Studies on the Anti-inflammatory Effect of S-Adenosyl-L-methionine

Nicholas Charles Matthews

Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

April 1991

The University of Aston in Birmingham

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SUMMARY

1. S-adenosyl-L-methionine (SAMe) had no effect on cytochrome C reduction by superoxide generated from xanthine oxidase except at high concentrations. This was due to direct inhibition of the enzyme.
2. SAMe inhibited the neutrophil respiratory burst , measured by luminol enhanced chemiluminescence, to FMLP and zymosan A but not to PMA.
3. Adenosine and methylthioadenosine (MTA) inhibited the respiratory burst elicited by FMLP.
4. SAMe inhibited the phagocytosis of latex particles by neutrophils at high concentrations but methionine and S-adenosyl-L-homocysteine had no effect.
5. Treatment with SAMe had no effect on cell infiltration or PGE_2 production in 6-day air pouches.
6. Treatment with SAMe at the optimum dose of 50mg/kg inhibited the early phases of carrageenan induced rat hind paw inflammation but had a lesser effect on the secondary response.The antiinflammatory effect was sustained after inhibiton of polyamine synthesis.
7. SAMe increased liver putrescine levels in the presence and absence of inflammation Spermidine levels were increased in the presence of inflammation but spermine levels were unaffected by any of the treatments.
8. MTA and adenosine increased liver putrescine and spermidine levels
9. Treatment with SAMe had no effect on the polyamine status of blood.
10.Treatment with SAMe had no effect on the levels of glutathione in liver or blood.
11.SAMe and MTA inhibited histamine and platelet-activating factor (PAF) induced hind paw inflammation but had no effect on inflammation induced by dextran, zymosan, compound 48/80, 5-hydroxytryptamine, arachidonic acid or glucose oxidase. MTA was more effective than SAMe.
12. PAF-induced rat hind paw inflammation was inhibited by isoprenaline and verapamil. Combinations of these drugs with SAMe or MTA had no further enhancement of effect.
13. Incubation of rat PMNLs with [^{14}C] SAMe increased the intracellular levels of S-adenosyl-L-homocysteine in a dose dependent manner, but had no effect on the intracellular levels of SAMe, adenosine or MTA.
14. Pharmacokinetic studies of plasma SAMe following a single dose of the drug (50mg/kg) i.p. demonstrated that SAMe is rapidly absorbed and metabolised

Key Words:

S-Adenosyl-L-methionine, Inflammation, Polyamines, Neutrophil

Dedication

For my family

Acknowledgements

I would like to thank Dr. David A. Lewis for constant advice and helpful criticism throughout the course of this study. I would also like to thank fellow travellers from this laboratory Dr. Rob Hyde and Dr. Graham Shaw for guidance and encouragement.

I wish to thank the following people who have helped me in various ways : Mel Gamble, Kevin Hughes, Andy Keates, Dr Mike Thompson and Chris the technician.

I wish to acknowledge my friends and family for their support , patience and understanding.

I am indebted to Lisa Tweedie. Without her love, support and advice this thesis would not have been realised.

1.0 Introduction

1.1 History of S-adenosyl-l-methionine

A study of transmethylation reactions by Cantoni (1951) established that the co-factor adenosine triphosphate (ATP) was an essential prerequisite for the formation of 'active methionine'. The reaction was described thus:

$$\text{L-methionine} + \text{ATP} \xrightarrow[Mg2+]{GSH} \text{active methionine} + 3Pi$$

The product was distinguished from methionine by its ability to donate methyl groups in the absence of ATP. The active intermediate was designated S-adenosyl-l-methionine (SAMe) and was described by the author as 'an addition product of methionine and the adenosine moiety of ATP'. The compound was believed to be a labile intermediary metabolite but development of sensitive and specific assay systems showed SAMe to be present in μg /g quantities in mammalian tissues particularly liver and brain (Baldessarini 1975). Indeed wider investigations have revealed the cofactor to be ubiquitous and essential to life in all its manifestations from viruses to man.

The chemically labile nature of SAMe precluded it from any therapeutic investigations until a group of investigators led by Stramentinoli formulated a stable p-toluene sulphonate complex of SAMe sulphate which could be exploited as a drug. Parenteral administration of the compound to depressives produced significant, rapid and beneficial effects (Agnoli et al 1976). However by chance it was further discovered that for those patients who had their misery exacerbated by osteoarthritis , the symptoms of this debilitating condition were also relieved (Cecatto etal 1976). These were the first observations of the anti-inflammatory effect of SAMe. Over a decade later it is administered as as an antidepressant and as a treatment for osteoarthritis in Europe and has undergone extensive clinical trials in the United States. Open and double blind clinical studies have indicated that SAMe has an efficacy comparable to conventional non steroidal anti- inflammatory drugs (Fassbender 1987). In contrast, patients treated with SAMe reported no more side effects than the

placebo group (Berger and Novak 1987). Indeed SAMe has been demonstrated to have a cytoprotective effect on the gastric mucousa (Laudano 1987), a facet of its action which has stimulated interest in the use of SAMe as a novel anti-ulcer drug.

The full repertoire of the pharmacological actions of SAMe will be described later in the text. The definitive mode of action of SAMe remains unknown but the diverse and complex biochemistry of the cofactor is the key to understanding how critical SAMe is to the body.

1.2 Biochemistry of SAMe

1.2.1 Enzymatic synthesis of SAMe

The sulphonium compound SAMe is formed at the expense of ATP by SAMe synthetase [ATP: L-methionine-s-adenosyltransferase, EC 2.5.1.6.] in the presence of Mg^{2+} and K^{+}. The reaction consists of the complete dephoshorylation of ATP and nucleophilic transfer of the 5'-deoxyadenosyl moiety to one of the free pairs of electrons of the thioether sulphur of l-methionine. Enzyme bound tripolyphosphate (PPPi) is an intermediate of the reaction (Mudd et al 1966) which upon hydrolysis yields SAMe and PPi and Pi thus the enzyme can recycle.

Investigations of highly purified SAMe synthetase from rat liver have revealed 2 distinct isozymes designated α and ß of 110 and 210 KDa (Suma et al 1986) and consisting of 4 and 2 subunits of 48.5 KDa each.Cabrero et al (1987) reports that the peptide maps of the 2 forms are virtually identical.The tetramer has a low Km for methionine of 2-20μM and is inhibited through product inhibition by SAMe and pyrophosphate, the dimer has a high Km of 600-1000 μM and has a dose dependent response to SAMe being either activated or inhibited but pyrophosphate has no effect.Thus the 2 synthetases have different specific activities at physiological concentrations of methionine and provide a means of regulation by methionine and by the products of the reaction.

The proportion of the isozymes change during the foetal stage to maturity (Okada et al 1980).In rat there is a dramatic decrease in tissue levels in SAMe after the neonatal period (Eloranta 1966) followed by a gradual decrease in SAMe levels during maturation and

aging (Gharib et al 1982 and Stramentinoli et al 1977), the activity of the enzyme however shows little change from youthful levels in liver and brain (Eloranta 1966). The activity of the tetramer can be increased by the administration of ethionine and adenine (Tsukada et al 1980) and by feeding on a high methionine diet (Matsumoto et al 1984). It was observed that the quantity of S-adenosyl-l -ethionine and SAMe in rat liver increased in parallel with the change in activity of the α form (Matsumoto et al 1984).Compared to other tissues the level of SAMe is highest in the liver and concentrations of SAMe could be increased by 10 fold using a methionine rich diet (Finkelstein and Martin,1986).Baldessarini (1966) increased SAMe levels in brain by administering large exogenous doses of methionine since under normal physiological conditions SAMe synthetase is non-saturated. However the administration of high oral doses of methionine to man does not induce an increase in plasma levels of SAMe (Baldessarini et al 1975) and has no anti inflammatory effect (Stramentinoli 1987).

The close relationship between l-methionine and SAMe established by Baldessarini and Kopin showed that SAMe levels are controlled by rates of synthesis rather than rates of use, and that the rate of synthesis was limited by tissue levels of methionine (Lombardini et al., (1971) which was less than the Km of the adenosyl -transferases for l-methionine. The enzyme is inhibited by cycloleucine (1-aminocyclopentane-1-carboxylic acid) a drug which has been shown to restrain the growth of microorganisms (Lombardini et al 1970) and tumours (Lombardini et al 1971). It has also been reported that this inhibitor can suppress aspects of the immune system (Coulter et al 1974). In vivo studies with Sprague-Dawley rats and DBA mice treated with cycloleucine by Lombardini and Talalay (1973) showed that tissue methionine levels were increased 2-4 fold within minutes and sustained for many hours.This was accompanied by a decrease of 25-40% in tissue levels of SAMe except for the liver where a doubling in SAMe content was observed.The critical contribution of SAMe metabolism to the mechanics of the immune system is illustrated by the role of the cofactor in human peripheral blood lymphocyte ontogeny.

Human lymphocytes proliferate in response to M protein (streptococcal M protein is the M5 protein of group A streptococci).It is a specific mitogen and recent studies have shown that exposure of lymphocytes to M protein leads to an increased generation of cytotoxic T cells.

The rate of SAMe turnover in resting T lymphocytes was 5 fold greater than other tissues with the exception of liver.Most significantly the rate of SAMe use increased 10 fold after stimulation of the cells with lectins and the activity of SAMe synthetase was doubled (Kotb et al 1987)

1.2.2 Pharmacokinetics of SAMe

S-adenosyl-l-methionine (SAMe) is metabolised very quickly.Studies in man following intravenous injections of SAMe gave half life values of 81± 8 and 101± 7 minutes at doses of 100 and 500mg respectively (Guilidori et al 1984).Stramentinoli (1987) has confirmed that SAMe is effective by the oral route with a 6 fold increase in the plasma levels of SAMe being obtained 3 hours after the ingestion of 200mg of the drug.This confirmed animal studies where 100mg doses in enteric coated tablets delivered to dogs generated a well defined peak of absorption 1-2 hours after treatment. An oral dose of 400mg administered to patients at 8 hour intervals for 3 days raised plasma levels of SAMe from 58 ng/ml to 140 ng/ml and more significantly, since SAMe is a treatment for degenerative joint disease, increased SAMe levels in synovial fluid from 40 ng/ml to 120 ng/ml. An audit of the metabolism of exogenous SAMe using the methyl -[^{14} C] derivative revealed that 15.5 % of the dose was excreted in urine and 23.5 % was lost in faeces which suggests that about 60 % was incorporated into stable pools. Guilidori et al (1984) observed little evidence of binding or sequestration of the drug since the apparent volume of distribution was 0.4 litre / kg. Dosing by the intramuscular route showed the bioavailability to be 85 % but the oral bioavailability was approximately 1%.Once administered the drug is metabolised rapidly with approximately 70% conversion to metabolites during the first hepatic portal circuit (Stramentinoli et al 1977).

Contrary to previous belief, although SAMe is both polar and chemically labile, it can cross the blood brain barrier and reach the central nervous system (Taylor and Randall 1975).The administration of a single large intravenous dose of SAMe (100mg/kg) to rats doubled levels of SAMe in brain tissue within 5 minutes and decayed with a half life of 1-2 hours (Stramentinoli et al 1977). Intramuscular administration of the drug increased brain SAMe content by 46% but had a slower rate of decay (Stramentinoli et al 1978). To date it

has not been established whether SAMe treatment by the oral route can increase human brain SAMe levels but Bottigleri et al (1984) has reported that doses of 200-275 mg i.v can increase SAMe levels in cerebrospinal fluid (by 17-29 %) but without any detectable effect on the plasma concentration of the compound.

1.2.3. Transport of SAMe in Mammalian Cells

Stekol et al (1958) observed that rat liver slices incubated in the presence of SAMe synthesised choline and creatinine more effectively than in the presence of methionine thus providing indirect evidence for an in vivo mechanism of uptake of exogenous SAMe.Mizoguchi et al., (1972) showed by autoradiography that labelled SAMe was incorporated into isolated rat pancreas cells.Direct evidence was presented by Stramentinoli et al., (1978) where rabbit erythrocytes were incubated with exogenous labelled SAMe.Although total radioactivity increased in the cells the actual activity associated with unmodified SAMe remained constant and did not accumulate. Approximately 10% of the total radioactivity was associated with methylated derivatives of phosphatidylethanolamine. Details of the uptake mechanism were established using isolated rat hepatocytes (Pezzoli et al., 1979).There were 2 phases consisting of an initial fast uptake completed in 15 seconds and a second slower phase which probably represented intracellular metabolism with a Km of 8.33 μM and Vmax of 10.6 pmol / mg protein / minute.Uptake was both temperature and pH dependent and sensitive to dinitrophenol thus suggesting an active uptake mechanism.Similarly in this tissue whilst total radioactivity increased specific radioactivity associated with SAMe remained constant. Intracellular metabolism of the compound was indicated by $^{14}CO_2$ formation by preparations incubated with [^{14}COOH] labelled SAMe.In vivo SAMe transport has been described by Zappia et al., (1978) using a liver perfusion technique. A comparison of SAMe with structurally similar compounds revealed that the sulphonium ion partially retarded the transport of the molecule since methionine and methylthioadenosine were absorbed more effectively than the sulphonium compounds SAMe , decarboxylated SAMe and dimethylthioadenosine. The results suggested a high affinity permease for thioethers and a low affinity uptake system for sulphonium compounds. It was concluded that despite the charged sulphonium ion which limits SAMe

membrane permeability exogenous SAMe can be taken by mammalian cells in vivo and in vitro.

1.3. Metabolism of SAMe

S-adenosyl-l-methionine is metabolised via three pathways (fig 1.3.1.).These are primarily transmethylation followed by transsulphation, decarboxylation and the minor metabolic pathway of cleavage.

1.3.1.Transmethylation

The adenosylation of methionine to SAMe converts the stable sulphur atom in the thioether linkage into the highly reactive sulphonium derivative.In this form the methyl group is highly labile and can be donated to any of a number of different methyl group acceptors, including proteins, phospholipids, nucleic acids and neurotransmitters, leaving S-adenosyl-l-homocysteine as the demethylated product. There have been over 40 documented transmethylations (Stramentinoli, 1987) and it has been estimated that 85 % of these reactions occur in the liver (Mudd and Poole, 1975). Investigations of SAMe metabolism in vivo suggest that 30% of SAMe is consumed in transmethylation reactions (Guilidori et al., 1984). Studies with erythrocytes in vitro estimated that up to 70% of exogenous SAMe was devoted to phospholipid methylation (Pezzoli et al., 1978).

1.3.1.1. Phospholipid methylation

Hirata and Axelrod (1980) have reported the isolation of two distinct phosholipid methyl -transferases from adrenal medulla. They established that phospholipid methylation occurs in two stages each catalysed by the two enzymes which are distributed asymmetrically in membranes. The first methyltransferase (MT1) is located on the cytoplasmic side of the plasma membrane and catalyses the conversion of phosphatidyletanolamine (PE) into phosphatidyl- N-monomethylethanolamine (PME).The enzyme requires Mg^{2+} for full activity, has a pH optimum of 7.0 and a low km of 2 μM for SAMe.The product is translocated from the inside to the outside of the membrane. The second (MT 2) is located on the outer face of the membrane and successively methylates PME to phosphatidylcholine

(PC). This enzyme has no requirement for Mg^{2+}, has a pH optimum of 10 and a high km for SAMe of 100 μM. Since initial location of the enzymes in the microsomes and mitochondria of the adrenal medulla the enzyme system has been reported in brain, erythrocytes , lymphocytes , mast cells and poly -morphonuclear leukocytes (Crews et al.,1980; Hirata and Axelrod, 1978; Hirata et al., 1980; Hirata et al., 1979; Hirata et al., 1979).

The first methylation step and subsequent vectoral rearrangement led to a decrease in membrane viscosity (Kahlenberg et al., 1974) and increased the lateral mobility of ligand receptors and transduction machinery eg ß adrenceptors with adenylate cyclase.Further methylation had little effect on membrane viscosity but was observed to stimulate a 30-50% increase in the number of ß adrenoceptor sites.Incubation of cells with the demethylated product SAHc abolished both the decreased microviscosity and the number of ß adrenoceptor sites. Thus both methyltransferases exhibit a sensitivity to SAHc and are inhibited by product feedback inhibition. Hirata et al., (1980) purport that phospholipid methylation is an initial common pathway for the transduction of many receptor mediated signals.

1.3.1.2. Protein carboxy-O-methylation

Protein-O-carboxymethyltransferase (EC 2.1.1.24) methylates the free carboxyl groups of aspartyl and glutamyl residues of protein at physiological pH thus neutralising the negative charge (Dilberto et al., 1976). This covalent modification can cause a rapid alteration of a substrate's conformation , charge or hydrophobicity and thus provides a mechanism for fast changes in its functional properties.Protein carboxymethylation is believed to be an important regulatory mechanism for functions as diverse as neurosecretion to chemotaxis (O'Dea et al., 1978). Correlations between protein carboxymethyltransferase activity and cellular events include stimulus - secretion coupling in adrenal medulla (Dilberto et al., 1976), repair and removal of aged proteins in erythrocytes (Barber and Clarke 1984), chemotaxis of leukocytes (O'Dea et al., 1978), dopamine stimulation of synaptosomes (Billingsley et al., 1982), thrombin activation of platelets (O'Dea et al., 1978b) and differentiation of rat brain cells (Gregor et al., 1983) and neuroblastoma cells (Kloog et al.,

1983).

The enzyme has a ubiquitous tissue distribution but the highest specific activities have been found in neural tissue and secretory cells (O'Dea et al., 1978). Its presence in synaptosomes and evidence of axonal transport suggests a role in synaptic and secretory function.Billingsley et al., (1982) have proposed a mechanism where the act of charge neutralisation of the cytosolic surfaces of the vesicle and plasma membrane would promote exocytotic secretion of exportable product. The enzyme is primarily cytosolic (Billingsley et al., 1982) but Saido et al., (1987) have isolated and characterised a membrane bound form from chicken erythrocytes. Although the total activity of the membrane derived protein carboxymethyltransferase was less than in the cytosol it had a greater ability to carboxymethylate the endogenous methyl acceptor protein (MAP). The 3 fold difference in affinity for SAMe with Km values of 0.7 and 2.1 μM for the membrane and cytosolic forms of the enzyme, respectively , suggests that the two enzymes have different physiological roles in cellular processes. Methyl acceptor proteins are as equally ubiquitous in distribution. Dilberto and Axelrod (1974) have reported the presence of the substrate in the pituitary gland where it was associated primarily with neurosecretoty storage granule lysates (Gagnon and Axelrod 1979). These were identified as two minor proteins of molecular mass 32 and 55 KDa (Gagnon et al., 1978).

The relationship between protein carboxymethylation of methyl acceptor protein substrate and function has been described by Strittmatter et al., (1978) using the rat parotid gland preparation. Treatment with isoproterenol a potent ß adrenoceptor agonist induced a rapid and reversible activation of both protein carboxymethyltransferase (PCM) and methyl acceptor protein. The time course of the increase in PCM activity and methyl acceptor protein capacity stimulated by the agonist paralleled the secretion of amylase.

Calmodulin has been shown to be a critical modulator of calcium dependent cell functions (eg calcium dependent ATPase, cyclic nucleotide degradation, assembly of cytoskeletal units (Means and Dedman 1980)). Gagnon et al (1980) reported that calmodulin is a substrate for PCM. Carboxymethylation of calmodulin inhibits calcium dependent phosphorylation in rat brain membranes (Billingsley et al., 1983) there being a direct correlation between the extent of methylation and the degree of calmodulin inhibition. It has

been further reported that carboxymethylation directly inhibits calmodulin target protein eg calcium stimulated cyclic GMP phosphodiesterase and calmodulin dependent protein kinase (Billingsley et al., 1984). Such results suggest that PCM may modulate calcium dependent activities in the cell.

Regulation of cell function by PCM works in conjunction with protein methyl esterase (PME) which provides the 'off' reaction. It had been thought that the de-esterification of carboxy- methylated proteins was a non-enzymatic process at physiological process at physiological pH (Dilberto & Axelrod, 1976). Gagnon et al (1979) has reported the presence of the enzyme in many mammalian tissues with the highest specific activity present in the kidney. Protein carboxymethyltransferase shares with other methyltransferases an absolute requirement for SAMe and a sensitivity to product feedback inhibition by SAHc (Borchardt 1977) with reported Ki values of 0.3 - 0.7μM.

1.3.2. S-Adenosyl-l-homocysteine

S-Adenosyl-l-homocysteine (SAHc) was first characterised by Cantoni and Scarano (1954) as the demethylated product of 'active methionine'. Its structure was unequivocally established through total synthesis by Baddiley and Jamieson in 1955. It is one of the products of all transmethylations involving SAMe and it is a potent inhibitor of almost all methyltransferases (Zappia et al., 1969, Kerr 1972 and Pegg 1971).

The metabolic fate of exogenous SAHc in vivo has been determined by Duerre et al (1969) by administering S-[^{3}H-homocysteine]adenosyl-l-homocysteine i.v to rats. The compound was rapidly cleared from the circulation with a t $1/2$ of about 20 minutes. After 4 hours less than 1% of the label was incorporated into the protein methionine content of liver, kidney and small intestine. About 50-60% of the tracer was excreted in urine within 4 hours. This consisted of approximately 10% α-ketobutyrate with the remaining fraction identified as S-adenosyl-γ- thio-α-ketobutyrate which is formed through the following reaction:

$$SAHc + O_2 + H_2O \text{ ----------> } \text{S-adenosyl-}\gamma\text{-thio-}\alpha\text{-ketobutyrate} + NH_3 + H_2O_2$$

The reaction is catalysed by L-amino acid oxidase (L-amino acid: oxygen oxidoreductase,

EC 1.4.3.2.). The limited extent to which exogenous SAHc was metabolised by the rat suggests that cells are relatively impermeable to this compound. Rat liver perfusion studies with SAHc failed to demonstrate any detectable hydrolysis or deamination of the thioether but perfusion of tissues with equimolar concentrations of adenosine and homocysteine caused a pronounced elevation of the intracellular SAHc content (Miller and Duerre 1969).

The primary means of removal of SAHc is by hydrolysis by the enzyme S-adenosyl-l-homocysteine hydrolase (EC 3.3.1.1.). First identified by Dela Haba and Cantoni (1954) in rat liver it catalyses the condensation of adenosine and homocysteine to SAHc and is thus fully reversible. The equilibrium favours condensation but SAHc will be hydrolysed if adenosine and homocysteine are removed.

The distribution of the enzyme in the rat has been surveyed by Finkelstein and Harris (1973).It is present in all major organs studied except intestinal mucousa. The highest levels were found in the liver, pancreas and kidney and the lowest in the brain and heart. In all systems studied synthesis was favoured over hydrolysis. The reaction is very complex and shows both substrate and product inhibition in the condensation reaction and product inhibition in the hydrolytic reaction (Finkelstein and Harris 1973; Walker and Duerre 1975). In addition the condensation reaction is also inhibited by SAMe (Finkelstein and Harris 1973). The enzyme in rat brain has been characterised by Schatz et al (1979) and appears to be a multimer of 180 KDa consisting of 48KDa subunits with a Km of 36 μM.From in vivo and in vitro data SAHc hydrolase is a bioregulatory enzyme which functions in maintaining the intracellular level of SAHc. The activity of SAHc hydrolase is higher than that of SAMe synthetase and the concentration of SAMe is at least 4 fold greater than that of SAHc (Eloranta 1977). From these observtions Cantoni et al (1979) have suggested that the cellular concentration of SAHc or the SAMe / SAHc concentration ratio could play a key role in the regulation of the utilisation of SAMe. The kinetics of the reaction have however been exploited with specific antagonists of the enzyme.Chiang and Cantoni (1979) have reported that the administration of the SAHc blocker 3-deazaadenosine to rats elevated the intracellular levels of SAMe, SAHc and created a 3-deazaadenosine derivative of homocysteine. This event correlated with a significantly reduced rate of synthesis and excretion of creatinine and decreased rate of incorporation of the methyl

group of labelled methionine into liver lipids.The increase in intracellular levels of SAHc induced by 3-deazaadenosine caused potent inhibition of SAMe dependent RNA methylation and has been harnessed by Robert-Gerro et al (1975). The drug has been shown to be an effective anti-viral drug suppressing the growth and replication of Rous Sarcoma virus and Herpes I and II.

1.3.3. Transsulphation

The hydrolysis of SAHc liberates homocysteine which is the beginning of the transsulphation pathway it is however a metabolic crossroads since the compound is the substrate for three enzymes two of which are involved in the recycling of homocysteine to methionine and thus constitute salvage pathways. Betaine: L-homocysteine-s-methyltransferase [EC 2.1.1.5] transfers a methyl group from betaine (a derivative of choline) to homocysteine with the formation of methionine and N,N-dimethylglycine (Finkelstein et al., 1975). This reaction conserves but does not enhance the methyl group pool. It may be considered a minor metabolic route since the enzyme is only found in substantial quantities in the liver.

The second methionine forming enzyme , 5-methyltetrahydrofolate: homocysteine methyl-transferase (MTHF, EC 2.1.1.13) uses a methyl group synthesised de novo via the Folate pathway .However this requires methylated vitamin B_{12} as a coenzyme for the reaction. It is activated by SAMe requiring N^5-methyltetrahydrofolate-homocystine(cobalamin)-methyl transferase (Harvey and Dev. 1975). Thus as pointed out by Finkelstein (1979) this is the junction point for three metabolic pathways-methionine,vitamin B_{12} and folate.The third pathway involves reaction with serine to form cystathionine catalysed by cystathionine-ß-synthase (EC 4.2.1.22). The enzyme is unidirectional in vivo therefore once commited to this reaction it can no longer be recycled to methionine. The last reaction in the transsulphation pathway is the hydrolysis of cystathionine to cysteine and homoserine. The enzyme cystathionine-γ-lyase (EC 4.4.1.1.) has a broad range of substrates and is reversible.Both enzymes require pyridoxal phosphate as a co-factor (Finkelstein 1979).

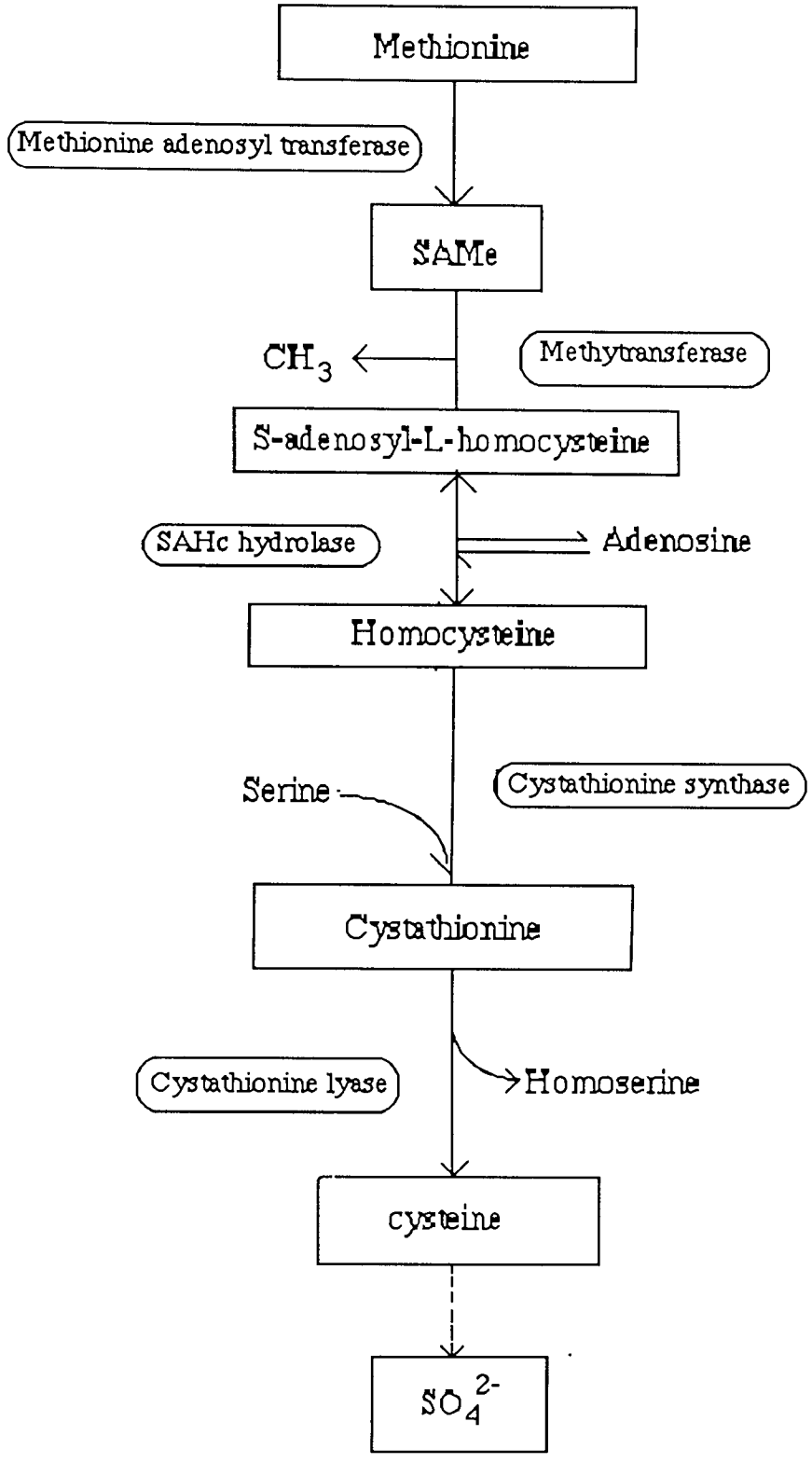


Fig 1.3.3.1. The Transsulphation pathway

The enzymes of transsulphation have a Km at least ten fold greater than those of homocysteine transmethylation. Therefore at low levels of substrate enzymes involved in

methionine conservation have the greater advantage. Conversely, at high levels of substrate an increasing proportion of the substrate would be devoted to transsulphation. The system of regulation is highly complex. Product feedback inhihibition by methionine inhibits both homocysteine methyltransferases and activates cystathionine synthesis. S-Adenosyl-l-homocysteine reverses inhibition of MTHF by SAMe but itself inhibits the the use of MTHF in homocysteine remethylation and activates cystathionine synthesis. Thus although thermodynamics favour the condensation of SAHc from homocysteine and adenosine, SAHc is maintained at low concentrations. The relative proportion of homocysteine converted to methionine is dependent upon the developmental and dietary status of the animal.Studies in vitro with cultured malignant lymphoid cells by German et al (1983) suggest that only a few percent of homocysteine is remethylated to methionine. The distribution of enzymes concerned with methionine conservation is ubiquitous (Finkelstein et al 1973) but the specific activity varies greatly. The highest activities in the rat are found in the kidney, brain, testes, lung and adrenal gland.

Sulphurous derivatives of cystathionine and cysteine can be metabolised further but the ultimate product is oxidation to sulphate (Guilidori et al 1984).

1.3.4. Decarboxylation

The second major route of SAMe metabolism is initiated by decarboxylation of the methionine moiety by SAMe decarboxylase (EC 4.1.1.50). The product S-adenosyl-5'-deoxy (5')-3-methylthiopropylamine (decaSAMe) is the substrate for aminopropyltransferases which donate the aminopropyl group to polyamine acceptors (reviewed by Pegg et al 1987).The enzyme has been isolated and characterised and consists of 2 subunits of 32 KDa (Shirahata et al 1985) formed from a 37 KDa precursor (Pegg and Shirahata 1986). Each subunit contains a covalently bound pyruvate prosthetic group and requires putrescine for activation (Kay and Lindsey, 1973) by accelerating the conversion of the precursor to the active enzyme (Kameji and Pegg 1987).The enzyme is rapidly induced and in mouse kidney has a half life of 50-120 minutes (Tabor and Tabor 1984). The enzyme has a Km of 30 μM for SAMe and the decarboxylation of the compound is the rate limiting step for polyamine synthesis (Pegg and Hibasami, 1979). The enzyme is

present at low concentrations in rat liver constituting only 4 ng/mg of soluble protein (Pegg and Hibasami, 1979). Similarly the intracellular content of decaSAMe is also very low with values of 0.9 - 2.5 nmol /g/ wet weight in resting rat liver (Hibasami et al 1980) which approximates to 2-4% of the content of SAMe in the same tissue (67± 7.1nmol / g/ wet weight). Changes in SAMe decarboxylase activity can be brought about by stimuli such as androgen treatment and polyamine depletion which increase the amount of enzyme protein (Shirahata et al 1985, Pegg 1984). Mitogenic stimulation of bovine lymphocytes increased the rate of synthesis and the half life of SAMe decarboxylase (Seigfried et al., 1982). Elevation of spermidine and spermine decrease SAMe decarboxylase content. Conversely when polyamine metabolism is inhibited the amount of SAMe decarboxylase protein increases (Mamont et al., 1981).

1.3.5.1. Polyamine synthesis

Polyamine synthesis is initiated by the decarboxylation of ornithine by ornithine decarboxylase (L-ornithine decarboxylase EC 4.1.1.17,) to form putrescine (Russell and Snyder 1968). In eukaryotes the enzyme is primarily cytosolic and plays a major role in the regulation of cell growth. The basal level of activity in resting cells is extremely low (Pegg et al 1982). It has been estimated that in such cells ornithine decarboxylase (ODC) amounts to less than 1 part per 10^6.This enzyme has the fastest turnover rate of any other mammalian enzyme with a half life of 10 to 30 minutes (Tabor and Tabor 1984). It is activated by hormones , drugs and mitogens (Bachrach 1984) and it is thought that cyclic nucleotides mediate its induction (Russell 1976). Ornithine decarboxylase has been isolated from a variety of mammalian tissues and appears to consist of two 53 KDa subunits (Seely et al 1985). All known ODC requires pyridoxal phosphate for activity. Mammalian ODC are highly unstable proteins and need dithiols for full activity (Janne and Williams 1971) by preventing polymerisation which renders the enzyme inactive. More than one form of ODC may be present in the same tissue (Obenrader and Prouty 1977; Seely et al., 1985). Ornithine decarboxylase shows high selectivity for L-ornithine as substrate but the decarboxylation of lysine (a higher homologue of ornithine) to cadaverine has been reported for ODC from rat liver (Pegg and McGill 1979) and mouse kidney (Persson

1977). The reaction is much less efficient though the Km for lysine being 100 fold greater. The regulation of ODC is highly complex. The short half life of ODC is due in part to product feedback inhibition by putrescine (Pegg 1977). In addition a macromolecular inhibitor called ODC antizyme has been shown to be induced by exposing cells to exogenous polyamines (Canellakis et al., 1979) which are thought to release the antizyme from subcellular components (Fujisawa and Kitani, 1985). Other proposed mechanisms include post translational modifications such as phosphorylation (Atmar and Keuhn 1981) and transglutimination (Russell 1981). Sertich and Pegg (1987) have suggested that the primary mode of regulation of ODC is at the level of translation. Of particular interest is the presence of a long 5' leader sequence on the mRNA transcript (Kahana and Nathans 1984) which when removed caused a 40 fold increase in the translation of ODC (Kahana and Nathans 1985). Inhibition of ODC translation by polyamines could be due to interaction with this 5' leader sequence (Sertich and Pegg 1987). However other workers contend that most of the loss of ODC protein appears to be mediated by an increased rate of enzyme degradation rather than by a reduction in the rate of synthesis (Persson et al 1984, Murakami et al., 1985; Dirks et al., 1986).

Transaminopropylation is conducted by aminopropyltransferases namely spermidine and spermine synthase which transfer the aminopropyl group to putrescine and spermidine respectively.The reaction consists of nucleophilic attack by putrescine or spermidine on the methylene C_3 adjacent to the positively charged sulphonium centre of decaSAMe (Tabor and Kellogg 1970). Each aminopropylation results in the formation of stoichiometric amounts of methylthioadenosine (MTA). The two aminopropyltransferases have markedly different affinities for decaSAMe. Pajula et al (1979) reports a km of 0.6 μM for spermine synthase whilst a Km of 10 - 75 μM has been reported for spermidine synthase (Pegg and Conover 1976). This suggests that spermine synthesis is favoured when the availability of decaSAMe is low.

Spermidine synthase (EC 2.5.1.16) has been isolated from bovine brain, rat prostrate (Raina et al 1984), rat liver (Samejima et al 1982) and pig liver (Yamanoha et al., 1984). It consists of two subunits of 35-37 KDa and has no known cofactors. The enzyme has been explored as a target for the inhibition of polyamine synthesis (reviewed by Pegg , 1987).

S-adenosyl-1, -8-diamino-3-octane (AdoDato) is the most potent compound with a reported IC_{50} value of less than 20 nM for mammalian spermidine synthase (Coward et al., 1982). The drug has little effect on spermine synthase. The administration of AdoDato to cultured cells caused a dramatic reduction in spermidine levels but led to an increase in the levels of putrescine , spermine and decaSAMe (Pegg et al., 1982). Most physiologically relevant is the observation that the enzyme is strongly inhibited by an excess of decaSAMe (Hibasami et al., 1980). The converse is true for spermine synthase (Pajula et al., 1983) but it is strongly inhibited by the product, MTA (Pajula and Raina, 1979) and by high doses of SAHc (Hibasami and Pegg 1978).Spermine synthase has been purified to homogeneity from bovine brain and consists of two identical subunits of 44 KDa (Pajula, 1983). Like spermidine synthase it has no known cofactors or prosthetic groups.

1.3.5.2. Polyamine interconversion

Polyamine interconversion has been reviewed by Seiler (1987).The aminopropylations which form spermidine and spermine are essentially irreversible but they be converted back to the preceding polyamine by the combined actions of a spermine / spermidine acetyl transferase and polyamine oxidase (Seiler et al., 1981). The reaction occurs in two stages; Firstly, polyamine degradation is initiated by the formation of N^1-acetylspermine and N^1-acetylspermidine. This step is catalysed by a cytosolic acetyltransferase which requires acetyl CoA as a cofactor (Della Ragione and Pegg, 1982). Secondly , the acetamidopropyl moiety of the monoacetylated derivative is removed (Bolkenius and Seiler, 1981) in an oxidative cleavage reaction catalysed by polyamine oxidase (Hölttä, 1977) to yield putrescine or spermidine.Polyamine oxidase has been found in all tissues thus far examined which suggests that the enzyme is involved in normal polyamine turnover (Seiler et al., 1980) The reaction is enhanced whenever polyamines have to be removed from cells probably as a means of detoxification (Seiler et al., 1981b, Matsui et al., 1982).Spermidine N^1 acetyltransferase is induced by exposure of cells to toxins such as carbon tetrachloride concomitant with stimulation of ODC (Pegg et al., 1981), however polyamine oxidase seems to be constitutive.

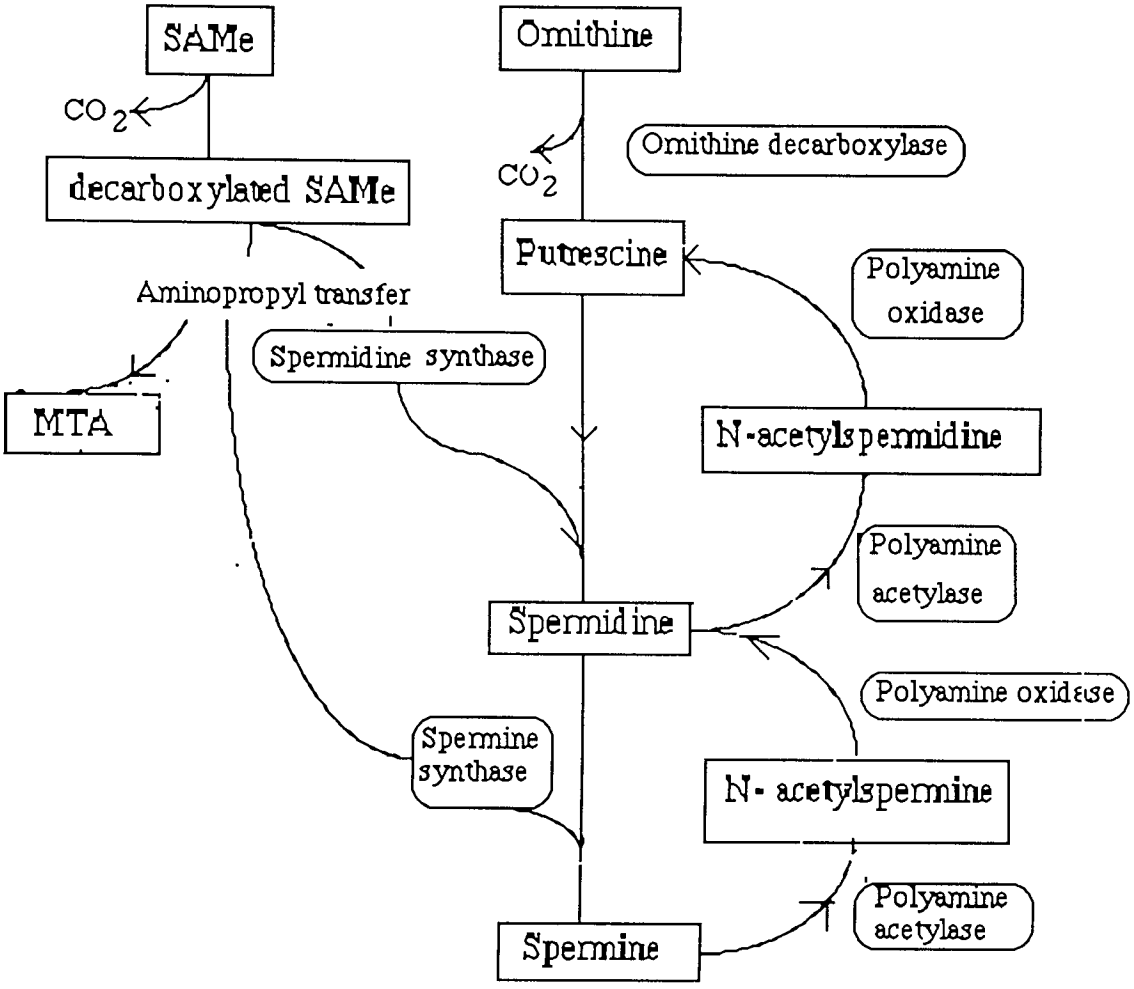
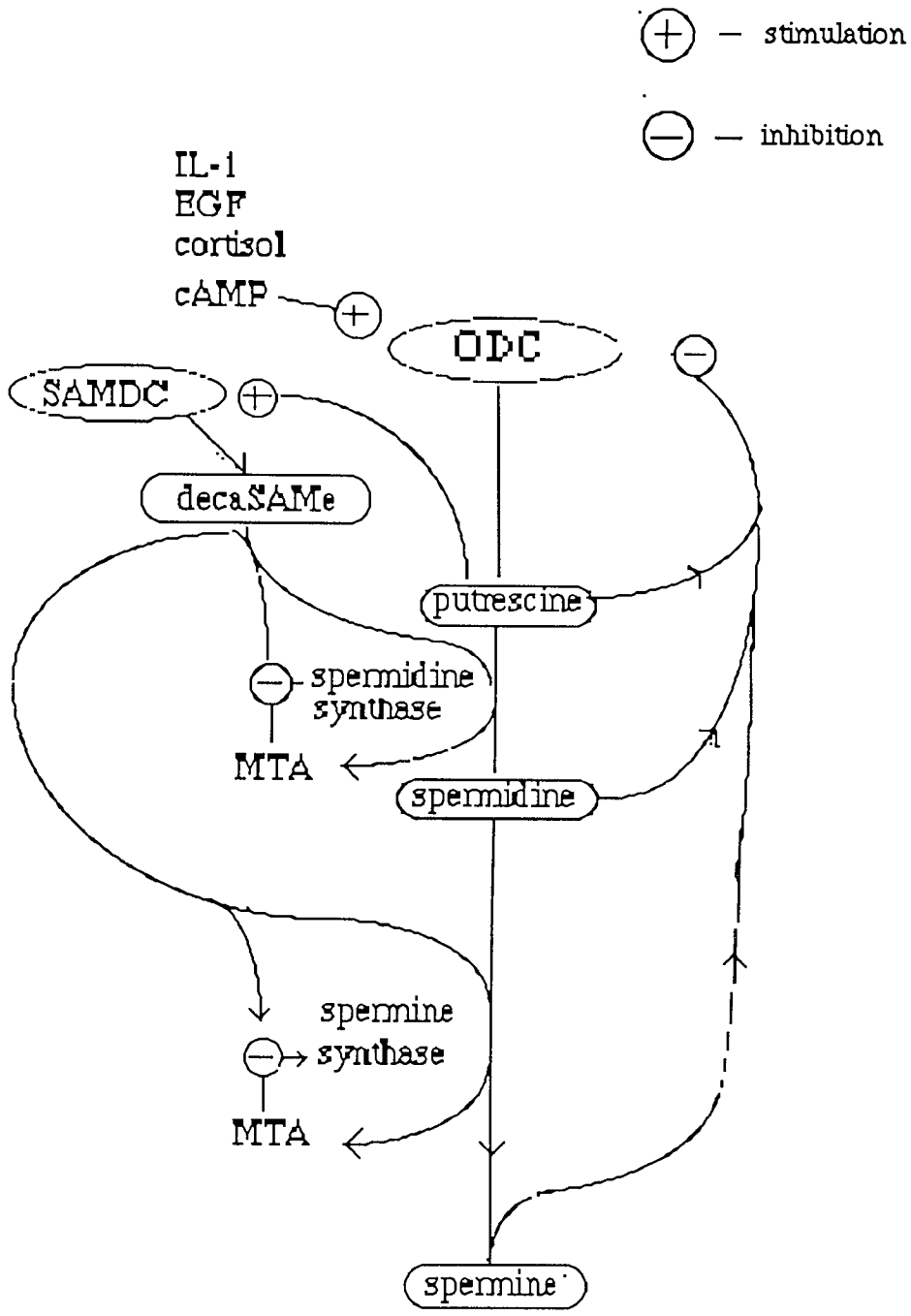


Fig 1.3.5.1. Pathways of polyamine synthesis and interconversion



A second terminal oxidation route for polyamines has been identified (Seiler et al ., 1985a) in which terminal amino groups are oxidatively deaminated to aldehydes by copper amine oxidases.Compounds formed from this pathway are irreversibly removed from the polyamine metabolic cycle. This is illustrated by the alternative metabolism of putrescine which can be oxidised by diamine oxidase.Diamine oxidase activity is increased in tissues with enhanced growth such as regenerating liver, hypertrophic heart and kidney (Perin et al .,1983; Desidero et al., 1982 ; Sessa et al., 1982) which suggests that diamine oxidase is

involved in the regulation of putrescine levels in growing tissue (Perin et al., 1984). A further minor pathway for putrescine has been established in vertebrate brain where putrescine is monoacetylated to form a substrate for monoamine oxidase (Seiler and Al-Therib, 1974).

