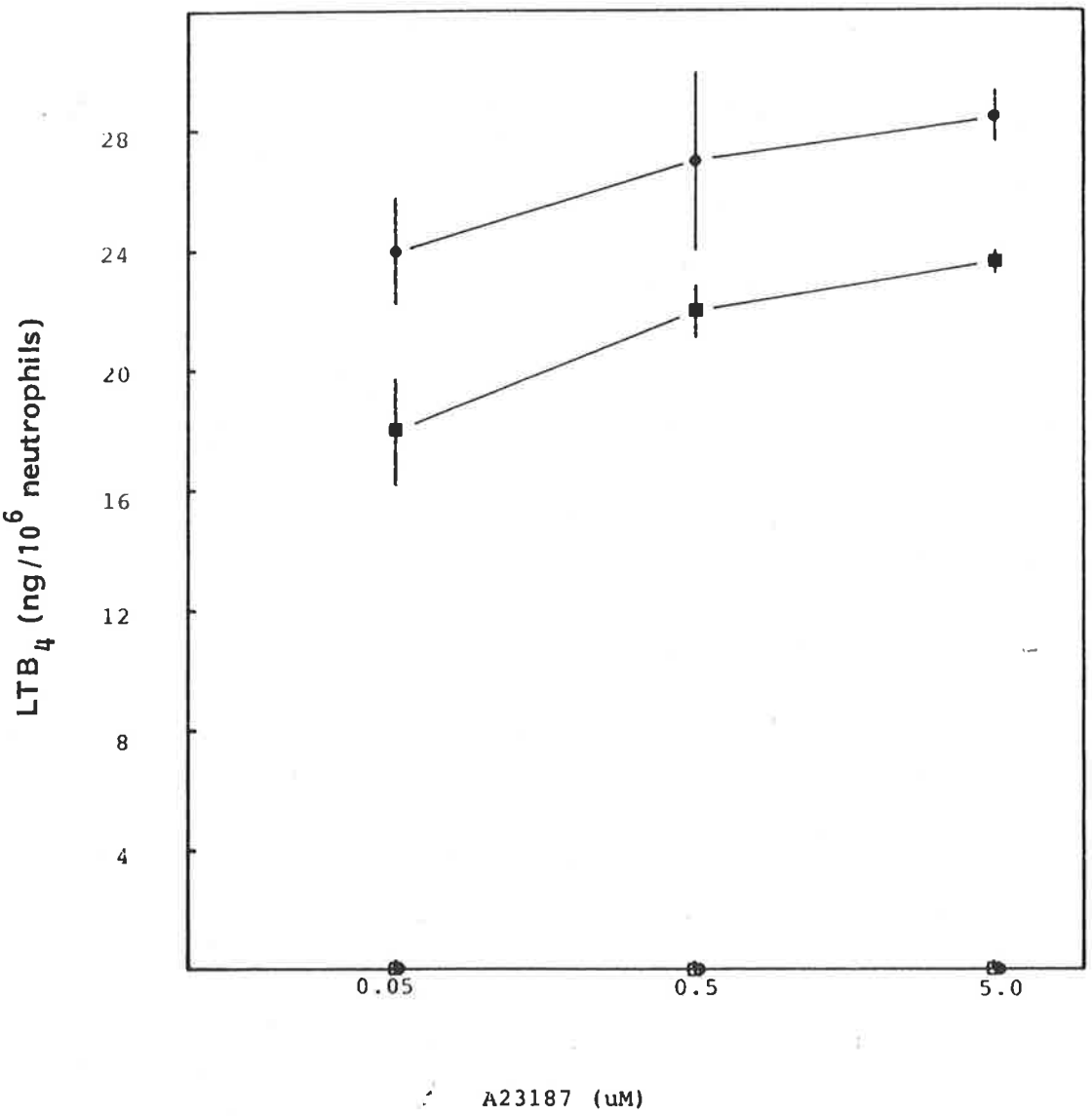


Figure 4.31 Effect of 10 min preincubation with 2 mM EDTA on the production of LTB_4 by neutrophils. Cells were stimulated for 5 min with three different doses of A23187 either alone or with 2.5 uM AA. Symbols represent A23187 alone (■), A23187 + AA (●), A23187 + EDTA (□) or A23187 + AA + EDTA (○).

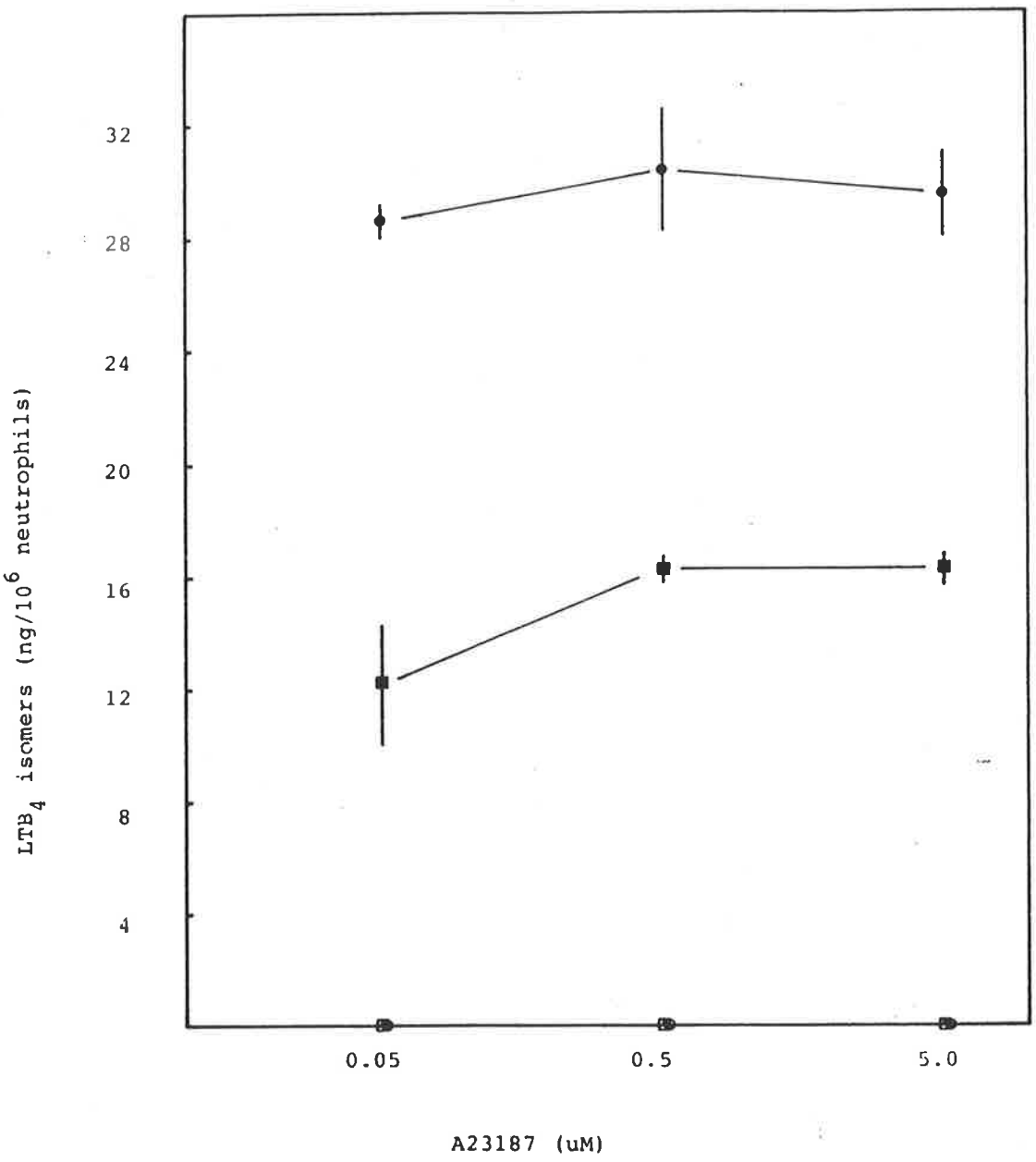


Figure 4.32 Effect of 10 min preincubation with 2 mM EDTA on the production of LTB_4 isomers by neutrophils. Cells were stimulated for 5 min with three different doses of A23187 either alone or with 2.5 uM AA. Symbols represent A23187 alone (■), A23187 + AA (●), A23187 + EDTA (□) or A23187 + AA + EDTA (○).

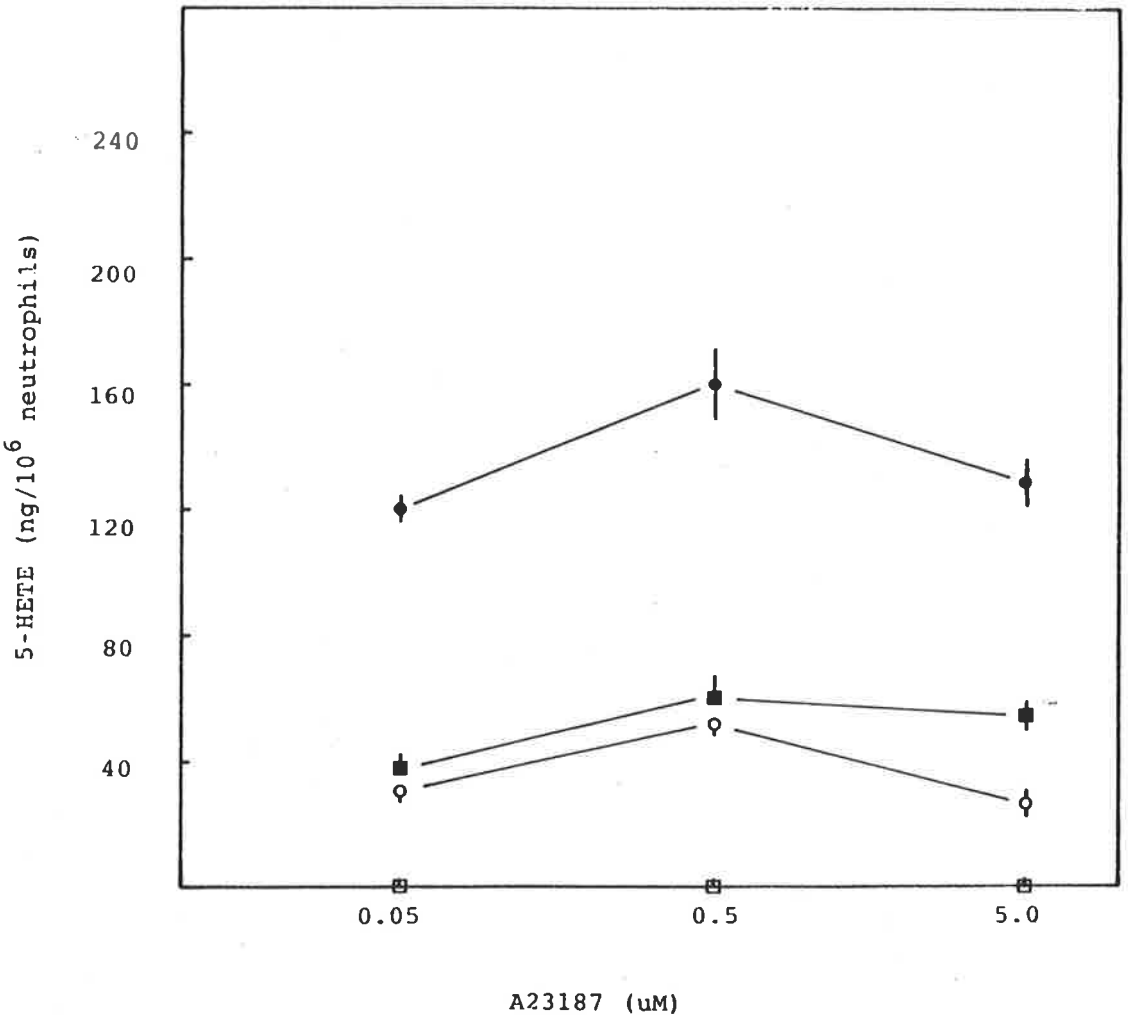


Figure 4.33 Effect of 10 min preincubation with 2 mM EGTA on the production of 5-HETE by neutrophils. Cells were stimulated for 5 min with three different doses of A23187 either alone or with 2.5 uM AA. Symbols represent A23187 alone (■), A23187 + AA (●), A23187 + EGTA (□) or A23187 + AA + EGTA (o).

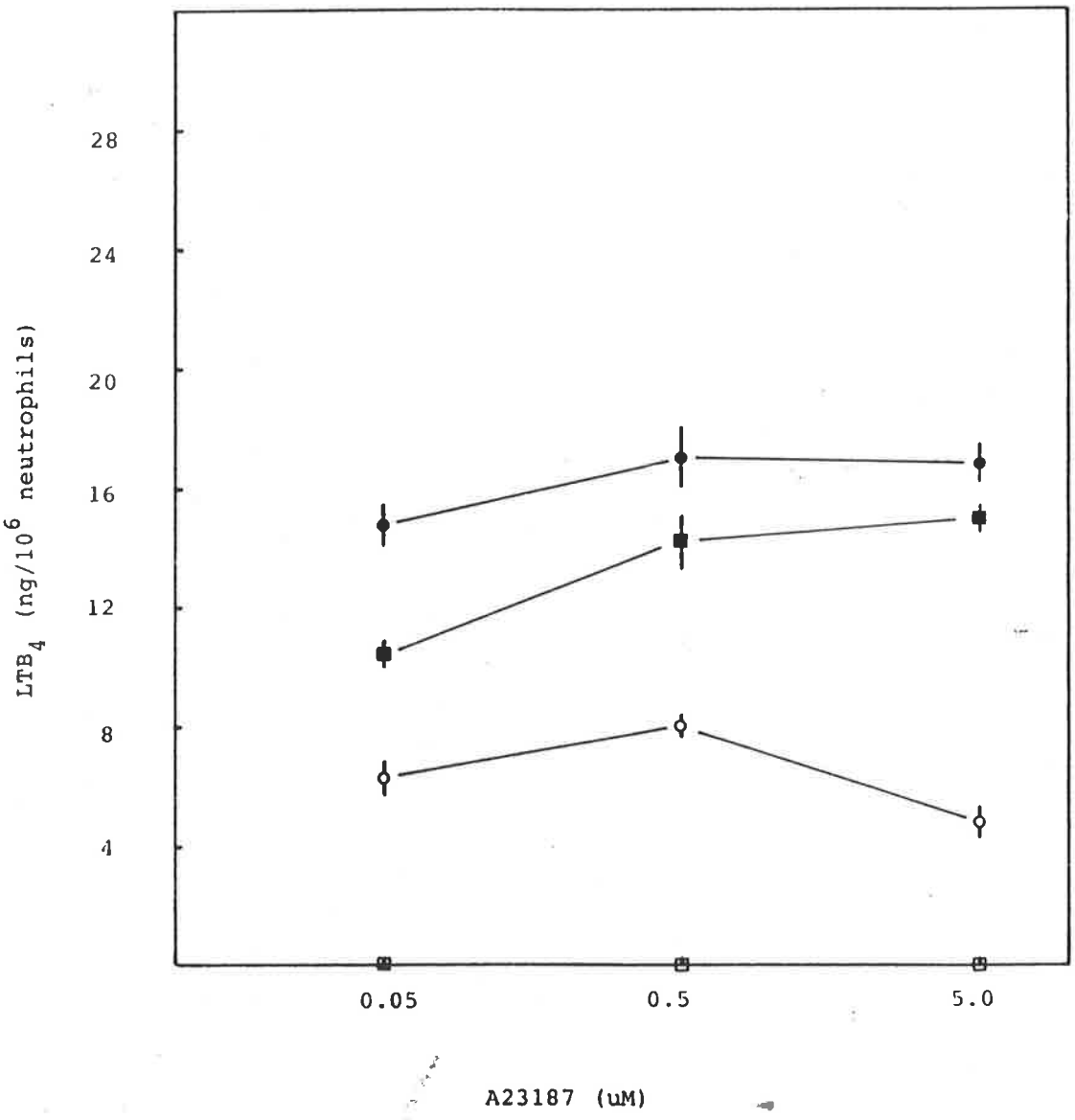


Figure 4.34 Effect of 10 min preincubation with 2 mM EGTA on the production of LTB_4 by neutrophils. Cells were stimulated for 5 min with three different doses of A23187 either alone or with 2.5 uM AA. Symbols represent A23187 alone (■), A23187 + AA (●), A23187 ± EGTA (□) or A23187 + AA + EGTA (○).

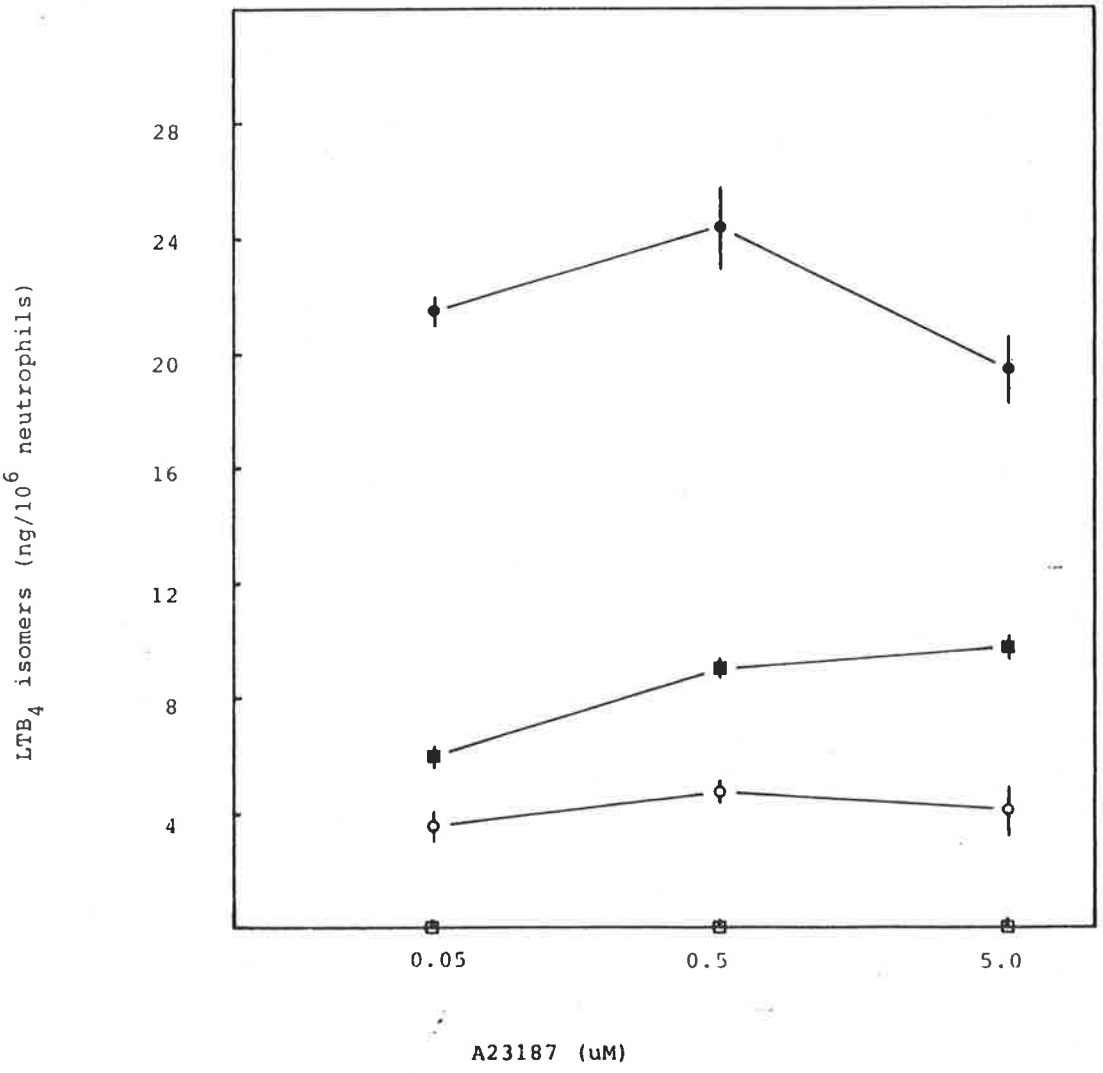


Figure 4.35 Effect of 10 min preincubation with 2 mM EGTA on the production of LTB_4 isomers by neutrophils. Cells were stimulated for 5 min with three different doses of A23187 either alone or with 2.5 uM AA. Symbols represent A23187 alone (■), A23187 + AA (●), A23187 + EGTA (□) or A23187 + AA + EGTA (○).

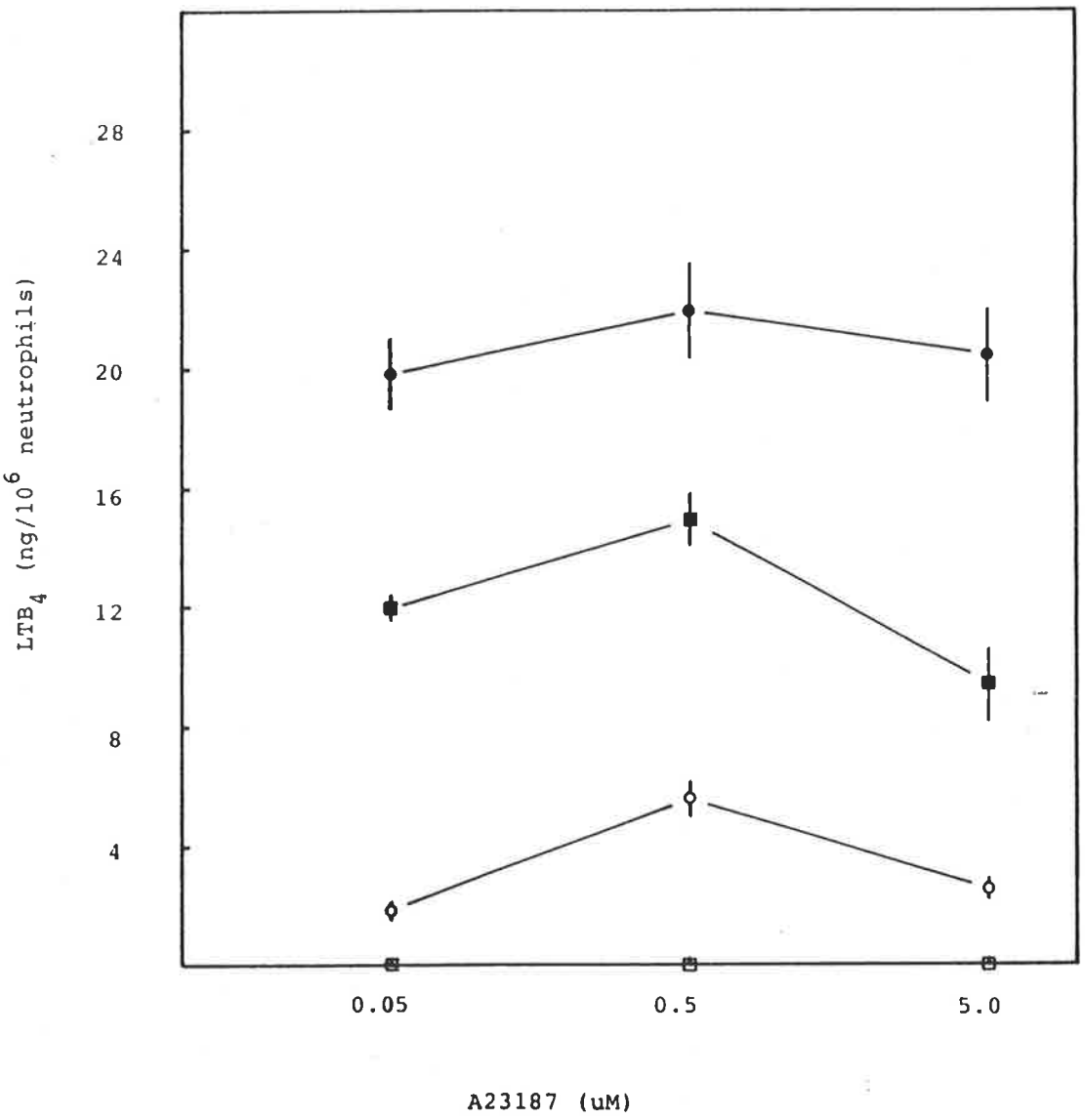


Figure 4.36 Effect of 10 min preincincubation with 65 uM TMB-8 on the production of LTB_4 by neutrophils. Cells were stimulated for 5 min with three different doses of A23187 either alone or with 2.5 uM AA. Symbols represent A23187 alone (■), A23187 + AA (●), A23187 + TMB-8 (□) or A23187 + AA + TMB-8 (○).

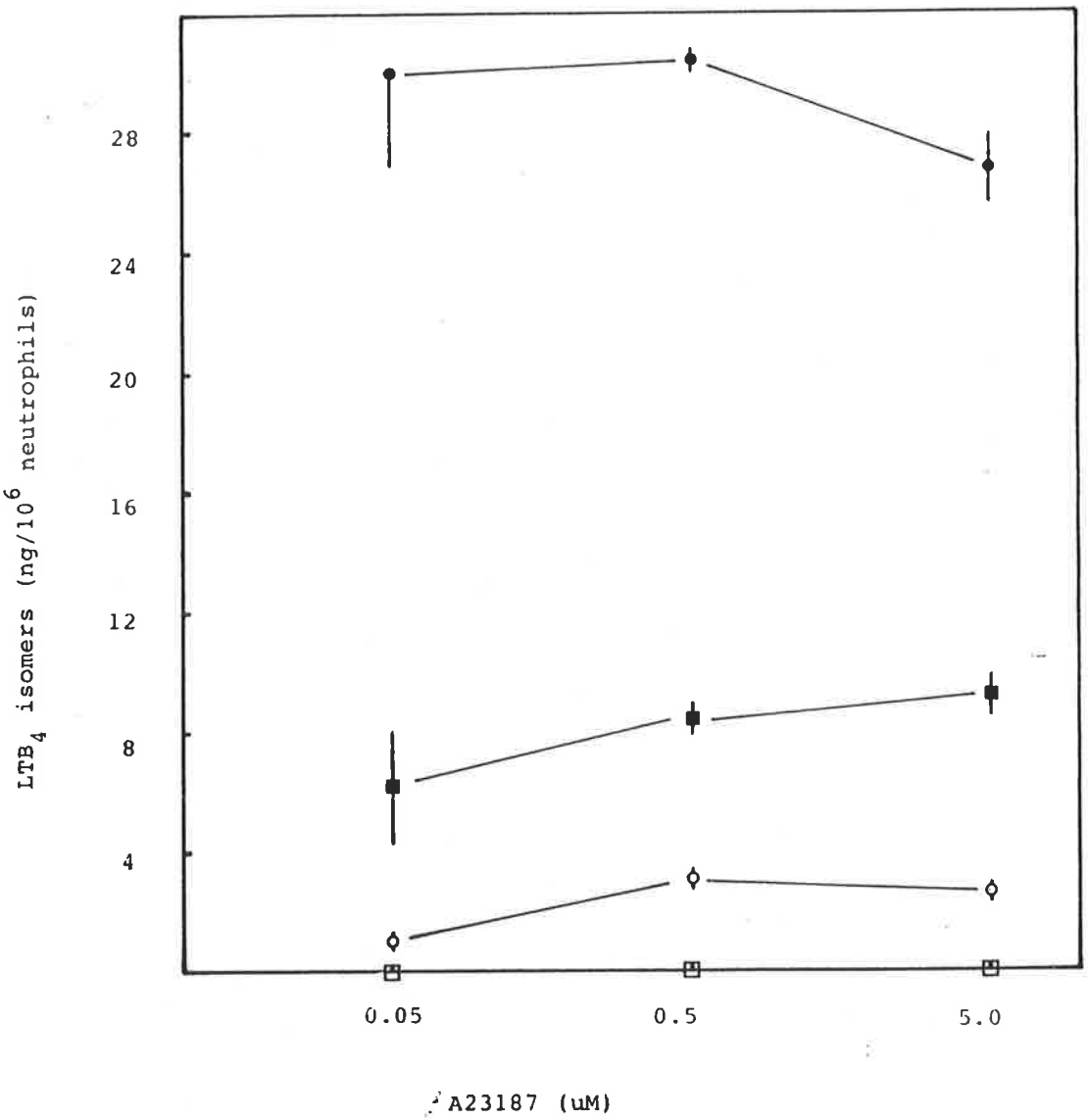


Figure 4.37 Effect of 10 min preincubation with 65 uM TMB-8 on the production of LTB_4 isomers by neutrophils. Cells were stimulated for 5 min with three different doses of A23187 either alone or with 2.5 uM AA. Symbols represent A23187 alone (■), A23187 + AA (●), A23187 + TMB-8 (□) or A23187 + AA + TMB-8 (○).

CHAPTER 5: THE IN VITRO METABOLISM OF EPA BY HUMAN NEUTROPHILS.

5.1 INTRODUCTION.

This chapter describes (a) methods for identification and quantitation of EPA-derived 5-LO metabolites and (b) the effect of exogenous EPA on metabolism of AA by the neutrophil 5-LO system.

5.2 RESULTS.

5.2.1 Identification of EPA 5LO-metabolites of human neutrophils.

Because no standards for LTB_5, LTB_5 isomers or 5-HEPE were available for these studies, identification was made indirectly. Two methods were used to achieve this. Firstly, to neutrophil suspensions, 0.1 uCi of either ^{14}C-EPA, or ^{3}H-AA mixed with unlabelled AA (5 uM final) was added, followed by 5 uM A23187. The cells were stimulated at 37^{o}C for 5 min. The extraction procedure was the same as described previously (Section 2.2.4). HPLC elution fractions were collected every 20 sec and the amount of radioactivity was determined by liquid scintillation counting.

There are three radioactive peaks in each sample and the respective position of each peak in each sample is similar (Figures 5.1 & 5.2). The major difference between the two samples is that the peaks in the sample to which EPA was added elute slightly earlier than the corresponding peaks in the sample with added AA.

The other method of identifying the EPA metabolites involved performing dose response curves by stimulating neutrophils with 5 uM A23187 and increasing amounts of EPA. Dose-related increases in peak heights with k's corresponding to those observed in the radiolabel experiment were observed suggesting that these peaks are EPA metabolites (Table 5.1). Production of all three peaks and the AA-derived peaks was inhibited upon pre-incubation with the 5-LO blocker NDGA (data not shown).

Although positive identification is impossible without pure standards or GC-MS analysis of elution peaks, extrapolation from published reports indicates that peak I is made up of the isomers of LTB_5, peak II is LTB_5 and peak III is 5-HEPE (Prescott, 1984).

Another problem encountered in running this assay when EPA was used as a substrate was that LTB_5 coeluted with PGB_2. Fortunately, 5-HEPE eluted before 15-HETE so provided assay system 2 was used for these experiments, PGB_2 could be omitted and 15-HETE used as internal standard for both the 5-hydroxy-acids and LTB and its isomers. The problem of quantifying the production of these compounds without pure standards was overcome using the standard curves of the AA-metabolites (Lands, personal communication).

5.2.2 Comparison of production of EPA and AA 5-LO metabolites by human neutrophils.

To compare the production of their respective metabolites, dose-response curves to increasing amounts of EPA and AA using 0.05 uM A23187 as stimulus were performed. Fatty acid was added to cell samples at 37°C followed rapidly by A23187 and the reaction was stopped by the addition of 10 ul of 100 mM citric acid (Figures 5.3, 5.4 & 5.5). The lowest dose of AA (0.31 uM) did not cause a detectable increase in the amount of LTB_4 produced by neutrophils when stimulated by 0.05 uM A23187 (Figure 5.3). However, a dose-dependent rise in production was observed from 0.63 uM AA up to 2.5 uM AA after which production decreased. In contrast, the lowest dose of EPA added (0.31 uM) caused production of LTB_5 while having no effect on the production of LTB_4. The amount of LTB_5 produced was increased by addition of up to 10 uM EPA, after which production decreased. When EPA was added at concentrations greater than 1.25 uM, production of detectable amounts of endogenously-derived LTB_4 was inhibited (Figure 5.3).

The maximal production of LTB_4 was 20-25 ng/10^6 neutrophils and occurred at 2.5 and 5.0 uM AA. In contrast, maximal LTB_5 production was 10-12 ng/10^6 neutrophils and occurred at 10.0 uM EPA. These findings indicate that exogenous EPA is not converted as efficiently as AA to LTB and also inhibits production of LTB_4 from endogenous sources.

Production of 5-HETE from exogenous AA followed a similar pattern to that of LTB_4 production (Figure 5.4). The lowest concentration of AA (0.31 uM) did not enhance the 5-HETE production above that caused by A23187 alone, however production was increased from 0.63 - 2.5 uM in a dose-dependent manner after which production decreased. Inhibition of 5-HETE production from endogenous AA was observed at the same doses of EPA which caused inhibition of LTB_4 production, and maximal 5-HEPE production was observed at 10 uM (same as maximal LTB_5 production). However, unlike production of LTB, maximal 5-HEPE production was larger than that of 5-HETE. These findings suggest that 5-HPEPE is converted to 5-HEPE more effectively than 5-HPETE is converted to 5-HETE. Alternatively, 5-HPEPE may be converted less effectivley to LTA_5 than 5-HPETE is to LTA_4.

The dose-response curves for the production of the all-trans isomers of LTB have similar characteristics to those of 5-HETE (Figure 5.5). A dose of 2.5 uM of AA was required for maximal production of LTB_4 isomers (similar to LTB_4 and 5-HETE), giving a maximum production of 30-35 ng/10^6 neutrophils. Increased production of LTB_4 isomers above that caused by A23187 alone (approximately 10 ng/10^6 cells) was observed from 0.63 uM AA (the same as LTB_4 and 5-HETE), at which the production of the isomers rose to 15 ng/10^6 cells. In contrast, production of the isomers of LTB_5 occurred with the addition of 0.31 uM EPA and increased up to 10.0 uM (maximum production was 40-45 ng/10^6 neutrophils). 100 % inhibition of production of LTB_4 isomers from endogenous AA was observed with 1.25 uM EPA or greater.

Exogenous EPA inhibits production of endogenously derived LTB_4, LTB_4 isomers and 5-HETE by human neutrophils. Furthermore, exogenous EPA is metabolised less effectively than exogenous AA to LTB in favour of the LTB isomers and 5-hydroxy acid. This probably reflects a decreased ability of LTA_4 hydrolase to convert LTA_5 to LTB_5 (Prescott, 1984).

5.2.3 Competitive inhibition of exogenous AA metabolism in neutrophils by EPA.

Neutrophils were provided with a fixed dose of exogenous AA (2.5 uM), and increasing doses of EPA were added. The cells were then stimulated for 5 min with 0.05 uM A23187. 2.5 uM AA was chosen because this dose produced maximal generation of AA-derived metabolites (Section 5.2.2). EPA was added in the range of 0 - 25 uM final concentration giving final EPA:AA ratios 0, 0.1, 0.5, 1.0, 5.0, 10.0. Production of the EPA-derived metabolites with increasing doses of EPA followed similar patterns to the corresponding dose-response curves described in section 5.2.2 (Figures 5.3, 5.4 and 5.5). Interestingly, levels of LTB_5, LTB_5 isomers and 5-HEPE was still high when 25 uM EPA was added. This contrasted with the previous experiments where production of the same compounds was minimal at 20 uM.

As the ratio of EPA:AA increased, less AA-derived metabolites were formed. At a ratio of 1.0, production of LTB_4 was inhibited by 34% while production of the LTB_4 isomers and 5-HETE were both inhibited by 18%. In contrast, Prescott (1984) found 68% inhibition

of LTB_4 production at a ratio of 1.0, using 10 uM AA. However, no details of production of LTB_4 isomers or 5-HETE was given.

The addition of increasing doses of EPA in the presence of exogenous AA caused small changes in terms of % inhibition of AA-derived products (Figure 5.9). The ID_{50} for inhibition of LTB_4 (8.5 uM) was lower than for LTB_4 isomers or 5-HETE (11.5 uM and 19.0 uM respectively), supporting the theory that inhibition occurs at the LTA_4 hydrolase. However, the exogenous EPA probably inhibits production of all exogenous AA-derived metabolites to approximately the same extent. It is likely that to observe an inhibitory effect at the LTA hydrolase level a smaller amount of exogenous AA is required.

5.3 DISCUSSION.

LTB_5 is less potent in a variety of assays than its AA-derived counterpart LTB_4. It was 10 - 30 times less potent than LTB_4 as a neutrophil aggregant, degranulator or chemokinetic stimulus and when enhancing bradykinin-induced plasma exudation (Terano et al., 1984b). LTB_5 was 30 times less potent than LTB_4 as a chemotaxin but equipotent as a human neutrophil degranulating agent (Goldman et al., 1983). Furthermore, LTB_5 bound to the high-affinity class of LTB receptors with 500-fold less affinity than LTB_4 while both compounds bound to the low-affinity receptor class with equal affinity (Goldman et al., 1983).

EPA is a good inhibitor of the PGH synthetase pathway (Needleman et al., 1979; Lands and Hanel, 1983) as is evident by the smaller

amounts of EPA-derived metabolites compared with those produced by the same doses of AA. This inhibition appears to be related to the requirement of PGH synthetase for lipid peroxide activator (Lands and Hanel, 1983). In addition, some of the prostaglandin metabolites of EPA have significantly reduced activity (Whitaker et al., 1979; Needleman et al., 1979). The effect of EPA on the 5-LO pathway seems on the surface to mimic its inhibitory effect on the PGH synthetase pathway, however, more detailed analysis reveals that while LTB_5 (Goldman et al., 1983; Prescott, 1984; Terano et al., 1984b) and LTC_5 (Hammarstrom, 1980) are less active than corresponding AA-metabolites, larger amounts of other products of the 5-LO pathway are generated (for example, 5-HEPE and LTB_5 isomers) (Prescott, 1984). This indicates a more selective effect than an inhibition of the 5-LO enzyme.

In this study, metabolism of exogenous EPA by the 5-LO system of human neutrophils led to inhibition of production of both endogenous and exogenously-derived AA metabolites and led to production of EPA-derived metabolites. The major difference was that both 5-HEPE and LTB_5 isomers were produced in larger amounts than their exogenous AA-derived counterparts, implying that EPA is a good substrate for 5-LO (see also Jakschik et al., 1980; Murphy et al., 1981). In contrast, LTB_5 production was much smaller than that of LTB_4, implying that LTA hydrolase does not convert LTA_5 to LTB_5 as efficiently as it does LTA_4 to LTB_4 (see also Prescott, 1984; Lee et al., 1984). Interestingly, addition of exogenous eicosatrienoic acid (20:3, ETA) to neutrophils stimulated with A23187 results in the production of less LTB_3 compared to LTB_4 from the same concentration of AA while

production of LTB_3 isomers is greater and is possibly caused by inhibition of LTA hydrolase by LTA_3 (Stenson et al., 1984).

Ex vivo findings on the metabolism of EPA have generally not supported the in vitro findings. Equivalent amounts of LTB_4 and LTB_5 have been reported when cells from animals or humans fed EPA-supplements have been stimulated (Murphy et al., 1981; Prescott et al., 1985; Strasser et al., 1985). Furthermore, the amount of LTC_5 produced by mastocytoma cells from mice fed an EPA rich diet from birth was 1/10 that of LTC_4, indicating an inhibition site at LTC synthetase (Murphy et al., 1981). It appears therefore that in vitro and in vivo metabolism of EPA is different.

Table 5.1: Effect of increasing doses of EPA on heights of peaks I, II and III.

	Peak Height (mm)		
Peak	I	II	III
EPA (uM)			
0	-	-	-
0.5	10.5±1.0	6.5±2.1	24.0± 1.8
2.5	27.1±0.8	10.8±2.5	29.0± 5.1
5.0	48.1±5.4	13.3±1.5	129.2±12.4

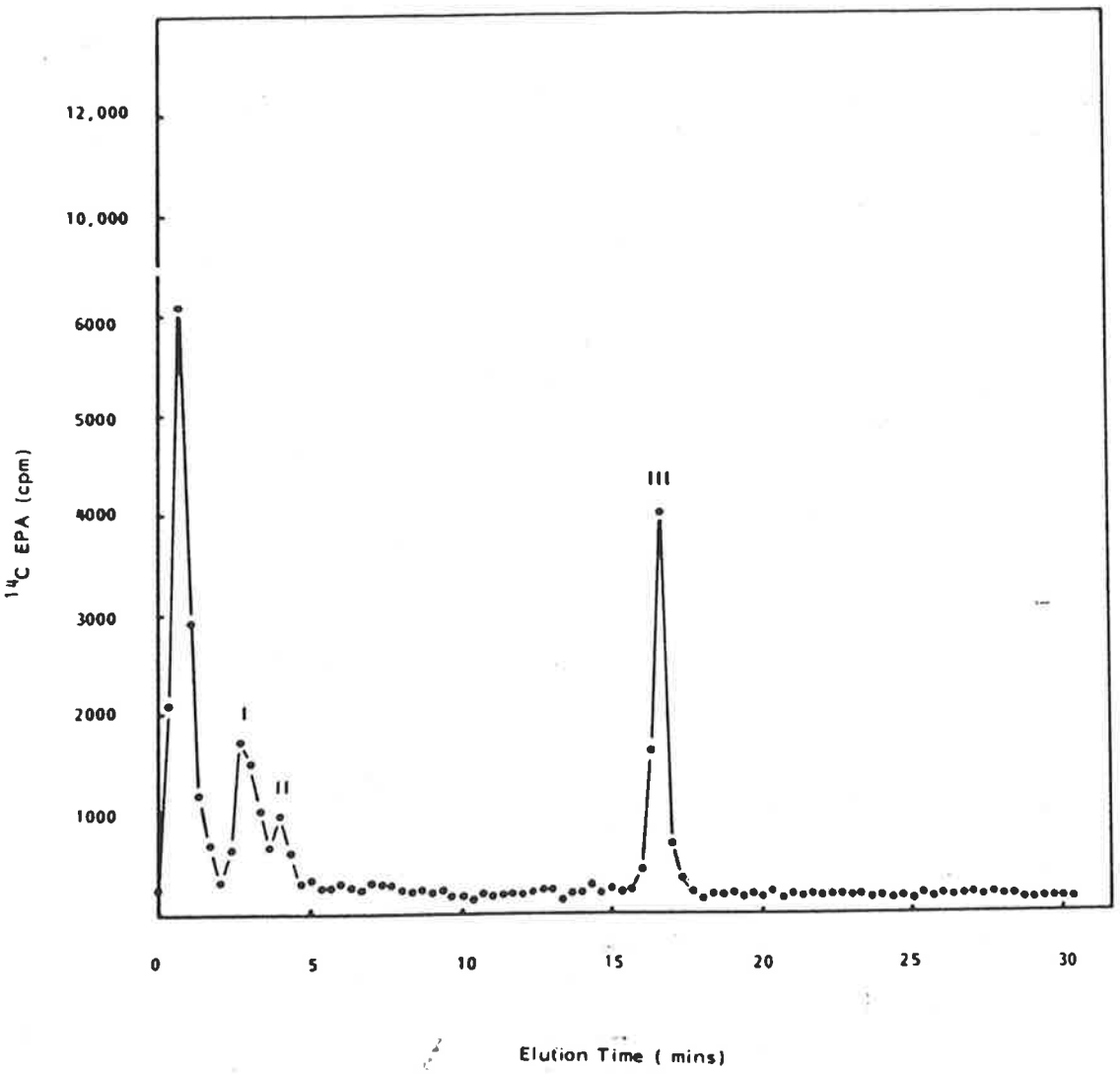


Figure 5.1 Radiochromatogram of ^{14}C-EPA metabolites of neutrophils stimulated with 0.5 uM A23187 for 5 min. 0.1 uCi of ^{14}C-EPA was added simultaneously with A23187. After 5 min, the reaction was stopped, the leukotrienes were extracted from the samples and HPLC analysis was performed (see Chapter 2 for details). HPLC elution fractions were collected every 20 sec. Peak I consists of the LTB_5 isomers, peak II is LTB_5 and peak III is 5-HEPE.

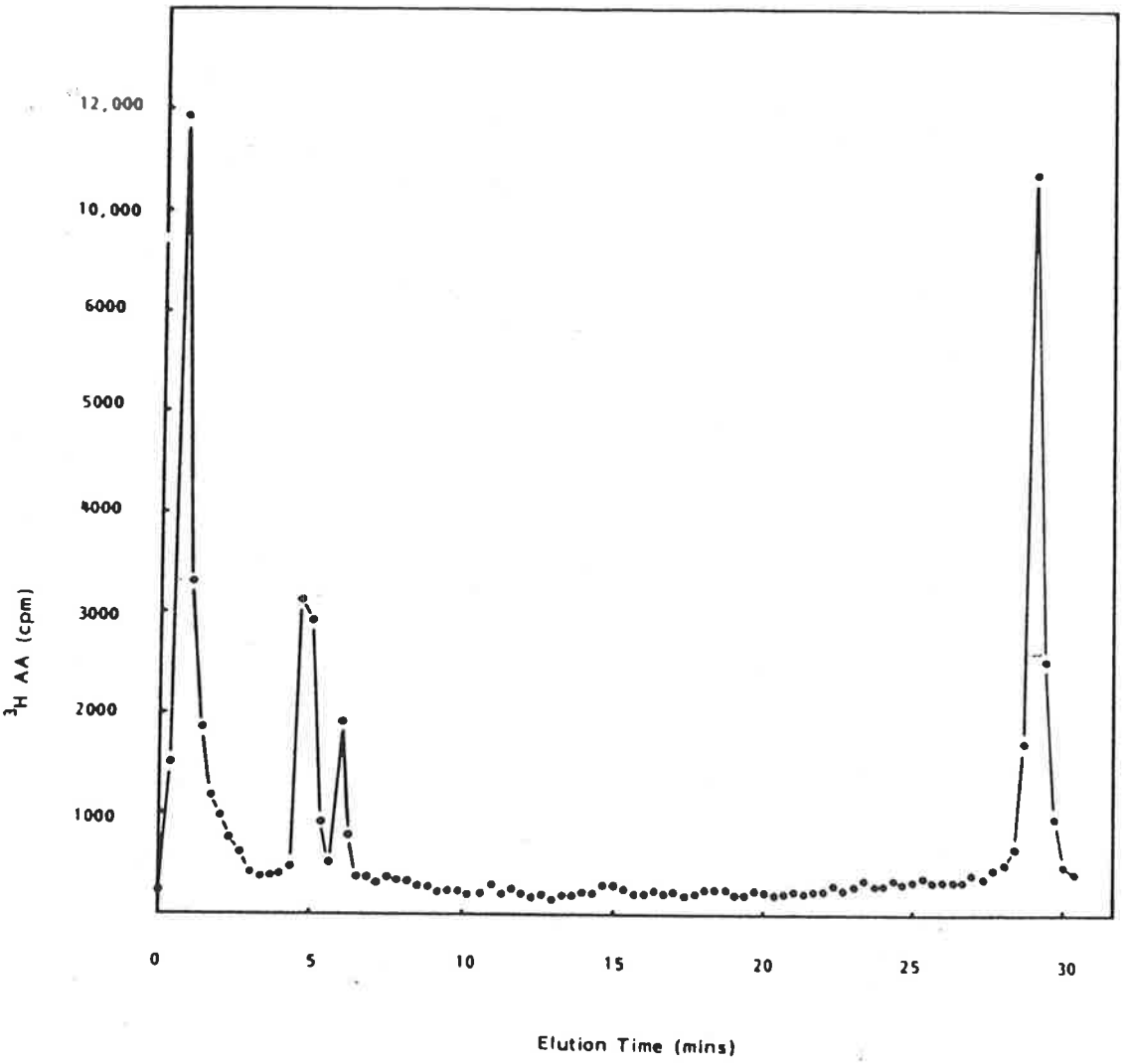


Figure 5.2 Radiochromatogram of ^{3}H-AA metabolites of neutrophils stimulated with 0.5 uM A23187 for 5 min. 0.1 uCi of ^{3}H-AA was added simultaneously with A23187. After 5 min, the reaction was stopped, the leukotrienes were extracted from the samples and HPLC analysis was performed (see Chapter 2 for details). HPLC elution fraction were collected every 20 sec.