Thus there exists a futher means of control of cellular polyamine levels independent of the activities of the biosynthetic decarboxylases. Therefore polyamine synthesis can proceed in the absence of SAMe or ornithine.

1.3.6.1. Methylthioadenosine

Each aminopropylation results in the formation of 5'-deoxymethylthioadenosine (MTA).This thioether compound is present in all cells but it is maintained at very low levels. Tissue concentrations are typically less than 10nmol / g even in tissues such as rat prostrate gland which has a prestigous rate of polyamine synthesis (Pegg & Williams-Ashman 1969). Methylthioadenosine is also formed by the enzymatic cleavage of SAMe by SAMe lyase into MTA and homoserine lactone (Baxter and Coscia 1973). Polyamine synthesis and SAMe cleavage accounts for almost all MTA formation in eukaryotes but prokaryotes have at least three further independent means of MTA production (Stoner and Eisenberg 1975).Non accumulation of MTA in higher non-malignant cells is due to the rapid metabolism of the compound by an enzyme system which allows recycling of the metabolically valuble methylthio group (reviewed by Williams-Ashman et al 1982; Schlenk 1983; Williams-Asman 1985).The metabolism and removal of MTA by the cell is both valuble and desirable since the nucleoside has cytostatic activity (Vandenbark et al 1980) and has been reported to have critical enzyme regulatory properties (Reviewed by Williams-Ashman et al 1982).

In mammalian cells methylthioadenosine is rapidly degraded by MTA phosphorylase to methylthioribose-1-phosphate (MTR-1-P) and adenine (Schlenk 1983).The reaction is fully reversible and strongly inhibited by adenine. This enzyme has been purified to homogeneity from rat liver by Ferro et al (1979) and has a relative molecular mass of 90 KDa and exhibits a Km of 0.47 μM for MTA and 0.2 mM for phosphate. The enzyme has been shown to be induced in rat and human lymphocytes stimulated by lectins (Christa et

al.1983) and reaches a peak of activity during the exponential growth phase (Sunkara et al. 1985). Hormonal regulation of the enzyme in vivo has been reported by Nicolette et al (1980). Castration of rats reduced prostrate gland enzyme activity by 95% which could only be partially restored by testosterone treatment. In female rats ovarectomy reduced uterine MTA phosphorylase activity but this was restored to normal levels by treatment with 17-ß-oestradiol.Neither castration nor 17-ß-oestradiol treatment affected MTA phosphorylase activity in female rat lung or liver.

The adenine moiety liberated by the reaction is readily converted to 5'-adenosine monophosphate (5-AMP) by reaction with phosphoribosyl pyrophosphate in a process catalysed by adenine phosphoribosyltransferase (APRT) (Kamatani and Carson 1981).Further phosphorylation steps yield ADP and ATP. Thus the ATP invested in SAMe synthesis can be salvaged. Evidence in support of this comes from Kamatani and Carson (1981) who showed that proliferating lymphoblastoid WIL2 cells deficient in APRT but endowed with MTA phosphorylase produced adenine at an equivalent rate to the formation of MTA by an MTA phosphorylase deficient human lymphoblastoid CCRF-CM cell line. From these results they estimated that 85% of adenine derived from MTA was from MTA produced during transaminopropylation reactions. Certain malignant murine leukemia cell lines deficient in MTA phosphorylase accumulate and excrete the nucleoside (Kamatani et al 1981). Toohey (1978) has reported that this deficiency results in an absolute dependence on exogenous methionine for growth.

Methylthioribose-1-phosphate can be isomerised to 5'-methylthioribulose-1-phosphate (Trackman and Abeles 1983) and via a series of reactions is converted to 2-keto-4-S-methyl thiobutyrate (KMTB) which in turn can be transaminated with either glutamine or asparagine to form l-methionine. The definitive mechanism for the formation of KMTB from 5'-methylthioribulose-1-phosphate has not been established but results from Backlund and Smith (1981) using cell free homogenised liver extracts, have indicated the transfer of 4 carbon atoms from the ribose moiety of MTR-1-P into the aminobutryl chain of methionine. Other mechanisms have been suggested by Trackman and Abeles (1981) and Schlenk (1983) which require a dephosphorylation and opening of the ribose ring.

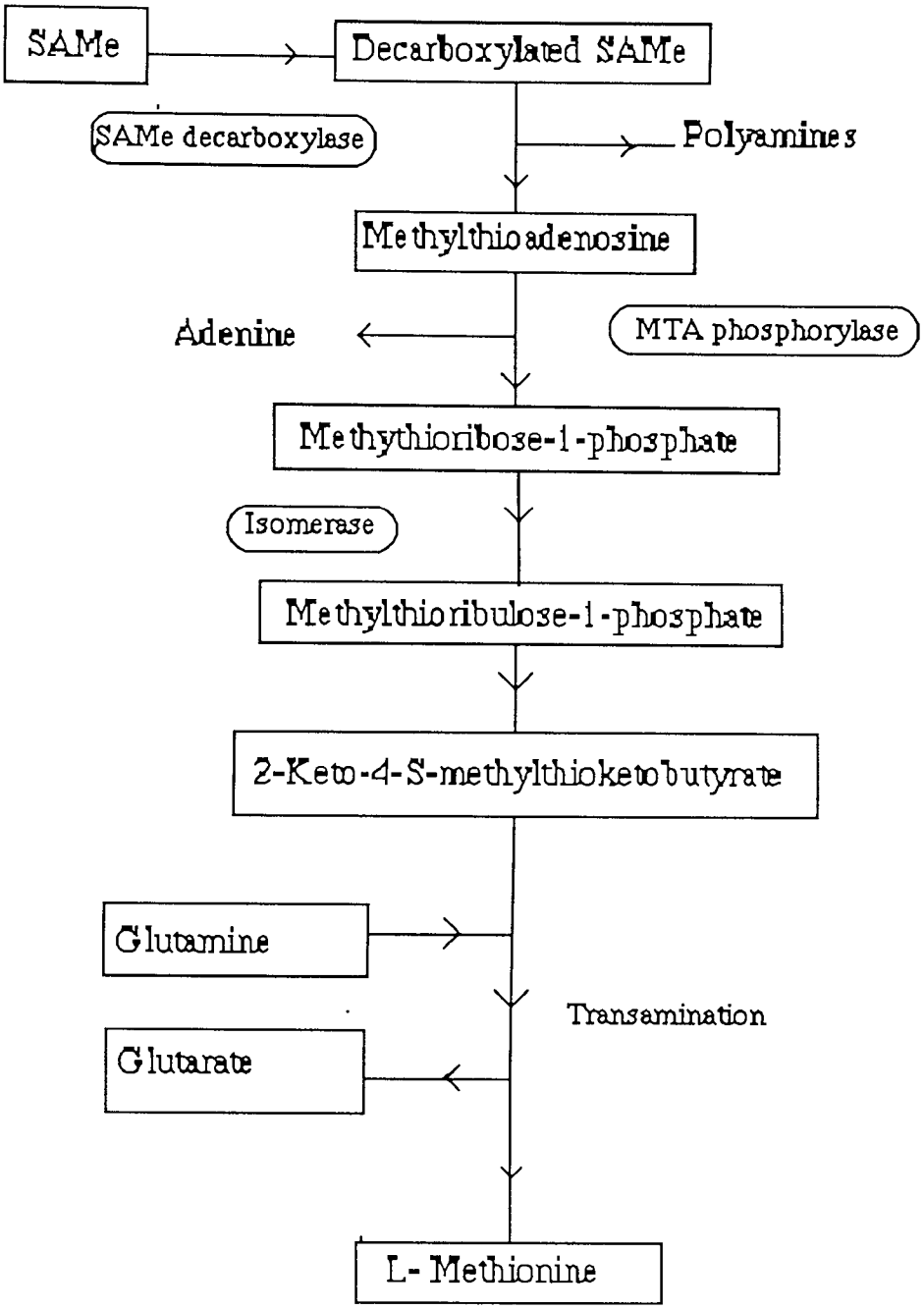


Fig 1.3.6.1. Methylthioadenosine (MTA) salvage pathway

Experiments investigating methionine formation from MTA by rat bone marrow cells by Deacon et al (1990) have revealed the release of formate from carbon atom 1 of MTR-1-P which is transferred by tetrahydrofolate as a single carbon unit for the de novo synthesis of adenine and guanine.

As pointed out by Pegg and Williams-Ashman (1987) many cell types that contain MTA

phosphorylase can recycle MTA into methionine. However Christa et al (1986) have shown that although CCL39 cells (derived from Chinese hamster lung fibroblasts) could incorporate exogenous MTA into methionine, SAMe and protein, the nucleoside could not support cell growth in the absence of exogenous methionine. Stimulation of resting cells by 100 μM methionine caused a doubling of the rate of incorporation of MTA into methionine and SAMe which was equivalent to the incorporation of MTA into extracellular methionine. In other words methionine salvaged from MTA was exported from the cell and used preferentially for SAMe synthesis. In contrast, methionine derived from MTA was poorly used for protein synthesis (Christa et al.1988). These workers contend that the metabolic route:

SAMe -----> MTA-----> methionine -----> SAMe

comprises a cycle which can provide the precursor and remove the potentially toxic by product of polyamine synthesis. Thus the reactions of MTA metabolism allow salvage of ATP and methionine used in cellular SAMe production and conserve those parts of the decarboxylated SAMe molecule not used for polyamine synthesis. It follows therefore that those cells with the full complement of enzymes for MTA catabolism can synthesise polyamines de novo without any net consumption of methionine.

1.3.6.2 MTA transport

Zappia et al (1982) studied the transport and metabolism of MTA in human erythrocytes. The nucleoside was incorporated rapidly into the cell and metabolised to MTR-1-P and adenine. The former was dephosphorylated and secreted from the cell. Kinetic studies by Carteni-Farini et al.(1983) showed that the transport mechanism had high capacity but low affinity, was not energy dependent or highly specific and appeared to rely on facilitated diffusion. These findings were in direct contrast with the results from an investigation of MTA transport in isolated and perfused rat liver which described an active high affinity permease specific for thioethers (Zappia et al. 1978). Evidence of a nucleoside mediated carrier system has been revealed in the myeloid leukaemic HL 60 cell line (Stoekler and Li 1987). Carrier mediated influx of MTA was strongly inhibited by adenosine, deoxyadenosine and the nucleoside transport inhibitors nitrobenzylthioinosine and dipyrimadole. Loss of nucleoside transport capacity during differentiation of HL 60 cells

was accompanied by a corresponding decrease in the rate of MTA influx. At high concentrations there was a considerable passive diffusion component which accounted for over half the influx of exogenous MTA. In proliferating HL 60 cells passive diffusion of MTA was rapid enough to be non-rate limiting for the conversion of MTA to methionine. Iizaza et al. (1984) has noted that the high lipophilicity of MTA allows influx or efflux rates that exceed those of the enzymes that degrade or produce the nucleoside. This characteristic may allow it to permeate not only the plasma membrane but also intracellular structures.

1.3.6.3. Function and enzyme regulation by MTA

The replenishment of ATP and methionine pools by the requisite salvage pathways sustains the high rate of polyamine synthesis observed during cell proliferation and keeps intracellular levels of MTA low. However, some cells deficient in MTA salvage enzyme systems excrete MTA to prevent accumulation of the nucleoside to levels where it can suppress growth (Vandenbark et al 1980). The addition of high concentrations of MTA has been reported to inhibit lectin induced lymphocte transformation (Vandenbark et al.1980), virus induced chick embryo fibroblast transformation (Enouf et al.1979) and lymphoid cell proliferation (Wolford et al. 1981). The mechanisms by which this could occur include inhibition of spermidine and spermine synthases (Pegg et al 1980; Raina et al. 1982), inhibition of certain methyltransferases (reviewed by Williams-Ashman et al. 1982) and inhibition of SAHc hydrolase (Della-Ragione and Pegg, 1983).

Zappia et al (1969) has demonstrated that MTA (0.5mM) inhibited the methylation of histamine and acetylserotonin by 20 and 29%, respectively. Casellas and Jeanteur (1978) have reported that protein carboxyl-O-methyltransferase in Krebs II ascites cells is effectively inhibited by MTA with a Ki of 35 μM.Similar results were obtained by Galletti et al (1981) where MTA inhibited PCM activity in human erythrocytes. A strong inhibitory effect of MTA on human erythrocyte SAHc hydrolase has been reported by Ferro et al.(1981). It is unclear whether the interruption of transmethylation reactions is due to direct inhibition of the methylases by MTA or to elevated SAHc levels as a result of SAHc hydrolase inhibition. Schlenk (1983) has suggested that these observations may explain some of the cytostatic effects of MTA.

In addition to confirmed cytostatic effects, MTA has been reported to inhibit pokeweed mitogen induced immunoglobulin secretion , B cell differentiation (Padova et al.1985) and lymphocyte mediated cytolysis in a reversible, dose dependent manner (Wolberg et al. 1982). The IC_{50} for the effect of the nucleoside on the latter lymphocyte function was 375 μM and correlated with an elevation of cAMP through inhibition of cAMP phosphodiesterase (3',5'-cyclic-nucleotide 5'-nucleotidohydrolase, EC 3.1.4.17). The elevation of basal lymphocyte cAMP levels by MTA was modest (< 2 fold) but marked potentiation of the cAMP response to PGE_1 and cholera toxin was observed in the presence of the nucleoside. Concommitant with the increase in cAMP levels was a 3 fold increase in SAHc. It is worth noting that the processes of virus induced cell transformation , mitogen stimulated lymphocyte transformation and proliferation that are reported to be inhibited by MTA (Pegg et al. 1981; Backlund and Smith, 1981), are also inhibited by elevated cellular levels of cAMP (Otten et al. 1972; Smith et al.1971; Coffino et al.1975). Further, de Ferra and Baglioni (1984) have demonstrated a correlation between the anti-proliferative effect of interferon γ and elevated intracellular levels of MTA in human lymphoblastoid Daudi cells.

1.3.7. Function of polyamines

Polyamines are required for optimal growth in all cells investigated. Numerous studies have demonstrated that rapidly growing cells have higher levels of ODC and polyamines than slowly growing or quiescent cells. When resting cells are stimulated there is a dramatic surge of ODC activity and polyamine production followed by increased levels of DNA, RNA and protein. However, as stated by Tabor and Tabor (1984), there are considerable difficulties in trying to define which particular biochemical mechanisms are influenced by cellular polyamine status.

1.3.7.1. Interaction of polyamines with nucleic acids

Polyamines have been found associated with nucleic acids isolated from viral, bacterial and mammalian cells (reviewed by Tabor and Tabor, 1984; Bachrach, 1973) and have been reported to induce precipitation and provide protection against denaturation and mechanical

shearing. The nature of the interactions between polyamines and tRNA was predicted by Cohen et al (1971) and confirmed in crystallographic studies by Quigley et al (1978) which indicated that the 'cloverleaf' shape of tRNA was stabilised by two spermine molecules. Evidence that polyamines facilitate DNA conformational changes in vitro has been provided by Behe and Felsenfield (1981) that spermine and spermidine promote the B-A transition and a spermine to nucleotide ratio of 40-50: 1 can effect the B-Z transition.These conformational changes are thought to be critical to the control of DNA function (Behe and Felsenfield 1981; Brahmachari et al.1985; Nordhiem and Rich,1983)

Hung et al.(1983) has showed that the conformation of DNA in 9L rat brain tumour cells depleted of putrescine by the ODC inhibitor difluoromethylornithine (DFMO) was altered and that this alteration could be normalised by exogenous putrescine.Studies conducted by Fillingame, et al.(1975), showed that polyamine depleted lymphocytes stimulated by mitogen initiated DNA replication at the same time as control cells, but the rate of [^{3}H] thymidine incorporation and entry into the mitotic cycle was greatly reduced. It has since been confirmed that polyamines are needed for DNA replication , but not for entry into the S phase (Morris and Harada,1980; Heby and Jänne, 1981).Polyamine depletion of HL-60 cells caused an extension of the S phase but this could be due to effects on the enzyme systems involved in DNA synthesis such as helicases, DNA binding proteins, primases and topoisomerases (Kornberg, et al.1982). Eukaryote topoisomerases are strongly stimulated by polyamines (Srivenugopal and Morris, 1985). These enzymes regulate the superhelical density of DNA (reviewed by Wang, et al. 1985) and hence chromosome structure (Earnshaw and Heck, 1985).

1.3.7.2. Effect of polyamines on protein synthesis

Evidence has been reported that suggests that polyamines may be directly involved at nearly every stage in protein synthesis (reviewed by Marton and Morris, 1987). Protein synthesis in exponentially growing HTC cells was clearly blocked by DFMO, as indicated by a decreased incorporation of radioactive amino acids and by changes in the pattern of polysomes (Rudkin, et al. 1984). However, when quiescent lymphocytes are stimulated by mitogen there is a marked increase in polyamine levels and the rate of polyamine synthesis

as the cells move from the G_0 to the G_1 phase (Fillingame and Morris, 1973). The increase in polyamine synthesis preceded DNA replication and was accompanied by an increase in the rate of [^{3}H] leucine incorporation (Morris, et al. 1977). Inhibition of polyamine synthesis had little effect on protein synthesis during the G_0 - G_1 phase, but protein synthesis was highly sensitive to polyamine depletion once the cells had fully entered the mitotic cycle (Igarashi and Morris, 1984). The authors inferred that the basal levels of polyamines in quiescent cells were sufficient for enabling protein synthesis in the early stages of mitogenic stimulation, but as cells continued to grow in the presence of polyamine inhibitors, the polyamine requirement for protein synthesis became clear.

Polyamines affect protein synthesis by a direct participation in translation , affecting both the rate of initiation of translation and the rate of elongation of the nascent polypeptide chain (Morris, 1981). Depletion of cellular polyamines caused a reduction in the rate of elongation that was directly proportional to the suppression of cell growth. Kuroda, et al.(1982) has reported that the activity of protein kinase 380, which inhibits eukaryotic factor 2-α, is itself inhibited by polyamines. Further, the initiation factor, eIF-4D is bound to the polyamine metabolite hypusine and this conjugate is formed upon mitogenic stimulation (Cooper, et al. 1983).

In addition to the effect of polyamines on the rate of protein synthesis there is evidence that polyamines have a significant effect on translational fidelity i.e the frequency with which amino acids are correctly incorporated into a polypeptide in response to a given codon (Hirishima, et al. 1979; Morch and Benicourt, 1980; Hryniewicz and Vonder Haar, 1983) and ribosome structure (Cohen and Leichenstein, 1960).

Studies with mammalian cells have indicated that polyamines stimulate the synthesis of specific proteins, generally of high RMM rather than low RMM (Atkins, et al. 1975). This could be interpreted as being due to the promotion of elongation by polyamines or slow, inaccurate termination of protein in the absence of polyamines. In support, Igarashi and Morris (1984) have reported that polyamine depletion affects the synthesis of specific proteins in mitogen activated lymphocytes.

1.3.7.3. Effect of polyamines on membrane structure and function

Polyamines have many characteristics of Ca^{2+} and Mg^{2+} but instead of representing point charges, protonated polyamines have positive charges at fixed length and can thus bridge critical distances (Liquori, et al. 1967). Electrostatic interaction of exogenous polyamines with negatively charged membrane components has been shown to stabilise protoblasts or spheroblasts isolated from bacteria (Tabor, 1962), mitochondria (Tabor, 1960) and lysosomes (Powell and Reidenberg, 1982), against osmotic shock. Tadolini, et al. (1985) has reported that the binding of polyamines to polar head groups protects membranes from lipid peroxidation.The mechanics of the stabilisation process has been studied using a fluorescence polarisation technique (Schindler, et al. 1980). A dose dependent inhibition of lipid and transmembrane glycoprotein mobility in erythrocyte membranes was observed with spermine. There was no decrease in membrane lipid microviscosity . Farmer , et al. (1985) has suggested that membrane stabilisation by polyamines is achieved by bridging proteins and lipids and by promoting protein -protein links between the plasma membrane and the cytoskeleton.

Modulation of surface charge by polyamines could play an important role in the regulation of membrane associated enzymes dealing with charged substrates (Wojtczak and Nalecz, 1979) such as phospholipids.Sechi, et al.(1978) has reported the inhibition of the hydrolytic activity of phospholipase A_2 and C on phospholipid vesicles and mitochondrial membranes.

Spermine has been found to stimulate the activity of enzymes involved in the biosynthesis of glycerolipids and phospholipids (Bates and Saggerson,1981). Jamdar and Osborne (1983) have reported that the activity of microsomal Mg^{2+} dependent phosphatidate phosphohydrolase was potentiated by polyamines through facilitating the translocation of the enzyme from the cytosol to the membrane.Jamdar, et al. (1987) have suggested that polyamines can mediate membrane construction during cell growth. This proposal has been further strengthened by the finding that galactosyltransferases involved in the synthesis of glycoproteins are also stimulated by polyamines (Baker and Hillegass, 1974; Navaratnam, et al., 1986).

The activities of Na^+, K^+-ATPases derived from many tissues have been found to be

modulated by polyamines, particularly by spermine (Heinrich-Hirch, et al. 1977). Spermine activated the enzyme at low K^+ and ATP levels whereas an inhibition was apparent at high K^+ and ATP concentrations (Tashima, et al. 1981). The mode of modulation is complex and ill defined, but a proposal has been made that the regulatory role of spermine in nucleotide dependent reactions could be due to mediating the intracellular levels of free and cation bound nucleotides (Schuber, 1989). Thus polyamines may regulate ion transport.

1.3.7.4. Polyamines and calcium homeostasis

The regulation of calcium homeostasis by polyamines has been reviewed by Schuber, (1989). Spermine and spermidine have been found to stimulate calcium transport in mitochondria isolated from rat liver (Nicchitta and Williamson, 1984), brain (Jensen, et al.1987) and permeabilised hepatocytes (Nicchitta and Williamson, 1984). The effect was accomplished at submillimolar doses and was linked to an increase of the affinity of the uniporter for calcium.

Rapid mobilisation of polyamines and intracellular calcium was elicited by testosterone in kidney cortex tissue (Koenig, et al.1983a). Testosterone mediated processes such as calcium flux across the plasma membrane and efflux from intracellular storage organelles were linked to a rapid and sustained increase in cellular polyamines provoked by a rapid and transitory elevation in ODC activity. Similar effects have been observed with the ß adrenoceptor agonist isoproterenol (Koenig,1983b), insulin (Goldstone et al.1985) and in K^+ depolarised synaptosomes (Iqbal and Koenig, 1985). The authors suggest that polyamines may act as intracellular signals for elevation of cytoplasmic calcium.Inhibition of polyamine synthesis by DFMO blocked the effect of isoproterenol on isolated rat ventricular myocytes (Koenig et al.1988) and the release of neurotransmitters from K^+ depolarised synaptosomes (Iqbal and Koenig, 1985).

1.3.7.5. Polyamines and polyphosphoinositide metabolism

Polyamines have been demonstrated to affect the formation and and the metabolism of diacylglycerol and inositol (1,4,5) trisphosphate (IP_3). These intracellular second

messengers are products of the cleavage of phosphatidylinositol bisphosphate by a calcium dependent phospholipase C and constitute a major pathway of signal transduction (reviewed by Berridge, 1987). Diacylglycerol activates protein kinase C (Ca^{2+} / phospholipid-dependent protein kinase, EC?), whereas IP_3 causes the release of Ca^{2+} from intracellular stores.

Physiological concentrations of spermidine and spermine inhibited the phospholipase C-dependent hydrolysis of phosphoinositides in rat brain (Eichberg et al.,1981) and platelets (Nahas and Graff, 1982). Investigations by Wojcikiewicz and Fain, (1988) with rat anterior pituitary cells have confirmed that polyamines inhibit GTPγS analogue and Ca^{2+} stimulated polyphosphoinositide hydrolysis. The authors suggest that the inhibition results from electrostatic interaction of the amino groups of the polyamines with the phosphate groups of the phosphoinositides. In addition to these effects, polyamines have also been shown to stimulate phosphatidylinositol (PI) and phosphatidylinositol monophosphate (PIP) kinase activities (Vogel and Hoppe, 1986).At low Mg^{2+} concentrations spermidine and spermine enhanced phosphorylation of PI and PIP by 2-4 fold but had no effect at high Mg^{2+} concentrations.Smith and Snyderman , (1988) suggest that polyamines activate phosphoinositide kinases by removing the requirement for high, superphysiological levels of Mg^{2+}. Polyamines inhibit PIP and IP_3 dephosphorylation. Inositol (1,4,5)P_3 5-phosphatase is a key enzyme in the signal cascade which terminates the effect of IP_3 on calcium mobilisation (Seyfried et al., 1984).

1.3.7.6. Effect of polyamines on protein kinase C

Protein phosphorylation and dephosphorylation is a fundamental regulatory mechanism of cell function. Protein kinase C (PKC) has been discovered to have a critical role in this (reviewed by Nishizuka et al., 1984).Activation of the enzyme requires physical translocation from the cytosol to the plasma membrane where it forms a complex with membrane phosphatidylserine and Ca^{2+}. Qi et al., has reported that the activity of PKC from various tissues was inhibited by spermine, non-competitively with respect to phosphatidylserine and Ca^{2+}.In addition , activation of PKC by phorbol esters, Ca^{2+} and

phosphatidylserine was also inhibited by spermine and spermidine (Thams et al., 1986).Meers et al., (1986) have demonstrated in liposomes that polyamines can compete with Ca^{2+} for binding to phosphatidylsrine. This observation is supported by Moruzzi et al. (1987), who have shown that micromolar concentrations of spermine can interfere with the formation of the PKC - phosphatidylserine complex. In addition , millimolar concentrations of spermine can strongly inhibit the phosphorylating activity of the enzyme (Mezzetti et al., 1988).

1.4. Inflammation

Inflammation has been defined as the local response of vascularised tissue to injury (Robbins and Cotran, 1979) be it bacterial, viral, thermal, physical, or chemical in nature. The inflammatory response is characterised by heat, redness, pain , swelling and pain. These features represent pathophysiological effects; heat and redness are manifestations of vasodilation and increased blood flow.The sensation of pain is induced by stimulation of local thermal, mechanical and chemoreceptors. The swelling of the inflammatory lesion is a result of enhanced vascular permeability combined with extravascular leakage and cell migration. The disturbance of function of the inflammed site is equated with both tissue destruction by toxic cell products and the development of more chronic lesions (Bray, 1986).

The inflammatory response is regulated by an array of mediators produced by a variety of resident and migratory cells. The inflammatory response to a given inflammatory stimulus proceeds through a number of phases.

1.4.1. Vasoactive mediators in the early inflammatory response

1.4.1.1. Histamine

Histamine is contained in mast cells and basophils. On injury, the inflammatory response consists of a series of local vascular changes as histamine released from activated mast cells acts on vascular smooth muscle to promote vasodilatation and endothelial cells to increase vascular permeability. Upon stimulation histamine causes a transient vasoconstriction followed by vasodilatation of arterioles, capillaries and venules (Movat et al., 1979). Vasodilatation leads to an increase in both the volume and the amount of blood flow through the capillary network. During vasodilatation blood flow is at first increased but later slows progressively. Histamine causes increased vascular permeability by directly stimulating contractile elements within endothelial cells to open at cell junctions. This exposes the basement membrane which is freely permeable to plasma proteins and fluid (Owen et al., 1980).Oedema results from the leakage of fluid from post capillary venules into interstitial spaces at a rate which exceeds the capacity of the local lymphatic vessels.

histamine on the vasculature are mediated via H_1 and H_2 receptors. Using specific H_1 and H_2 histamine receptor agonists, Owen et al. (1980) demonstrated that vasodilation and increased vascular permeability were mediated through H_1 receptors but H_2 agonists produced vasodilation with minimal effect on oedema formation. In addition , histamine has been reported to induce the expression of the GMP 140 cell adhesion molecule on the endothelial cell surface . It is believed this molecule may have a role in leukocyte-endothelial interactions (Geng et al., 1990). The contribution of histamine to the initial phase of inflammation is demonstrated by the effects of tissue depletion of histamine with compound 48/80 (classical mast cell degranulating substance) which has been reported to delay the inflammatory response to injection of bacteria in guinea pigs (Shelden and Bauer, 1960), and the beneficial effects of antihistamine drugs in the treatment of urticaria and allergic rhinitis. Histamine has also been implicated in anaphylactic shock and immediate hypersensitivity reactions (Roitt et al., 1985).

1.4.1.2. 5-Hydroxytryptamine

In addition to histamine, rat and mouse mast cells secrete 5-hydroxytryptamine (5-HT). This compound has been postulated as the primary mediator of rat and mouse anaphylaxis (Gershon et al., 1975). 5-HT causes vasoconstriction of vascular smooth muscle by activating 5-HT_2 type receptors in both veins and arteries thus generating increased local blood pressure. It is a potent stimulant of sensory nerve endings and induces marked pain. The inflammatory effect of histamine and kinins is potentiated by 5-HT at physiological concentrations (Richardson et al., 1985) and can thus enhance vascular permeability and capillary dilation. Although 5-HT is not released from human mast cells it is released on degranulation of platelets and thus may contribute to vascular permeability changes in man (Fearon and Austen, 1979).

1.4.1.3. The plasma enzyme system

The inflammatory effect of histamine in the early phase of inflammation is reinforced by a series of interacting plasma enzyme systems. These include the clotting system, the kinin system, the fibrinolytic system and the complement system (Roitt et al., 1985). On tissue

injury enzymes are released and surfaces are exposed which activate Hageman factor XII and mast cell histamine release. Activated Hageman factor (XIIa) activates, and is reciprocally activated by, factor XIa and kallikrien. Kallikrien, itself formed through proteolytic cleavage of prekallikrien, cleaves bradykinin from the plasma precursor kininogen. In addition kallikrien activates the fibrinolytic system to produce plasmin, an enzyme which can activate Hageman factor and complement components, and splits fibrin to produce fibrinopeptide chemotactic factors.

Bradykinin induces pain, vascular permeability and vasodilation by acting on blood vessels to activate phospholipase A_2 metabolism (Wiggins et al., 1984). It can also stimulate further release of histamine from mast cells (Fearon and Austen, 1979),thus maintaining and enhancing the original inflammatory response.

1.4.1.4. Anaphylotoxins

The complement components C3a and C5a comprise the anaphylotoxins. Both of these complement fragments have properties that are dependent on the possession of the carboxyterminal arginine (Vallota and Müller-Eberhard, 1973). C3a causes smooth muscle contraction in a variety of animal tissues. Administration intravenously or intradermally causes an immediate erythema and oedema. The potency of C5a is 10-20 fold more than C3a. It is a potent mediator of vascular permeability and is a major chemotactic factor for neutrophils liberated during complement activation (Henson et al., 1978; Fernandez et al., 1978). Both C3a and C5a have indirect inflammatory effects through stimulating mast cell degranulation (Roitt et al., 1985). The vasoactive mediators described above mediate the first 30-60 minutes of the inflammatory response to noxious stimuli. These activate the synthesis of, and are superseded by, longer lived mediators such as prostaglandins , platelet activating factor and the leukotrienes.

1.4.2. Products of arachidonic acid metabolism.

1.4.2.1. Prostaglandins

The role of prostaglandins in inflammation has been reviewed by G.P. Lewis, (1983). Prostaglandins are synthesised and released on stimulation by all major inflammatory cells.

The rate limiting step for prostaglandin synthesis is the release of arachidonic acid from membrane phospholipids by phospholipase A_2 or by the action of diacylglycerol lipase which can cleave arachidonic acid from diacylglycerol , the endogenous substrate of protein kinase C (Nishizuka 1984). Arachidonic acid is the substrate for cyclooxygenase a ubiquitous enzyme which generates the cyclic endoperoxides PGG and PGH. These products are highly unstable and are subsequently converted to prostaglandins and thromboxanes depending on the tissue of origin.

The major producer of prostaglandins, with respect to inflammmation, is the macrophage (Gemsa et al., 1981).Prostaglandin production has been reported to be elicited by a variety of agents including opsonised zymosan, immune complexes, mitogens such as phytohaemagglutinin and concanavalin A and interleukin 1 (Gilman et al., 1988).The major macrophage prostaglandins are PGE_2 and PGI_2. Both products have been reported to have potent vasodilator activity (Humes et al., 1977) and the release is sustained over a long period. In addition, prostaglandins have been reported to cause oedema by increasing vascular permeability through the induction of vascular leakage at post capillary venules. In general prostaglandins are most potent at promoting vasodilation and less so at oedema.

These findings were confirmed and extended by the classic study of Williams (1979). The administration of PGE_2 and PGI_2 intradermally to guinea pigs stimulated vasodilation but had little effect on plasma exudation. The intradermal injection of arachidonic acid had the same effect. However, the co-administration of arachidonic acid and bradykinin induced vasodilation and marked plasma exudation. This effect was blocked by indomethacin (cyclooxygenase blocker). Thus the inflammatory effects of prostaglandins such as pain and oedema are not direct but are the result of the potentiation of the inflammatory effects of the early mediators such as histamine, bradykinin and complement C5a.

Prostaglandins may be considered mediators of inflammation in the short term, but studies have indicated that prostaglandin release over longer intervals may have anti-inflammatory properties (Lewis, 1983). The formation of pannus in adjuvant arthritic rats has been reported to be inhibited by PGE_2 (Zurier and Quagliata, 1971). PGE_2 has also been reported to interfere with cotton pellet induced granuloma in rats (Bonta and Parnham, 1978). The anti-inflammatory effect of prostaglandins may be linked with the elevation of

cyclic adenosine monophosphate (cAMP) elicited by these substances in many cell types (Bourne et al., 1974). Prostaglandins elevate cAMP via receptor interaction with stimulatory G proteins (receptor associated GTP binding proteins) and adenyl cyclase. Increased intracellular cAMP in inflammatory cells has been associated with the inhibition of inflammatory functions such as lymphocyte activation , cell mediated cytotoxicity and the release of inflammatory mediators (Weissmaa et al., 1980). Recently Smith (1989) has suggested that prostaglandins may also interact with receptors linked to inhibitory G proteins to inhibit cAMP synthesis.

Parnham et al., (1978) showed that during development of adjuvant arthritis in the rat, the PGE_2 levels in samples of paw perfusate paralelled the paw volume, and that the highest levels of PGE_2 correlated with elevated cAMP levels.In vitro studies with cultured synovial cells derived from rheumatoid arthritis patients showed a similar pattern with both elevated PGE_2 and cAMP levels (Robinson et al., 1975). In support of this finding, prostaglandins have also been found in synovial fluid samples from patients with arthritis (Bonta and Parnham, 1978). However, 90% of the cell content from articular effusions from rheumatoid patients is neutrophils (Palmer, 1968). Prostaglandin release from neutrophils is stimulated by a wide range of stimuli but is comparatively short lived (Gemsa et al., 1981). Exposure of neutrophils to PGE_1, PGE_2 and PGI_2 has been reported to inhibit neutrophil function.

Gordon et al (1976) first suggested that prostaglandins may play a role in modulating macrophage-lymphocyte interaction by interfering with cytokine production. It has since been reported that prostaglandins inhibit monocyte interleukin 1 secretion by elevating intracellular levels of cAMP (Knudsen et al., 1986). Kunkel et al (1988) has also demonstrated that prostaglandins inhibit macrophage tumour necrosis factor gene expression.Conversely, stimulation of synovial cells with IL-1 and TNF induces a slow release of prostaglandins (Godfrey et al., 1988) and the systemic effects of injected IL-1 and TNF can be inhibited by pretreatment with indomethacin.

1.4.2.2. Leukotrienes.

Leukotrienes are formed via the second major pathway of archidonic metabolism catalysed

Leukotrienes are formed via the second major pathway of arachidonic metabolism catalysed by a series of enzymes called the lipoxygenases . The two classes of leukotrienes are formed from a 5, 6 epoxide precursor (LTA $_4$) and consist of 5, 12 dihydroxy-eicosatetranoic acid (LTB_4) and a series of C-6 amino-acid substituted hydroxy fatty acids designated LTC_4, LTD_4 and LTE_4 (Chakrin and Bailey 1984). Leukotrienes are potent mediators of inflammation (reviewed by Bray, 1986). In general, the inflammatory effects of the peptido-leukotrienes are on smooth muscle whilst LTB_4 is a potent chemotactic factor (Smith et al., 1980)

The major producers of leukotrienes are activated neutrophils, eosinophils and macrophages although different cell types produce different proportions of different leukotrienes. Interrelationships exist between various mediators and the leukotrienes; for example the formyl peptide FMLP and opsonised zymosan stimulate LTB_4 release from human neutrophils (Palmer et al., 1983; Salari et al., 1985). Lin et al (1982) reports that platelet activating factor (PAF) has the same stimulatory effect thus maintaining and amplifying the original inflammatory response. Similar interactions have been reported for the mouse macrophage which generates LTC_4 on intraperitoneal challange with *C.parvum* (Scott et al., 1982), and LTC_4 and LTB_4 on stimulation with zymosan (Rouzer et al., 1980). Differential release of leukotrienes is further demonstrated by cultured human eosinophils which exclusively release LTC_4 on stimulation with opsonised zymosan (Bruynzeel et al., 1985), and LTB_4 on stimulation by C5a (Goetzl 1980) PAF, FMLP and calcium ionophore A23187 induce LTB_4 and LTC_4 release (Weller et al., 1983). Indeed both migratory and resident cells have the ability to respond to inflammatory stimuli by releasing leukotrienes. Examples of resident cells reported to generate leukotrienes include mucosal mast cells, dermal keratinocytes, vascular endothelium and lung epithelium (Bray, 1986). The major cell type for leukotriene generation in the human lung is the mast cell which on anti-IgE stimulation generates LTB_4 and LTC_4, LTD_4 (Peters et al., 1984).The alveolar macrophgage responds to IgE immune complexes by releasing LTC_4 and prostaglandins (Rouzer et al., 1982).

Pages 54 missing

endothelial culture have suggested that LTB_4 stimulated leukocyte - endothelium adhesion was endothelium dependent (Gimbrone Jr et al., 1984). LTB_4 may also be chemokinetic for lymphocytes and enhance T-suppressor cell activity and interleukin-1 formation (reviewed by Goetzl et al., 1984; Rola-Pleszczynski, 1985).

In common with other mediators of PMNL activation such as C5a and FMLP, LTB_4 has been reported to stimulate the respiratory burst and lysosomal enzyme release (Serhan et al., 1982). However the latter feature was relatively weak and required the presence of cytochalsin B. LTC_4 has also been reported to stimulate superoxide and hydrogen release from guinea-pig macrophages (Hartung, 1983).

Injection of LTB_4 into human skin increases plasma exudation but has a weak effect on blood flow. Both effects could be potentiated by the co-administration of PGE_2, PGD_2 and PGI_2 (Camp et al., 1983 investigate!) and were abolished by prior treatment with indomethacin. Feurstein et al., (1981) has reported that LTC_4 and LTD_4 stimulates PGE_2 and thromboxane release from rat peritoneal macrophages, and PGI_2 release from endothelium (Cramer et al., 1983). Such effects may enhance the vasodilator effects of LTC_4 and LTD_4 in vivo.

1.4.2.3. Platelet activating factor (PAF)

Platelet activating factor (1-O-alkyl -2-acetyl sn-glycero-3-phosphorylcholine, PAF) was first identified in the supernatant of rabbit basophils challenged with antigen (Benveniste et al., 1972). It is a potent inflammatory mediator with a wide range of biological activities (O'Flaherty and Wykle, 1983). Tissue sources of PAF include the liver (Buxton et al., 1984), the lungs (Camussi et al., 1983), phagocytic cells of the blood and spleen (Arnoux et al., 1982) and cells involved in inflammation and allergy including mast cells, neutrophils, macrophages, monocytes, basophils , eosinophils (Camussi et al., 1981; Camussi et al., 1987), and endothelial cells (Camussi et al., 1983; Bussolino et al., 1986). The release of PAF by mast cells and basophils is stimulated by antigen or anti-IgE challenge. Monocytes and macrophages respond to phagocytic stimuli such as bacteria,

opsonised zymosan or immune complexes, and to the calcium ionophore A23187. Neutrophils and eosinophils respond additionally to C5a, phorbol esters and FMLP. Endothelial cells release PAF in response to thrombin, vasopressin, angiotensin II and interleukin-1 (Braquet and Rola-Pleszczynski, 1987). There are two pathways of synthesis.In most cell types the basal level of PAF in resting cells is thought to be maintained at low steady state levels through the transfer of phosphocholine from cytosine diphosphate choline to 1-O alkyl-2-acetyl-sn-glycerol.The majority of PAF is formed upon membrane stimulation (Snyder, 1987) through the acetylation of the lyso-PAF precursor which is itself released through phospholipase A_2 activation. Upon generation PAF is readily degraded by PAF acetylhydrolase , an enzyme isolated from both cells and plasma (Farr et al., 1980). The level of PAF in plasma is consequently low. The activity of PAF is also controlled by C-reactive protein and other liver derived proteins. It is believed that this may be an important non-specific mechanism for controlling PAF induced activation of platelets (Vigo, 1985).

The biological activities of PAF in human tissue include platelet aggregation and secretion (McManus et al., 1981), bronchoconstriction (Gateau et al., 1984), chemotaxis of monocytes (Czarnetski , 1983) and the activation of PMNL chemotaxis, chemokinesis, aggregation and degranulation (O'Flaherty et al., 1981; Shaw et al., 1981). It may also have a key role as a regulator of cellular immune responses (reviewed by Braquet and Rola-Pleszczynski, 1987).

The effect of PAF in vivo has been investigated by Pipkorn et al., (1984). Inhalation of PAF had little effect on the vasculature of the nasal mucousa but intradermal injection caused a wheal and flare reaction with some subjects showing an early and late response (O'Flaherty et al., 1981). Subsequent analysis of biopsy of the skin reaction showed a massive infiltration of inflammatory cells.

In addition to release of PAF into the extracellular milieu , vascular endothelial cells have been reported to generate intracellular quantities of PAF in response to exposure to histamine,bradykinin, ATP (McIntyre et al., 1985) thrombin (Zimmerman et al., 1986), and oxidant induced damage (Lewis et al., 1988). Under normal tissue culture conditions most of this PAF was not released. In thrombin stimulated or oxidant damaged

endothelium there was a concentration response relationship which correlated with time courses for PAF production and and enhanced capability for neutrophil adherence. It is believed that intracellular PAF and PAF receptors within the cell may mediate this effect (Meade et al., 1991).

It is unclear as to whether platelets can synthesise PAF since they lack the necessary acetyl transferase and the major product is the precursor lyso-PAF. However, platelets can form PAF in synergy with PMNLs which are rich in the enzyme. On PAF stimulation platelets aggregate and secrete thromboxanes, 5-HT and adenine nucleotides. These mediators can modulate vascular permeability and endothelial cell adhesion properties (Meade et al., 1991).

Diseases associated with increased levels of PAF in plasma and tissues include septic shock (Bussolino et al., 1987), anaphylactic shock (Meade et al., 1991), late phase astmha (Nakamura et al.,1987) and systemic lupus erythromatosus (SLE). Interleukin-1 and TNF are thought to play an important role in septic shock (Tracey et al., 1987) and since these peptides are known to stimulate endothelial PAF release (Camussi et al., 1987), this could provide an explanation for increased levels of plasma PAF . In addition, PAF has been shown to stimulate IL-1 and TNF production by monocytes (Pignol et al., 1987) and pre-exposure to TNF and endotoxin prime the effect of PAF in vivo (Heuer et al., 1990). This could be a possible mechanism for positive feedback.

1.4.3. The role of cytokines in inflammation.

Cytokines are low molecular weight glycoproteins with critical roles in inflammation, the immune response, haematopoeisis and fibrosis (Feldmann et al., 1990).Regulatory factors embraced by this category include the interleukins, tumour necrosis factors, the interferons, bone marrow colony stimulating factors and a variety of growth factors (fibroblast, epidermal, transforming and platelet derived growth factors, Hopkins, 1990).They may act locally in an autocrine or paracrine mode but can also have major effects systemically. Of particular interest are:

interleukin 1

interleukin 6

interleukin 8

interferon γ

tumour necrosis factor α

since recent evidence suggests that these cytokines have a pivotal role in inflammation.

1.4.3.1. Interleukin 1

The major source of interleukin 1 (IL-1) is from the activated macrophage but it has been reported to be produced by fibroblasts, endothelial cells, smooth muscle cells, keratinocytes and Langerhans cells (reviewed by Mizel, 1989). IL-1 release is induced by antigen, immune complexes and by other cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF), transforming growth factor β, tumour necrosis factor α , interferon γ and IL-1 itself (Akira et al., 1990). In an immunological situation the major stimulant of IL-1 secretion is through interaction of activated T lymphocytes with antigen presenting cells.

IL-1 is secreted in two forms, designated α and β of 13 and 17 KDa respectively, each is processed from a common precursor of 33 KDa (Giri et al., 1985). It is thought that processing of the precursor molecule occurs during or after secretion. The inflammatory effects of IL-1 are at both the local and systemic level. Targets of IL-1 include chondrocytes, fibroblasts, synovial cells, hepatocytes and osteoclasts. Arguably, the most

crucial effects are on T and B cell activation and maturation with the subsequent induction of IL-2 secretion and IL-2 receptor expression (Mizel, 1988; Dinarello, 1984; Shirakawa et al., 1986,). Feldmann (1990) has reported high levels of IL-1 in synovial fluid samples from rheumatoid arthritis patients. Administration of IL-1 to to rabbits caused a chronic erosive arthritis (Pettipher et al., 1986.). At the local level, IL-1 activates the secretion of neutral proteases from neutrophils, fibroblasts and chondrocytes and osteoclasts which could account for the potent bone and cartilage degrading activity of the cytokine reported in vivo and in vitro studies (Allison ,1985), and the expression of endothelial leukocyte adhesion molecules (ELAM, Gimbrone et al., 1989). Systemic effects attributed to IL-1 are the promotion of neutrophil release from bone marrow (Sipe et al., 1982) and the mediation of the acute phase response via the induction of, and synergising with, IL-6 (Gauldie et al., 1987). It can also act as a pyrogen since the administration of IL-1 can cause a considerable elevation of body temperature (Dinarello, 1984). A key characteristic of IL-1 on all target cells is the activation of arachidonic acid metabolism (as has already been described in section 1.4.2.1 and 1.4.2.3.). Inflammatory features of IL-1 such as the release of plasminogen activator by synovial cells have been found to be regulated by eicosanoid synthesis and bone resorption induced by IL-1 can be inhibited by indomethacin (Dewhirst et al., 1987).