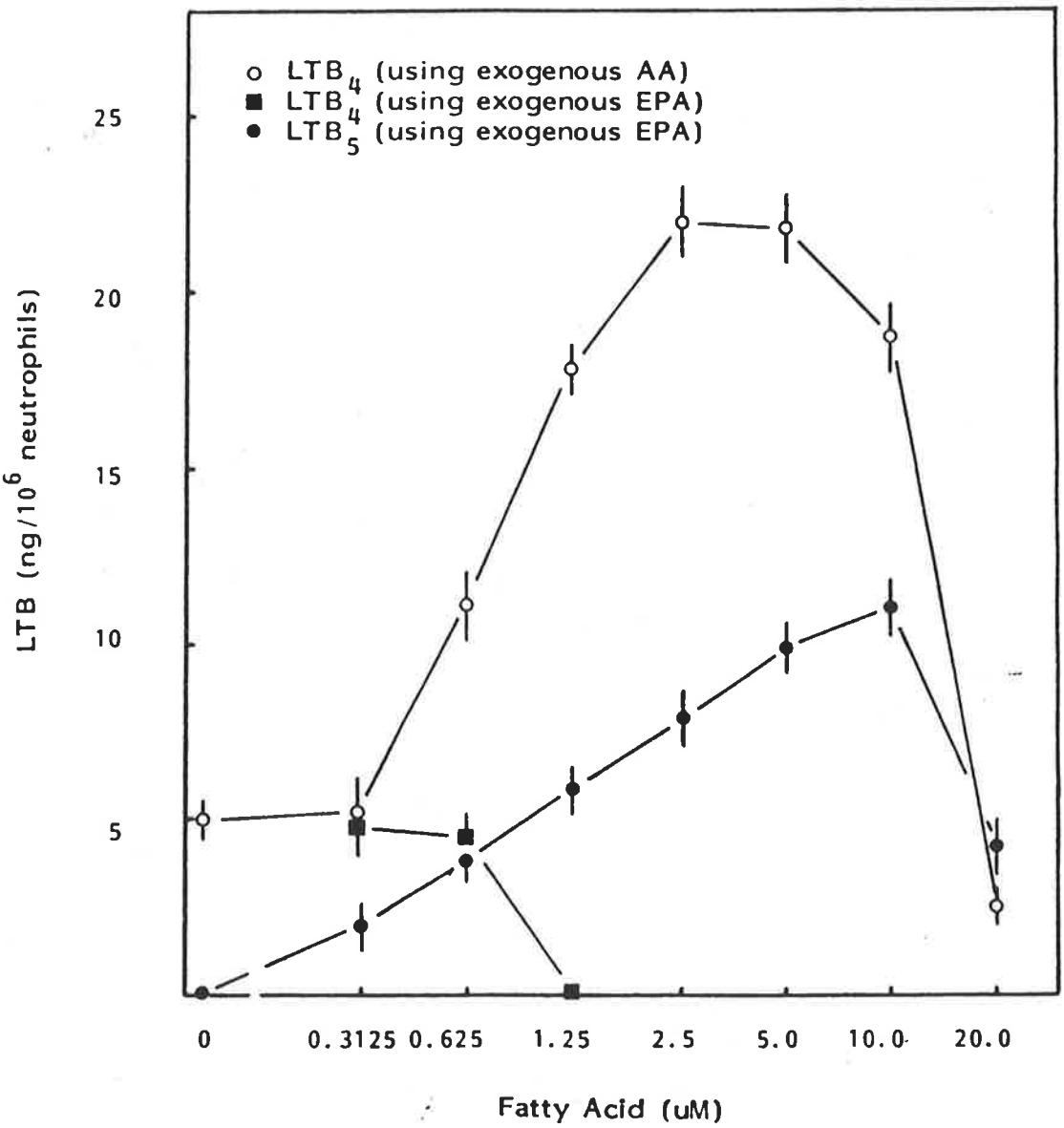


Figure 5.3 Dose response curves of production of LTB metabolites of AA or EPA by neutrophils stimulated for 5 min with 0.05 uM A23187 and increasing doses of either AA or EPA.

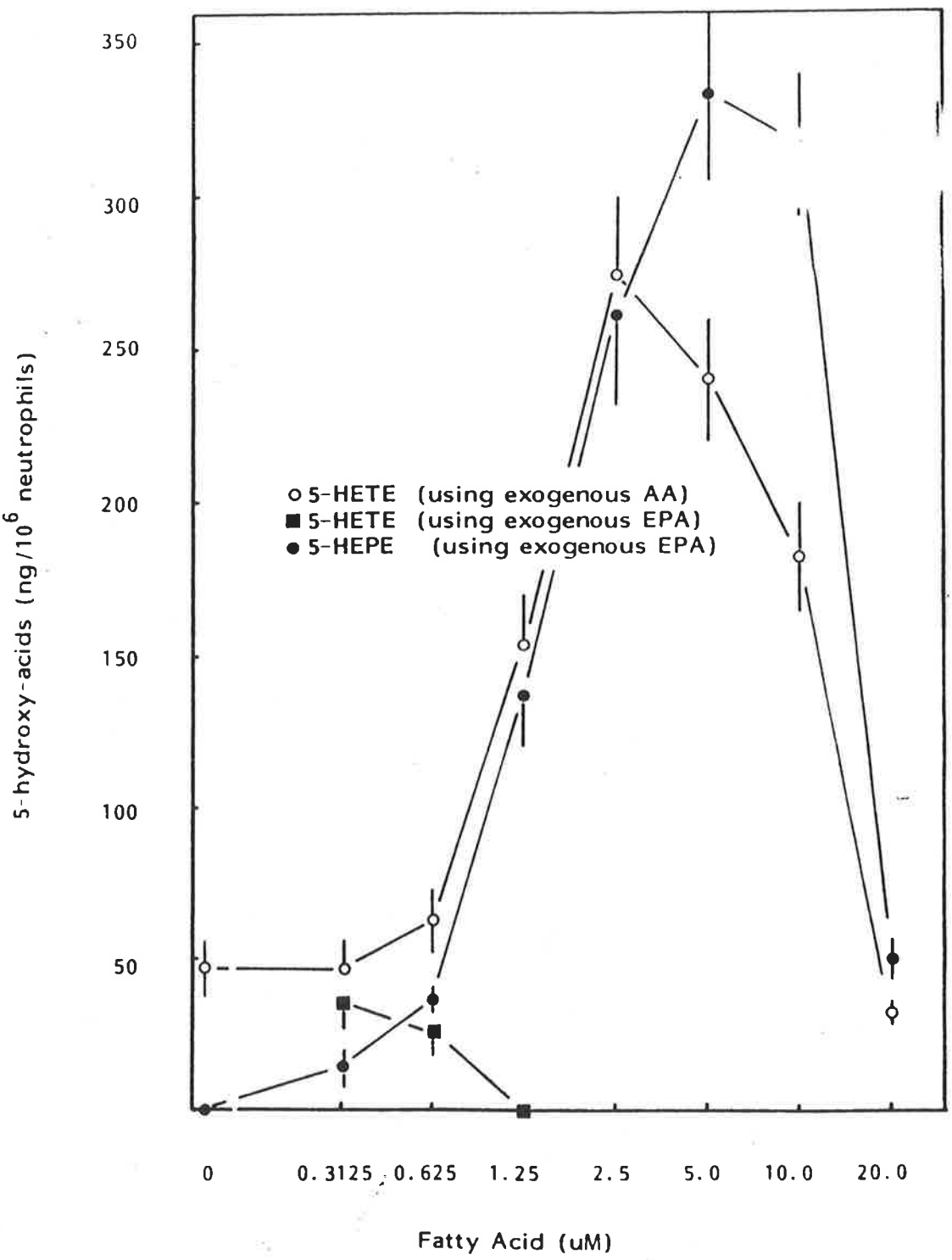


Figure 5.4 Dose response curves of production of 5-hydroxy acid metabolites of AA or EPA by neutrophils stimulated for 5 min with 0.05 uM A23187 and increasing doses of either AA or EPA.

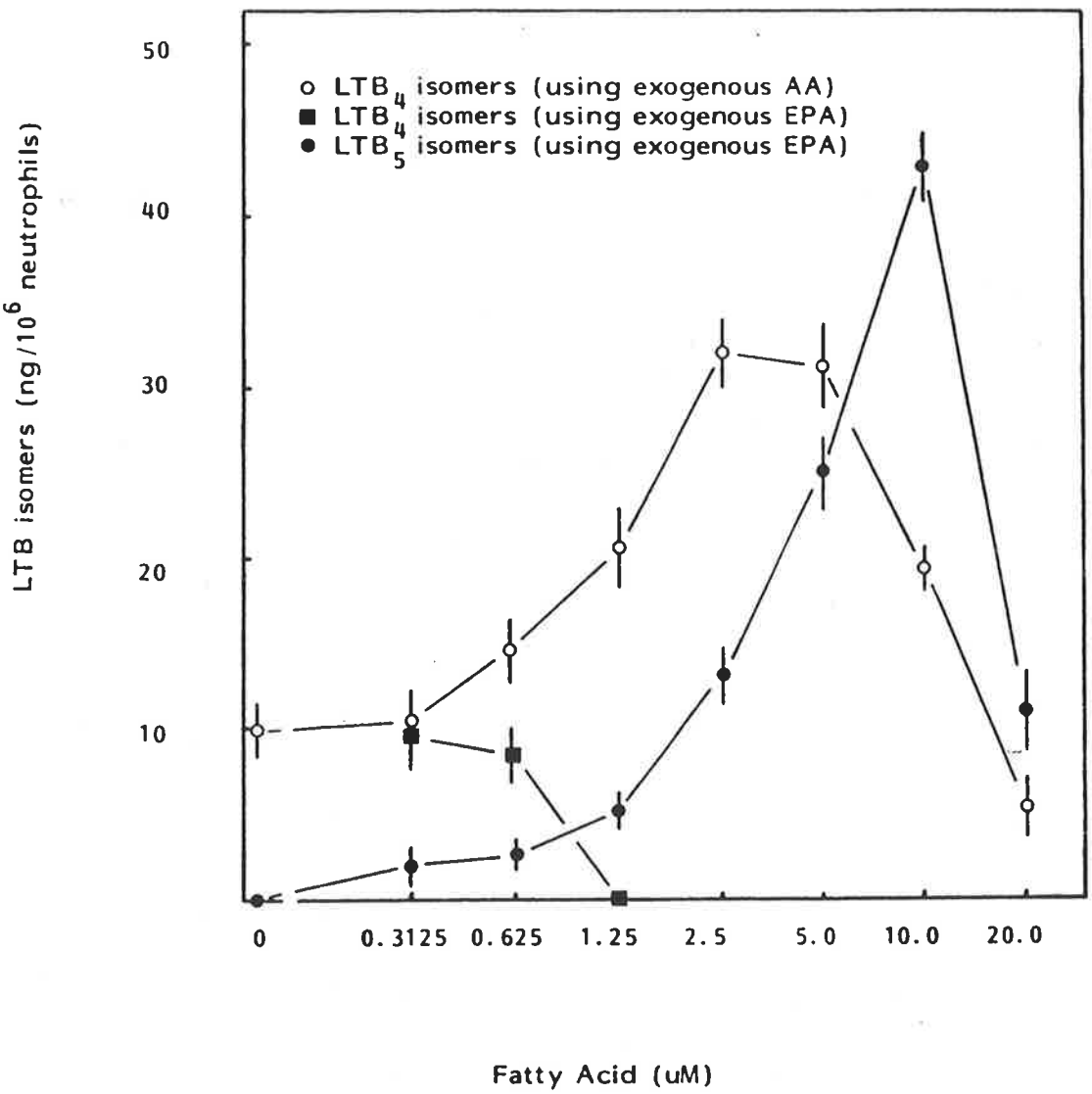


Figure 5.5 Dose response curves of production of LTB isomer metabolites of AA or EPA by neutrophils stimulated for 5 min with 0.05 uM A23187 and increasing doses of either AA or EPA.

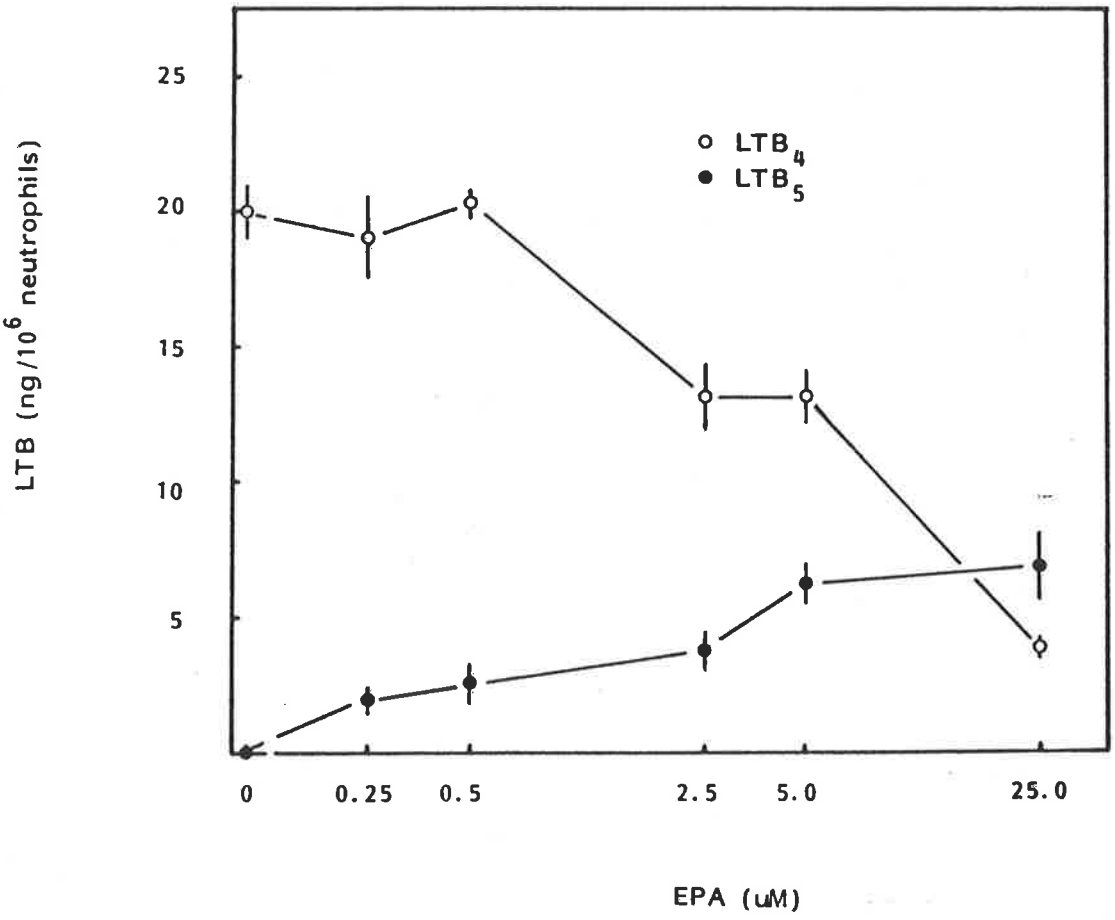


Figure 5.6 Inhibition of LTB_4 production by EPA. Neutrophils were stimulated for 5 min with 0.05 uM A23187 and 2.5 uM AA in the presence of increasing doses of EPA.

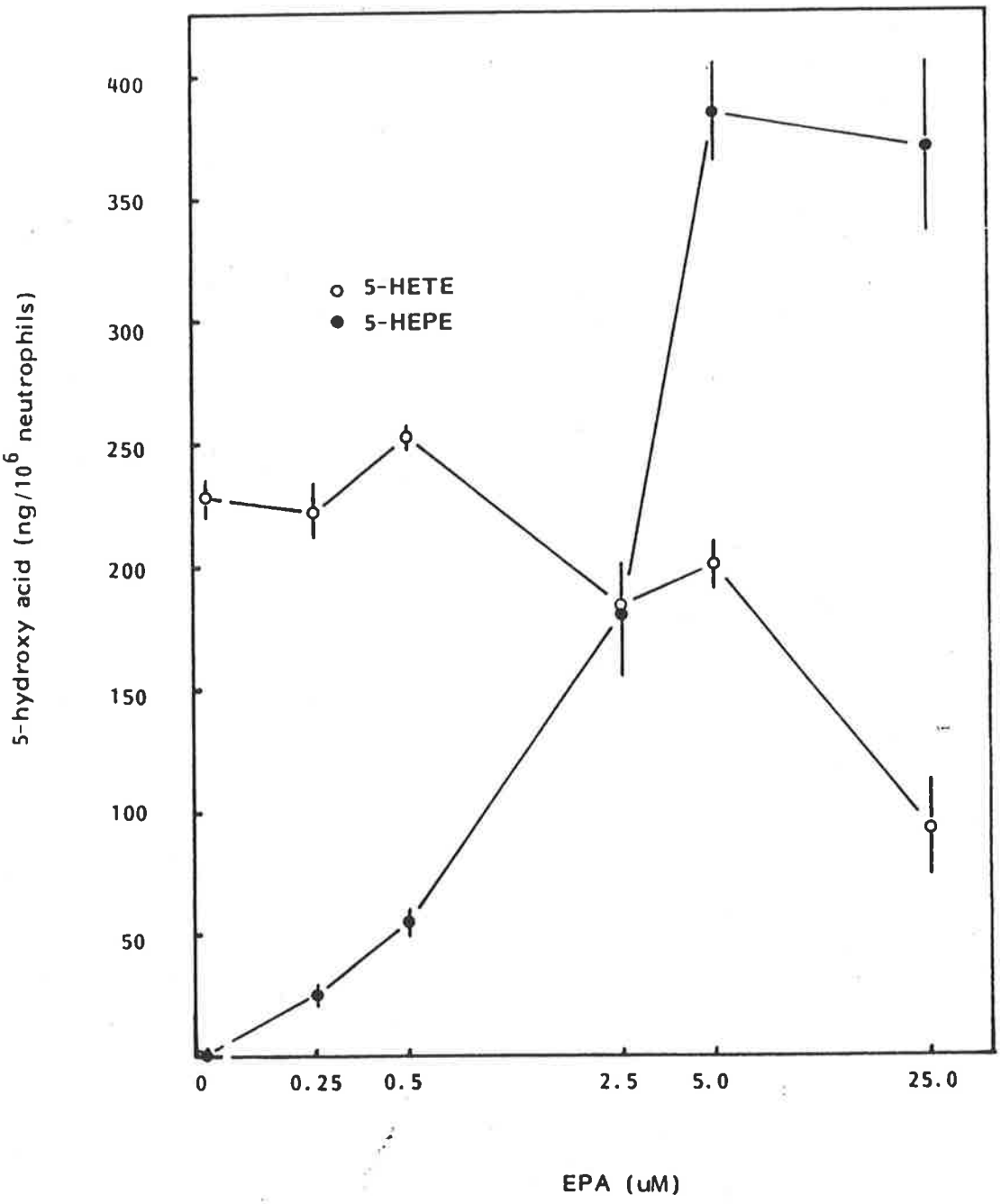


Figure 5.7 Inhibition of 5-HETE production by EPA. Neutrophils were stimulated for 5 min with 0.05 uM A23187 and 2·5uM AA in the presence of increasing doses of EPA.

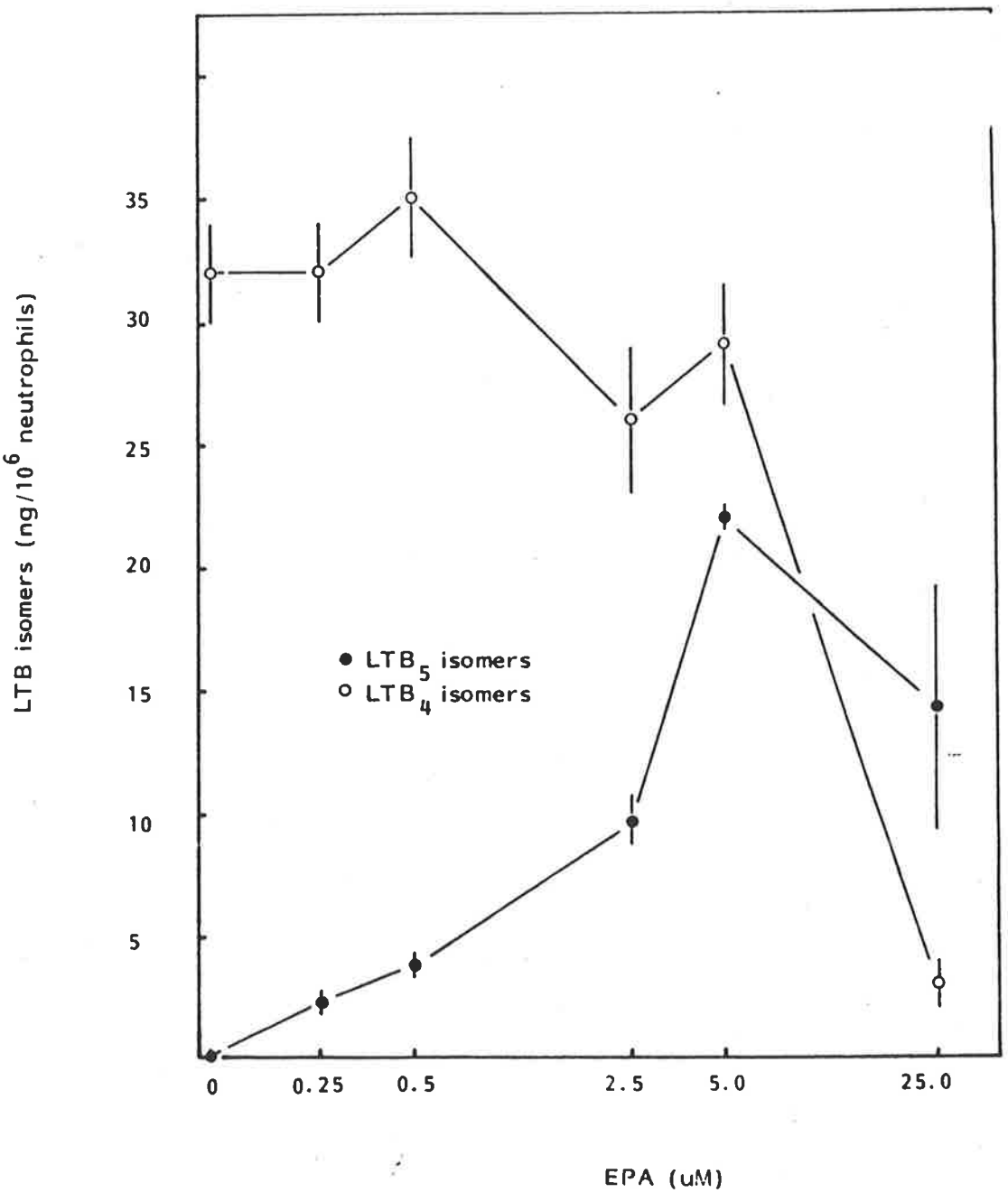


Figure 5.8 Inhibition of LTB_4 isomer production by EPA. Neutrophils were stimulated for 5 min with 0.05 uM A23187 and 2.5 uM AA in the presence of increasing doses of EPA.

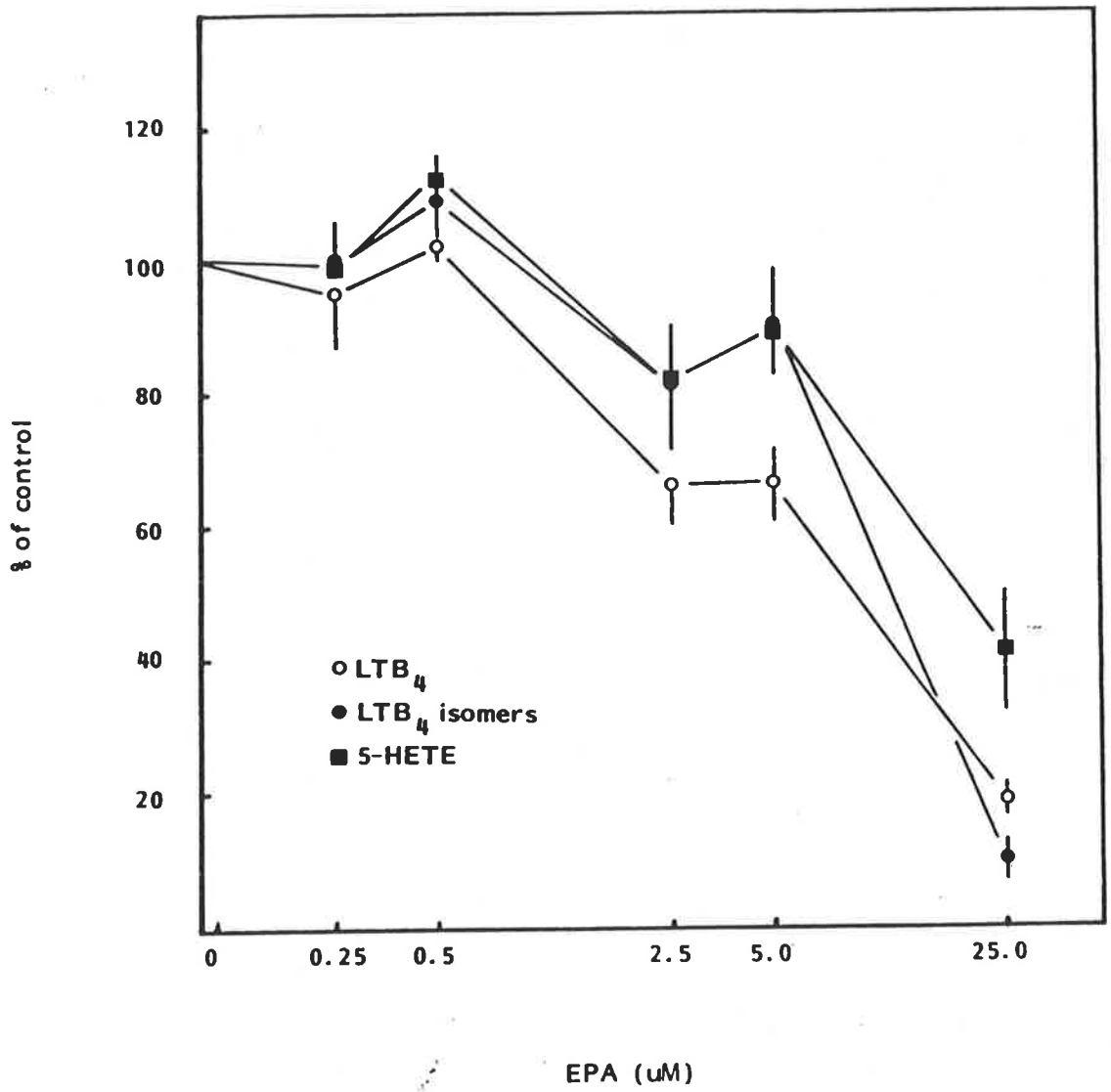


Figure 5.9 Inhibition of production of LTB_4, LTB_4 isomers and 5-HETE by EPA shown as % of control.

CHAPTER 6: EFFECT OF DIETARY SUPPLEMENTATION WITH EPA ON ANIMAL MODELS OF INFLAMMATION.

6.1 INTRODUCTION.

The experiments described in this chapter were designed to examine the effect of modifying dietary polyunsaturated fatty acids, particularly EPA on inflammation in rats. Three initial experiments were performed to evaluate which of three experimental models of inflammation (paw oedema, granulomatous sponge or adjuvant arthritis model) was most likely to yield useful information. After completion of the first three experiments, a more detailed analysis of the adjuvant arthritis model was undertaken and over a further 6 experiments the effect of dietaty modification on this model in two separate rat strains was examined (Table 6.1).

6.2 RESULTS.

6.2.1 GLC analysis of PUFA content of oils and diets.

GLC analysis of the long-chain fatty acids present in sunflower oil (SO), MaxEPA (FO) and linseed oil (LO) are shown in Table 6.2. SO provided a large percentage of w6 PUFA (59.4%) compared to 4.1% in FO and 15.2% in LO. The major w6 PUFA in all three oils was linoleic acid. In contrast, LO and FO contained large percentages of w3 PUFA (54.9 and 36.8 respectively) whereas SO contained only 1.1%. The major w3 PUFA in LO was γ-linolenic acid, whereas FO contained mainly

EPA and some DHA.

The distribution of long-chain fatty acids in the four diets is shown in Table 6.3. The control diet (Diet C, which contained 4% fat by weight compared to the oil supplemented diets which contained 16% total fat), consisted primarily of w6 PUFA (32.6%), the majority of which was linoliec acid, while the SO diet contained linoleic acid (56.9%) as the only detectable w6 PUFA. Neither of these diets contained a large percentage of w3 PUFA. Rats fed diets supplemented with CLO (experiments 1 & 2) received approximately 1.4 g EPA and 1.8 g DHA/day, whereas rats fed diets supplemented with MaxEPA (experiments 3-9) received 2.8 g EPA and 1.8 g DHA/day. The diets were essentially isocaloric as determine by bomb calorimetry; 18.5, 17.6, 17.3 and 16.5 ($\times 10^{-2}$ Kj/g) for diets C, S, F, and L respectively.

6.2.2 Group homogeneity.

In all of the experiments described below no significant differences between groups on the basis of weight were found prior to induction of inflammation. All rats were weighed weekly and average group weights compared. Typical growth patterns for each strain of rat are shown in Figure 6.1 & 6.2.

6.2.3 Experiment 1: Carrageenan paw swelling.

DA rats were used in this experiment and rats in group C were fed the unsupplemented diet and rats in groups S and F were fed diets

supplemented with sunflower-seed oil and cod liver oil respectively (Table 6.1). After 4 weeks of feeding, the animals were given an injection of 1% carrageenan in one paw. The group of rats fed fish oil (group F) had significantly less paw swelling than rats fed the unsupplemented diet (group C). Group S rats also had less paw swelling than group C rats, however, the difference was not significant (Table 6.4).

6.2.4 Experiment 2: Granulomatous sponge.

DA rats were also used for this experiment and fed the same three diets (Table 6.1). After 4 weeks of feeding, sponges impregnated with M. Tuberculosis were implanted into each flank of the dorsum of each rat and carefully removed 4 days later. The sponges were dried and weighed to determine the weight gain due to cellular infiltration. There was no difference between the three dietary treatments with respect to sponge exudate weight (Table 6.5).

6.2.5 Experiment 3: Adjuvant-induced arthritis.

This was the final experiment of the initial phase of the project. DA rats were again used for this experiment which involved substitution of the initial cod liver oil supplemented diet with a diet supplemented with another cod liver oil preparation, MaxEPA (Table 6.1). Rats fed this fish oil diet will also be referred to as group F rats. MaxEPA was used as the cod liver oil supplement in all subsequent experiments. After four weeks of feeding, adjuvant arthritis was induced by an intradermal tail injection of M.

Tuberculosis dispersed in squalane. Assessment of arthritis was made 12 days after the injection. No significant differences between the groups with respect to tail or paw swelling were observed (Table 6.6). Group F rats exhibited significantly less weight loss and lower disease scores, and the least paw and tail swelling compared with either group S or group C rats (Table 6.6). These findings indicate that w3 PUFA supplementation reduces the severity of adjuvant-induced arthritis in DA rats.

6.2.6 Experiment 4: Adjuvant-induced arthritis.

A second strain of rat was incorporated into the study at this stage to ascertain whether the decrease in arthritis in DA rats fed w3 PUFA observed in the previous experiment could be duplicated in another rat strain. A fourth dietary treatment containing linseed oil (fed to group L) was included for this and all subsequent experiments (Table 6.1). After four weeks of feeding, adjuvant disease was induced by intradermal injection of M. Tuberculosis dispersed in squalane. All groups showed similar degrees of tail and paw swelling (Table 6.7). In contrast to the findings of the previous experiment, group F rats showed significantly higher disease scores than all other groups while group S rats had significantly lower disease scores than all other groups (Table 6.7). Group F rats also showed significantly greater paw swelling than either group C or S rats (Table 6.7). These findings imply that the two rat strains respond to the dietary lipids in different ways. An interesting result was that the severity of arthritis in rats fed linseed oil (group L) was similar to those fed the baseline diet (group C). The linseed oil diet provides a

potential metabolic precursor of EPA and may have been expected to have similar effects to the fish oil diet.

6.2.7 Experiment 5: Adjuvant-induced arthritis.

This experiment, carried out concurrently with experiment 4, was performed on DA rats fed the four diets described previously (Table 6.1). However, triolein, a trioleate glycerol, was used as adjuvant for M. Tuberculosis. Previous reports have shown that the DA and hooded rat strains vary in their response to squalane and triolein (Garret et al., 1985). These authors found that the polyarthritis caused by squalane was more severe than triolein. We therefore sought to determine whether the anti-inflammatory effect of MaxEPA in the DA strain was still observed when a less severe arthritis was induced.

There were no significant differences between the treatments with respect to tail swelling, or weight change. However, group S and L rats had significantly less paw swelling than group C rats while group F rats had smaller disease scores than the other groups (see Table 6.8). These results are in general agreement with those of experiment 3, indicating that dietary supplementation with w3 fatty acids reduces severity of adjuvant arthritis in DA rats.

6.2.8 Experiment 6: Adjuvant-induced arthritis.

This experiment was performed to complete the second phase of the project. WH rats were fed the same four diets as in previous experiments, however, triolein was used as adjuvant. In contrast to

the previous experiment using WH rats, no differences were observed between the four groups with respect to any of the parameters assessed (Table 6.9). Very severe disease was induced in all groups and a disease enhancing effect of fish oil could not have been observed.

In order to reexamine the reproducibility of the above findings in the adjuvant arthritis model, three of the latter four experiments were repeated.

6.2.9 Experiment 7: Adjuvant-induced arthritis.

This experiment was designed to repeat experiment 3 (Section 6.2.5) with the exception that the fourth dietary group (supplemented with linseed oil) was included. Adjuvant arthritis was induced by intradermal injection of M. Tuberculosis with squalane as adjuvant. Assessments were made on days 10 and 12 after the injection. No significant differences between any of the four groups were observed with respect to any of the parameters assessed were observed (Table 6.10).

6.2.10 Experiment 8: Adjuvant-induced arthritis.

This and the following experiment were performed in an attempt to discern any effect of the diets upon the rate of developement of disease after adjuvant injection. DA rats were injected with M. Tuberculosis dispersed in triolein. Assessments were performed on days 0,3,5,7,9,10,11,12 after induction of disease. After day 12, rats were used for neutrophil studies (described below). The time

course of assessment provided a more complete set of data than any of the previous experiments. However, it was not until the final day of assessment that any significant difference was observed. Group L showed less disease scores than group C rats. No other differences were observed at any of the time points (Table 6.11). No severe disease was observed until days 10 - 12, where paw swelling occurred and articular lesions developed as has been reported in previous studies (Pearson and Wood, 1959). In contrast, tail swelling and weight loss began immediately. No difference in the rate of disease developement was observed. Severe disease was observed in all groups.

6.2.11 Experiment 9: Adjuvant-induced arthritis.

This was the final experiment performed in this project. Basically designed to repeat experiment 6, disease induced by triolein in WH rats, (Section 6.3.8), this incorporated a time course of disease assessment as performed in experiment 8 (see Section 6.3.11). There was a trend towards more severe disease in paw and tail swelling and disease activity scores although the differences did not reach statistical significance (Table 6.12). No difference in rate of disease developement was observed,

6.3 DISCUSSION.

The majority of animal studies examining the effect of dietary PUFA modification have used two diets - menhaden oil and beef tallow. Fernandes et al., 1986 have recently reported that a diet high in

saturated fat enhances murine lupus. This creates difficulties when interpreting effects of fish oil diets when compared with beef tallow diets. Furthermore, diets high in saturated fat enhance symptoms of the lupus in NZB x NZW/F_1 and MRL/1pr/1pr mice (Morrow et al., 1985a; 1985b; Morrow et al., 1986). In designing the experiments described in this chapter, an attempt was made to include a diet which was as close as possible to the "normal" diet of the rats. This diet was termed baseline or reference diet and fed to group C. MaxEPA (fish oil) was selected to provide a diet rich in EPA whereas the main PUFA provided by the safflower oil diet was linoleic acid. Thus with the maxEPA diet the rats were provided with the w3 substrate for 5-LO, while the rats fed safflower oil were provided with a precursor for the w6 substrate (AA). With the linseed oil diet, precursors for both EA (eg: linolenic acid), and AA (eg: linoleic acid) were presented in the supplement. Fatty acid analysis by GLC showed that all diets caused the predicted changes in the PUFA content of cellular lipids after four weeks (Pazios, personal communication). For example, rats fed fish oil and linseed oil had increased levels of w3 PUFA (particularly EPA) while the safflower oil-fed rats had increased levels of w6 PUFA (particularly AA). Studies on the 5-lipoxygenase products of peritoneal neutrophils of both rats strains indicated that after four weeks on the diets, the neutrophils of rats fed MaxEPA and linseed oil produced the EPA-metabolites LTB_5 and 5-HEPE (Pazios, personal communication).

Swelling in the carrageenan paw oedema model 4 hrs after injection is largely attributable to production of eicosanoids (Velo et al., 1973). Therefore, the results from this study support the

hypothesis that w3 PUFA can reduce inflammation by interfering with AA-derived eicosanoid synthesis. Supplementation of male Wistar rats with 240 mg EPA/day for 4 weeks significantly reduced carrageenan-induced oedema and levels of TXB_2 and PGE_2 but had no effect on levels of LTB_4 (Terano et al., 1986). In contrast, there was no difference in paw oedema in female Sragues-Dawley rats fed 100 mg EPA/day for 10 weeks (Sametz and Juan 1985). Furthermore, there was no reduction in levels of PGI_2 or PGE_2. It is appears therefore that reduction in levels of prostaglandins of the 2 series is necessary to reduced swelling and that 100 mg EPA/day is not sufficient to achieve such a reduction (Sametz and Juan, 1985). Moreover, the level of LTB_4 does not correlate with inhibition of paw swelling in this model (Terano et al., 1986).

The results from the study on the granulomatous sponge model support the findings of Terano et al., (1986) demonstrating that feeding rats 240 mg EPA/day had no effect on either cellular infiltration into carrageenan-soaked sponges or production of LTB_4, while levels of PGE_2 and TxB_2 were reduced (Terano et al., 1986). It is possible that a significant reduction in levels of LTB_4 is required to significantly reduce the extent of cellular infiltration into carrageenan-soaked sponges.

During the course of these experiments, variability in response to the same adjuvant by the same rat strain was observed. For example, in experiment 3 (DA/squalane), disease activity scores on day 12 ranged from 7.2 - 11.2, whereas when this experiment was repeated 8 months later (experiment 7), the disease scores ranged from 12.1 -

12.6. Similar observations were made between experiments 5 & 8, when DA rats were given arthritis using triolein. These experiments were performed 6 months apart. Furthermore, similar variation was observed between experiments 6 & 9 when WH rats were given arthritis using triolein. The reasons for this variability are unclear, however, it is possible that a single rat strain could respond differently under varying conditions at the time of study, for example, changes in basal diet (see below), seasonal changes or intercurrent infections.

When effects of the dietary PUFA modification on adjuvant arthritis were observed (for example, experiments 3,4 and 5), opposite results were obtained depending on the strain of rat. This possibly indicates that the two strains metabolise dietary lipids differently. Jeffrey and Redgrave, (1982) reported similar findings when comparing chylomicron metabolism of albino and hooded rats and concluded that the genetic factors regulating lipoprotein metabolism in the rat may be linked to the hh geneotype responsible for Hooded pigmentation. Garret et al., (1985) found different responses to gold therapy when DA and several hooded rat strains were given adjuvant arthritis, thereby demonstrating that different strains respond differently to the same therapeutic agents. Immunological differences between rat strains have been demonstrated, and hooded rat strains have impaired Ia expression (Mayrhofer et al., 1983). Such immunological differences could provide a possible basis for different responses to adjuvant disease and theraputic agents.

Lack of efficacy of the dietary treatment can be related to severity of disease. In general, where no significant effect of

dietary modification was observed (for example, experiments 6,7 and 8), very severe disease was present. It is possible that discrimination between the effect of dietary treatments would not be observed under these circumstances other mechanisms may override the influence of eicosanoids.

It is important to note that during the course of these experiments, significant variation in the contents of rat chow purchased from Milling Industries was experienced. For several months during the latter section of experimentation, (particularly experiments 6 and 7), severe problems were encountered in producing large litters of rats. The problem was traced to the presence of a 10-fold excess of ethoxyquin in the standard rat chow. Apart from the effect this diet had on litter size, the presence of this amount of antioxidant may have masked a beneficial effect of the dietary treatments.

Table 6.1: Details and order of experiments in Chapter 6.

Experiment	Model	Diets	Rat strain	Adjuvant
1.	Paw swelling	C,S,F	DA	NA
2.	Granulomatous sponge	"	"	NA
3.	Ajuvant arthritis	"	"	squalane
4.	"	C,S,F,L	WH	"
5.	"	"	DA	triolein
6.	"	"	WH	"
7.	"	"	DA	squalane
8.	"	"	DA	triolein
9.	"	"	WH	"

Diets: C = control diet, S = sunflower oil diet, F = fish oil diet, L = linseed oil diet. DA = dark agouti; WH = wistar-hooded.

Table 6.2: Fatty acid analysis* of oils used as supplements.

Fatty acid	Common name	Sunflower oil	Fish oil#	Linseed oil
14:0	Myristic	-	6.5	-
15:0	-	-	0.5	-
16:0 (iso)	-	-	0.1	-
16:0	Palmitic	6.3	16.9	6.4
17:0	Margaric	0.1	2.1	0.1
18:0	Stearic	4.4	3.5	4.6
20:0	Arachidic	0.4	1.1	0.3
24:0	Lignoceric	0.3	0.3	0.4
Total Saturates		11.5	31.0	11.5
14:1	Myristoleic	-	0.3	-
16:1	Palmitoleic	-	9.3	-
18:1	Oleic	27.7	15.8	17.7
20:1	Gondoic	0.3	2.5	0.6
24:1	Nervonic	-	0.2	0.1
Total Monoenes		28.0	28.1	18.4
18:2	Linoleic	59.3	1.9	14.9
18:3	-linolenic	-	0.3	-
20:2	-	0.1	0.3	0.2
20:3	Dihomo- -linolenic	-	0.2	-
20:4	Arachidonic	-	1.0	0.1
22:4	Adrenic	-	0.4	-
Total w6 acids		59.4	4.1	15.2
18:3	-linolenic	0.4	0.7	53.9
20:5	Timnodonic	-	18.1	0.9
22:5	Docosapentaenoic	0.7	2.8	-
22:6	Clupanodonic	-	11.1	0.1
Total w3 acids		1.1	36.8	54.9

* Results expressed as a percentage of total fatty acids.
# MaxEPA oil

Table 6.3: Fatty acid analysis* of test diets**.

Fatty Acid	Diet C	Diet S	Diet F#	Diet L
14:0	1.4	0.3	5.3	0.4
15:0	0.3	-	0.6	-
16:0 (iso)	-	-	0.3	-
16:0	19.2	7.8	17.4	9.3
17:0	0.7	0.3	1.9	0.4
18:0	6.6	4.6	5.2	5.4
20:0	0.6	0.5	1.7	-
24:0	0.6	0.6	0.6	0.3
Total Saturates	29.4	14.1	32.9	15.8
14:1	-	-	0.4	-
16:1	2.6	0.5	7.5	0.6
18:1	22.4	26.4	17.2	18.8
20:1	3.2	0.5	2.5	0.6
24:1	0.8	-	0.6	0.2
Total Monoenes	29.1	27.4	28.2	20.2
18:2	32.3	56.9	8.8	19.1
18:3	-	-	0.3	-
20:2	0.3	-	0.4	0.2
20:3	-	-	0.3	-
20:4	-	-	0.9	0.2
22:4	-	-	0.8	-
Total w6 acids	32.6	56.9	11.8	19.5
18:3	2.8	-	1.4	42.6
20:5	4.2	0.3	14.1	0.9
22:5	-	-	2.2	0.2
22.6	1.9	0.5	9.3	0.8
Total w3 acids	8.9	0.8	27.0	44.5
Total w6/w3	3.7	71.2	0.4	0.4

* Results expressed as a percentage of total fatty acids.

** Rat chow from a commercial supplier was crushed, mixed with or without supplementation and then repelleted:
Diet C fed to Group C = rat chow repelleted without supplementation.
Diet S fed to Group S = rat chow supplemented with sunflower oil.
Diet F fed to Group F = rat chow supplemented with fish oil.
Diet L fed to Group L = rat chow supplemented with linseed oil.

\# MaxEPA supplemented diet.

Table 6.4: Experiment 1: Effect of dietary PUFA modification on the degree of carrageenan-induced paw swelling in DA rats.

Group	n	water displaced (g)
C#	5	0.66±0.05
S	9	0.41±0.07
F	10	0.29±0.03*

Paw swelling was determined as the amount of water displaced from a standard 12 x 75 mm test tube by the immersion of the paw up to the anatomical hairline.
* Significantly different from group C at $p < 0.05$.
# Group C = group fed control diet, S = sunflower oil diet and F = fish oil diet. Values are expressed as mean±sem.