The IL-1 receptor has been reported by Dower (1989) to exist in two forms of 80KDa and 68 KDa although several groups have reported that IL-1 α and β share the same receptor (Mizel 1989). Evidence has accumulated that the main second messenger for IL-1 in some cell types may be cAMP. Treatment of murine thymocytes and human rheumatoid synovial cells with IL-1 results in the activation of adenylate cyclase and the elevation of intracellular cAMP (Shirakawa et al., 1989). In support of this finding the activation of these cells by IL-1 could be blocked by drugs that preferentially inhibited cAMP dependent protein kinase A, but not by those drugs that specifically inhibit protein kinase C (Chedid et al., 1989).

1.4.3.2. Interleukin 6

Interleukin 6 (IL-6) is a glycoprotein of 26-30 KDa depending on the degree of glycosylation (Mizel, 1989). Like IL-1, it is produced by a wide range of cell types

including fibroblasts, monocytes / macrophages, endothelial cells, mesangial cells and tumour cells. It has a broad spectrum of cell targets and thus can influence over a variety of immune and inflammatory responses in vitro and in vivo (Mizel 1989). It has been referred to by various designations ; plasmacytoma growth factor, interferon β2 and hybridoma growth factor (Billiau et al., 1989) which reflect the range of effects of this interleukin. Except for certain tumour cells, IL-6 has to be induced; it is not produced constitutively by 'normal' cells. Reported stimulants of IL-6 production include TNFα, PDGF, viral infection , double stranded RNA and IL-1 (Mizel 1989). IL-6 synergises with IL-1 in the induction of T cell proliferation (Elias et al., 1989), differentiation of cytotoxic T cells (Tosato et al., 1986), and enhances B cell growth and differentiation to antibody secreting plasma cells (Kishimoto and Hirano, 1988). IL-6 and IL-1 synergise the production of acute phase proteins from hepatocytes as part of the acute phase response to injury or infection (Gauldie et al., 1987). However, IL-6 unlike IL-1 can mediate changes in the full array of acute phase reactants - C-reactive protein, fibrinogen and serum amyloid protein A both in vivo and in vitro (Gauldie et al., 1987; Ramadori et al., 1988). It also acts as a pyrogen and has been isolated from the synovia of patients with rheumatoid arthritis. From this observation it has been suggested that IL-6 may contribute to the aetiology of connective tissue disease. However, IL-6 does not stimulate cartilage or bone catabolism or induce arachidonic acid metabolism (Guerne et al., 1989). Indeed as pointed by Hopkins (1990), since IL-6 levels rise rapidly after even minor trauma, and as a major regulator of the acute phase response (an anti-inflammatory mechanism), it is entirely appropiate that IL-6 does not stimulate inflammatory mediators.

1.4.3.3. Tumour necrosis factor

Tumour necrosis factor (TNF) exists in two forms, designated α and β of 17 and 25 KDa, respectively. Only the β form is glycosylated (Akira et al.,1990). It is produced by activated macrophages challenged with endotoxin and by activated T lymphocytes. TNF β has both cytostatic and cytotoxic effects. The name originates from a factor derived from the serum of mice challenged with endotoxin which caused the necrosis of various tumours

in vivo. In vivo TNF α mediates septic shock in concert with IL-1 and PAF (Heuer et al., 1990), synergises with IL-1 in the induction of fever (Mizel, 1989), induces the production of IL-6 and thus regulates the acute phase response (Hopkin 1990) and amplifies the effects of IL-1 in diseases characterised by increased IL-1 production. Locally, TNF has been reported to enhance neutrophil adhesive properties and endothelial cell toxicity (Akira et al., 1990). TNFα is found in rheumatoid synovial fluid samples and can induce collagenase and metalloproteinase secretion (Feldmann et al., 1990). Injection of TNF α causes less inflammation than IL-1 but in synergy with IL-1, induces significant cartilage and bone degradation in vitro (Feldmann, 1990). TNF like IL-1 causes a slow release of prostaglandins from synovial cells over many hours (Hopkins 1990). The effect of TNF α on fibroblast proliferation (in combination with IL-1) can be inhibited by indomethacin

1.4.3.4. Interferon γ

Interferon gamma (IFN-γ) was characterised through its potent antiproliferative and antiviral properties (Bonvini et al., 1985). It is a protein of 20-25 KDa depending on the degree of posttranslational glycosylation (Biondi et al., 1984). It has a wide range of immunoregulatory functions including the induction of class II antigen (HLA-Dr Ia) expression by myeloid and lymphoid cells (Wong et al., 1983) and endothelial cells (Dustin and Springer, 1988), augmented phagocytic ability and Fc receptor expression by mouse macrophages (Fertsch and Vogel 1984), and activation of the repiratory burst by human monocytes (Nathan et al., 1983) with the release of both reactive oxygen intermediates and reactive nitrogen intermediates (Ding et al., 1988). IFN-γ exerts its action through a cell surface receptor of 90-110 KDa molecular mass, the presence of which has been demonstrated on the surface of murine macrophages (Celeda and Schrieber., 1985). Interferon gamma is mainly produced by activated T helper cells on stimulation by antigen, mitogen and phorbol esters (Biondi et al., 1984). Production of IFN-γ is augmented by IL-1 and IL-2 (Farrar et al., 1982). In vitro IFN-γ has been reported to inhibit IL-1 induced

bone degradation and IL-1 induced IL-1 secretion (Schindler et al., 1989). In addition it has been reported to synergise with TNFα in the induction of macrophage mediated antibody-dependent cytotoxicity and in the inhibition of fibroblast proliferation (Feldmann et al., 1990). The antiproliferative properties could be due to inhibition of ornithine decarboxylase since the cytokine suppresses the induction of ornithine decarboxylase activity by serum in growth arrested mouse and human fibroblasts (Sekar et al., 1983).

Interferon γ stimulates an increase in eicosanoid metabolism in many cells but has been reported to inhibit prostaglandin synthesis in monocytes (Hopkins, 1990) This may explain its efficacy in the treatment of rheumatoid arthritis (Browning, 1987). This is contentious though since other workers have failed to find inhibition by IFN-γ of IL-1 and TNF α induced prostaglandin synthesis (Hart et al., 1989). Levels of IFN-γ are beyond detection in synovial fluid samples from rheumatid arthritis patients and Feldmann et al., (1990) have reported that the level of IFN-γ mRNA in mononuclear cells from rheumatoid joints is downregulated and recovers on rest in culture. Thus in this disease a deficiency of IFN-γ could be of pathological significance.

1.4.3.5. Interleukin 8

Although lacking direct activity on neutrophil chemokinesis, human recombinant IL-1α was found to be capable of inducing the production of a chemokinetic factor from human synovial fibroblasts (Watson et al., 1987). Since 1989 this potent neutrophil activating factor, of 6.7 KDa molecular mass, has been designated interleukin-8 (Westwick et al., 1989). IL-8 gene transcription is rapidly induced (<30 minutes) by IL-1 and TNFa in peripheral blood mononuclear cells, dermal fibroblasts, endothelial cells and keratinocytes (reviewed by Matsushima and Oppenhiem, 1989). Schroeder et al (1988) have also reported that the mitogen PHA activates the production of IL-8 by T cells as does double stranded RNA in fibroblasts (Van Damme et al., 1989) and chondrocytes (Van Damme et al., 1990).

The immunoregulatory functions of IL-8 are many and varied. The factor is chemotactic for

neutrophils, basophils and T cells and stimulates the release of histamine by basophils derived from both normal and atopic individuals (Matsushima and Oppenhiem, 1989). It has no reported effects on eosinophils. Both Schroeder et al., (1987) and Peveri et al., (1988) have demonstrated that IL-8 stimulates the neutrophil respiratory burst and lysosomal enzyme release in the presence of cytochalasin B. However the ED_{50} for this effect was 10 fold greater than the optimal dose for chemotaxis in vitro. The T cell was shown to be 10 fold more sensitive to IL-8 as a chemotactic agent than neutrophils (Matsushima and Oppenhiem, 1989)

The effect of IL-8 in vivo has been investigated. An injection of IL-8 i.p in mice caused a rapid increase in the number of neutrophils followed by an increase in the numbers of neutrophils extravasating into the peritoneum (Furtura et al., 1989). Administration of IL-8 intravenously to rabbits caused a rapid onset of plasma leakage (Matsushima and Oppenhiem, 1989). A more detailed in vivo investigation by Larsen et al., (1989) showed that a subcutaneous injection of IL-8 caused margination and and emigration of both lymphocytes and neutrophils from post-capillary venules in a dose dependent manner. In vitro, lower doses (0.01 ng) of IL-8 were strongly chemotactic for lymphocytes whereas higher doses (1 ng) promoted a predominance of neutrophils at the site of injection (Larsen et al., 1989).In vitro IL-8 has been reported to induce the expression of the CD18 adhesion molecule complex by neutrophils without de novo protein synthesis (Farina et al., 1989). This probably accounts for the increased adhesion of neutrophils to vascular endothelial cells and thus the margination of neutrophils at IL-8 injection sites. On the other hand, Gimbrone et al., (1989) have reported a potentially anti-inflammatory facet of IL-8 action since at nanomolar concentrations IL-8 inhibits neutrophil adhesion to cytokine activated endothelial cells and protects them from neutrophil mediated damage. It is suggested that the chemotactic and adhesion inhibitory properties are coordinated responses since IL-1, TNFα cause sustained upregulation of IL-8 gene expression in endothelial cells (Gimbrone et al, 1989). Thus the vascular endothelium can be protected within a time scale that extends beyond the initial inflammatory stimulus.

Feldmann et al (1989) have reported the presence of IL-8 in synovial fluid effusions from patients with rheumatoid arthritis and inflammatory cells from the same source expressed

high levels of mRNA. A different form of IL-8 has been isolated by Schroeder et al (1986) in psoaritic scale. A recent investigation by Sietz et al (1991) has revealed that the spontaneous production of IL-8 from peripheral blood mononuclear cells (PBMC) of rheumatoid arthritis patients was 10 fold greater than normal and was even greater in cells derived from synovial fluid. Interferon γ significantly reduced both the spontaneous and stimulated production of IL-8 by peripheral blood and synovial fluid mononuclear cells from normal and rheumatoid arthritis afflicted individuals. The production of IL-8 in vivo was abolished by intraarticular injections of betamethasone but indomethacin actually increased IL-8 yield from PBMC in culture, suggesting that prostaglandins may inhibit IL-8 production.

The expression of the IL-8 receptor by neutrophils has been studied by Samanta et al (1989). Ligand binding studies suggest that the receptor is dynamically regulated by the ligand itself and that the rapid recycling of the receptor may be needed for the chemotactic response. Data from investigations of the mode of transduction of the IL-8 signal suggest that in common with other peptide chemoattractants , IL-8 induces a rapid elevation of intracellular calcium and is protein kinase C dependent (Thelens et al., 1988).

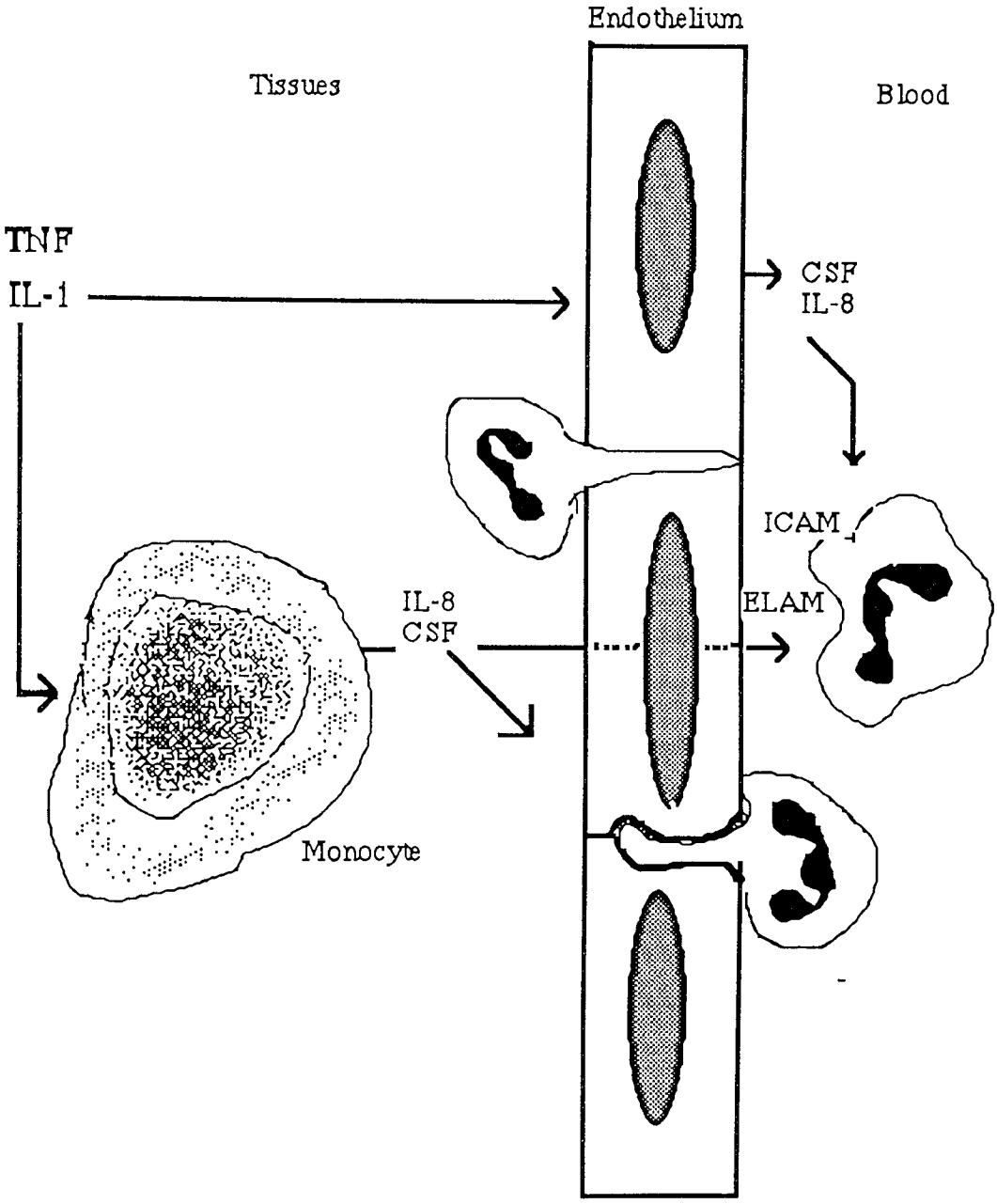


Fig 1.4.3.1 Cytokine-endothelial interactions in leukocyte extravasation

IL-1 and TNF are not directly chemotactic but elicit leukocyte extravastion by inducing the expression of the adhesion molecules intercellular adhesion molecules (ICAM-1 and 2) and endothelial leukocyte adhesion molecules (ELAM), and the production of colony stimulating factors (CSF) and IL-8 by endothelial cells, monocytes and tissue cells.

1.4.4. The role of the neutrophil in inflammation

Neutrophils represent the first line of defence against infection by bacteria and fungi. In the circulation the cells move by mass flow but when adherent to the vessel wall the cells move by amoeboid motion. The speed and direction of movement can be influenced by chemical factors in the environment. Movement along a chemical gradient towards the source of the signal is the process of chemotaxis.

The first step in the heirarchy of responses is the vasoactive response which results in the reduction of blood flow and then local vasodilation.Neutrophils arrive and adhere to the endothelial vessel wall via specific intercellular adhesion molecules. This is followed by dipedesis through the internedothelial cell junctions (Ryan and Majno, 1977) and migration through the interstitial tissue to the focus of injury. At the source of the chemotactic signal the cell reponds with a series of actions characterised by degranulation of degradative enzymes and the oxidative burst.

1.4.4.1. Intercellullar adhesion

The role of cell adhesion molecules has been recently rviewed by Albeda and Buck (1990). Adherence of the neutrophil to endothelial cells in vitro is increased by stimulation of the neutrophils by chemotactic factors such as PAF, FMLP, and IL-8 or by activation of endothelial cells with cytokines or endotoxin (Pohlman et al., 1986). Studies with monoclonal antibodies indicate that the adherence of activated neutrophils is dependent on CD11a / CD18 (LFA 1) and CD11b / CD18 (MAC 1) surface adhesion molecules (Pohlman et al., 1986; Gamble et al., 1985; Smith et al., 1989). However, the adherence of unactivated neutrophils to the inflamed endothelium depends on interaction between LFA-1 on the neutrophil and endothelial intercellular adhesion molecule (ICAM-1), it has been suggested that this occurs in concert with ELAM-1 and a CD18 independent neutrophil adhesion molecule called Lectin adhesion molecule 1 (LECAM-1, Smith et al., 1991). Both the endothelial molecules are present at very low levels in resting cells and are rapidly upregulated on stimulation by cytokines. Smith et al (1991) have proposed that the initial binding event of the unstimulated neutrophil in circulation to the activated endothelium under flow is mediated by LECAM-1. Once bound and exposed to chemotactic factors, the LECAM-1 complex is rapidly shed from the cell. This

downregulation is coordinated with the upregulation of MAC-1 expression and function. It is thought that MAC-1 is important for reinforcing adhesion between the cells and for promoting transendothelial cell migration (Smith et al., 1991). In addition, the MAC-1 has a ligand repertoire which includes complement C3bi, fibrinogen and endotoxin (Albelda and Buck, 1990).

1.4.4.2. Chemotaxis

It has been established that chemotactic factors such as PAF, LTB_4, C5a, FMLP and IL-8 are all capable of activating neutrophils via membrane receptors. In the intensely investigated response to FMLP, Snyderman et al., (1983) have proposed that high affinity receptors transduce chemotaxis while low affinity sites transduce oxidant production and degranulation. It is thought that the number and affinity of FMLP receptors is modulated by GM-CSF (Weisbart et al., 1986). Goldman and Goetzl (1984) have reported a similar pattern of receptor heterogeneity for LTB_4 and the dose dependence of the functional responses to IL-8 has already been described (section 1.4.3.5.).

Upon exposure to chemoattractants the neutrophil changes shape as intracellular components such as microtubules are orientated to form a pseudopod in the direction of the chemoattractant. Ultrastructural studies show that the nucleus is shifted to the rear of the cell (Roberts et al., 1983). The neutrophil squeezes between the endothelial cells by diapedesis and penetrates the basement membrane (Hoover et al., 1978).Through a process of locomotion generated by the sliding of interdigitating actin and myosin (Stossel, 1978) the neutrophil moves under the influence of chemotactic factors into extravascular tissues. Once in the tissues the neutrophils phagocytose and digest foreign material and demolish host tissue releasing lysosomal enzymes, active oxygen species, and generating prostaglandins and chemotactic factors including PAF and LTB_4 (Westwick and Poll, 1986).

1.4.4.3. Phagocytosis

Neutrophils engage opsonised particles via IgG Fc and complement C3b receptors or by non-specific receptors if the particle is non-opsonised. Upon binding the plasma membrane becomes activated, extends pseudopods and invaginates to form a phagosome (Zucker et al., 1964). Digestion of the engulfed particle by lysosomal enzymes is initiated by fusion of lysosomal membranes with the phagosome thus forming the phagolysosome. However, as in the case of type II hypersensitivity reactions where host cells are coated with opsonins and are too large for the neutrophil to digest , or in type III hypersensitivity reactions where the neutrophil tries to phagocytose immune complexes trapped in tissues, phagocytosis is "frustrated" and leakage of lysosomal enzymes to the extracellular environment occurs (Becker and Henson, 1973-report). Lysosomal enzyme and mediator release elicited by high concentrations of inflammatory mediator binding to low affinity neutrophil receptors (as in the case of FMLP and LTB_4), or the same receptor (for C5a, PAF and IL-8) in the absence of phagocytosis is called degranulation (Sklar, 1986). This event has potential for causing condiderable damage and exacerbation of the original inflammatory response.

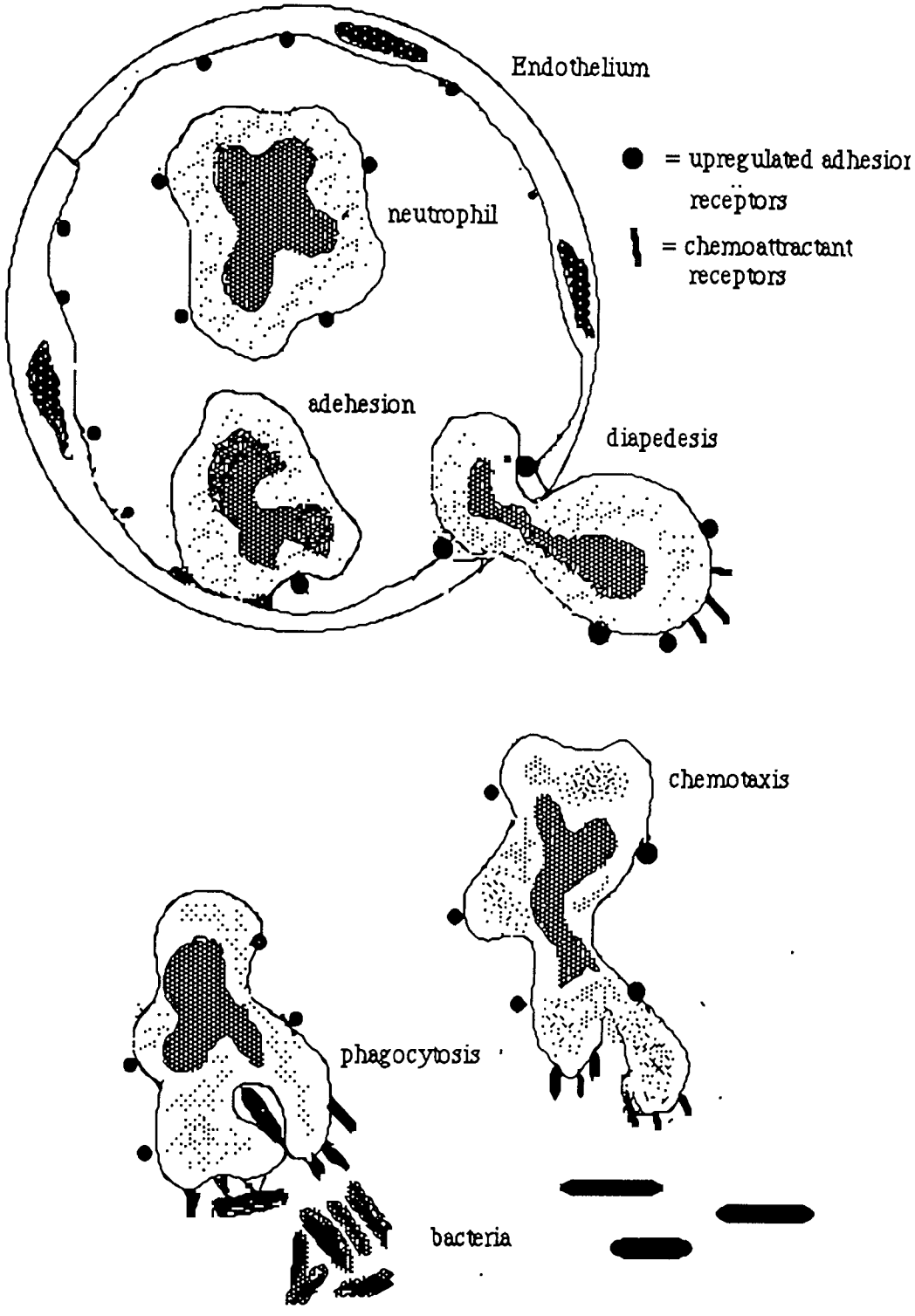


Fig 1.4.4.1 Role of adhesion and chemoattractant receptors in inflammation

Mediators released by activated macrophages or mast cells stimulate the expression of adhesion molecules such as ICAM-1, ELAM-1 and GMP-140 on the surface of endothelial cells and upregulate expression of ICAM-1 on leukocytes within the the vascular lumen. Leukocytes adhere to the activated endothelial cells, migrate through the endothelium and enter areas of tissue damage or infection via chemotaxis. Once at the inflammatory site neutrophils phagocytose bacteria or damaged tissue and generate superoxide.

The neutrophil lysosome contains at least 20 different enzymes (lipases, glucoronidase, esterases, acid phosphatases and proteases). Many of these enzymes function at the acid pH of the phagolysosome. The function of these enzymes is primarily antibacterial and they are inactivated at physiological pH. However, neutral proteases secreted from the neutrophil are active and may attack host tissue (Smolen and Weissmann, 1979). The most significant of these, with respect to inflammation, are collagenases and elastase. Both these enzymes can mediate the destruction of connective tissue and extracellular matrix. More specifically, elastase can degrade the elastin component of blood vessels (Krane, 1979). Other neutrophil enzymes of note are cathepsin G and cathepsin D. Both these enzymes can degrade proteoglycans and other proteins. Cathepsin D is only active at acid pH (Krane, 1979). Neutral proteases are thought to be responsible for much of the extracellular proteolytic activity found in rheumatoid arthritis (McGuire et al., 1982). Protease activity is regulated by a complex series of antiproteases (Weiss et al., 1984). During phagocytosis a series of oxidative reactions are triggered resulting in a respiratory burst.

1.4.4.4. The respiratory burst

Membrane stimulation by particulate activators such as bacteria, zymosan or latex particles, or high concentrations of neutrophil receptor ligands, such as IgG, FMLP, C5a, IL-8 and PAF , result in the respiratory burst. The respiratory burst generates large amounts of superoxide and subsequent active oxygen species of varying toxicity (Badwey et al., 1980). The respiratory burst is primarily an essential step in host defence against microorganisms (Baboir et al., 1973) but as is the case with inappropiate lysosomal enzyme release, the exposure of host tissue to active oxygen species can cause considerable damage and is thus believed to be involved in the aetiology of immune complex mediated disease.

The respiratory burst is characterised by increased oxygen consumption and activation of the hexose monophosphate shunt. Molecular oxygen is reduced by electrons donated by nicotinamide adenine dinucleotide phosphate (NADPH) during the oxidation of glucose. The primary product superoxide ($O_2^{\cdot -}$) is converted to hydrogen peroxide which subsequently interact to form highly toxic products such as hydroxyl radical ($OH^{\cdot}$), singlet

subsequently interact to form highly toxic products such as hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl) (Badwey et al., 1980).

The enzyme, dormant in resting cells , exists as a membrane associated NADPH oxidase in activated cells (Cohen et al., 1980). The NADPH binding site faces the cytoplasm , whereas the superoxide product is released on the extracellular or intravacuolar space. Baboir et al., 1981). NADPH oxidase is believed to be a dissociable enzyme complex composed of a low potential cyochrome b_{-245} which can directly reduce oxygen to superoxide (Segal and Jones, 1978), two cytosolic proteins called p47 and p67 which have affinity for GTP, a flavoprotein and possibly ubiquinone (Segal, 1989). The cytochrome b consists of 2 subunits which have been located in azurophilic granules (Garcia and Segal, 1984). It has been suggested that respiratory burst stimulants activate the translocation of the granule associated cytochrome b to the plasma membrane to form the active enzyme (Borregaard et al., 1983). However, as pointed out by Clark (1990) the plasma membrane of unstimulated neutrophils contains a significant amount of viable oxidase, so it is more likely that translocation from granule stores would sustain rather than initiate the respiratory burst. Borregaard et al., (1983) contend that the plasma membrane is the ideal location for cytochrome b since as this invaginates to form the phagocytic vacuole, cytochrome b contained within the vacuole wall can then reduce the oxygen within the phagosome.

1.4.4.5. Neutrophil generated superoxide and Inflammation

The relationship between superoxide and inflammation has been reviewed by Weiss (1986). The cytotoxicity of superoxide and its postulated role as a mediator of inflammation results through evoked tissue response and damage caused by superoxide metabolites. Superoxide may have a direct role in phagocyte mediated capilllary permeability by inducing the formation of a chemoattractant. Unlike C5a or FMLP it does not stimulate degranulation or the respiratory burst (Petron et al., 1980). Damage induced by superoxide includes the degradation of cartilage and proteoglycan (Greenwald et al., 1976), and hyalauronic acid (Saline and McCord, 1975). In addition superoxide promotes the conversion of the collagenase precursor to the active enzyme (Burkhardt et al., 1986). The

hydroxyl radical through the Fenton modified Haber Weiss reaction (McCord and Day, 1978). Oxygen radicals can damage membranes of erythrocytes (Fenton and Ward, 1982), human PMNLs (Saline and McCord, 1975) and cultured human endothelial cells by initiating lipid peroxidation (Sacks et al., 1978). Such interactions between oxidative species and the polyunsaturated lipid elements of membranes result in loss of membrane structural integrity and function. In vitro studies have shown that lipid peroxidation promotes the release of lysososmal enzymes and that antioxidants inhibit this (Younes and Seigers, 1984). Oxidative species can denature IgG. Lunec and Blake (1984) have reported that structural changes in IgG exposed to active oxygen species attack was identical to IgG isolated from rheumatoid serum and synovial fluid. Denatured IgG stimulates further generation of free radicals by neutrophils thus providing a positive feedback mechanism for active oxygen species release and tissue damage mediated by neutrophils.

Hydrogen peroxide is formed primarily by the enzymatic dismutation of superoxide by superoxide dismutase (McCord et al., 1973). It is cytotoxic to human lymphocytes (Allen et al., 1986) and induces the lysis of erythrocytes (Stocks and Dormandy, 1973). Cell death occurs after several hours at doses of 250-300 μM (Schraufstätter et al., 1990). At least 30-40% of H_2O_2 is converted to hypochlorous acid via myeloperoxidase (Foote et al., 1983) This compound is potently cytotoxic and induces cell lysis within 1 hour (Schraufstätter et al., 1990).

All aerobic cells generate basal levels of superoxide through normal cellular processes. In addition to NADPH oxidase superoxide is generated by xanthine oxidase and hydrogen peroxide is produced by glucose oxidase (McCord et al., 1971). Thus all aerobic cells are equipped with protective intracellular antioxidant enzyme systems comprising of superoxide dismutase (McCord et al., 1971), catalase and glutathione peroxidase (Fridovich, 1976). Vitamin E is the main non-enzymatic chain breaking antioxidant (Witting et al., 1980). Extracellular protection against free radical attack is afforded by caeruloplasmin , a copper glycoprotein acute phase reactant (Goldstein et al., 1979).

1.4.5. Transmethylation in phagocyte function

The integrity of phagocyte function requires transmethylation reactions mediated by SAMe. These functions include the production of the oxidative burst in macrophages (Pike and Snyderman, 1982; Pick and Mizel, 1982), chemotaxis in polymorphonuclear leukocytes and monocytes (Snyderman et al., 1980), phagocytosis by macrophages (Leonard et al., 1978) and the binding of activated macrophages to neoplastic target cells (Adams et al., 1981). Inhibition of these facets of phagocyte function can be achieved by elevating the intracellular content of S-adenosyl-l-homocysteine (SAHc) thus inhibiting methyltransferase reactions sensitive to the metabolite.Pike et al., (1980) have reported that treatment of human monocytes with the specific adenosine deaminase blocker erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) in the presence of adenosine and homocysteine thiolactone elevated the intracellular concentration of SAHc by 1500 fold. Concomitant with the increase of SAHc was a massive reduction of protein carboxy-O-methylation and a marked inhibition of chemotactic responsiveness. However, in contrast to the findings of Leonard et al., (1978), they reported that phagocytic ability was resistant to the drug treatment.

The stimulation of phospholipid methylation in rabbit neutrophils with the chemotactic peptide FMLP was linked to calcium influx and arachidonic acid metabolism (Baries et al., 1982).Increased calcium availability activates phospholipase A_2 (Shier, 1979) generating arachidonic acid from phospholipids some of which arise from methylation. Incubation of cells with 3-deazaadenosine (SAHc hydrolase inhibitor) in the presence of homocysteine thiolactone , leading to an elevation of intracellular SAHc, blocked FMLP stimulated arachidonic acid release but not calcium ionophore A23187 induced release.chemotactic peptide stimulated calcium influx was also partially blocked suggesting that methyltransferase reactions are required for these FMLP activated functions.

Chemotactic peptides stimulate a rapid and transient increase in protein carboxy-O-methylation in phagocytes.This process is also highly sensitive to inhibition by elevated intracellular concentrations of SAHc (Snyderman et al., 1980). There was no effect on ligand receptor binding but the chemotactic responsiveness of human monocytes

was depressed by 92%.Yet it seems that methylation reactions are not needed for motility as such, since random migration was unaffected by the drug treatment. Protein carboxymethylation is reversed by protein methylesterase (PME). Protein methylesterase activity in PMNLs was stimulated by chemotactic peptides. Half maximal stimulation of this activity was induced by 3nM FMLP, a concentration close to that neeeded for a half - maximal chemotactic response (1nM). Specific chemotactic peptide antagonists depressed the PME response in neutrophils. The rapid transient nature of these phenomena were linked to the earliest events in the leukotactic response which suggests that the turnover of protein methylesters is involved in the transduction of the chemotactic signal into directed migration of the cell.

Bonvini et al., (1986) have reported that treament of mouse macrophages with interferon γ increased the intracellular content of SAMe and was associated with the acquisition of tumouricidal activity. Exposure to bacterial lipopolysaccharide also increased SAMe levels and activated cytotoxicity. Depletion of intracellular SAMe with cycloleucine made the macrophages refractory to the induction of cytotoxicity by interferon γ or lipopolysaccharide. This suggests a critical role for SAMe in macrophage activation.

1.4.6. Polyamines in inflammation

Polymines have been found to be anti-inflammatory in animal models of acute and chronic inflammation (Bird et al., 1983; Oyanagui, 1984). Polyamines have effects which are analagous to those of glucocorticoids. A link between the anti-inflammatory action of dexamethasone and putrescine has been established (Bartholeyns, 1980).The anti-inflammatory effect of dexamethasone is manifested through the induction of the gene for, and the expression of, proteins called lipocortins (Tsurufuji et al., 1979). The expression of this protein has been shown to correlate with the inhibition of eicosanoid generation (Parente et al., 1984). Recombinant lipocortin has been reported to inhibit the release of thromboxane A_2 from activated guinea-pig lungs (Cirino et al., 1987) and prostacyclin production by human endothelial cells (Cirino and Flower, 1987). Moreover,

mouse lipocortin impaired the generation of leukotriene B_4 and platelet activating factor by inflammatory rat neutrophils in vitro (Fradin et al., 1988).Lipocortin has been demonstrated to have potent anti-inflammatory activity in vivo, suppressing the generation of prostaglandin E_2, LTB_4 and the chemotactic ability of isolated inflammatory rat neutrophils (Errasfa and Russo-Marie, 1989).

Dexamethasone stimulated hepatic ODC activity but inhibition of ODC with difluoromethylornithine (DFMO, a competitive irreversible blocker of ODC) abolished the anti-inflammatory effect of the drug (Bartholeyns et al., 1980). A further study by Bartholeyns et al., (1981) confirmed that de novo synthesis of putrescine was required for induction of the lipocortin. Stimulation of liver ODC by hepatectomy, carbon tetrachloride (Meilens, 1969) or thioacetamide (Bird, 1979) inhibited inflammation. Both ODC and SAMe decarboxylase activity was elevated in rat liver during inflammation induced by turpentine as part of the acute phase response, which leads to the synthesis of the acute phase proteins (Scalabrino et al., 1987).Polyamine synthesis was preceded by prostaglandin synthesis. Blockade of cyclooxygenase by indomethacin reduced the response of the polyamine biosynthetic decarboxylases to the inflammatory stimulus. This suggests a requirement for arachidonate metabolism as part of the acute phase response.

Many potential anti-inflammatory effects have been attributed to polyamines: Kafy and Lewis (1986) have reported that polyamines protected guinea pig liver lysosomes from superoxide induced lysis.Polyamines can scavenge superoxide (Vanella et al., 1979; Vanella et al., 1980a) and inhibited the reduction of cytochrome C by the hypoxanthine-xanthine oxidase superoxide generating system.Thus polyamines could protect cells from oxidative injury (Vanella et al 1980b). Tadolini et al., (1984) has reported that the protective mechanism could be through inhibiting lipid peroxidation. In addition , polyamines inhibit phospholipase A_2 and phospho- lipase C (Sechi et al., 1978; Nahas and Graff, 1982).

Ferrante (1986) has demonstrated that the metabolites of the polyamine - polyamine oxidase system can inhibit the oxidative burst of neutrophils in vitro by decreasing the activity of the hexosemonophosphate shunt. These polyamine metabolites can also be produced in the presence of superoxide released from neutrophils during the respiratory burst (Kafy and

Lewis, 1987).Ferrante et al., (1986) suggests that this mechanism could constitute a self regulating switch for terminating the respiratory burst response. Further, these metabolites inhibit human neutrophil chemotaxis (Ferrante et al., 1985).

1.5. Clinical Applications of SAMe

1.5.1 S-Adenosyl-methionine in the treatment of primary fibromyalgia

Fibromyalgia is a chronic non-articular rheumatic disease characterised by pain and stiffness in areas of the musculoskeletal system accompanied by increased tenderness at specific anatomic sites known as trigger points (Campbell et al., 1983). The disease symptoms also include neurotic and psychopathic disturbances such as depression and paranoia (Payne et al., 1982).The condition is resistant to treatment with analgesic, steroidal and non-steroidal anti-inflammatory drugs (Goldenberg et al., 1986; Clark et al., 1985) but does respond to treatment with tricyclic muscle relaxants (Campbell et al., 1984) and antidepressant drugs such as chloripramine and amitriptyline (Goldenberg et al., 1986). Tanvoni et al., (1987) have reported some encouraging results with SAMe. Daily intramuscular administration of 200mg of SAMe significantly improved the depressive state of the patients and reduced the number of trigger sites.

1.5.2. S-Adenosylmethionine and affective disorder

The first clinical application of SAMe was reported by Fazio et al., (1974) for the treatment of schizophrenia and depression.The drug was found to have a beneficial mood altering effect. Double-blind placebo controlled trials (Agnoli et al., 1976; Caruso et al., 1984) and controlled trials with amitriptyline and chloripramine (Kufferle and Grunberger 1982) showed that SAMe was as equally effective as standard antidepressants and worked very quickly , typically within 3-7 days. However, Carney et al., (1983) have reported that SAMe can precipitate mood swings into mania / hypomania.

A study by Bottigleri et al., (1984) revealed no significant difference between pretreatment and posttreatment levels of plasma SAMe but did show a significant rise in the concentration of SAMe in cerebrospinal fluid (CSF). Further, the increase in CSF SAMe levels was associated with elevated levels of 5-hydroxyindole acetic acid and a 91% decrease in plasma prolactin levels, a result not found in the placebo group, thus indicating that SAMe may influence monoamine neurotransmitter metabolism. The results indicated that patients with endogenous depression had a greater response to SAMe treatment than those with neurotic depression (Carney et al., 1983; Carney et al., 1986).

The mechanism for the anti -depressant effect of SAMe is unclear but wide ranging in vitro studies have revealed a variety of neuropharmacological effects which may provide some insight into the problem.

The local application of SAMe to rat cerebral cortex induced slowly appearing excitatory effects in 94% of all cells examined (Philis, 1981). The latency of appearance of neuronal firing was 30-60 seconds and was maintained for 1-4 minutes. The effect was dose dependent and could not be substituted by l-methionine , homocysteine or adenosine.S-Adenosylhomocysteine was only weakly active.These observations suggest that SAMe may have a selective excitatory effect on cortical neurones of mammalian brain. The effect of SAMe on monoamine metabolism in rodents has been examined by Algeri et al., (1977). The administration of SAMe to isolated cerebral nerves failed to block the uptake and subsequent inactivation of catecholamines and 5-hydroxytryptamine (5-HT).SAMe has weak and ill defined effects on monoamine oxidase causing increased activity in the heart and brain and inhibition in the liver (Bidard et al., 1977). It may also increase noradrenaline and dopamine turnover (Bidard et al., 1977). However , Algeri et al., (1977) have reported that SAMe decreased the activity of tyrosine hydroxylase (the rate limiting step of catecholamine synthesis) at low doses and stimulated activity at high doses.The turnover of 5-HT has also been reported to be increased by SAMe (Curcio et al., 1978).

Reynold et al., (1983) have suggested that changes in neurotransmitters, receptor sensitivity, or endocrine function may be mediated by disturbances in membrane function and modified by the administration of SAMe. The effects of SAMe (at 1- 100 μM) on neuronal membrane function in vitro correlated with the synthesis of phosphatidylcholine and included increased binding of labelled benzodiazepine or gamma amino butyric acid (GABA) and ß2 adrenoceptor agonists but not α–adrenoceptors dopaminergic D_2 or opiate receptors (DiPerri et al., 1983). These effects were antagonised by SAHc and other inhibitors of methylation.

1.5.3. S-Adenosylmethionine ameliorates cerebral ischaemia

A potential clinical application of SAMe for cerebral ischaemia might result from studies which have shown that SAMe reversed the changes in lipid metabolism (Trovarelli et al.,

1983), prevented brain oedema (Kozuka et al., 1988) and neuronal death induced by transient ischaemia (Matsui et al., 1987). The effects of SAMe on ischaemic brain metabolism has been investigated by Kozuka and Iwata (1989). Ischaemia was induced by bilateral carotid artery occlusion in hypertensive rats. A dose of 100 mg/kg of SAMe was administered i.p every 30 minutes from the beginning of the occlusion for 3 hours.SAMe significantly mitigated the depletion of creatine phosphate, ATP and lactate and the increase in GABA caused by the ischaemia. The drug also significantly suppressed the increase in brain water content. The authors suggest that the protective effect of SAMe may be due in part to restoring neuronal membrane phospholipid composition. Alternatively, the high and frequent doses of SAMe may also promote membrane fluidity in erythrocytes thus increasing erythrocyte fluidity. This would increase cerebral blood flow and ameliorate the detrimental changes in brain metabolism due to restricted supplies of oxygen and glucose. Indeed, Matsui et al., (1989) have reported that the administration of SAMe at a dose of 100mg/kg hourly for 5 hours inhibits calcium accumulation and ameliorates glucose metabolism disturbed by transient ischaemia in rats.

1.5.4. The cytoprotective effect of SAMe against gastric ulcers

In an investigation to evaluate the possible interference of SAMe with eicosanoid dependent cytoprotective mechanisms of the gut Gualano et al., (1983) discovered that even at a dose of 200mg/kg SAMe failed to cause haemorrhage or ulceration in rats. Szabo et al., (1984) has reported that ethanol induced haemorrhagic gastric lesions were associated with a rapid decrease in the gastric concentration of non-protein sulphydryl groups and that cysteine and synthetic sulphydryls such as cysteamine were protective against ethanol toxicity.

The cytoprotective effect of SAMe against ethanol, aspirin and stress induced gastric damage was compared with misoprostol, a PGE1 analogue. Administration of SAMe (100mg/kg) significantly increased the non-protein sulphydryl content in gastric and duodenal mucousa and was as effective as misoprosol in protecting against gastric damage by the above necrotising agents. The cytoprotective effect was maintained in the presence of indomethacin which suggests that the protection was independent of endogenous prostaglandin synthesis. Prior administration of mercury chloride , a non-protein

sulphydryl inhibitor abolished the cytoprotective effect of SAMe.Laudano (1987) has hypothesised that exogenous SAMe provides a source of sulphur which through transsulphuration reactions may promote the synthesis of those non-protein sulphydryl groups which confer cytoprotection.

1.5.5. Protective role of SAMe against hepatotoxicity

The administration of SAMe to rats antagonised liver damage caused by galactosamine and acetaminophen (Stramentinoli et al., 1978; Stramentinoli et al., 1979). These agents cause accumulation of liver triglycerides, hyperplasia of the smooth endoplasmic reticulum and cell necrosis (Koff et al., 1971). The liver damage was associated with reduced levels of hepatic SAMe but had no effect on plasma SAMe levels. The activity of hepatic methionine adenosyl transferase was reduced by half and the depletion of SAMe levels could be due to impairment of this enzyme (Stramentinoli et al., 1978). Similar liver damage and SAMe depletion has been reported with hepatotoxins such as carbon tetrachloride, selenite and diethylnitrosamine (Stramentinoli et al., 1975; Hoffman, 1977; Buehring et al., 1976, respectively).

Feo et al., (1982a) has reported that SAMe prevented liver steatosis in animals fed a choline deficient diet and accelerated the spontaneous recovery from this steatosis following choline supplements. Clinical trials conducted by Mazzanti et al., (1979) have suggested that SAMe effectively reduces fat deposition in the liver of patients suffering from chronic liver disease complicated from steatosis and a history of ethanol abuse. Maintenance of a high hepatic SAMe pool prevented fatty liver induced in rats by acute ethanol intoxication and accelerated recovery from steatosis after ethanol withdrawl (Feo et al., 1982b). Prophylactic treatment with SAMe at a dose of 25mg/kg to ethanol intoxicated rats restored 85% of the depleted hepatic glutathione content and reduced liver triglyceride content and liver and blood acetaldehyde concentration. Drugs which prevented phosphatidylcholine synthesis abolished the protective effect of SAMe against ethanol induced liver steatosis and glutathione depletion.

1.5.6. The treatment of osteoarthritis with SAMe

Osteoarthritis is a joint disease characterised by a loss of joint cartilage and hypertrophy of

adjacent bone. The disease is most prevalent among the elderly with nearly 85% of people over 70 showing radiologic evidence of osteoarthritis (Moskowitz et al., 1987). The exact mechanism of cartilage loss remains to be elucidated but the loss of compressive strength of the tissue is associated with a reduction in the size and rate of synthesis of proteoglycans and an increased rate of degradation (Brandt, 1985).This is coupled with an increase in chondrocyte metabolism and cell mitosis.The rate of degradation causes a net loss of cartilage. Inadequate cartilage cover and subsequent increased wear and tear on joints results in fatigue fractures. The repair of these fractures is manifested as hypertrophy of the bone (Dieppe et al., 1986).