Table 6.5: **Experiment 2: Effect of dietary PUFA modification on fluid exudate accumulation and inflammatory cell migration into sponges implanted in the dorsal flanks of DA rats.**

Group	n	dry wt. gain (mg)
C#	10	29±2
S	11	26±2
F	11	33±3

Weight measurements determined by subtraction from pre-experimental dry weight of sponges. # Group C = group fed control diet; S = sunflower oil diet and F = fish oil diet. Values expressed as mean±sem.

Table 6.6: Experiment 3: Effect of dietary PUFA modification on the severity of adjuvant-induced arthritis in DA rats using squalane as adjuvant.

Group	n	Paw(mm)	Tail(mm)	Wt(g)	Disease(/14)
C#	9	7.1±0.4	10.1±0.2	175±7	11.2±0.8
S	10	6.7±0.3	10.4±0.3	176±5	10.8±1.0
F	10	6.3±0.3	9.9±0.2	199±8*	7.2±1.1*

Arthritis was induced by an intradermal tail injection of heat-killed Mycobacterium Tuberculosis dispersed in squalane. Paw and tail swelling were determined using digital calipers. Wt. refers to whole body weight and disease scores were determined as described in Section 2.2.14. # Group C = group fed control diet; S = sunflower oil diet and F = fish oil diet. * Significantly different from group C and S at $p < 0.05$. Results expressed as mean±sem.

Table 6.7: Experiment 4: Effect of dietary PUFA modification on the severity of adjuvant-induced arthritis in WH rats using squalane as adjuvant.

Group	n	Paw(mm)	Tail(mm)	Wt(g)	Disease(/14)
C#	9	7.8±0.3	11.7±0.1	220± 3	8.9±1.0
S	8	7.0±0.3	11.6±0.3	190±14	5.1±1.0+
F	9	8.7±0.3*	11.3±0.3	238± 6	11.0±0.9**
L	8	7.9±0.3	11.5±0.4	215±10	8.8±1.0

Arthritis was induced by an intradermal tail injection of heat-killed Mycobacterium Tuberculosis dispersed in squalane. # Group C = group fed control diet; S = sunflower oil diet; F = fish oil diet and L = linseed oil diet. * Significantly different from groups C and S at $p < 0.05$. ** Significantly different from groups C,S and L at $p < 0.05$. + Significantly different from groups C, F and L at $p < 0.05$. Results expressed as mean±sem.

Table 6.8: Experiment 5: Effect of dietary PUFA modification on the severity of adjuvant-induced arthritis in DA rats, using triolein as adjuvant.

Group	n	Paw(mm)	Tail(mm)	Wt(g)	Disease(/14)
C#	10	5.7±0.2	10.0±0.2	194± 9	5.8±0.7
S	10	6.6±0.3	10.8±0.2	200±10	6.2±0.9
F	10	6.4±0.3	10.3±0.3	179± 6	3.9±0.7**
L	8	7.0±0.4*	10.6±0.2	181± 5	5.8±1.3

Arthritis was induced by an intradermal tail injection of heat-killed Mycobacterium Tuberculosis dispersed in triolein. # Group C = group fed control diet; S = sunflower oil diet; F = fish oil diet and L = linseed oil diet. * Significantly different from group C at $p < 0.05$. ** Significantly different from groups C and S at $p < 0.05$. Results expressed as mean±sem.

Table 6.9: Experiment 6: Effect of dietary PUFA modification on the severity of adjuvant-induced arthritis in WH rats, using triolein as adjuvant.

Group	n	Paw(mm)	Tail(mm)	Wt(g)	Disease(/14)
C#	6	7.9±0.4	10.5±0.4	182± 7	12.2±1.6
S	6	7.9±0.7	10.6±0.3	188± 3	11.8±1.4
F	6	7.3±0.4	10.5±0.1	175± 5	11.2±0.6
L	6	7.4±0.4	10.7±0.2	188±10	10.2±1.7

Arthritis was induced by an intradermal tail injection of heat-killed Mycobacterium Tuberculosis dispersed in triolein. # Group C = group fed control diet; S = sunflower oil diet; F = fish oil diet and L = linseed oil diet. Results expressed as mean±sem.

Table 6.10: Experiment 7: Effect of dietary PUFA modification on the severity of adjuvant-induced arthritis in DA rats, using squalane as adjuvant.

Group	n	Paw(mm)	Tail(mm)	Wt(g)	Disease(/14)
a) Day 10					
C#	7	4.6±0.4	9.7±0.1	194± 7	1.4±1.2
S	7	5.9±0.6	9.9±0.2	199± 6	5.1±1.8
F	5	5.3±0.6	9.9±0.1	200± 7	2.6±1.3
L	5	4.4±0.4	9.9±0.1	181±13	3.0±1.3
b) Day 12					
C		6.8±0.4	10.0±0.3	163± 6	12.1±0.8
S		7.9±0.5	10.4±0.3	171± 6	12.6±0.8
F		6.9±0.6	9.6±0.2	169± 7	12.2±1.5
L		6.7±0.4	9.8±0.3	172±11	12.4±1.2

Arthritis was induced by an intradermal tail injection of heat-killed Mycobacterium Tuberculosis dispersed in squalane. # Group C = group fed control diet; S = sunflower oil diet; F = fish oil diet and L = linseed oil diet. Results expressed as mean±sem.

Table 6.11: Experiment 8: Time course of effect of dietary PUFA modification on the severity of adjuvant-induced arthritis in DA rats, using triolein as adjuvant.

Group	n	Paw(mm)	Tail(mm)	Wt(g)	Disease(/14)
a) Day 0					
C#	6	5.4±0.2	7.3±0.3	212±16	-
S	8	5.5±0.2	7.6±0.3	204±13	-
F	9	5.3±0.2	7.4±0.4	202±20	-
L	9	5.3±0.2	7.4±0.3	191±12	-
b) Day 3					
C		4.9±0.1	9.2±1.0	187±13	-
S		4.9±0.1	9.4±0.9	180±12	-
F		4.9±0.2	9.6±0.7	190±21	-
L		4.9±0.2	9.0±0.9	171±14	-
c) Day 5					
C		5.0±0.4	9.7±1.6	190±17	-
S		5.0±0.2	9.7±0.5	182±13	-
F		4.8±0.6	10.1±0.6	192±20	-
L		5.2±0.3	9.3±0.4	176±14	-
d) Day 7					
C		5.0±0.1	9.8±0.9	195±15	-
S		5.0±0.2	9.1±0.5	188±12	-
F		5.0±0.2	10.3±0.4	200±20	-
L		5.1±0.3	9.5±0.7	182±15	-

e) Day 10

C	5.7±0.4	9.8±0.9	194±20	5.8±1.1
S	5.7±0.5	9.3±1.2	178±70	6.3±1.3
F	5.8±0.7	10.1±0.6	204±25	6.9±1.0
L	5.7±0.9	9.4±0.6	192±25	4.6±1.4

f) Day 11

C	7.0±0.1	9.4±0.3	182± 5	10.3±0.8
S	6.5±0.3	9.3±0.1	200± 5	7.9±1.4
F	6.6±0.2	9.7±0.2	191± 7	9.8±1.2
L	6.5±0.4	9.4±0.2	180± 5	7.2±1.4

g) Day 12

C	8.1±0.2	9.4±0.2	177± 5	13.5±0.5
S	7.6±0.4	9.6±0.1	180± 6	10.9±1.0
F	7.3±0.3	9.8±0.1	179± 7	11.6±0.6
L	7.0±0.5	9.1±0.3	176± 5	10.0±1.2*

Arthritis was induced by an intradermal tail injection of heat-killed Mycobacterium Tuberculosis dispersed in triolein. # Group C = group fed control diet; S = sunflower oil diet; F = fish oil diet and L = linseed oil diet. * Significantly different from group C at $p < 0.05$. Results expressed as mean±sem.

Table 6.12: Experiment 9: Time course of effect of dietary PUFA modification on severity of adjuvant-arthritis in WH rats using triolein as adjuvant.

Group	n	Paw(mm)	Tail(mm)	Wt(g)	Disease(/14)
a) Day 0					
C#	9	5.9±0.1	7.7±0.1	222± 8	-
S	11	5.9±0.1	7.6±0.1	207± 5	-
F	7	5.7±0.1	7.6±0.1	199± 5	-
L	9	5.9±0.1	7.8±0.1	214± 3	-
b) Day 3					
C		5.7±0.1	8.6±0.2	206± 8	-
S		5.7±0.1	9.3±0.2	195± 4	-
F		5.6±0.1	8.8±0.3	195± 6	-
L		5.7±0.1	9.3±0.2	193± 6	-
c) Day 5					
C		5.5±0.1	10.2±0.2	203± 8	-
S		5.6±0.1	10.4±0.1	199± 5	-
F		5.5±0.1	10.4±0.2	194± 5	-
L		5.4±0.1	10.3±0.2	208± 5	-
d) Day 7					
C		5.7±0.1	9.2±0.2	210± 9	-
S		5.7±0.1	9.8±0.2	204± 6	-
F		5.7±0.1	9.3±0.4	209± 7	-
L		5.6±0.1	9.6±0.2	216± 5	-

e) Day 10

C	6.0±0.1	9.2±0.2	222± 9	0.6±0.4
S	6.3±0.2	9.6±0.1	209± 7	1.8±0.7
F	6.0±0.1	9.6±0.2	228± 3	0.4±0.2
L	5.9±0.1	9.5±0.3	223± 6	1.0±0.6

f) Day 11

C	6.5±0.3	9.7±0.1	222± 9	3.2±0.9
S	6.8±0.4	9.8±0.2	208± 8	4.2±1.1
F	6.6±0.2	10.1±0.2	227± 3	3.0±1.0
L	6.5±0.2	9.7±0.1	226± 5	2.9±0.9

g) Day 12

C	6.8±0.3	9.8±0.1	223± 9	5.2±1.7
S	7.1±0.4	10.1±0.3	210± 7	6.0±1.7
F	7.3±0.3	10.7±0.3	223± 4	8.4±2.1
L	6.9±0.3	10.1±0.2	225± 6	5.8±1.7

Arthritis was induced by an intradermal tail injection of heat-killed Mycobacterium Tuberculosis dispersed in triolein. # Group C = group fed control diet; S = sunflower oil diet; F = fish oil diet and L = linseed oil diet.
Results expressed as mean±sem.

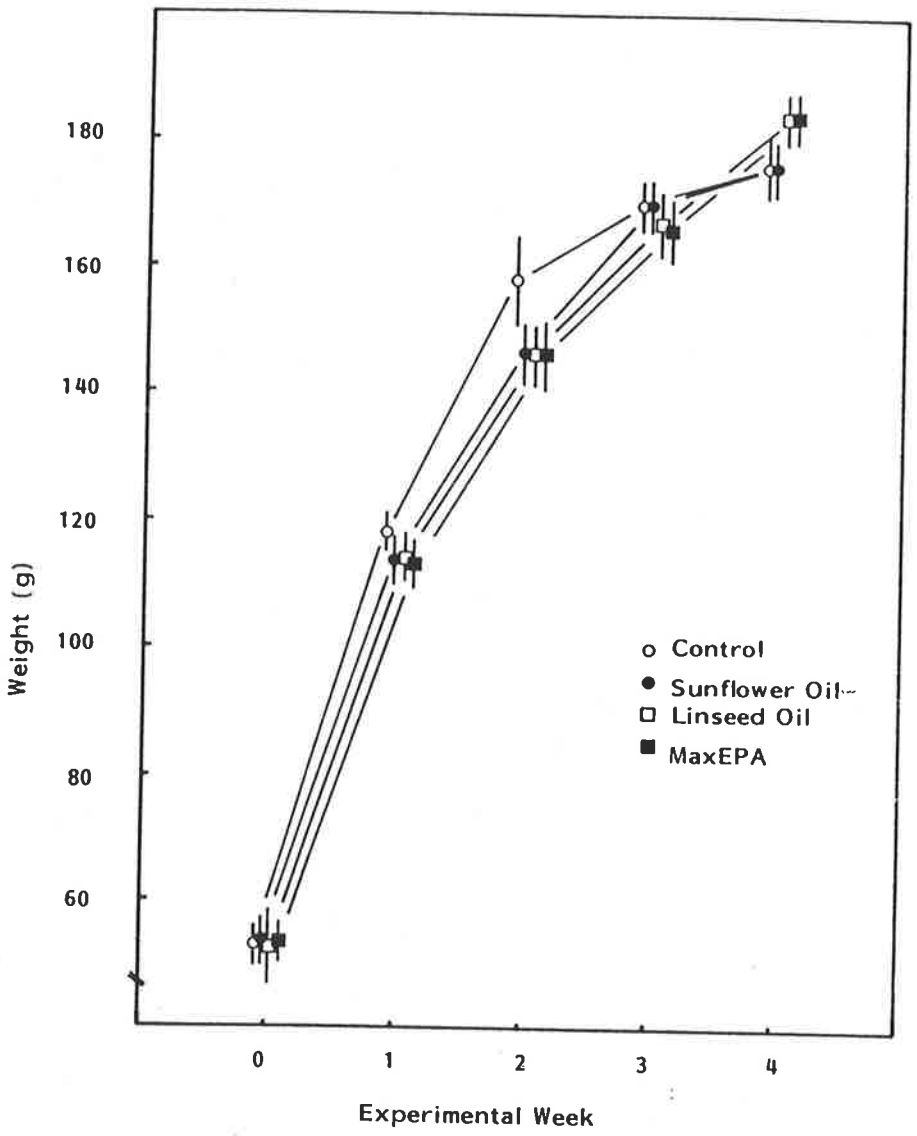


Figure 6.1 Growth patterns of DA rats fed four different diets for four weeks. Group weights were assessed at each time point by one-way analysis of variance and no significant differences were found. Results are expressed as mean ± sem.

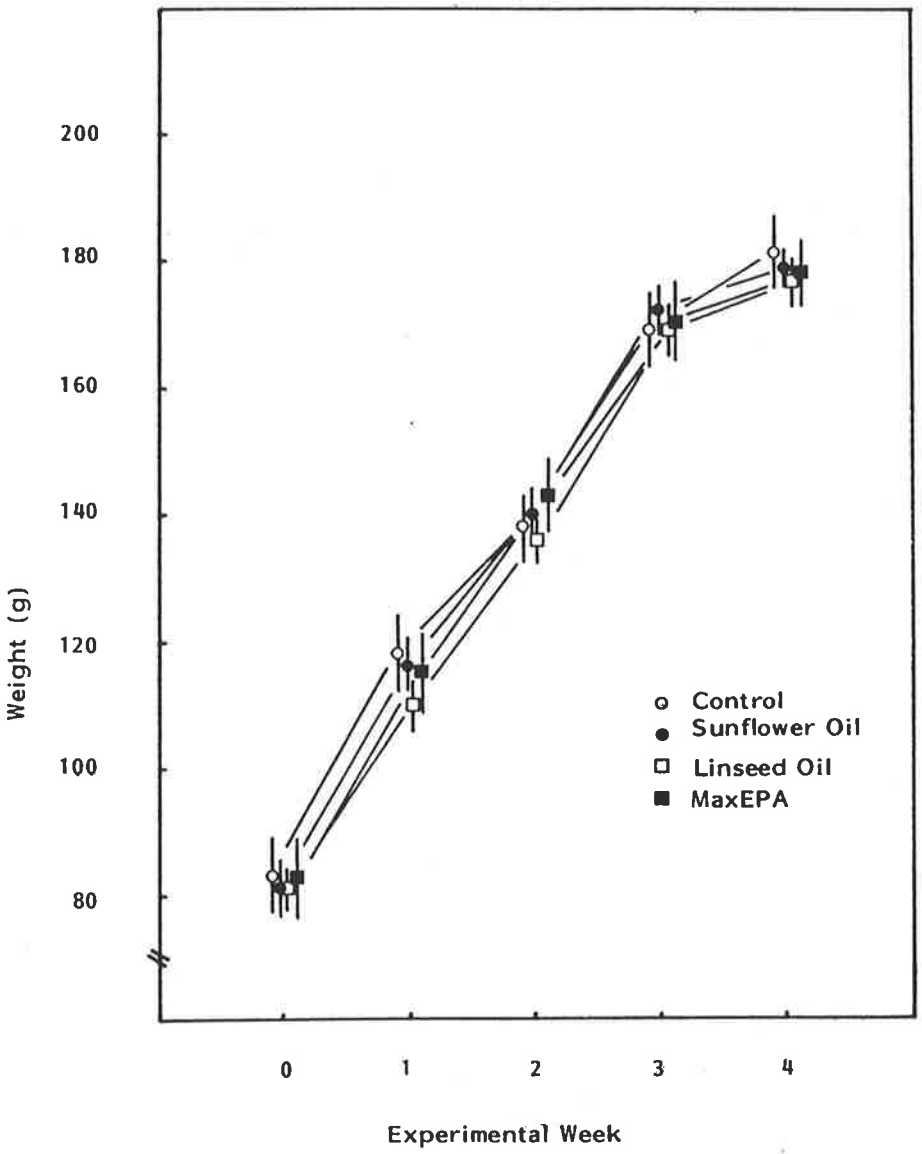


Figure 6.2 Growth patterns of WH rats fed four different diets for four weeks. Group weights were assessed at each time point by one-way analysis of variance and no significant differences were found. Results expressed as mean ± sem.

CHAPTER 7: MODULATION BY PMA OF A23187 OR FMLP AND ARACHIDONIC ACID STIMULATED LEUKOTRIENE PRODUCTION IN NEUTROPHILS.

7.1 INTRODUCTION.

Although there have recently been major breakthroughs in the understanding of the biochemical pathways involved in O_2^- generation by phagocytes (Section 1.3.7), little is understood of the biochemical events leading to leukotriene production in spite of the fact that some agonists stimulate both pathways (for example: A23187 and FMLP).

Synergism of biological response occurs between PMA (a potent stimulator of O_2^- production) and A23187 (a potent stimulator of leukotriene synthesis). For example, pretreatment of phagocytes with sub-stimulatory doses of A23187 enhances the rate of superoxide production induced by PMA, and decreases the lag time associated with stimulation by PMA prior to generation of superoxide (Robinson et al., 1984; French et al., 1987). PMA can also enhance A23187-induced effects. For example, PMA strongly enhances the AA-mobilising effect of A23187 in human platelets (Halenda et al., 1985). PMA enhances the A23187-induced production of LTC_4 by mouse macrophages (Tripp et al., 1985), and enhances the A23187-induced release of AA from rabbit peritoneal neutrophils (Volpi et al., 1985). It is believed that PMA stimulates PKC-dependent regulation of AA release through regulation of lipomodulin activity (Hirata et al., 1984).

The experiments described in this chapter were designed to examine the effect of phorbol myristate acetate on leukotriene production by human neutrophils stimulated by a) A23187 and b) FMLP and AA.

7.2 RESULTS

7.2.1 Effect of PMA on A23187-induced leukotriene production.

These experiments were performed to determine the effect of PMA on A23187-induced leukotriene production. Neutrophils were stimulated for 5 min by A23187 in the range of 0.05 to 10 uM as described previously. No detectable amounts of LTB_4 or 5-HETE were produced by PMA in the range 0-100ng/ml. When PMA (100 ng/ml) was added simultaneously with A23187, production of LTB_4 and 5-HETE was enhanced compared with A23187 alone (Figures 7.1 & 7.2). The largest percentage enhancements were observed with 10 uM A23187 and were 80 ± 15 ($x\pm sd$, n=3) and 50 ± 7 for LTB_4 and 5-HETE respectively. Average increases along the dose response curve to A23187 were 44% and 33 % for LTB_4 and 5-HETE respectively.

Further experiments to refine this data were performed using 100 ng/ml PMA and 0.5 uM A23187. Production of LTB_4, LTB_4 isomers and 5-HETE were significantly increased (Students t-test $p < 0.05$) when PMA was added with A23187 (Table 7.1). Percentage enhancements were 45 ± 14, 23 ± 2.0 and 33 ± 8 ($x\pm sd$, n=4 for LTB_4, LTB_4 isomers and 5-HETE respectively.

The biologically inert phorbol ester 4-αphorbol didecanoate (it does not stimulate protein phosphorylation or superoxide production), did not enhance the A23187-induced production of LTB_4, LTB_4 isomers or 5-HETE, indicating that a likely mechanism of the effect of PMA is via phosphorylation of protein/s.

7.2.2 Time course of the effect of PMA.

To determine whether preincubation with PMA prior to addition of A23187 had any effect on the enhancement by PMA, neutrophils were preincubated with PMA (100 ng/ml) for up to 10 min. The time of preincubation with PMA had no effect on the enhancement of the A23187-induced release of LTB_4 (Figure 7.3).

7.2.3 Dose-dependence of the effect of PMA.

To determine whether the enhancement of A23187-induced production of 5-LO metabolites by PMA was dose-dependent, neutrophils were stimulated by simultaneous addition of 0.5 uM A23187 with increasing doses of PMA (0.001 - 10 ug/ml). The dose of PMA which stimulated maximal enhancement of LTB_4 production was 50 ng/ml (Figure 7.4) and was used in all subsequent experiments.

7.2.4 Effect of exogenous AA on the synergism between PMA and A23187.

There existed two possibilties to explain the enhancing effect of PMA on the A23187-induced production of leukotrienes by neutrophils. PMA could be enhancing the activity of the 5-lipoxygenase enzyme, thus

increasing the amount of LTB_4, LTB_4 isomers and 5-HETE produced, or it could be enhancing release of AA from membrane phospholipid.

To test the hypothesis that PMA was enhancing 5-LO activity, neutrophils were stimulated with A23187 (0.5 uM) in the presence and absence of PMA (50 ng/ml), with or without of exogenous AA (2.5 uM). Addition of exogenous AA effectively prevented the enhancement of A23187-induced LTB_4, LTB_4 isomer and 5-HETE production by PMA (Table 7.2) indicating that PMA was not acting by directly stimulating the 5-lipoxygenase enzyme.

7.2.5 Incorporation of ^{3}H-AA into and release from neutrophil membrane phospholipid.

To test the hypothesis that PMA was increasing the release of AA from the membrane, it was first necessary to determine that ^{3}H-AA was incorporated into membrane phospholipids, and to set up an assay to measure ^{3}H-AA release. Lipid extracts from neutrophils which had been preincubated with ^{3}H-AA at 37^{o}C for up to 1 hr were separated into major phospholipid classes by TLC (Section 2.2.6). The time course of incorporation of ^{3}H-AA into the major phospholipid classes is shown in Figure 7.5. An incubation time of 1 hr. was chosen for subsequent experiments because at this time the majority of the ^{3}H-AA had been incorporated into both PC and PI (Figure 7.5).

After preincubation for 1 hr with ^{3}H-AA, neutrophils were stimulated by A23187 (0.5 uM) alone or PMA (50 ng/ml) alone for 5 min. and their lipids were then extracted and concentrated prior to HPLC

and elution fraction collection. The HPLC assay for AA was identical to Assay System 1 (Section 2.2.7) except that solvent conditions were 95/5/0.08 (methanol/water/acetic acid). Radiochromatographs show that the amount of ^{3}H-AA released by PMA was equivalent to that released by the unstimulated sample, indicating that PMA alone does not stimulate release of endogenous AA (Figures 7.6 & 7.7). In contrast, A23187 caused release and metabolism of ^{3}H-AA (Figure 7.8). In subsequent experiments, neutrophils were preincubated with NDGA (10 uM) for 15 min prior to stimulation. This prevented metabolism of released ^{3}H-AA by 5-lipoxygenase (Figure 7.9). Thus it was unnecessary to separate the samples by HPLC.

PMA had no effect on the release of ^{3}H-AA from neutrophil phospholipids into the supernatant whereas A23187 stimulated release of 570 $\pm$ 65 cpm of ^{3}H-AA in 5 min (Table 7.3). When PMA was added with A23187, the amount of ^{3}H-AA released was increased by more than 2-fold (1530 $\pm$ 286 cpm). This implies that PMA enhances the A23187-induced release of AA from membrane phospholipid.

7.2.6 Effect of PMA on the release of leukotrienes from human neutrophils stimulated by FMLP and AA.

To determine whether PMA enhances leukotriene generation by FMLP and AA, neutrophils were collected and separated as described previously. All incubations were carried out in a modified Dulbecco°s phosphate buffered saline (138 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 7.5 mM glucose). Cells stimulated for 5 min with 1 uM FMLP and 5 uM AA produced 3.5$\pm$0.3, 5.4$\pm$0.7 and

52.9±5.9 ng/10^6 cell of LTB_4, LTB_4 isomers and 5-HETE respectively (Table 7.4). Simultaneous addition of 50 ng/ml PMA increased production of LTB_4 by 77±14% (x±sd, n=4), and reduced production of the LTB_4 isomers by 54±9% (both significant at $p < 0.05$). 5-HETE production was unaffected (94±1% of control).

7.2.7 Phosphorylation by PMA of neutrophil proteins.

To determine which proteins are phosphorylated upon stimulation of neutrophils with PMA, neutrophils were preincubated with ^{32}P (Section 2.3.8), after which the cells were stimulated by PMA. The proteins were then separated by PAGE and the gel was exposed to x-ray film for two days (Section 2.2.9). The major protein bands phosphorylated are 38-40 kD (Figure 7.10) and 68-70 kD (Figure 7.11).

7.3 DISCUSSION.

The data presented in this study, demonstrating that PMA enhances A23187-induced AA metabolism by increasing the A23187-induced release of AA from membrane phospholipids, are consistent with previous reports using platelets (Halenda et al., 1985), or rabbit neutrophils (Volpi et al., 1985). However, this study showed that PMA enhanced AA release and metabolism to a smaller extent than previously demonstrated (2-fold compared with 4 to 5-fold). It is possible that this reflects differences in endogenous lipomodulin levels in different cell types.

Upon stimulation with PMA, neutrophils phosphorylated a protein with an Mr of approximately 37-40 kDa (Figure 7.10). Lipomodulin is a 40 kDa in rat neutrophils (Wallner et al., 1986), 37 kDa in rat peritoneal exudates (Pepinski et al., 1986) or 47 kDa in platelets (Touqui et al., 1986), although Crouch and Lapetina, (1986), have recently questioned that lipomodulin is a 47 kDa protein in platelets. Lipomodulin is a 40 kDa protein in rabbit neutrophils (Hirata, 1981) and murine thymocytes (Hirata et al., 1984).

The data presented here support the hypothesis that the mechanism of synergism between PMA and A23187 in human neutrophils occurs through phosphorylation of lipomodulin by PKC. Other reports, using platelets (Halenda et al., 1985), macrophages (Tripp et al., 1985) and rabbit peritoneal neutrophils (Volpi et al., 1985) also provide indirect evidence. The only direct evidence of this effect of PKC comes from studies on murine thymocytes (Hirata et al., 1984). In this study, immunoprecipitable lipomodulin was inactivated upon phosphorylation of tyrosine residues by a tyrosine kinase, the activity of which was stimulated by PKC and inhibited by PKA (cyclic AMP-dependent protein kinase A). Similar studies in human neutrophils have not been reported (Hirata et al., 1984).

The control by PKC of the activity of lipomodulin has important implications. Purified lipomodulin inhibits growth of cultured U937 cells, implying that lipomodulin inhibits a step in mitogenesis by inhibiting the action of phospholipases (Hirata et al., 1984). Further evidence supports this conclusion. A fully active PLA_2 (after lipomodulin has been inactivated) could lead to accumulation of

lysophosphatidylcholine (lysoPC). This product enhances Na^+ and glucose uptake (Rozengurt et al., 1981) and influences the Na^+/K^+-ATPase. LysoPC could also serve as an acceptor of PUFAs such as AA or oleic acid the rate of which is controlled by Acyl-CoA synthetase. The action of this enzyme is regulated by lysoPC (Szamel and Resch, 1981). All of these events occur following stimulation of T lymphocytes with mitogens.

It is thought that the biochemical chain of events leading to activation of PKC is initiated by a receptor-ligand interaction which, by activating PLC, stimulates PI turnover and liberates diacyl glycerol (DAG) (Hurst, 1987). Phorbol esters mimic the effect of DAG which is an endogenous activator of PKC (Nishizuka, 1984). Neither DAG nor PMA alone can stimulate the release or metabolism of AA, and although they can cause platelets to aggregate, they do not stimulate a detectable rise in cytosolic Ca^{++} (White et al., 1974). Furthermore, the enhancing effect of PMA on the A23187-induced leukotriene release occurs independently of PI turnover, indicating that the effect is mediated via PLA_2, a Ca^{++}-dependent enzyme (Halenda et al., 1985). It is possible therefore that the amount of DAG normally produced by receptor-linked stimulation of PI turnover, amplifies the effectiveness of Ca^{++} as an activator of PLA_2-mediated AA release and metabolism to leukotrienes.

The effect of PMA on the FMLP and AA-induced leukotriene synthesis had two differences to that of PMA on the synthesis induced by A23187. Firstly, A23187-stimulated generation of all products (LTB_4, LTB_4 isomers and 5-HETE) was increased by PMA, and secondly,

the effect was abolished in the presence of exogenous AA (see Table 7.1). Both points imply that PMA exerts an effect on release of AA from the membrane. In contrast, when PMA is added with FMLP and AA, while LTB_4 production increased, production of 5-HETE was unaffected and production of the LTB_4 isomers was reduced. This points to a more selective effect than simply an increased release of AA. The observed enhancement of LTB_4 production, with a concommitant decrease in production of LTB_4 isomers is consistent with a stimulatory effect on LTA hydrolase. It is therefore possible that PKC phosphorylates LTA_4 hydrolase, thereby increasing its activity.

LTA hydrolase is a cytosolic protein which has a molecular weight of 68-70 kDa (Radmark et al., 1984). Upon stimulation with PMA, human neutrophils which had been prelabelled with ^{32}P phosphorylated a protein in the region of approximately 68kDa (see Figure 7.11). Although this evidence is indirect, further evidence on the identity of this protein band is provided by phosphorylation studies on monocytes and platelets. Human monocytes possess LTA hydrolase and when stimulated by PMA, phosphorylate a 68 kDa protein (Figure 7.12). In contrast, platelets do not possess LTA hydrolase and do not phosphorylate a 68 kDa protein when stimulated by PMA (Figure 7.13).

Little is understood regarding the control of leukotriene synthesis in phagocytes (Section 1.3.7). It is known that both PLA_2 and 5-LO are Ca^{++}-dependent enzymes, however, the factors controlling the other enzymes in the 5-LO pathway are unknown. Experiments performed in chapter 4 have demonstrated that considerable variation in production of the various AA-metabolites occurs. For example, on

some occasions, the amount of LTB_4 produced is greater than that of the LTB_4 isomers or vice versa. Control of the amount of LTB_4 produced is of major physiological importance as this leukotriene is believed to possess the greatest pro-inflammatory effect. A stimulatory influence of PKC on this enzyme may be of major importance in the control of LTB_4 production.

Table 7.1 The effect of PMA on the A23187-induced generation of LTB_4, LTB_4 isomers and 5-HETE by human neutrophils. Results are expressed as mean ± sd. * significantly different from control at $p < 0.05$, students t-test.

	Amount generated ($ng/10^6$ neutrophils)		
Condition	LTB_4	LTB_4 isomers	5-HETE
A23187	4.9±0.4	3.1±0.2	49.9±3.9
A23187 + PMA	7.1±0.7*	3.8±0.005*	66.2±4.1*

Table 7.2 The effect of exogenous AA on the enhancement of A23187-induced LTB_4, LTB_4 isomer and 5-HETE production by PMA. Results are expressed as mean ± sd. * significantly different from control at $p < 0.05$, students t-test.

	Amount generated (ng/10^6 neutrophils)		
Conditions	LTB_4	LTB_4 isomers	5-HETE
A23187	15.6±0.8	12.7±1.8	64.5±11.3
A23187 + PMA	24.7±2.4*	17.6±1.5*	107.1±11.3*
A23187 + AA	23.1±1.0	28.6±2.2	208.3±27.1
A23187 + AA + PMA	23.7±1.5	26.4±0.2	227.7±22.4

Table 7.3 The effect of PMA on the A23187-induced release of ^{3}H-AA from human neutrophils. * ^{3}H-AA release determined as (cpm from stimulated samples - cpm from control samples). All samples were stimulated for 5 min. Results expressed as mean ± sem of three experiments).

Condition	^{3}H-AA release*
Control (methanol, DMSO)	0
PMA (100 ng/ml)	0
A23187 (0.5 uM)	570± 65
PMA + A23187	1530± 286

Table 7.4 The effect of PMA on the FMLP and AA-induced generation of LTB_4, LTB_4 isomers and 5-HETE by human neutrophils. Results are expressed as mean ± sd. * significantly different from control at $p < 0.05$, students t-test.

	Amount generated (ng/10^6 neutrophils)		
Condition	LTB_4	LTB_4 isomers	5-HETE
FMLP + AA	3.5±0.3	5.4±0.7	52.9±5.0
FMLP + AA + PMA	6.2±0.5*	2.9±0.5*	49.5±0.4

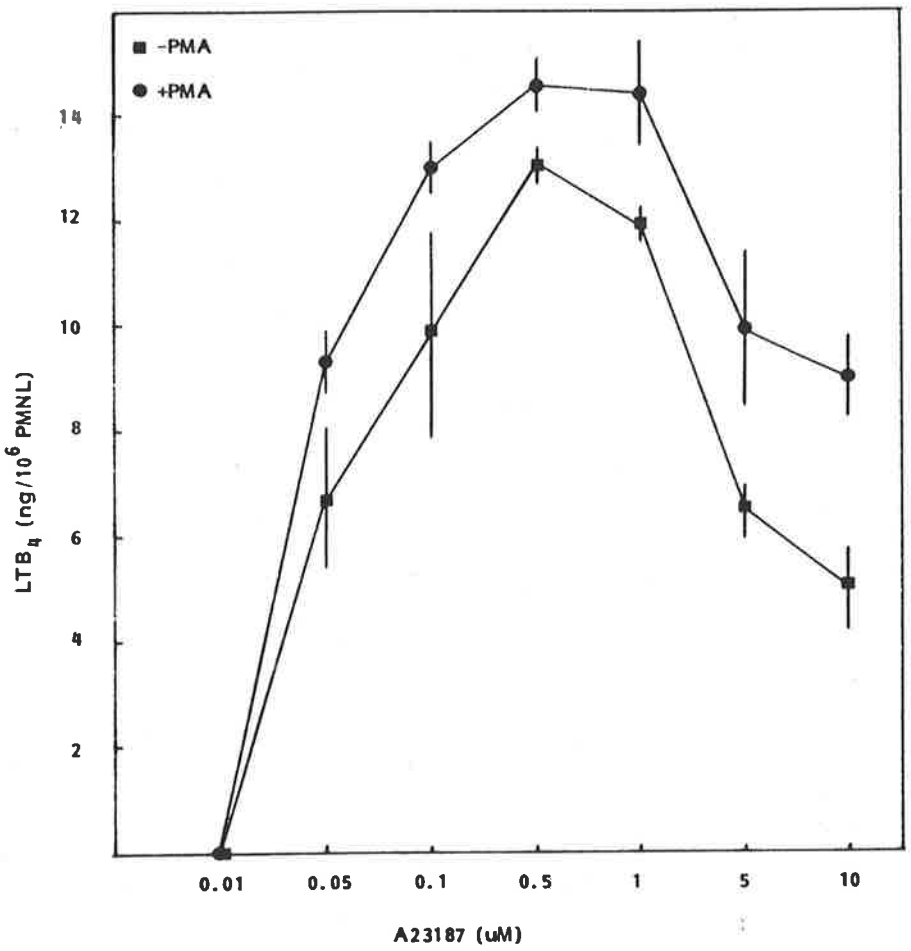


Figure 7.1 Effect of PMA on the A23187-induced synthesis of LTB_4 by neutrophils. Cells were stimulated for 5 min with increasing doses of A23187 either alone or with 100 ng/ml PMA.

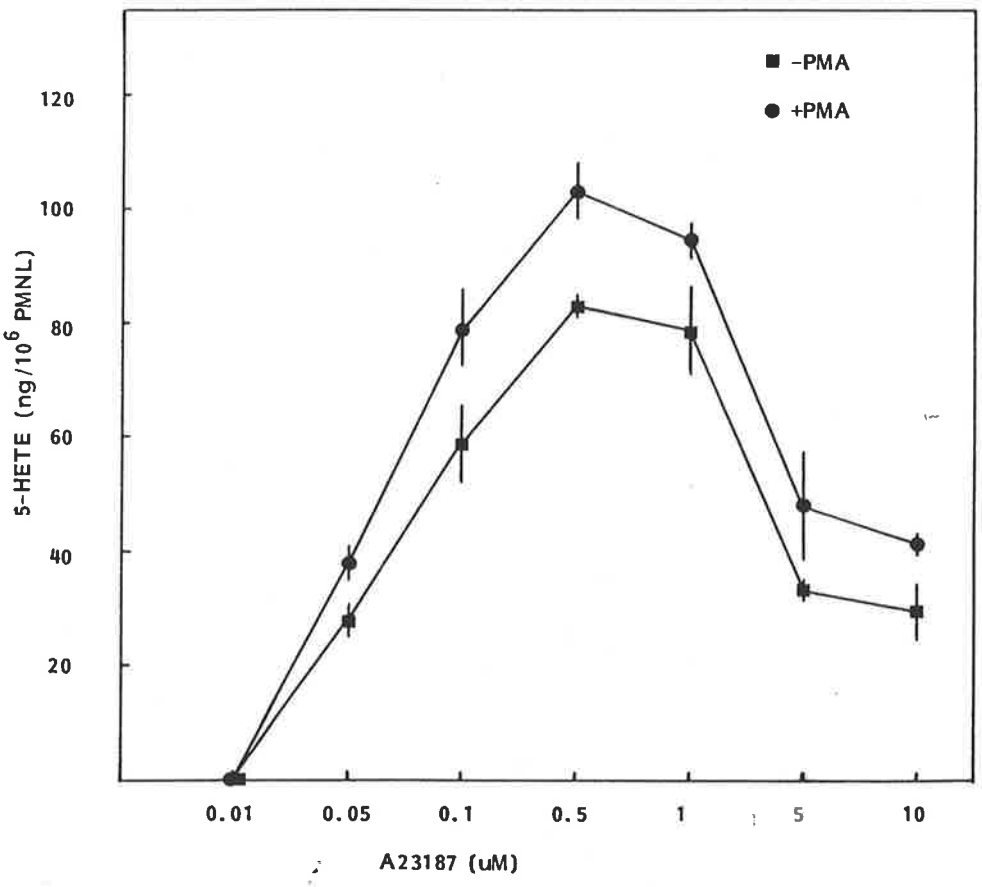


Figure 7.2 Effect of PMA on the A23187-induced synthesis of 5-HETE by neutrophils. Cells were stimulated for 5 min with increasing doses of A23187 either alone or with 100 ng/ml PMA.

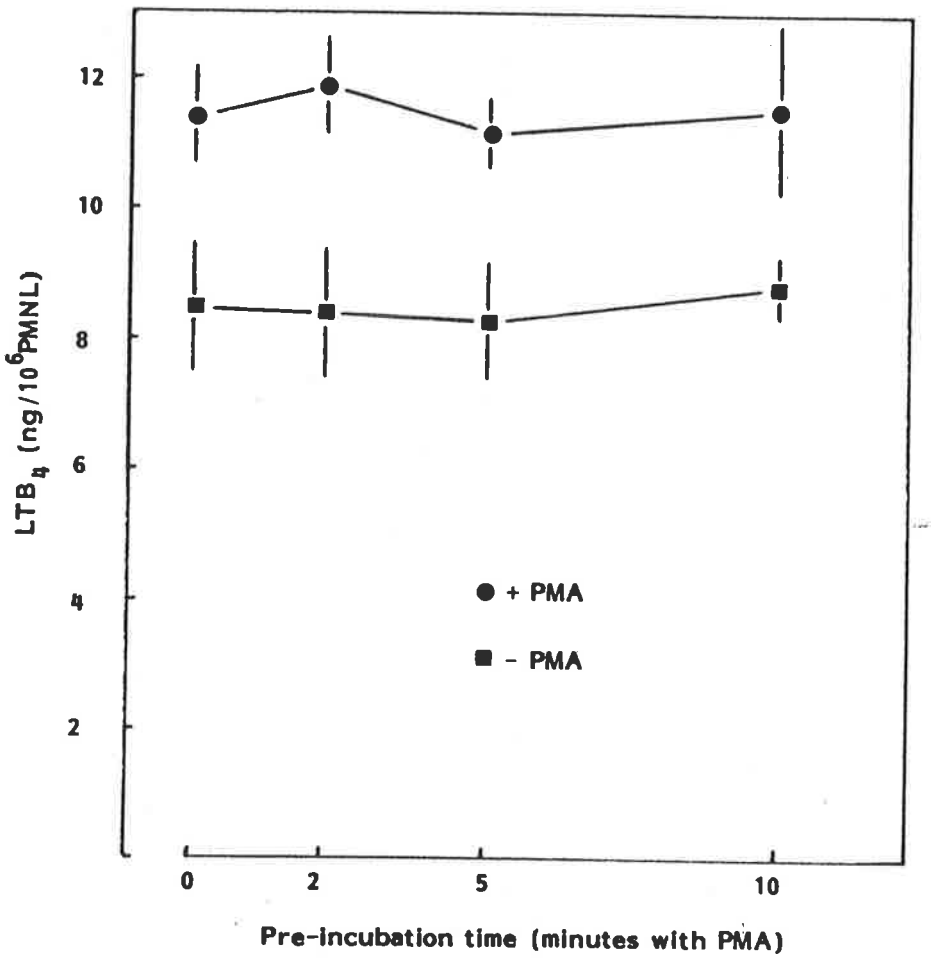


Figure 7.3 Time-dependence of the enhancing effect of PMA on LTB_4 synthesis by neutrophils. Cells were preincubated with 100 ng/ml PMA for up to 10 min prior to addition of A23187 for 5 min.

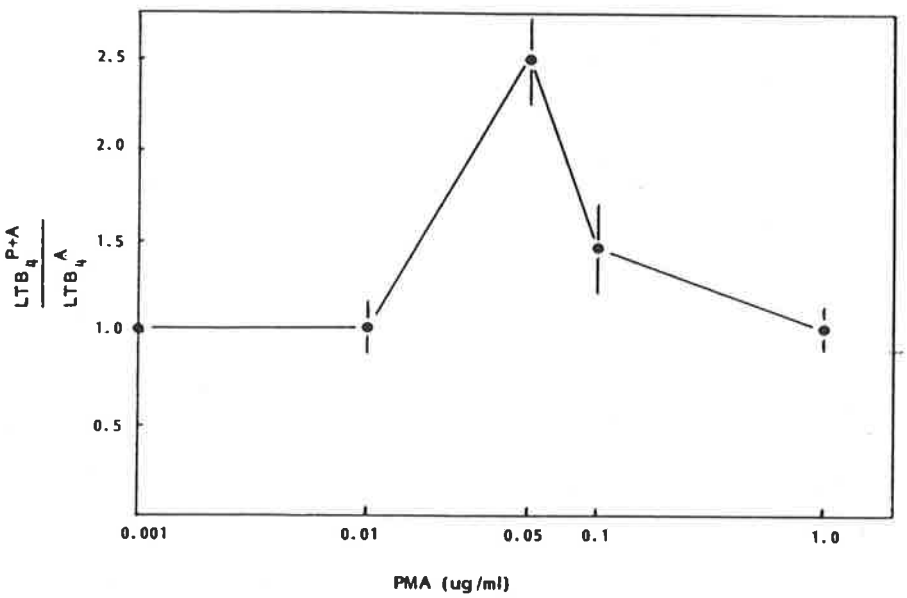


Figure 7.4 Dose-dependence of the enhancing effect of PMA on A23187-induced LTB_4 synthesis by neutrophils. Cells were stimulated for 5 min by 0.5 uM A23187 with increasing concentrations of PMA.

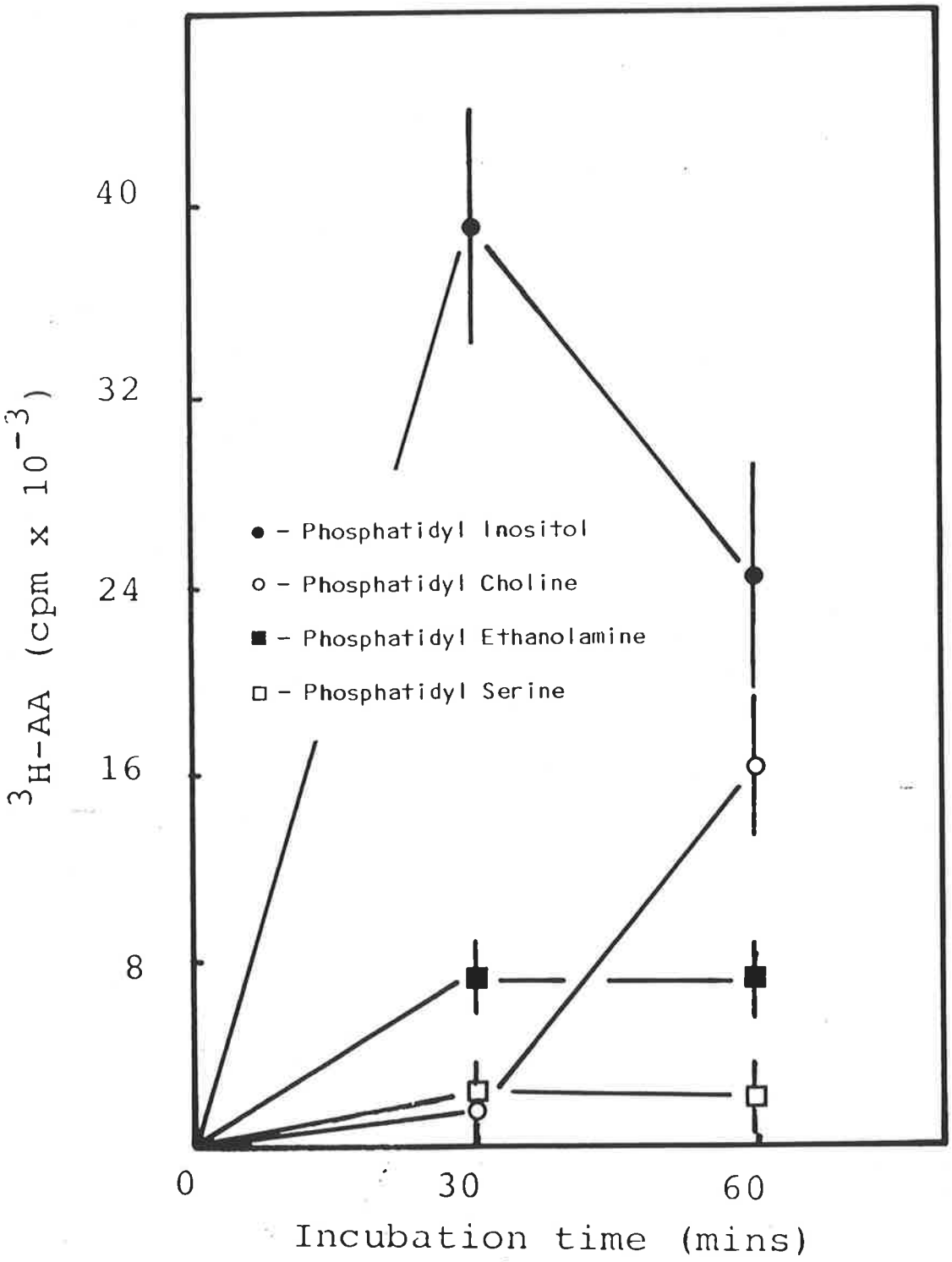


Figure 7.5 The effect of time on the incorporation of ^{3}H-AA into neutrophil membrane phospholipids. Cells were preincubated with 0.1 uCi of ^{3}H-AA for up to 60 min. After the desired preincubation time, the samples were treated with citric acid, the lipids were extracted, separated by TLC and the amount of ^{3}H-AA incorporated was determined by liquid scintillation counting as described in Chapter 2.

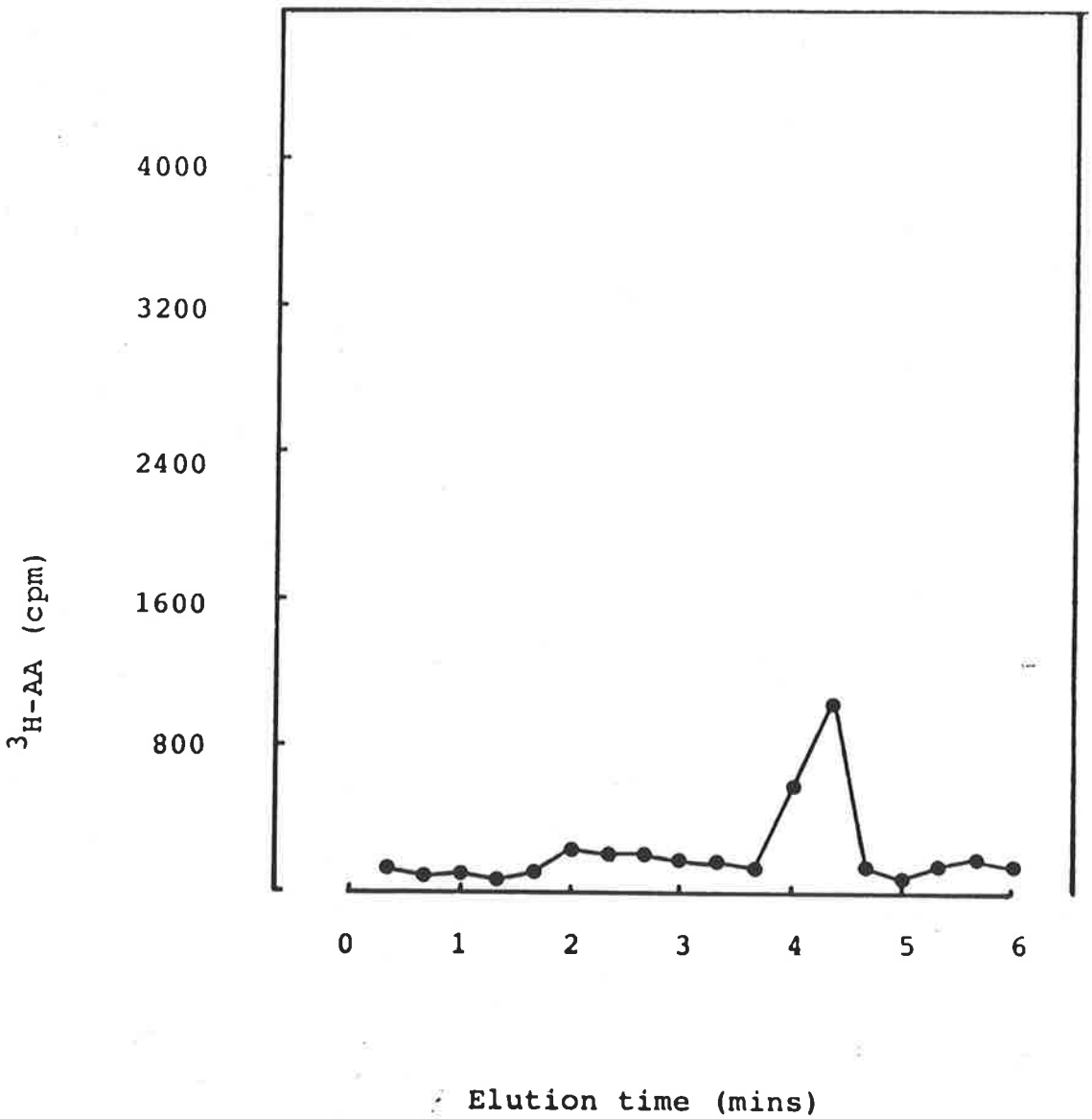


Figure 7.6 Radiochromatogram showing the amount of ^{3}H-AA released from neutrophil phospholipids following incubation for 5 min with HBSS. Neutrophils were preincubated with 0.1 uCi of ^{3}H-AA for 60 min, washed twice and resuspended in HBSS, and incubated with 10 ul of HBSS for 5 min.

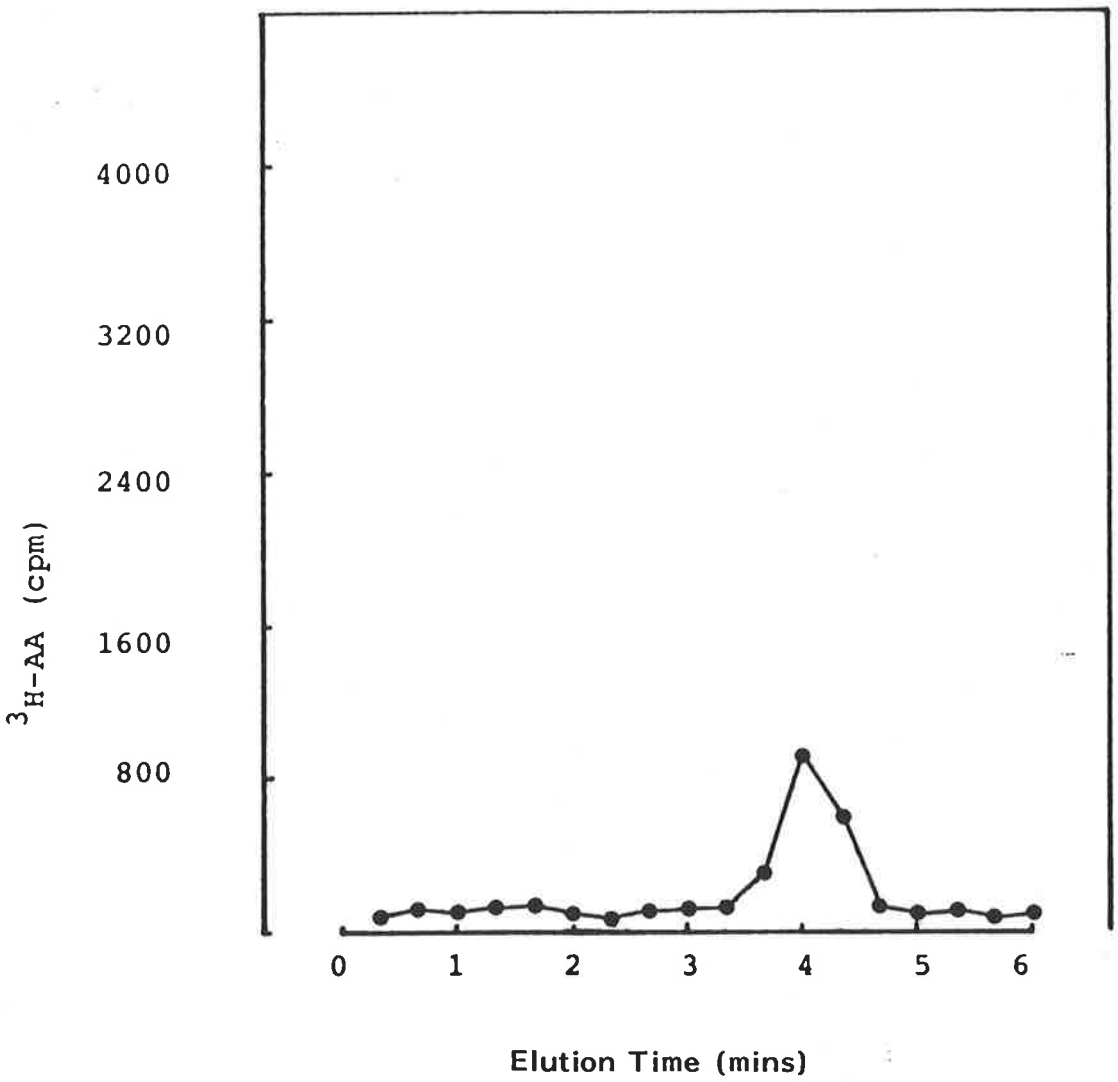


Figure 7.7 Radiochromatogram showing the amount of ^{3}H-AA released from neutrophil phospholipids following incubation for 5 min with 50 ng/ml PMA. Neutrophils were preincubated with 0.1 uCi of ^{3}H-AA for 60 min, washed twice and resuspended in HBSS, and incubated with PMA for 5 min.

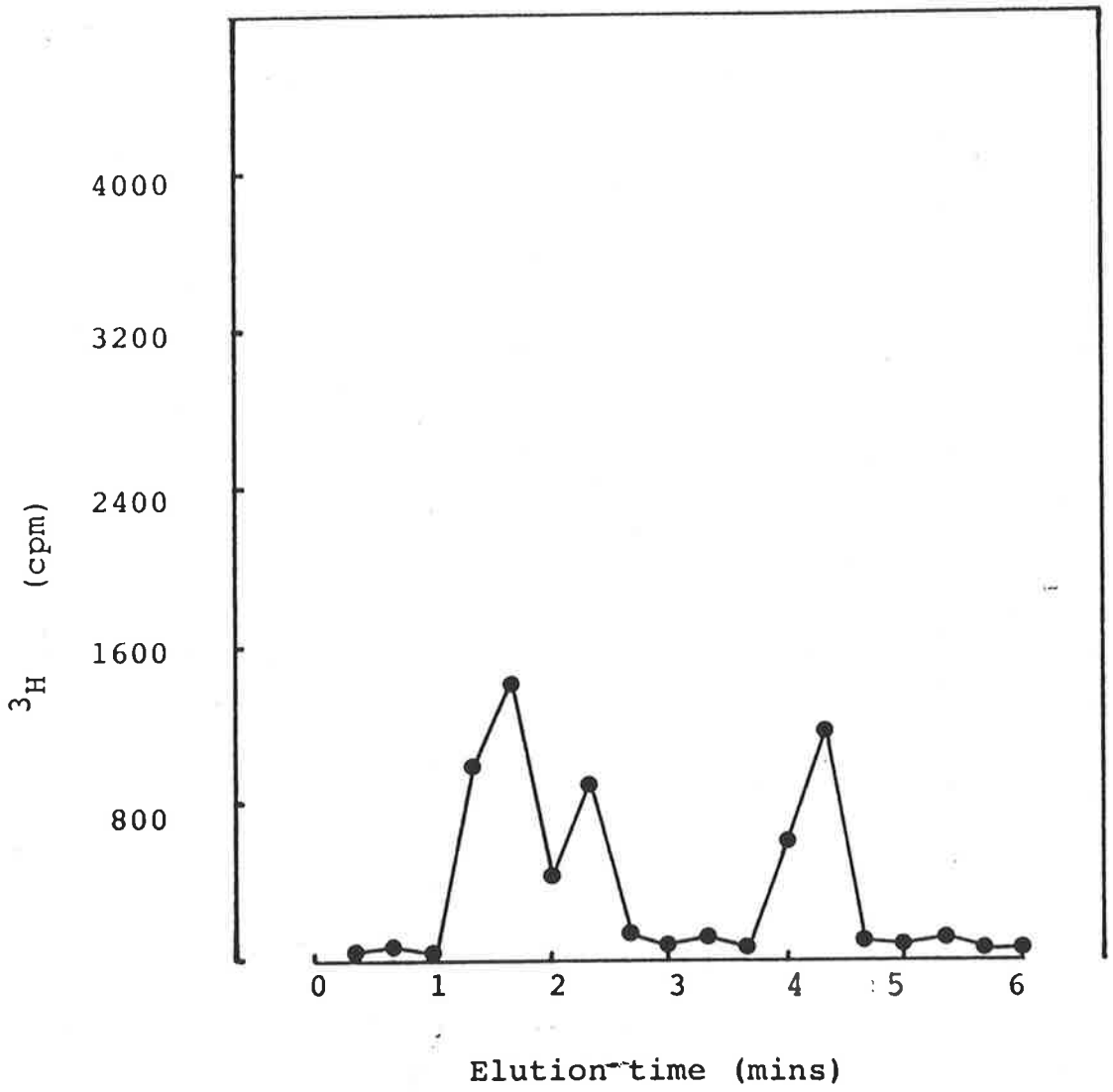


Figure 7.8 Radiochromatogram showing the amount of ^{3}H-AA released from neutrophil phospholipids following incubation for 5 min with 0.5 uM A23187. Neutrophils were preincubated with 0.1 uCi of ^{3}H-AA for 60 min, washed twice and resuspended in HBSS, and incubated with A23187 for 5 min.

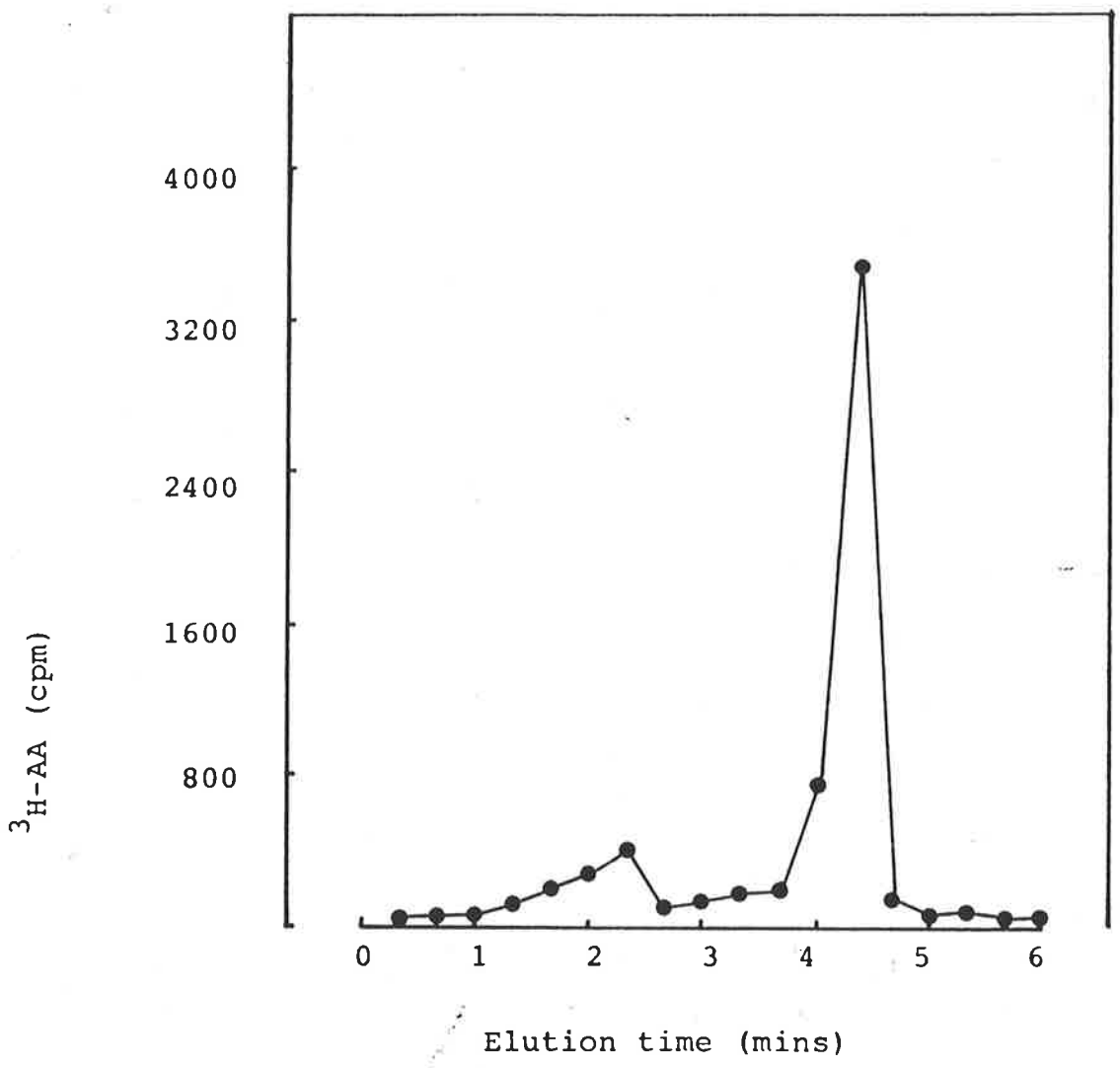


Figure 7.9 Radiochromatogram showing the amount of ^{3}H-AA released from neutrophil phospholipids following incubation for 5 min with 0.5 uM A23187. Neutrophils were preincubated with 0.1 uCi of ^{3}H-AA for 60 min. 10 uM NDGA was added after preincubation of 45 min. After 60 min, the cells were washed twice and resuspended in HBSS, and incubated with A23187 for 5 min.

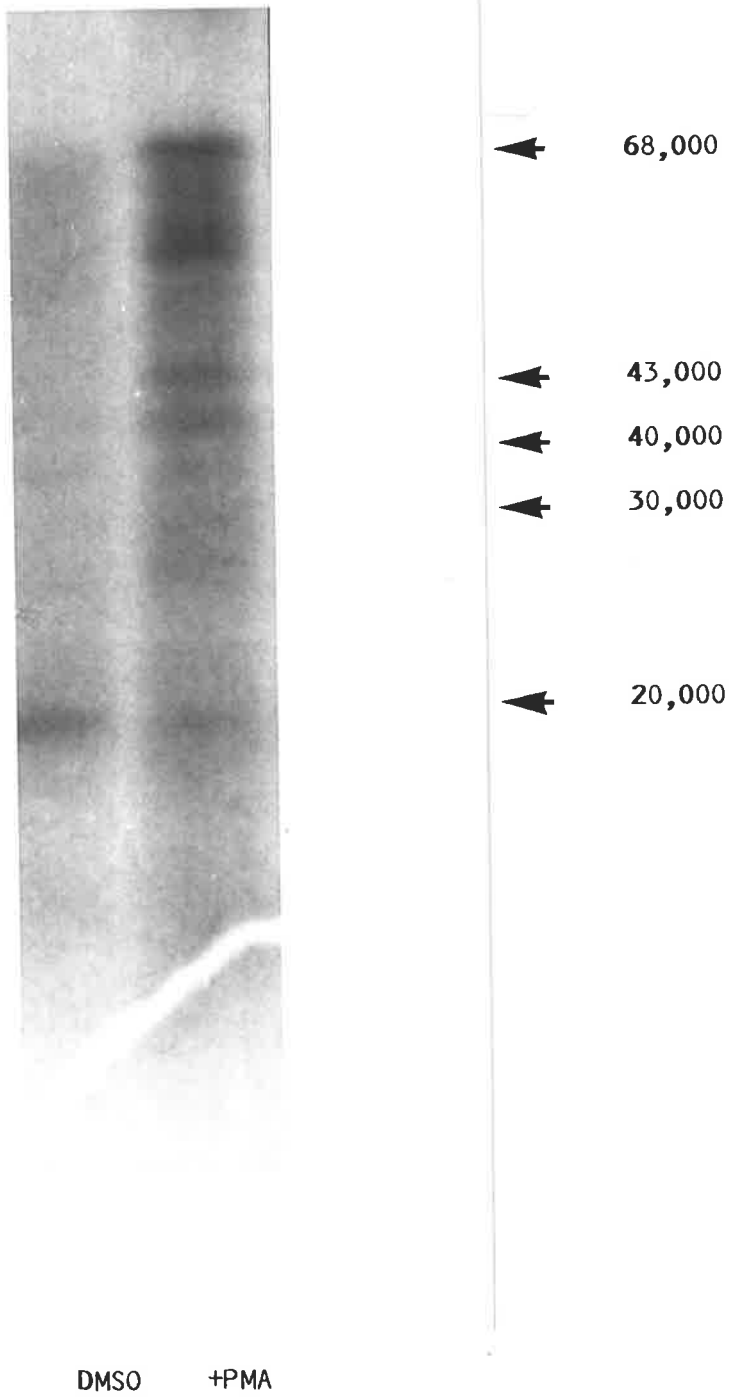


Figure 7.10 Autoradiograph of a polyacrylamide gel of neutrophil proteins showing phosphorylation of a protein band of approximately 38-40 kD. Neutrophils were preincubated with ^{32}P for 1 hr and stimulated with PMA for 5 min.

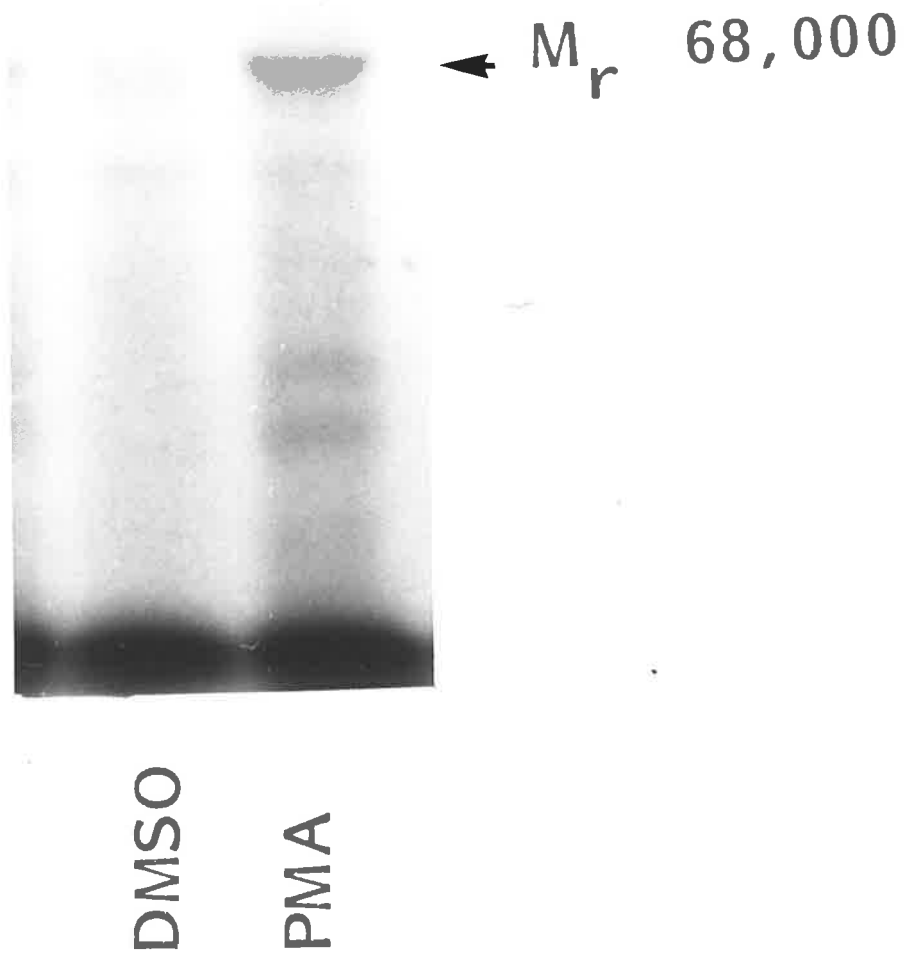


Figure 7.11 Autoradiograph of a polyacrylamide gel of neutrophil proteins showing phosphorylation of a protein band of approximately 68-70 kD. Neutrophils were preincubated with ^{32}P for 1 hr and stimulated with PMA for 5 min.

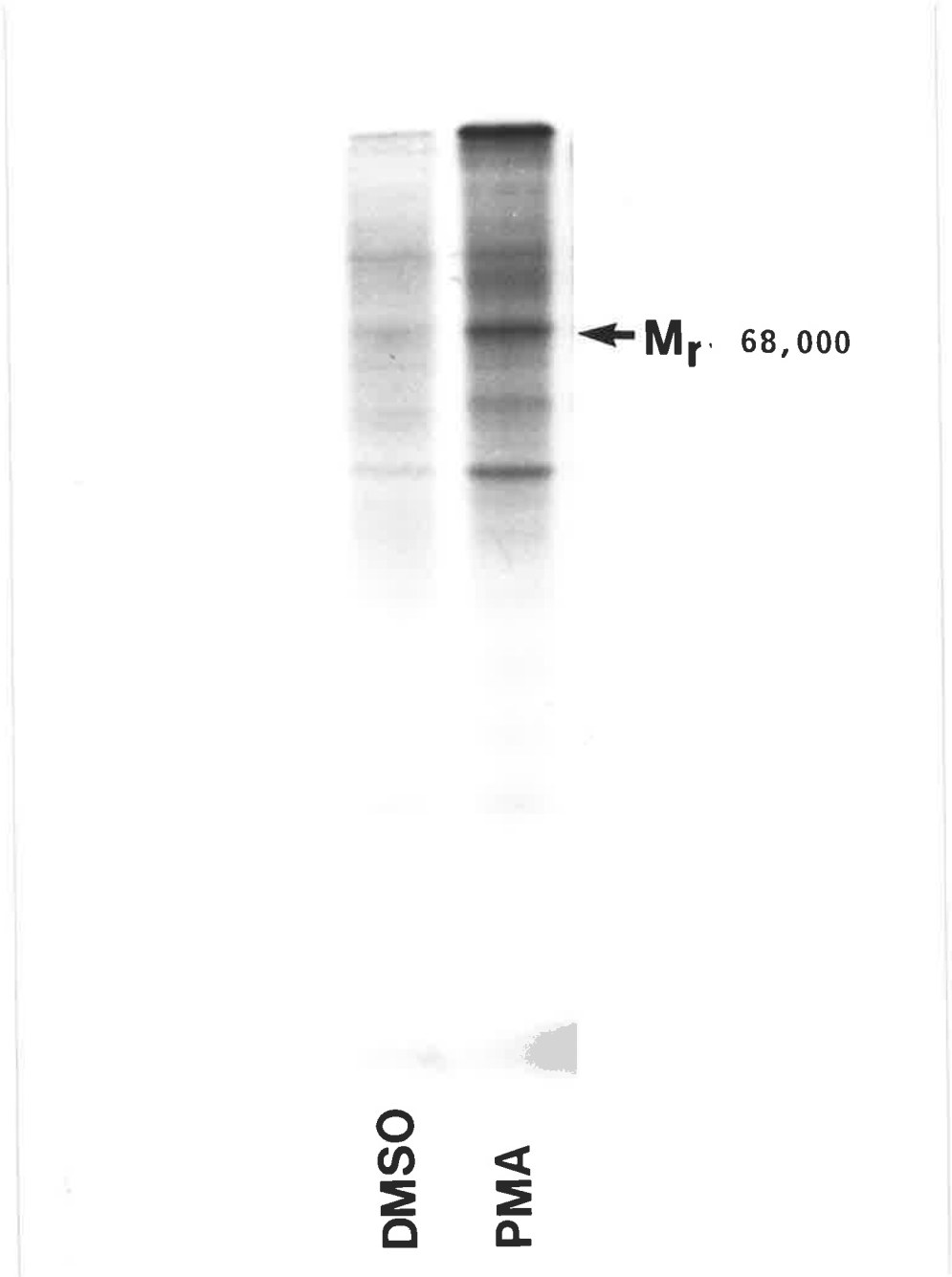


Figure 7.12 Autoradiograph of a polyacrylamide gel of human monocyte proteins. Monocytes were preincubated with ^{32}P for 1 hr prior to stimulation with PMA for 5 min.

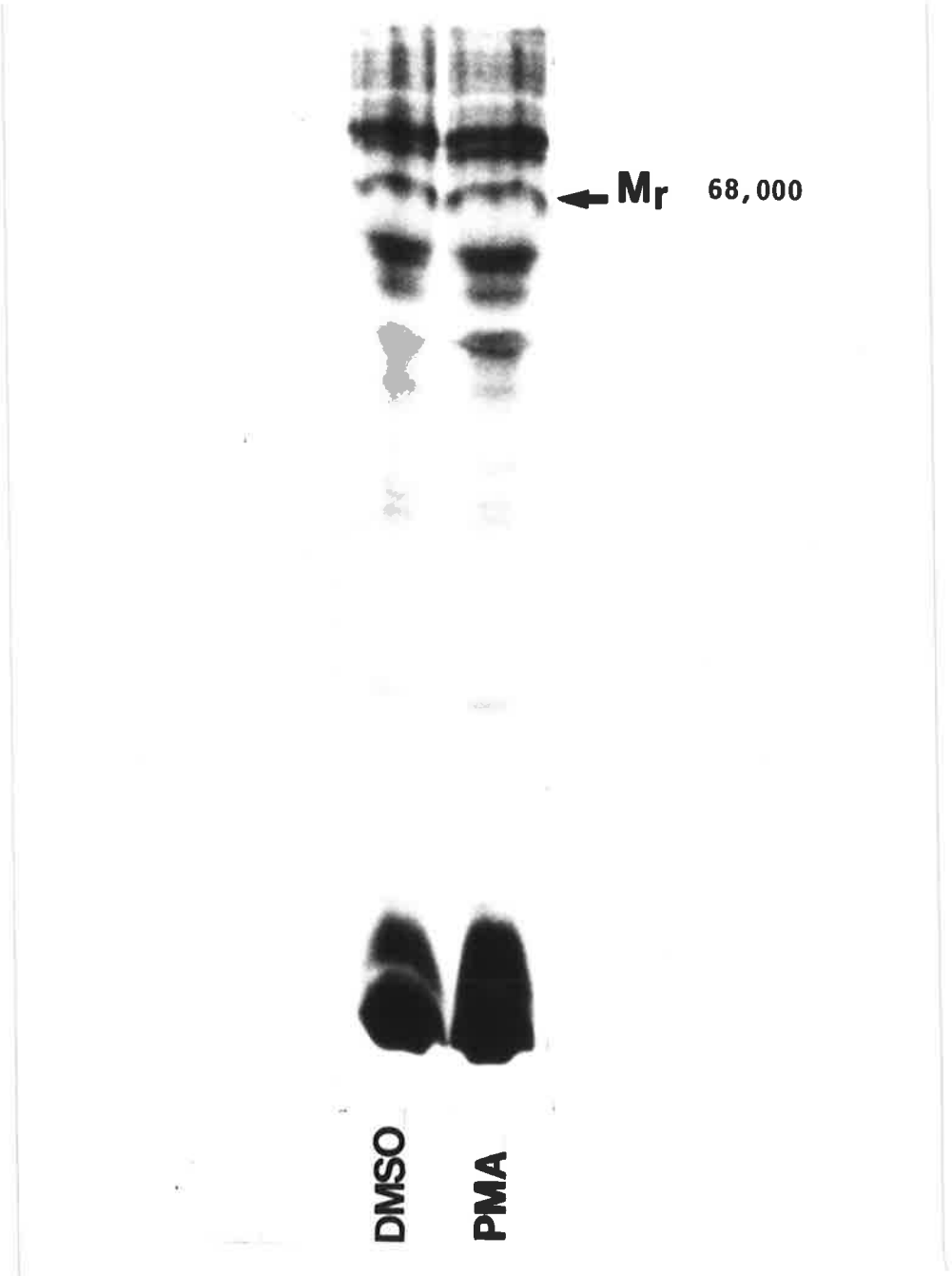


Figure 7.13 Autoradiograph of a polyacrylamide gel of human platelet proteins. Platelets were preincubated with ^{32}P for 1 hr prior to stimulation with PMA for 5 min.

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSION.

8.1 INTRODUCTION.

To quote the Nobel laureate Bengt Samuelsson;

"That the synthesis of leukotrienes by leucocytes should be tightly controlled is consistent with the proposed role of this compounds in inflammation and hypersensitivity. Clearly the delineation of this regulatory mechanism is the key to our future understanding of the capacity of the leucocyte to respond to inflammatory stimuli with leukotriene production. It is conceivable that such knowledge will provide valuable information concerning the more general aspects of stimulus response coupling in the leucocyte." (Rouzer et al., 1986b)

In this quotation Samuelsson highlights the problems facing research into leukotriene biosynthesis. Leukotrienes activate phagocytes in vitro (Samuelsson, 1983), and have been detected in inflammatory loci in conditions such as rheumatoid arthritis, gout, psoriasis, asthma and urticaria (Bray, 1986). A major factor contributing to the lack of understanding of leukotriene metabolism is the inability of pathophysiological substances such as chemotactic peptides, complement components, immunoglobulins and cytokines to stimulate phagocyte leukotriene synthesis. However, inflammatory cells such as neutrophils, eosinophils, macrophages and mast cells

produce leukotrienes upon stimulation by the calcium ionophore A23187 (Bray, 1986). Another problem has been the lack of specific inhibitors of leukotriene metabolism. For example mepacrine, is a PLA_2 inhibitor but it also inhibits other biochemical pathways including calmodulin- and PKC mediated events. This lack of specificity of metabolic inhibitors has also hindered elucidation of the pathways of activation of other neutrophil functions such as superoxide release.

8.2 ROLE OF EXOGENOUS ARACHIDONIC ACID IN LEUKOTRIENE SYNTHESIS.

The requirements for leukotriene production both in vitro and in vivo are sufficient AA and an active 5-LO system. Within cells, AA is usually esterified in the sn-2 position of membrane phospholipids and its release from phospholipids is controlled by PLA_2 (Irvine, 1982). Alternatively, neutrophils can metabolize AA from other sources. For example, Marcus et al., (1982; 1984) have demonstrated that neutrophils can synthesise LTB_4 using AA from platelets. Other possible sources of exogenous AA include macrophages, (Humes et al., 1982) and damaged tissue (Hammarstrom et al., 1875). In fact, non-esterified AA has been measured at concentrations as high as 100 uM in inflamed tissue (Hammarstrom et al., 1975).

There are two possible ways in which neutrophils can use exogenous AA for leukotriene synthesis. These are either as a stimulus or as a substrate.

8.2.1 Exogenous AA as a stimulus of leukotriene synthesis.

AA has been shown to stimulate both prostaglandin and leukotriene synthesis in platelets (Lagarde et al., 1984). High concentrations of AA also stimulate superoxide production in neutrophils either by directly activating PKC (Murakami and Routtenberg, 1985) or by acting as a Ca^{++} ionophore (Wolf et al., 1986). Either of these two mechanisms is a possible basis for a stimulatory effect of AA on leukotriene synthesis by neutrophils. However, the actual evidence that exogenous AA acts as a stimulus of leukotriene synthesis in neutrophils is controversial.

In the studies described in this thesis I demonstrated that AA (up to 20 uM) did not stimulate synthesis of LTB_4, its isomers or 5-HETE by human neutrophils. These data are in agreement with several other publications (Sun & McGuire, 1984; Hendriks et al., 1986). However, using a more sensitive assay (RIA), Palmer and Salmon, (1983) were able to detect 130 pg $LTB_4/10^6$ neutrophils stimulated with approximately 33 uM AA. Also, using larger concentrations of exogenous AA (250 uM), Clancy et al., (1983) demonstrated synthesis of 1 ng $LTB_4/10^6$ neutrophils. These levels are small and must be considered to be on the limits of sensitivity of each of the two assays. Furthermore, the RIA for LTB_4 is subject to cross reactivity with the platelet-derived 12-HETE and 5s,12s diHETE (Haines et al., 1987). Therefore the presence of small numbers of platelets could possibly account for the level of LTB_4 detected in the study by Palmer and Salmon.

Several other reports have demonstrated formation of large quantities of mono-HETEs (5-,12- or 15-HETE), but no LTB_4, LTB_4 isomers or metabolites upon incubation of neutrophils with exogenous AA (Haines et al, 1986; Borgeat and Samuelsson, 1979; Serhan et al., 1984). Although I occasionally detected small levels of monoHETEs on my HPLC traces, I was able to demonstrate that they were present in the exogenous AA since addition of AA to "dead" cells or to buffer alone, or direct injection of AA onto the HPLC column, still led to their detection. This finding is in agreement with a report stating that AA can spontaneously oxidise to several monoHETEs even if it is stored at -20^{o}C (Johnson et al, 1983). It is therefore possible that the monoHETEs measured in the reports stated above were due to addition of oxidised AA to the cell suspensions.

Alternatively, it is possible that the doses of AA used in these experiments (usually greater than 100 uM) were toxic to the cells. It has been shown that cell damage unmasks 15-lipoxygenase activity in neutrophils (McGuire et al., 1985) a finding which could account for the presence of 15-HETE. A further possibility is that detection of 12-HETE could be due to contamination with platelets. In another study (McColl, unpublished observations), I demonstrated that detectable levels of 12-HETE can be produced by 10^6 platelets. Furthermore, I demonstrated that in Percoll, Ficoll and Metrizamide cell separations, there are routinely approximately one or more platelets per neutrophil. Since most of the studies cited above used cell suspensions of approximately 10^7 neutrophils/ml, it is possible

that sufficient platelets were present to account for synthesis of 12-HETE.

Thus, unlike the platelet where exogenous AA does stimulate both cyclooxygenase and lipoxygenase pathways (Lagarde et al., 1984), exogenous AA alone does not act as a stimulus of the 5-lipoxygenase pathway of neutrophils.

8.2.2 Exogenous AA as a substrate for leukotriene synthesis.

In the studies carried out in this thesis I demonstrated that addition of exogenous AA enhanced production of LTB_4, LTB_4 isomers and 5-HETE by neutrophils stimulated by A23187. The minimum dose of AA required to cause this enhancement was approximately 0.65 uM. A dose-related increase was then observed until a dose in the range of approximately 2.5-5.0 uM was added. Progressive inhibition occurred thereafter (Section 4.2.1). Similarly, 0.65 uM AA caused detectable synthesis of LTB_4, its isomers and 5-HETE by 1 uM FMLP. FMLP alone (0.01-100 uM), did not stimulate detectable leukotriene release because it did not stimulate detectable AA release (Section 8.2.4). Furthermore, using FMLP as a stimulus, a similar dose response curve to increasing doses of AA was observed to that with A23187.

The enhancing effect of exogenous AA on leukotriene production has been reported in neutrophils stimulated by A23187 (Borgeat and Samuelsson, 1979d; Sun & McGuire, 1984; Haines et al., 1987), STZ (Palmer and Salmon, 1983; Haines et al., 1987), C5a (Clancy et al.,

1983; 1985), IgG (Haines et al., 1987; Smith et al., 1986) and monosodium urate crystals (Serhan et al., 1984).

I have demonstrated that addition of 2.5 uM AA to cells stimulated with 0.5 uM A23187 enhanced production of 5-HETE by approximately 2-4 fold, LTB_4 isomers by approximately 3 fold and leukotriene B_4 by approximately 2 fold. These increases are similar to those reported by Sun and McGuire, (1984), although they used higher concentrations of both AA (50 uM) and A24187 (10 uM). Interestingly, in the latter study maximal production of LTB_4 5-HETE and LTB_4 isomers was observed using 100 uM AA, a concentration approximately 20 times greater than the dose which achieves maximal release in my experiments. However, the ratio of AA to cell number when maximal enhancement was observed in the study of Sun and McGuire was identical to mine (5 nmoles AA/10^6 neutrophils).

It is possible that the large concentrations of exogenous AA added with other stimuli may not simply be acting as substrate (Haines et al., 1987). Doses of 50 uM or greater have often been used to enhance stimulation with FMLP, STZ and IgG and these doses have been shown to activate protein kinase C thereby initiating phosphorylation of a variety of proteins (Murakami and Routtenberg, 1985). My results showing significant leukotriene synthesis with doses as low as 1 uM AA added with FMLP do not support this theory as such low doses of AA do not increase protein kinase C activity (Murakami and Routtenberg, 1985). However, I have demonstrated that PMA, a specific activator of PKC can modulate the 5-lipoxygenase pathway (Chapter 7).

Thus, exogenous AA can act as a substrate for 5-lipoxygenase, enhancing leukotriene synthesis stimulated by agonists such as A23187, STZ and FMLP. In addition, at high concentrations of AA, it is possible that factors including activation of PKC may also be involved (Chapter 7; Section 8.4).

8.3 THE ROLE OF Ca^{++} IN LEUKOTRIENE SYNTHESIS.

The PLA_2 responsible for release of esterified AA from cell membranes is Ca^{++}-dependent (Lapetina, 1984). Similarly, activation of the 5-LO pathway is also Ca^{++}-dependent (Jakschik and Kuo, 1980; Parker and Aykent, 1982). For example, addition of Ca^{++} to the medium is sufficient to stimulate homogenized RBL-1 cells to produce leukotrienes (Jakschik and Kuo, 1980; Parker and Aykent, 1982). Under these conditions maximal stimulation occurs with approximately 0.5 mM Ca^{++} (Parker and Aykent, 1982). Furthermore, a purified human leukocyte enzyme possessing both 5-LO and LTA synthetase activities may be activated by addition of Ca^{++} to the medium (Rouzer et al., 1986a). The Ca^{++}-dependence of the 5-LO pathway appears to be limited to the 5-LO and LTA synthetase and not the LTA hydrolase, since addition of 5-HPETE to intact cells initiates leukotriene synthesis without A23187 (Parker et al., 1980), and LTC_4 and LTB_4 may be synthesised from LTA_4 by homogenised RBL-1 cells in a Ca^{++}-free medium (Jakschik and Kuo, 1983).

A23187 is a calcium ionophore which forms complexes with divalent cations, consisting of two molecules of A23187 per cation (Betts, personal communication). Thus, the complex is uncharged and being lipophilic, crosses the cell membrane (Betts, personal communication). A23187 thus promotes rapid exchange of divalent cations between the intra and extra cellular environment and thus may act as a potent stimulator of 5-LO product formation in several cell types (Parker, 1984). The major role attributed to Ca^{++} in leukotriene synthesis is to activate both PLA_2 (thereby stimulating release of AA) and enzymes of the 5-LO pathway. Although A23187 has proven a useful tool for investigation of leukotriene biosynthesis because of its ability to stimulate both release and metabolism of large amounts of leukotrienes, the Ca^{++} flux produced by A23187 is not representative of a physiological or pathophysiological stimulus (Irvine, 1982).

I have demonstrated that leukotriene synthesis stimulated by A23187 is both time and dose dependent. I found the optimal time for leukotriene stimulation by A23187 was 5 min which is in general agreement with the published literature (Clancy et al., 1985; Sun and McGuire, 1984; Bonser et al., 1981). I found the optimal dose range for leukotriene synthesis was between 0.5 and 1 uM A23187, a result which is not in agreement with several other reports. For example, Jorg et al., (1982) found that 20 uM A23187 was required for maximal release of LTC_4 by equine eosinophils. Lee et al., (1985) reported maximal leukotriene release (LTB_4, LTB_4 isomers and 5-HETE) by human neutrophils and monocytes using between 10 and 20 uM A23187. A23187 slowly polymerises in aqueous solution and it is possible there is a

greater bioavailability of A23187 when added in methanol compared to aqueous solutions. Both Jorg et al., (1982) and Lee et al., (1985) added aqueous dilutions of A23187 to cells whereas, because of its polymerisation properties, I used A23187 dissolved in methanol and added it directly to the cells (final concentration 0.5% methanol).

A further explanation may be that the cell concentration used by Jorg et al., (1982) was approximately 5×10^6/ml and I have demonstrated that at this cell concentration the relationship between leukotriene synthesis and concentration of A23187 is not linear (section 4.2.1). Therefore, use of 5×10^6 cells/ml or greater shifts the dose response curve to A23187 (Figures 4.7-4.12) to the right. It is possible that factors such as cell concentration and the form in which the stimulus is added to cell suspensions may have an impact on the absolute amounts of product detected.

Theoretically, the Ca^{++} flux caused by A23187 alone should stimulate both release and subsequent metabolism of AA by activating both PLA_2 and the 5-LO pathway. However, I was able to demonstrate that at low concentrations of A23187 (less than 0.03 uM) leukotriene synthesis was only observed if exogenous AA was added. This indicated that the Ca^{++} flux caused by the low doses of A23187 was sufficient to activate the 5-LO pathway but not PLA_2, and implied that PLA_2 was more Ca^{++} dependent than the 5-LO pathway.

Confirmation of this theory was obtained by stimulating cells in medium which contained Ca^{++} levels ranging from 0-1.6 mM. Cells in

medium which contained no Ca^{++} failed to synthesise detectable levels of leukotrienes in response to A23187 alone: a minimum of 0.05 mM exogenous Ca^{++} was required. Leukotriene synthesis was maximal when the extracellular Ca^{++} concentration was greater than 0.8 mM. In contrast, in the presence of AA, A23187 stimulated leukotriene synthesis even if the cells were placed in a medium which contained no Ca^{++}. Maximal leukotriene synthesis using both AA and A23187 was found when the extracellular Ca^{++} concentration was greater than 0.05 mM. These data confirmed that PLA_2 was more dependent on Ca^{++} than the 5-LO pathway. Furthermore, these data suggest that activation of the 5-LO pathway was either not dependent on Ca^{++} or that the intracellular mobilization of Ca^{++} caused by A23187 was sufficient to activate the 5-LO pathway.

These two possibilities were investigated in the studies using EDTA, EGTA and TMB-8. EDTA, which chelates both intra- and extracellular Ca^{++} (Williams and Cole, 1981) completely inhibited stimulation of leukotriene synthesis caused by A23187 alone and A23187 with 5 uM AA, indicating that both PLA_2 and the 5-LO pathway required Ca^{++} for activation. Furthermore, the extracellular Ca^{++} chelator, EGTA (Smolen and Boxer, 1983) inhibited stimulation of leukotriene synthesis by A23187 alone but not by A23187 with AA, indicating that intracellular mobilization of Ca^{++} by A23187 in the absence of extracellular Ca^{++} was sufficient to activate the 5-LO pathway, but not PLA_2. Finally, in the presence of extracellular Ca^{++}, the specific intracellular Ca^{++} inhibitor TMB-8 (Lad et al., 1985) inhibited leukotriene synthesis by A23187 alone but not with AA,

suggesting that the extracellular flux of Ca^{++} was enough to raise intracellular Ca^{++} to sufficient levels to activate the 5-LO pathway but not PLA_2.