Padova (1987) has reported that up to 1987 over 21,000 patients had received SAMe treatment for osteoarthritis. The dosage varied between 400mg per day and 1200mg per day and all trials to date have shown that SAMe improved both the objective and subjective parameters of the condition with an efficacy comparable to conventional non steroidal anti inflammatory drugs.

A double-blind study in Italy showed that SAMe (1200mg/day) was as effective an analgesic as naproxen (750mg/day) but was tolerated significantly better (Caruso and Pietrogrande, 1987).A double blind study by Maccagno et al., (1987) comparing SAMe with piroxicam showed that patients treated with SAMe maintained clinical improvement at the end of treatment longer than patients on piroxicam. Similar results have obtained with comparative trials of SAMe with ibuprofen (Capretto et al., 1985) and indomethacin (Vetter, 1987). A long term 24 month open trial by Konig (1987) affirmed that SAMe is very well tolerated , could be administered for up to 2 years without side effects and reduces the incidence of relapse of the disease.

Animal models of osteoarthritis have revealed that SAMe at a dose of 30-60 mg/kg per day for 12 weeks prevented surgically induced experimental osteoarthritis and antagonised degenerative joint lesions following the procedure (Wiemayer et al., 1987). It was discovered that SAMe treated animals had significantly thicker cartilage and higher counts of chondrocytes. The effect of SAMe on human articular chondrocyte differentiation in vitro has been studied by Harmand et al., (1987).The effect of SAMe (2.5, 25 and 250μM) on cell proliferation and protein and proteoglycan synthesis was evaluated using a thick layer culture of normal and osteoartritic chondrocytes (Harmand et al., 1982). Chondrocyte

cultures incubated with SAMe showed no change in cell size, shape or proliferation pattern in normal and osteoarthritic chondrocytes but doses of 2.5 and 25 μM SAMe increased protein and proteoglycan synthesis but the maximal response was obtained at 25 μM where total protein synthesis was increased by 50% and proteoglycan synthesis by 60%. Incubation of chondrocytes in 250 μM SAMe was ineffective and actually inhibited the synthesis of certain proteoglycans subfractions. The authors suggested that SAMe was unlikely to serve as a source of inorganic sulphate since 250 μM SAMe was inhibitory and 65 μM methionine was included in the medium.They hypothesise that SAMe has a specific stimulatory effect on chondrocyte metabolism. Agents such as forskolin (Takigawa et al., 1981) and parathyroid hormone (Malemud and Papay, 1984) which both work through cAMP, stimulated sulphated proteoglycan synthesis by chondrocytes in vitro.

1.6. The acute anti-inflammatory effect of SAMe

Gualano et al., (1983) has reported that a single subcutaneous dose of SAMe inhibited carrageenan induced paw oedema in rats. The inhibition was only statistically significant at 100 mg/kg which exerted an effect equivalent to 9 mg/kg of indomethacin. The same inhibitory effect was observed with nystatin induced

oedema. Intraduodenal administration of SAMe in gelatine capsules suppressed carrageenan induced oedema at 50 mg/kg. The anti-inflammatory effect of SAMe was not dependent on conversion to methionine since the administration of methionine at doses equimolar to 25, 50 and 100mg/kg was completely ineffective at reducing carrageenan induced rat paw swelling (Stramentinoli, 1987).

The analgesic effect of SAMe reported by investigators involved in clinical trials for the treatment of osteoarthritis has been confirmed in animal studies (Gualano et al., 1983). The intravenous administration of SAMe to mice significantly reduced writhings in mice in response to phenylquinone and acetic acid. Oral doses of SAMe were also found to be effective in the mouse tail flick test (D'Amour and Smith, 1941) where a dose of 100mg/kg doubled reaction time to the stimulus (Stramentinoli, 1987).

Evidence has been provided by Gualano et al., (1985) that SAMe may interfere with the

eicosanoid system. Purified residues extracted from inflammatory exudates derived from polyester sponges implanted into rats were measured for prostaglandin - like activity by bioassay. Extracts from animals treated with 100 mg/kg SAMe (s.c) had significantly reduced prostaglandin -like activity. A similar pattern of inhibition by SAMe treatment was found with a study of thromboxane A_2 generation by guinea pig lungs challenged with histamine. The inhibition was dose dependent but with a potency 1500 fold less than indomethacin. Results obtained by selected ion monitoring analysis of the prostanoid components of the exudates (thromboxane B_2 and 12-hydroxyeicosatetranoic acid) showed that SAMe treatment had negligible effect on eicosanoid generation (Stramentinoli, 1987). The mechanism by which SAMe exerts anti-inflammatory and analgesic activity is unclear.

Rationale and aims of study

Osteoarthritis is a degenerative joint disease but it usually becomes clinically manifest when a secondary synovitis develops (Fassbender, 1987). SAMe is an effective treatment for osteoarthritis. It has antiiflammatory, analgesic and antidepressant activity but its pharmacological mode of action is unknown.

The acute antiinflammatory activity of SAMe has been demonstrated by Gualano et al (1983). Since the neutrophil is the prime cell mediator of acute inflammation , the effect of SAMe on neutrophil function was investigated. Special emphasis was given to the effect of SAMe on the respiratory burst since oxidative free radicals are implicated in inflammatory joint disease. SAMe is a labile substance and is quickly metabolised by cells. Therefore the intracellular metabolism of SAMe was investigated to determine candidate metabolites with possible antiinflammatory activity.

A major metabolic pathway of SAMe catabolism is polyamine synthesis. Polyamines are endogenous antiinflammatory compounds induced by inflammatory stimuli. A major part of this study was designed to assess whether the antiinflammatory effect of SAMe upon the carrageenan induced rat hind paw model of inflammation was linked to changes in tissue polyamine status.

The inflammatory response to carraggenan consists of distinct phases regulated by a variety

of inflammatory mediators. Therefore the effect of SAMe on these components of the inflammatory response was determined. The duration of the antiinflammatory effect of SAMe was compared with the pharmacokinetics of the drug.

2.0. Materials and Methods

2.1 The Effect of SAMe on the reduction of Cytochrome C by the Hypoxanthine Xanthine Oxidase Superoxide generating system

S-Adenosyl-l-methionine sulphate -p-toluenesulphonate (Bioresearch SpA) was tested for its effect on the reduction of cytochrome C by superoxide produced by the hypoxanthine / xanthine oxidase superoxide generating system.The method of Kafy and Lewis (1986) was used.

A stock solution of phosphate buffer (0.1 M pH 7.4) containing 50mM HEPES (Sigma Chemical Co, Poole, Dorset , U.K.), 0.5mM hypoxanthine (Sigma Chemical Co,) and 50 nmol of cytochrome C (Horse heart type III,Sigma Chemical Co) was prepared. SAMe was added to the reaction mixture at concentrations of 10μm- 5mM in plastic cuvettes .The reaction was initiated by the addition of 0.2 units (8μl) of xanthine oxidase (grade III chromatographically purified from butter milk, Sigma Chemical Co). The reduction of cytochrome C was followed at 550nm in a spectrophotometer (Pye Unicam SP500) over 10 minutes at 25^0C in the absence or presence of the SAMe concentrations above.The blank consisted of the same reaction mix but xanthine oxidase was replaced by an 8μl aliquot of phosphate buffer (0.1M, pH 7.4). The effect of SAMe was compared with superoxide dismutase (200 units) and catalase (600 units,Sigma Chemical Co).

2.2 The Effect of SAMe on Urate formation from the Hypoxanthine / Xanthine Oxidase reaction

S-Adenosyl-l-methionine was tested for its effect on urate formation from the hypoxanthine/xanthine oxidase reaction. The stock solution described in section 2.1 was prepared but cytochrome C was omitted.S-adenosyl-l-methionine was tested for any effect on enzyme activity in the concentration range 10μM-5mM.The reaction was initiated by the addition of 0.2 units of xanthine oxidase .The formation of urate was followed at 295nm in a spectrophotometer over 10 minutes at 25^0C in the absence or presence of the concentrations of SAMe above. The blank consisted of the same sample but the enzyme

was replaced with an equal volume of phosphate buffer (0.1M,pH 7.4). The effect of SAMe was compared with superoxide dismutase.

2.3 The Effect of SAMe on the Aerobic photoreduction of Nitroblue Tetrazolium

SAMe was tested for any inhibitory effect on the aerobic photoreduction of nitroblue -tetrazolium (NBT).The method described by Kafy et al (1986) was used .The reaction mixture consisted of 5 x 10^{-5}M NBT ,10^{-2}M methionine , 1.17 x10^{-6}M riboflavin , 2 x10^{-5}M sodium cyanide in phosphate buffer (0.02M) pH 7.8. SAMe was added over a range of concentrations (10μM-5mM) and omitted from the control.The reaction mixtures were incubated at 30°C and illuminated for 6 minutes by a Shandon -Southern photopol lamp . The absorbance was measured at 560nm using the SP500 spectrophotometer.Superoxide dismutase was tested as a positive control at concentrations of 50-400 units /ml.

2.4 The effect of SAMe on cytochrome C reduction by rat neutrophils

2.4.1 Animals

Adult male wistar rats (200-300g) obtained from Bantin and Kingman (Hull,U.K.) were the source of rat neutrophils used throughout these studies.Animals were housed at 20°C and maintained on Rat and Mouse Breeding diet (code 422),(Pilsburys Ltd,Birmingham, U.K.) with water ad libitum.

2.4.2 Sterilisation of glassware and reagents

All glassware, pipette tips, Eppendorf tubes and millipore filters were sterilised by autoclaving at 115°C for 20 minutes at 15 pounds per square inch (psi).Hanks balanced salt solution (HBSS)(Gibco Ltd , Paisley, Scotland) was sterilised by filtration through a sterile millipore filter (0.22μm pore size) and stored at 4°C.

2.4.3 Anaesthesia

Halothane Anaesthesia was induced in the experimental animals using Boyles apparatus before inoculating with irritant and harvesting of the inflammatory exudate.Oxygen ($95\%O_2/5\%CO_2$) and nitrous oxide were mixed at flow rates of 200 and 300ml/min , respectively with halothane (ICI Pharmaceutical Division, Alderley edge, Macclesfield, U.K.) at 4% for initial anaesthesia and 1.5-2% for maintenance.Animals were sacrificed with an overdose of halothane followed by cervical dislocation.

2.4.4 Elicitation of peritoneal rat neutrophils

Peritoneal neutrophils were elicited by injection of 10ml of sodium caesinate (12%w/v) NaCl (0.9% w/v) into the peritoneal cavity.Animals were killed 12 hours later and the peritoneal membrane was exposed.An incision was made along the ventral midline andthe inflammatory exudate was recovered from the peritoneum by lavage with 40ml of ice cold heparinised HBSS (10 units / ml)preservative free heparin . The leukocyte rich fluid was harvested using a plastic pasteur pipette and collected in sterile universals (Sterilin,Teddington, Middlesex, U.K.) and kept on ice.

2.4.5. Cell purification procedure

Cells from the lavage fluid were recovered by centrifugation at 200g for 5minutes at room temperature, pooled and any contaminating erythrocytes lysed by osmotic shock with ice cold distilled water (9 volumes) for 30 seconds. Isotonicity was restored by addition of 10x concentrated HBSS (1 volume).After washing at 200g for 5minutes the cell pellet was resuspended in 8ml HBSS. The cell suspension was then carefully layered onto 3ml Histopaque-1077 (1.077g/ml) in a 15ml conical plastic centrifuge tube (Sterilin) and centrifuged for 30 mins at 400g.

The mononuclear cell fraction was isolated at the histopaque/buffer interface leaving a pellet of neutrophils.The pellet was washed 3 times in ice cold HBSS for 5 minutes at 200g prior to performing viability,total and differential counts.]

2.4.6 Cell viability, total and differential counts

Cell viability : was assessed prior to drug treatment and was based on the trypan blue (1%w/v in distilled water) exclusion test.

Total leukocyte count : was determined using the 'Improved Neubauer' haemocytometer.

Differential count : The proportion of neutrophils was determined using the Haema -Gurrs stain technique. A smear of cell suspension was air dried then stained with Haema-Gurrs stain (BDH Diagnostics, Poole, U.K.).The cells were examined under an oil immersion lens at x1000 magnification and approximately 200 cells were examined in 4-5 random fields.

2.4.7 Preparation of FMLP

N-formyl-l-methionine-l-leucyl-l-phenylalanine (FMLP, Sigma chemical Co, Poole, Dorset) was made up in dimethylsulphoxide (DMSO) to a concentration of 10mM. The peptide was further diluted with buffered HBSS. Bovine serum albumin (1mg/ml) was added to each 1.5 ml aliquot of FMLP at the relevant concentration (10μM) needed for each set of experiments. These were stored at -20^{o}C in sterile Eppendorf tubes.Each aliquot was only used once since the methionine sulphur of FMLP tends to oxidise over time (Wilkinson 1982).

2.4.8 Measurement of cytochrome C reduction by PMNLs

The method of McCord and Fridovich (1969) was adapted for evaluating the effect of SAMe on the reduction of cytochrome C by FMLP stimulated rat PMNLs. The reference cuvette contained 50 nmol cytochrome C, 20 nmol FMLP and 1000 units of superoxide dismutase in 1ml of buffered HBSS. The sample cuvette contained the above with the exception that superoxide dismutase was replaced by 10 μl buffer. The reaction was started by the addition of 1x 10^6 neutrophilsto both cuvettes . The neutrophilshad been incubated for 1 hour at 37°C in the presence or absence of SAMe (10 μM-1mM). The absorbance change at 550nM was continuously recorded with an SP500 spectrophotometer .Superoxide dismutase was tested for comparison as a positive control on the reduction of cytochrome C by FMLP stimulated neutrophilsin the dose range 150-1200 units.

2.5 The effect of SAMe on luminol dependent chemiluminescence response of stimulated rat PMNLs

2.5.1 Preparation of Luminol

Luminol (5-amino-2,3-dihydro -1,4-phthalazinedione, Sigma Chemical Co) was dissolved in DMSO to a concentration of 20mM and stored at 4°C in darkness. This stock was diluted to 200μM with buffered HBSS prior to use.

The effect of SAMe on stimulated rat PMNL luminol dependent chemiluminescence was tested using FMLP, phorbol myristate acetate and unopsonised Zymosan A.

2.5.2 Preparation of phorbol myristate acetate

Phorbol myristate acetate (PMA, Sigma Chemical Co) was dissolved in DMSO to a concentration of 10mM. Aliquots of 50μl were stored at -20°C in sterile Eppendorf tubes.PMA was diluted prior to use to 100nM with buffered HBSS.

2.5.3 Preparation of Zymosan A

Zymosan A (Sigma chemical Co) from *Sacchromyces cerevisae* was suspended in buffered HBSS to a concentration of 5mg/ml. To ensure even distribution the suspension was ultrasonicated for 15 minutes.Each Zymosan A suspension was prepared just before use.

2.5.4 Measurement of chemiluminescence

Chemiluminescence was detected in a model 1250 LKB Wallac luminometer.The reaction mixture consisted of 800μl buffered HBSS containing 200μM luminol and $1x10^6$ cells.Chemiluminescence was stimulated by the addition of 200μl of activator. neutrophilswere prepared as detailed in section 2.4.1 .Aliquots of $1x10^7$ PMNls were incubated in the presence or absence of SAMe, in the dose range 1μM-1mM, for 1hour at 37°C. Aliquots of 100μl containing $1x10^6$ neutrophilswere added to the vials and the innate chemiluminescence at 37°C with stirring ie in the absence of exogenous activator was recorded for 3 minutes.Stimulated chemiluminescence was initiated by the addition of 200μl activator to the vials and the response in mV was continuously measured on a Gallenkamp data trace potentiometric recorder.

2.55 Effect of SAMe on PMA stimulated luminol dependent rat PMNL chemiluminescence

Rat neutrophils isolated as in section 2.4.1 and treated with SAMe (see section 2.54) were activated by 200µl 100nM PMA.Superoxide dismutase was tested for its effect on PMA stimulated luminol dependent rat PMNL chemiluminescence at the doses reported in section 2.4.

2.5.6 Effect of SAMe on FMLP stimulated luminol dependent rat PMNL chemiluminescence

Rat neutrophilsisolated as in section 2.4.1 and treated with SAMe (see section 2.5.4) were activated by 200µl 10µM FMLP. Catalase (EC 1.11.1.6, from Bovine liver . 1 unit will decompose 1µmol H_2O_2 per minute at pH 7 and 25°C) in the dose range 150-1200 units was assayed for its effect on FMLP stimulated luminol dependent rat PMNL chemiluminescence.

2.5.7 Effect of SAMe on Zymosan A stimulated luminol dependent rat PMNL chemiluminescence

Rat neutrophilsisolated as in section 2.4.1 and treated with SAMe (section 2.5.4) were activated by 200µl 5mg/ml zymosan A suspension .

2.6 The effect of Adenosine and Methylthioadenosine on FMLP stimulated luminol dependent rat PMNL chemiluminescence

Adenosine and methylthioadenosine (Sigma Chemical Co) were dissolved in DMSO and diluted to the required concentration in buffered HBSS.Rat neutrophilsisolated as in section 2.4.1 were treated for 1 hour at 37°C in the absence or presence of each drug in the dose range 1nM-10µM. Aliquots of $1x10^6$ neutrophilswere added to the reaction mixture described in section 2.5.4 .

2.7 Effect of SAMe on phagocytosis by rat neutrophils in vitro

Phagocytosis was assessed by a modified version of a simple fluorometric assay (Oda and Maeda 1986).Rat neutrophils treated in the absence or presence of SAMe (1μM-1mM) were incubated for 1 hour at 37°C in a shaking water bath with 1.1μm fluoresbrite carboxylated polystyrene latex microspheres (2.5% solids latex, Polysciences , Warrington , UK). The microspheres were dispersed by immersion in a sonic bath 2 minutes prior to dilution to the required particle concentration . The number of particles per ml in the stock particle suspension was determined using the formula (Polysciences 1987):

$$\frac{N = 6\omega \times 10^{12}}{\pi \times p \times d^3}$$

where ω= % solids

d= diameter (μm)

p= density of bulk polymer (g/ml)

The cell suspension was mixed with the particle suspension to give a particle to cell ratio of 10:1. After incubation the cell suspension was transferred to polystyrene centrifuge tubes and non-cell associated particles were removed during the course of 3 washes in buffered HBSS by centrifugation at 400g for 10 minutes. After the last cell wash each sample was counted to account for any cell loss. The final cell suspension was solubilised in 0.2% sodium dodecyl sulphate (Sigma Chemical Co) in 0.1M tris HCl buffer pH 8.5. Bead fluorescence was measured in a Perkin Elmer LS-5 spectrofluorimeter. Excitation was measured at 458nm and emission at 540nm. The mean number of particles per cell was calculated from a bead calibration curve .

The assay was also conducted with cells incubated in 1mM S-adenosyl-l-homocystiene and methionine. Further, cytochalasin B , a potent phagocytosis inhibitor at a final concentration of 8μg/ml was used as a positive control.

2.8 The effect of SAMe on prostaglandin E_2 production by rat neutrophils stimulated with zymosan

Rat neutrophils were prepared as described in section 2.4.1 .Aliquots of 8.5x106 neutrophils were suspended in 1ml buffered HBSS containing 5mg sonicated suspension of zymosan A. The cell preparations were incubated for 2 hours at 37°C in a shaking water

bath in the absence or presence of 1mM SAMe

Following incubation the cells were pelleted by centrifugation at 400g for 10 minutes.The supernatant was decanted and deproteinised with 100µl 1M HCl then centrifuged at 200g for 5 minutes . The clear supernatant was extracted twice with 1.5ml ethylacetate (BDH chemicals)and dried under nitrogen. The extract was then subjected to PGE_2 radioimmunoassay.

2.9 Effect of SAMe in vivo on rat neutrophils in six day air pouches

2.91 Preparation of air pouches

Air is injected subdermally to create a symmetrial air pouch on the back of the animal , thus creating a space into which a chemotactic factor or inflammatory agent can be introduced. The inflammatory cells can be harvested from the pouch and counted and the inflammatory exudate can be assayed for inflammatory mediators. The structure of the pouch is analogous to that of the synovial lining (Edwards et al 1981) and has been exploited as a model for the synovium to study the development of acute and chronic inflammatory diseases . In this investigation the air pouch was used as in vivo chemotaxis assay. Air pouches were prepared using the method of Edwards et al 1981.

Animals

Male Wistar rats (150-175g) obtained from Bantin and Kingman (Hull) were the animals of choice since younger aniamals had less subcutaneous fat .Animals were maintained as in section 2.4.1

Induction of air pouch

Rats were anaethesised using halothane (see section 2.4.3) and the back of each rat was shaved with electric clippers. The pouch was formed by the injection of 20ml air through a 25 gauge needle inserted subcutaneously between the scapulae. The air pouch was prevented from covering the head and neck by gently pressing over the neck.The air pouch was refilled with 10ml air 3 days later.The animals were challenged 6 days after the formation of the pouch since this was the time needed for sufficient development of the pouch lining and vasculature (Sedgewick et al 1983)

2.9.2 Effect of SAMe on cell infiltration into the six day air pouch elicited by FMLP

The chemoattractant FMLP was used as the irritant in this assay. Before innoculating with FMLP, animals were treated 30 minutes before with SAMe (50mg/kg) or pyrogen free saline injected intraperitoneally. Inflammation in the air pouch was induced by 2ml of 10nM FMLP injected directly into the pouch. The animals were sacrificed at 6 hours post inoculation (maximum infiltration of PMNL- Dr R.Hyde personal communication) by halothane overdose. An incision was made across the dorsal surface to expose the air pouch. This was carefully dissected away from the subcutaneous / connective tissue. The pouch was punctured and lavaged with 30ml of ice cold heparinised (10 units/ml) buffered HBSS. The lavage samples were pelleted by centrifugation at 100g for 5 minutes. The supernatant was decanted and stored at -20^{o}C.The pellet was resuspended in 5ml buffered HBSS then total , differential and viability counts were performed (see section 2.4.6).

2.10 The effect of SAMe on carrageenan induced rat hind paw inflammation

Hind paw inflammation was induced in male wistar rats (100-120g) by the method of Winter et al (1962). A 2% (w/v) solution of carragenan (type IV lambda carrageenan Sigma Chemical Co) was made up in sterile saline. Experimental animals were anaethesised (see section 2.4.3) weighed and injected intraperitoneally with SAMe dissolved in 0.5ml saline or the same volume of saline 30 minutes before innoculation.Oedema was induced during light anaesthesia by the sub-plantar injection of 50 µl carrageenan solution into the right hind paw of the rat.

The inflammatory response elicited by the injection of carrageenan into the paw was determined by measuring the change in volume.The hind foot was immersed to the hair line in a 25ml mercury bath linked to a pressure transducer and a Washington recorder. The recorder was calibrated using a 2ml standard. The foot of each animal was measured before the innoculation and at various time intervals after the carrageenan injection.The % increase in foot volume was derived by dividing the change in foot volume by the

preinoculation volume. The antiinflammatory effect of the drugs was assessed by comparing the mean % increase in the foot volume of the drug treated animals with those of the saline treated controls. Rats were allowed access to food and water throughout.

2.10.1 Dose response study

Experimental animals were treated with SAMe at doses of 0, 25, 50 and 100 mg/kg 30 minutes before carrageenan innoculation. The response was measured at time = 0, 3 and 5 hours. Indomethacin (10mg/kg I.P) was used as a positive control.

2.10.2 Time course study

Experimental animals were treated as described in section 2.10.Hind paw inflammation was measured at discrete intervals for 5 hours.

2.10.3 The effect of SAMe on carrageenan induced rat hind paw inflammation in animals pretreated with polyamine metabolism inhibitors

Experimental animals were pretreated with specific inhibitors of polyamine metabolism at 12 hour intervals for a period of 48 hours before innoculation.

2.10.3.1 Preparation of difluoromethylornithine and methyl bisguanylhydrazone

DL-α-difluoromethylornithine (DFMO,Merrel Dow research, Strasbourg,France) is an irreversible competitive inhibitor of ornithine decarboxylase (Metcalf et al 1982).It was dissolved in 0.9% sterile saline at a concentration of 100mg/ml.Experimental animals received DFMO at a dose of 500mg/kg I.P.

Methylbisguanylhydrazone (MBGB,Sigma Chemical Co), a SAMe decarboxylase inhibitor (Pegg and McGill, 1978) was dissolved in 0.9% sterile saline at a concentration of 40mg/ml .Experimental animals received MBGB at a dose of 20mg/kg.

Carrageenan oedema was induced as described in 2.10.The effect of DFMO and MBGB on the antiiflammatory effect of SAMe was determined using the following experimental groups:

1.control-saline treated

2.control-SAMe treated (50mg/kg)

3.DFMO (500mg/kg) and SAMe (50mg/kg)

4.DFMO,MBGB(20mg/kg) and SAMe

5.SAMe and MBGB

6.DFMO only

7.MBGB only

There were 5 rats in each group. The statistical difference between test groups was calculated by unpaired 't' test

2.10.4 The effect of the SAMe metabolites methylthioadenosine and adenosine on carrageenan induced hind paw inflammation

Experimental animals were pretreated with either Methylthioadenosine or adenosine (Sigma Chemical Co) at a dose of 50mg/kg 30 minutes before innoculation with 2% carrageenan (see section 2.81)

2.11 Effect of SAMe on polyamine levels in liver and blood in the presence or absence of carrageenan induced hind paw inflammation

2.11.1 Preparation of tissues:

Rats (experimental conditions see section 2.8.1) dosed with SAMe or sterile 0.9% saline with or without carrageenan induced hind paw inflammation were killed by halothane overdose followed by cervical dislocation 5.5 hours after drug treatment.The livers were quickly removed and washed in saline to remove excess blood.Each sample was dried then weighed before being crudely chopped with a scalpel.The tissue was homogenised in 1M perchloric acid (1: 5 w/v) in a glass Potter homogeniser using 20 strokes at 3000rpm. The homogenates were centrifuged at 4000 rpm for 15 minutes.The crude supernatant was decanted and centrifuged again at 4000 rpm for 30 minutes. The final supernatant was eiher assayed immediately or stored overnight at -20°C.The concentration of putresine,spermidine and spermine was determined in each indvidual liver sample.

Liver samples from animals treated with inhibitors of polyamine metabolism, MTA or adenosine were prepared using the same methodology.

2.11.2 The determination of polyamine content in rat liver

Polyamine levels were determined using a modification of the Dansylation method of Bird et al 1983.This consists of isolating the fluorescent dansyl polyamine derivative by thin layer chromatography followed by quantitative estimation of polyamine content by fluorimetry.

An aliquot of 1ml of rat liver supernatant was added to 2.5ml of 1-dimethylaminonapthalene-5-sulphonylchloride (DANS-Cl,Sigma Chemical Co)solution (4mg/ml in acetone).The mixture was saturated with Na_2CO_3 $10H_20$ was allowed to react overnight at room temperature under darkness.Sixteen hours later 10mg of D-proline solution was aded to the mixture to remove excess DANS-Cl.After a further two hours the dansylated amine was extracted from the dansylate mixture with 2x10ml aliquots of toluene. The toluene was evacuated by evaporation under vacuum.The extract remaining was dissolved in 0.5ml of toluene / ethyl acetate (v/v 7:3).Aliquots of 20µl were applied,in duplicate, to 200 x 200 mm silica gel (250µm gel thickness) thin layer chromatography (TLC) plates (Sigma Chemical Co).The same quantity of putrescine,spermidine and spermine dansylated standards were also applied to each plate.The plates were developed in a mobile phase of ethyl acetate / cyclohexane (v/v 1:1) with 1% triethylamine.

After development the TLC plates were dried and examined under a u/v lamp. Spots with an Rf value corresponding to the relevant polyamine standards were indicated with a soft pencil to prevent damage to the gel.The plate was cut into columns and the polyamine spots were scraped out with a scalpel into glass test tubes.The dansylated amine was extracted from the silica with 3ml toluene:ethylacetate:acetone (v/v 1:1:1).

The tubes were sonicated in a sonic bath for 15 minutes then centrifuged for 5 minutes at 200g.The fluorescence of the extracts was measured at 500 nm emission and 365 nm excitation in a Perkin Elmer LS-5 spectrofluorimeter.The concentration of polyamine in the sample was determined from standard curves for each of the three polyamines.

2.12 Preparation of blood samples for the determination of polyamines

Light halothane induced anaesthesia was in experimental animals (section 2.4.3).Samples of blood (1ml) were removed by cardiac puncture and carefully injected into a heparinised blood tube. An aliquot of 0.5ml of 1M perchloric acid was added to the sample. The tube was vortexed for 10 seconds then centrifuged at 400g for 10 minutes.The deproteinised supernatant was analysed for polyamine content using the the dansylation-TLC method detailed in section 2.11.2.

2.13 Effect of methylthioadenosine and adenosine on polyamines in rat liver

Polyamine content in rat liver after MTA or adenosine treatment was determined as in section 2.11.2.

2.14 Effect of SAMe in the presence and absence of carrageenan induced rat hind paw inflammation on glutathione in rat liver and blood

Liver and blood samples from experimental animals dosed with SAMe (50mg/kg) or saline i.p in the presence or absence of carrageenan induced hind paw inflammation were prepared as in section 2.11 and 2.11.1 respectively.

2.15 Effect of MTA and adenosine on glutathione in rat liver

Deproteinised liver extracts from animals dosed with MTA or adenosine (50mg/kg) in the presence of carrageenan induced hind paw inflammation were prepared as in section 2.11.1

2.16 Determination of total glutathione "GSH" in rat liver

Glutathione content in rat liver was determined using the spectrophotometric method of Tieze (1969).In this analysis both the reduced (GSH) and the oxidised (GSSG) forms of glutathione are measured thus the data is expressed in "GSH" equivalents GSH and 2GSSG.Aliquots of 300µl of deproteinised rat liver extracts were neutralised by 150µl of 2M potassium hydroxide and vortexed for 10 seconds.An aliquot of 100µl of the

neutralised mixture was diluted to 1ml with reaction buffer which consisted of 125mM Na_2HPO_4 and 6.3mM Na_2EDTA pH 7.5 .The reation mixture consisted of 700µl of 0.3mM nicotinamide dinucleotide diphosphate reduced (NADPH), 100µl of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, 2.38mg/ml, Sigma chemical Co) and 200µl of the diluted neutralised liver extract.The reaction was started by the addition of 0.5 units of glutathione reductase (E.C.1.6.4.2, 1unit will reduce 1µmol of CoA-S-S-G per minute at pH 5.5 and 25°C).The reaction was carried out at 30°C in a Cecil 2112 spectrophotometer.The change in absorbance was read at 405nm against a blank of 700µl buffer, 200µl acid-base mix and 100 µl DTNB solution.The gradient of the initial enzyme reaction was measured and glutathione content was determined from a glutathione calibration curve (1.56-100 nmol).

2.17 Determination of total glutathione in rat blood

Rat blood was removed by cardiac puncture (see section 2.12).A 10µl aliquot of blood was haemolysed in 990 µl cold phosphate -EDTA buffer pH 7.5.For analysis 25 µl of haemolysate was added to the standard glutathione assay mixture described in section 2.16.

2.18 Effect of SAMe on rat hind paw inflammation induced by inflammatory mediators and irritants

Carrageenan oedema is a complex event involving the interaction of many inflammatory mediators in the early phase and a later phase characterised by inflammatory cell infiltration (Di Rosa et al 1971). In this study the effect of SAMe and its metabolite MTA on hind paw inflammation induced by the isolated inflammatory mediators implicated in carrgeenan induced oedema has been assessed.

The experimental animals for this study were as described in section 2.10 and prepared for drug treatment and inflammatory mediator innoculation as detailed in section 2.4.3. Hind paw oedema is defined as the difference in hind paw volume (ml) measured plethysmographically by mercury displacement prior to and after mediator / irritant injection.

2.18.1 Effect of SAMe on arachidonic acid induced rat hind paw inflammation

The method of DiMartino et al (1987) was used to study the effect of SAMe on inflammation induced by arachidonate.A solution of 0.5% arachidonic acid (from porcine liver, Sigma Chemical Co) was prepared in 0.2 M carbonate buffer pH 9.2. Experimental animals were treated I.P with SAMe or sterile saline. Thirty minutes after drug treatment an aliquot of 100µl of arachidonate solution was injected into the sub plantar region of the right hind paw. Foot volume was measured at intervals over a period of 3 hours.Dexamethasone (0.1 mg/kg, Sigma Chemical Co) was used as a positive control.

Dose response study : Experimental animals were dosed with 25,50 and 100mg/kg SAMe 30 minutes before arachidonic acid injection.The maximal response was measured at 30 and 60 minutes.

2.18.2 Effect of MTA on arachidonic acid induced rat hind paw inflammation

The effect of MTA on arachidonic acid induced rat hind paw inflammation was determined using the methodology described in section 2.18.1 .Experimental animals were pretreated with MTA or with vehicle (DMSO/ sterile distilled water 4:1 v/v) 30 minutes before arachidonic acid injection. The dose effect of MTA was determined at doses of 25, 50 and 100mg/kg.The maximal response was measured at 30 and 60 minutes.Dexamethasone (0.1mg/kg) was used as a positive control.

2.18.3 Effect of SAMe on histamine induced rat hind paw inflammation

A solution of histamine dihydrochloride (2mg/ml, Sigma Chemical Co) was prepared in sterile saline. Rat hind paw oedema was induced by the injection of 100µl of histamine solution (200µg) into the sub plantar region of the hind paw .Experimental animals were dosed I.P. 30 minutes before innoculation with sterile saline or SAMe (50 mg/kg) .Mepyramine (mepyramine maleate, May and Baker , Dagenham, England) at a dose of 5mg/kg was used as a positive control. The foot volume was measured over a period of 90

minutes.

Dose response study : Experimental animals were dosed with 10, 25 and 50 mg/kg SAMe 30 minutes before histamine injection.The maximal response was measured.

2.18.3.2 Effect of MTA on histamine induced rat hind paw inflammation

Experimental animals were pretreated with MTA (50 mg/kg) or with vehicle 30 minutes before histamine injection (section 2.18.3.1).The dose response relationship was assessed by pretreatment with 10, 25 and 50mg/kg.The maximal response was measured.

2.18.3.3 Effect of SAMe on dextran induced rat hind paw inflammation

A suspension of 1% dextran (RMM 440,000 , Sigma Chemical Co) was prepared in 0.9% sterile saline.Experimental animals were pretreated i.p with SAMe (50 mg/kg) or sterile saline 30 minutes before dextran innoculation.The inflammatory response was measured at 0, 30, 60 and 180 minutes.

2.18.3.4 Effect of SAMe on Zymosan A induced rat hind paw inflammation

A suspension of 1% Zymosan A (from Sacchromyces cerevisae, Sigma Chemical Co) was prepared in 0.9% sterile saline then sonicated for 10 minutes .Experimental animals were pretreated with SAMe (50mg/kg) or respective vehicles I.P. 30 minutes prior to the sub plantar injection of 100µl Zymosan A suspension. The inflammatory response was measured plethysmographically at 0, 30, 60, 180 and 300 minutes.

2.18.3.5 Effect of SAMe and MTA on compound 48/80 induced rat hind paw inflammation

A solution of compound 48/80 (200 µg/ml), a condensation product of N-methyl-p-methoxy-phenylethylamine with formaldehyde (Sigma Chemical Co) was prepared in 0.9% sterile saline.Experimental animals were pretreated with SAMe, MTA (50 mg/kg) or respective vehicles I.P. 30 minutes prior to the sub plantar injection of 100 µl

compound 48/80 solution.The inflammatory response was measured plethysmographically at 5, 10, 15, 30 and 45 minutes.

2.18.4 Effect of SAMe on 5-hydroxytryptamine induced rat hind paw inflammation

A solution of 5-hydroxytrptamine creatinine sulphate (5-HT, Sigma Chemical Co) at a concentration of 100 µg/ml was prepared in 0.9% sterile saline. Experimental animals were pretreated with SAMe (50 mg/kg) or vehicle I.P. 30 minutes prior to the sub plantar injection of 100 µl (10 µg) 5-HT solution.The inflammatory response was measured plethysmographically at 0, 5, 10, 15, 30 and 45 minutes.

2.18.5 Effect of SAMe on glucose oxidase rat hind paw inflammation

The method of Spillert et al (1987) was adapted.A solution of glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*, Sigma Chemical Co) was prepared in 0.9% sterile saline to a concentration of 100 units /ml. Experimental animals were pretreated with SAMe (50 mg/kg) or the vehicle I.P. 30 minutes prior to the sub plantar injection of 100 µl glucose oxidase solution. The inflammatory response was measured plethysmographically at 30, 60 and 180 minutes

2.18.6 Effect of SAMe and MTA on platelet activating factor induced rat hind paw inflammation

2.18.6.1

Platelet activating factor (PAF, L-α-phosphatidylcholine -ß-acetyl-γ–o–alkyl, Sigma Chemical Co) was prepared in a buffer solution consisting of 150mM NaCl,10mM Tris and 0.25% Bovine serum albumen to a final concentration of 2 µg/ml. Experimental amimals were pretreated with SAMe, MTA (50 mg/kg) or the respective vehicles i.p 30 minutes prior to the sub plantar injection of 100 µl of PAF solution. The inflammatory response was measured at 30, 60, 90 and 120 minutes.The dose response relationship was assessed by pretreating experimental animals with doses of 5, 12.5, 25 and 50 mg/kg

response was measured at 30, 60, 90 and 120 minutes.The dose response relationship was assessed by pretreating experimental animals with doses of 5, 12.5, 25 and 50 mg/kg SAMe or MTA.

2.18.6.2 Effect of SAMe and MTA in combination with isoprenaline on PAF induced rat hind paw inflammation

Experimental animals were pretreated with SAMe or MTA (50 mg/kg) in combination with isoproterenol (- isoprenaline hydrochloride, Sigma Chemical Co) i.p 30 minutes prior to the sub plantar injection of 100µl PAF solution.The effect of isoproterenol with these compounds was determined at doses of 0.5, 1, 2, 4 and 8 mg/kg. SAMe, MTA and isoproterenol at the same doses were used as controls.

2.18.6.3 Effect of SAMe and MTA in combination with verapamil on PAF induced rat hind paw inflammation

Experimental animals were pretreated with SAMe or MTA (50 mg/kg) in combination with verapamil (± verapamil hydrochloride, Sigma Chemical Co) i.p 30 minutes prior to the sub plantar injection of 100 µl PAF solution .The effect of verapamil in combination with SAMe and MTA was determined at doses of 5 and 10 mg/kg.SAMe,MTA and verapamil at the same doses were assessed individually as controls.

2.19 Effect of SAMe on rat neutrophils in vitro : Analysis of uptake and metabolites by high performance liquid chromatography (HPLC)

2.19.1 HPLC apparatus

The HPLC analysis apparatus consisted of an injection module incorporating a 100 µl loop to a Gilson 302 pump coupled to a Gilson 802 manometric module. Chromatographic separation was performed by a Thames 10 µm Partisil SCX column (4.6 x 250 mm) protected by a 30mm guard column. The eluent was analysed by a Cecil CE 212 ultravoilet spectrophotometer incorporating an ultra voilet absorbance flow cell (manufacturer ?).Absorbance was recorded by a Gallenkamp Euroscribe potentiometric recorder.

The mobile phase consisted of 100mM ammonium dihydorgen orthophosphate solution pH 2.6.This was filtered through a 0.2 μm membrane filter and degassed by nitrogen for 15 minutes before use.The mobile phase was pumped at a flow rate of 1ml/minute.

2.19.2 Analysis of SAMe metabolism by rat neutrophils in vitro

Rat neutrophilswere elicited by 12% sodium caseinate (see section 2.4.4).Aliquots of 10^7 cells were incubated at 37°C in a shaking water bath in the presence or absence of ^{14}C SAMe labelled at the C_2 position of the methionine carbon chain (Amersham PLC, specific activity 59mCi / mmol).Cell metabolism was arrested by immersion in an ice bath.The cells were washed 3 times in ice cold HBSS and centrifuged at 1000g in an Eppendorf microfuge.The supernatant was decanted and the amount of radioactivity at each washing stage was counted with the cell samples.

The final cell pellet was deproteinised by 100 μl of 1M perchloric acid and subjected to 15 minutes sonication in a sonicator bath. The suspension was neutralised by 1M KOH and the subsequent $KClO_4$ precipitate was removed by centrifugation.The clear supernatant was injected into the HPLC system described in section 2.19.1.The eluent was collected at 1 minute intervals by an LKB Redirac fraction collector.A 0.5 ml aliquot from each 1minute fraction was added to 10 ml Optiphase hisafe II scintillant (LKB Wallac). Each vial was shaken to disperse the precipitate.The amount of radioactivity was determined by a Beckman LS-230 beta counter.

Dose response study : Rat PMNL were incubated at 37°C in SAMe at concentrations of 0.004, 0.02, 0.05, 0.25, 1.25 μCi / ml for 60 minutes. The amount of radioactivity was determined by the radio-HPLC method described above. Non radioactive metabolites were determined by retention time and quantified by calibration curves relating peak area absorbance to concentration.

Time course study : Rat neutrophils (10^7) were incubated at 37°C in 1.5ml Eppendorf tubes with 0.25 μCi SAM and were analysed at 5, 15, 30, 60 and 120 minutes.The cell samples were analysed by HPLC (section 2.19.2).

Metabolite standards: Standards of adenosine, S-adenosyl-l-homocysteine, adenine, MTA and SAMe were prepared in 1M perchloric acid and neutralised by 1M KOH. The neutral supernatant was analysed by HPLC (section 2.19.1) and the retention times for each standard was determined.Standard curves were established for each metabolite in the concentration range 0.1- 100 μM corresponding to 10 pmol- 10 nmol in the loop.

2.20 Analysis of SAMe pharmacokinetics by HPLC

Experimental animals were anaethesised by halothane (section 2.4.3) and blood was withdrawn from the tail tip into 1.5 ml Eppendorf tubes.A sample was taken during anaesthesia before SAMe administration. Each animal (n=4) was dosed i.p with SAMe (50 mg/kg).Blood was withdrawn at 0, 30, 60, 120, 180 and 300 minutes after drug treatment. The blood samples were centrifuged at 1000g for 3 minutes in an Eppendorf microfuge and 50 μl of plasma was removed.The plasma sample was deproteinised by 100 μl 1M perchloric acid , vortexed for 10 seconds and recentrifuged at 1000g for 2 minutes.The acidic supernatant was neutralised by 1M KOH and the precipitate removed by further centrifugation at 1000g for 2 minutes.A sample of the final supernatant was analysed by the HPLC method described in section 2.19.2 and the concentration of SAMe in the plasma was determined by reference to the SAMe standard curve.The half life, clearance and distribution volume of the drug was determined. Further, the pharmacokinetics of SAMe was analysed in the presence of 2% carrageenan induced hind paw inflammation (see section 2.10)

3.0. Results

3.1.1 Effect of SAMe on cytochrome C reduction by the hypoxanthine xanthine oxidase superoxide generating system

The effect of SAMe on the reduction of cytochrome C by superoxide produced by the cell free hypoxanthine xanthine oxidase system is shown in fig 3.1.1. The rate of cytochrome reduction was rapid and reached equilibrium within 3 minutes.The amount of cytochrome C reduction was calculated using a molar extinction coefficient of 15,500 M^{-1} cm^{-1}. The results show that up to a concentration of 1mM SAMe had no significant effect on the rate of cytochrome C reduction. The rate of cytochrome reduction in the absence of SAMe was 5.09± 0.15 μmol / min (n=5).The highest concentration of SAMe 5mM caused a modest but significant reduction of the maximal rate of cytochrome C reduction by 17.5± 6.1% (n=5, P< 0.05).

The effect of SAMe at this concentration was compared with the endogenous antioxidants superoxide dismutase and catalase. Fig 3.1.2 shows that superoxide dismutase (500 units)reduced the rate of cytochrome C reduction to 46.8±8.6 % (n=5) of the control but catalase (600 units) in comparison only reduced the rate of reation by 21%. This degree of decrease was comparable to 5 mM SAMe ($p < 0.05$)

3.2 Effect of SAMe on urate produced by the hypoxanthine xanthine oxidase reaction

The rate of cytochrome C reduction is dependent on the amount of superoxide available for reaction.Superoxide can be removed by antioxidant scavengers or by inhibition of the superoxide generating system.The effect of SAMe on uric acid production by the hypoxanthine xanthine oxidase system was used to determine whether the inhibition at 5mM was due to superoxide scavenging or to a direct effect on the enzyme. Fig 3.2.1 shows that up to a concentration of 100 μM SAMe had no significant effect on the rate of uric acid production but at higher concentrations there was a subsequent dose dependent inhibition of uric acid production and thus the superoxide generating enzyme.The highest

dose used (5 mM) significantly inhibited the rate of uric acid production by 33.2 ± 6.3 % ($p < 0.001$).

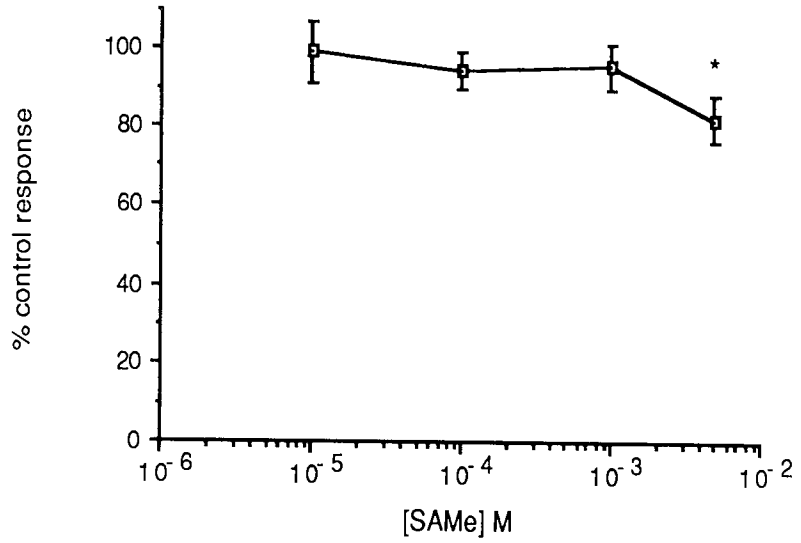


Fig 3.11 Effect of SAMe on the rate of cytochrome C reduction by the hypoxanthine xanthine oxidase reaction .Results are expressed as % control response , means ± SEM (n=5). Significant inhibition at 5mM * $P<0.05$.