Taken together, these data demonstrate that PLA_2 is more Ca^{++}-dependent than the 5-LO pathway. For complete activation of the 5-LO pathway, a minimum of 0.05 mM extracellular Ca^{++} and normal intracellular Ca^{++} levels are required. In contrast, for activation of PLA_2, a minimum of 0.05 mM extracelluar Ca^{++} and physiological intracellular Ca^{++} levels are required, and for maximum activation of PLA_2, greater tha 0.8 mM extracellular Ca^{++} and physiological intracellular Ca^{++} is required. Depletion either extra- or intracellular Ca^{++} prevents release of AA by A23187. For determination of the absolute levels of Ca^{++} required for activation of PLA_2 and the 5-LO pathway, studies to measure extracellular and intracellular Ca^{++} fluxes in response to stimulation should be performed.

8.4 THE ROLE OF RECEPTOR LIGANDS IN LEUKOTRIENE SYNTHESIS.

The fact that PLA_2 activity is masked under conditions of low intracellular Ca^{++} may explain why other more physiological agonists (eg: FMLP, STZ or IgG) do not appear to stimulate detectable leukotriene production. These stimuli may raise the intracellular Ca^{++} sufficiently to activate the 5-LO pathway but not PLA_2. Thus, in vitro, these stimuli will cause leukotriene synthesis if exogenous

AA is supplied. Similarly, these agonists may stimulate leukotriene synthesis in vivo if AA was supplied from an endogenous source. An obvious candidate for this is the platelet which releases AA in response to several endogenous stimuli for example, thrombin ADP and adrenalin (Marcus et al., 1982; 1984).

Chemotactic peptides, complement components and immunoglobulins activate chemotaxis, phagocytosis, degranulation and the respiratory burst in neutrophils after binding to specific receptors on a neutrophil membrane. These compounds, are among the obvious candidates for stimulation of leukotriene synthesis in vivo. Leukotriene synthesis in response to these stimuli added with exogenous AA has been demonstrated (Palmer and Salmon, 1983; Clancy et al, 1983). Although these agonists have been shown to stimulate leukotriene synthesis alone, they do so to a far smaller degree than that achieved by A23187. For example, human monocytes release approximately 1 ng of $LTB_4/10^6$ cells (Ferreri et al., 1986) and 0.7 ng $LTC_4 \times 10^6$ cells (Williams., et al 1984) in 15 minutes in response to aggregated IgG. STZ ellicited synthesis of approximately 8 ng of $LTC_4/10^6$ eosinophils in 15 minutes (Bruynzeel et al 1985) and 1 ng $LTB_4/10^6$ neutrophils in 5 minutes (Palmer & Salmon, 1983). The chemotactic peptide, FMLP has been shown to stimulate leukotriene synthesis in neutrophils and monocytes. For example, 1 uM FMLP stimulated release of approximately 350 pg of $LTB_4/10^6$ neutrophils in 10 minutes (Salari et al 1985), 8 ng of $LTC_4/10^6$ eosinophils in 5 minutes (Owen et al 1987) and 2.5 ng of $LTB_4/10^6$ monocytes in 15 minutes (Williams et al 1986). All the amounts cited in these reports

are far below those detected upon stimulation of cells with A23187.

In the study described in section 4.2.4, I found no evidence of release or metabolism of AA by FMLP alone. Furthermore, neutrophils which had been pre-labelled with ^{3}H-AA for 1 hr failed to produce ^{3}H-LTB_4 in response to FMLP. Stimulation with A23187 however, resulted in synthesis of ^{3}H-LTB_4, indicating that the cells were still capable of LT synthesis after the 1 hr preincubation. Moreover, neutrophils which had been pre-incubated for 1 hr in buffer, metabolized exogenous ^{3}H-AA when it was added with FMLP indicating that the receptor for FMLP was still functional after the 1 hr pre-incubation. I found that 1 uM FMLP and exogenous AA will stimulate significant leukotriene synthesis. These findings are supported in other publications (see also Clancy et al., 1983; 1985; Bruynzeel et al., 1985; Palmer & Salmon, 1983). Possible reasons for the inability to detect synthesis of leukotriene in response to FMLP include lack of assay sensitivity, although in some of the publications, radio-immuno assays were used (Palmer & Salmon, 1983). These should have detected the levels reported by Salari et al., (1985). Alternatively, expression of the FMLP receptor may have been altered during cell separation.

During completion of this thesis, a paper examining leukotriene synthesis by neutrophils in response to specific receptor ligands was published (Haines et al., 1987). The ligands used activated receptors specific for the C3b component of complement, (STZ), the Fc portion of IgG (heat aggregated IgG) and the synthetic chemotactic peptide

(FMLP). These workers found barely detectable amounts of LTB_4 synthesised in response to these stimuli although all stimulated release of significant amounts when they were added with exogenous AA. While these findings are in general agreement with my results using FMLP there are several important differences between my results and those of Haines et al., (1987). Firstly, the amounts of 5-HETE generated are far less than LTB_4 a finding which no other published reports agree (Borgeat and Samuelsson, 1979c; Sun and McGuire, 1984; Clancy et al., 1983;1985). A likely explanation for this is that the retention time for 5-HETE in the HPLC assay used by Haines et al 1987 is approximately 50 minutes which may contribute to a substantial decrease in detection. Secondly, there is apparently no internal standard for this assay. This also may contribute to problems with accurately measuring compounds which elute from the column very late. Thirdly, a dose of 50 uM AA was required for significant leukotriene synthesis in response to FMLP (these authors claim that doses of 1 and 10 uM have no effect). The requirement for this amount of exogenous AA is in sharp contrast to my findings where I have consistently observed a significant production of LTB_4, LTB_4 isomers and 5-HETE when approximately 1 uM AA was added with FMLP. Furthermore, doses above 5-10 uM caused inhibition. These differences in observed levels of products are typical examples of the variability reported in leukotriene research. I have demonstrated that there is considerable variability within the leukotriene generating system. Section 4.2.7. However, this variability is not sufficient to explain all differences in observations. It is likely that differences in methodology for example assay sensitivity, extraction procedures,

conditions of stimulation or cell separation procedures or combinations of some or all of these factors may account for these observed differences.

8.5 POSSIBLE ROLE OF PKC IN LEUKOTRIENE SYNTHESIS.

Protein kinase C (PKC) is a protein which is both cytoplasmic and membrane bound (Wolfson et al). Several factors are required for complete activation of PKC. For example, it must be translocated and bound to the membrane, - a process which is greatly enhanced by Ca^{++}. Furthermore, once bound to the cell membrane, phosphatidylserine acts as a co-factor further enhancing activation (Konig et al., 1985). The active form of PKC phosphorylates a number of proteins including transferrin (Stratford et al., 1984), interleukin 2 receptors (Shackleford and Trowbridge, 1984) and lipomodulin (Hirata et al., 1984)

Putative mechanisms of action involving protein phosphorylation have now been postulated for many of the stimuli used to generate superoxide production by phagocytes. For example, receptor ligands (FMLP, STZ), stimulate superoxide production by a biochemical pathway that is believed to involve guanine neucleotide binding proteins, phosphoinostide turnover, PKC mobilization from the cytosol to the membrane, and subsequent phosphorylation of NADPH oxidase (Figure 1.6) (Downes and Michell, 1985; Genaro et al., 1985; Heyworth and Segal., 1986). Phorbol esters enter the pathway by directly activating PKC whereas ionophores appear to activate a different pathway which is

Ca^{++} and possibly calmodulin dependent (Nishizuka, 1984). These are not the only pathways leading to superoxide production in phagocytes. There are some alternative pathways possibly involving activation of other protein kinases which have recently been put forward (Rossi et al., 1986).

Although there have recently been major breakthroughs in the understanding of the biochemical pathways involved in superoxide generation by phagocytes, little is understood of the biochemical events leading to leukotriene production. This is in spite of the fact that several agonists are common to both pathways (eg: A23187, FMLP, STZ and AA). In this thesis, I have shown that PMA the most potent stimulus of superoxide production (French et al., 1987b) does not stimulate detectable leukotriene synthesis or release of AA. Others have also demonstrated this (Volpi et al., 1985; Halenda et al., 1985). Although high concentrations of AA stimulate superoxide production (possibly by directly activating PKC) (Badwey et al., 1981; Murakami and Routtenberg, 1985) AA does not stimulate leukotriene synthesis (Section 8.2.1). Moreover, FMLP and STZ, also potent stimuli of superoxide production (French et al., 1987b) appear to require exogenous AA to stimulate significant leukotriene synthesis. In contrast, the ionophore A23187 is a weak superoxide stimulus (French et al., 1987b) but a powerful stimulator of leukotriene synthesis. These data suggest that phosphorylation of the enzymes in the 5-LO pathway by PKC would not necessarily lead to their activation as other factors are involved (eg: changes in intracellular Ca^{++}). However, experiments performed in chapter 7 have indicated that the

activity of at least two enzymes involved in leukotriene synthesis are possibly regulated by PKC.

Effect of PKC on PLA_2 activity.

The phorbol ester PMA enhanced production of LTB_4, LTB_4 isomers and 5-HETE by neutrophils stimulated with A23187. This effect was not observed using 4 αphorbol didecanoate, a phorbol ester which does not activate PKC, suggesting that the synergistic effect of PMA was PKC-dependent. The effect of PMA was abolished by addition of exogenous AA with the A23187, suggesting that activation of PKC increased release of AA from a neutrophil membrane. This hypothesis was confirmed by demonstrating that neutrophils pre-incubated with ^{3}H-AA for 1 hr. released more ^{3}H-AA into the supernatant when PMA was added with A23187 than with A23287 alone (see also Volpi et al., 1985; Halenda et al., 1985).

Previous reports have demonstrated that PKC phosphorylates the PLA_2 inhibitory protein lipomodulin, thus decreasing lipomodulin activity (Hirata et al., 1984). Therefore, upon stimulation with A23187, more AA can be released. I have demonstrated that upon stimulation with PMA, a protein of the same approximate molecular weight as lipomodulin (approximately 40 KDa) is phosphorylated (Hirata et al., 1984).

Effect of PKC on LTA hydrolase activity.

LTA hydrolase is the enzyme which converts LTA_4 to LTB_4 (Borgeat and Samuelsson, 1979d). The activity of this enzyme is therefore critical to the amount of LTB_4 produced by inflammatory cells. Control of LTB_4 production is potentially of major physiological importance as it is the most pro-inflammatory neutrophil activator of the leukotriene family (Feinmark et al., 1981). The other major 5-lipoxygenase neutrophil products 5-HETE and the all-trans isomers of LTB_4 are considerably less active (Feinmark et al., 1981).

In this study I have presented evidence that the ratio of production of LTB_4 to LTB_4 isomers is variable (Section 4.2.3). This variability is possibly due to day-to-day changes in the activity of LTA hydrolase. When the enzyme is less active more isomers are produced and vice versa (see also Sun and McGuire, 1984; Jakschik and Kuo, 1983). I have also demonstrated that addition of PMA to neutrophils stimulated with FMLP and AA, enhanced production of LTB_4, reduced production of LTB_4 isomers but had no effect on production of 5-HETE (section 7.2.6).

These observations imply that treatment with PMA enhances the activity of LTA hydrolase. Since PMA stimulates PKC-dependent protein phosphorylation, a likely mechanism of this effect is via PKC-dependent phosphorylation of LTA hydrolase. I have shown that upon stimulation with PMA, neutrophils and mononuclear cells (both of which possess an LTA hydrolase), not only phosphorylate a 40 kDa protein, but also a 68 kDa protein. This is the same molecular weight as that reported for human LTA

hydrolase (Radmark et al., 1984). The effect of PMA on FMLP and AA-induced leukotriene synthesis was not mediated through lipomodulin as the co-addition of 5 uM exogenous AA ensured that PLA_2 activity was not rate-limiting.

8.6 CONCLUSION.

The main aims of the Thesis were achieved: to characterise the 5-lipoxygenase pathway of human neutrophils and to investigate factors regulating that pathway. However, as is the case with most research, the experiments I performed raised as many questions as they answered. Further studies into the involvement of PKC and other kinases will be required to enhance the understanding achieved by the work performed in this Thesis.

APPENDICES.

During the course of this thesis, various studies were carried out on leukocytes which proved to have no direct relationship with the theme of the thesis. These are now in press and are presented in appendices II and III. The remaining six appendices are other publications in support of this thesis.

APPENDIX I

MODULATION OF HUMAN NEUTROPHIL LTA HYDROLASE ACTIVITY BY PHORBOL 12, 13 MYRISTATE ACETATE.

Shaun R. McColl*, Nigel P. Hurst, W. Henry Betts and Leslie G. Cleland.

The Queen Elizabeth and Royal Adelaide Hospital Rheumatology Unit, Woodville Road, Woodville, South Australia, 5011. * To whom correspondence is to be sent at Unite de Recherche en Immunologie et Rhumatologie, Le Centre Hospitalier de l'Universite Laval, Boulevarde Laurier, Quebec, Que. G1V 4G2.

SUMMARY: The phorbol ester, phorbol 12,13 myristate acetate enhanced leukotriene (LT) B_4 production stimulated by formyl-methionyl-leucyl-phenylalanine and arachidonic acid and reduced the production of the all-trans isomers of LTB_4 by human neutrophils. Production of 5-hydroxyeicosatetraenoic acid was unaffected. These observations are consistent with a stimulatory effect on LTA hydrolase, the enzyme which catalyses the conversion of LTA_4 to LTB_4. We demonstrate that a protein of the same molecular weight as LTA hydrolase is phosphorylated upon stimulation of neutrophills with phorbol myristate acetate. These data suggest that the activity of LTA hydrolase may be regulated by protein kinase C-dependent phosphorylation.

INTRODUCTION: Upon stimulation by the calcium ionophore A23187, neutrophils synthesise and release products of the 5-lipoxygenase (5-LO) enzyme system, notably LTB_4, the all-trans isomers of LTB_4 and 5-hydroxyeicosatetraenoic acid (5HETE) [1]. The Ca^{++} flux caused by A23187 probably initiates leukotriene synthesis by stimulating both phospholipase A_2 (PLA_2)-dependent release of esterified arachidonic acid (AA) and its subsequent metabolism by 5-LO [2]. In contrast, little is known of the factors controlling the activity of other enzymes of the leukotriene generating pathway such as the LTA synthetase and the LTA hyrolase which catalyse production of LTA_4 and LTB_4 respectively (see Figure 1).

Phorbol myristate acetate (PMA) is a synthetic diacyl glycerol

analogue which activates the Ca^{++}/phospholipid-dependent protein kinase C (PKC) thereby initiating phosphorylation of proteins involved in a number of cellular functions [3]. We have recently demonstrated that PMA enhanced the A23187-induced release and metabolism of AA in neutrophils, an effect which has also been demonstrated in several other cell types [4,5,6,7]. This effect of PMA is probably mediated by PKC-dependent phosphorylation of lipomodulin [8]. Direct [8] and indirect [4-7] evidence for this mechanism has been presented.

In contrast, little is known about the effect of PMA on the release of leukotrienes stimulated by FMLP and AA. In this study we report that co-addition of PMA with FMLP and AA enhances production of LTB_4, reduces production of the LTB_4 all-trans isomers but has no effect on 5-HETE production.

MATERIALS AND METHODS: Human neutrophils [9], mononuclear cells [10] and platelets [11] were isolated as described previously. For the leukotriene studies incubations were carried out in a modified Dulbecco's phosphate buffered saline (DPBS, 138 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 7.5 mM glucose at pH 7.4). FMLP, PMA and AA were obtained from Sigma Chemical Company, St. Louis, MO. FMLP and PMA were dissolved in DMSO at 20 uM and 1 mg/ml stock solutions respectively, and AA was dissolved at 1 mM in methanol. FMLP was stored at 4^oC, PMA at -10^oC and AA at -70^oC under nitrogen. LTB_4, LTB_4 isomers and 5-HETE were assayed using a previously published method [9].

For the phosphorylation studies on MNC and platelets, cells were incubated with 100 uCi of ^{32}P in a phosphate free buffer for 30 min. The cells were washed twice and resuspended in Hank's balanced salt solution at 37^oC prior to addition of stimuli. Phosphorylation was halted by the addition of 5 mls of cold DPBS after which the samples were washed twice. 400 ul of lysis buffer (0.5% triton X-100, v/v in water) and 4 ul of 100 mM PMSF in acetone was added, the samples were mixed and allowed to stand at 4^oC for 45 min. For the phosphorylation studies on neutrophils, the buffer and lysis conditions were altered according to a previously published method [12]. Briefly, The PO_4^{3-}-free buffer consisted of 30 mM HEPES, pH 7.4, 110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM L-glutamine and 2 mg/ml BSA). After labelling with 100 uCi of ^{32}P, the cells were washed twice and resuspended in buffer before adding the appropriate stimuli. Phosphorylation reactions were stopped and the cells were lysed by the addition of an equal volume of ice-cold 2x RIPA buffer (2% NP-40. 0.2% SDS, 0.15 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 0.68

M sucrose, 5 mM EDTA, 10 mM NaF, 5mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM ATP, 10 mM DFP, 100 ug/ml DNase, 5% FCS, pH 7.2). This lysis buffer maximally inhibits neutrophil phosphatase and protease activities. After centrifugation at 1000 x g for 5 min, 200 ul of the samples were removed for SDS-PAGE. The samples and gels were prepared, run and stained according to the method of Laemlli [13]. After the gels were dried, they were exposed to x-ray film for two days.

RESULTS: Co-addition of 50 ng/ml PMA to neutrophils stimulated with 1 uM FMLP and 5 uM AA for 5 min, caused a significant increase in LTB_4 production and a significant decrease in production of the all-trans LTB_4 isomers. 5-HETE production was unchanged (Table 1). Co-addition of 50 ng/ml PMA to neutrophils stimulated with 0.5 uM A23187 led to an increase in the production of all three compounds (Table 2).

Stimulation of neutrophils with PMA resulted in enhanced phosphorylation of several proteins including one of Mr approximately 68,000 (Figure 2). A protein of similar molecular weight is phosphorylated in MNC (Figure 3) but not platelets (Figure 4) stimulated with PMA.

DISCUSSION: In a previous report we demonstrated that PMA enhanced the production of LTB_4 and 5-HETE by neutrophils stimulated with A23187 and that the mechanism of this effect was via increased release of AA [4]. In this study show that production of the LTB_4 isomers is also enhanced by PMA and that addition of exogenous AA with A23187 abolishes this effect. It has been postulated that PMA enhances AA release by activating PKC-mediated phosphorylation of lipomodulin, thereby reducing its PLA_2-inhibitory effect [8].

In contrast, when PMA was added with FMLP and AA, LTB_4 production increased, 5-HETE was unaffected and production of the LTB_4 isomers was reduced. This phenomenon was observed in the presence of

exogenous AA, excluding an effect mediated by lipomodulin. These data are consistent with an activating effect of PMA on LTA hydrolase. It is therefore possible that PMA stimulates PKC-dependent phosphorylation of LTA hydrolase.

LTA hydrolase has recently been isolated and reported to have a molecular weight of 68,000-70,000 [14]. We have demonstrated that upon stimulation with PMA, neutrophils and MNC (which possess LTA hydrolase) but not platelets (which do not possess LTA hydrolase) phosphorylate a protein with a molecular weight of approximately 68,000. These data are consistent with the hypothesis that the activity of LTA hydrolase is regulated by PKC-dependent phosphorylation.

ACKNOWLEDGEMENTS: We are grateful to Ms. L. Gorjatschko and Mr. A Wozniak for their technical assistance. This work was supported by grants from the National Health & Medical Research Council of Australia, the Arthritis Foundation of Australia and the Arthritis Foundation of Australia (South Australia). S.R. McC. is the recipient of a Mary Paxton-Gibson Postgraduate Research Scholarship from the Arthritis Foundation of Australia.

ABBREVIATIONS: PMA, phorbol 12-myistate, 13-acetate; LTB_4, leukotriene B_4; 5-HETE, 5-hydroxyeicosatetraenoic acid; AA, arachidonic acid; PKC, protein kinase C; FMLP, formyl-methionyl-leucyl-phenylalanine; DAG, diacylglycerol; MNC, mononuclear cells; PMSF, phenyl methyl sulphonyl fluoride; DMSO, dimethyl sulfoxide.

REFERENCES

1. Sun, F.F. and McGuire, J.C. (1984) Biochim. Biophys. Acta 794:56-64.
2. Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. and Lefkowitz, J. (1986) Ann. Rev. Biochem. 15:69-102.
3. Hurst, N.P. (1987) Ann. Rheum. Dis. in press.
4. McColl, S.R., Hurst, N.P. and Cleland, L.G. (1986) Biochem. Biophys. Res. Commun. 141:399-404.
5. Tripp, C.S., Mahoney, M. and Needleman, P. (1985) J. Biol. Chem. 260:5895-5898.
6. Halenda, S.P., Zavoico, G.B. and Feinstein, M.B. (1985) J. Biol. Chem. 260:12484-12491.
7. Volpi, M., Molski, T., Naccache, P. H., Feinstein, M.B. and

Sha'afi, R. I. (1985) Biochem. Biophys. Res. Commun. 128:594-600.
8. Hirata, F., Matsuda, K., Notsu, Y., Hattori, T. and Del Carmine, R. (1984) Proc. Natl. Acad. Sci. U.S.A. 81:4717-4721.
9. McColl, S.R., Betts, W.H., Murphy, G.A. and Cleland, L.G. (1986) J. Chromatogr. 378:444-449.
10. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21:(Suppl 97) 77.
11. Tapley, P.M. and Murray, A.W. (1984) Biochem. Biophys. Res. Commun. 118:835-841.
12. Changelian, P.S. and Fearon, D.T. (1986) J. Exp. Med. 163:110-115.
13. Laemmli, U.K. (1970) Nature (London) 227:680-685.
14. Radmark, O., Shimizu, T., Jornvall, H. and Samuelsson, B. (1984) J. Biol. Chem. 259:12339-12345.

Table 1: The effect of PMA on the FMLP and AA-induced generation of LTB_4, LTB_4 isomers and 5-HETE by human neutrophils.

	Amount generated (ng/10^6 neutrophils)		
Condition	LTB_4	LTB_4 isomers	5-HETE
FMLP + AA	3.5±0.3	5.4±0.7	52.9±5.0
FMLP + AA + PMA	6.2±0.5*	2.9±0.5*	49.5±0.4

Cells were stimulated for 5 min with 1 uM FMLP + 5 uM AA with or without simultaneous addition of 50 ng/ml PMA. Results are expressed as mean ± sd, from one typical experiment of three experiments, each performed in quadruplicate. * significantly different from control at $p < 0.05$, students t-test.

Table 2: The effect of PMA on A23187-induced LTB_4, LTB_4 isomer and 5-HETE production.

	Amount generated (ng/10^6 neutrophils)		
Condition	LTB_4	LTB_4 isomers	5-HETE
A23187	15.6±0.8	12.7±1.8	64.5±11.3
A23187 + PMA	24.7±2.4*	17.6±1.5*	107.1±11.3*

Cells were stimulated for 5 min with 0.5 uM A23187 with or without simultaneous addition of 50 ng/ml PMA. Results are expressed as mean ± sd, from one typical experiment of three experiments each performed in quadruplicate. * significantly different from control at $p < 0.05$, students t-test.

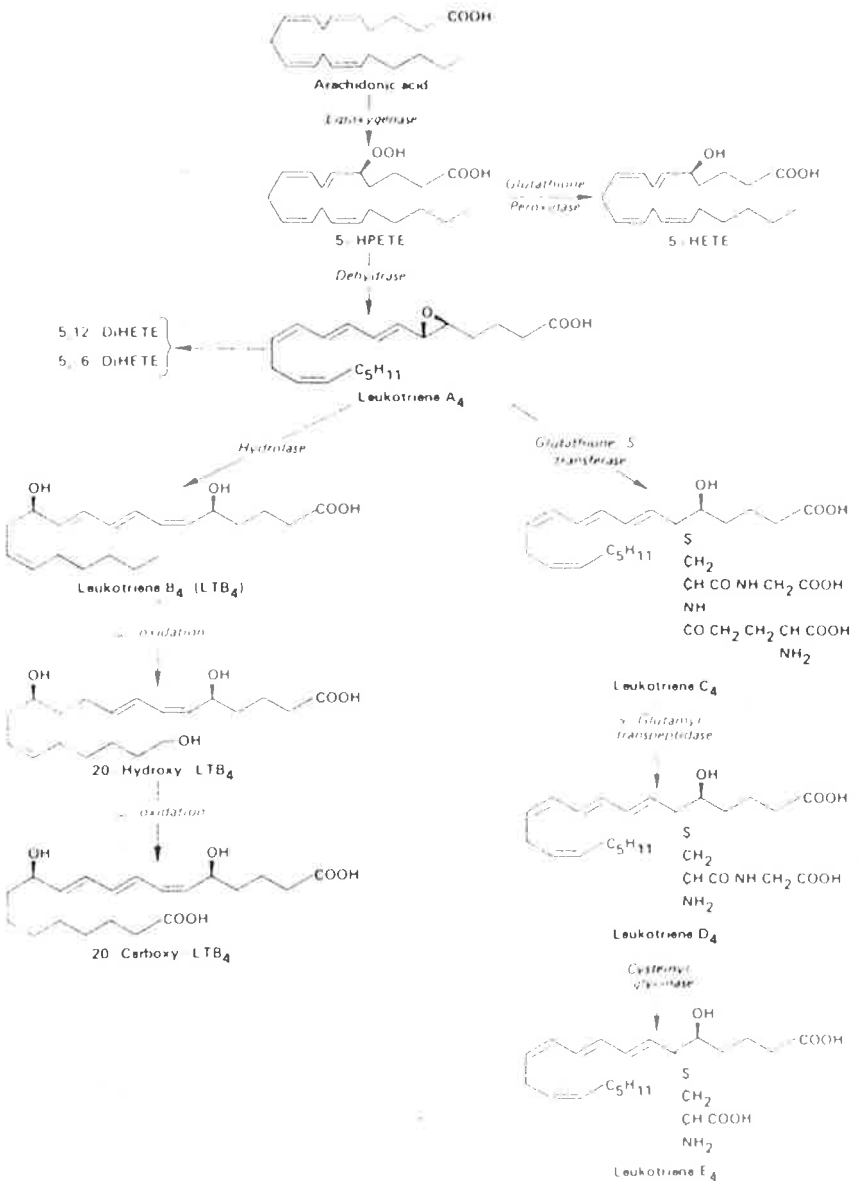


Figure 1: The 5-lipoxygenase pathway of leukocytes.

See Figure 7.11

Figure 2: Autoradiograph of polyacrylamide gel of neutrophil proteins. Neutrophils were preincubated with ^{32}P for 1 hr and stimulated with PMA for 5 min.

See Figure 7.12

Figure 3: Autoradiograph of polyacrylamide gel of human monocyte proteins. Monocytes were preincubated with ^{32}P for 1 hr prior to stimulation with PMA for 5 min.

See Figure 7.13

Figure 4: Autoradiograph of polyacrylamide gel of human platelet proteins. Platelets were preincubated with ^{32}P for 1 hr prior to stimulation with PMA for 5 min.

APPENDIX II

A COMPARISON BETWEEN THE INFLAMMATORY MEDIATORS PRODUCED BY THE BLUE-TONGUE LIZARD (*TILIQUA SCINCOIDES*) AND HUMAN WHITE BLOOD CELLS.

Shaun R. McColl* and Christopher B. Daniels#

* The Queen Elizabeth Hospital Rheumatology Unit, Adelaide, South Australia, 5011 and # Department of Physiology, School of Medicine, Flinders Medical Centre, Bedford Park, South Australia.

ABSTRACT

Human white blood cells, particularly neutrophils and macrophages produce a number of biologically active molecules which are involved in mechanisms of host defence. These molecules include oxygen-derived free radicals and some of the metabolites of arachidonic acid. White blood cells of the lizard *T. scincoides* produce certain derivatives of arachidonic acid which include prostaglandins, thromboxane and 12- and 15-hydroxyeicosatetraenoic acid. The ability to produce these compounds indicates that these animals possess the enzymes cyclooxygenase, 12- and 15-lipoxygenase. *T. scincoides* white blood cells did not produce leukotriene B_4 or 5-hydroxyeicosatetraenoic acid indicating that unlike human white blood cells they do not possess a 5-lipoxygenase enzyme. The *T. scincoides* cells are also capable of producing the oxygen-derived free radical superoxide.

INTRODUCTION

The white blood cells (WBC) of higher vertebrates produce a number of biologically active molecules which are involved in host defence. These molecules include the phagocyte-derived oxygen-radicals and metabolites of arachidonic acid (eicosatetraenoic acid; AA). The latter include prostaglandins, thromboxane, leukotrienes and mono-hydroxyeicosatetraenoic acids (HETEs) and are collectively termed eicosanoids (Granstrom et al., 1982; Johnson et al., 1983). In human phagocytes, oxygen-radical production can be elicited by a number of stimuli. For example, N-formyl-methionyl-leucyl- phenylalanine (FMLP) which is a peptide segment from bacterial cell walls, stimulates superoxide production after binding to a specific receptor on the cell surface (Figure 1). Alternatively, stimuli such as phorbol myristate acetate (PMA) stimulate superoxide production by binding to protein kinase C (PKC) which in turn stimulates NADPH oxidase (Figure 1) (Volpi et al., 1985).

AA released from phospholipids by phospholipases, may be metabolized to eicosanoids (Figure 2) by two enzyme systems - cyclooxygenase or lipoxygenase. Cyclooxygenase metabolizes AA to prostaglandins and thromboxane, whereas lipoxygenase enzymes (eg: 5-, 12- or 15-lipoxygenase) metabolize AA to HETEs and leukotrienes (Granstrom et al., 1982; Johnston et al., 1983). Eicosanoids have important physiological actions. PGI_2 (usually termed prostacyclin) inhibits platelet aggregation and causes vasodilation. In contrast, TXA_2 promotes platelet aggregation and is a vasoconstrictor. These compounds are involved in the control of haemostasis (Moncada et al.,

1982; Johnston et al., 1983). The leukotrienes and HETEs, particularly leukotriene B_4 (LTB_4), are potent pro-inflammatory agents. LTB_4 is a neutrophil chemotaxin which mediates neutrophil aggregation and degranulation (Ford-Hutchinson et al., 1980; Feinmark et al., 1980), whereas 5-, 12- and 15-HETE may synergize with other stimuli to enhance inflammatory responses. For example, 5-HETE increases superoxide production by human neutrophils which have been pretreated with FMLP (Beckman et al., 1985). On the other hand, prostaglandins can inhibit neutrophil activation by inhibiting production of LTB_4 (Ham et al., 1983).

The aim of this study was to compare the inflammatory mediators of the WBC of a reptile with those of humans. Several analyses were performed. WBC were stimulated with FMLP and PMA and production of superoxide was determined as an indicator of the presence of NADPH oxidase. The lipids of erythrocytes and WBC were assayed to compare the percentage of linoleic and arachidonic acid because these compounds are the precursors for eicosanoids). Several AA-metabolites were assayed to indicate the presence of key enzymes for AA metabolism. TXB_2 and 6-keto-$PGF_{1\alpha}$ were assayed because their production indicates the presence of cyclooxygenase (TXB_2 and 6-keto-$PGF_{1\alpha}$ are the stable breakdown products of TXA_2 and PGI_2 respectively and so are usually assayed to indicate their production). 12-HETE and 15-HETE were assayed as products of 12- and 15-lipoxygenase respectively, and LTB_4 and 5-HETE to indicate the presence of the 5-lipoxygenase enzyme.

MATERIALS & METHODS

Four adult male Australian Blue Tongue lizards Tiliqua scincoides (Scincidae)(264 ± 8 mm SVL: 255 ± 13 g) were collected locally (Adelaide, Australia) in summer 1985-86 and experiments were performed in May 1986 (late fall). All lizards were maintained together in outdoor enclosures until the experiment. They were fed minced fruit and vegetables and were provided with water ad libitum.

Calcium ionophore (A23187), FMLP, PMA, EDTA, arachidonic acid and citric acid were from Sigma Chemical Company (St. Louis, USA). Brietal Sodium was from Lilly (Indianapolis, USA). All chemicals used in buffers were from Ajax Chemical Company (Auburn, Australia). All solvents were HPLC grade (Waters-Millipore, Milford, USA). Antisera to 6-ketoPGF$_1$ was from Seragen Corp. (Boston, USA) and ^{3}H-labelled 6-ketoPGF$_{1\alpha}$ and TXB$_2$ was from Amersham (Buckinghamshire, England). Unlabelled standards for 6-keto PGF$_{1\alpha}$ and TXB$_2$ were from Cayman Chemical Co. (Ann Arbor, USA). Antisera to TXB$_2$ was prepared from rabbits immunized with TXB$_2$ conjugated to thyroglobulin (James, personal communication).

Lizards were anaesthetized by intraperitoneal injection with 500 ul of Brietal sodium (20 ug/kg methohexitone). A ventral incision was made along the midline from the cloaca to the throat and the abdominal aorta was cannulated (polyethylene catheter tubing 1.45 mm o.d.). Blood was allowed to collect in a polypropylene test tube containing 4.5% EDTA (5:1 v/v). Human peripheral blood was collected from four healthy volunteers by venepuncture and mixed with 4.5% EDTA (5:1 v/v).

The lizard blood (approximately 4 mls) was spun slowly (150 x g)

for 10 min. and the white blood cell rich supernatant (buffy coat) was removed. Pelleted erythrocytes and 500 ul of the WBC were retained for fatty acid analysis. The remainder of the buffy coat was washed twice in a Reptilian Ringer's Solution (155mM NaCl, 4mM KCl and 2mM PO_4 at pH 7.4) (Gleeson et al., 1980) by spinning at 450 x g for 5 min. After a final wash, the WBC pellet was resuspended in 5 mls of Hank's Balanced Salt Solution (137mM NaCl, 5.4mM KCl, 1.3mM $CaCl_2$, 0.35mM Na_2HPO_4, 0.44mM KH_2PO_4, 0.08mM $MgSO_4.7H_2O$, 5.5mM Glucose and 4.1mM $NaHCO_3$). The final cell concentration determined by haemocytometer was 5 x 10^6 WBC/ml. The human blood was treated in a similar fashion except cell washing was performed in Dulbecco's Phosphate Buffered Saline (140mM Nacl, 2.7mM KCl, 8.1mM Na_2PO_4 and 1.4mM KH_2PO_4) rather than in Reptilian Ringer's Solution.

Superoxide anion production was determined by the superoxide dismutase-inhibitable reduction of horse heart ferricytochrome C as previously described (Weening et al., 1975). Reduction of cytochrome C was monitored at 550 nm UV on an LKB spectrophotometer. Cells were stimulated for 10 min. and then placed on ice. All experiments were performed in triplicate.

Lipid samples were converted to their constituent fatty acid methyl esters which were extracted with petroleum spirit and analysed by gas liquid chromatography (Gibson, 1983). A 1.5 m column (2 mm id) packed with 5% SP2310 (Supelco Inc., Bellefonte, PA) served as the stationary phase. Chromatographic conditions were as follows; injection port temperature 200°C, flame ionization detector temperature 300°C, initial oven temperature 125° rising to 225°C at 4°C/min and holding for 20 min. The carrier gas used was nitrogen at a flow rate of 16-20 ml/min.

The cells were stimulated at room temperature (19°C) for 15 min. by simultaneous addition of 0.1 uM A23187 (final concentration) and 10 uM arachidonic acid (final concentration). The reaction was stopped by addition of 50 ul of 100 mM citric acid and 50 ng of PGB_2 was added as internal standard. The AA metabolites were extracted with 2.5 mls of chloroform/methanol (7:3 v/v) and following centrifugation (900 x g) for 5 min, the chloroform layer was removed and evaporated in a savant centrifugal evaporator. The samples were reconstituted in 100 ul of methanol and either injected onto the HPLC for leukotriene analysis or assayed for prostaglandins by RIA.

The HPLC system comprised a Waters chromatographic system centred around a Nova-PAK C_{18} column. The mobile phase was methanol/water/acetic acid (71/29/0.08, pH 6.2 with ammonium hydroxide) run at 1 ml/min. Detection was by UV spectrophotometry (270 nm for LTB_4 and 234 nm for mono-HETEs) (McColl et al., 1986).

PGI_2 and TXA_2 are usually measured by the assay of their stable breakdown products 6-ketoPGF_1 and TXB_2 (Moncada et al., 1982). For RIA analysis, samples in methanol were evaporated with nitrogen and reconstituted in 0.3 ml of Na_2CO_3 solution (1 mM). 0.1 ml aliquots of this solution were used for the analysis.

RESULTS

When stimulated with PMA, WBC from T. scincoides produced 0.10±0.05 (mean ± sem) nmoles of superoxide/10^6 cells/min. Under the same conditions, human white blood cells produced 3.6±0.1 nmoles of superoxide/10^6 cells/min. When FMLP was used as a stimulus, human cells produced 3.2±0.1 nmoles superoxide/10^6 cells/min, however the

lizard cells did not respond. The human and reptilian blood cells both contain large percentages of linoleic (18:2 w6) and arachidonic (20:4 w6) acids, the precursor molecules for prostaglandin and leukotriene synthesis (Table I). Erythrocytes and WBC from T. scincoides contained a greater percentage of these than did human cells. Upon stimulation with 0.1 uM A23187 and 10 uM AA for 15 mins, T. scincoides WBC produce 12- and 15-HETE. There was no evidence of either 5-HETE or LTB_4 (Table II). By contrast, human white blood cells produce all four of these compounds. A23187 and AA also stimulated both human and lizard cells to produce 6keto$PGF_{1\alpha}$ and TXB_2 indicating production of PGI_2 and TXA_2 respectively.

DISCUSSION

There is some knowledge of production of certain inflammatory mediators by non-mammalian vertebrates. For example, amphibia produce PGE_2 (Tyler, 1976) and chicken erythrocytes produce 12-HETE (Kobayashi et al., 1983). However, it must be stressed that this comparison is between human cells and those of only one non-mammalian species and all observations were made at one point in time (May) using T. scincoides which provided constant yields of WBC (approximately 5 x 10^6WBC/ml of blood taken).

With the notable exception of products of the 5-lipoxygenase system (5-HETE, LTB_4), the WBC of T. scincoides produce the same types of chemical mediators as human cells, i.e. they produce AA metabolites (prostaglandins, thromboxane, 12- and 15-HETE) and the oxygen-derived free radical superoxide.

Reptiles possess a similar range of WBC to that of humans

including lymphocytes, monocytes and granulocytes (Saint-Girons, 1970). Reptilian granulocytes are of four types; eosinophils, basophils, azurophils and neutrophils (Saint-Girons, 1970) although their neutrophils are monocytic and not polymorphic as in humans. Relative proportions of reptilian granulocytes vary with age, sex, pregnancy and pathological disorders (Duguy, 1970). Moreover, the total WBC count of a lizard can vary 6-10 fold depending on the season (Duguy, 1970). For these reasons we performed the study at one point in time which provided a constant WBC count.

In general, reptilian blood has fewer neutrophils and more eosinophils and basophils than human blood (Andrew et al., 1974). In humans, the leukocytes capable of oxygen-radical production are neutrophils, eosinophils and monocytes and these comprise approximately 70% of total leukocytes (not counting platelets)(Lentner, 1984). In contrast, in three species of Mabuya (family Scincidae) this combination comprises approximately 40% of total leukocytes (Duguy, 1970). This creates difficulties when comparing the rate of superoxide produced by the WBC of T. scincoides with that of human buffy coat cells. If the oxygen-radical producing leukocytes of T. scincoides were directly comparable with those of human cells, we would expect approximately 60% of the rate of superoxide production. In fact in response to PMA, the rate of superoxide production by lizard cells was only 3% of that by human leukocytes. We do not know whether this difference relates to the leukocytes in T. scincoides having differential abilities to produce superoxide or being generally less capable of superoxide production than their human counterparts.

FMLP and PMA are potent stimuli of superoxide production in human

leukocytes. In this study, FMLP failed to stimulate production of superoxide by T. scincoides WBC, implying that the receptor for FMLP is not present. However, the ability of PMA to stimulate superoxide production by T. scincoides leukocytes shows that these cells possess an enzyme capable of converting molecular oxygen to superoxide and that they possess a PKC which probably phosphorylates this enzyme. The human NADPH oxidase is a cyanide insensitive, b-type cytochrome. Further investigation to identify these characteristics in this reptilian enzyme is therefore warranted before we can be certain it is the same enzyme.

In humans, generation of at least two AA-metabolites has been demonstrated in all of the WBC present in a buffy coat preparation (Johnson et al., 1983; Hansson et al., 1983). Therefore in order to demonstrate the presence of all the key enzymes of the AA-cascade in T. scincoides (eg; cyclooxygenase and the lipoxygenases), we stimulated buffy coat preparations. The problem of comparing the quantities produced by the human and lizard cells did not arise here as in this section of the study we sought only to demonstrate the existence of certain pathways.

The erythrocytes and WBC of T. scincoides have both linoleic and arachidonic acids, the fatty acid precursors for eicosanoids. T. scincoides cells generated the cyclooxygenase products 6-keto$PGF_{1\alpha}$ and TXB_2 indicating the production of PGI_2 and TXA_2 respectively. Whether PGI_2 and TXA_2 have the same physiological effects in T. scincoides as in humans is unknown. T. scincoides WBC also produced 12- and 15 HETE. In humans, 12-HETE is produced by platelets whereas 15-HETE is produced by monocytic cells. In humans, these compounds have some inflammatory effects (Goetzl et al., 1980).

Detection of other eicosanoids (eg: TXA_2, 12-HETE) from cells stimulated with A23187 indicates that lack of production of 5-HETE and LTB_4 by T. scincoides relates to a deficiency of 5-lipoxygenase and not to methodology. The major product of the 5-lipoxygenase enzyme is 5-HETE. For example, human neutrophils stimulated by A23187 for 5 minutes produce 30 times as much 5-HETE as they do LTB_4 (McColl unpublished observations). The same applies for LTC_4 and LTD_4 production by basophils (Jakschik et al., 1980). Therefore the absence of 5-HETE implies the absence of the 5-lipoxygenase enzyme. The leukotrienes are 2-3 orders of magnitude more potent than the HETEs and unlike the HETEs, specific receptors for them have been identified (Kriesle et al., 1983; Hogaboom et al., 1983). In humans, LTB_4 is produced mainly by neutrophils and its actions are very specific for neutrophils (Ford-Hutchinson et al., 1980; Feinmark et al., 1980; Hansson et al., 1983). In humans, the neutrophil represents approximately 95% of total granulocytes (Lentner, 1984). In contrast, this cell makes up less than 5% of lizard leukocytes (Duguy, 1970). It is possible therefore, that the absence of LTB_4 correlates with the absence of the neutrophil in T. scincoides blood. Howevever, this may not be the case because 10-30% of lizard leukocytes are azurophillic granulocytes which are difficult to distinguish either morphologically or functionally from neutrophils in these animals (Saint Girons, 1970). LTC_4 and LTD_4 (also known as slow reacting substance of anaphylaxis) modulate pulmonary function (Dahlen et al., 1980). Interestingly, in humans, basophils and monocytes produce LTC_4 and LTD_4 (Creticos et al., 1984; Goldyne et al., 1984) and although these cell types exist in T. scincoides, these compounds are not produced.

ACKNOWLEDGEMENTS

We would like to thank Drs. M James, WH Betts, NP Hurst, JK French, LG Cleland, and RA Gibson for their assistance in this project. Animals were collected under National Parks and Wildlife Permit No. Q05048. SR McColl is a recipient of a Mary Paxton-Gibson Research Scholarship from The Arthritis Foundation of Australia.

REFERENCES

Andrew, W. and Hickman, C. (1970) Phylogenetic survey of the blood. In: Andrew, W. and Hickman, C. (eds) Histology of the vertebrates. The C.V. Mosby Co., St. Louis. pp.166-181.

Beckman, J.K., Gay, J.C., Brash, A.R., Lukens, J.N. and Oates, J.A. (1985) Stimulation by lipoxygenase products of superoxide anion production in FMLP-treated neutrophils. Lipids 20:318-321.

Creticos, P.S., Peters, S.P., Adkinson, N.F., Nacherio, R.M., Hayes, E.C., Norman, P.S. and Lichtenstein, L.M. (1984) Peptide leukotriene release after antigen challenge in patients sensitive to ragweed. New Engl. J. Med. 310:1626-1630.

Dahlen, S.E., Hedquist, P., Hammarstrom, S. and Samuelsson, B. (1980) Leukotrienes are potent constrictors of human bronchi. Nature 288:484-486.

Duguy, R. (1970) Numbers of blood cells and their variation. In: Gans,

C. and Parsons, T.S. (eds) Biology of the Reptilia. Academic Press, New York. 3:93-110.

Feinmark, S.J., Lindgren, J.A., Claesson, H.E., Malmsten, C. and Samuelsson, B. (1980) Stimulation of human leukocyte degranulation by leukotriene B_4 and its w-oxidised products. FEBS Lett. 136:141-144.

Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, M.E. and Smith, M.J.H. (1980) Leukotriene B, a potent chemotactic and aggregating substance released from polymorphonuclear leukocytes. Nature 286:264-265.

Gibson, R.A. (1983) Australian fish - an excellent source of both arachidonic and w-3 polyunsaturated fatty acids. Lipids 18:743-752.

Gleeson, T.T., Putnam, R.W. and Bennet, A.F. (1980) Histochemical, enzymatic, and contractile properties of skeletal muscle fibers in the lizard Dipsosaurus dorsalis. J. Exp. Zool. 214:293-302.

Goetzl, E.J. and Pickett, W.C. (1980) The human PMN leukocyte chemotactic activity of complex hydroxy-eicosatetraenoic acids (HETEs). J. Immunol. 125:1789-1791.

Goldyne, M.E., Burrish, G.F., Poubelle, P. and Borgeat, P. (1984) Arachidonic acid metabolism among human mononuclear leukocytes. J. Biol. Chem. 259:8815-8819.

Granstrom, E. and Hedquist, P. (1982) Prostaglandins, thromboxanes and leukotrienes. In: Nossel, H.R. and Vogel, H.J. (eds) Pathobiology of the Endothelial Cell. Academic Press, New York. pp. 287-300.