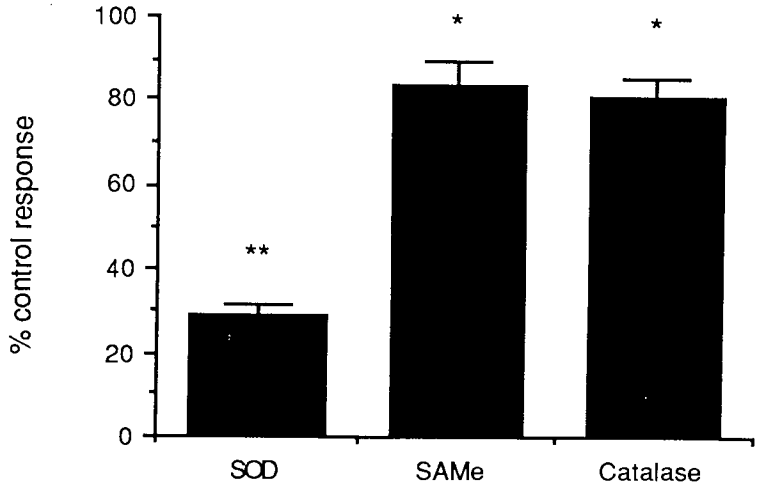


Fig 3.1.2 Comparison of the antioxidant effects of superoxide dismutase (SOD, 200 units), catalalase (600 units) and SAMe 5mM.Results are expressed as % control response mean±SEM (n=5). * $P<0.05$, **$P<0.001$

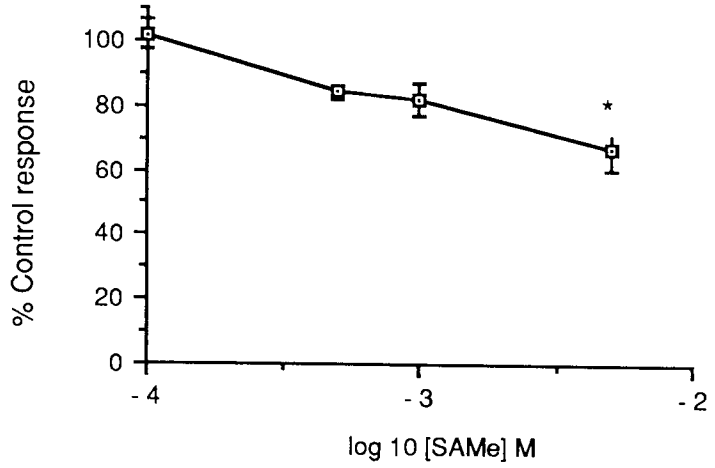


Fig 3.2.1 Effect of SAMe on the rate of uric acid production from the hypoxanthine xanthine oxidase reaction Results are presented as % control response means ± SEM (n=5).Significant inhibition at 5mM * P<0.001.

3.3 Effect of SAMe on the aerobic photoreduction of Nitroblue tetrazolium (NBT)

Fig 3.3.1 shows that SAMe in the dose range 1μM - 5mM had no significant effect on the photoreduction of NBT. This is a reaction which produces superoxide via an enzyme free system.

The maximum concentration of SAMe employed in the assay (5mM) had no significant effect on the assay causing a marginal inhibition of only 7.4 ± 2.3 % (n=6). In contrast fig 3.3.2 demonstrates a dose dependent inhibition of the photoreduction of NBT by superoxide dismutase. The maximum dose of SOD (1200 units)reduced the response by 79.1 ± 3.7 % (n=4). From this data the IC_{50} was estimated to be 456 ± 9 units.

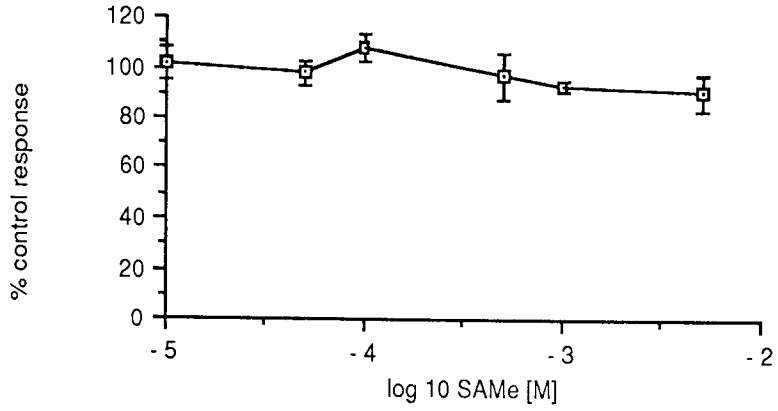


fig 3.3.1 The effect of SAMe on the aerobic photoreduction of NBT .Results are expressed as % control response (maximum absorbance), means ±SEM (n=6).

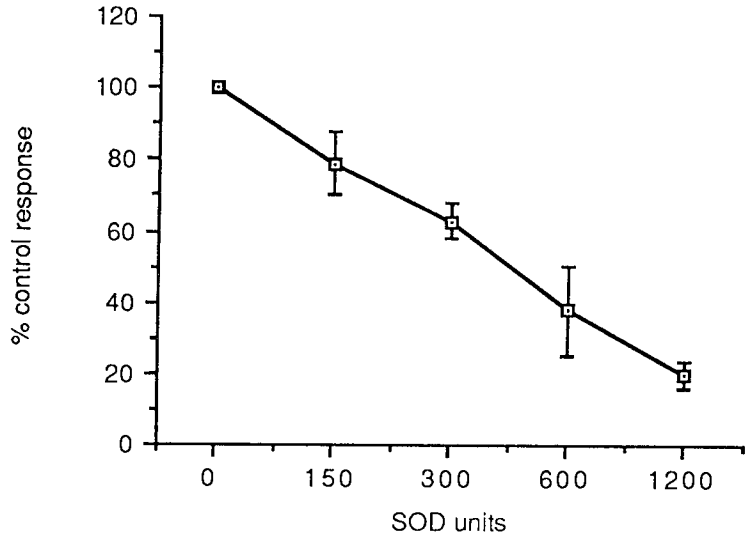


Fig 3.3.2 Superoxide dismutase dose dependent inhibition of the aerobic photoreduction of NBT.Results are expressed as % control response (maximum absorbance), means ± SEM (n=3).The IC_{50} for SOD = 456±9 units.

3.4 Effect of SAMe on cytochrome C reduction by rat neutrophils stimulated with FMLP

The effect of SAMe on the oxidative burst of rat neutrophils activated by FMLP was studied using the reduction of cytochrome C as an index of superoxide generating activity. Fig 3.4.1 shows that in the concentration range 1μM-10μM incubation with SAMe had no significant effect on the rate of cytochrome C reduction .Drug treated cells generated 717 ± 120 pmol O_2^- / min / 10^6 cells (n=4) compared to 800 ± 90 pmol O_2^- / min / 10^6 cells

(n=6) for the buffer only control. The control response was corrected to 100% maximal response and the results are expressed as % control response.Significant reduction of the rate of superoxide production was recorded at a dose of 100 μM where the rate of cytochrome C reduction was reduced by 32±9 % (n=4 , P< 0.01). The highest dose of 10 mM massively reduced the rate of cytochrome C reduction by 73 ±7 % (n=4). Examination of cell viability by the Trypan blue exclusion test showed the viability to be slightly less than the control but was still 90 %. Further, the relatively high concentration of HEPES (50mM) required to buffer the strongly acidic SAMe preparation had no effect on cell viability or cellular response in comparison with cells incubated in standard HBSS buffer preparations.

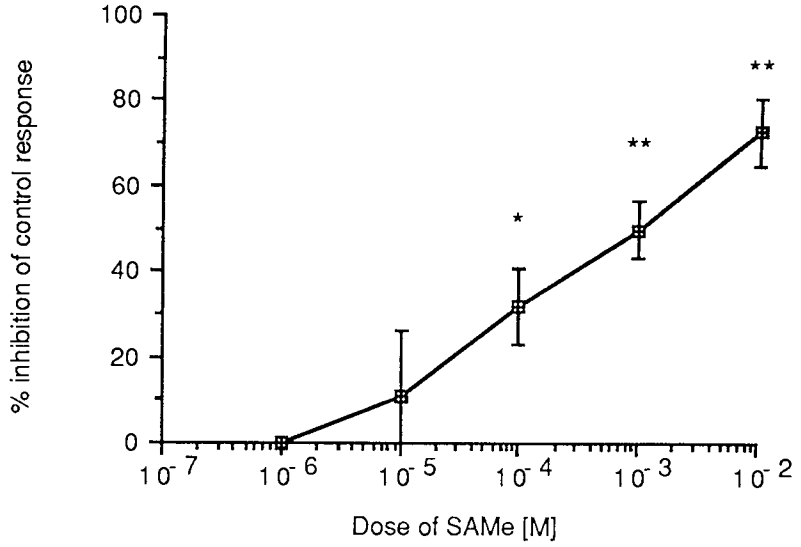


Fig 3.4.1 The dose dependent inhibition by SAMe of the rate of cytochrome C reduction (nmol / min / 10^6 cells) by rat neutrophils stimulated with 1μM FMLP. Results are expressed as mean ± SEM % inhibition of control response (n=4).*P<0.01, **P<0.001.

3.5.1 Effect of SAMe on PMA activated rat neutrophils luminol dependent chemiluminescence

The results shown in fig 3.5.1.1 indicate that PMA (10 nM) stimulated cells were highly resistant to SAMe treatment . The chemiluminescent response was hardly dampened even at doses as high as 10mM where the maximal response was 83.6± 11% (n=3). The time course showed a latency of typically 2 minutes followed by a massive exponential chemiluminescent response quite untypical of any other activator tested. The mean reponse was 65 ± 9 mV / 10^6 cells (n=7) and it was noted that cells previously refractory to other activators showed a similar explosive oxidative burst response. Chemiluminescence by PMA stimulated rat neutrophils was effectively inhibited in a dose dependent manner by superoxide dismutase (fig 3.5.1.2). The IC_{50} was estimated to be 226 ± 14 units. The maximum inhibition was to 18.1 % by 1200 units.

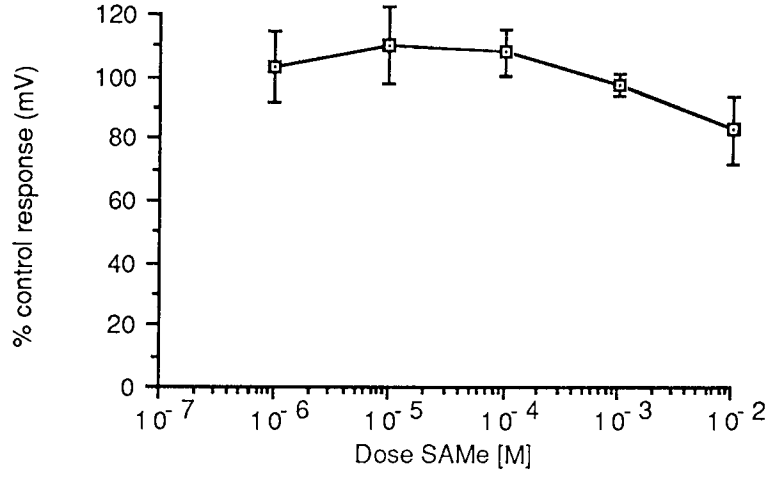


Fig 3.5.1.1 Effect of SAMe on PMA induced rat neutrophils chemiluminescence (mV/10^6 cells).Results are expressed as % control response , means ± SEM (n=3)

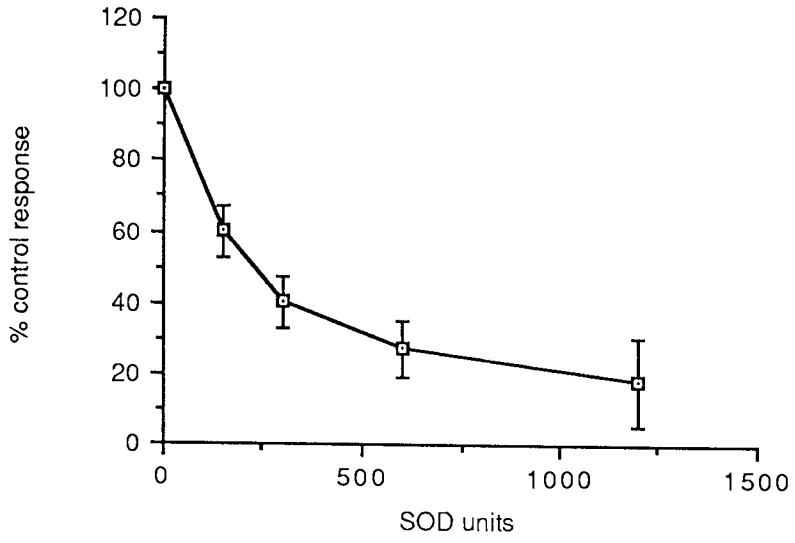


Fig 3.5.1.2 Dose dependent inhibition of PMA stimulated rat neutrophils chemiluminescence (mV/10^6 cells) by SOD. Results are expressed as % control response , means ± SEM (n=3). The IC_{50} for SOD = 226±14.4 units.

3.5.2 Effect of SAMe on FMLP activated luminol dependent rat neutrophils chemiluminescence

The chemiluminescent response to FMLP showed a bimodal response (Dahlgren et al 1987) characterised by a sharp transitory peak within 30 - 60 seconds followed by a second slow peak after about 6 minutes. The effect of SAMe treatment on the fast and slow peaks is shown in fig 3.5.2.1 and 3.5.2.2 respectively. The chemiluminescent response to 1 μM FMLP was not significantly affected by SAMe at low concentrations (1- 10 μM). At higher concentrations the chemiluminescent response was significantly inhibited. The highest dose 10mM dramatically reduced the maximal response by 74.6 %. The IC_{50} of SAMe was 1.9 ± 0.4 mM for the first peak and 4 ± 0.8 mM for the second slow peak. The fast peak was consistently and significantly more sensitive to SAMe than the latter peak ($P < 0.05$) where the effect was highly variable. Consequently the inhibitory effect of SAMe on the latter peak showed significant difference from the control and the fast peak at the maximum dose of 10 mM only.

Catalase quenched the FMLP induced respiratory burst in buffer control and drug treated

cells (1mM SAMe). Fig 3.5.2.3 indicates that the fast transitory peak was significantly more sensitive to catalase than the slow peak with an IC_{50} of 289 ± 16 units as compared to 1179 ± 18 units respectively. Drug treated cells were more sensitive to catalase than the control (fig 3.5.2.4). The IC_{50} of catalase for the fast peak was significantly reduced to 149± 3 units (n=5, $P < 0.001$). Similarly the slow peak was significantly more sensitive than the control but significantly less than the initial fast peak ($P < 0.001$).

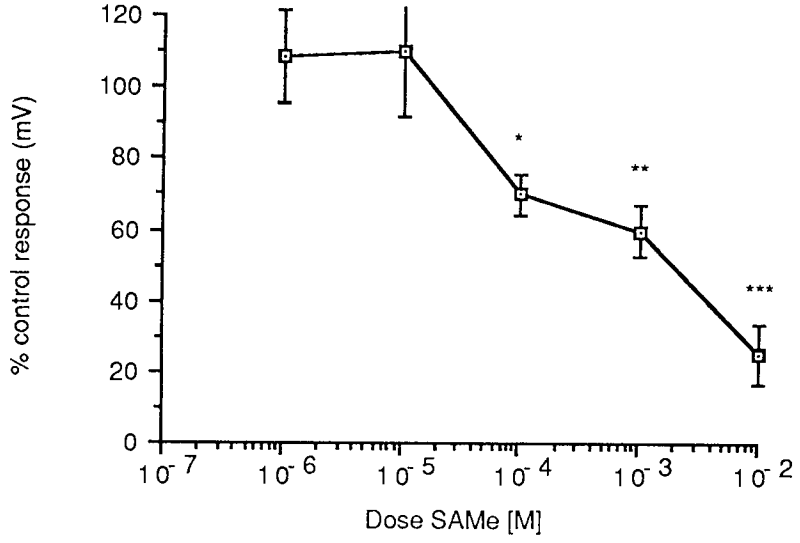


Fig 3.5.2.1 Effect of SAMe on the fast peak of FMLP stimulated rat neutrophils chemiluminescence (mV/ 10^6 cells). Results are expressed as % control response Means ±SEM (n=6).* P<0.05, ** P<0.01, *** P<0.001.The IC_{50} for SAMe = 1.9±0.38 mM

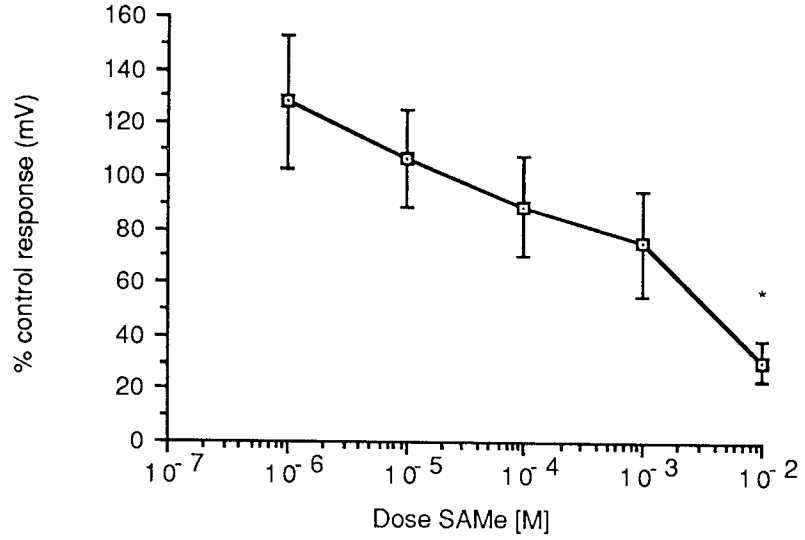


Fig 3.5.2.2 Effect of SAMe on the slow peak of FMLP stimulated rat neutrophils chemiluminescence (mV / 10^6 cells). Results are expressed as % control response , means ± SEM (n=6).Significant inhibition was only at 10mM * P<0.001.The IC_{50} for SAMe was 4 ± 0.83mM.

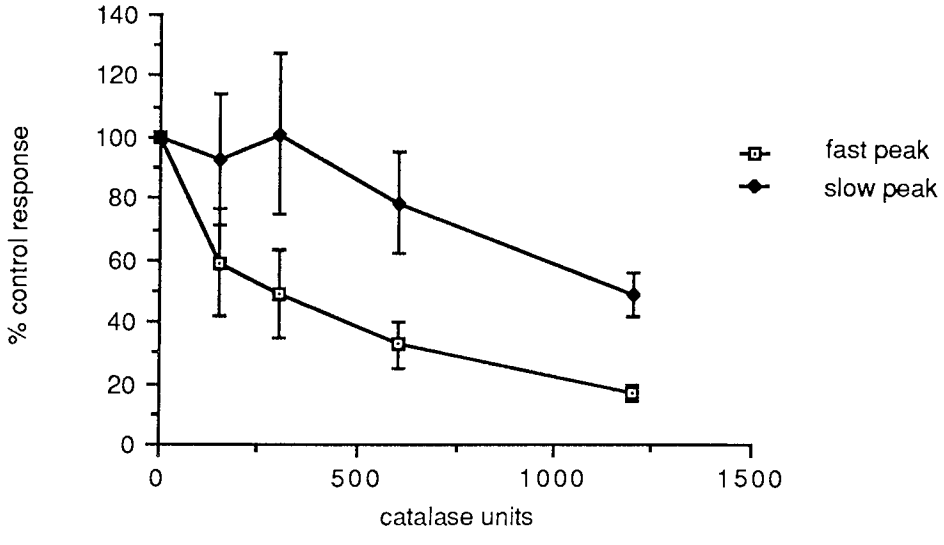


Fig 3.5.2.3. Effect of catalase on FMLP stimulated rat neutrophils chemiluminescence (mV/10^6 cells) Results are expressed as % control response, means ± SEM (n=3).

The IC_{50} of catalase for the fast peak was 289±16 units, for the slow : 1179±12 units.

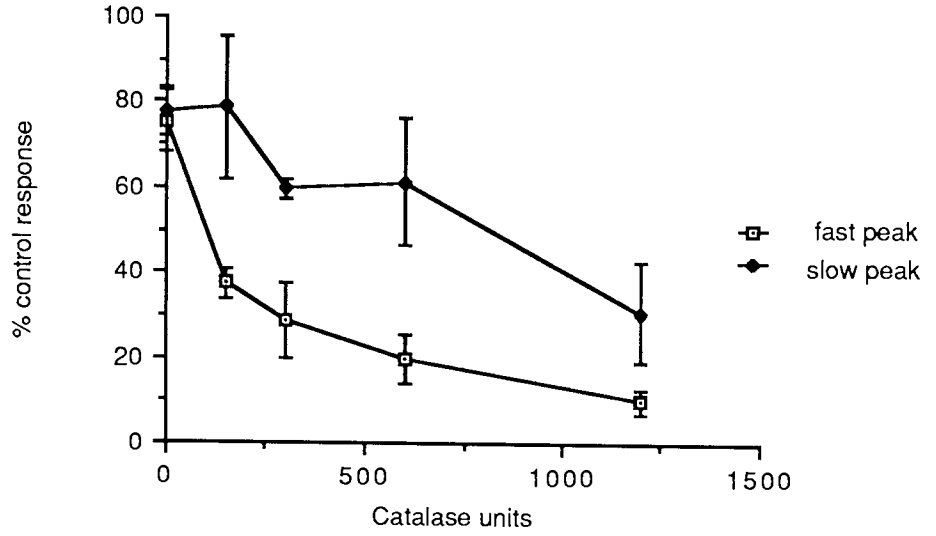


Fig 3.5.2.4 Effect of catalase on FMLP stimulated chemiluminescence (mV/10^6 cells) emitted by SAMe treated cells (1mM).Results are expressed as % control response , means ± SEM (n=3). The IC_{50} of catalase for the fast peak was 149±6.3 units and 1041± 23 units for the slow peak.

3.5.3 Effect of SAMe on zymosan activated rat neutrophils luminol dependent chemiluminescence

The time course profile of unopsonised zymosan A stimulated rat neutrophils chemiluminescence had a PMA type profile but had a latency and magnitude of response similar to that induced by FMLP. Initial studies of the effect of SAMe (1μM-10mM , fig 3.5.3.1) revealed dose dependency and significant inhibition at even the lowest dose except at 100μM where the response was highly variable but due to the wide standard deviation was not significantly different from the control.A more detailed dose response study (31.25 μM- 1mM) shown in fig 3.5.3.2 exhibited a similar highly variable peak at the dose of 125 μM (mean of 152 ± 39.5 % (n=5)).In this study the lowest dose of 31.25 μM inhibited chemiluminescence by 69.4±11% .

Since the rat neutrophils respiratory burst stimulated by zymosan A , a particulate activator, was particularly sensitive to SAMe treatment a study was conducted into the effect of cytochalasin B(an alkaloid reported to disrupt microtubule aggregation and thus inhibit phagocytosis) on the respiratory burst . Incubation of rat neutrophils with cytochalasin B

(8μg / ml) for 30 minutes virtually abolished the response (fig 3.5.3.3). The maximal control response from 12 ± 2.3 mV to 0.6± 0.2 mV (n=4) in contrast SAMe (1mM) reduced this to 3± 0.5 mV (n=4). The combination of SAMe and cytochalasin B had no further inhibitory effect. The addition of PMA (10 nM) to these preparations caused a massive oxidative burst (rate of 3.5 mV/ min) which suggests that the cellular machinery for the oxidative burst was intact.

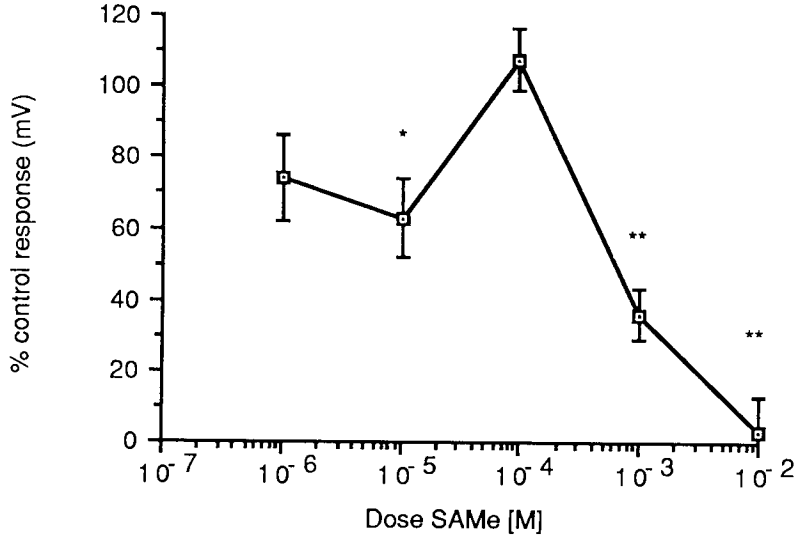


Fig 3.5.3.1 Effect of SAMe on zymosan stimulated rat neutrophils chemiluminescence ($mV/10^6$ cells). Results are expressed as % control response , means ± SEM (n=4), * P<0.025, **P<0.001.

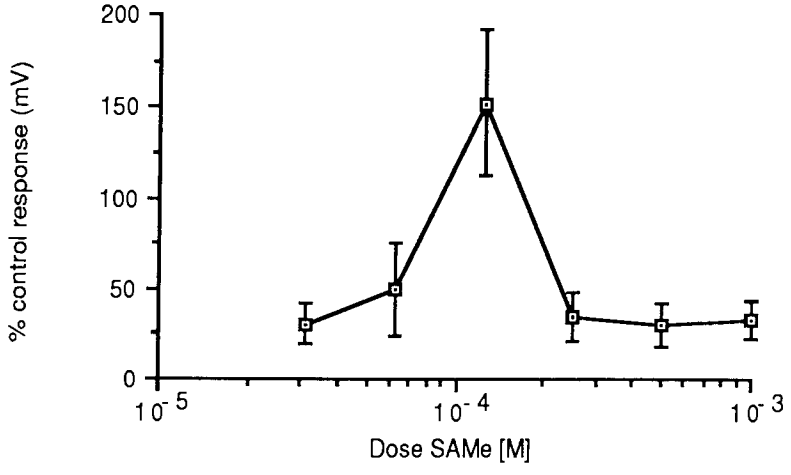


Fig 3.5.3.2 Effect of SAMe in the dose range 31.25μM-1mM on zymosan induced rat neutrophils chemiluminescence .A peak of the response is exhibited by cells incubated in 125μM SAMe.

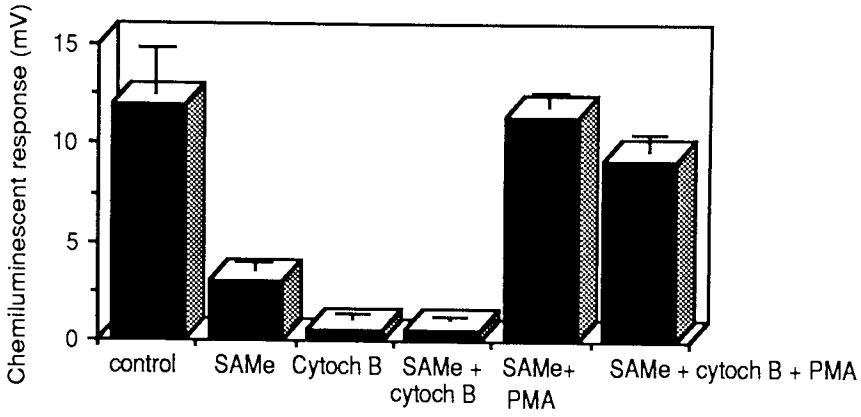


Fig 3.5.3.3 Inhibition of zymosan induced chemiluminescence by SAMe (1mM) and cytochalasin B (cytoch B, 8μg/ml). The response to PMA (10nM) was unaffected by both these drugs.

3.5.4 Effect of MTA and adenosine on FMLP activated rat neutrophils chemiluminescence

In contrast to the inhibitory effect of SAMe on FMLP induced rat neutrophils chemiluminescence the SAMe metabolite MTA inhibited both the fast transitory phase (a) and the slow sustained phase (b) equally effectively but in different parts of the dose range (1nM-100μM, fig 3.5.4.1). The fast peak was most effectively reduced by MTA at 10μM to 39±5.5 % (n=4) whereas the inhibition of the later peak was independent of concentration and was sustained at about 40% throughout the dose range.The viability of MTA treated cells was not significantly different from the control.

Adenosine in the same dose range inhibited both fast and slow phases (fig 3.5.4.2). As was the case with MTA adenosine inhibited the fast phase in a dose dependent manner but had no significant effect at doses of 10nM and less.Maximum inhibition was obtained at 100μM where the maximal response was reduced by 42±3% (n=4). The reverse was true for the slow phase which like MTA depressed the maximal response throughout the dose range but the strongest inhibition was at the lowest dose of 1nM (57.8±3.3% n=4).

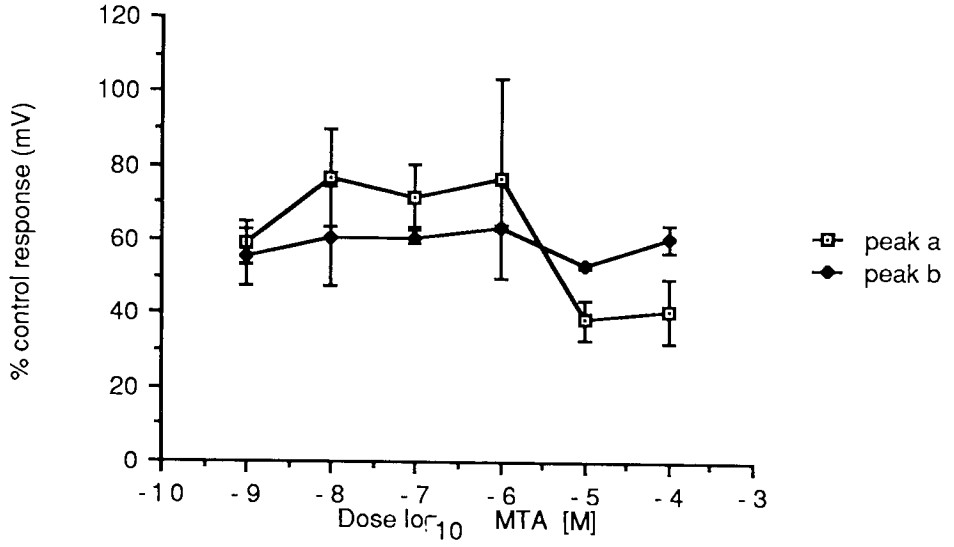


Fig 3.5.4.1 Effect of MTA on FMLP stimulated rat neutrophils chemiluminescence ($mV/10^6$ cells) , results expressed as % control response , means ± SEM (n=4).

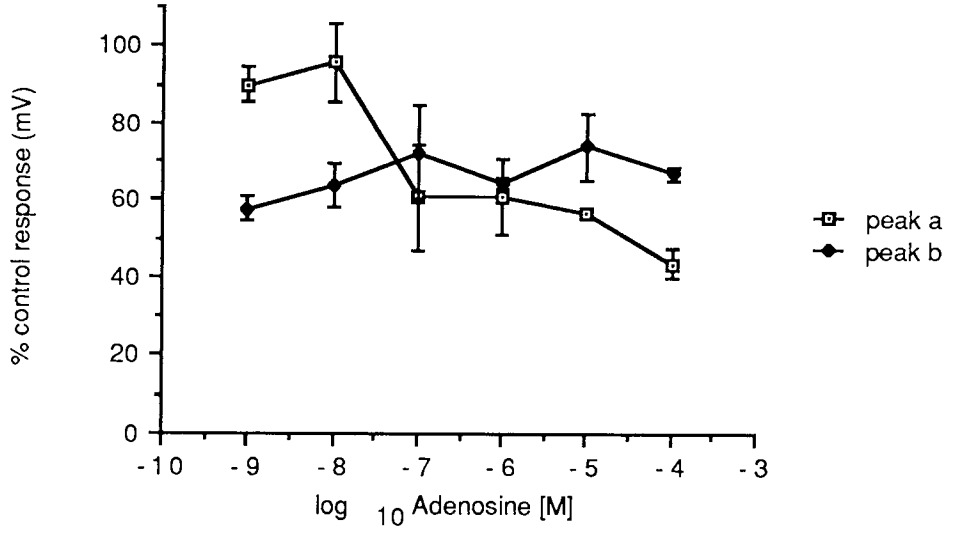


Fig 3.5.4.2 Effect of adenosine on FMLP stimulated rat neutrophils chemiluminescence ($mV/ 10^6$ cells), results expressed as % control response , means ± SEM (n=4).

3.6 Effect of SAMe on phagocytosis by rat neutrophils in vitro

Fluorescent microscopy showed that latex particles were avidly taken up rat neutrophils within the 1 hour incubation time.The mean particle uptake for the control was 5.33 ± 0.8 (n=6) particles per cell.Fig 3.6.1 shows that co-incubation with SAMe at doses of 1 and 10mM significantly inhibited phagocytosis with an IC_{50} of 6.6± 1 mM (n=6, P< 0.01). Concentrations of 1-100μM had no significant effect on phagocytosis but there is a suggestion of the peak observed at 100μM in the zymosan activated chemiluminescence study (see section 3.5.3).Cells incubated at this concentration showed a consistently greater mean uptake of particles than at any other concentration tested (96.3±16.3% n=6). Tests of cell viability by Trypan blue exclusion confirmed viability to be >93%.There was no significant difference between SAMe treated and control cells.

S-adenosyl-l-homocysteine (SAHc) and methionine (1mM) did not inhibit phagocytosis (fig 3.6.2) indeed SAHc treated cells showed a slight enhancementof uptake to 110±14% (n=6).Cytochalasin B (8μg/ml) significantly inhibited rat neutrophils phagocytosis of particles (P<0.01) to 61.8± 11.5 % (n=6) of the control.This degree of inhibition was comparable to treatment with 1mM SAMe.

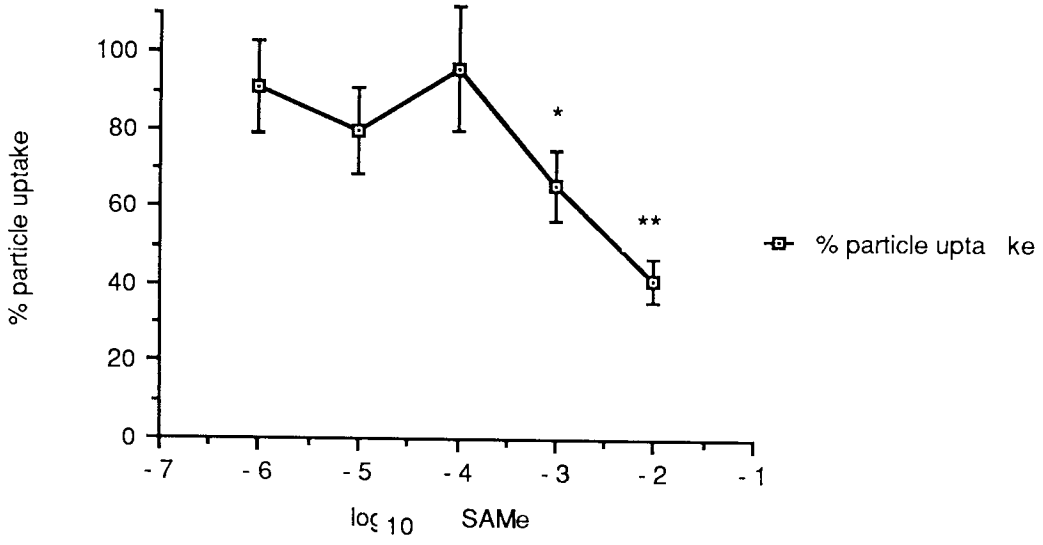


Fig 3.6.1 Effect of SAMe on phagocytosis by rat neutrophils of fluorescent latex paticles in vitro.Results are expressed as % control response (mean particles per cell) , means ± SEM (n=6).*P<0.01, **P<0.001, IC_{50} = 6.57 ± 0.95mM

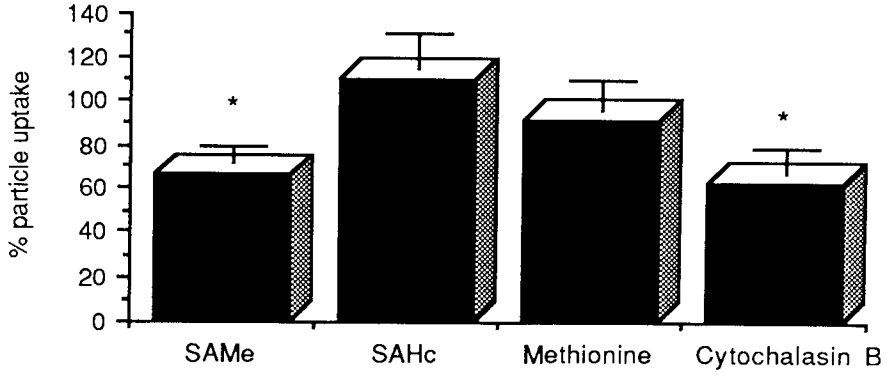


Fig 3.6.2 Effect of SAMe and metabolites (1mM) and cytochalasin B (8μg/ml) on phagocytosis of fluorescent latex particles in vitro. Results are expressed as % control response , means ± SEM (n=6).* P<0.01

3.7 Effect of SAMe on PGE_2 production by rat neutrophils stimulated with zymosan A

Rat peritoneal neutrophils elicited with caseinate were incubated in the presence of 1mM SAMe with sonicated zymosan A (5mg/ml).Control cells generated 90.1±3.8 pg PGE_2 / 10^6 cells (n=4) and SAMe treated cells produced 83.7±7.8 pg PGE_2 /10^6 cells. There was no significant difference between the two groups.

3.8 Effect of SAMe in vivo on infiltration of rat neutrophils into six day air pouches elicited by FMLP

The viability of neutrophils extracted from six day air pouches by the Trypan blue exclusion test was 98%. The results in table 3.8.1 show that dosing with 50mg/kg I.P had no effect on leukocyte infiltration.There were 4 animals per group.

	cells 10^6 / ml
saline treated	8.8 ± 0.7
SAMe treated	8.6 ± 0.5

3.9.1 Effect of SAMe on carrageenan induced rat hind paw oedema

The biphasic nature of carraggeenan induced rat hind paw oedema is illustrated in fig 3.9.1.1.The first phase reached a nadir between 90 and 120 minutes where there was no significant difference in swelling . This was succeeded by a second phase of inflammation which culminated in a maximum mean swelling after 300 minutes. However in most cases the actual point of maximal paw inflammation was probably beyond this time point. A comparison of the mean swelling values obtained after 240 minutes and 300 minutes showed no significant difference in both the control and the SAMe treated groups. Preliminary studies of the anti-inflammatory effect of SAMe on this model revealed that a single dose of 50mg suppressed oedema at both the 3 and 5 hour interval.

A more detailed time course study showed that SAMe had no significant effect during the first hour but from fig 3.9.1.1 it is clear that the swelling had been significantly reduced

after 90 minutes from the control of 27.5±0.12% (n=10) to 13.3± 1.03% (n=10, P<0.001).Maximum inhibitory effect was observed after 180 minutes where SAMe treated animals showed a mean swelling of 18.3±0.7% (n=13) compared to the control value of 39.6±3% (n=13, P<0.001).The anti-inflammatory effect was sustained up to 5 hours but was significantly less potent (P<0.01).

Indomethacin a potent cyclooxygenase inhibitor (Scalabrino 1987) 10mg/kg i.p. was considerably more potent than SAMe (50mg/kg). The mean oedema at the 3 hour interval was 9.2± 2.9% (n=6) and 26±5.3% (n=6) after 5 hours .The results of a dose response study are shown in fig 3.9.1.2.and suggest that 50mg/kg was the optimum anti-inflammatory dose since it was anti-inflammatory at both the 3 and 5 hour intervals.

Animals dosed with 25mg/kg SAMe showed slight but non-significant inhibition at the 3 hour interval.Indeed there was a sharp increase in the rate of swelling between 3 and 4 hours over the control resulting in no perceptible difference at all. Curiously the greater dose of 100mg/kg demonstrated a similar efficacy profile with significant anti-inflammatory activity only apparent at the 3 hour interval.Mean paw swelling doubled in 1 hour from 23.6± 6.8% to 48.2±11 % (n=5) but the variation was quite wide since some animals showed an enhanced oedema.Thus SAMe had anti-inflammatory action in this model of acute inflammation but dosage was critical.

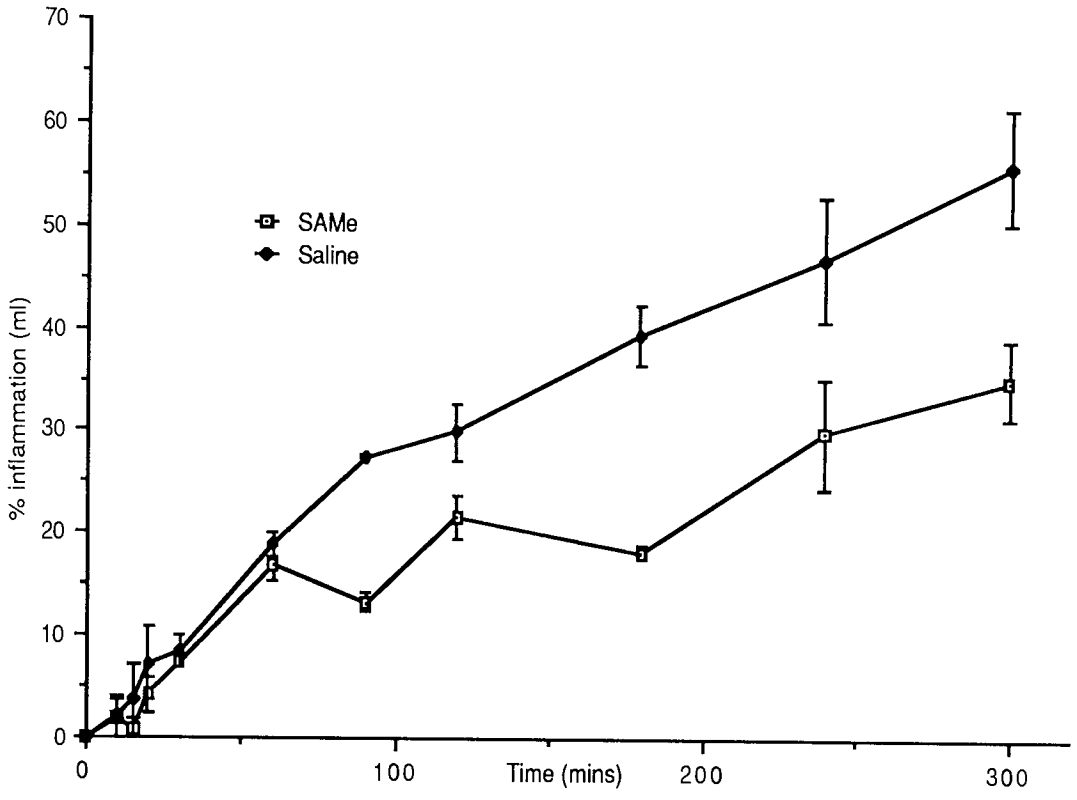


Fig 3.9.1.1. Time course of 2% carrageenan induced hind paw inflammation in saline and SAMe (50mg / kg) treated animals. Results are presented as % increase in foot volume per group (ml), means ± SEM (n=10).

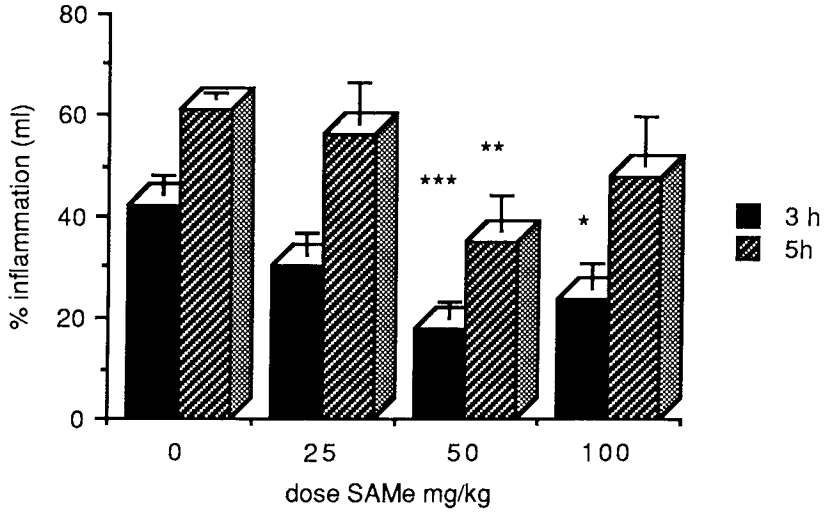


Fig 3.9.1.2 Dose dependent inhibition of carrageenan induced hind paw inflammation by SAMe.Results are expressed as % increase in foot volume per group (ml), means ±SEM (n=5). * P<0.05, **P<0.01, ***P<0.002.

3.9.2 Effect of SAMe on carrageenan induced rat hind paw oedema in animals pretreated with inhibitors of polyamine metabolism

Difluromethylornithine (DFMO) a competitive and irreversible ornithine decarboxylase blocker (Metcalf et al 1978) administered to animals at doses of 500mg/kg at 12 hour intervals for 48 hours had no obvious toxic effects.The results in fig 3.9.2.1 indicate that DFMO pretreatment had no significant effect on the anti-inflammatory effect of SAMe in the carrageenan model at the 3 hour interval but enhanced suppression of oedema at 5 hours.The actual increase in inflammation was considerably more severe in this study than was observed in section 3.9.1.At 3 hours post -inoculation the control group showed a mean increase in swelling of 85±1.5% (n=5) compared to the SAMe and DFMO treated group which showed a mean increase in swelling of 52.5 ±12% (n=5).The relative inhibition was about 40% a value that was not significantly different from that of SAMe alone.After 5 hours the control group showed a mean increase of 110±7.4 % but the swelling had been arrested in the SAMe and DFMO group at 48.2±10% ($P<0.001$).Animals treated with DFMO alone showed a modest but significant inhibition of carrageenan induced oedema at the 3 hour interval ($P<0.05$) but this was reduced to non significance by 5 hours (fig 3.9.2.2).

In order to further examine the possibility that the anti-inflammatory effect of SAMe may be due to its role as an aminopropyl group donor , some animals were also pretreated with methyl glyoxal bis guanyl hydrazone (MBGB) an inhibitor of SAMe decarboxylase (Warrell and Burchenal 1983). Fig 3.9.2.3. demonstrates that the anti-inflammatory effect of SAMe against carrageenan induced oedema was sustained in animals where polyamine metabolism had been theoretically arrested.The degree of inhibition was comparable at 3 hours with no significant difference betwen SAMe, SAMe and DFMO and SAMe,DFMO and MBGB treated groups.Unlike the SAMe and DFMO group there was no enhancement of the inhibition observed at 5 hours . Animals treated with MBGB alone (20mg/kg) showed slight relief from carrageenan induced oedema at 3 hours but the effect was highly variable (mean % swelling=33.8±18, n=5) and due to this variability was rendered non significant. This held for the 5 hour interval where some animals showed an actual increase

in swelling greater than the control (fig 3.9.2.4).

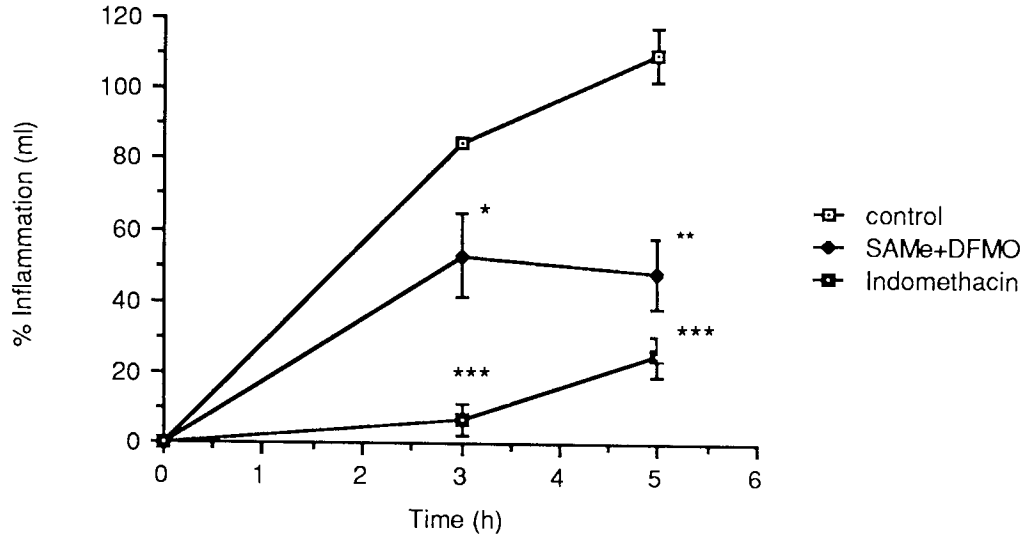


Fig 3.9.2.1. Effect of DFMO (500mg/kg) and SAMe (50mg/kg) on carrageenan induced inflammation.Results are expressed as % increase in inflammation , means ±SEM (n=5).Significant inhibition was maintained at 3 ($P<0.05$) and 5 hours ($P<0.002$).Indomethacin (10mg/kg) significantly inhibited oedema throughout ($P<0.001$).

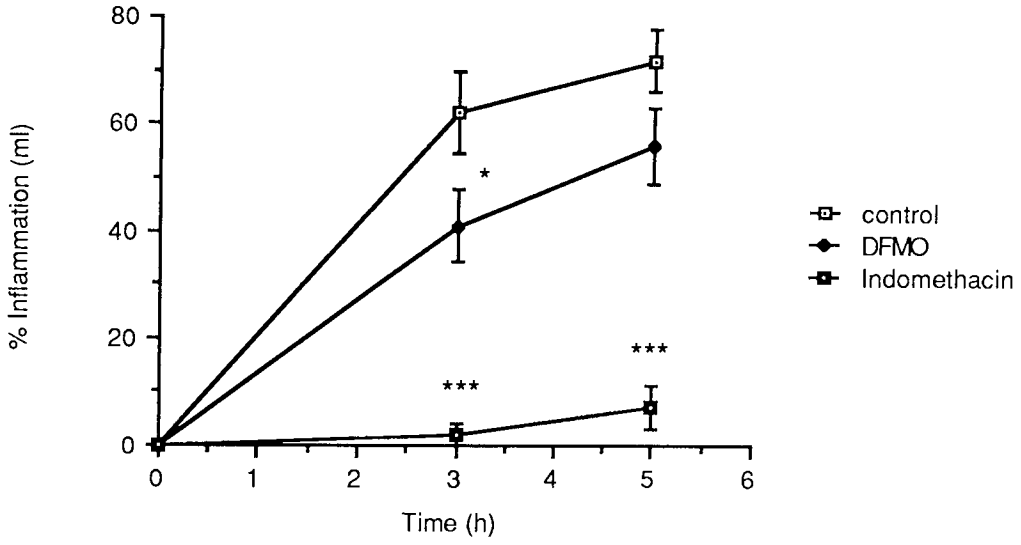


Fig 3.9.2.2. Effect of DFMO (500mg/kg) on carrageenan induced hind paw inflammation. Results are expressed as % increase in inflammation , means ± SEM (n=5).Significant inhibition shown at 3 hours (*$P<0.05$).

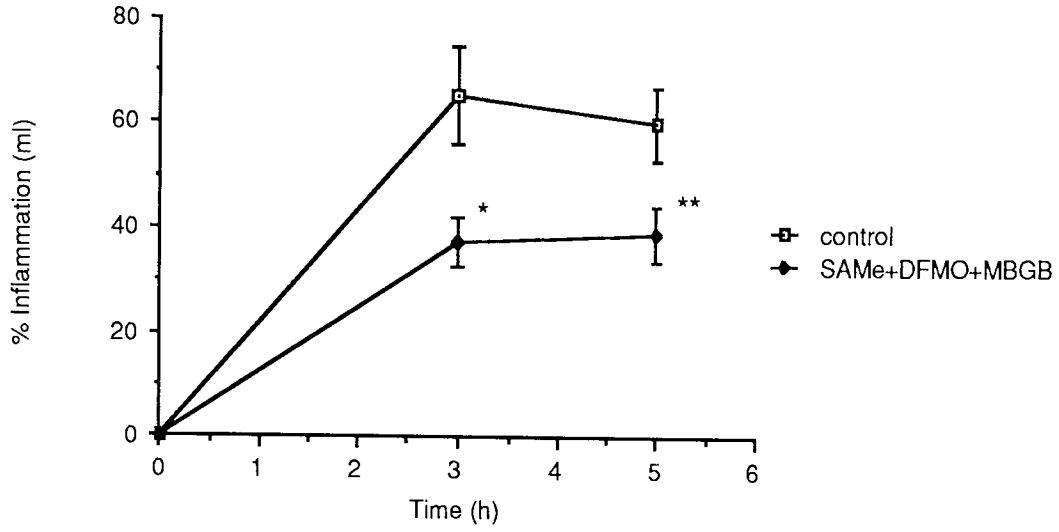


Fig 3.9.2.3. Effect of DFMO (500mg/kg), MBGB(20mg/kg) and SAMe (50mg/kg) on carrageenan induced inflammation. Results are expressed as % increase in inflammation (ml) , means ± SEM (n=10), * P<0.05, ** P<0.01.

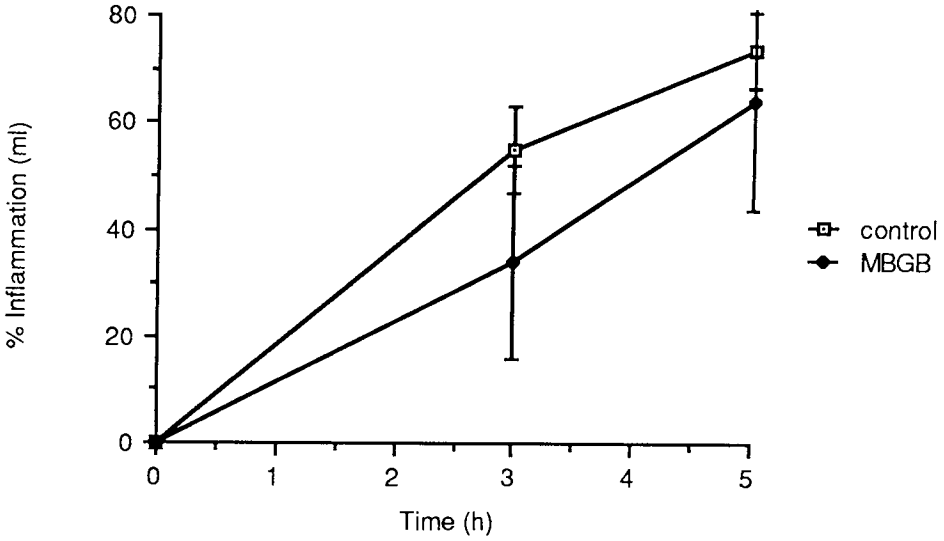


Fig 3.9.2.4. Effect of MBGB (20mg/kg) on carrageenan induced inflammation.Results are expressed as % increase in inflammation (ml), means ± SEM (n=5).

3.9.3 Effect of MTA and adenosine on carrageenan induced rat hind paw oedema

Initial studies showed that MTA at a dose of 50mg/kg significantly inhibited carrageenan induced hind paw oedema at both 3 and 5 hour intervals (fig 3.9.3.1).However like SAMe the anti-inflammatory effect whilst sustained at 5 hours ($P<0.05$) was less effective than at 3 hours. A dose response study showed that the anti-inflammatory effect of MTA at 3 hours was independent of dose (fig 3.9.3.2) in the dose range employed as there was no significant difference in oedema suppression in animals treated with 25, 50 and 100 mg/kg. After 5 hours the lowest dose of 25 mg/kg failed to have any inhibitory effect. The DMSO/distilled water vehicle for the drug had no effect on the time course or the magnitude of carrageenan induced hind paw oedema.

The same dose of adenosine (50mg/kg) significantly reduced hind paw swelling at 3 hours ($P<0.01$) from the control value of 55 ±5.7% (n=5) to 28.8±5.6% (n=5)but there was no significant difference betwwen the two groups after 5 hours (fig 3.9.3.3).

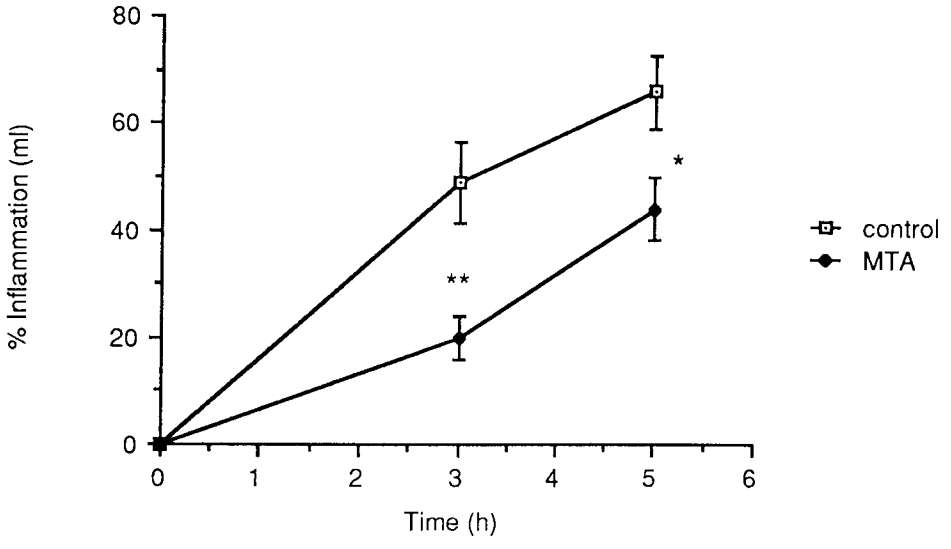


Fig 3.9.3.1. Effect of MTA (50 mg/kg) on carrageenan induced inflammation . Results are expressed as % increase in inflammation (ml), means ± SEM (n=8), *P<0.05, **P<0.01.

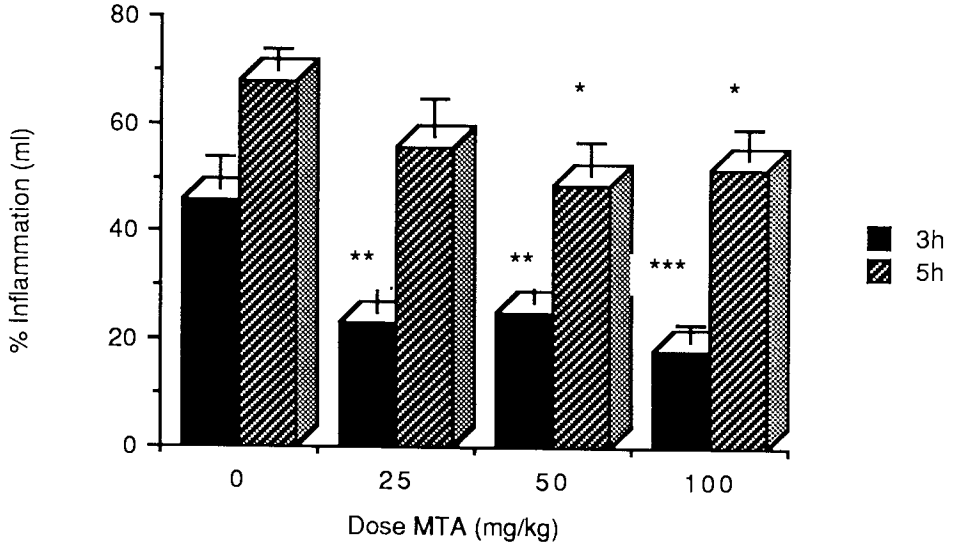


Fig 3.9.3.2. The anti-inflammatory effect of MTA in the carrageenan model is non-dose dependent. Results are expressed as % increase in inflammation , means ± SEM (n=5). *P<0.05,**P<0.01, **P<0.002.

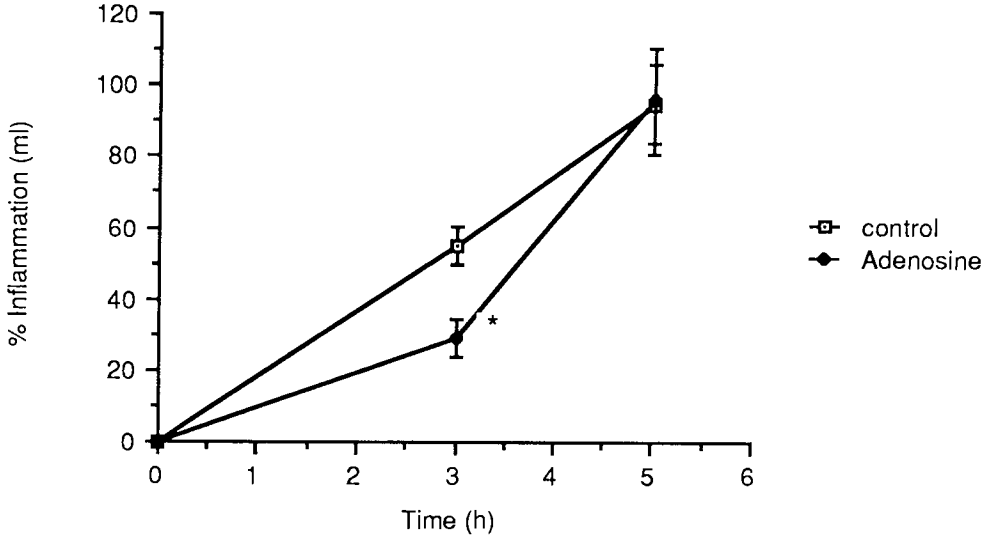


Fig 3.9.3.3. Effect of adenosine (50mg/kg) on carrageenan induced inflammation.Results are expressed as % increase in inflammation (ml), means ± SEM (n=5), * P<0.01.

3.10 Effect of SAMe on polyamines in liver and blood in the presence and absence of carrageenan induced rat hind paw oedema

The quantities of the physiological polyamines ; putrescine , spermidine and spermine in whole blood and liver perchloric acid extracts were determined by TLC-fluorimetry. The results in fig 3.10.1.1 show that carrageenan induced hind paw oedema had no significant effect on the basal levels of putrescine, spermidine or spermine in rat liver after 5 hours of inflammatory stress.

Animals treated with SAMe (50 mg/kg) showed a significant increase in liver putrescine levels ($P<0.05$) , in the presence or absence of inflammation , of about 30%.Spermidine levels were only significantly increased from the control value of 1098±22 nmol/g to 1281 ±97 nmol/g ($P<0.05$) under inflammatory stress.Spermine levels were unaffected by SAMe treatment or inflammatory stress.

An examination of the polyamine content of rat whole blood samples following carrageenan induced inflammatory stress in the presence or absence of SAMe (50mg/kg)showed no significant difference between the values of putrescine , spermidine or spermine (fig 3.10.1.2). Liver extracts from indomethacin treated animals showed a marked increase in putrescine content to 110±15 nmol/g ($n=5$, $P<0.01$).

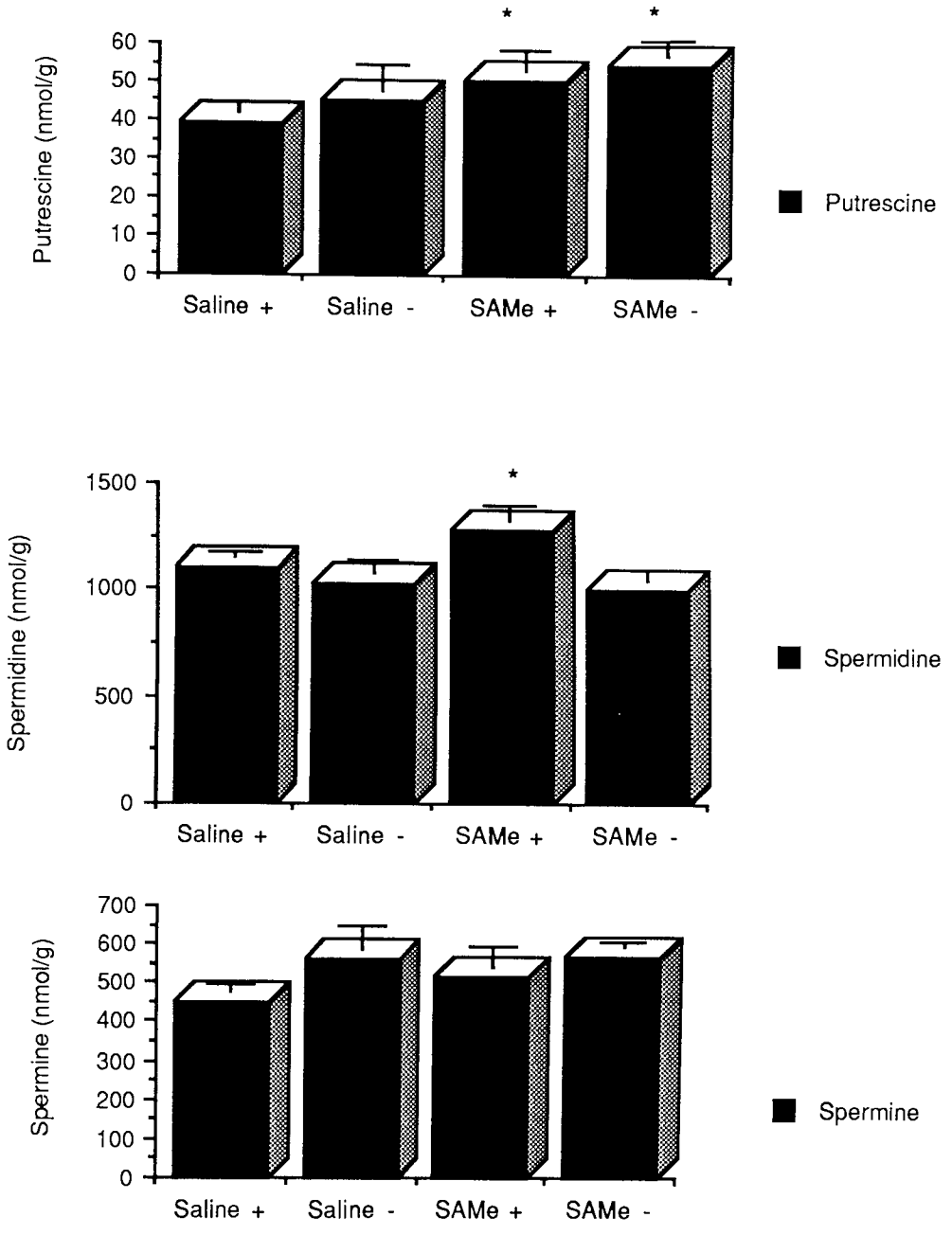


Fig 3.10.1.1. Effect of SAMe (50mg/kg) on the polyamine content of rat liver in the presence (+) or absence (-) of carrageenan induced inflammation. Results are presented as polyamine concentration (nmol per gram), means ± SEM (n=5).

Statistical analysis by 2x2 ANOVA, * $P<0.05$.

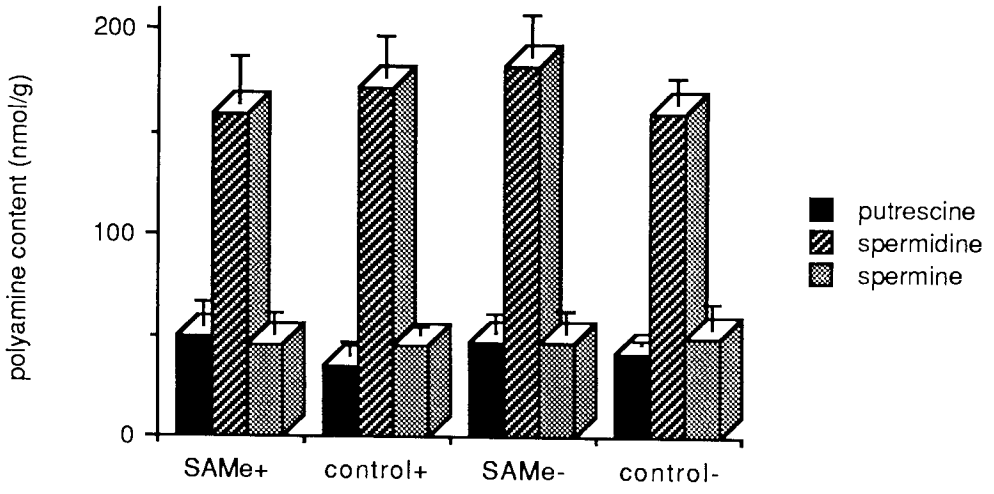


Fig 3.10.1.2. Effect of SAMe (50mg/kg) on polyamine content in rat whole blood (density 1.077g/ml) in the presence (+) or absence (-) of carrageenan induced inflammation.Results are presented as polyamine content (nmol/g), means ± SEM (n=5).Statistical analysis by 2x2 ANOVA.There was no significant difference in putrescine, spermidine or spermine content between the 4 groups.

3.10.2 Effect of SAMe on polyamine content in animals pretreated with inhibitors of polyamine metabolism

Fig 3.10.2.1 illustrates the effect of pretreatment with DFMO on polyamine status in rat liver following SAMe administration in animals subjected to carrageenan induced inflammatory stress.Putrescine content was massively reduced from 51.2±5 nmol/g (n=10) to 2.3±1.6 nmol/g (n=10, P<0.001).The SAMe stimulated increase in spermidine was abolished (971±6 nmol/g n=10) but there was no significant alteration in spermine levels . Treatment with DFMO only reduced putrescine levels to below detectable levels and significantly reduced spermidine content to 889±47 nmol/g (n=6,P<0.05).Spermine levels were unchanged.

The combination of MBGB with DFMO in SAMe treated animals restored putrescine to the same level as the saline treated control.Spermidine and spermine levels were not significantly different from the saline treated control.Animals treated with MBGB alone showed a doubling of putrescine content to 109 nmol / g (n=5, P<0.001) and paradoxically

since MBGB is a SAMe decarboxylase inhibitor, stimulated an elevation of spermidine. Spermine levels were not significantly affected.

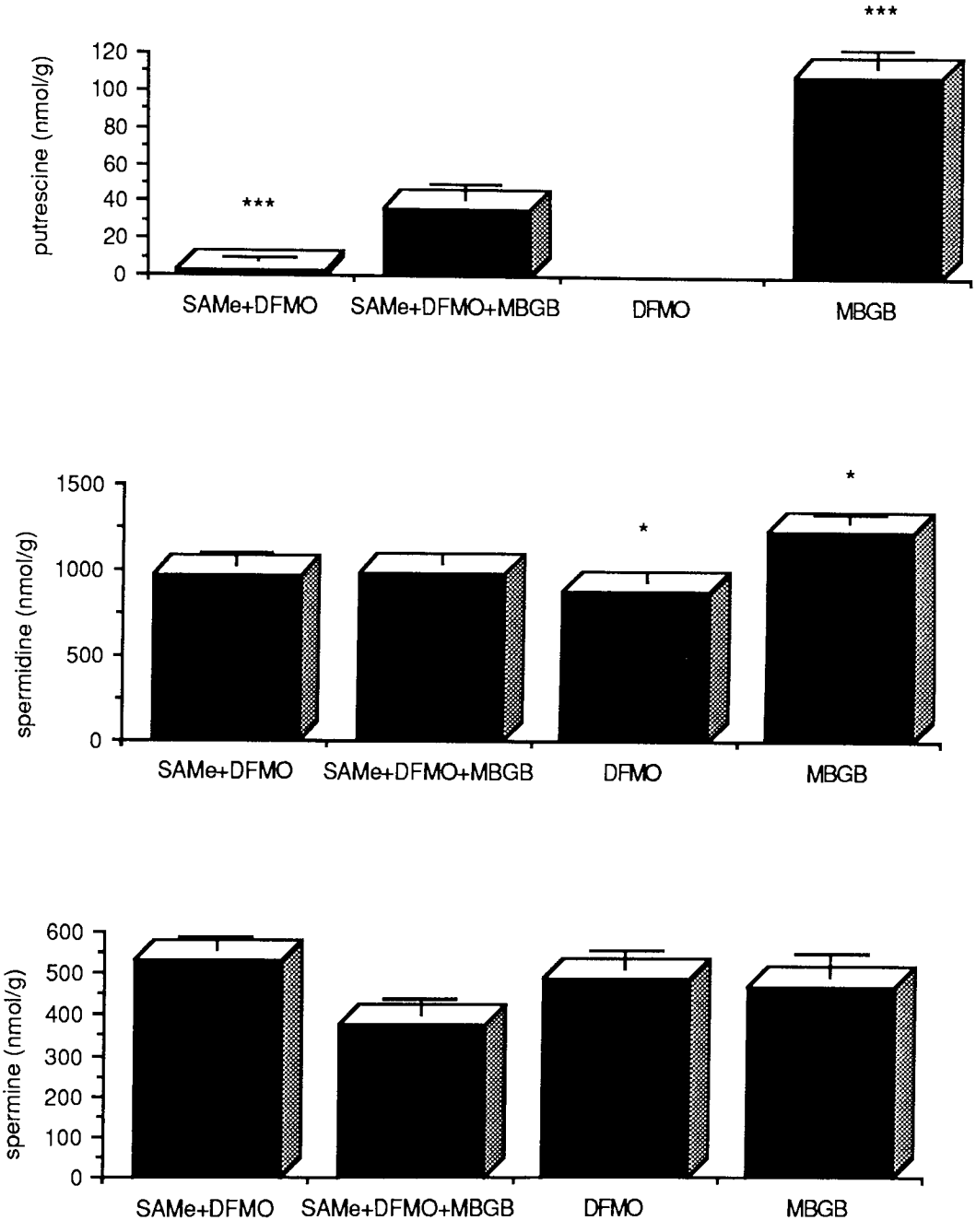


Fig 3.10.2. Effect of pretreatment with DFMO (500mg/mg) and MBGB (20mg/kg) on polyamine content in rat liver in the presence or absence of SAMe (50mg/kg) following carrageenan induced inflammation.Results are presented as nmol polyamine per g tissue, means ± SEM (n=5), * $P<0.05$, *** $P<0.001$.

3.11 Effect of MTA and adenosine on polyamine status in rat liver following inflammatory stress

Fig 3.11.1 shows that MTA (50mg/kg) strongly stimulated putrescine synthesis from the basal level of 39.6±2.2 nmol/g to 79±19 nmol/g (n=10 , $P<0.001$). Spermidine levels were also significantly increased, but not to the same extent as SAMe ($P<0.05$). Spermine levels were not significantly different from the DMSO/ distilled water control group.

Adenosine treatment (50mg/kg) significantly stimulated putrescine synthesis ($P<0.05$) to the same degree as SAMe but increased spermidine levels to 1371±44 nmol/g (n=6, $P<0.001$). Thus adenosine was more effective than SAMe in elevating spermidine . Mean spermidine levels were reduced to 373±44 nmol/g but this was not significantly different from the control.

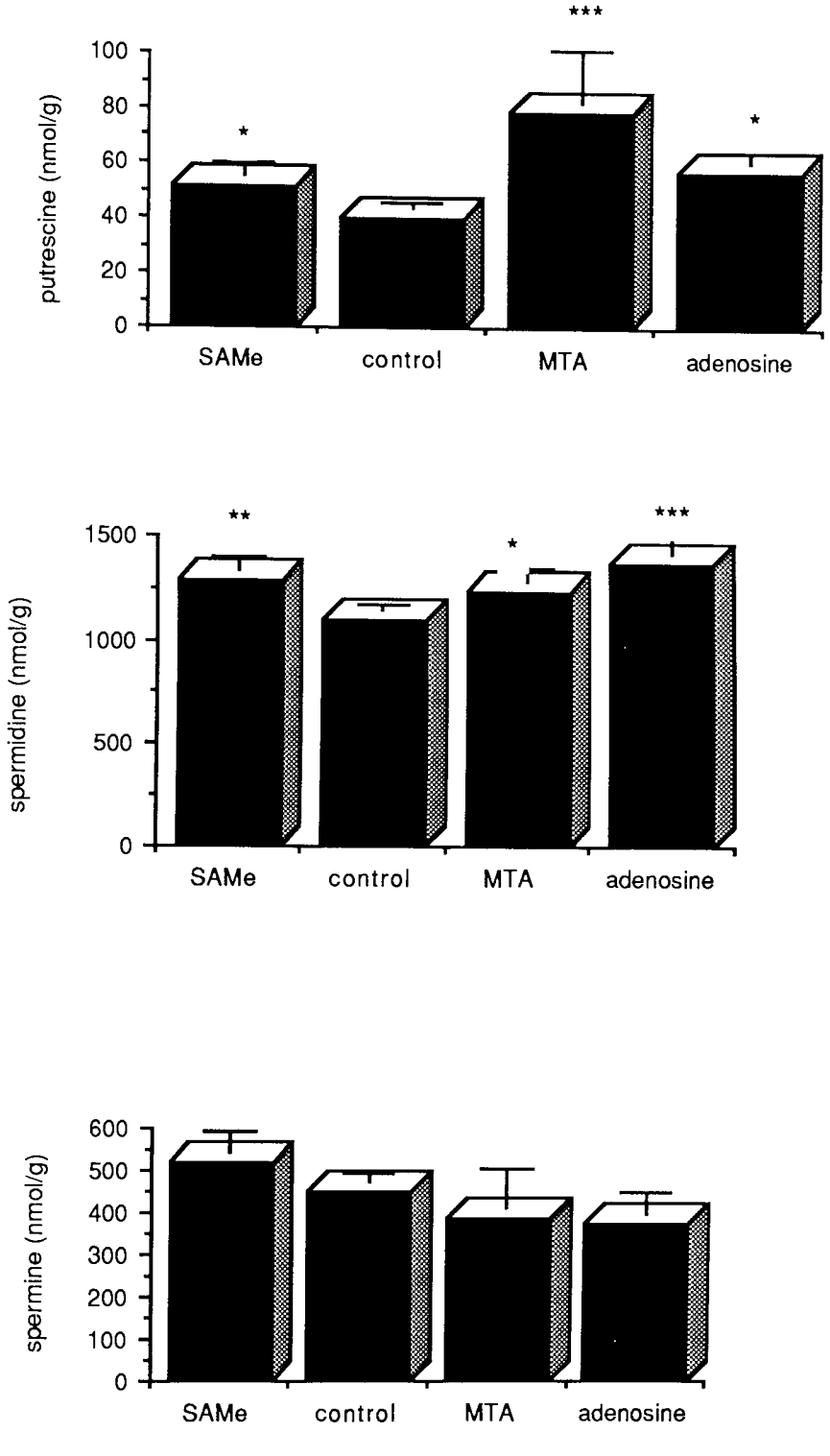


Fig 3.11.1. Effect of MTA and adenosine (50mg/kg) on polyamine levels in rat liver following carrageenan induced inflammation. Results are presented as nmol polyamine per gram of tissue, means ± SEM (n=5),* P<0.05, **P<0.0.1, ***P<0.001.

3.12 Effect of SAMe,MTA and adenosine on glutathione content in rat liver and blood in the presence and absence of carrageenan induced inflammation

Total glutathione content (the sum of both the oxidised and reduced forms of glutathione) in rat liver was estimated to be 1.97±0.17mg/g (n=5).Treatment with SAMe in the presence or absence of carrageenan induced inflammation caused no significant alteration of this parameter (1.83 ±0.16 and 2.1±0.08 mg/g, respectively).Liver glutathione content in animals treated with MTA showed a reduction to 1.65±0.17 mg/g (n=5) but this was not significant. Similarly, adenosine administration had no significant effect on hepatic glutathione content either.

Basal levels of total glutathione in whole rat blood was estimated at 326 ± 36.5 µg/g (n=5). Induction of carrageenan hind paw oedema in the presence or absence of SAMe (50mg/kg) had no significant effect (table 3.12.1).There was no correlation between blood glutathione content and the severity of hind paw swelling

Treatment	tissue	total glutathione content / g
Saline + inflammation	liver	1.97 ± 0.17 mg
Saline - inflammation	liver	1.94 ± 0.16 mg
SAMe + inflammation	liver	1.83 ± 0.16 mg
SAMe - inflammation	liver	2.10 ± 0.08 mg
Adenosine + inflammation	liver	1.85 ± 0.17 mg
MTA + inflammation	liver	1.65 ± 0.13 mg
Saline + inflammation	blood	326 ± 36 µg
Saline + inflammation	blood	343 ± 29 µg
SAMe + inflammation	blood	354 ± 23 µg
SAMe + inflammation	blood	334 ± 41 µg

table 3.12.1 Total glutathione content (GSH+GSSG) in liver and blood samples from animals treated with saline, SAMe (± carrageenan induced hind paw inflammation) MTA and adenosine (50mg/kg).Results are means ± SEM (n=5).

3.13 Effect of SAMe and MTA on inflammatory mediators and irritants

3.13.1.1 Effect of SAMe on arachidonic acid induced rat hind paw oedema

Arachidonic acid (0.5%) in carbonate buffer (0.2M, pH 8.3) injected into the sub-plantar region of the hind paw caused a severe oedema which as shown in fig 3.13.1.1 reached a maximum after 30 minutes of 78±11% (n=5).Table 3.13.1 shows that SAMe at doses of 50,75 and 100mg/kg i.p had no significant effect in reducing oedema.Dexamethasone (0.1 mg/kg) significantly reduced the maximum oedema to 35±3.4% (n=5, P<0.01).

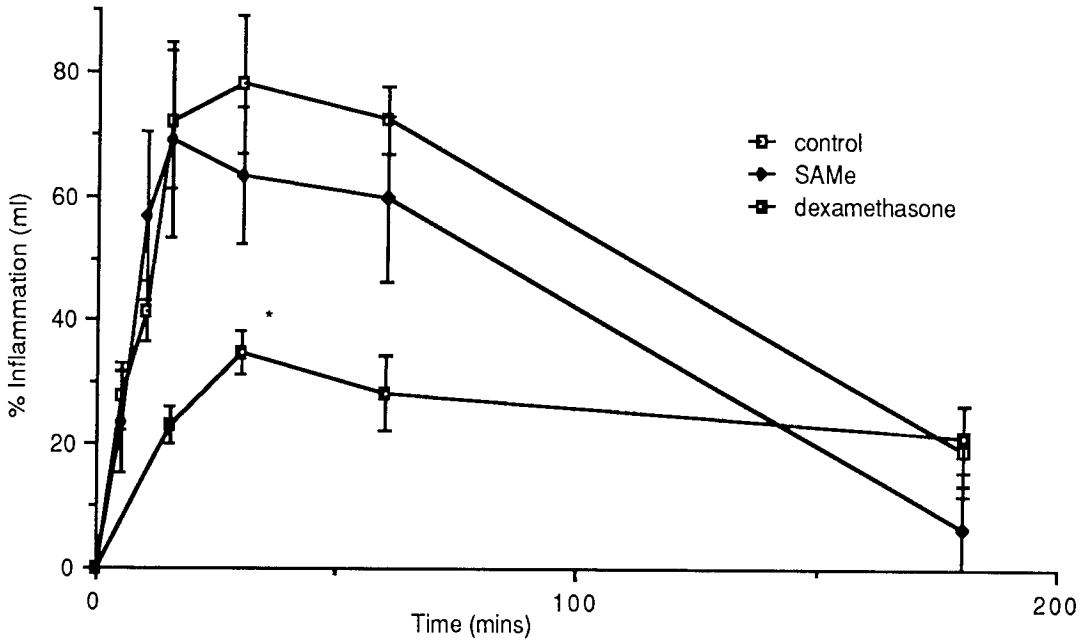


Fig 3.13.1.1. Effect of SAMe (50mg/kg) and dexamethasone (0.1mg/kg) on 0.5% arachidonic acid induced rat hind paw oedema. Results are presented as the mean % increase in inflammation (ml) ± SEM (n=5). Dexamethasone exerted significant inhibition of the maximum response (* P<0.01).

Dose SAMe (mg/kg)	maximum % inflammation (ml)
0	78 ± 11
50	65 ± 11
75	73 ± 15
100	71 ± 13

Table 3.13.1 Lack of inhibitory effect of SAMe in the dose range 50 - 100 mg/kg on 0.5% arachidonic acid induced rat hind paw inflammation .Results are means ± SEM (n=5)

3.13.1.2 Effect of MTA on arachidonic acid induced rat hind paw oedema

Table 3.13.2 shows that the administration of MTA at doses of 50 ,75, and 100mg/kg had no significant effect on arachidonic acid induced rat hind paw oedema.

Dose MTA (mg/kg)	Maximum % inflammation (ml)
0	69 ± 9
50	71 ± 10
75	73 ± 12
100	73 ± 14

Table 3.13.1.2 Lack of inhibitory effect of MTA in the dose range (50 - 100 mg/kg) on 0.5% arachidonic acid induced rat hind paw oedema. Results are means ± SEM (n=5).

3.13.2.1 Effect of SAMe on histamine induced rat hind paw oedema

Histamine (200μg) induced a rapid oedematous response which peaked at 30 minutes (fig 3.13.2.1).In contrast to arachidonic acid induced oedema approximately 70% of the maximal mean swelling of 65±8% (n=13) was generated within 5 minutes.The adminstration of SAMe (50mg/kg) had no significant effect on the first rapid 5 minute phase with a mean value of 41±10.5% (n=12) as compared to the mean saline value of 40±8.8% (n=13).However the SAMe group achieved maximum oedema at 10 minutes after which the swelling subsided.The two groups were significantly different after 20 minutes ($P<0.05$).After 60 minutes post inoculation the SAMe treated group exhibited a highly significant reduction of oedema to only 18.6±4% (n=12, $P<0.001$), approximately a third of the control value.Thus the results suggest that SAMe had no effect on the speed of induction or the magnitude of the first 5 minute phase of histamine oedema but the second phase was significantly inhibited and therefore recovery from the oedema was much faster. The drug displayed no crudely obvious analgesic effect but it was observed that the erythema stimulated by histamine was relieved faster than the control.Indeed paw colour was virtually normal after 60 minutes whereas the saline group typically showed obvious erythema.

The results of a dose response study are shown in fig 3.13.2.1.2. Animals were dosed with 10, 25 and 50mg/kg and the response was measured at 30 and 60 minutes.In contrast to the inhibitory effect of SAMe on carragenan induced inflammation SAMe doses of 10 and 25 mg/kg also significantly inhibited histamine induced oedema after 30 minutes ($P<0.05$). The degree of inhibition was not significantly different between doses.However after 60 minutes a dose dependent relationship was clear since the efficacy of SAMe at 50mg/kg was almost twice that of 10 and 25mg/kg.

Mepyramine (10mg/kg) a histamine H1 blocker suppressed histamine induced oedema after 30 minutes to 22.4±6.4% (n=5).The efficacy of SAMe (50mg/kg) was equivalent to mepyramine in this model but the potency was at least 1/5.

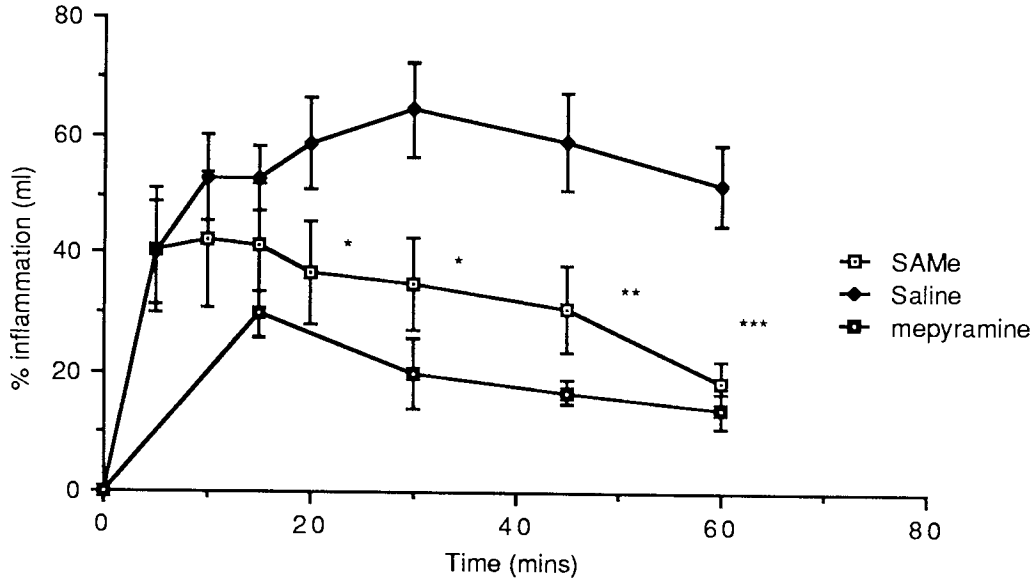


Fig 3.13.2.1.1 Time course of histamine induced rat hind paw oedema in saline , SAMe (50mg/kg) and mepyramine (10mg/kg) treated animals.The results are expressed as the mean % increase in inflammation (ml) in each group ± SEM (n=13).

* P<0.05,** P<0.01, ***P<0.001.

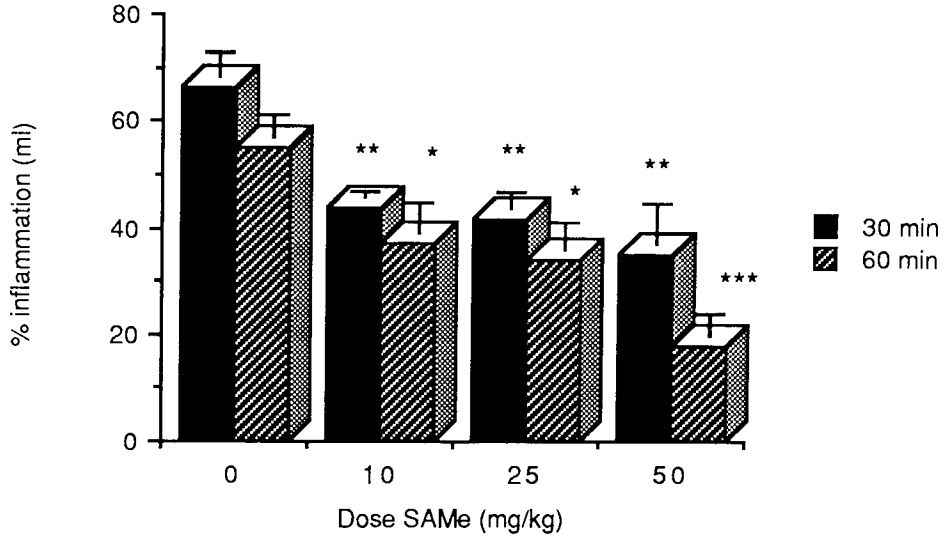


Fig 3.13.2.1.2. Dose dependent inhibition of histamine induced rat hind paw oedema by SAMe is only apparent after 60 minutes.Results are presented as the mean ± SEM % inflammation (ml) for each group. *P<0.05, **P<0.01, ***P<0.001

3.13.2.2 Effect of MTA on histamine induced hind paw oedema

Fig 3.13.2.2.1 illustrates the inhibitory effect of MTA (50mg/kg) on histamine induced hind paw oedema. In contrast to the action of SAMe MTA strongly inhibited the initial fast phase of the oedema by about 60%.The maximal response at 30 minutes was significantly reduced from 70.6±7% (n=7) to 27.4±6% (n=7, $P<0.001$)but was significant at all time points.The overall effect on oedema after 60 minutes was similar for both drugs; the suppression of oedema to 18.6±4% (n=12) by SAMe was comparable 17.8±4.8% (n=7) by MTA.Thus the main difference in action between the two drugs was that SAMe accelerated the subsidence of the initial oedema upon which it had no effect . Whereas MTA strongly inhibited the initial phase but the recovery from the initial oedema was comparatively slow as there was no significant difference between the oedema after 10 and 60 minutes.