Ham, E.A., Soderman, D.D., Zanetti, M.E., Dougherty, H.W., McGauley, E. and Kuehl, F.A. (1983) Inhibition by prostaglandins of leukotriene B_4 release from activated neutrophils. Proc. Natl. Acad. Sci. USA. 80:4349-4353.

Hansson, G., Malmsten, C. and Radmark, O. (1983) The leukotrienes and other lipoxygenase products. In: Pace-Asciak, C. and Granstrom, E. (eds) Prostaglandins and related substances. Elsevier, Amsterdam. pp. 127-169.

Hogaboom, G.K., Mong, S., Wu, H.L. and Crooke, S.T. (1983) Peptidoleukotrienes: Distinct receptors for leukotriene C_4 and D_4 in the guinea pig lung. Biochem. Biophys. Res. Commun. 116:1136-1143.

Jakschik, B.A., Sun, F.F., Lee, L.H. and Steinhoff, M.M. (1980) Calcium stimulation of a novel lipoxygenase. Biochem. Biophys. Res. Commun. 95:103-110.

Johnson, M., Carey, F. and McMillan, R.M. (1983) Alternative pathways of arachidonate metabolism: Prostaglandins, thromboxane and leukotrienes. In: Campbell, P.N. and Marshall, R.D. (eds) Essays in Biochemistry. Academic Press, New York. 19:41-141.

Kobayashi, T. and Levine, L. (1983) Arachidonic acid metabolism by erythrocytes. J. Biol. Chem. 258:9116-9121.

Kriesle, R.A. and Parker, C.W. (1983) Speific binding of leukotriene B_4 to a receptor on human polymorphonuclear leukocytes. J. Exp. Med. 157:628-641.

Lentner, C. (1984) Blood Cells. In: Lentner, C. (ed) Geigy Scientific Tables. Ciba-Geigy, Basle. 3:205-213.

McColl, S.R., Betts, W.H., Murphy, G.A. and Cleland, L.G. (1986) Determination of 5-lipoxygenase activity in human polymorphonuclear leukocytes using high-performance liquid chromatography. J. Chromatogr. 378:445-449.

Moncada, S. and Vane, J. (1982) The role of prostaglandins in platelet-vessel wall interactions. In: Nossel, H.L. and Vogel, H.J. (eds) Pathobiology of the Endothelial Cell. Academic Press, New York. pp. 253-285.

Saint Girons, M.C. (1970) Morphology of circulating blood cells. In: Gans, C. and Parsons, T.S. (eds) Biology of the Reptilia. Academic Press, New York. 3:73-92.

Tyler, M. (1976) In: Frogs. Collins, Sydney.

Volpi, M., Naccache, P., Molski, T., Shefcyk, J., Huang, C., Marsh,

M., Munoz, J., Becker, E. and Sha'afi, R. (1985) Pertussis toxin inhibits FMLP but not phorbol ester stimulated changes in rabbit neutrophils: Role of G-proteins in excitation response coupling. Proc. Natl. Acad. Sci. USA. 82:2708-2712.

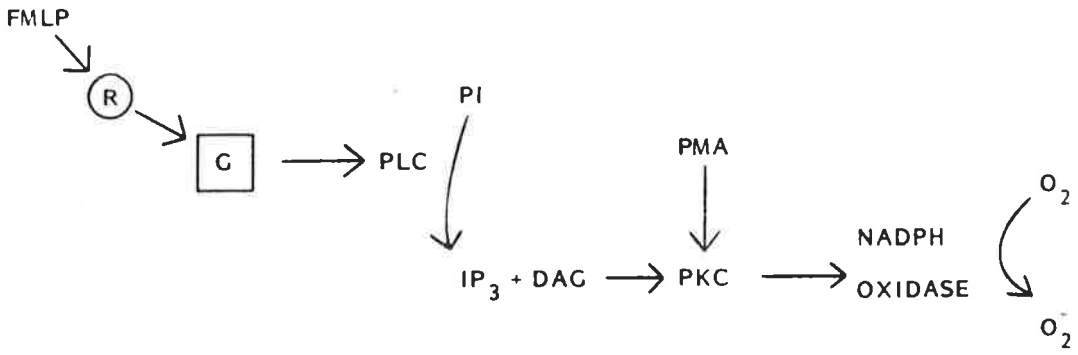
Weening, R.S., Wever, R. and Roos, D. (1975) Quantitative aspects of the production of superoxide radicals by phagocytosing human granulocytes. J. Lab. Clin. Med. 85:245-252.

Table I: Analysis of major fatty acids of human and lizard blood cells. Results expressed as a percentage of total fatty acids (mean±sem).

Fatty Acid	Common name	Erythrocytes	White blood cells
A) Human cells (n=4)			
16:0	Palmitic	24±4	18±2
18:0	Stearic	13±1	14±1
18:1 w9	Oleic	19±2	27±3
18:2 w6	Linoleic	13±1	10±1
20:4 w6	Arachidonic	10±1	11±1
B) Lizard cells (n=4)			
16:0	Palmitic	13±5	10±1
18:0	Stearic	10±3	13±1
18:1 w9	Oleic	30±6	32±4
18:2 w6	Linoleic	17±6	13±3
20:4 w6	Arachidonic	12+1	19±2

Table II: Production of eicosanoids by human and lizard white blood cells. WBC were stimulated for 15 min. with 0.1 uM A23187 and 10 um AA (see material and methods). Results expressed as ng/10^6 white blood cells (mean±sem).

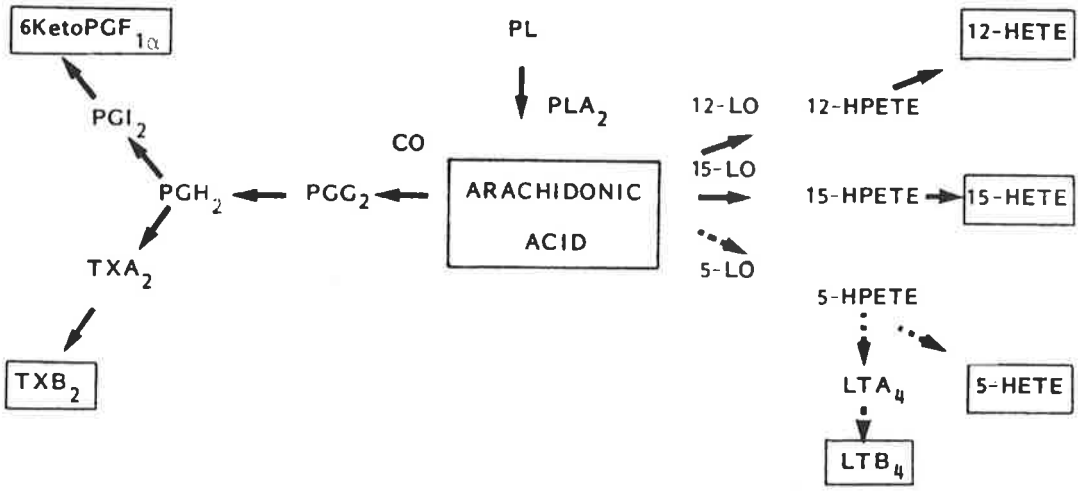
Eicosanoid	Human (n=4)	Lizard (n=4)
LTB_4	12±2	-
5-HETE	68±6	-
12-HETE	37±5	69±3
15-HETE	28±2	40±6
TXB_2	> 12.5	1.0±0.1
6-keto-$PGF_{1\alpha}$	0.6±0.1	> 12.5



Abbreviations :

FMLP	N-formyl-methionyl-leucyl-phenylalanine	IP_3	Inositol tris phosphate
R	FMLP receptor	DAG	Diacyl glycerol
G	Guanine nucleotide binding protein	PMA	Phorbol myristate acetate
PLC	Phospholipase C	PKC	Protein kinase C
PI	Phosphatidyl inositol		

Figure 1: Pathways leading to superoxide production in human phagocytes.



Abbreviations :

PL	-	Phospholipid	LT	-	Leukotriene
PLA_2	-	Phospholipase A_2	HPETE	-	Hydroperoxyeicosatetraenoic acid
LO	-	Lipoxygenase	HETE	-	Hydroxyeicosatetraenoic acid
CO	-	Cyclooxygenase			
PG	-	Prostaglandin			

→ Present in human and lizard white blood cells

⇢ Present only in human white blood cells

□ Products assayed in this study

Figure 2: The Arachidonic Acid Cascade.

APPENDIX III

EFFECTS OF PIROXICAM ON SUPEROXIDE GENERATION, PHOSPHOLIPID METHYLATION AND LEUKOTRIENE PRODUCTION BY HUMAN BLOOD MONONUCLEAR CELLS.

John K. French, Nigel P. Hurst, Shaun R. McColl, Leslie G. Cleland

Royal Adelaide Hospital-The Queen Elizabeth Hospital
Rheumatology Unit, Adelaide, South Australia.

ABSTRACT

The anti-inflammatory agent piroxicam *in vitro*, caused dose-dependent inhibition of N-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced monocyte superoxide release, but had no effect on the response to serum treated zymosan, phorbol myristate acetate or the calcium ionophore A23187. The inhibitory effect on the superoxide response to FMLP correlated with inhibition of specific ^{3}H-FMLP-binding. Piroxicam did not inhibit production of leukotriene B_4 stimulated either by A23187 or by FMLP with arachidonic acid. Piroxicam did not inhibit phospholipid methylation. Since piroxicam inhibited neither the superoxide response to zymosan, a membrane receptor mediated response, nor phospholipid methylation, the effect of this drug on FMLP receptor binding appears to be relatively selective. These data also exclude a significant effect of piroxicam

on activation of protein kinase C or the NADPH oxidase of mononuclear phagocytes.

INTRODUCTION

Stimulation of polymorphonuclear (PMN) and mononuclear phagocytes leads to activation of enzymes which produce mediators of inflammation. These enzymes include a membrane bound NADPH oxidase which generates superoxide ($O_2^{\cdot -}$) and a 5-lipoxygenase enzyme which catalyses the production of leukotrienes. Leukotrienes have potent chemotactic and degranulating actions [1]. The biological actions of superoxide and other oxyradicals in chronic inflammatory arthritides are complex, and whether their net effect is pro- or anti-inflammatory is yet to be established [2,3].

The modulation of phagocyte function is one potentially important mode of action of both nonsteroidal and slow acting antirheumatic anti-inflammatory agents [4-8]. Piroxicam is a cyclo-oxygenase inhibitor which also has inhibitory effects on human PMN functions [6], including inhibition of degranulation and superoxide release in response to certain stimuli. In these studies we have investigated the effect of piroxicam on blood mononuclear phagocytes, cells which are of key importance in the pathogenesis of chronic inflammatory disease such as rheumatoid arthritis [9]. In particular we have examined whether piroxicam causes selective or generalised inhibition of monocyte superoxide release or 5-lipoxygenase activity in response to different stimuli.

METHODS

Preparation of cell suspensions. Venous blood was drawn from healthy human volunteers, anticoagulated with 4.5% EDTA (pH 7.4) and mononuclear cells (MNC) separated on a Ficoll-Hypaque gradient [10]. The percentage of monocytes was determined by size distribution on a Coulter counter (Model ZF)[11]. Final cell suspensions were adjusted to between 0.3 and 0.8×10^6 monocytes/ml in indicator-free Hanks balanced salt solution (HBSS).

Preparation of solutions of piroxicam. Except where indicated, stock solutions of piroxicam were prepared in dimethyl sulphoxide (DMSO) and stored at -20°C. Solutions were diluted in Ca^{++}/Mg^{++}-free Dulbecco's phosphate buffered saline (DPBS) immediately prior to use and the pH adjusted slowly to 7.4 with 0.1 M NaOH.

Preparation of stimuli. To prepare serum treated zymosan (STZ), zymosan (Sigma) was incubated in fresh human serum (2:1 w/v) at 37°C for 30 minutes, washed twice, resuspended in HBSS (20 mg/ml) and stored frozen in aliquots. Phorbol myristate acetate (PMA) (Sigma) was dissolved in DMSO (1 mg/ml), stored at -10°C and diluted to 10 ug/ml in Ca^{++}/Mg^{++}-free DPBS (pH 7.4) immediately prior to use. FMLP (Sigma) was dissolved in DMSO (2 mg/ml), stored at 4°C and diluted with DPBS to 2 ug/ml immediately prior to use. A23187 and arachidonic acid (AA) (Sigma) were dissolved in methanol (1 mM and 500 uM respectively). A23187 was stored at 4°C whereas AA was stored under nitrogen at -70°C and 5 ul (or an equal volume of methanol) was added directly to the cells. The final concentrations of methanol or DMSO

did not exceed 0.5 or 0.1% respectively and had no effect on release or detection of superoxide or leukotrienes.

Measurement of superoxide production. Superoxide production was measured by reduction of cytochrome C using an extinction coefficient (550-540nm) of 21.1 $mM^{-1}.cm^{-1}$ [12]. MNC (700 ul) were preincubated at 37°C for 20 min. with each concentration of piroxicam before addition of 100 ul cytochrome C (Sigma Type III)(100 uM final) and appropriate stimulus (100 ul, except A23187). Cells were stimulated with FMLP (0.2 ug/ml) for 10 min., STZ (2 mg/ml) for 30 min., PMA (1 ug/ml) for 30 min. or 5 uM A23187 for 10 min. Each experiment was performed in quadruplicate (unless otherwise stated). Continuous time course experiments were performed on a Varian DM-100S double beam spectrophotometer attached to a Varian DS-15S computer. Results of inhibitory studies are expressed as percentage of control value (no piroxicam added). Cell viability was confirmed to be ↑95% by trypan blue exclusion. Control studies confirmed that contaminating lymphocytes did not affect the performance of the assay (not shown).

Measurement of phospholipid methylation. Phospholipid (PL) methylation was measured as described previously [13]. 450 ul of MNC ($2x10^6$/ml) were preincubated at 37°C with or without piroxicam at specified concentrations in quadruplicate for 20 min., then 10 uCi of L-[^{3}H-methyl]-methionine (New England Nuclear 80 Ci/mmol) were added and cells incubated for a further 60 min. The reaction was stopped by the addition of cold 10% trichloroacetic acid. The cells were washed twice, resuspended in 2 mls of DPBS and PLs extracted with 5 mls of chloroform/methanol (2:1 v/v). Aliquots of the lipid extract were

transferred to scintillation vials and evaporated to dryness before counting in 5 mls scintillation fluid (0.4% PPO; 70% toluene; 30% ethanol). Incorporation of ^{3}H-methyl from L-[^{3}H-methyl]-methionine into phosphatidyl choline was confirmed by thin layer chromatography, using authentic PL standards (Sigma) (14).

Measurement of ^{3}H-FMLP binding. To measure FMLP binding to monocytes, incubations were performed in quintuplicate at 37^{o} in 96 well filtration plates manufactured with an integral 5 um pore size filter at the bottom of each well (Millipore, STGV 09610). The plates were precoated with unlabelled FMLP, then washed and dried as below. MNC (100 ul, containing 1X10^{6} monocytes) were incubated in Tris buffer (containing 100 mM Tris; 10 mM NaN_3; 1.6 mM $CaCl_2$; 11,000 U/ml catalase; 5 ug/ml cytochalasin B; pH adjusted to 7.2 with HCl) for 20 min. with or without piroxicam. ^{3}H-FMLP (2 uM) with or without 1000x excess unlabelled FMLP was added, the cells incubated for a further 30 min., washed 5x with DPBS containing 0.01 M Tris at pH 7.2. Washing was performed by placing the Millipore plate over a vacuum manifold (Millipore, XX2809600) which draws the supernatant through the bottom of each well. Further buffer was added to the wells for each wash. After washing, the plate was dried at 37^{o}C, the filter from each well was cut out and bound ^{3}H-FMLP measured by liquid scintillation counting.

Measurement of leukotrienes. The 5-lipoxygenase products of MNC were quantified using modifications of a method published previously [15]. Briefly, leukotrienes were extracted in chloroform/methanol (7:3 v/v) and chromatographed by reverse-phase HPLC. All

chromatographic equipment was from Waters Millipore. The system comprised a Model 510 pump, 730 Data Module, 490 programmable UV detector and a 710B WISP autoinjector. Solvent conditions were methanol/water/acetic acid (71:29:0.02 v/v/v) at pH 5.6 (adjusted with concentrated NH_4OH). Separations were carried out using a Waters Nova-Pak C_{18} column (15 cm x 3.9 mm) at 1.5 ml/min. Detection of leukotriene B_4 (LTB_4) and prostaglandin B_2 was achieved at 270 nm. 50 ng of prostaglandin B_2 (Sigma) was added to cells as internal standard prior to leukotriene extraction.

RESULTS

Control studies:

Initial control studies were performed to establish times course of monocyte superoxide production. Typical time courses for monocyte superoxide release stimulated by PMA, STZ, A23187, and FMLP are shown (Figure).

Effect of piroxicam on monocyte superoxide release:

Piroxicam caused dose dependent-inhibition of FMLP-induced monocyte superoxide release, but did not affect superoxide release stimulated by STZ, PMA or A23187 (Table 1). As previous studies have used a variety of different solvents to dissolve piroxicam [6-8], we also examined the effect of piroxicam dissolved in either DMSO, sodium hydroxide or methanol. In each case the effect of piroxicam on FMLP-induced superoxide release was identical (data not shown).

Effect of piroxicam on ^{3}H-FMLP-binding to monocytes:

We next examined whether the effect of piroxicam on FMLP-induced superoxide release was due to an effect on receptor binding. 100 uM piroxicam inhibited ^{3}H-FMLP binding to monocytes by 38% - a similar extent to the inhibition of monocyte superoxide release (Table 2).

Effect of piroxicam on phospholipid methylation:

Preincubation of MNC for 20 min. with up to 100 uM piroxicam had no effect on basal rates of phospholipid methylation as measured by incorporation of ^{3}H-methyl groups into phospholipid (Table 3).

Effect of piroxicam on 5-lipoxygenase activity:

Preincubation of MNC for 20 min. with up to 100 uM piroxicam had no effect on LTB_4 production stimulated by either 5 uM A23187 or 1 uM FMLP with 2.5 uM AA. FMLP, PMA or AA alone do not stimulate production of LTB_4 (Table 4).

DISCUSSION

Recent advances in the understanding of the intracellular pathways leading to activation of the phagocyte NADPH oxidase and superoxide release have provided a conceptual model [16,17,18], for the investigation of potential sites of anti-inflammatory drug action. Receptor occupancy by an external stimulus or ligand (e.g. FMLP or STZ) leads to activation of phosphoinositidase C, which hydrolyses phosphatidyl inositol bisphosphate to generate two second messengers inositol trisphosphate (ITP) and diacylglycerol (DG). These molecules respectively raise intracellular Ca^{++} and activate PKC. Ca^{++} and DG act synergistically in the activation of PKC. Recent evidence

suggests that PKC directly phosphorylates and activates the NADPH oxidase [19,20]. Calcium ionophores and phorbol esters can be used to mimic the effects of ITP and DG respectively, thereby activating the NADPH oxidase independently of surface receptors or inositol lipid hydrolysis [21].

In these studies we investigated the effect of piroxicam on the monocyte superoxide response stimulated either by two surface receptor dependent agonists, FMLP and STZ, or by A23187 or PMA. We report that piroxicam inhibited FMLP-stimulated monocyte superoxide release but had no effect on the response to the other stimuli used in this study. Furthermore, piroxicam (100 uM) inhibited specific binding of ^{3}H-FMLP to the same extent as FMLP-induced superoxide release, suggesting an effect on membrane function. These data exclude any significant direct effect of piroxicam on the activation of PKC, or the NADPH oxidase. The effects of piroxicam on FMLP-binding and FMLP-induced superoxide release by monocytes are similar to those obtained using PMN [6,7]. However our data on PMA-stimulated monocytes differs from studies on PMN which showed that piroxicam inhibited PMA-stimulated superoxide release and PMA-dependent activation of the membrane NADPH-oxidase in PMN [8]. The reason for these differences between PMN and monocytes is not clear and may be technical or biological.

Interestingly, although piroxicam decreased FMLP-binding, it did not inhibit production of LTB_4 stimulated by FMLP with AA. This suggests that the 5-lipoxygenase and NADPH oxidase may either be linked to different classes of FMLP receptors, with different sensitivities to piroxicam, or that their requirements for receptor occupancy are different. The latter is supported by previous studies

which have shown that different proportions of total formyl peptide receptor occupancy are required for different cellular responses; while 20-30% receptor occupancy is required for superoxide release, less than 2% occupancy is required for maximal changes in cytosol calcium [22]. This suggests that 5-lipoxygenase, which is activated by calcium, may require a lower level of receptor occupancy for activation than the NADPH oxidase.

In addition to having no effect on 5-lipoxygenase activation stimulated by FMLP with AA, piroxicam did not inhibit LTB_4 release in MNC stimulated by A23187. By raising cytosol Ca^{++}, A23187 activates both phospholipase A_2-dependent release of AA as well as the 5-lipoxygenase enzyme. Thus piroxicam inhibits neither phospholipase A_2 nor 5-lipoxygenase.

Since the effect of piroxicam on ^{3}H-FMLP-binding may reflect an effect on membrane fluidity and disturbance of membrane architecture [6], we examined its effect on phospholipid methylation. PL methylation is a minor pathway of synthesis of phosphatidyl choline which proceeds via successive methylation of the N-terminal head group of phosphatidyl ethanolamine. Its possible importance includes the synthesis of an AA-rich pool of phosphatidyl choline [23], maintenance of membrane asymmetry and a role in transmembrane signalling [24]. We found that piroxicam had no effect on this pathway suggesting that piroxicam does not cause a generalised disturbance of membrane function. This contrasts with the antimalarial agents, mepacrine and chloroquine, both of which inhibit PL methylation[5].

In conclusion, these studies show that piroxicam selectively inhibits the superoxide response of monocytes to the chemotactic stimulus FMLP by interfering with receptor binding. Piroxicam had no effect on 5-lipoxygenase activity or phospholipid methylation. Further studies will be required to determine whether the effect on FMLP receptors contributes significantly to the mechanism of action of this anti-inflammatory agent *in vivo*.

ACKNOWLEDGEMENTS

The authors wish to thank Dr WH Betts for helpful advice, and Ms L Gorjatschko for technical assistance. This work was supported by grants from the National Health & Medical Research Council of Australia, Arthritis Foundation (Australia) and Arthritis Foundation (South Australia), and The Queen Elizabeth Hospital Research Foundation, and Pfizer Pty Ltd. JKF was supported by Dawes/Curren/Hughes and NH & MRC Postgraduate Medical Scholarships, and SRMcC by a Mary Paxton-Gibson Postgraduate Research Scholarship from The Arthritis Foundation of Australia.

REFERENCES

1. Hammarstrom S. Leukotrienes. Ann Rev Biochem 1983; 52: 355-77.

2. Halliwell B, Gutteridge JMC. The importance of free radicals and catalytic metals in human diseases. Molec Aspects Med 1985; 8: 89-193.

3. Hurst NP, Bell AL, Nuki G. Studies of the effect of D-penicillamine and sodium aurothiomalate therapy on superoxide anion production by monocytes from patients with rheumatoid arthritis: evidence for in vivo simulation of monocytes. Ann Rheum Dis 1986; 45: 37-43.

4. Simchowitz L, Mehta J, Spilberg I. Chemotactic factor-induced generation of superoxide radicals by human neutrophils. Arthritis Rheum 1979; 22: 755-763.

5. Hurst NP, French JK, Bell AL, Nuki G, O'Donnell ML, Betts WH, Cleland LG. Differential effects of mepacrine, chloroquine, and hydroxychloroquine on superoxide anion generation, phospholipid methylation and arachidonic acid release by human blood monocytes. Biochem Pharmacol 1986; 35: 3083-3089.

6. Kaplan HB, Edelson HS, Korchak HM, Given WP, Abramson S, Weissmann G. Effects of non-steroidal anti-inflammatory agents on human neutrophil functions in vitro and in vivo. Biochem Pharmacol 1984; 33: 371-378.

7. Minta JO, Williams MD. Some nonsteroidal anti-inflammatory drugs inhibit the generation of superoxide anions by activated polymorphs by blocking ligand-receptor interactions. J Rheumatol 1985; 12: 751-757.

8. Biemond P, Swaak AJG, Penders JMA, Beindorff CM, Koster JF. Superoxide production by polymorphonuclear leucocytes in

rheumatoid arthritis and osteoarthritis: in vivo inhibition by the antirheumatic drug piroxicam due to interference with the activation of the NADPH-oxidase. Ann Rheum Dis 1986; 45: 249-255.

9. Nathan CF, Cohn ZA. Cellular components of inflammation; monocytes and macrophages. In: Kelley WN, Harris ED, Ruddy S, Sledge CB, eds. "Textbook of Medicine" Philadelphia: WB Saunders, 1985: 144-69.

10. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 1968; 21 (supplement 97): 77-89.

11. Loos H, Blok-Schut H, Kipp B, van Doorn R, Meerhof L. Size distribution, electronic recognition and counting of human blood monocytes. Blood 1976; 48: 743-53.

12. Weening RS, Wever R, Roos D. Quantative aspects of the production of superoxide radicals by phagocytosing human granulocytes. J Lab Clin Med 1975; 85: 245-52.

13. Bonvini E, Bougnoux P, Stevenson HC, Miller P, Hoffman T. Activation of the oxidative burst in human monocytes is associated with inhibition of methionine- dependent methylation of neutral lipids and phospholipids. J Clin Invest 1984; 73: 1629-37.

14. Niwa Y, Sakane T, Taniguchi S. Phospholipid transmethylation in the membrane of human neutrophils and lymphocytes. Arch Biochem Biophys 1984; 234: 7-14.

15. McColl SR, Betts WH, Murphy GA, Cleland LG. Determination of 5-lipoxygenase activity in human polymorphonuclear leucocytes using high-performance liquid chromatography. J Chromatogr 1986; 378: 444-49.

16. Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 1984; 308: 693-98.

17. Berridge MJ, Irvine RF. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 1984; 312: 315-321.

18. Hurst NP, The molecular basis of phagocyte activation. Ann Rheum Dis 1986; in press.

19. Segal AW, Heyworth PG, Cockcroft s, Barrowman MM. Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a M_r-44000 protein. Nature 1985; 316 : 547-49.

20. Papini E, Grzeskowiak M, Bellavite J, Rossi F. Protein kinase phosphorylates a component of the NADPH oxidase of neutrophils. FEBS Lett 1985; 190: 204-208.

21. Takenawa T, Ishitoya J, Homma Y, Kato M, Nagai Y. Role of enhanced inositol phospholipid metabolism in neutrophil activation. Biochem Pharm 1986; 34: 1931-35.

22. Sklar LA, Hyslop PA, Oades ZG, Omann GM, Jesaitis AJ, Painter RG, Cochrane CG. Signal transduction and ligand-receptor dynamics in the human neutrophil. J Biol Chem 1985; 260: 11461-467.

23. Crews FT, Morita Y, McGivney A, Hirata F, Siragnian RP, Axelrod J. IgE-mediated histamine release in rat basophilic leukemia cells: receptor activation, phospholipid methylation, Ca^{++} flux and release of arachidonic acid. Arch Biochem Biophys 1981; 121: 561-571.

24. Hirata F, Axelrod J. Phospholipid methylation and biological signal transmission. Science 1980; 209: 1082-90.

Table 1. The effect of piroxicam on monocyte superoxide production.

Stimulus	n	Piroxicam (uM)		
		1	10	100
PMA	3	101 ± 3*	100 ± 3	98 ± 10
STZ	6	95 ± 7	103 ± 5	94 ± 8
A23187	2	95 ± 7	94 ± 6	97 ± 8
FMLP	5	95 ± 2	84 ± 9	69 ± 6

*Results expressed as mean (± sem) % of control (no drug added). n = number of separate experiments.

Table 2. The effect of piroxicam on ^{3}H-FMLP binding to monocytes.

Piroxicam (uM)	specific ^{3}H-FMLP binding
0	100
1	93 ± 9
10	92 ± 5
100	61 ± 12

^{3}H-FMLP binding to monocytes determined after 20 minutes preincubation with piroxicam. Results expressed as mean ± sem of control (n = 5 experiments).

Specific binding in control (mean ± sem) = 11.8 ± 3 fmoles FMLP/ 10^{6} monocytes

Table 3. The effect of piroxicam on phospholipid methylation in mononuclear cells.

Piroxicam (uM)	^{3}H methyl incorporation*
0	100
1	86 ± 5
10	100 ± 5
100	86 ± 3

After 20 minutes preincubation with piroxicam at 37°C, [^{3}H-methyl]-L-methionine was added, the incubation continued for a further 60 minutes, and ^{3}H-methyl incorporation into PL determined.
*Results expressed as mean ± sem of control (n = 2 experiments).
Methylation rate in control (mean ± sem) = 118 ± 4 fmoles/10^{6}.MNC.

Table 4. The effect of piroxicam on leukotriene B_4 production in mononuclear cells

Stimulus	Product	Concentration of Piroxicam (uM)			
		0	1	10	100
A23187	LTB_4	100*	92 ± 8	100 ± 3	104 ± 1
FMLP + AA	LTB_4	100*	108 ± 3	92 ± 3	100 ± 3

After 20 min preincubation with piroxicam, cells were stimulated with either 5 uM A23187 or 1 uM FMLP and 2.5 uM AA for 5 min and LTB_4 measured. * Results expressed as mean ± sem % of control (n=3 experiments). Control values were 10 ± 0.4 ng $LTB_4/10^6$ MNC stimulated by A23187 and 18 ± 0.2 ng LTB_4 stimulated by FMLP with AA. PMA, FMLP, or AA alone did not stimulate detectable LTB_4 release.

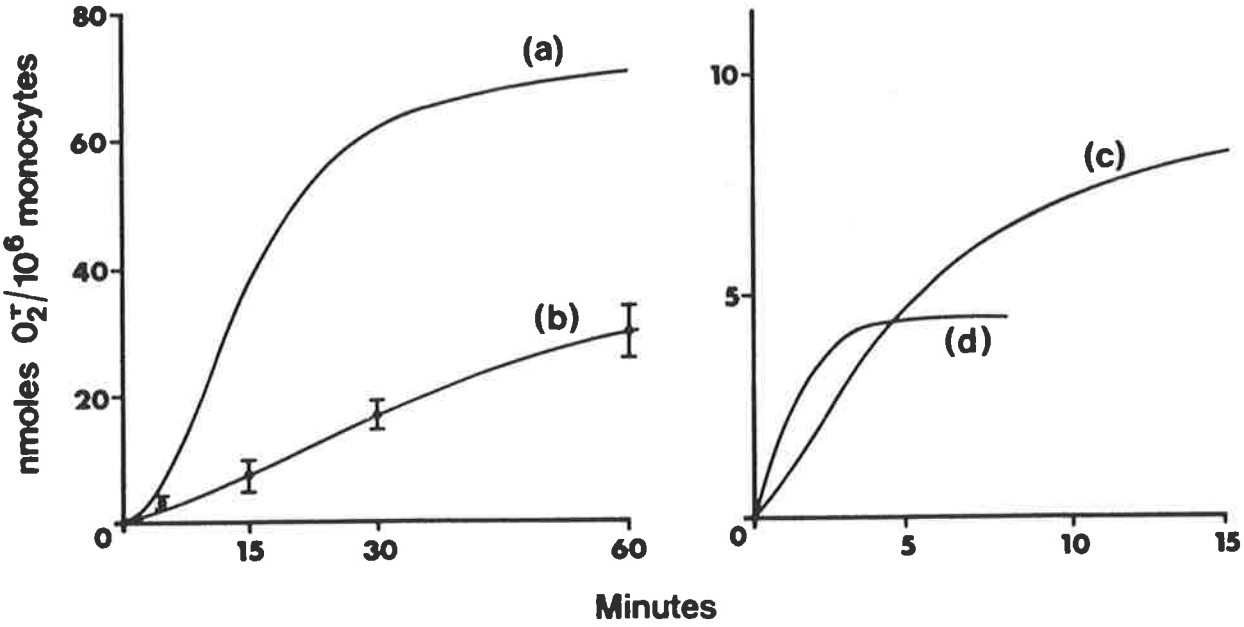


Figure: Time courses of monocyte superoxide release.

Monocyte superoxide production stimulated by (a) PMA (b) STZ (c) A23187 (d) FMLP. (a), (c) and (d) are continuous traces of superoxide production obtained from a double beam spectrophotometer. (b) represents measurements of superoxide release at separate time points (mean ± sem, n=6).

APPENDIX IV.

Journal of Chromatography, 378 (1986) 444—449
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3087

Note

Determination of 5-lipoxygenase activity in human polymorphonuclear leukocytes using high-performance liquid chromatography

S.R. McCOLL*, W.H. BETTS, G.A. MURPHY and L.G. CLELAND

Rheumatology Unit, The Queen Elizabeth Hospital, Woodville Road, Woodville, South Australia 5011 (Australia)

(First received October 1st, 1985; revised manuscript received January 2nd, 1986)

Products of arachidonate metabolism (prostaglandins, leukotrienes and thromboxanes) are of central importance in vascular/platelet homeostasis [1], reproduction [2], allergy [3—5] and inflammation [4—6]. A variety of analytical procedures have been used to quantify and/or isolate these products. The procedures used range from bioassay [7,8], radioimmunoassay (RIA) [9,10], thin-layer chromatography (TLC) [11,12], high-performance liquid chromatography (HPLC) [13—16] and gas chromatography—mass spectrometry (GC—MS) [13,15,17]. All methods suffer from disadvantages. For example, quantification in bioassay is difficult and may lack specificity. RIA, which affords high sensitivity, necessitates the use of radioisotopes and suffers occasionally from insufficient antibody specificity to discriminate between closely related compounds. TLC allows a broad range of products to be detected but is not suitable for quantification of biological samples without use of radiolabelled precursor molecules. GC—MS provides not only specific and very sensitive results but also structural information. However, constraints of cost, time involved in sample preparation and assay time limit this method to special studies. HPLC provides both specific and reproducible results combined with great sensitivity and is an ideal method for simultaneous analysis of compounds which are chemically closely related. Use of radioisotopes is not necessary in the case of compounds with favorable UV absorption or fluorescent characteristics.

EXPERIMENTAL

Chemicals

Calcium ionophore A23187, 15-lipoxygenase (soy-bean), sodium tetraborate

0378-4347/86/$03.50

and arachidonic acid (AA) were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium borohydride was purchased from Laboratory Chemicals (Ajax Chemicals, Auburn, Australia) and leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE) standards were a generous gift from Merck, Sharp and Frosst (Canada) and the Department of Organic Chemistry at the University of Adelaide. All solvents (HPLC grade) were purchased from Waters Millipore (Milford, MA, U.S.A.). 15-HETE was biosynthesised as described below.

Apparatus

All chromatographic equipment was from Waters Assoc. The system included a Model 510 pump, 730 data module, 490 variable-wavelength UV detector and 710B WISP autoinjector. The mobile phase for HPLC was methanol—water—acetic acid (72:28:0.08) and the pH was 6.2 (adjusted with ammonium hydroxide). The separations were carried out using a Waters Nova-Pak C_{18} (15 cm × 3.9 mm) column (5 μm average particle size) at a flow-rate of 1.0 ml/min. A C_{18} guard column (10 μm) was used at all times. Chromatography was carried out at room temperature and the mobile phase was constantly bubbled with helium to remove dissolved oxygen. Detection of HETEs was carried out at 234 nm UV at 0.02 a.u.f.s. and LTB_4 at 280 nm UV at 0.005 a.u.f.s. Identification of compounds was verified using pure standards.

Method for purification of blood cells

Human polymorphonuclear leucocytes (PMNLs) were isolated, stimulated and their 5-lipoxygenase products extracted as follows: whole blood was taken from healthy volunteers by venepuncture and mixed with 4.5% EDTA (5:1). Red blood cells were sedimented at 37°C by addition of dextran (5:1 of Dextraven 150). The white blood cell rich supernatant was carefully layered onto a double Percoll (Pharmacia, Uppsala, Sweden) gradient (specific gravities of 1.070 and 1.092) and spun at 450 *g* for 20 min. The PMNLs were collected from the interface between the two Percoll layers, washed twice in Dulbecco's phosphate-buffered saline before a final resuspension in Hank's balanced salt solution at the desired cell concentration (approximately $2 \cdot 10^6$ cells per ml). Using this procedure cell purity was greater than 97% as determined by gentian violet staining. Cells were stimulated by addition of 10 μl of 1 m*M* AA followed rapidly by 5 μl of 1 m*M* A23187 to 1 ml of cells at 37°C and the reaction was terminated after 2 min by addition of 5 ml of ethyl acetate. Internal standard (170 ng of 15-HETE) was added and the samples were vortexed for 15 s and centrifuged at 1000 *g* for about 5 min. The ethyl acetate layer was withdrawn and evaporated to dryness under vacuum using a centrifugal evaporator (Savant, Hicksville, NY, U.S.A.) and the samples were reconstituted in 100 μl of methanol. Aliquots of 25 μl were injected onto the column (Fig. 1). Standard curves were performed for each experiment.

Biosynthesis of 15-HETE

15-Lipoxygenase (10 μl, 7350 U) was added to 1 ml of sodium tetraborate buffer (200 m*M* at pH 9.0) at 25°C followed by 10 μl of 1 *M* AA. The reaction mixture was stirred for 20 min prior to addition of 10 μl of 100 mg/ml sodium

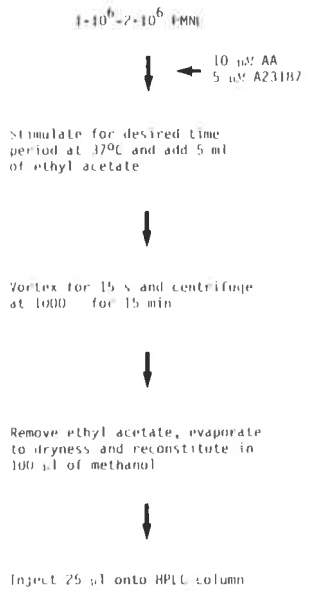


Fig. 1. Procedure for stimulation of cells and extraction of AA metabolites prior to HPLC.

borohydride [added to reduce 15-hydroperoxyeicosatetraenoic acid (15-HPETE) to 15-HETE]. The reaction mixture was then stirred continuously overnight. 15-HETE was extracted into ethyl acetate, evaporated to dryness and reconstituted in benzene (for storage) at a concentration of 0.19 m*M*. Aliquots were dried and reconstituted in methanol for use as internal standard. Quantification was performed in methanol using a spectrophotometer (E = 30 500 at 234 nm [14]). 15-HETE (170 ng) was added as internal standard.

Synthesis and determination of radiolabelled AA metabolites

Isolated neutrophils were stimulated for 2 min at 37°C with 0.1 μCi [^{14}C]AA (40—60 mCi/mmol) and 5 μM A23187. The AA metabolites were extracted, evaporated to dryness and reconstituted prior to HPLC (as described above). The samples were monitored by UV spectrophotometry and 1-min aliquots were collected for liquid scintillation counting.

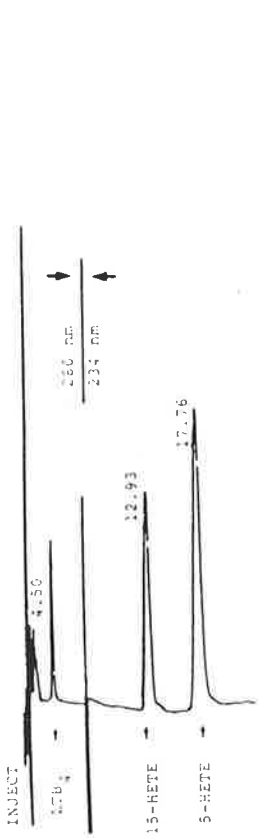
RESULTS

Chromatography

Figs. 2 and 3 are typical chromatograms of LTB_4, 5-HETE and 15-HETE. Fig. 2 shows a chromatogram of a mixture of pure standards and Fig. 3 shows a typical chromatogram of a sample from cells stimulated with A23187. The k' values for LTB_4, 15-HETE and 5-HETE were 3.2, 11.8 and 16.2 min, respectively.

Recovery

The procedure for stimulation, extraction and chromatographic preparation was found to be rapid and free from interferences. Recoveries were found to be



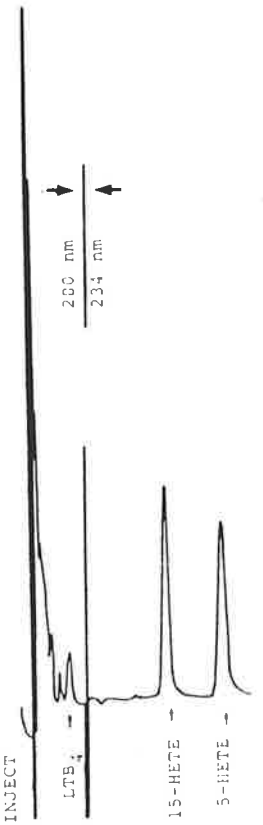


Fig. 2. Chromatogram of pure standards.

Fig. 3. Chromatogram of cell samples stimulated for 2 min with 5 μM A23187 and 10 μM AA (final concentrations).

58% for LTB_4 (range 0–50 ng) and 92% for the mono-HETEs (range 0–250 ng).

Standard curves and sensitivity

Standard curves were obtained for each experiment in the range 0–250 ng (5-HETE) and 0–50 ng (LTB_4). Using the linear regression analysis, the coefficients of correlation for 5-HETE and LTB_4 were 0.999 and 0.996, respectively. Sensitivity was less than 1 ng (as determined at a signal-to-noise ratio of 2:1) when extracted from $1 \cdot 10^6$–$5 \cdot 10^6$ cells.

Precision

Coefficients of variation for both intra- and inter-assay variability were calculated for both 5-HETE and LTB_4. Intra-assay variability was determined by measuring quadruplicate samples at high and low levels of the respective metabolites following production by cells. The low level (point A) was obtained by measuring products in cell samples stimulated for 30 s and the high level (point B) by measuring products after 5 min of stimulation (see Figs. 4 and 5). The coefficients of variation of the assay for 5-HETE at these two points were 6.5 and 5.9% and those for LTB_4 were 9.8 and 12.6% (points A and B, respective-

448

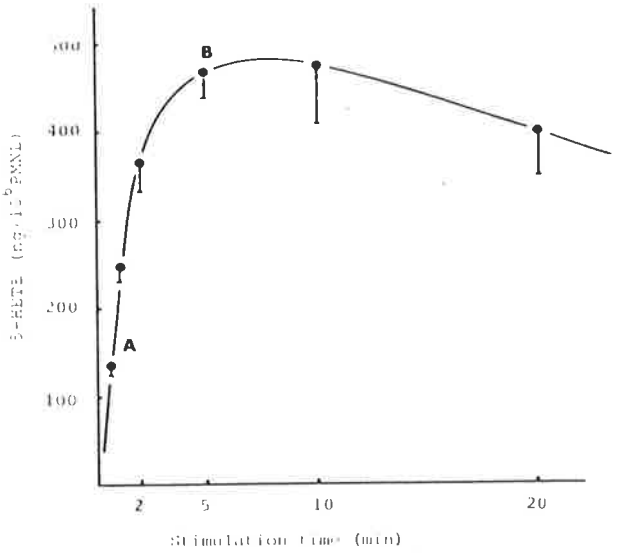


Fig. 4. Time course of production of 5-HETE. $1 \cdot 10^6$ PMNLs were stimulated with 5 μM A23187 and 10 μM AA for designated time periods.

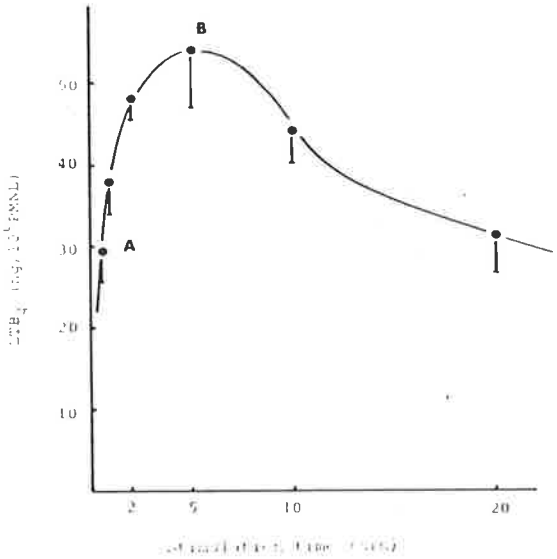


Fig. 5. Time course of production of LTB_4. $1 \cdot 10^6$ PMNLs were stimulated with 5 μM A23187 and 10 μM AA for designated time periods.

ly). The value for inter-assay variability was obtained by quadruplicate measurement of a known standard in separate assays over a period of twenty experimental days by two analysts. The coefficients of inter-assay variation were 5.6% for LTB_4 and 4.2% for 5-HETE.

Choice of internal standard

Cells stimulated with 5 μM A23187 and radiolabelled AA produced large quantities of ^{14}C-labelled LTB_4 and 5-HETE. No ^{14}C-labelled 15-HETE was produced under these conditions.

DISCUSSION

Several HPLC methods for analysis of leukotrienes and hydroxy fatty acids have been described [13—16]. The present assay was specifically designed to measure the two major eicosanoid products PMNLs, 5-HETE and LTB_4. A 10-ml volume of venous blood is sufficient to produce quantifiable amounts of these products using this method. The extraction and guard column clean-up procedures are simple and rapid compared to other methods, and the analysis time for assaying both LTB_4 and 5-HETE is ca. 20 min. Using this technique we experienced no difficulty in resolving LTB_4 from its isomers and ω-oxidation products. Sensitivity and precision are high.

Under the experimental conditions described, no 15-HETE was detected. There have been several previous reports indicating that PMNLs synthesize 15-HETE [18, 19] but these studies were not performed using intact PMNLs. Therefore, in an intact-PMNLs experimental system, 15-HETE is a suitable internal standard, providing the correct controls are performed.

The assay cannot be used to measure the isomers or the ω-oxidation products of LTB_4 or the peptidoleukotrienes without significantly increasing analysis time. Therefore, in its current form the assay is limited to measuring the two major biologically active 5-lipoxygenase products in inflammatory cells such as PMNLs.

ACKNOWLEDGEMENT

This work was supported in part by The Arthritis Foundation of Australia (S.A.).