A study of the effect of MTA at doses of 10, 25 and 50 mg/kg on the maximal swelling induced by histamine after 30 minutes revealed no significant difference in the degree of inhibition as shown in fig 3.13.2.2.2.However after 60 minutes there was a significant difference ($P<0.05$) between the inhibition exerted by 10mg/kg and 50 mg/kg with mean % swelling values of 28±3.3 (n=5) and 17.8±5 (n=7) , respectively.The pattern and magnitude of the inhibition of histamine induced oedema by MTA was comparable to that of 10mg/kg mepyramine.

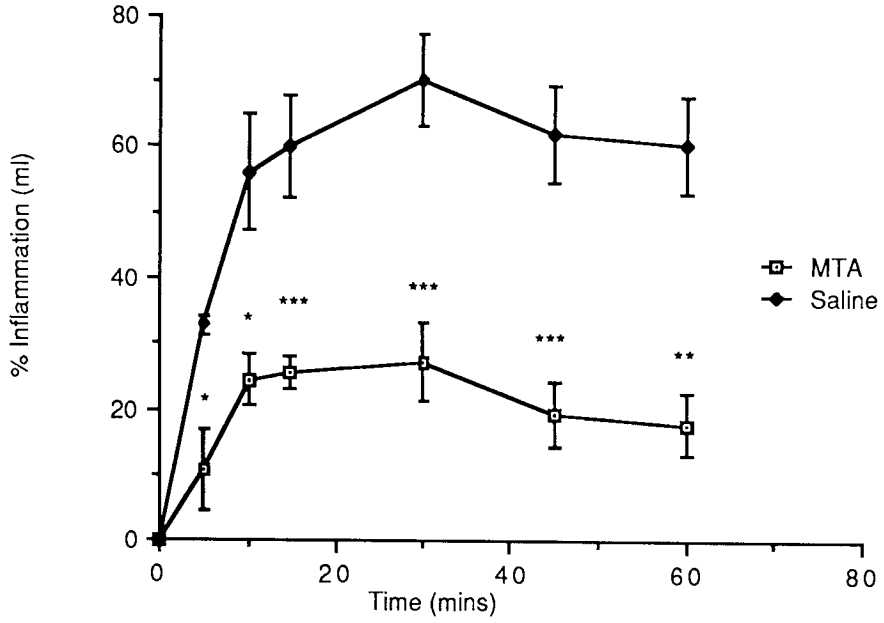


Fig 3.13.2.2.1 Time course of histamine induced rat hind paw inflammation in groups treated with saline (DMSO/distilled water) and MTA (50mg/kg).Results are presented as the mean % increase in inflammation ± SEM (n=7).* P<0.02, **P<0.002, ***P<0.001.

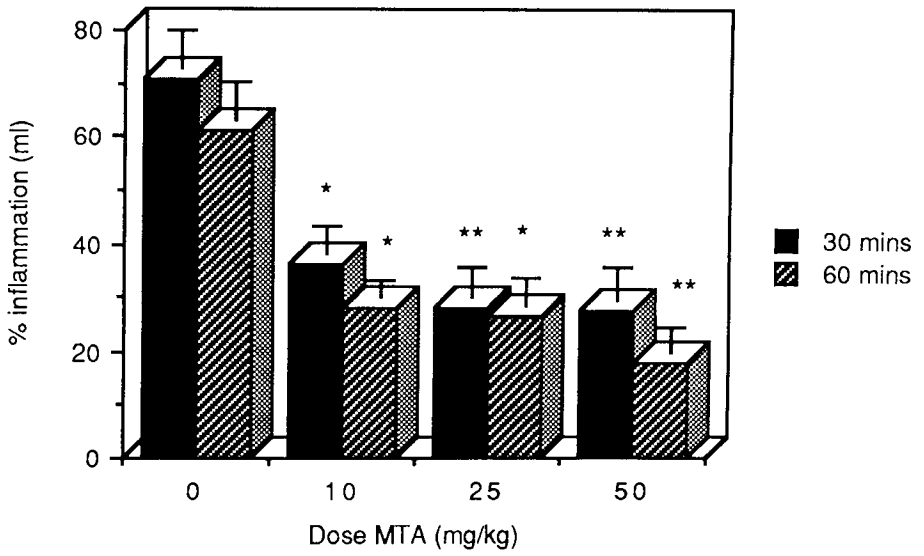


Fig 3.13.2.2.2 Inhibition of histamine induced rat hind paw inflammation by MTA in the dose range 10 - 50 mg/kg.Dose dependent inhibition is apparent after 60 minutes.Results are expressed as the mean % increase in paw inflammation ± SEM for each group (n=5).*P<0.02, **P<0.002.

3.13.3 Effect of SAMe and MTA on compound 48/80 induced rat hind paw oedema

Compound 48/80 (100μg) induced massive hind paw inflammation generating a mean % swelling of 160±16% (n=5) within 15 minutes (fig 3.13.3.1).Treatment with SAMe (50mg/kg i.p) caused an even greater oedematous response of 185±27 % (n=5) after the same interval but this was not significant.

The bolus of compound 48/80 was reduced to 20μg and gave a more typical magnitude of response.Maximal inflammation at 30 minutes was 110±5.3% (n=5).Treatment with MTA (50mg/kg) had no significant effect on the time course or the magnitude of the oedema (fig 3.13.3.2).

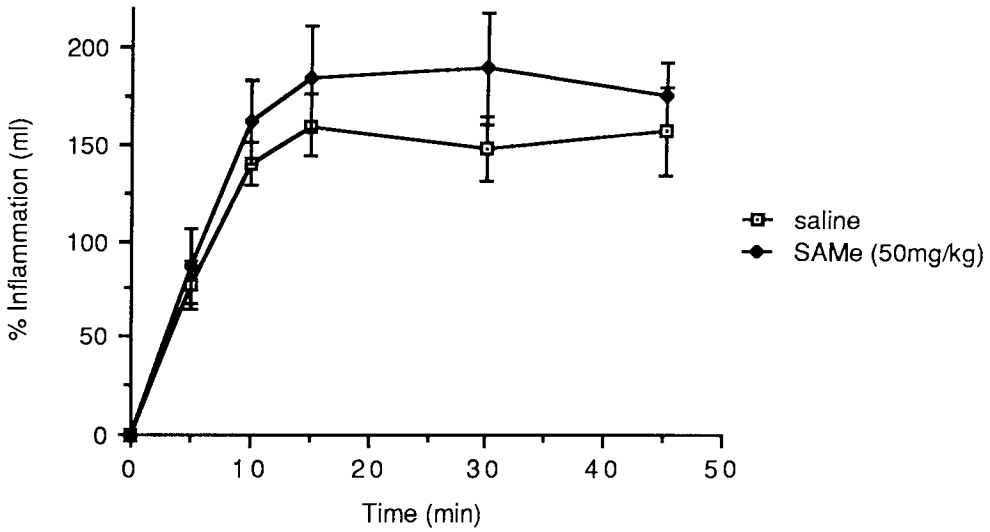


Fig 3.13.3.1 Effect of SAMe (50mg/kg) on compound 48/80 induced rat hind paw oedema.Results are expressed as mean % increase in paw inflammation ± SEM (n=5).

.

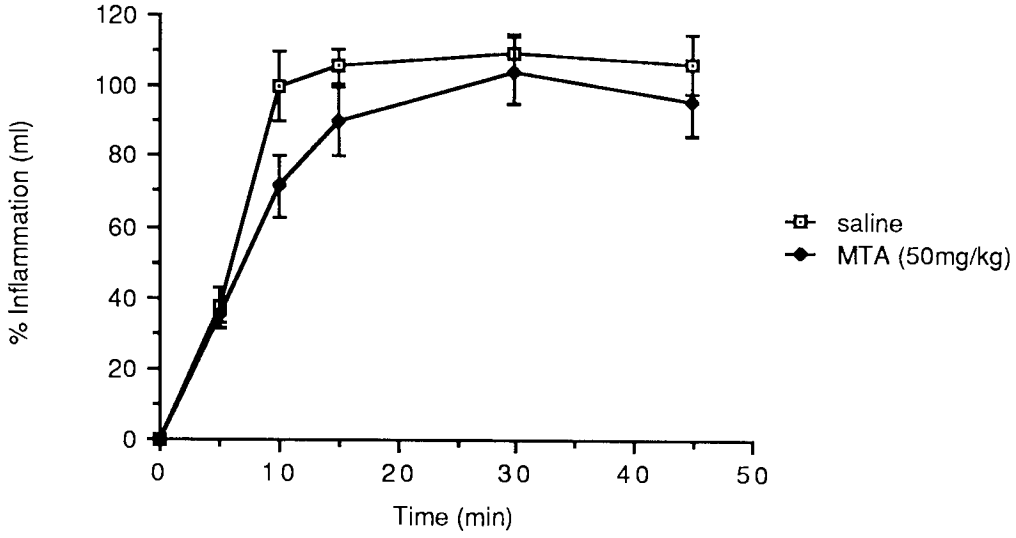


Fig 3.13.3.2 Effect of MTA on compound 48/80 induced rat hind paw oedema.Results are expressed as mean % increase in paw inflammation ± SEM (n=5).

3.13.4 Effect of SAMe on dextran induced hind paw inflammation

Dextran 1% (RMM=77,800) in 0.9% sterile saline induced a maximal swelling of 116±13% (n=8) within 30 minutes (fig 3.13.4). In contrast to the previous inflammagens studied the swelling was sustained over 3 hours.Treatment with SAMe (50mg/kg) significantly inhibited the inflammatory response at 15 minutes from 108±12% (n=8) to 66.6±13 % (n=8, $P<0.05$) but had no significant effect at any other time interval.

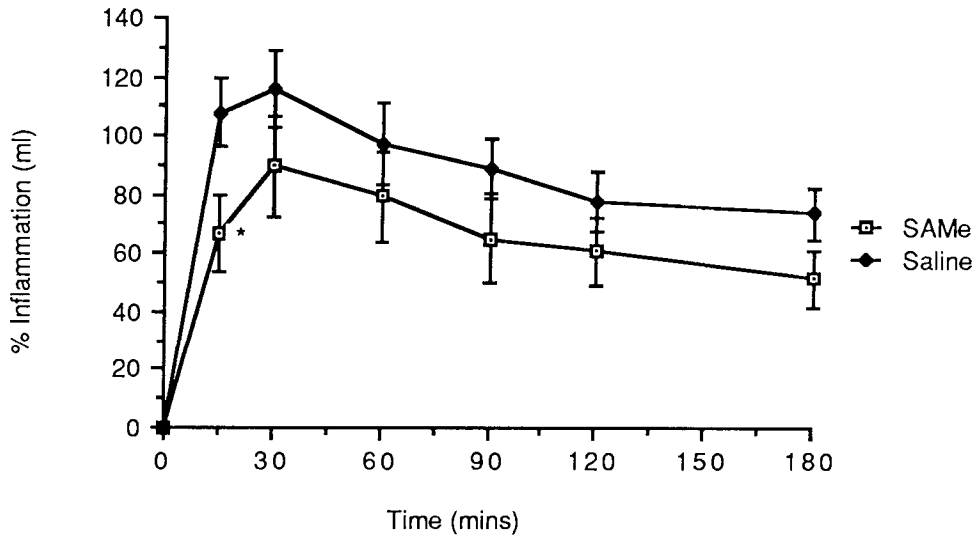


Fig 3.13.4. Effect of SAMe (50mg/kg) on 1% dextran induced rat hind paw inflammation.Results are expressed as mean % increase in inflammation (ml) ± SEM (n=5). Significant difference at 15 minutes *$P<0.05$.

3.13.5 Effect of SAMe on zymosan induced hind paw inflammation

Fig 3.13.5 shows that zymosan A (1mg) induced a massive inflammatory response of 144±10% (n=5) within 30 minutes of inoculation.Treatment with SAMe at the standard dose of 50mg/kg had no significant effect on the time course or the magnitude of the oedema.

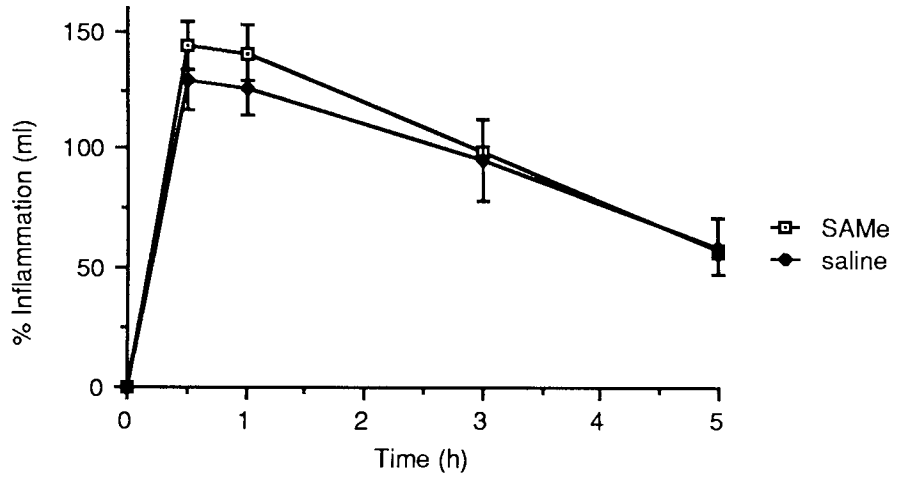


Fig 3.13.5 Effect of SAMe on zymosan (1mg) induced rat hind paw inflammation. Results are expressed as mean % increase in inflammation (ml) ± SEM (n=5).

3.13.6 Effect of SAMe on 5-HT induced hind paw inflammation

The potency of 5-HT (10μg) was approximately 15-20x greater than histamine in inducing rat hind paw oedema (fig 3.13.6). As was the case with histamine approximately 70% of the maximal oedema induced by 5-HT (137±14% , n=5) was generated within 15 minutes.The oedema development was greater and more sustained than that caused by histamine . Maximum response was achieved after 45 minutes .In comparison histamine induced oedema had already started to subside.

Drug treatment with SAMe (50mg/kg) had no significant effect on the first ten minutes of oedema development but exerted significant inhibition ($P<0.05$) after 15 minutes. The oedema was reduced from 119±9.5% (n=5) to 89±9% (n=5).The oedema was arrested beyond this time point and unlike the effect of SAMe on histamine induced

oedema showed little evidence of accelerating recovery from the oedema.There was however a significant difference in the mean oedema value between the two groups after 45 minutes ($P<0.05$).In comparison with the inhibitory effect of SAMe upon histamine induced oedema this was modest.

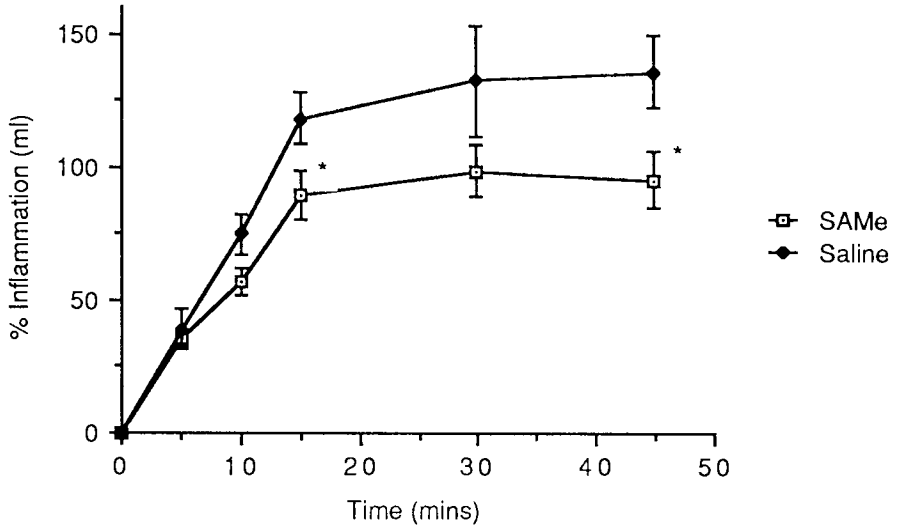


Fig 3.13.6 Time course of 5-HT induced rat hind paw oedema in animals treated with saline or SAMe (50mg/kg). Results are expressed as the mean % increase in inflammation (ml) ± SEM in each group (n=5).*$P<0.05$

3.13.7 Effect of SAMe on glucose oxidase induced hind paw inflammation

A 100µl aliquot of glucose oxidase solution (25 units/ml) invoked an inflammatory response equal to that of compound 48/80 or 5-HT (fig 3.13.7.).Approximately 90% of the maximal response of 96.6±13.6 (n=5) was generated within 30 minutes and was strongly sustained even over 72 hours (93±11.2%).Treatment with SAMe (50mg/kg) had no significant effect the time course or magnitude of the inflammatory episode.Co-administration of 100 units of catalase with glucose oxidase significantly inhibited the progress of the oedema ($P<0.005$) from 96.2±14% (n=5) to 42 ±6% in the first 30 minutes and was sustained throughout.

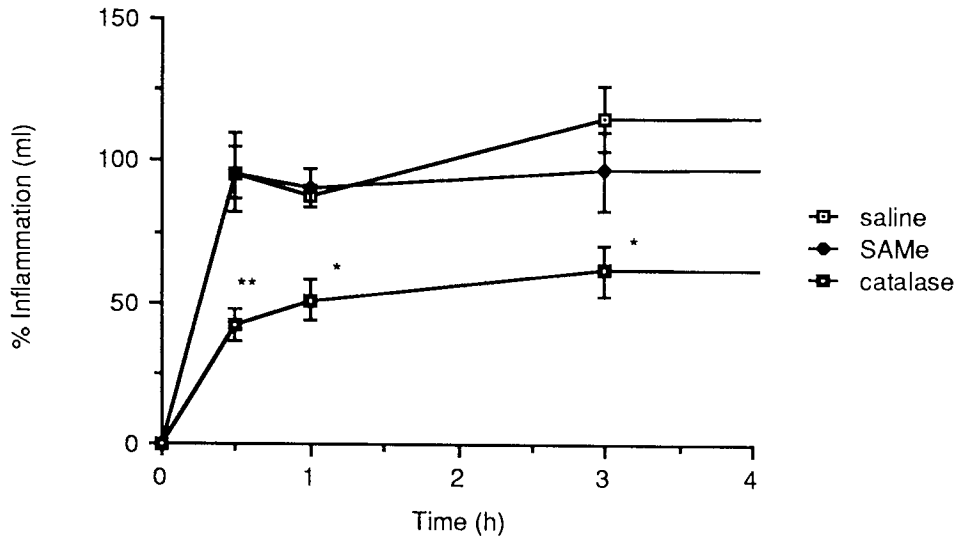


Fig 3.13.7 Time course of the effect of SAMe (50mg/kg) and catalase (100 units / ml) on glucose oxidase (25u / ml) induced rat hind paw oedema. Results are expressed as the mean % increase in inflammation (ml) ± SEM for each group (n=5).* P<0.01, **P<0.002.

3.13.8 Effect of SAMe and MTA on PAF induced hind paw inflammation

3.13.8.1 Effect of SAMe on PAF induced oedema

The inflammatory effect of PAF is illustrated in fig3.13.8.1.1. A 100µl aliquot (200ng) of PAF induced severe oedema equivalent to that induced by 5-HT.The potency of PAF was approximately 50x greater than 5-HT and 500-1000x greater than histamine.Maximum oedema of 86±6% (n=12) was generated within 30 minutes.After which the oedema gradually subsided over the course of 3 hours.However after this period a substantial oedema of 49.3±4% persisted.

Treatment with SAMe (50mg/kg) inhibited the magnitude and the time course of PAF induced oedema .The maximal response at 30 minutes was significantly reduced from 86±6% (n=12) to 57±3.5% (n=13, P<0.001) and was significantly less throughout the course of the inflammatory episode.Minimum inhibitory effect was observed after 180 minutes where the response of the control was reduced from 49.3±4% to 36.4±1.9% (n=12, P<0.02).

The result of a dose response study is shown in fig 3.13.8.1.2 .The inflammatory response to PAF was measured at 30 minutes.Doses of 12.5 and 25 mg/kg had no significant effect on the oedema magnitude or course of development . As was found with the anti-inflammatory effect of SAMe against carrageenan and histamine induced oedema the most effective dose of SAMe was 50mg/kg.

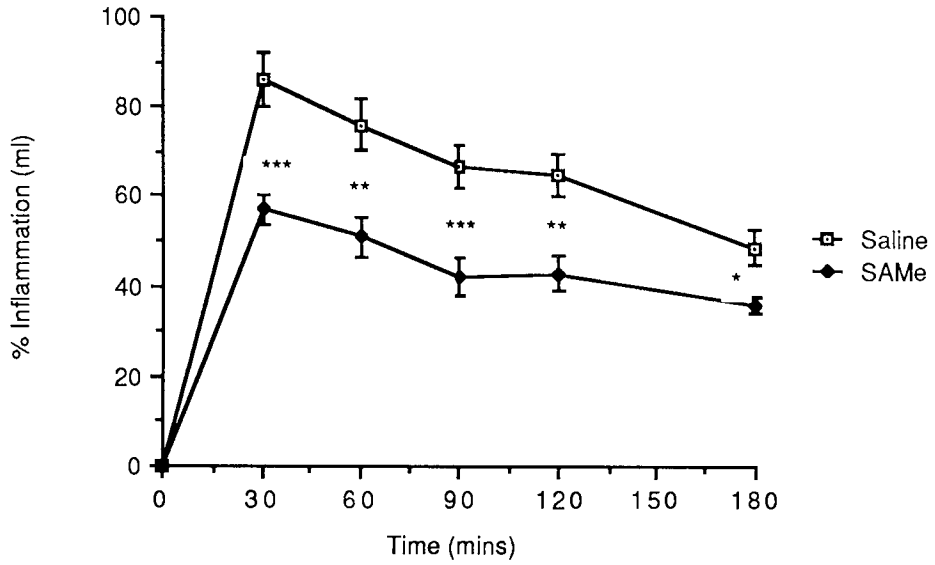


Fig 3.13.8.1.1 Time course of PAF induced rat hind paw oedema in animals treated with saline and SAMe (50mg/kg).Results are presented as mean % increase in inflammation (ml) ± SEM for each group (n=12 and 13 respectively). *P<0.02, **P<0.002, *** P<0.001.

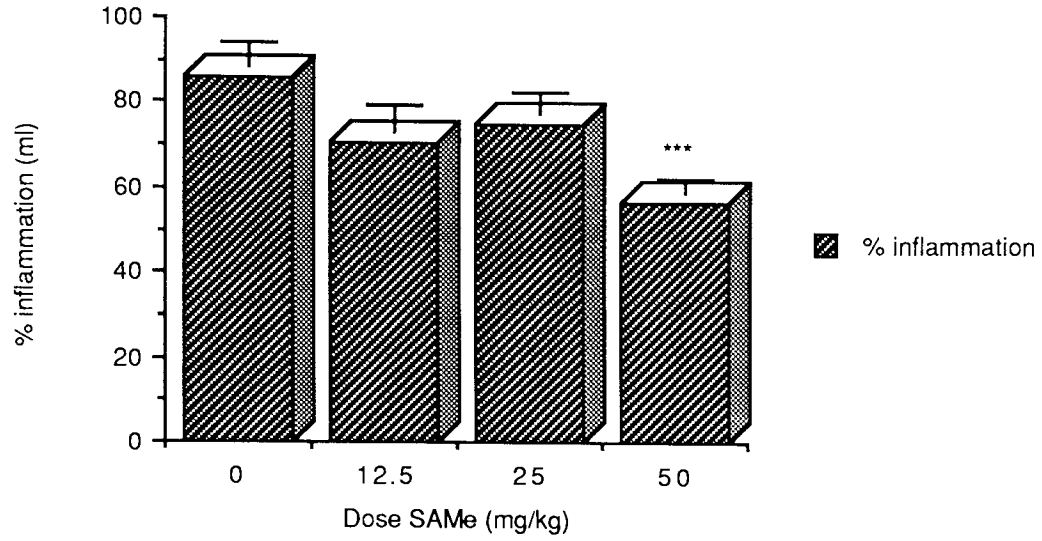


Fig 3.13.8.1.2. PAF induced rat hind paw oedema was only inhibited by SAMe at 50 mg/kg. Results are presented as maximum % increase in inflammation (ml) ± SEM for ecah group (n=13 for 50mg/kg and n= 5 for 12.5 and 25 mg/kg SAMe).

*** P<0.0.001

3.13.8.2 Effect of MTA on PAF induced hind paw oedema

Dramatic inhibition of PAF induced oedema by MTA (50mg/kg) is illustrated in fig 3.13.8.2.1.The maximal response was massively reduced from 85.1±4.3 (n=8) at 45 minutes to 36.6±4.6% (n=10, P<0.0005).Maximal oedema in MTA treated animals was retarded to 60 minutes by which time the control group had started to recover.The oedema was thus suppressed throughout the 3 hour assay.In comparison with SAMe the recovery of MTA treated animals from the oedema was rapid. The mean % oedema of 13.7±3.5% (n=10) after 3 hours was less than half of the SAMe treated group.

A comparison of the effectiveness of inhibition of the maximal response revealed MTA (50mg/kg) to be significantly more efficacious (P<0.001) than the same dose of SAMe.

An analysis of any possible effect of the DMSO/distilled water vehicle for MTA on PAF induced oedema showed no significant difference at any time point between this group and the control group treated with 0.5ml sterile saline i.p in the SAMe investigation.

Since the inhibitory effect of SAMe on PAF induced oedema was only apparent at 50mg/kg it was of interest to know if this narrow therapeutic window applied to MTA.Fig 3.13.8.2.2 shows the maximal % swelling induced by PAF in animals treated with 12.5, 25, 50 and 100mg/kg MTA i.p.The lowest dose of 12.5 mg/kg failed to inhibit oedema but 25mg/kg was significant ($P<0.05$) and suppressed the maximal % oedema to 59.6±8 (n=5).As illustrated in fig 3.13.8.2.3 the lower dose significantly enhanced recovery and at the 60 minute interval reduced the oedema to 45.3±7% (n=5). This value was not significantly different from the effect observed for the 50mg/kg group.Fig 3.13.8.2 and 3 both illustrate that the inhibitory effect of the maximum dose of 100mg/kg was not significantly different from that of 50mg/kg.Clearly MTA was both more potent and had a greater efficacy than SAMe since the inhibitory effect of the optimal dose of the latter was equivalent to that of 25mg/kg MTA.

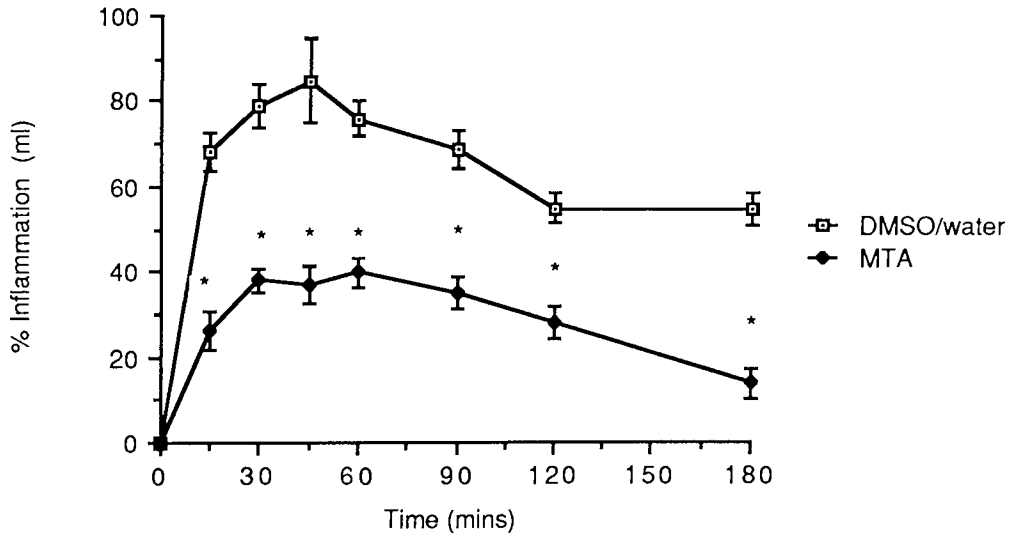


Fig 3.13.8.2.1. Time course of the effect of MTA (50mg/kg) on PAF induced rat hind paw oedema.Results are presented as mean % increase in inflammation ± SEM for each group (n=5).Significant inhibition *$P<0.001$ throughout.

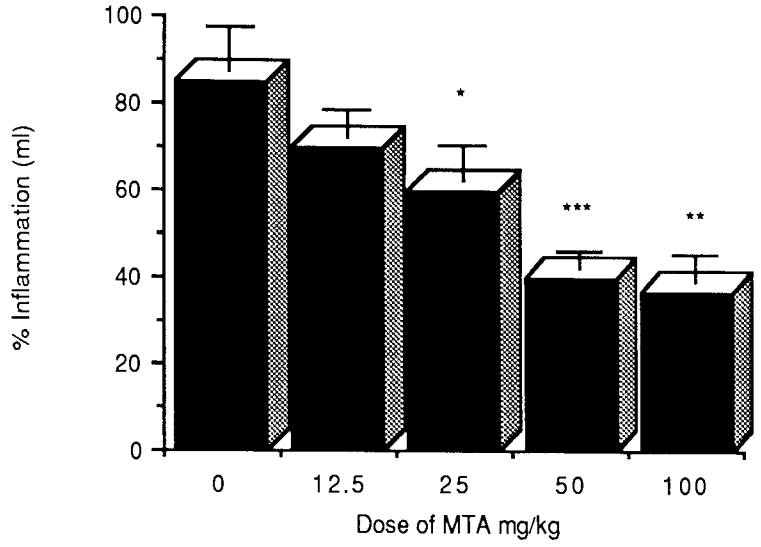


Fig 3.13.8.2.2 Dose dependent inhibition of PAF induced rat hind paw oedema by MTA.Results are presented as mean % increase in inflammation (ml) ± SEM for each group (n=5).*P<0.05, **P<0.002, ***P<0.001.

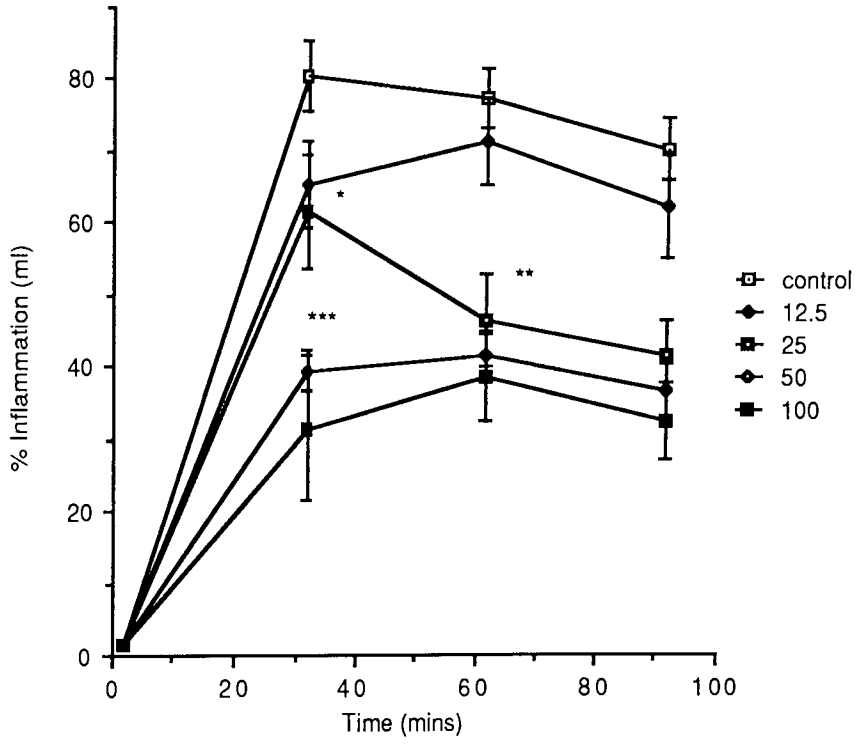


Fig 3.13.8.3.3.Time course of PAF induced rat hind paw inflammation in animals treated with doses of 12.5, 25, 50 and 100mg/kg MTA.Results are presented as mean % increase in inflammation (ml) ± SEM for each group (n=5). *P<0.05, **P<0.01, ***P<0.001.

3.13.8.3. Effect of isoprenaline on PAF induced hind paw oedema

Isoprenaline , a ß1+2 adrenoreceptor agonist with potent cardiovascular effects has been credited with anti-inflammatory effect in the carrageenan hind paw model (Mohd-Hidir and Lewis, 1984). The release of PAF-like materials has been implicated in the aetiology of the first phase of carrageenan induced hind paw oedema (Hwang et al 1986). Thus it was of interest to know whether isoprenaline would also inhibit PAF induced hind paw oedema.

The results in fig 3.13.8.3.1 show that isoprenaline 8mg/kg i.p strongly suppressed the maximal response from 99.5±6.8% (n=5) to 34.5±1.6%. Recovery was slow and was held at a plateau over the subsequent hour.The potency of the drug was most striking. Significant inhibition of oedema (P<0.001) was observed at doses as low as 0.5mg/kg and the efficacy was equivalent to 50mg/SAMe.The results of a dose response study illustrated in fig 3.13.8.3.2 show that the optimum inhibitory dose was 4mg/kg.

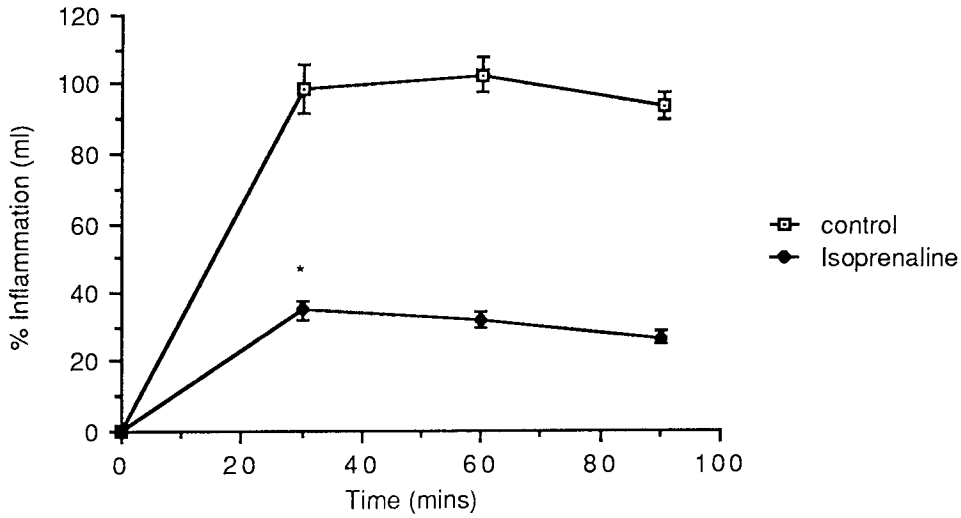


Fig 3.13.8.3.1 Time course of the inhibition of PAF induced rat hind paw oedema by isoprenaline (8mg/kg).Results are expressed as mean % increase in inflammation (ml) ± SEM in each group (n=5), *P<0.001.

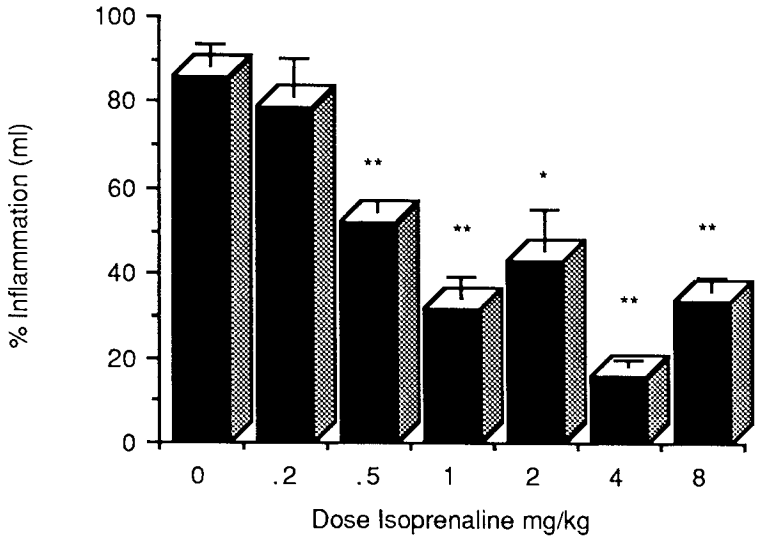


Fig 3.13.8.3.2. Dose response relationship between isoprenaline (0.2-8 mg/kg) and PAF induced rat hind paw inflammation.Results are presented as the mean % increase in paw inflammation (ml) ± SEM for each group (n=5),*P<0.01, **P<0.001.

3.13.8.4 Effect of SAMe in combination with isoprenaline on PAF induced hind paw oedema

Isoprenaline and SAMe were both found to inhibit PAF induced oedema. It was of interest to know if SAMe in combination with isoprenaline would have an enhanced anti-inflammatory effect in this model.The effect of the co-administration of SAMe (50mg/kg) with isoprenaline (0.2 - 8 mg/kg) is illustrated in fig 3.13.8.4. The mix of SAMe and isoprenaline (8mg/kg) was significantly different from SAMe alone but not from isoprenaline in isolation.At the optimal dose of 4mg/kg the SAMe and isoprenaline group showed a significant increase in maximal swelling of 36±7.5% compared to 16±1.2% for the isoprenaline control (n=5, P<0.05) but was not significantly different from the effect of the greater dose of 8mg/kg.The combination of SAMe with isoprenaline at 2mg/kg exerted an inhibitory effect significantly different from that observed for isoprenaline alone (P<0.05) but this was lost after 90 minutes.Doses of isoprenaline of 1mg and less in combination with SAMe showed no further enhancement of the anti-inflammatory effect of SAMe alone.

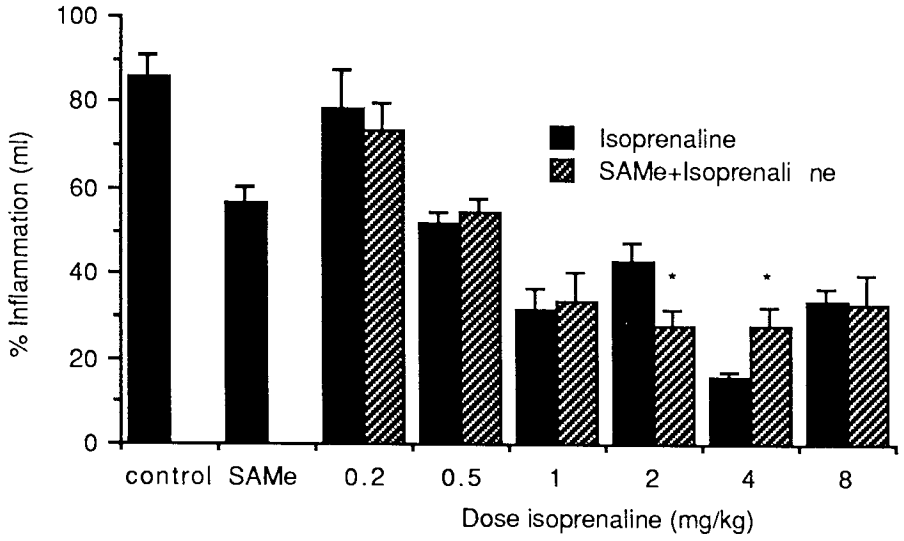


Fig 3.13.8.4. Comparison of the anti-inflammatory effect of isoprenaline alone and in combination with SAMe (50mg/kg).Results are expressed as the mean % inflammation (ml) ± SEM for each group (n=5) ,* P<0.05.

3.13.8.5 Effect of MTA in combination with isoprenaline on PAF induced oedema

Co-administration of the subthreshold dose of isoprenaline (0.2mg/kg) with MTA (50mg/kg) shown in fig 3.13.8.5 caused no enhancement of the inhibitory effect of MTA upon PAF induced hind paw oedema alone.

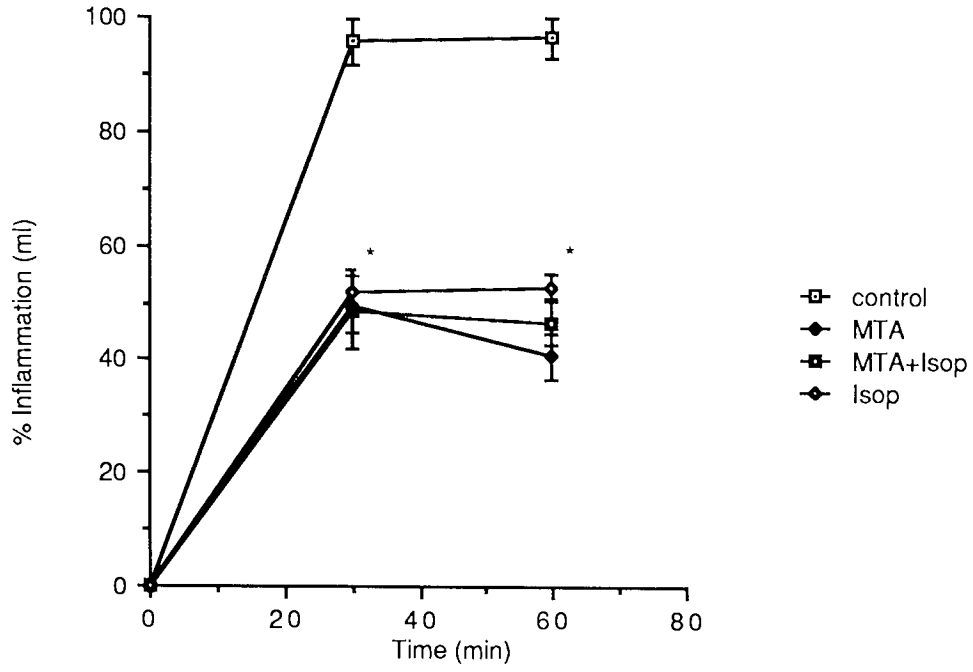


Fig 3.13.8.5. Time course of the effect of MTA (50mg/kg) and isoprenaline (0.5mg/kg) alone and in combination on PAF induced rat hind paw inflammation.Results are presented as the mean % increase in inflammation (ml) ± SEM (n=5),* P<0.001.

3.13.8.6 Effect of SAMe and MTA in combination with verapamil on PAF induced oedema

Administration of verapamil (10mg/kg) a calcium channel blocker , as shown in fig 3.13.8.6.1 significantly suppressed maximal PAF induced oedema from 81±5 to 49.6±4.8% (n=5, P<0.002).Co-administration with SAMe caused no further significant suppression of PAF oedema by these drugs.The combination with MTA (fig 3.13.8.6.2) however had no further inhibitory effect during the first hour but prevented the recovery from the oedema at 90 minutes promoted by the drugs in isolation .The mean % swelling after 90 minutes was not significantly different from the control (56±3% n=5) but was significantly greater than the MTA treated group (45.5±1.7, n=5, P<0.01) and the verapamil treated group (36.1±3.5% , n=5, P<0.001).

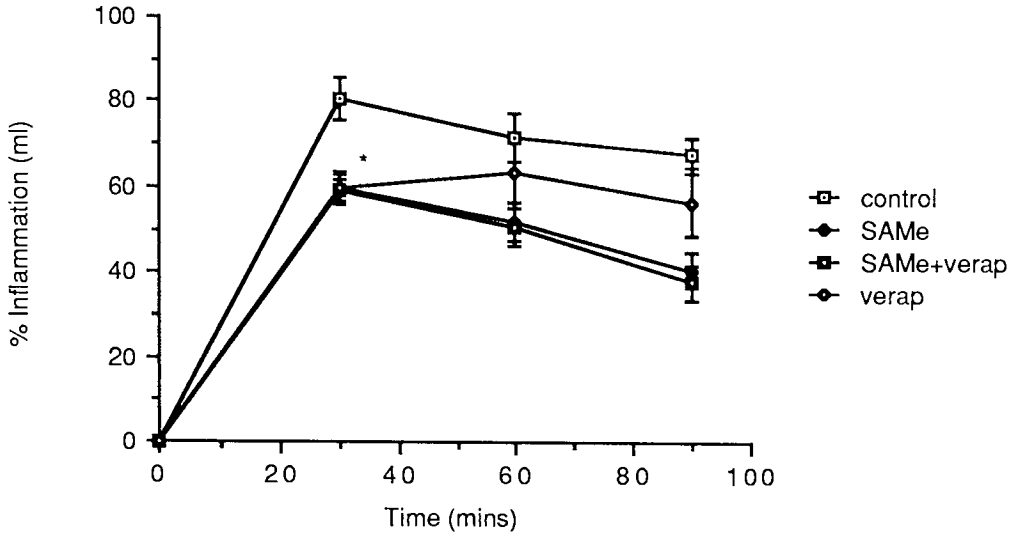


Fig 3.13.8.6.1.Time course of the effect of SAMe (50mg/kg) and verapamil (10mg/kg) on PAF induced inflammation.Results are expressed as mean % increase in inflammation (ml)±SEM for each group (n=5), *P<0.002

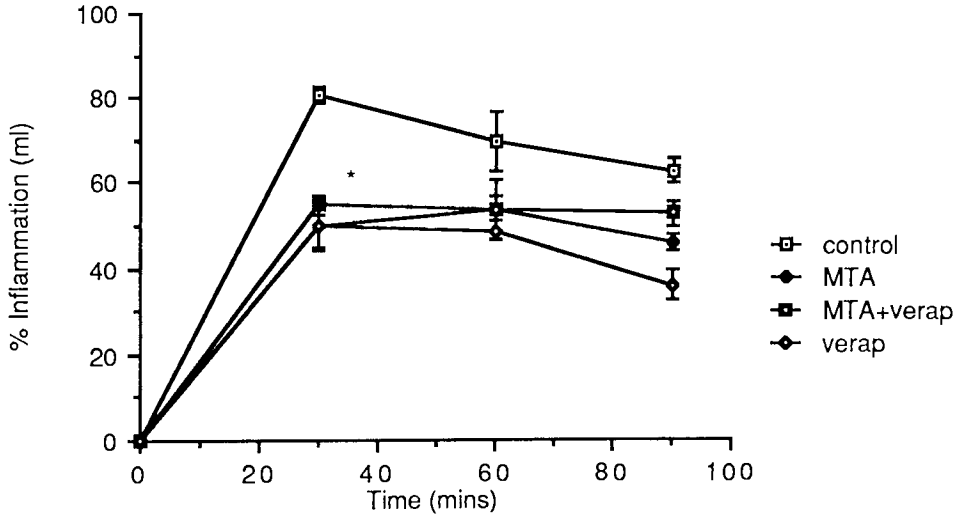


Fig 3.13.8.6.2 Time course of the effect of MTA (50mg/kg) and verapamil (10mg/kg) alone and in combination on PAF induced rat hind paw oedema. Results are presented as the mean % increase in inflammation (ml) ± SEM (n=5), * P<0.001.