REFERENCES

1 S. Moncada and J. Vane, in H. Nossel and H. Vogel (Editors), Pathobiology of the Endothelial Cell, Academic Press, New York, 1982, p. 253.
2 A. Norstrom, Prostaglandins, 23 (1982) 361.
3 P. Borgeat and P. Sirois, J. Med. Chem., 24 (1981) 121.
4 J. O'Flaherty, Lab. Invest., 47 (1982) 314.
5 B. Samuelsson, Int. Arch. Allergy Appl. Immunol., 66 (Suppl. 1) (1981) 98.
6 G. Weissmann, J. Smolen and H. Korchak, N. Engl. J. Med., 303 (1980) 27.
7 R. Murphy, S. Hammarstrom and B. Samuelsson, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 4275.
8 M. Palmer, R. Stepney, G. Higgs and K. Eakins, Prostaglandins, 20 (1980) 411.
9 J. Salmon, P. Simmons and R. Palmer, FEBS Lett., 146 (1982) 18.
10 J. Salmon, P. Simmons and R. Palmer, Prostaglandins, 24 (1982) 225.
11 W. Stenson, C. Parker and T. Sullivan, Biochem. Biophys. Res. Commun., 96 (1980) 1045.
12 B. Jakschik and C. Kuo, Prostaglandins, 25 (1983) 767.
13 B. Jakschik, A. Morrison and H. Sprecher, J. Biol. Chem., 258 (1983) 12797.
14 F. Sun and J. McGuire, Biochim. Biophys. Acta, 794 (1984) 56.
15 P. Borgeat and B. Samuelsson, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 2184.
16 T. Lee, J. Mencia-Huerta, C. Sih, E. Corey, R. Lewis and K. Austen, J. Clin. Invest., 74 (1984) 1922.
17 J. MacDermot, C. Kelsey, K. Waddell, R. Richmond, R. Knight, P. Cole, C. Dollery, D. Landon and I. Blair, Prostaglandins, 27 (1984) 163.
18 E. Goetzl and F. Sun, J. Exp. Med., 150 (1979) 406.
19 J. McGuire, J. McGee, N. Crittenden and F. Fitzpatrick, J. Biol. Sci., 260 (1985) 8316.

Vol. 141, No. 2, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 15, 1986 Pages 399-404

MODULATION BY PHORBOL MYRISTATE ACETATE OF ARACHIDONIC ACID RELEASE AND LEUKOTRIENE SYNTHESIS BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES STIMULATED WITH A23187

Shaun R. McColl*, Nigel P. Hurst, and Leslie G. Cleland

The Queen Elizabeth and Royal Adelaide Hospital Rheumatology Units, Adelaide, South Australia

Received September 25, 1986

SUMMARY: Phorbol myristate acetate augmented the release of ^{3}H-AA and the synthesis of leukotriene B_4 and 5-hydroxyeicosatetraenoic acid by human polymorphonuclear leukocytes stimulated by A23187. PMA alone had no effect. Enhancement of the response to A23187 was not seen when the inactive phorbol ester 4-alpha phorbol didecanoate was added with A23187. These data are consistent with the hypothesis that activation of protein kinase C enhances AA release and metabolism in stimulated polymorphonuclear leukocytes.

INTRODUCTION: Activation of PMNL by A23187 stimulates release of AA and subsequent metabolism by 5-LO to LTB_4 and 5-HETE. Addition of exogenous AA enhances the A23187-induced synthesis of leukotrienes [1]. In contrast, PMA even in the presence of exogenous AA does not stimulate 5-LO activity in human PMNL although it is a potent activator of other PMNL functions such as superoxide anion release [2]. Most studies of leukotriene synthesis by human PMNL have involved stimulation of cells with 5-10 uM A23187 alone or in the presence of AA (10-250 uM). Although there is a requirement for a rise in cytosolic Ca^{++} and a supply of either exogenous or endogenous AA as substrate, the sequence of physiological signals involved in production of leukotrienes in intact PMNL remains uncertain. Furthermore, it is not clear whether the addition of exogenous AA

*To whom correspondence should be addressed: Rheumatology Unit, The Queen Elizabeth Hospital, Woodville, South Australia, 5011.

ABBREVIATIONS: PMA, phorbol 12-myristate, 13-acetate; PMNL, polymorphonuclear leukocyte; LTB_4, 5s,12r dihydroxyeicosatetraenoic acid; 5-HETE, 5 hydroxyeicosatetraenoic acid; AA, arachidonic acid; PDD, 4-alpha phorbol didecanoate; 5-LO, 5-lipoxygenase; PKC, protein kinase C; PLA_2, phospholipase A_2; LDH, lactate dehydrogenase

0006-291X/86 $1.50

stimulates leukotriene production by providing additional substrate, or by some other mechanism such as increasing "peroxide tone" [3] or stimulation of PKC [4].

A number of studies on activation of PMNL via surface receptors have implicated the bifurcating signalling pathway involving phosphoinositidase C-dependent hydrolysis of phosphatidylinositol 4,5 bisphosphate and release of diacylglycerol and inositol polyphosphates [5]. These two second messengers are believed respectively to activate PKC and to stimulate Ca^{++} release from internal stores. Their effects can be mimicked by phorbol esters and A23187. In a variety of cellular systems, including PMNL, these agents act synergistically to elicit functional responses [6]. The A23187-induced rise in cytosol Ca^{++} is believed to have a synergistic effect on PKC activation by promoting translocation and binding of PKC to the cell membrane and both signals appear to be required for complete cellular responses [7,8,].

Previous reports using platelets [9] and macrophages [10] have shown that PMA enhances the A23187-induced synthesis of leukotrienes. This effect may be mediated by increased AA release secondary to phosphorylation and inhibition of lipomodulin [9,10]. In view of these reports, we have examined the effect of PMA on the release and metabolism of AA in isolated human PMNL stimulated with A23187.

MATERIALS & METHODS: A23187 (1 mM stock solution in methanol), PMA and PDD (1 mg/ml stock solution in DMSO), EDTA, DMSO and PGB_2 were from Sigma Chemical Co. (St. Louis, Mo, USA), ^{3}H-AA (80-135 Ci/mmol) was from Amersham, (Buckinghamshire, England), Dextraven 150 from Fisons Ltd. - Pharmaceutical Division, (Loughborough, England), Percoll from Pharmacia AB, Laboratory Separation Division, (Uppsala, Sweden), solvents from Waters Associates, (Milford, Mass., USA) and citric acid from Univar, Ajax Chemicals, (Auburn, Australia). LTB_4 and 5-HETE standards were a generous gift from Merck, Sharp and Frosst, Canada. 15-HETE was biosynthesized as described previously [11].

Purified human PMNL were prepared from human venous blood (anticoagulated with 4.5% EDTA) by dextran sedimentation followed by centrifugation on a double Percoll gradient as described previously [11]. After washing in Dulbecco's balanced salt solution (pH 7.4), PMNL were resuspended at $1\text{-}2\times10^6$/ml in Hank's balanced salt solution. PMNL (1 ml) were incubated with each stimulus in polypropylene tests tubes at 37°C as indicated. Reactions were stopped by addition of 50 ul of 100 mM citric acid. All experiments were performed in triplicate.

5 ul A23187, 10 ul PMA or PDD or equivalent volumes of solvent were added to PMNL suspensions at concentrations required to achieve desired

final concentrations. The maximum final concentrations of methanol (0.5%) and DMSO (0.01%) had no effect on LTB_4 and 5-HETE synthesis in control experiments (data not shown).

The 5-LO products of PMNL were quantified using modifications of a method published previously [11]. Briefly, leukotrienes were extracted in chloroform/methanol (7:3 v/v) and chromatographed by reverse-phase HPLC. All chromatographic equipment was from Waters Millipore. The system comprised a Model 510 pump, 730 Data Module, 490 programmable UV detector and a 710B WISP autoinjector. Solvent conditions were methanol/water/acetic acid (70:30:0.08 v/v/v) at pH 6.2 (adjusted with conc. NH_4OH). Separations were carried out using a Waters Nova-Pak C_{18} column (15 cm x 3.9 mm) at 1 ml/minute. Detection of LTB_4 and PGB_2 was performed at 270 nm at 0.005 AUFS and HETEs at 234 nm at 0.02 AUFS. 50 ng of PGB_2 and 170 ng of 15-HETE were added to cells before extraction of leukotrienes as internal standards.

Cell viability was assessed by LDH release measured spectrophotometrically [12], using a Technicon Random Access Discrete Analyser, RA-1000 (Technicon Instruments Corporation, Tarrytown, New York, USA).

PMNL (1 ml) were incubated with ^{3}H-AA (0.1 uCi) for 1 hr. At 15 min. prior to washing, nordihydroguaiaretic acid (NDGA, 10 uM) was added to prevent conversion of released AA to leukotrienes. The cells were washed twice and incubated with stimuli for 5 min. Reactions were terminated as described and spun at 1000 x g for 5 min. 100 ul aliquots from each supernatant were taken for measurement of released ^{3}H-AA by scintillation counting.

RESULTS & DISCUSSION: Incubation of PMNL with A23187 (0.05 uM) for 5 min caused release of ^{3}H-AA from PMNL. When 100 ng/ml PMA (a dose which stimulates maximum rates of PMNL superoxide anion release) was added simultaneously with A231287, release of ^{3}H-AA was increased about 180%. PMA alone had no effect (Table 1).

A23187 up to 0.5 uM, also stimulated production of both LTB_4 and 5-HETE in a dose-dependent manner (Figure 1). Higher doses caused

Table 1

Effect of PMA and A23187 on release of ^{3}H-AA from human PMNL

Condition	^{3}H-AA release*
Control (methanol, DMSO)	0
PMA (100 ng/ml)	0
A23187 (0.5 uM)	570 ± 65
PMA + A23187	1530 ± 286

* ^{3}H-AA release determined as (cpm from stimulated samples - cpm from control samples). All samples stimulated for 5 min. (mean ± sem of three separate experiments).

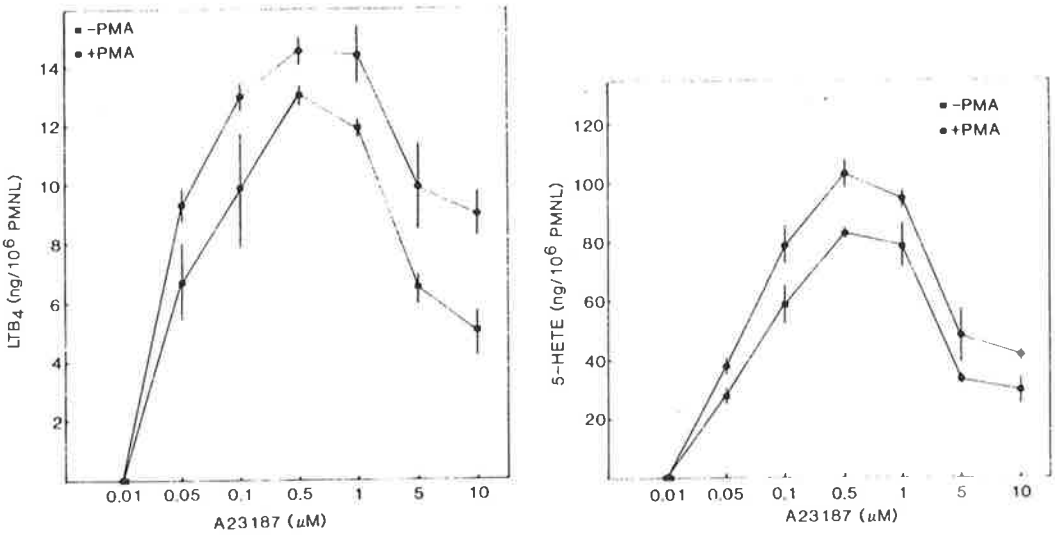


Figure 1. Effect of PMA on A23187-induced LTB_4 and 5-HETE synthesis. PMA (100 ng/ml) enhances LTB_4 and 5-HETE synthesis by human PMNL stimulated with increasing doses of A23187 (mean ± sem of 3 separate experiments).

progressive inhibition of LTB_4 and 5-HETE synthesis. The mechanism of this inhibition is unclear but may reflect decreased production and/or increased metabolism. Threshold levels of A23187 required to stimulate measurable amounts of 5-HETE and LTB_4 were between 0.01 and 0.05 uM (Figure 1). PMA alone did not stimulate leukotriene production, but enhanced A23187-induced synthesis of LTB_4 and 5-HETE (Figure 1). At 0.05 uM A23187, PMA increased synthesis of LTB_4 by approximately 50% and 5-HETE by 30%.

Preincubation of PMNL with PMA for up to 10 min prior to addition of A23187 did not cause any further enhancement of LTB_4 production (Figure 2). The biologically inactive phorbol ester PDD at a concentration of 100 ng/ml had no augmenting effect on LTB_4 and 5-HETE release stimulated by 0.05, 0.5 or 5.0 uM A23187. This phorbol ester does not activate PKC or exhibit tumour promoting effects [13]. PDD did not enhance the A23187-induced release of ^{3}H-AA (data not shown). Thus the augmenting effect of PMA on LTB_4 and 5-HETE release may result from activation of PKC. No evidence of increased LDH release was detected at the maximum concentration of any stimulus used.

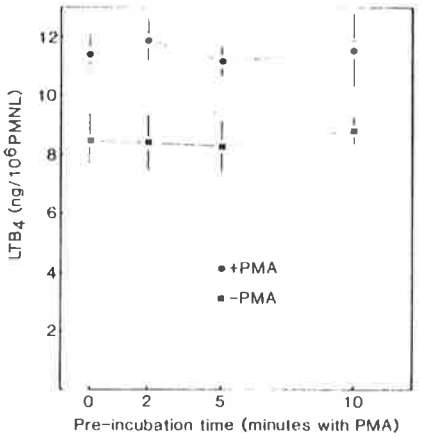


Figure 2. Time-dependence of augmenting effect of PMA on LTB_4 synthesis. Preincubation of cells with PMA (100 ng/ml) for up to 10 min. before addition of A23187 has no further enhancing effect on LTB_4 synthesis.

These data are consistent with previous reports using platelets [9] or macrophages [10] which show that PMA enhances A23187-induced AA metabolism by increasing the A23187-induced release of AA from phospholipid. However, although PMA increases the A23187-induced release of AA, and production of leukotrienes and prostaglandins by platelets 9-fold and the production of LTC_4 by macrophages 50-fold, our data shows that in human PMNL, PMA enhances the A23187-induced production of leukotrienes and the release of ^{3}H-AA from phospholipid to a smaller extent (<2-fold).

The mechanism by which PMA augments synthesis of leukotrienes is unclear, but a likely mechanism is via phosphorylation of a protein(s) involved directly or indirectly in lipoxygenase activation. For example, phosphorylation of lipomodulin purified from rabbit peritoneal PMNL results in loss of its PLA_2 inhibitory activity [14]. The increase in AA release suggests that this is the likely mechanism of the augmenting effect of PMA in our system. Alternatively, PKC activation may cause phosphorylation of cytoskeletal elements [15] which in turn may result in alteration of the cellular location of 5-lipoxygenase and its accessibility to substrate. It is important to emphasise that we have noted batch to batch variation in

the augmenting effect of PMA; this may reflect variation in PMNL lipomodulin activity or levels.

We have shown that PMA acts synergistically with A23187 in human PMNL to stimulate LTB_4 and 5-HETE synthesis and that this effect is mediated via an increase in release of AA. Our data supports the hypothesis that activation of PKC is required for this effect. These data may also be relevant to understanding mechanisms of leukocyte activation by agents such as N-formyl-methionyl-leucyl-phenylalanine which utilise the phosphoinositide pathway of signalling. Thus activation of PKC during stimulation by FMLP may augment the Ca^{++}/PLA_2 dependent release of AA from phospholipid.

ACKNOWLEDGMENTS: We are grateful to Dr. J. Buttery and Mr. C.R. Milner (Department of Clinical Chemistry, The Queen Elizabeth Hospital) for measuring LDH levels. S.R. McColl was supported by a Mary Paxton Gibson Research Scholarship (The Arthritis Foundation of Australia). Support was also given by The Royal Adelaide Hospital Research Foundation.

REFERENCES

1. Borgeat, P., and Samuelsson, B. (1979) Proc. Natl. Acad. Sci. USA. 76, 2148-2152.
2. DeTogni, P., DellaBianca, V., Grzeskowiak, M., and Rossi, F. (1985) Biochim. Biophys. Acta. 338, 12-22.
3. Cleland, L. (1984) J. Rheumatol. 11, 725-726.
4. Murakami, K., and Routtenberg, A. (1985) FEBS Lett. 192, 189-193.
5. Downes, P., and Michell, R. (1985) Molecular Mechanisms of Transmembrane Signalling; Molecular Aspects of Cellular Regulation Vol 2, pp 3-56, Elsevier, Amsterdam, New York.
6. Nishizuka, Y. (1984) Nature 308, 693-698.
7. Wolf, M., Levine, H., May, W., Cuatrecasas, P., and Sahyoun, N. (1985) Nature 317, 546-549.
8. May, W., Sahyoun, N., Wolf, M., and Cuatrecasas, P. (1985) Nature 317, 549-551.
9. Halenda, S., Zavoico, G., and Feinstein, M. (1985) J. Biol. Chem. 260, 12484-12491.
10. Tripp, C., Mahoney, My. and Needleman, P. (1985) J. Biol. Chem. 260, 5895-5898.
11. McColl, S., Betts, W., Murphy, G., and Cleland, L. (1986) J. Chromatogr. 378, 445-449.
12. Wacker, W., Ulmer, D., and Vallee, B. (1956) New Engl. J. Med. 255, 449-456.
13. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.
14. Hirata, F. (1981) J. Biol. Chem. 256, 7730-7733.
15. Feuerstein, N., Monos, D., and Cooper, H. (1985) Biochem. Biophys. Res. Commun. 126, 206-213.

APPENDIX VI.

Effect of Dietary Polyunsaturated Fatty Acid (PUFA) Supplementation on Adjuvant Induced Polyarthritis in Rats

SHAUN R. McCOLL, LESLIE G. CLELAND, MICHAEL W. WHITEHOUSE and BARRIE VERNON-ROBERTS

Abstract. **Dietary treatment with fish oil reduced the severity of adjuvant induced polyarthritis in Dark Agouti rats but enhanced disease severity in Wistar-Hooded rats. The difference in effects of fish oil between strains was not related to differences in severity of disease induced in untreated rats. Genetically determined factors may influence the effects of fish oil supplementation on severity of inflammatory diseases. (*J Rheumatol 1987;* 14:)**

Key Indexing Terms:
ADJUVANT ARTHRITIS
DIETARY MODIFICATION
FISH OIL
RATS

Modification of dietary polyunsaturated fatty acids (PUFA) alters immunological and inflammatory responses in humans and laboratory animals[1,2]. Sprague Dawley rats fed an essential fatty acid (EFA) deficient diet developed a milder adjuvant disease than rats fed an EFA sufficient diet[1]. Within 3 weeks of supplementing EFA deficient rats with corn oil (58% linoleic acid, 1% linolenic acid, 0.2 ml daily), the cutaneous signs of EFA deficiency disappeared and inflammation was expressed.

Recently, attention has been focused upon the ways in which the omega-3 PUFA found in marine fish oils may influence the metabolism of the essential omega-6 PUFA from which the immunologically active dienoic prostaglandins and tetraenoic leukotrienes are derived[2]. Dietary supplementation with oils rich in omega-3 PUFA delayed development of the spontaneous lupus nephritis in 2 mouse strains[3,4]. By contrast, the frequency of induction of arthritis induced by innoculation with type II collagen was increased in Sprague Dawley rats fed a diet supplemented with omega-3 PUFA, compared to rats fed a diet supplemented with beef tallow[5].

Rat strains are known to differ in their susceptibility to adjuvant disease[6], and in aspects of lipid metabolism[7]. Our studies were undertaken to assess the effect of dietary supplementation with oils rich in omega-3 and omega-6 PUFA upon adjuvant disease in 2 rat strains (Dark Agouti, PVG Wistar-Hooded).

From the Queen Elizabeth and Royal Adelaide Hospital Rheumatology Unit and Department of Pathology, University of Adelaide, Adelaide, South Australia.

Supported by grants from The Arthritis Foundation of Australia (SA) and The Royal Adelaide Hospital Research Foundation. S.R.McC. is a recipient of a Mary Paxton-Gibson Research Scholarship (Arthritis Foundation of Australia).

S.R. McColl, B Sc(Hons); L.G. Cleland, MD, FRACP, Queen Elizabeth and Royal Adelaide Hospital Rheumatology Unit; M.W. Whitehouse, DPhil.; B. Vernon-Roberts, DPhil, MD, FRCPA FRCPath, Department of Pathology.

Address requests for reprints to Dr. L.G. Cleland, Rheumatology Unit, Queen Elizabeth Hospital, Woodville Rd., Woodville, South Australia, 5011.

Submitted April 3, 1986 revision accepted October 27, 1986.

MATERIALS AND METHODS

Animals. Male weanling rats from the South Australian Department of Agriculture Gilles Plains animal house were used for all 3 experiments. Experimental groups comprised 8-10 rats each. Dark Agouti (DA) rats were used in Experiments 1 and 3 and PVG Wistar-Hood rats (WH) in Experiment 2. Each group was fed exclusively one of 4 diets (outlined below) from 4 weeks of age until termination of the experiment 6 weeks later. In preliminary studies, daily food consumption was monitored and we established that rats ate similar amounts of each diet. These studies were carried out using cages with wire bottoms to allow collection of uneaten food. In all subsequent studies, rats were housed in large cages with sawdust bedding, up to 10 per cage and fed diet and water "ad libitum." Similar growth patterns from weaning to induction of disease were seen in all groups (Figure 1).

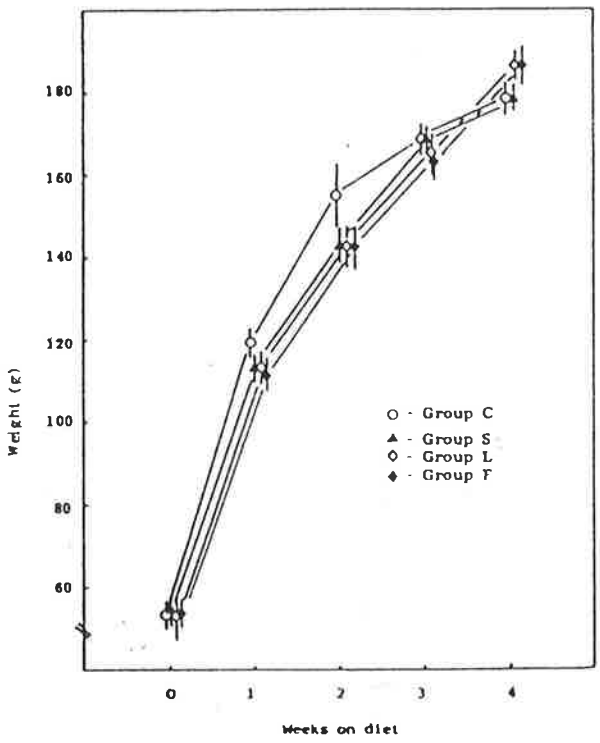


Fig. 1. Growth patterns of DA rats fed 4 different diets for 4 weeks. Group weights were assessed at each time point by one way analysis of variance and no significant differences were found. Similar results were obtained in all 3 experiments.

Diets. A commercially available rat chow (Milling Industries Australia Pty. Ltd.), containing 4% fat w/w, was crushed, repelleted without supplementation and used as a baseline diet (fed to Group C). This chow was also used as a basis for diets supplemented with the following oils (12% w/w): sunflower seed oil (fed to Group S); fish oil (fed to Group F); and linseed oil (fed to Group L). The oils were added to crushed chow, mixed, repelleted and stored at −20°C before use in animal experiments. Sunflower seed oil was obtained from Nuttelex Food Products Pty. Ltd. Australia, MaxEPA (fish oil) was a gift from R.P.Scherer Pty. Ltd. and unboiled linseed oil was obtained from Diggers Trading, Adelaide, Australia. Upon delivery, butylated hydroxy-toluene (0.05% w/v)(Sigma Chemical Company, St. Louis, MO, USA) was added to oils as antioxidant. Samples of oils and isopropanol/chloroform extracts of complete diets were analyzed for lipid content by gas liquid chromatography (GLC) (Tables 1 and 2). The diets were essentially isocaloric as determined by bomb calorimetry: 18.5, 17.6, 17.3 and 16.5 ($\times 10^{-2}$ Kj/g) for diets C, S, F and L, respectively.

GLC analysis of fatty acids. GLC was performed as described[8]. Briefly, lipid samples were converted to their constituent fatty acid methyl esters by heating in a solution of 1% H_2SO_4 in methanol in a sealed tube under nitrogen at 70°C for 3 h. The esters were extracted with petroleum spirit (bp 40–60°C). Analysis was performed on 1.5 m columns (2 mm id) packed with 5% SP2310 (Supelco Inc., Bellefonte, PA). The chromatographic conditions were as follows: injection port temperature 200°C, flame ionization detector temperature 300°C, initial oven temperature 125° rising to 225°C at 4°C/min and holding for 20 min. The carrier gas used was nitrogen at a flow rate of 16–20 ml/min.

Lipid analysis. Venous blood (5 ml) was collected from 4 rats chosen at random from each group at the termination of Experiment 3. Erythrocytes were pelleted by centrifugation, washed twice in saline and lipids extracted with isopropanol/chloroform 1:2 (HPLC grade Waters Associates, Milford, MA, USA). The lipids were converted to constituent fatty acid methyl esters and quantified by GLC.

Induction of adjuvant arthritis. After 4 weeks of feeding, a complete Freund's adjuvant containing heat killed *Mycobacterium tuberculosis* (M.Tb., Weybridge Laboratories, Ministry of Agriculture, UK) dispersed in squalane (Fluka A.G., Buchs, Switzerland) or triolein (B.D.H. Chemicals Ltd., Poole, England) at a final concentration of 10 mg/ml was injected intradermally into the tail of the rats. In Experiments 1 and 2 the vehicle was squalane, and in Experiment 3, triolein. Assessment of inflammation was made 2 weeks later.

Assessment of adjuvant disease. Four variables were used to quantify the severity of the disease: (1) rear paw swelling between the dorsum and the sole of the foot measured at the midpoint between the posterior border of the calcaneum and the 5th metatarsophalangeal joint (using digital calipers); (2) widest diameter of swelling of the tail in the region of injection (using digital calipers); (3) weight change and (4) disease activity score (total of 0–14 points) determined as follows: for each rear paw (0–4 points) where 0 = no localized articular lesion or swelling, 1 = localized articular lesions, or ankle swelling, 2 = localized articular lesions and ankle swelling, 3= moderate generalized ankle and foot swelling, and 4 = gross generalized ankle and foot swelling: for each forepaw (0–3 points) where 0 = no localized articular lesion or swelling, 1 = localized articular lesions or wrist

Table 1. *Fatty acid analysis* of oils used as supplements*

Fatty acid	Common Name	Sunflower Oil	Fish Oil	Linseed Oil
14:0	Myristic	—	6.5	—
15:0	—	—	0.5	—
16:0 (iso)	—	—	0.1	—
16:0	Palmitic	6.3	16.9	6.4
17:0	Margaric	0.1	2.1	0.1
18:0	Stearic	4.4	3.5	4.6
20:0	Arachidic	0.4	1.1	0.3
24:0	Lignoceric	0.3	0.3	0.4
Total Saturates		11.5	31.0	11.5
14:1	Myristoleic	—	0.3	—
16:1	Palmitoleic	—	9.3	—
18:1	Oleic	27.7	15.8	17.7
20:1	Gondoic	0.3	2.5	0.6
24.1	Nervonic	—	0.2	0.1
Total Monoenes		28.0	28.1	18.4
18.2	Linoleic	59.3	1.9	14.9
18.3	γ-linolenic	—	0.3	—
20:2	—	0.1	0.3	0.2
20:3	Dihomo-γ-linolenic	—	0.2	—
20:4	Arachidonic	—	1.0	0.1
22.4	Adrenic	—	0.4	0.1
Total w6 acids		59.4	4.1	15.2
18:3	α-linolenic	0.4	0.7	53.9
20:5	Timnodonic	—	18.1	0.9
22:5	Docospentaenoic	0.7	2.8	—
22:6	Dupanodonic	—	11.1	0.1
Total w3 acids		1.1	36.8	54.9

* Results expressed as a percentage of total fatty acids.

Table 2. *Fatty acid analysis* of test diets***

Fatty Acid	Group C	Group S	Group F	Group L
14:0	1.5	0.3	5.3	0.4
15:0	0.3	—	0.6	—
16:0 (iso)	—	—	0.3	—
16:0	19.2	7.8	17.4	9.3
17:0	0.7	0.3	1.9	0.4
18:0	6.6	4.6	5.2	5.4
20:0	0.6	0.5	1.7	—
24:0	0.6	0.6	0.6	0.3
Total Saturates	29.4	14.1	32.9	15.8
14:1	—	—	0.4	—
16:1	2.6	0.5	7.5	0.6
18:1	22.4	26.4	17.2	18.8
20:1	3.2	0.5	2.5	0.6
24:1	0.8	—	0.6	0.2
Total Monocnes	29.1	27.4	28.2	20.2
18:2	32.3	56.9	8.8	19.1
18:3	—	—	0.3	—
20:2	0.3	—	0.4	0.2
20:3	—	—	0.3	—
20:4	—	—	0.9	0.2
22:4	—	—	0.8	—
Total w6 Acids	32.6	56.9	11.8	19.5
18:3	2.8	—	1.4	42.6
20:5	4.2	0.3	14.1	0.9
22:5	—	—	2.2	0.2
22:6	1.9	0.5	9.3	0.8
Total w3 Acids	8.9	0.8	27.0	44.5
Total w6/w3	3.7	71.2	0.4	0.4

* Results expressed as a percentage of total fatty acids.

** Rat chow from a commercial-supplier was crushed, mixed with or without supplementation and then repelleted:
Diet fed to Group C = rat chow repelleted without supplementation.
Diet fed to Group S = rat chow supplemented with sunflower oil.
Diet fed to Group F = rat chow supplemented with fish oil.
Diet fed to Group L = rat chow supplemented with linseed oil.

swelling, 2 = localized articular lesions and wrist swelling and 3= gross generalized paw and wrist swelling. All assessments of disease activity were undertaken by an observer who was unaware of the treatments applied.

Statistical analysis. One way analysis of variance was performed for analysis of parametric variables (verification of group homogeneity before induction of disease, paw swelling, tail swelling and weight change). When significant variance was found using this method, Student's t tests were performed to identify the source of the variance. Disease activity scores were assessed nonparametrically using a Kruskal-Wallis one way analysis of variance. If significant variance was found using this test, a Mann-Whitney U test was performed.

RESULTS

Effect of diet on severity of adjuvant arthritis. Adjuvant disease was induced in all rats irrespective of dietary treatment given. In Experiment 1, rats fed fish oil (Group F) had lower mean disease activity score and less mean weight loss than rats fed the baseline diet (Group C) or sunflower oil diet (Group S) (Table 3). In Experiment 2, which was extended to include rats fed linseed oil (Group L), all groups fed lipid supplemented diets gains weight, whereas Group C loses weight. Group F had the highest mean disease activity score and the largest rear paw swelling of all groups. In Experiment 3, Group F had the lowest mean disease activity score and the mean highest weight gain. In this experiment increased paw swelling was seen in Group L and, to a lesser extent Group S, compared with Group C.

Effect of dietary treatment on erythrocyte lipids. Dietary supplementation produced profound alterations in the fatty acid content of erythrocyte lipids (Table 4). Group S had an increase in arachidonic acid (C 20:4 w6, AA) content relative to Group C. The erythrocytes from Groups F and L exhibited a substantial increase in timnodonic acid (commonly referred to as eicosapentaenoic acid, C 20:5 w3, EPA) and reduction in AA. The change in EPA content was greatest in Group F. Changes in the ratios of AA/EPA were generally congruent with changes in total w6/w3 PUFA ratios.

DISCUSSION

Adjuvant disease in rats has been used as a model of generalized polyarthritis with systemic inflammation. This disease has features in common with rheumatoid arthritis and Reiter's syndrome, but doesn't exactly mimic any single human inflammatory disorder[9].

In our study, DA rats developed more severe disease than WH rats. Garrett, *et al*[6] have recently reported similar findings regarding greater severity of disease in DA rats compared to a hooded strain. These authors also established the greater arthritogenicity of M.Tb/squalane compared to M.Tb/triolein. The latter arthritogen was not used in WH rats because it had been found not to induce a consistently measurable disease (M. Whitehouse, unpublished findings). Why rat strains vary in their susceptibility to arthritogenic adjuvants and response to disease suppressing agents is unknown.

Disease activity score was the most sensitive variable chosen to monitor the effects of diet on adjuvant disease. As shown in Experiment 2, weight change may be a less reliable indicator when lipid supplemented diets are compared with baseline diets (diets not supplemented with lipid). The fish oil supplemented diet was associated with the lowest mean disease activity scores in DA rats, and the highest mean disease activity score in WH rats. These findings indicate that rat strains may respond differently to the same dietary manipulation and dictate caution in generalizing from observations made using a single rat strain. It is also possible that a single strain could respond differently under varying conditions at the time of study (e.g. seasonal changes, intercurrent infections).

WH rats fed sunflower oil showed less disease activity than rats on the baseline diet (Experiment 2). An explanation may

Table 3. *Effect of PUFA dietary supplements on severity of adjuvant disease in 2 rat strains with 2 adjuvants. (mean ± SEM)*

Group	Paw Size (mm)	Tail Swelling (mm)	Wt. Change (g)	Disease Score (/14)
Experiment 1 (DA rat, M.Tb/squalane)				
C (n= 9)	7.1 ± 0.4	10.1 ± 0.2	−38 ± 6.7	11.2 ± 0.8
S (n=10)	6.7 ± 0.3	10.4 ± 0.3	−33 ± 4.4	10.8 ± 1.0
F (n=10)	6.3 ± 0.3	9.9 ± 0.2	−14 ± 5.3*	7.2 ± 1.1*
Experiment 2 (WH rat, M.Tb/squalane)				
C (n= 9)	7.9 ± 0.3	11.7 ± 0.1	−10 ± 2.0	8.0 ± 1.0
S (n= 8)	6.9 ± 0.3	11.5 ± 0.3	7 ± 3.5*	5.1 ± 1.0*
F (n= 9)	8.7 ± 0.3	11.3 ± 0.3	9 ± 4.0*	11.0 ± 0.9*
L (n= 8)	7.9 ± 0.3	11.4 ± 0.3	9 ± 4.6*	8.8 ± 1.0
Experiment 3 (DA rat, M.Tb/triolein)				
C (n=10)	5.7 ± 0.2	10.1 ± 0.2	21 ± 2.8	5.8 ± 0.8
S (n=10)	6.6 ± 0.4*	10.8 ± 0.2	16 ± 3.5	6.2 ± 0.9
F (n=10)	6.4 ± 0.4	10.4 ± 0.4	23 ± 3.5	3.9 ± 1.0*
L (n= 8)	7.3 ± 0.3*	10.7 ± 0.2	9 ± 3.9	7.8 ± 0.9

* Significantly different compared to Group C at $p<0.05$

Noninflamed footpad thickness (mm) at 10 weeks of age: DA 5.3±0.05; WH 6.1±0.10

(See Materials and Methods section for details of statistical analyses)

be that sunflower oil inhibits the adjuvant disease promoting effects of EPA (present in the baseline diet) in this rat strain.

The presence of a small amount of EPA and the lack of measurable AA in unsupplemented rat chow suggests that fish meal or fish oil had been incorporated during manufacture. These observations demonstrate the need to characterize baseline diets to which supplements are added. The observed activity of all lipid enriched diets in at least one of the test variables illustrates the value of a nonsupplemented reference diet in studies of this nature. Several previous studies have compared a fish oil supplement with saturated fat enriched diets without establishing that the reference diet was inert in the system under study[3,5]. Fernandes, *et al* have shown that dietary supplementation with saturated fat may enhance murine lupus[10] and accordingly, tallow supplemented diets may not be suitable reference diets. Ideally, studies should compare multiple diets to allow test treatments to be more meaningfully assessed for the effects of total fats as well as the proportion of specific PUFA.

Alterations in proportions of PUFA in erythrocyte membranes were examined as a convenient means of monitoring incorporation of fatty acids into cellular lipids. The extent to which changes in erythrocyte fatty acid profiles reflect those of leukocytes[11] remains to be fully established. The differences between the total content of EPA in erythrocytes of DA rats fed the linseed oil diet or the fish oil diet (Experiment 3) indicate that linseed oil (with its high proportion of linolenic acid) is a less efficient means of increasing cellular EPA than fish oil. There was, however, a similar depression in AA content in erythrocytes with both diets. The linseed oil and fish oil diets also exhibited differing effects on disease activity in DA rats (linseed oil fed rats, disease enhanced; fish oil fed rats, disease suppressed). These findings suggest that increasing the cellular EPA content may affect disease severity independently of a concommitant depression in cellular AA.

The divergent effect of fish oil upon severity of adjuvant arthritis in the 2 rat strains suggest potential difficulties in predicting the outcome of PUFA dietary therapy in human rheumatic diseases. Variation within patient populations could take the form of differences in disease susceptibility and severity or aspects of fatty acid metabolism. Patient subgroups responsive to PUFA dietary therapy could remain unidentified in studies designed to distinguish differences in mean antiinflammatory effect in large groups of patients. It is therefore possible that a biochemical method for identifying such patients may be necessary before the efficacy of this mode of therapy can be established.

Table 4. *Fatty acid analysis* of total lipids of erythrocytes from Dark Agouti rats receiving test diets** (mean ± SEM)*

Fatty Acid	Group C	Group S	Group F	Group L
14:0	—	—	—	—
15:0	—	—	—	—
16:0	31.0 ± 1.0	25.0 ± 0.8	30.7 ± 0.7	27.4 ± 1.2
17:0	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
18:0	17.4 ± 0.5	18.2 ± 0.4	14.8 ± 0.2	20.7 ± 0.4
20:0		—	—	—
24:0	0.8 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	3.4 ± 0.2
Total Saturates	49.6	45.0	47.7	52.3
14:1	—	—	—	—
16:1	—	—	—	—
18:1	11.1 ± 0.4	10.9 ± 0.1	12.7 ± 0.1	10.9 ± 0.2
20:1	1.1 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	1.5 ± 0.1
24:1	0.9 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	1.2 ± 0.1
Total Monoenes	13.5	12.5	13.9	13.6
18:2	10.5 ± 0.4	14.1 ± 0.6	6.6 ± 0.1	14.0 ± 0.4
18:3	1.1 ± 0.1	—	—	—
20:2	0.3 ± 0.1	0.6 ± 0.1	—	0.4 ± 0.1
20:3	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	1.1 ± 0.1
20:4	13.3 ± 0.9	16.0 ± 1.4	6.3 ± 0.3	9.3 ± 0.7
22:4	—	—	—	—
Total w6 Acids	25.5	31.1	13.8	24.8
18:3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	1.4 ± 0.1
20:5	0.8 ± 0.2	0.1 ± 0.1	6.3 ± 0.3	2.5 ± 0.2
22:5	1.8 ± 0.2	1.0 ± 0.1	2.9 ± 0.3	2.5 ± 0.2
22:6	2.5 ± 0.5	2.2 ± 0.1	4.5 ± 0.2	2.1 ± 0.2
Total w3 Acids	5.2	3.4	13.8	8.5
Total w6/w3	4.9	9.1	1.0	2.9
20:4w6/20:5w3	16.6	160.0	1.0	3.7

* Results expressed as percentage of total fatty acids.

** Rats fed diets from weaning (4 weeks of age). Adjuvant disease induced after 28 days of feeding and blood taken at time of sacrifice 14 days later.

ACKNOWLEDGMENT

Thanks are extended to the following for assistance in this project: Ms. J. Hawkes; Mr. I.R. Garrett; Dr. J.K. French; Dr. R. Gibson; Staff at the Animal House, I.M.V.S., Adelaide, S.A.; Dr. J. Charnock, C.S.I.R.O. Division of Human Nutrition, Glenthorne, S.A.

REFERENCES

1. Denko CW: Modification of adjuvant inflammation in rats deficient in essential fatty acids. *Agents Actions 1976;*6/5:636–641.
2. Lee TH, Hoover RL, Williams JD, *et al:* Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on *in vitro* neutrophil and monocyte leukotriene generation and neutrophil function. *N Engl J Med 1985;*312:1217–1223.
3. Prickett JD, Robinson DR, Steinberg AD: Effects of dietary enrichment with eicosapentaenoic acid upon autoimmune nephritis in female NZB×NZW/F_1 mice. *Arthritis Rheum 1983;*26:133–139.
4. Kelley VE, Ferretti A, Izui S, *et al:* A fish oil diet rich in eicosapentaenoic acid reduces cyclooxygenase metabolites and suppresses lupus in MRL-lpr mice. *J Immunol 1985;*134:1914–1919.
5. Prickett JD, Trentham DE, Robinson DR: Dietary fish oil augments the induction of arthritis in rats immunised with type II collagen. *J Immunol 1984;*132:725–729.
6. Garrett IR, Whitehouse MW, Vernon-Roberts B, *et al:* Ambivalent properties of gold drugs in adjuvant induced polyarthritis in rats. *J Rheumatol.* (In press).
7. Jeffery F, Redgrave TG: Chylomicron catabolism differs between hooded and albino laboratory rats. *J Lipid Res 1982;*23:154–160.
8. Gibson RA: Australian fish — an excellent source of both arachidonic and w-3 polyunsaturated fatty acids. *Lipids 1983;*18:743–752.
9. Pearson CM, Wood FD: Studies of polyarthritis and other lesions induced in rats by injection of mycobacterial adjuvant. I. General clinical and pathological characteristics and some modifying factors. *Arthritis Rheum 1959;*2:440–459.
10. Fernandes G, Sandberg L, Khare A, *et al:* Comparison of calorie restriction against high dietary fats of animal, vegetable and fish origin on murine lupus disease. *Arthritis Rheum 1986;*29:suppl.S40.
11. Von Schacky C, Fischer S, Weber P: Long-term effects of dietary marine w-3 fatty acids upon plasma and cellular lipids, platelet function and eicosanoid formation in humans. *J Clin Invest 1985;*76:1626–1631.

French, J. K., McColl, S. R. & Cleland, L. G. (1986). Re: Fish oils and coronary disease 1. *New Zealand Medical Journal*, v.99, 962-963

French, J. K., McColl, S. R. & Betts, W. H. (1987). Re: Fish oils and coronary disease. *New Zealand Medical Journal*, in press.

BIBLOGRAPHY.

Ahmed, A.A. and Holub, B.J. (1984) Lipids 19:617-624.

Angaard, E. and Bergstrom, S. (1963) Acta. Physiol. Scand. 58:1-12.

Anderson, F.L., Jubiz, W. and Tsagaris, T.J, (1976) Am. J. Physiol. 231:426-429.

Anonymous. (1973) Annual reports of the chief medical officer in Greenland for the years 1973-1976. Ministry of Greenland, Copenhagen.

Arai, Y., Konno, M., Shimoji, K., Konishi, Y., Niwa, H., Toda, M. and Hayashi, M. (1982) Chem. Pharmacol. Bull. 30:379-382.

Augstein, J., Farmer, J.B., Lee, T.B., Sheard, P. and Tattersall, M.L. (1973) Nature 245:215-217.

Bach, M.K. (1984) In: Chakrin, L.W. and Bailey, D.M. (eds.). The Leukotrienes. Academic Press, New York. pp. 163-194.

Bach, M.K. and Brasher, J.R. (1986) Biochem. Pharmacol. 35:425-433

Bach, M.K., Brasher, J.R. and Gorman, R.R. (1977) Prostaglandins 14:21-38

Badwey, J.A., Curnutte, J.T. and Karnovsky, M.L. (1981) J. Biol. Chem. 256:12640-12643.

Bainton, D.F. Ullyot, J.L. and Farquar, M.G. (1971) J. Exp. Med. 134:907-934

Bang, H.O. and Dyerberg, J. (1972) Acta Med. Scand. 192:85-94

Bang, H.O., Dyerberg, J. and Nielsen, A.B. (1977) Lancet i:1143-1146

Bang, H.O., Dyerberg, J. and Sinclair, H.M. (1980) Am. J. Clin. Nutr. 33:2657-2661.

Bass, D.A., O'Flaherty, J.T., Goetzl, E.J., DeChatelet, L.R. and McCall, C.E. (1981) Prog. Lipid Res. 20:735-737.

Beckman, J.K., Gay, J.C., Brash, A.R., Lukens, J.N. and Oates, J.A. (1985) Lipids 20:318-321.

Bell, R.L., Kennerly, D.A., Stanford, N. and Majerus, P.W. (1979) Proc. Natl. Acad. Sci. U.S.A. 76:3238-3241.

Bergstrom, S., Danielsson, H. and Samuelsson, B. (1964) Biochim. Biophys. Acta 90:207-210.

Bergstrom, S., Dressler, F., Krabisch, L., Ryhage, R. and Sjovall, J. (1962a) Arkiv. Kemi. 20:63-66.

Bergstrom, S., Dressler, F., Ryhage, R., Samuelsson, B. and Sjovall, J. (1962b) Arkiv. Kemi. 19:563-576.

Bernstrom, K. amd Samuelsson, B. (1981) J. Biol. Chem. 256:9579-9582.

Berridge, M.J. and Irvine, R.I. (1984) Nature 312:315-321.

Bhattacherjee, P. and Eakins, K.E. (1984) In: Chakrin, L. and Bailey, D.M. (eds.). The Leukotrienes. Academic Press, New York. pp 195-214.

Bhattacherjee, P., Hammond, B., Salmon, J.A., Stepney, R. and Eakins, K.E. (1981) Eur. J. Pharmacol. 73:21-28.

Billah, M.M., Lapetina, E.G. and Cuatrecasas, P. (1980) J. Biol. Chem. 255:10227-10231.

Billah, M.M., Lapetina, E.G. and Cuatrecasas, P. (1981) J. Biol. Chem. 256:5399-5403.

Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257:5196-5200.

Bisgaard, H., Lercho, A. and Kristensen, J.K. (1985) J. Inv. Dermatol. 84:427-429.

Black, K.L., Culp, B., Madison, D., Randall, O.S. and Lands, W.E.M. (1979) Prost. Med. 3:257-268.

Blackwell, G.J. and Flower, R.J. (1978) Prostaglandins 16:417-425.

Blackwell, G.J., Carnaccio, R., DiRosa, M., Flower, R.J., Longham, C.S.J., Parente, L., Persico, P., Russell-Smith, N.C. and Stone, D. (1982) Br. J. Pharmacol. 76:185-194.

Bokoch, G.M. and Reed, P.W. (1980) J. Biol. Chem. 255:10223-10226.

Bokoch, G.M. and Reed, P.W. (1981) J. Biol. Chem. 256:4156-4159.

Bonser, R.W., Siegel, M.I., Chung, S.M., McConnell, R.T. and Cuatrecasas, P. (1981) Biochemistry 20:5297-5301.

Boraschi, D., Consini, S., Bertalini. M. and Tagliabne, A. (1985) J. Immunol. 135:502-505.

Borgeat, P. and Samuelsson, B. (1979a) J. Biol. Chem. 254:2643-2646.

Borgeat, P. and Samuelsson, B. (1979b) Proc. Natl. Acad. Sci. U.S.A. 76:3213-3217.

Borgeat, P. and Samuelsson, B. (1979c) Proc. Natl. Acad. Sci. U.S.A. 76:2148-2152.

Borgeat, P. and Samuelsson, B. (1979d) J. Biol. Chem. 254:7865-7869.

Borgeat, P., Hamberg, M. and Samuelsson, B. (1976) J. Biol. Chem. 251:7816-7820.

Borgeat, P., Fruteau de Laclos, B., Picard, S., Drapeau, J., Vallerand, P. and Corey, E.J. (1982) Prostaglandins 23:713-724.

Borgeat, P., Fruteau de Laclos, B., Rabinovitch, H., Picard, S., Braquet, P., Herbert, J. and Laviolette, M. (1984) J. All. Clin. Immunol. 74:310-315.

Boxer, L.A. and Stossel, T.P. (1976) J. Clin. Invest. 57:964-976.

Boxer, L.A., Allen, J.M., Schmidt, M., Yoder, M. and Baeher, R.L.

(1980) J. Lab. Clin. Med. 95:672-678.

Bray, M.A. (1986) Agents Actions 19:87-99.

Bray, M.A., Cunningham, F.M., Ford-Hutchinson, A.W. and Smith, M.J.H. (1981) Br. J. Pharmacol. 72:483-486.

Brocklehurst, W.E. (1953) J. Physiol. (Lond.) 129:16p-17p.

Brocklehurst, W.E. (1962) Prog. Allergy 6:589-558.

Bruynzeel, P.L.B., Kok, P.T.M., Hamelink, M.L., Kijne, A.M. and Verhagen, J. (1985) FEBS Lett 189:350-354.

Busse, W.D., Mardin, M., Gruetzmann, R., Dunn, J.R., Theodoreau, M., Sloane, B.F. and Honn, K.V. (1982) Fed. Proc. 41:Abstr. 8464.

Carr, S.E., Higgs, G.A., Salmon, J.A. and Spayne, J.A. (1981) Br. J. Pharmacol. 73:253p-254p

Changelian, P.S. and Fearon, D.T. (1986) J. Exp. Med. 163:110-115.

Chapnick, B.M., Feigen, L.P., Hyman, A.L. and Kadowitz, P.J. (1978) Am. J. Physiol. 235:4326-4332.

Chang, J., Skowronek, M.D. and Lewis, A.J. (1985a) Inflammation 9:395-406.

Chang, J., Blazek, E., Kreft, A. and Lewis, A.J. (1985b) Biochem. Pharmacol. 34:1571-1575.

Christiansen, N.O., Larsen, C.S. and Juhl, H. (1986) Biochim. Biophys. Acta 882:57-62.

Claesson, H.E., Lindgren, J.A. and Gustafsson, B. (1985) Biochim. Biophys. Acta 836:361-367.

Clancy, R.M. and Hugli, T.E. (1983) Anal. Biochem. 133:30-39.

Clancy, R.M., Dahlnden, C.A. and Hugli, T.E. (1983) Proc. Natl. Acad. Sci. U.S.A. 80:7200-7204.

Clancy, R.M., Dahinden, C.A. and Hugli, T.E. (1985) Prog. Biochem.

Pharmacol. 20:120-131.

Clarke, A.K., Vernon-Roberts, B. and Currey, H.L. (1975) Ann. Rheum. Dis. 34:326-332.

Clyman, R.I., Wong, L., Heymann, M.A. and Rudolph, A.M. (1978) Prostaglandins 15:231-325.

Cockrell, C.S. and Ellis, E.F. (1984) J. Chromatogr. 308:316-321.

Corey, E.J., Clark, D.A. Goto, G., Marfat, A., Mioskowski, C., Samuelsson, B. and Hammarstrom, S. (1980) J. Am. Chem. Soc. 102:1436-1439

Corey, E.J. and Munroe, J.E. (1982) J. Am. Chem. Soc. 104:1752-1754.

Creticos, P.S., Peters, S.P., Adkinson, N.F., Naclerio, R.M., Hayes, E.C., Norman, P.S. and Lichtenstein, L.M. (1984) New. Engl. J. Med. 310:1626-1630.

Crouch, M.F. and Lapetina, E, G. (1986) Biochem. Biophys. Res. Commun. 141:459-465.

Culp, B.R., Titus, B.G. and Lands, W.E.M. (1979) Prost. Med. 3:269-278.

Culp, B.R., Lands, W.E.M, Lucchesi, B.R., Pitt, B. and Romson, J. (1980) Prostaglandins 20:1021-1031.

Dahinden, C.A., Clancy, R.M. and Hugli, T.E. (1984) J. Immunol. 133:1477-1482.

Dahlen, S.E., Bjork, J., Hedquist, P., Afors, V.E., Hammarstrom, S., Lindgren, J.A. and Samuelsson, B. (1981) Proc. Natl. Acad. Sci. U.S.A. 78:3887-3891.

Daniel, W.W. (1983) In: Biostatistics: A Foundation For Analysis In The Health Sciences. John Wiley and Sons, New York.

Davidson, E.M., Rae, S.A. and Smith, M.J.H. (1982) J. Pharm.

Pharmacol 34:410.

Denko, C.W. (1976) Agents Actions 6:636-641.

Dirosa, M., Giroud, J.P. and Willoughby, D.A. (1971) J. Path. 104:15-29.

Di Virgillio, F., Lew, D.P. and Pozzan, T. (1984) Nature 310:691-693.

Doig, M.V. and Ford-Hutchinson, A.W. (1981) Prostaglandins 20:1007-1019.

Downes, C.P. and Michell, R.H. (1985) In: Cohen, P. and Houslay, M.D. (eds.). Molecular Aspects of Cellular Regulation Vol. 4. Elsevier Scientific Publications, B.V., Amsterdam. pp 3-56.

Dyer, R.D., Stafford, S.E. and Carter, G.W. (1982) Fed. Proc. 41:Abstr. 7490.

Dyerberg, J. and Bang, H.O. (1979) Lancet ii:433-435.

Dyerberg, J., Bang, H.O. and Hjorne, N. (1975) Am. J. Clin. Nutr. 28:958-966.

Dyerberg, J., Bang, H.O., Stofferson, E., Moncada, S. and Vane, J.R. (1978) Lancet ii:117-119.

Dyett, D.E., Malawista, S.E., Naccache, P.H. and Sha'afi, R. (1986) J. Clin. Invest. 77:34-37.

Dvorak, A.M., Dvorak, H.F., Peters, S.P., Shulman, E.S., MacGlashan, D.W., Pyne, K., Harvey, V.S., Galli, S.J. and Lichtenstein. L.M. (1983) J. Immunol. 131:2965-2976.

Eling, T., Warnock, R., Dick, D. and Tainer, B (1980) Prost. Med. 5:345-355.

Engineer, D.M., Niederhauser, U., Piper, P.J. and Sirois, P. (1978) Br. J. Pharmacol. 62:61-66.

Fahey, J.V., Ciosek, C.P. and Newcombe, D.S. (1977) Agent Actions 7:255-264.

Feigen, L.P., Chapnick, B.M., Fleming, J.E., Fleming, J.M. and Kadowitz, P.J. (1977) Am. J. Physiol. 233:4573-4579.

Feigen, L.P., Chapnick, B.M., Gorman, R.R., Hyman, A.L. and Kadowitz, P.J. (1978) Prostaglandins 16:803-813.

Feinmark, S.J., Lindgren, J.A., Claesson, H.E., Malmsten, C. and Samuelsson, B. (1981) FEBS Lett 136:141-144.

Feldberg, W. and Kellaway, C.H. (1938) J. Physiol. (London) 94:187-226.

Feldberg, W. and Milton, A.S. (1978) In: Vane, J.R. and Ferreira, S.H. (eds.) Handbook of Exp. Pharmacol. Vol. 50. Springer-Verlag, Berlin. pp 615-656.

Fernandes, G., Sandberg, L., Khare, A. and Troyer, D. (1986) Arthritis Rheum. 29 Suppl:172.

Ferreira, S.H. (1972) Nature 240:200-203.

Ferreri, N.R., Howland, W.C. and Spielberg, H.L. (1986) J.Immunol. 136:4188-4193.

Fleisch, L., Kennedy, I. and Whelan, C.J. (1982) J. Pharm. Pharmacol. 34:586-588.

Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, M.E. and Smith, M.J.H. (1980) Nature 286:264-265.

Franson, R., Beckerdite, S., Wang, P., Waite, M. and Elsbach, P. (1973) Biochim. Biophys. Acta 296:365-373.

French, J.K., Hurst, N.P., Zalewski, P., Valente, L. and Forbes. I.J. (1987) FEBS Lett 212:242-246.

French, J.K., Hurst, N.P., McColl, S.R. and Cleland, L.G. (1987b) J.

Rheumatol., in press.

Galloway, J.H., Cartwright, I.J., Woodcock, B.E., Greaves, M., Russel, G.G. and Preston, F.E. (1985) Clin. Sci. 68:449-454.

Garrett, I.R., Whitehouse, M.W., Vernon-Roberts, B. and Brooks, P.M. (1985) J. Rheumatol. 12:1079-1082.

Gay, J.C., Beckman, J.K., Brash, A.R., Oates, J.A. and Lukens, J.N. (1984) Blood 64:780-785.

Genaro, R., Florio, C. and Romeo, D. (1986) FEBS Lett 180:185-190.

Gibson, R.A. (1983) Lipids 18:743-752.

Goetzl, E.J. (1981a) Biochem. Biophys. Research. Commun. 101-344-350.

Goetzl, E.J. (1981b) Med. Clin. North. Amer. 65:809-828.

Goetzl, E.J. (1982) Biochem. Biophys. Res. Commun. 101:344-350.

Goetzl, E.J. and Goldstein, I.M. (1985) In: Kelley, W.N., Harris, E.D., Ruddy, S. and Sledge, C.B. (eds.). Textbook of Rheumatology Vol. 1. W.B. Saunders Co. pp 115-144.

Goetzl, E.J. and Pickett, W.C. (1980) J. Immunol. 125:1789-1791.

Goetzl, E.J. and Sun, F.F. (1979) J. Exp. Med. 150:406-411.

Goetzl, E.J., Weller, P.F. and Valone, F.H. (1979) Advances In Inflammation Research. 14:157-167.

Goetzl, E.J., Brash, A.R., Tauber, A.I., Oates, J.A. and Hubbard, W.C. (1980a) Immunology 39:491-501.

Goetzl, E.J., Weller, P.F. and Sun, F.F. (1980b) J. Immunol. 124:926-933.

Goetzl, E.J., Boeynaems, J.M., Oates, J.A. and Hubbard, W.C. (1981) Prostaglandins 22:279-288.

Goldberg, V.J. and Ramwell, P.W. (1975) Physiol. Rev. 55:325-351.

Goldman, D.W. and Goetzl, E.J. (1982) J. Immunol. 129:1600-1604.

Goldman, D.W., Pickett, W.C. and Goetzl, E.J. (1983) Biochem. Biophys. Res. Commun. 117:282-288.

Goldyne, M.E., Burrish, G.F., Poubelle, P. and Borgeat, P. (1984) J. Biol. Chem. 259:8815-8819.

Gordon, D., Bray, M.A. and Morley, J. (1976) Nature 262:401-402.

Granstrom, E. and Kindahl, H. (1983) In: Samulesson, B. and Ramwell. P. and Paoletti. R. (eds.). Advances in Prostaglandin Thromboxane and Leukotriene Research Vol. 11. Raven Press, New York. pp173-179.

Griffin, F.M. and Silverstein, S.C. (1974) J. Exp. Med. 139:323-336.

Griffin, F.M., Griffin, J.A., Leider, J.E. and Silverstein, S.C. (1975) J. Exp. Med. 142:1263-1282.

Hafstrom, I., Palmblab, J., Malmsten, C., Radmark, O. and Samuelsson, B. (1981) FEBS Lett. 130:146-148.

Haines, K.A., Giedd, K.N. and Weissmann, G. (1986) Biochem. J. 233:583-588.

Haines, K.A., Gield, K.N., Rich, A.M., Korchak, H.M. and Weissmann, G. (1987) Biochem. J. 241:55-62.

Halenda, S.P., Zavoico, G.B. and Feinstein, M.B. (1985) J. Biol. Chem. 260:12484-12491.

Hallett, M.B. and Campbell, A.K. (1983) Immunology. 50:487-495.

Hamazaki, T., Tateno, S. and Shishido, H. (1984) Lancet i:1017-1018.

Hamberg, M. and Hamberg, G. (1974) Biochem. Biophys. Res. Commun. 95:1090-1097.

Hamberg, M. and Samuelsson, B. (1973) Proc. Natl. Acad. Sci. U.S.A. 70:899-903.

Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72:2994-2998.

Hammarstrom, S. (1980) J. Biol. Chem. 255:7093-7094

Hammarstrom, S., Hamberg, M., Samuelsson, B., Duell, E., Stawski, M. and Voorhees, J.J. (1975) Proc. Natl. Acad. Sci. U.S.A. 72:5130-5134.

Hansen, T.M. Lerche, A., Kassis, V., Lorenzen, I. and Sondergaard, J. (1983) Scand. J. Rheumatol. 12:85-88.

Hansson, G., Lindgren, J.A., Dahlen, S.E., Hedquist, P. and Samuelsson, B. (1981) FEBS Lett. 130:107-112.

Harvey, J. and Osborne, D.J. (1983) J. Pharm. Methods. 9:147-155.

Hedquist, P., Dahlen, S.E. and Bjork, J. (1982) In: Samuelsson, B. and Paoletti, R. (eds.). Leukotrienes and other lipoxygenase products. Raven Press, New York. pp 187-200.

Hendricks, P.A.J., van der Tol, M.E., Engels, F., Nijkamp, F.P. and Verhoef, J. (1986) Inflammation 10:37-47.

Higgs, G.A. and Youlten, L.J.F (1972) Br. J. Pharmacol. 44:330

Higgs, G.A., Bunting, S., Moncada, S. and Vane, J.R. (1976) Prostaglandins 12:749.

Higgs, G.A., Eakins, K.E., Mugridge, K.G., Moncada, S. and Vane, J.R. (1980) Eur. J. Pharmacol. 66:81-86.

Higgs, G.A., Palmer, R.M.J., Eakins, K.E. and Moncada, S. (1981) Mol. Aspects. Med. 4:275-301.

Hirata, F. (1981) J. Biol. Chem. 256:7730-7733.

Hirata, F., Schifmann, E., Venkatasubramanian, K., Solomon, D. and Axelrod, J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77:2533-2536.

Hirata, F., Matsuda, K., Notsu, Y., Hattori, T. and Del Carmine, R.

(1984) Proc. Natl. Acad. Sci. U.S.A. 81:4717-4721.

Hoffstein, S.T., Korchak, H.M., Smolen, J.E. and Weissmann, G. (1982) In: Karnovsky, M.L. and Bolis, L. (eds.). Phagocytosis - past and future. Academic Press, New York. pp 47-62.

Hope, W.C., Welton, A,F., Fiedler-Nagy, C., Batula-Bernado, C. and Coffey, J.W. (1983) Biochem. Pharmacol. 32:367-371.

Hsueh, W. and Sun, F.F. (1982) Biochem. Biophys. Res. Commun. 106:1085-1091.

Hsueh, W., Sun, F.F. and Henderson, S. (1985) Biochim. Biophys. Acta 835:92-97.

Humes, J.L., Sadowski, S., Galavage, M., Goldberg, M., Subers, E., Bonney, R.J. and Kuehl, F.A. (1982) J. Biol. Chem. 257:1591-1594.

Hurd, E.R., Johnston, J.M., Okita, J.R., MacDonald, P.C., Ziff, M. and Gillian, J.N. (1981) J. Clin. Invest. 67:476-485.

Hurst, N.P. (1987) Ann. Rheum. Dis., in press.

Irvine, R. (1982) Biochem. J. 204:3-16.

Jakschik, B.A. and Kuo, C.G. (1982) In: Samuelsson, B., Paoletti, R. and Ramwell, P.W. (eds.). Advances in Prostaglandin, Thromboxane and Leukotriene Research Vol. 11. Raven Press, New York. pp 141-145.

Jakschik, B. A. and Kuo, C.G. (1983) Prostaglandins 25:767-782.

Jakschik, B.A. and Kuo, C.G. (1984) In: Chakrin, L.W. and Bailey, D.M. (eds.). The Leukotrienes. Academic Press, New York. pp 139-162.

Jakschik, B.A., Sims, A.R., Sprecher, H. and Needleman, P. (1980a)

Prostaglandins 20:401-409.

Jakschik, B.A., Sun, F.F., Lee, L.H. and Steinhoff, M.M. (1980b) Biochem. Biophys. Res. Commun. 95:103-110.

Jeffrey, F. and Redgrave, T.G. (1982) J. Lipid Res. 23:154-160.

Johnson, H.G., McNee, M.L. and Sun, F.F. (1985) Am. Rev. Respir. Dis. 131:917-922.

Johnson, M., Davison, P. and Ramwell, P.W. (1972) J. Biol. Chem. 247:5656-5658.

Johnson, M., Carey, F. and McMillan, R.M. (1983) In: Campbell, P.N. and Marshall, R.M. (eds.). Essays in Biochemistry Vol. 19. Academic Press, New York. pp 40-141.

Jorg, A., Henderson, W.R., Murphy, R.C. and Klebanoff, S.J. (1982) J. Exp. Med. 155:390-402.

Jubiz, W., Radmark, O., Malmsten, C., Hansson, G., Lindgren, J.A., Palmblad, J., Uden, A.M. and Samuelsson, B. (1982) J. Biol. Chem. 257:6101-6110.

Kaplowitz, N., Kuhlenkamp, J. and Clifton, G. (1975) Proc. Soc. Exp. Biol. Med. 149:234-237.

Kawaoka, E.J., Miller, M.E. and Cheung, A.T.W. (1981) J. Clin. Immunol. 1:41-44.

Kelley, V.E., Ferretti, A., Izui, S. and Strom, T.B. (1985) J. Immunol. 134:1914-1919.

Kingsbury, K.J., Morgan, D.M., Aylott, C. and Emmerson, R. (1961) Lancet i:739.

Kinsell, L.W., Michaels, G.D., Walker, R.E and Visintine, R.E. (1961) Diabetes 10:316-319.

Klebanoff, S.J. (1980) Ann. Intern. Med. 93:480-489.

Klickstein, L.B., Shapleigh, C. and Goetzl, E.J. (1980) J. Clin. Invest. 66:1166-1170.

Konig, B., Di Nitto, P.A. and Blumberg, P.M. (1985) J. Cell. Biochem. 29:37-44.

Koyabashi, T. and Levine, L. (1983) J. Biol. Chem. 258:9116-9121.

Koshihara, Y., Murota, S.I., Petasis, N.A. and Nicolaou, K.C. (1982) FEBS Lett. 143:13-16.

Koshihara, Y., Neichi, T., Murota, S., Lao, A., Fujimoto, Y. and Tatsumo, T. (1983) FEBS Lett. 158:41-44.

Koshihara, Y., Neichi, T., Murota, S., Lao, A., Fujimoto, Y. and Tatsumo, T. (1984) Biochim. Biophys. Acta 792:92-97.

Knapp, H.R., Reilly, I.A.G., Allesandri, P. and Fitzgerald, G.A. (1986) New Engl. J. Med. 314:937-942.

Kreisle, R.A. and Parker, C.W. (1983) J. Exp. Med. 157:628-641.

Kremer, J.M., Michalek, A.V., Liniger, L., Huyck, C., Biganotte, J., Timchalk, M.A., Rynes, R.I., Ziminski, J. and Bartholomew, L.E. (1985) Lancet i:184-187.

Kromhout, D., Bosschieter, E.B. and Coulander, C. (1985) New Engl. J. Med. 312:1205-1209.

Kuhl, P., Borbe, H.O., Romer. A., Fischer, H. and Parnham, M. (1985) Agents Actions 17:366-367.

Kurzok, R. and Lieb, C.C. (1930) Proc. Soc. Biol. Med. 28:268-272.

Lad, P.M., Olson, C.V., Grewal, I.S. and Scott, S.J. (1985) Proc. Natl. Acad. Sci. U.S.A. 82:8643-8647.

Laemmli, U.K. (1970) Nature 227:680-685.

Lagarde, M., Croset, M., Boukchacre, D., Greffe, A., Dechvanne, M. and Renaud, S. (1984) Prost. Leuk. Med. 13:61-66.

Lapetina, E.G. (1984) Ann. Rep. Med. Chem. 19:213-221.

Lands, W.E.M. and Hanel, A.M. (1983) In: Pace-Asciak, C. and Granstrom, E. (eds.). Prostaglandins and related substances. Elsevier Science Publishers B.V., pp 203-223.

Lazzari, K., Proto, P.J. and Simmons, E.R. (1986) J. Biol. Chem. 261:9710-9713.

Lee, T.H., Mencia-Huerta, J.M., Shih, C., Corey, E.J., Lewis, R.A. and Austen, K.F. (1984) J. Clin. Invest. 74:1922-1933.

Lee, T.H., Hoover, R.L., Williams, J.D., Sperling, R.I., Ravalese, J., Spur, B., Robinson, D.R., Corey, E.J., Lewis, R.A. and Austen, K.F. (1985) New Engl. J. Med. 312:1217-1224.

Leitch, A.G., Lee, T.H., Ringel, E.W., Prickett, J.D., Robinson, D.R., Pyne, S.G., Corey, E.J., Drazen, J.M., Austen, K.F. and Lewis, R.A. (1984) J. Immunol. 132:2559-2565.

Lentner, C. (1984) In: Lentner, C. (ed.) Geigy Scientific Tables Vol 3. Ciba Geigy, Basle. pp 205-213.

Leslie, C.A., Gonnerman, W.A., Ullman, M.D., Hayes, K.C., Franzblau, C. and Cathart, E.S., (1985) J. Exp. Med. 162:1336-1349.

Lew, P.D., Dayer, J.M., Wollheim, C.B. and Pozzan, T. (1984) FEBS Lett. 166:44-48.

Lewis, R.A., Goetzl, E.J., Wasserman, S.I., Valone, F.H., Rubin, R.H. and Austen, K.F. (1975) J. Immunol. 114:87-92.

Lippielo, L., Yammamoto, K., Robinson, D.R. and Mankin, H.J. (1978) Arthritis Rheum. 21:909-917.

Loos, H., Blok-Schut, H., Kipp, B., van Dorn, R. and Meerhof, L. (1976) Blood 48:743-745.

Luderitz, T., Schade, U. and Rietschel, E. (1986) Eur. J. Biochem.

155:377-386.

McColl, S.R., Betts, W.H., Murphy, G.A. and Cleland, L.G. (1986) J. Chromatogr. 378:444-449.

McGuire, J., McGee, J., Crittenden, N. and Fitzpatrick, F. (1985) J. Biol. Chem. 260:8316-8319.

Malmsten, C.L., Palmblad, J., Uden, A., Radmark, O., Engstedt, L. and Samuelsson, B. (1980) Acta Physiol. Scand. 110:449-451.

Manthorpe, R., Peterson, S.H., and Prause, J.U. (1984) Rheumatol. Int. 4:165-167.

Marcus, A.J., Brockman, M.J., Safier, L.B., Ullman, H.H. and Islam, N. (1982) Biochem. Biophys. Res. Commun. 109:130-137.

Marcus, A.J., Safier, L.B., Brockman, M.J., Ullman, H.L., Islam, N., Sorrell, T.C., Serhan, C.N., Weissmann, G., Oglesby, T.D. and Gorman, R.R. (1984) J. All. Clin. Immunol. 77:336-342.

Marom, Z., Shelamer, J.H., Sun, F.F. and Kaliner, M. (1983) J. Clin. Invest. 72:122-127.

Mathe, A.A., Hedquist, P., Holmgren, A and Svanborg, N. (1972) Adv. Biosci. 9:241-245.

Mathews, W.R. and Murphy, R.C. (1982) Biochem. Pharmacol. 31:2129-2132.

Mathews, W.R., Rokach, J. and Murphy, R.C. (1981) Anal. Biochem. 118:96-101.

Mayhofer, G., Pugh, C.W. and Barclay, A.M. (1983) Eur. J. Immunol. 13:112-122.

Michelassi, F., Landa, L., Zapol, W.M., Hill, R.D., Huttmeier, P. and Watkins, W.D. (1983) In: Samuelsson, B., Paoletti, R. and Ramwell, P.W. (eds.). Advances In Prostaglandin, Thromboxane and

Leukotriene Research Vol. 11. Raven Press, New York. pp 397-400.

Moncada, S., Gryglewski, R., Bunting, S. and Vane, J.R. (1976) Nature 263:663-665.

Morris, H.R., Piper, P.J., Taylor, G.W. and Tippins, J.R. (1979) Br. J. Pharmacol. 66:452p

Morris, H.R., Taylor, G.W., Piper, P.J., Sanhoun, M.N. and Tippins, J.R. (1980) Prostaglandins 19:185-201.

Muller, M. and Sorrel, T. (1985) J. Chromatogr. 343:213-218.

Murakami, K. and Routtenberg, A. (1985) FEBS Lett. 192:189-193.

Murphy, R.C., Pickett, W.C., Culp, B.R. and Lands, W.E.M. (1981) Prostaglandins 22:613-622.

Naccache, P.H., Sha'afi, R.I., Borgeat, P. and Goetzl, E.J. (1981) J. Clin. Invest. 67:1584-1587.

Nagy, L., Lee, T.H., Goetzl, E.J., Pickett, W.C. and Kay, A.B. (1982) Clin. Exp. Immunol. 47:541-547.

Nakano, J. (1973) In: Cuthbert, M.F. (ed.). The Prostaglandins. Pharmacological and Therapeutic Advances. William Heinen Med. Books, London. pp 23-124.

Needleman, P., Raz, A., Minkes, M.S., Ferendelli, J.A. and Sprecher, H. (1979) Proc. Natl. Acad. Sci. U.S.A. 76:944-948.

Needleman, P., Turk, J., Jakschik, B.A., Morrison, A. and Lefkowitz, J. (1986) Ann. Rev. Biochem. 15:69-102.

Neichi, T., Koshihara, Y. and Murota, S. (1983) Biochim. Biophys. Acta 753:130-132.

Nevalainen, T.J. (1980) Scand. J. Gastroenterol. 15:641-650.

Nordoy, A., Davenas, E., Giavitti, M. and Renaud, S. (1985) Biochim. Biophys. Acta 835:491-500.

Nishizuka, Y. (1984) Nature 308:693-698.

Nugteren, D.H. (1978) Agents Actions 8:296-298.

Nugteren, D.H. and Halzelhof, E. (1973) Biochim. Biophys. Acta 326:448-461.

O'Donell, M. and Welton, A.F. (1982) Fed. Proc. 41:Abstr.3221.

Ohl, V.S. and Litwack, G. (1977) Arch. Biochem. Biophys. 180:186-190.

Oliw, E., Granstrom, E. and Angaard, E. (1983) In: Pace-Asciak, C. and Granstrom, E. (eds.). The Prostaglandins and Related Substances. Elsevier, Science Publishers B.V. pp1-44.

Orange, R.P., Valentine, M.D. and Austen, K.D. (1967) Science 157:318-319.

Orange, R.P., Valentine, M..D. and Austen, K.F. (1968) J. Exp. Med. 127:767-782.

Orange, R.P., Stechschulte, D.J. and Austen, K.F. (1969) Fed. Proc. 28:1710-1715.

Orange, R.P., Murphy, R.C., Karnovsky, M.L. and Austen, K.F. (1973) J. Immunol. 110:760-770.

Orning, L., Hammarstrom, S. and Samuelsson, B. (1980) Proc. Natl. Acad. Sci. U.S.A. 77: 2014-2017.

Osborne, D.J., Peters, B.J. and Meade, C.J. (1983) Prostaglandins 26:817-832.

Pace-Asciak, C. (1977a) Prostaglandins 13:649-660.

Pace-Asciak, C. (1977b) Prostaglandins 13:661-668.

Palmblad, J., Malmsten, C.L., Uden, A.M., Radmark, O., Engstedt, L. and Samuelsson, B. (1981) Blood 58:658-661.

Palmer, R.M.J. and Salmon, J.A. (1983) Immunology 50:65-73.

Palmer, R.M.J., Stepney, R.J., Higgs, G.A. and Eakins. K.E. (1980) Prostaglandins 20:411-418.

Panosslan, A., Hamberg, M. and Samuelsson, B (1982) FEBS Lett. 150:511-513.

Parker, C.W. (1984) In: Chakrin, L.W. and Bailey, D.M. (eds.). The Leukotrienes. Academic Press, New York. pp 125-137.

Parker, C.W. and Aykent, S. (1982) Biochem. Biophys. Res. Commun. 109:1011-1016.

Parker, C.W., Stenson, W.F., Huber, M.G. and Kelly, J.P. (1979) J. Immunol. 122:1572-1577.

Parker, C.W., Falkenheim, S.F. and Huber, M.M. (1980a) Prostaglandins 20:863-886.

Parker, C.W., Koch, D., Huber, M.O. and Falkenheim, S.F. (1980b) Biochem. Biophys. Res. Commun. 94:1037-1043.

Patterson, R., Pruzansky, J.J. and Harris, K.E. (1981) J. Allergy Clin. Immunol. 67:444-449.

Pattison, N.R. (1981) Biochem. Biophys. Res. Commun. 102:403-410.

Pawlowski, N.A., Scott, W.A., Andreach, M. and Cohn, Z.A. (1982) J. Exp. Med. 155:1653-1664.

Payan, D. and Goetzl, E.J. (1983) J. Immunol. 131:551-553.

Payan, D., Goldman, D.W. andGoetzl, E.J. (1984) In: Chakrin, L.W. and Bailey, D. (eds.). The Leukotrienes. Academic Press, New York. pp. 231-245.

Pepinsky, R.B., Sinclair, L.K., Browning, J.L., Mattaliano, R.J., Smart, J.E., Pinchang Chow, E., Falbel, T., Ribolini, A., Garwin, J.L. and Wallner, B.P. (1986) J. Biol. Chem. 261:4239-4246.

Peters, S.P., Kagey-Sabotka, A., MacGlashan, D.W., Siegel, M.I. and

Lichtenstein, L.M. (1982) J. Immunol. 129:797-803.

Peters, S.P., MacGlashan, D.W., Schulman, E.S., Schleimer, R.P., Hayes, E.C., Rokach, J., Adkinson, N.F. and Lichtenstein, L.M. (1984) J. Immunol. 132:1972-1979.

Piper, P.J. and Temple, D.M. (1981) J. Pharm. Pharmacol. 33:384-386.

Powell. W.S. and Solomon, S. (1978) J. Biol. Chem. 253:4609-4016.

Prescott, S.M. (1984) J. Biol. Chem. 259:7615-7621.

Prescott, S.M., Zimmerman, G.A. and Morrison, A.R. (1985) Prostaglandins 30:209-227.

Prickett, J.D., Robinson, D.R. and Steinberg, A.D. (1981) J. Clin. Invest. 68:556-559.

Prickett, J.D., Robinson, D.R. and Steinberg, A.D. (1983) Arthritis Rheum. 26:133-139.

Prickett, J.D., Trentham, D.E. and Robinson, D.R. (1984) J. Immunol. 132:725-729.

Quesenberry, P. and Levitte, L. (1979) New Engl. J. Med. 301;pts. i,ii & iii; 755-760, 819-823, & 868-872.

Radmark, O., Malmsten, C.L., Samuelsson, B., Clark, D.A., Goto, G., Marfat, A. and Corey, E.J. (1980a) Biochem. Biophys. Res. Commun. 92:954-961.

Radmark, O., Malmsten, C. and Samuelsson, B. (1980b) Biochem. Biophys. Res. Commun. 96:1679-1687.

Radmark, O., Shimizu, T., Jornvall, H. and Samuelsson, B. (1984) J. Biol. Chem. 259:12339-12345.

Randall, R.W., Eakins, K.E., Higgs, G.A., Salmon, J.A. and Taterson, J.E. (1980) Agents Actions 10:553-555.

Robert, A. (1976) In: Samuelsson, B. and Ramwell, P.W. (eds.).

Advances In Prostaglandin , Thromboxane. Research Vol. 2. Raven Press, New York. pp 507-520.

Robinson, D.R. (1985) In: Kelley, W.N., Harris, E.D., Ruddy, S. and Sledge, C.B. (eds.). Textbook of Rheumatology Vol. 1. W.B. Saunders Company. pp 71-82.

Robinson, D.R., McGuire, M.B. and Levine, L. (1975) Ann. N.Y. Acad. Sci. 256:318:329.

Robinson, D.R., Dayer, J.M. and Krane, S.M. (1979) Ann. N.Y. Acad. Sci. 322:279-294.

Robinson, J.M., Badwey, J.A., Karnovsky, M.L. and Karnovsky, M.J. (1984) Biochem. Biophys. Res. Commun. 122:734-739.

Robinson, D.R., Prickett, J.D., Polisson, R., Steinberg, A.D. and Levine, L. (1985) Prostaglandins 30:51-75.

Robinson, D.R., Prickett, J.D., Makoul, G.T., Steinberg, A.D. and Colvin, R.B. (1986) Arthritis Rheum. 29:539-546.

Rola-Pleszcynsky, M. and Lemaire, I. (1985) J. Immunol. 135:3958-3961.

Rola-Pleszcynski, M., Borgeat, P. and Sirois, P. (1982) Biochem. Biophys. Res. Commun. 108:1531-1537.

Rola-Pleszcynsky, M., Gagnon, M.L. and Sirois, P. (1983) Biochem. Biophys. Res. Commun. 113:531-537.

Rollins, T., Zanolari, B., Springer, M., Guindon, Y., Zamboni, R. Lau, C. and Rokach, J. (1983) Prostaglandins 25:281-289.

Root, R.K. and Cohen, M.S. (1981) Rev. Infect. Dis. 3:565-598.

Rossi, F., Grzeskowiak, M. and Della Bianca, V. (1986) Biochem. Biophys. Res. Commun. 140:1-11.

Rouzer, C.A., Scott, W.A., Cohn, Z.A., Blackburn, P. and Manning, J.M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77:4928-4932.

Rouzer, C.A., Scott, W.A., Hamill, A.L., Liu, F., Katz, D.H. and Cohn,

Z.A. (1982) J. Exp. Med. 156:1077-1086.

Rouzer, C.A., Matsumoto, T. and Samuelsson, B. (1986a) Proc. Natl. Acad. Sci. U.S.A. 83:857-861.

Rouzer, C.A., Matsumoto, T., Shimizu, T. and Samuelsson, B. (1986b) In: Zor, U., Naor, Z. and Kohen, F. (eds.). Advances in Prostaglandin, Thromboxane and Leukotriene Research Vol. 16. Raven Press, New York. pp. 3-16.

Rozengurt, E., Geleherter, T.D., Legg, A. and Pettican, P. (1981) Cell 23:781-788.

Salari, H., Borgeat, P., Fournier, M., Hebert, J. and Pelletier, G. (1985a) J. Exp. Med. 163:1904-1915.

Salari, H., Braquet, P., Naccache, P.H. and Borgeat, P. (1985b) Inflammation 9:127-138.

Salmon, J.A., Simmons, P.M. and Palmer, R.M.J. (1982) Prostaglandins 15:383-397.

Samuelsson, B. and Hammarstrom, S. (1980) Prostaglandins 19:645-648.

Sanders, T.A.M. and Younger, K.M. (1981) Br. J. Nutr. 45:613-616.

Saynor, R. and Veral, D. (1980) I.R.C.S. Med. Sci. 8:378-379.

Seeger, W., Suttorp, N., Hellwig, A. and Bhakdi, S. (1986) J. Immunol. 137:1286-1293.

Sekiya, K., Okuda, H. and Arichi, S. (1982) Biochim. Biophys. Acta 713:68-72.

Serhan, C.N., Lundberg, U., Weissmann, G. and Samuelsson, B. (1984) Prostaglandins 27:563-581.

Shackleford, D.A. and Trowbridge, I.S. (1984) J. Biol. Chem. 259:11706-11712.

Sheard, P. (1981) In: Piper, P.J. (ed.). SRS-A and Leukotrienes.

Wiley, New York. pp 209-218.

Sheard, P., Holroyde, M.C., Ghelani, A.M., Bantik, J.R. and Lee, T.B. (1982) In: Samuelsson, B. and Paoletti, R. (eds.). Leukotrienes and other lipoxygenase products. Raven Press, New York. pp 229-235.

Showell, H.J., Naccache, P.H., Borgeat, P., Picard, S., Vallerand, P., Becker, E.L. and Sha'afi, R.I. (1982a) J. Immunol. 128:811-816.

Showell, H.J., Otterness, I.G., Marfat, A. and Corey, E.J. (1982b) Biochem. Biophys. Res. Commun. 106:741-747.

Siegel, M., McConnell, R., Abrahams, S., Porter, N. and Cuatrecasas, P. (1979) Biochem. Biophys. Res. Commun. 89:1273-1280.

Siegel, M., McConnell, R.T., Bonser, R.W. and Cuatrecasas, P. (1981) Prostaglandins 21:123-132.

Sirois, P., Borgeat, P., Jeanson, A., Roy, S. and Girard, G. (1980) Prost. Med. 5:429-444.

Smith, J.B. and Willis, A.L. (1971) Nature 231:235-237.

Smith, M.J.H., Ford-Hutchinson, A.W. and Bray, M.A. (1980) J. Pharm. Pharmacol. 32:517-518.

Smith, R.J. and Iden, S.S. (1979) Biochem. Biophys. Res. Commun. 91:263-271.

Smith, R.J., Yein, F.S., Speziale, S.C. and Bowman, B.J. (1986) Biochem. Biophys. Res. Commun. 136:310-315.

Smolen, J.E. and Boxer, L.A. (1983) Clin. Lab. Med. 3:779-800.

Smolen, J.E., Korchak, H.M. and Weissmann, G. (1981) Biochim. Biophys. Acta 667:512-520.

Snyderman, R. and Goetzl, E.J. (1981) Science 213:830-837.

Sok, D., Han, C., Pai, J. and Sih, C.J. (1982) Biochem. Biophys. Res.

Commun. 107:101-108.

Stenson, W.F. and Parker, C.W. (1980) J. Immunol. 124:2100-2104.

Stenson, W.F. and Lobos, E. (1982) J. Clin. Invest. 69:494-497.

Stenson, W.F., Nichells, M.W. and Atkinson, J.P. (1983) Prostaglandins 26:253-264.

Stenson, W.F., Prescott, S.M. amd Sprecher, H. (1984) J. Biol. Chem. 259:11784-11789

Strandberg, K. and Uvnas, B. (1971) Acta Physiol. Scand. 82:358-374.

Strasser, T., Fischer, S. and Weber, P.C. (1985) Proc. Natl. Acad. Sci. U.S.A. 82:1540-1543.

Stratford May, W., Jacobs, S., and Cuatrecasas, P. (1984) Proc. Natl. Acad. Sci. U.S.A. 81:2016-2020.

Sun, F.F. and McGuire, J.C. (1984) Biochim. Biophys. Acta 794:56-64.

Swingle, K.F., Jaques, L.W. and Kvam, D.C. (1969) Proc. Soc. Exp. Biol. Med. 132:608-612.

Szamel, M. and Resch, K. (1981) J. Biol. Chem. 256:11623-11681.

Terano, T., Salmon, J.A. and Moncada, S. (1984a) Biochem. Pharmacol. 33:3071-3076.

Terano, T., Salmon, J.A. and Moncada, S. (1984b) Prostaglandins 27:217-232

Traynor, J.R. and Authi, K.S. (1981) Biochim. Biophys. Acta 655:571-577.

Touqui, L., Rothut, B., Shaw, A.M., Fradin, A., Vargaftig, B.B. and Russo-Marie, F. (1986) Nature 321:177-180.

Tripp, C.S., Mahoney, M. and Needleman, P. (1985) J. Biol. Chem. 260:5895-5898.

Turk, J., Maas, R.L., Brash, A.R., Roberts, L.J. and Oates, J.A. (1982) J. Biol. Chem. 257:7068-7076.

Turner, S.R., Tainer, J.A. and Lynn, W.S. (1975) Nature 257:680-681.

Uden, A.M., Palmblad, J., Lindgren J.A. and Malmsten, C. (1983) Int. Arch. Allergy. Appl. Immunol. 72:91-93.

Vadas, P. and Hay, J.B. (1983) Life Sciences 26:1721-1729.

Vadas, P. and Pruzanski, W. (1984) In: Otterness, I., Capetola, R. and Wong, S. (eds.). Advances In Inflammation Research Vol. 7. Raven Press, New York. pp 51-59.

Vadas, P., Wasi, S., Movat, H.Z. and Hay, J.B. (1981) Nature 293:583-585.

Van Den Bosch, H. (1980) Biochim. Biophys. Acta 604:191-246.

Vanderhoek, J.Y., Bryant, R.W. and Bailey, J.M. (1980) J. Biol. Chem. 255:10064-10065.

Vanderhoek, J.Y., Tare, N.S., Bailey, J.M., Goldstein, A.L. and Pluznik, D.H. (1982) J. Biol. Chem. 257:12191-12195.

van Dorp, D.A. Beethins, R.K., Nugteren, D.H. and Vonkeman, H. (1964) Biochim. Biophys. Acta 90:204-207.

Vane, J.R. (1971) Nature 231:232-235.

Van Gent, C.M., Luten, J.B., Ruiter, A., Brongeest, A. and Schoute, H.C. (1979) Lancet ii:1249-1250.

VanRollins, M., Ho, S.H.K., Greenwald, J.E., Alexander, M., Dorman, N.J., Wong, L.K. and Horrocks, L.A. (1980) Prostaglandins 20: 571-577.

Velo, P., Dunn, C.J., Giroud, J.P., Tunsit, J. and Willoughby, D.A. (1973) J. Path. 111:149-149.

Verhagen, J., Walstra, P., Veldnik, G. and Vliegenthart, J. (1984)

Prost. Leuk. Med. 13:15-20.

Volpi, M., Molski, T., Naccache, P.H., Feinstein, M.B. and Sha'afi, R.I. (1985) Biochem. Biophys. Res. Commun. 128:594-600.

von Euler, U.S. (1934) Arch. Exp. Pathol. Pharmakol. 175:78-84.

von Euler, U.S. (1935) Klin. Wochenschrift 14:1182-1183.

von Schacky, C. and Weber, P. (1986) J. Clin. Invest. 76:3446-2450.

von Schacky, C., Seiss, W., Fischer, S. and Weber, P. (1985a) J. Lipid. Res. 26:457-464.

von Schacky, C., Fischer, S. and Weber, P. (1985b) J. Clin. Invest. 76:1626-1631.

Wade, B.H. and Mandell, G.L. (198?) Am. J. Med. 74:686-603.

Walker, J.R., Boot, J.R., Cox, B. and Dawson, W. (1980) J. Pharm. Pharmacol. 32:866-867.

Wallner, B.R., Mattliano, R.J., Hession, C., Cate, R.L., Tizard, R., Sinclair, L.K., Foeller, C., Pingchang Chow, R., Browning, J.L., Ramachandran, K.L. and Pepinsky, R.B. (1986) Nature 320:77-81.

Walsh, C.E., Waite, B.M., Thomas, M.J. and DeChatelet, L.R. (1980) J. Biol. Chem. 256:7228-7234.

Wasserman, M.A. (1975) Prostaglandins 9:959-973.

Wedmore, C.C. and Williams, T.J. (1981) Nature 289:646-650.

Weiner, B.H., Ockene, I.S., Levine, P.H., Cuenoud, H.F., Fisher, M., Johnson, B.F., Daoud, A.S., Jarmolych, J., Hosmer, D., Johnson, M.H., Natale, A., Vaudreuil, C. and Hoogasian, J.J. (1986) New Engl. J. Med. 315:841-846.

Weiner, T.W. and Sprecher, H. (1984) Biochim. Biophys. Acta 792:293-303.

Westcott, J.Y. and Murphy, R.C. (1983) Prostaglandins 26:223-239

Whitaker, M.O., Wyche, A., Fitzpatrick, F., Sprecher, H. and Needleman, P. (1984) Proc. Natl. Acad. Sci. U.S.A. 767:5919-5923.

White, J.G., Rao, G.H.R. and Estenson, R.D. (1974) Am. J. Pathol. 75:301-312.

Whitehouse, M.W., Orr, K.J., Beck, F.W.J. and Pearson, C.M. (1974) Immunology 27:311-330.

Whitaker, M.O., Wyche, A., Fitzpatrick, F., Sprecher, H. and Needleman, P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76:5919-5923.

Wightman, P.D., Dahlgren, M.E., Davies, P. and Bonney, R.J. (1981) Biochem. J. 200:441-444.

Williams, A.J. and Cole, P.J. (1981) Immunology 44:847-858.

Williams, J.D., Czop, J. and Austen, K.F. (1984) J. Immunol. 132:3034-3040.

Williams, J.D., Lee, T.H., Lewis, R.A. and Austen, K.F. (1985) J. Immunol. 134:2624-2630.

Williams, J.D., Robin, J.L., Lewis, R.A., Lee, T.H. and Austen, K.F. (1986) J. Immunol. 136:642-648.

Wilson, D.B., Prescott, S.M. and Majerus, P.W. (1982) J. Biol. Chem. 257:3510-3515.

Wolfson, M., McPhail, L.C., Nasrallan, V.N. and Snyderman, R. (1985) J. Immunol. 135:2057-2062.

Yamamoto, S., Ishii, M., Nakadate, T., Nakaki, T. and Kato, R. (1983) J. Biol. Chem. 258:12149-12152.

Yin, H.L. and Stossel, T.P. (1982) In: Karnovsky, M.L. and Bolis, L. (eds.). Phagocytosis - past and future. Academic Press, New York. pp 13-27.

Yoshimoto, T., Kunikawa, M., Yamamoto, S., Horie, T. and Watanabe-Kohno, S. (1983) Biochem. Biophys. Res. Commun. 116:612-618.

Yecies, L., Johnson, S., Wedner, J. and Parker, C. (1979) J. Immunol. 122:2090-2095.

Zigmond, S.H. and Hirsch, J.G. (1973) J. Exp. Med. 137:387.

Ziltener, H.J., Chavaillaz, P.A. and Jorg, A. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364:1029-1037.

----------000----------