3.14. Effect of SAMe on rat neutrophils in vitro : analysis of uptake and metabolites by HPLC

3.14.1 Effect of SAMe on intracellular levels of SAMe

The order and retention times of standards of SAMe and relevant metabolites are shown in table 3.14.1.Intracellular SAMe content of rat neutrophils was evaluated by HPLC analysis of perchloric acid soluble material from cells treated with buffered HBSS or [^{14}C] SAMe.The intracellular content of rat neutrophils incubated for 60 minutes in buffered HBSS was determined to be 36.5± 0.8 pmol / 10^6 cells (n=6). Fig 3.14.1.1 demonstrates that incubation of rat neutrophils with 0.25μCi SAMe (4.05μM) over the course of two hours had little effect on intracellular SAMe levels.After 120 minutes incubation ^{14}C SAMe the mean SAMe content was 38± 0.72 pmol / 10^6 cells (n=4).

Analysis of scintillation counts from 1 minute eluent fractions indicated the time course of uptake.Fig 3.14.1.2 shows that maximum uptake of SAMe occured after 15 minutes but that the isotope was within the intracellular SAMe pool within 5 minutes of exposure.The maximum amount of 'free' [^{14}C]- SAMe was 0.23 ± 0.087 pmol / 10^6 cells. After 120 minutes incubation this had decreased to 0.141 ± 0.015 pmol / 10^6 cells.Such minute changes in uptake were clearly beyond the limits of resolution of the HPLC assay.

A dose response study of SAMe uptake in the concentration range 0.004 - 1.25 μCi (equivalent to 68nM-21.4μM) illustrated in fig 3.14.1.3 shows that only the cells incubated in 1.25 μCi SAMe had significantly enhanced intracellular levels of SAMe to 43.7 ± 3.56 pmol / 10^6 cells (n=4 $P<0.05$).The data from HPLC and liquid scintillation counting provides evidence that SAMe is taken up in vitro by rat neutrophils but does not accumulate.

	retention time (mins)
adenosine	7.30
S-adenosyl-l-homocysteine	8.10
adenine	11.15
methylthioadenosine	14.20
S-adenosyl-l-methionine	28.00

Table 3.14.1 Retention times for SAMe and metabolite standards determined by cation exchange HPLC.The buffer system was 100mM ammonium dihydrogen phosphate pH 2.6

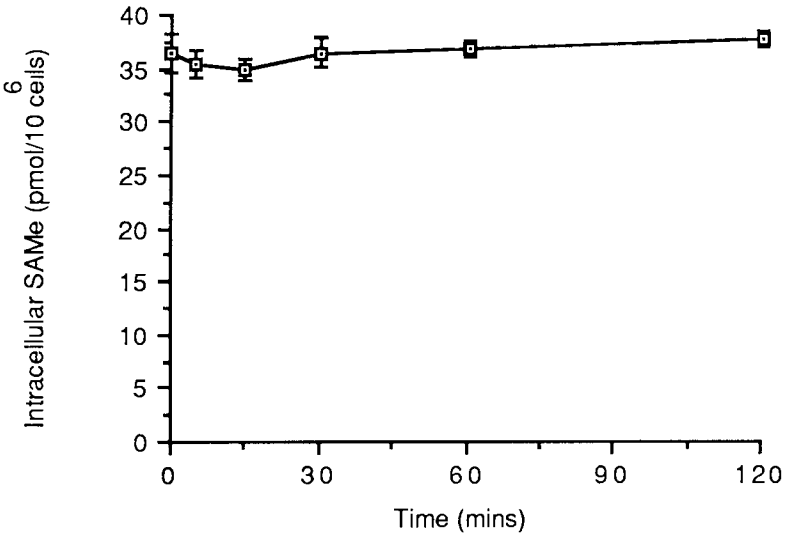


Fig 3.14.1.1. The effect of incubation of rat neutrophils with SAMe (4.05μM) on intracellular levels of SAMe (pmol / 10^6 cells) determined by HPLC.Results are presented as means ± SEM (n=4)

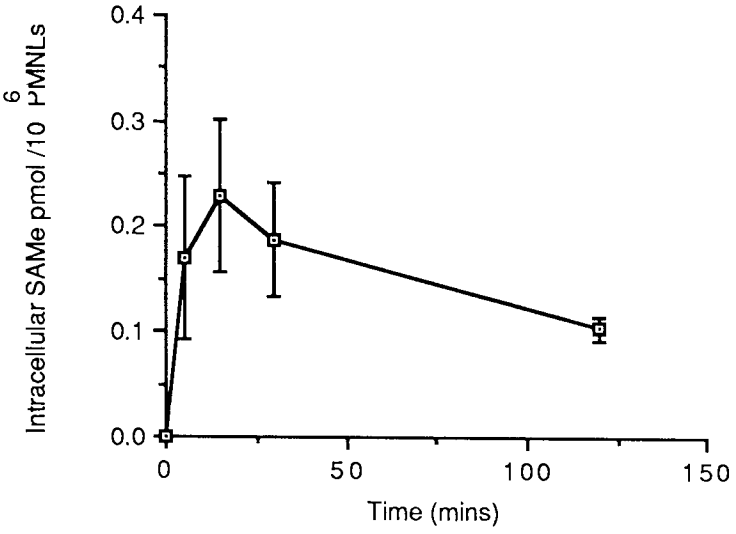


Fig 3.14.1.2. Time course of uptake of [^{14} C] SAMe by rat neutrophils in vitro.Results are expressed as means ± SEM (n=4).

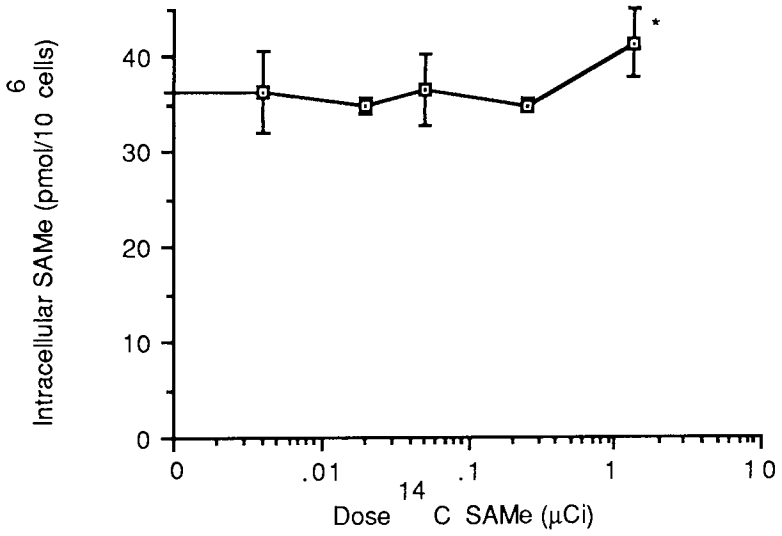


Fig 3.14.1.3. Effect of [^{14}C] SAMe (0.004 - 1.25μCi) on intracellular SAMe in rat neutrophils in vitro by HPLC. Results are expressed as means ± SEM (n=4), * P<0.05.

3.14.2 Effect of SAMe on intracellular levels of SAHc

The isocratic HPLC assay employed gave good distinct separation of standard SAHc from other SAMe metabolites but in practice with perchloric acid cell extracts the SAHc peak was lost in the peaks corresponding to adenosine and adenine.Therefore the data presented was solely from liquid scintillation counting of HPLC separated 1 minute fractions corresponding to the retention time for SAHc.For this reason also the basal level of SAHc was not successfully established.

Evidence from radio-HPLC analysis of rat PMNL incubated in [^{14}C] SAMe (0.25μCi) suggests that SAHc was formed from exogenous SAMe. A time course study (fig 3.14.2.1) revealed that the formation and accumulation of SAHc was time dependent and roughly linear over the first 30 minutes of incubation. Labelled intracellular [^{14}C] S-adenosyl-l-homocysteine was evident within 5 minutes of incubation and increased to 2.15 pmol / 10^6 cells (n=4) after 120 minutes.

Fig 3.14.2.2 demonstrates that the formation of [^{14}C] SAHc from SAMe was dependent on dose.Cells incubated in 1.25 μCi SAMe formed 9.52 pmol ± 0.85 pmol /10^6 cells of SAHc within 60 minutes.The formation of [^{14}C] SAHc was thus rapid and directly proportional to the concentration of exogenous SAMe.

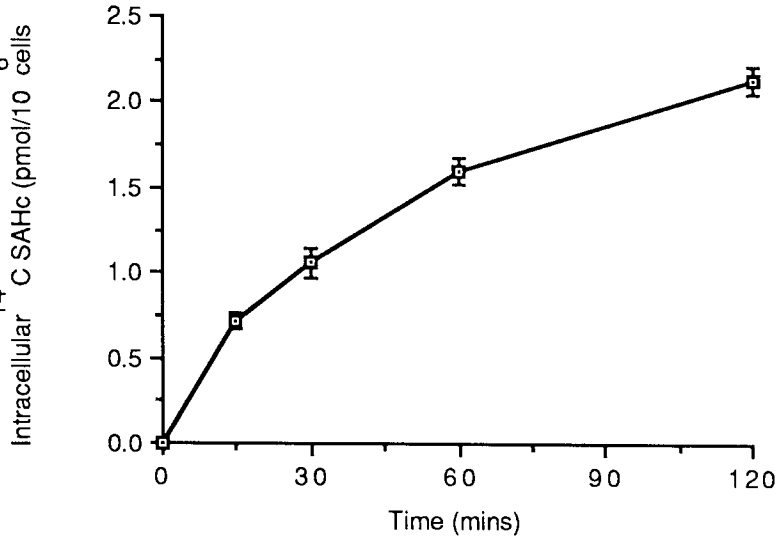


Fig 3.14.2.1. Time course of [^{14}C] SAHc formation in rat neutrophils incubated in SAMe (0.25μCi) in vitro. Results are expressed as means ± SEM (n=4).

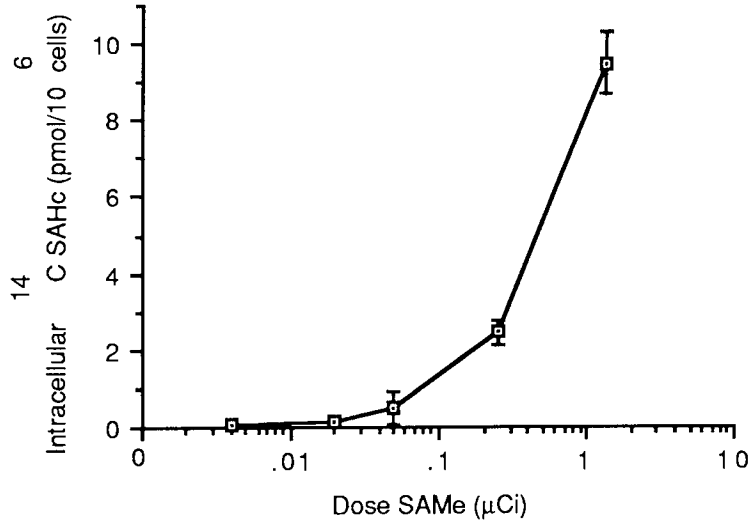


Fig 3.14.2.2. Dose dependent relationship between [^{14}C]- SAHc formation by rat neutrophils in vitro and [^{14}C]- SAMe concentration (0.004 - 1.25μCi). Results are expressed as means ± SEM (n=4).

3.14.3 Effect of SAMe on intracellular levels of adenosine

The basal level of adenosine in rat neutrophils was estimated by HPLC analysis to be 235±36 pmol /10^6 (n=6) cells.Incubation with 0.25μCi [^{14}C] SAMe had no significant effect on adenosine levels over 2 hours (fig 3.14.3.1.).Treatment with SAMe in the dose range (0.004 - 1.25 μCi) failed to significantly alter intracellular adenosine content (fig 3.14.3.2.)

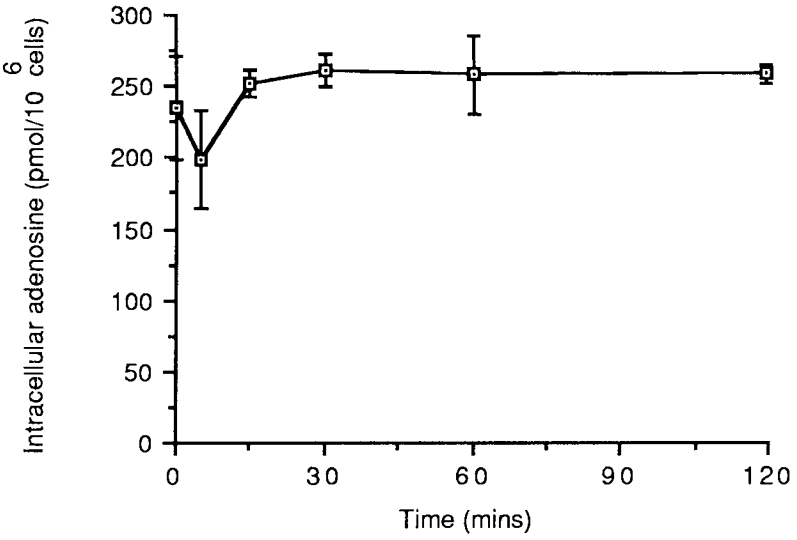


Fig 3.14.3.1. Time course of effect of [^{14} C] SAMe (0.25μCi) on intracellular adenosine content in rat neutrophils determined by HPLC.Results are expressed as means ± SEM (n=4)

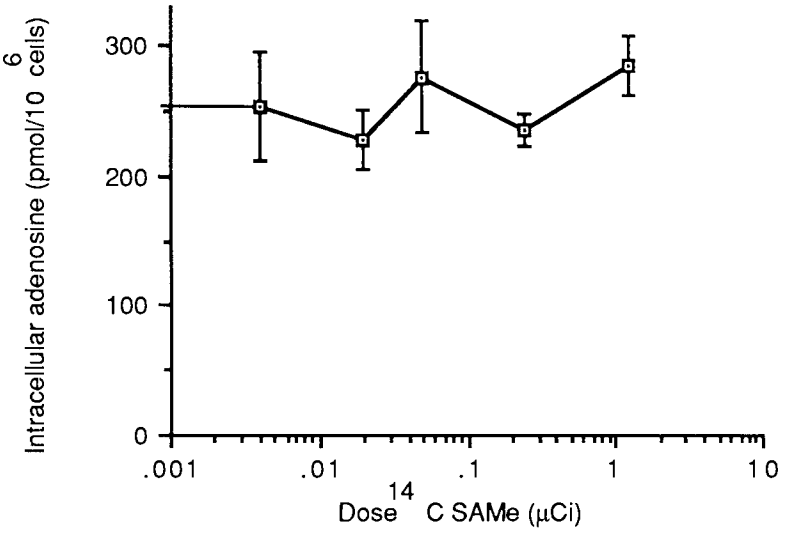


Fig 3.14.3.2.Lack of dose dependent effect of [^{14}C] SAMe (0.002 -1.25μCi) on intracellular adenosine content in rat neutrophils.Results are presented as means ± SEM (n=4).

3.14.4 Effect of SAMe on intracellular levels of MTA

The intracellular concentration of MTA was estimated by HPLC to be 13.4 ± 0.17 pmol / 10^6 cells (n=4). Incubation with 0.25 μCi [^{14} C]- SAMe had no significant effect on intracellular MTA content over 2 hours (fig 3.14.4.1). However a dose response study (fig 3.14.4.2) revealed that the MTA concentration in cells incubated in 1.25 μCi [^{14}C] SAMe had nearly doubled to 25.7 ± 0.82 pmol / 10^6 cells (n=4).

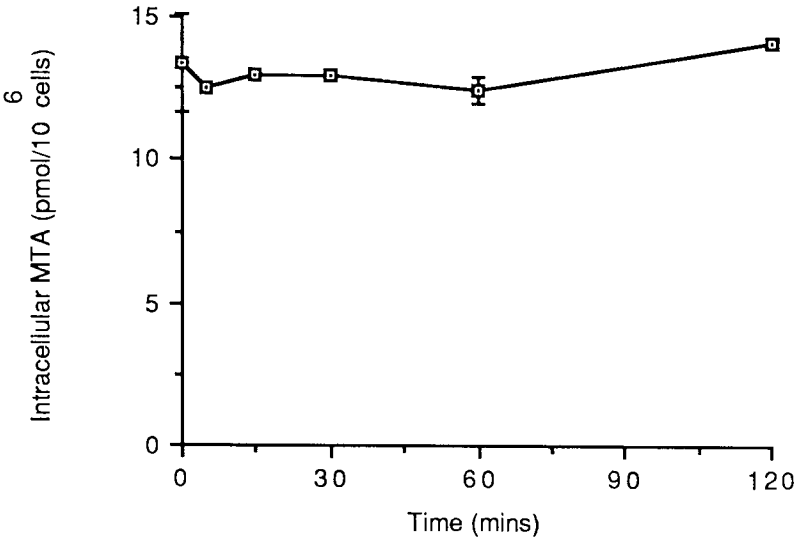


Fig 3.14.4.1. Time course of MTA metabolism in rat neutrophils incubated in 0.25μCi [^{14}C] SAMe.Results are expressed as means ± SEM (n=4)

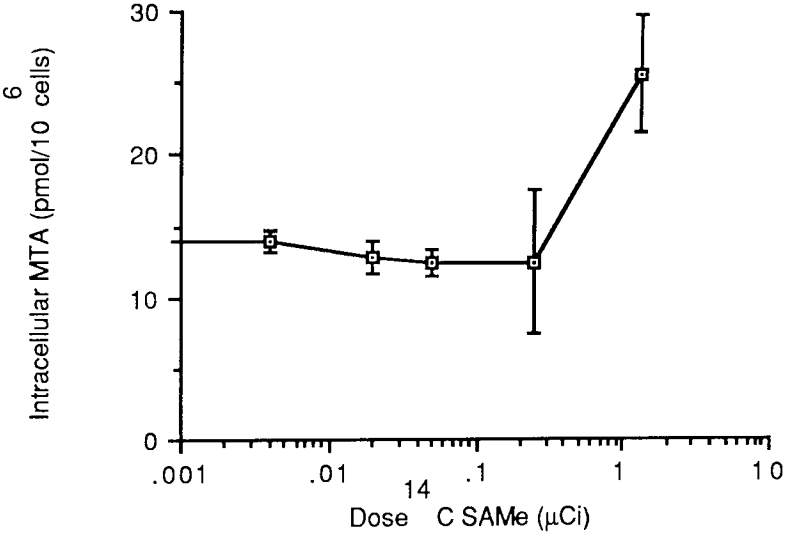


Fig 3.14.4.2. Effect of [^{14}C] SAMe (0.002 - 1.25μCi) on intracellular MTA in rat neutrophils incubated for 1hour.Results are presented as means ± SEM (n=4).

3.15 Analysis of SAMe pharmacokinetics by HPLC

Fig 3.15.1 illustrates the pharmacokinetics of SAMe (50mg/kg) administered intraperitoneally to male Wistar rats of 440-490g.The absorption of the single 0.5ml bolus of SAMe was rapid and reached a peak plasma concentration of 136.6 ± 37 nmol / ml (n=4) after 30 minutes.Since the basal level of plasma SAMe was 1.096 ± 0.116 nmol / ml this represents a 130 fold increase in SAMe concentration . The speed of clearance of SAMe from the circulation was as equally striking. From the pharmacokinetic data for 1animal (452g) the following parameters were established:

half life $t_{1/2}$:	27 minutes
Distribution volume :	305 ml
Clearance rate :	417ml / min

Table 3.15. Pharmacokinetics of SAMe *in vivo* .

After 3 hours the plasma concentration of SAMe had been reduced to 17±3.7nmol / ml and had returned to baseline after 5 hours. A comparison of animals treated with SAMe in the presence or absence of 2% carrageenan induced inflammatory stress failed to reveal any apparent difference in the pharmacokinetics of SAMe (fig 3.15.2). The plasma concentration of SAMe in saline treated control animals remained constant throughout the inflammatory episode.

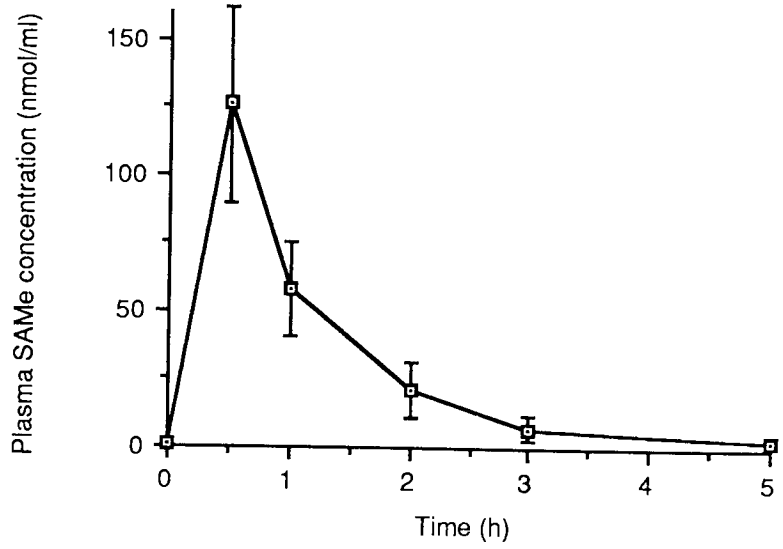


Fig 3.15.1 Time course of plasma SAMe concentration following the single intraperitoneal administration of SAMe (50mg/kg).Results are presented as means ± SEM (n=4).

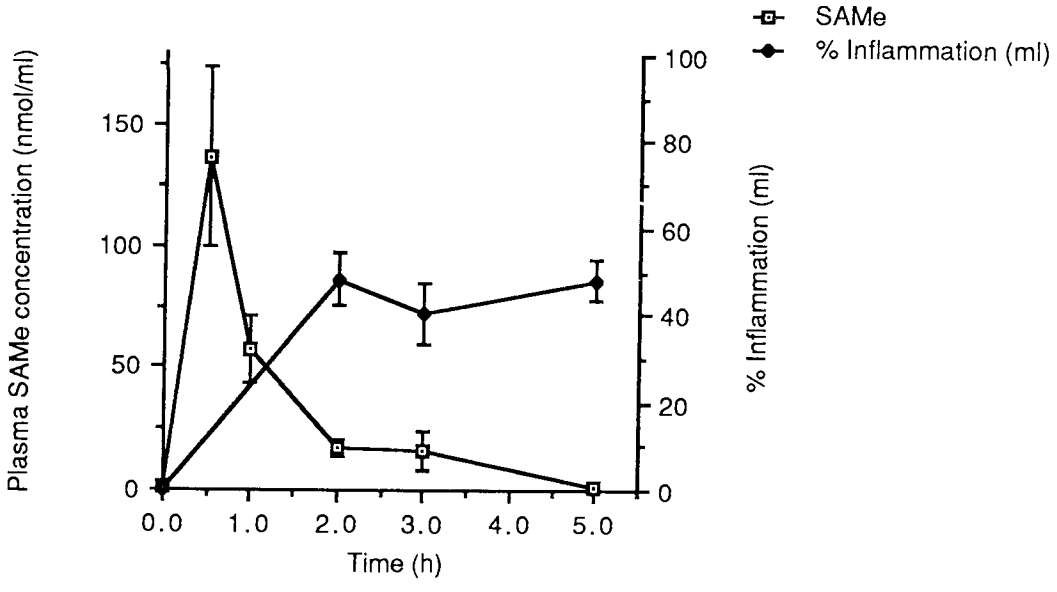


Fig 3.15.2 Effect of carrageenan induced hind paw inflammation on SAMe pharmacokinetics.There was no effect upon SAMe clearance and no correlation between mean plasma SAMe concentration (n=4) and the anti-inflammatory effect of the drug.

4.0. Discussion

S-adenosyl-l-methionine at a maximum concentration of 5mM significantly inhibited the reduction of cytochrome C by superoxide generated by the hypoxanthine xanthine oxidase reaction. However, since urate production was inhibited by 33% by the same concentration of SAMe, the observed significant effect was not due to any antioxidant nature of the drug but to a direct inhibitory effect on xanthine oxidase. The kinetics of the inhibition by SAMe were not investigated in detail but may have resulted from direct blockade of the xanthine binding site by the adenine moiety of the drug or through reaction with the sulphate-p-toluenesulphonate component. The lack of any influence of buffered SAMe on the aerobic photoreduction of NBT provides further evidence that SAMe is probably not an antioxidant. This finding was in contrast with other methionine derivatives such as methionine sulphoxide and methionine sulphone which have been reported to be excellent free radical scavengers (Urikrishnan, and Rao, 1990).It would be of interest to verify whether SAMe itself is altered chemically by exposure to superoxide and if these metabolites have pharmacological activity distinct from SAMe.

An investigation of the effect of SAMe on cytochrome C reduction by rat neutrophils stimulated with FMLP revealed a dose dependent inhibition of the rate of superoxide production. A significant reduction of the rate of superoxide generation of approximately 1/3 was achieved at a dose of 100 μM, a dose which had no significant effect on the reduction of cytochrome C by xanthine oxidase. This inhibition was paralleled by the finding that SAMe caused a dose dependent inhibition of FMLP and zymosan stimulated rat neutrophil chemiluminescene. In contrast SAMe did not inhibit the response to PMA.

Rat neutrophil chemiluminescence activated by FMLP displayed a characteristic bimodal pattern (Dahlgren, 1987) with a sharp transitory peak of activity within a minute of stimulation followed by a slow second peak after about 6 minutes. Incubation of neutrophils with SAMe for 1 hour inhibited both components of the response at high concentrations but had no significant effect at lower concentrations. The initial fast peak was significantly more sensitive to SAMe than the second peak. Dahlgren (1987) reports that the transitory initial peak is associated with extracellular chemiluminescence and the second peak with intracellular activity (Brihem et al., 1984).The observation that catalase, a

large protein that is unlikely to enter the cell, gave a dose dependent suppression of the initial fast peak of FMLP stimulated chemiluminescence provides further support that the inhibition by SAMe is probably of extracellularly derived chemiluminescence. The fact that SAMe and catalase reduced the peak associated with intracellular activity suggests that extracellular chemiluminescence contributes to the second peak. Conversely, the fact that the first peak was not totally eliminated suggests that there is a contribution from intracellular chemiluminescence. Extracellular oxidant secretion is believed to be necessary to kill non-phagocytosable pathogens and may also be important in the stimulus or deactivation of proinflammatory and anti -inflammatory regulatory components (Nurcombe and Edward 1989). The role of intracellular chemiluminescence may be restricted to the phagolysosome in order to target their production towards the ingested pathogen, but as the data shows soluble stimuli such as FMLP initiate intracellular chemiluminescence. The biological role of this production is unknown but it is unlikely to have a direct role in host tissue damage during inappropiate neutrophil activation in inflammation (Nurcombe et al., 1991).

DeChatelet et al., (1982) report that the generation of chemiluminescence is dependent on the myeloperoxidase-H_2O_2-Cl^- system. Myeloperoxidase is secreted from the neutrophil upon degranulation and mediates the formation of hypochlorous acid from H_2O_2 which reacts with luminol to form an active amino- phthalate ion that produces light upon relaxation to the ground state (Allen and Loose, 1976). Chemiluminescence data with cells obtained from patients with myeloperoxidase deficiencies have indicated that luminol dependent chemiluminesecence is dependent on this enzyme (Dahlgren and Stendahl, 1983). Lucinigen dependent chemiluminescence and cytochrome C reduction are assays which only measure oxidant secretion independent of myeloperoxidase activity (Nurcombe et al., 1991).Both superoxide dismutase and catalase inhibit luminol enhanced chemiluminescence.

It has been reported that human PMNLs stimulated with latex particles have high levels of oxygen consumption and high H_2O_2 release but little superoxide. However, in the same study cells treated with opsonised zymosan with the same level of oxygen consumption release massive amounts of superoxide (Curnette and Tauber, 1983). Hence the relative contribution of the products of MPO and NADPH oxidase may be dependent on the kind of

activator used.

Badwey (1980) reports that the site of active oxygen species production is at the plasmalemma and the phagosome. Respiratory burst activators such as FMLP, PMA and IgG coated latex particles stimulate the transfer of the granule associated cytochrome b to the plasma membrane (Borregaard, 1983).

SAMe had no inhibitory effect on PMA induced chemiluminescence which was strongly inhibited by SOD. The specificity of SOD has been questioned since Dahlgren (1987) has reported that SOD effectively scavenged peroxide produced in a cell free peroxidase system. The data suggest that PMA induced superoxide production but the relative contribution of superoxide and peroxides has yet to be established. Pure superoxide produced in a cell free xanthine oxidase system does not generate chemiluminescence (deChatelet et al., 1982). However chemiluminescence can occur in combination with other active oxygen species.The catalase insensitive portion of the FMLP chemiluminescence response might be attributed to superoxide production or be of intracellular origin.

PMA and FMLP are thought to act at different stages of the same transduction mechanism (Matsumoto et al.,1987). Nishizuka (1984) proposed that stimulus - secretion coupling in many cell types involved two pathways - activation of protein kinase C by diacylglycerol (DAG) and an increase in cytosolic calcium. The pathways are thought to act in synergy. Both events arise from the hydrolysis of the membrane phospholipid phosphatidylinositol bisphosphate by calcium dependent phospholipase C to give DAG and inositol triphosphate which can mobilise intracellular calcium. Penfield and Dale (1984) have reported synergism of the two pathways in human PMNLs. PMA acts directly on protein kinase C which phosphorylates a 31 KDa protein component of NADPH oxidase (Papini et al., 1985). Specific protein kinase C inhibitors have been reported to prevent the oxidative burst response to FMLP and opsonised zymosan suggesting that membrane and soluble stimulants share the same pathway (Wilson et al., 1986). The activation of phospholipase C by ligand-receptor binding is mediated by a guanine nucleotide binding regulatory protein (G-protein , Verghese et al., 1985). Inhibition of this G-protein prevents FMLP activated superoxide generation and degranulation. Hence PMA may activate the respiratory burst directly by activating protein kinase C while membrane stimulants act by a receptor mediated transduction system. The results suggest that SAMe inhibits the respiratory burst

elicited by membrane receptor stimulants. Administration of PMA to SAMe treated cells previously refractory to FMLP or zymosan induced a massive respiratory burst response, suggesting that SAMe had no direct effect on the translocation or assembly of the NADPH oxidase complex.Since SAMe did not inhibit PMA induced luminol -myeloperoxidase dependent chemiluminescence it it may be induced that SAMe treatment did not interfere with the functional activity of myeloperoxidase or degranulation of the enzyme as SOD, a large protein unlikely to be imported into the cell , suppressed PMA induced extracellular chemiluminescence with an IC_{50} of about 230 units (Dahlgren, 1987). Further, the lack of effect of SAMe on this stimulator suggests that SAMe had no inhibitory effect on protein kinase C. Thus it is probable that the inhibitory effect of SAMe is through interference with the signal transduction mechanism downstream of protein kinase C.

This model has become increasingly more complicated as recent studies have reported novel mechanisms of neutrophil activation that are phosphatidylinositol (4,5) bisphosphate and phospholipase C independent. Traynor et al., (1989) have reported transient increases in phosphatidylinositol (3,4) bisphosphate and phosphatidylinositol (3,4,5,) trisphosphate (PIP_3) in human neutrophils stimulated with chemoattractant. The rapid time course of the production of PIP_3 and PIP_2(3,4) paralleled the duration of oxidant production. Of interest is the observation that the maximum PIP_3 production occured within the 45-60 second period associated with the fast transitory peak of FMLP activation. In the same study the weak respiratory burst stimulated by LTB_4 , which invokes the same degree of calcium mobilisation,was associated with a much shorter duration of PIP_3 production.The workers proposed that the phosphatidyl inositol-3 kinase may contribute to neutrophil activation via tyrosine kinase through a G-protein linked to chemotactic peptide receptor. This may explain how neutrophil activation can occur in calcium depleted cells in response to certain stimulants (Twomey et al., 1990). Other PIP_2 (4,5) independent mechanisms include the generation of phosphatidic acid by phospholipase D (PLD) which can be converted to DAG by phosphohydrolase (Shukla and Halenda, 1991). This enzyme is activated by FMLP, calcium ionophore A23187, and PMA. It may be regulated by a G-protein either directly or secondarily to phospholipase C activation or increased cytosolic calcium. Although neither of the latter is necessary for receptor mediated activation of the

enzyme. The effect of PMA was blocked by staurosporine, a PKC inhibitor, which suggests that PLD may be activated by PKC, however the response to FMLP was potentiated by the same drug (Reinhold et al., 1990). It is thought that PLD may provide a more sustained supply of DAG over and above of that supplied by the transient acivity of PLC (Shukla and Halenda, 1990). Neutrophil stimulants may therefore elicit reponses through PKC dependent and independent mechanisms. But this is contentious since Twomey et al., (1991) have recently reported that PKC is involved in PIP_2 -independent mechanisms of neutrophil activation by a variety of stimuli (Twomey et al., 1990). With respect to the inhibitory effect of SAMe on the transitory phase of the FMLP response, one may speculate that SAMe treatment may intervene in the generation of these novel phosphoinositides since SAHc has been reported to inhibit neutrophil kinase PI kinase (Pike and DeMeester, 1988), but these authors did not distinguish between PI-kinase subtypes.

The oxidative burst in PMNLs and mononuclear cells is blocked by phospholipase A_2 inhibitors (Smolen and Weissman, 1980). Arachidonic acid is cleaved from membrane phosphatidylcholine suggesting the need for phospholipid methylation as part of the requirement for the elicitation of an oxidative burst, by those agents which need activation of a phospholipase. Baries et al., (1982) and Pick and Mizel (1982) have demonstrated that the inhibition of transmethylation blocked FMLP induced functions. Elevated levels of intracellular SAHc blocked transmethylation and Pike and Snyderman (1982) report that the inhibition of superoxide production in human PMNLs was paralleled by the inhibition of phospholipid methylation. Pick and Mizel (1982) have proposed that the induction of an oxidative burst by membrane stimulants is dependent upon the integrity of SAMe mediated phospholipid methylation leading to the formation of phosphatidylcholine and its effect on receptor expression (Hirata, 1980). Phosphatidylcholine is the primary substrate for neutrophil phospholipase D (Billah et al., 1989). Decreased phosphatidylcholine substrate availability as a result of the inhibition of phospholipid transmethylation reactions may result in an attenuated PLD mediated response, however PLD can probably use other phospholipid substrates (Shukla and Halenda, 1991).Oxidative burst stimulants acting via a specific membrane receptor were highly sensitive to inhibition of transmethylation but agents such as PMA were highly resistant. Oxidative burst responses elicited by

concanavalin A and wheat germ agglutinin were more sensitive than those to FMLP (Pick and Mizel, 1982).

The results for the inhibition by SAMe of FMLP and zymosan induced chemiluminescence are compatible with the above observations.Unopsonised zymosan induced CL was reduced by micromolar concentrations of SAMe within an hour (except at 100 μM). Neutrophil activation by unopsonised zymosan is mediated through the binding of the glucan component with the complement CR3 receptor (Czap, 1988). The marked sensitivity of the response to this particulate activator is consistent with the results for the inhibition of the sugar specific Con A and WGA lectin stimulated macrophage oxidative burst by elevated levels of SAHc (Pick and Mizel,1982). Elevation of intracellular SAHc would be expected to inhibit methyltransferases. Pick and Mizel (1982) suggest that the integrity of phospholipid methylation is required for the respiratory burst, but SAMe can deactivate calmodulin (Gagnon, 1983) and inhibit cyclic AMP phosphodiesterase (Billingsley, 1984) directly by protein carboxymethylation. This would lead to an accumulation of cAMP and an interference in calmodulin-mediated cellular events, both of which could inhibit the respiratory burst (Lehmeyer and Johnston, 1978).The elevation of SAHc in lymphocytes was linked with increased cAMP levels (Zimmerman et al., 1980). Elevation of intracellular cyclic AMP inhibits FMLP stimulated neutrophil function (Hills et al., 1975). Transmethylation is also blocked by MTA (Di Flore et al., 1984) by irreversibly inactivating SAHc hydrolase and therefore elevating SAHc (Della Ragione and Pegg, 1983). Both MTA and SAHc inhibit PI kinase from human neutrophils, thus depleting PIP_2 (4,5) membrane content. PLC stimulation would result in reduced levels of IP_3 and DAG , thus the transduction of the intracellular signal would be attenuated. Pike and DeMeester (1988) report that chemoattractant induced increases in IP_3 were reduced by increased intracellular SAHc content. Thus SAHc may regulate the phosphoinositide pathway.

Studies of the intracellular metabolism of [^{14}C]-SAMe herein demonstrate that incubation of neutrophils with exogenous SAMe increases the intracellular levels of SAHc in a time and dose dependent manner. The highest dose of 21 μM increased the levels of SAHc to 9.6± 0.9 pmol /10^6 cells after 1hour. Although the basal SAHc level was not successfully established in this study, Pike et al., (1978) report that the basal concentration was <0.4

nmol /10^9 monocytes and <0.37 nmol /10^9 cells in human neutrophils (Pike and DeMeester, 1988). If similar levels exist in rat neutrophils then this would represent a 24 fold increase in SAHc levels. The uptake of SAMe and conversion to SAHc was rapid. A dose of 4.2 μM doubled the assumed levels of SAHc within 5 minutes. In agreement with the results of Stramentinoli and Pezzoli (1979), intracellular levels of SAMe were not increased by exogenous SAMe up to a dose of 4.2 μM. Although SAMe levels were significantly increased by 21 μM (1.25 μCi) SAMe. This might be accounted for by SAHc inhibition of transmethylation reactions. Under these conditions SAMe supply would exceed the rate of consumption. Zimmerman et al., (1979) found that deazaadenosine treatment (inhibitor of SAHc hydrolase) of human lymphocytes increased the intracellular levels of SAMe by 79% in the absence of homocysteine and to 186% in the presence of the amino acid. A dose of 10 μM deazaadenosine increased the intracellular levels of SAHc to 4240 pmol / 10^7 cells and inhibited chemotaxis and cytolysis. An increase of monocyte SAHc content to just 0.7nmol /10^9 cells caused a 49% decrease in protein carboxy-methylation (Pike et al., 1978). Increasing SAHc to 159 nmol / 10^9 cells depressed the same function by 84% and was accompanied by a 55% reduction in chemotactic responsiveness with no change in random mobility. The amount of [^{14}C]-SAHc generated by [^{14}C]-SAMe was directly proportional to SAMe concentration and approximated to:

$$Y = 0.1628 + 0.4468X \qquad \text{where X=concentration of SAMe (μM)}$$

Assuming that the increase in SAHc remained constant the predicted amount of SAHc after treatment with 1mM SAMe would be 447 pmol / 10^6 cells. As described above this concentration of intracellular SAHc has been achieved with drugs that inhibit SAHc hydrolase and adenosine deaminase (at much lower doses) and causes marked effects on cell functions such as chemotaxis and cytolysis through inhibition of phospholipid metabolism and stimulation of cAMP production. The results in this study indicate that high intracellular levels of SAHc can be generated in neutrophils incubated in solutions of SAMe *in vitro* . In contrast, the intracellular concentration of adenosine was unaffected by SAMe treatment as was MTA except at the highest dose of 21 μM. This result was a surprise since neutrophils contain substantial amounts of MTA phosphorylase. The excess may have resulted from cleavage of SAMe by SAMe lyase due to excess concentrations or to the diversion of SAMe to polyamine synthesis, this is unlikely though since the neutrophil is

fully differentiated and cannot proliferate and therefore has a small requirement for polyamines. It is possible that the elevated amounts of MTA could contribute to the inhibitory effects of elevated intracellular SAHc achieved at high SAMe concentrations. MTA and adenosine both inhibited the FMLP stimulated neutrophil respiratory burst and were each significantly more potent than SAMe. Adenosine inhibition of the primary peak was dose dependent with maximal inhibition of 56% at 100 μM, a result consistent with the findings of Cronstein et al., (1983) and Pick and Mizel (1982), but the secondary peak was suppressed throughout the dose range. The inhibitory effect of adenosine is mediated by adenosine A_2 receptors (Cronstein et al., 1985) but although this receptor is adenylate cyclase linked, inhibition of superoxide generation was associated with but was not dependent on cAMP elevation or interference with calcium metabolism (Cronstein et al., 1988; Neilson and Vestal, 1989). Instead it appears that adenosine treatment promotes the binding of the internalised FMLP ligand receptor complex to the cytoskeleton which in some ill defined manner terminates oxidant generation (Cronstein et al., 1990). The signalling via A_2 receptors was insensitive to pertussis toxin and microtubule independent. This is in marked contrast to the characteristics of the adenosine A_1 receptor which is reported to mediate FMLP activated neutrophil chemotaxis (Crostein et al., 1985). It has been proposed that the inhibition of the respiratory burst by adenosine could constitute a defence mechanism against oxidant damage to endothelial cells by neutrophils en route to inflammatory sites (Cronstein et al., 1986).

Methylthioadenosine had a similar inhibitory effect to adenosine but had a less well defined dose response relationship. The primary peak of chemiluminescence was inhibited by over 60 % but the secondary peak was suppressed by about 50% throughout the dose range. Unlike adenosine which is thought to be an endogenous mediator of neutrophil function and is present in plasma at concentration of 0.3 μM, MTA is usually at very low concentrations due to the action of MTA phosphorylase . Exogenous MTA has been reported to be cytostatic for lymphocytes (Vandenbark et al., 1980), and inhibits lymphocyte mediated cytolysis by elevating intracellular cAMP through blocking of cAMP phosphodiesterase (Wolberg et al., 1982). A combination of effects including the inhibition of PI kinase (Pike and DeMeester, 1988), elevation of SAHc through inhibition of SAHc hydrolase (Della Ragione and Pegg, 1983) contributing to the direct elevation of cAMP

levels and inhibition of transmethylation (DiFlore , 1984). These are events which could deactivate the neutrophil and attenuate the response to FMLP. The greater potency of adenosine and MTA over SAMe may be due to the fact that SAMe has to be specifically transported into the cell, whereas adenosine can exert effects through cell surface receptors and MTA can enter the cell through passive diffusion (Iizaza et al., 1984).

Zymosan activation was most sensitive to SAMe treatment possibly due to a greater dependence of the zymosan receptor on SAMe methylation. It would be interesting to know the sensitivity of opsonised zymosan activation to SAMe inhibition. It was observed that the zymosan chemiluminescent profile mirrored that of the effect of SAMe on phagocytosis, particularly with regard to the lack of effect at 0.1 mM. A subsequent further study of the same system in the dose range 31.25 μM - 1mM showed a highly variable response at a dose of 125 μM. These results may indicate a biphasic effect of SAMe treatment where at certain doses the function of the cell is enhanced by SAMe rather tan inhibited. Pacheco (1985) reports that this concentration enhanced spontaneous and specifically induced histamine release from human basophils but lower doses had no significant effect. It could be that 0.1 mM is a pivotal concentration in the function of leukocytes in vitro. Specifically induced histamine release from basophils incubated in SAHc was significantly reduced.Whether, and how much SAHc enters the intracellular pool is open to argument since Morita et al., (1981) report the non-effect of exogenous SAHc on histamine release by basophils due to its low penetration.

Incubation with SAMe at doses of 1 and10 mM significantly inhibited phagocytosis, the inhibition at 1mM was of the same degree of significance as incubation wth 8μg/ml cytochalasin B. Methionine and SAHc had no significant effect. The intracellular accumulation of SAHc induced by deazaadenosine inhibited phagocytosis by guinea pig macrophages but had little effect on the phagocytic ability of human monocytes (Snyderman 1980). The results suggest that phagocytosis by rat neutrophils is relatively insensitive to exogenous SAMe treatment. The non effect of SAHc may be explained by its low penetration into the cell. The lack of effect of methionine suggests that the inhibitory effect of SAMe was not due to conversion to methionine. On the other hand although imported methionine was probably converted to SAMe and subsequently SAHc the products could probably not obtain concentrations that inhibited cell function since

methionine adenosyl transferase would probably be inhibited by SAMe product feedback inhibition (Suma et al., 1986). It may be that the apparant lack of potent effect of cytochalasin B is due to an over estimation of the amount of particles ingested by the cells. The fluorescent technique employed may have counted particles that had adhered to the cell membrane but had not actually been phagocytosed. A rigorous procedure of cell washes was incorporated into the method to remove loose particles. The fluorescence of the supernatant from the last wash was typically so low as to be nearly beyond detection. Cytochalasin B inhibits phagocytosis by disrupting microfilament assembly (Cronstein et al., 1983) thus those latex particles attached to the cell membrane may not be destined for phagocytosis. Alternatively a more physiological substrate such as fluorescently labelled bacteria or zymosan at higher particle : cell ratios could provide more meaningful information as to whether phagocytosis is inhibited by SAMe or not. If the result for zymosan induced chemiluminescence is a measure of phagocytosis as well as a measure of C3b receptor activation, the preliminary data presented would suggest that SAMe may inhibit phagocytosis at micromolar concentrations *in vitro* .

In contrast, SAMe at 1mM had no effect on the production of PGE_2 by neutrophils stimulated by zymosan. This finding was consistent with the evidence reported by Stramentinoli (1987) and Laudano (1987). Both workers report that had SAMe no effect on prostaglandin production *in vivo* . However, it may be relevant to the observed suppression of zymosan induced chemiluminescence that neutrophil PGE_2 receptors are linked to adenylate cyclase and are associated with deactivation of the cell (Sklar , 1986). It may be speculated that the resultant elevation of cAMP may synergise with the inhibitory effect of SAMe metabolites and enhance the overall inhibitory effect of the drug. In addition Czap (1988) has reported that incubation of human neutrophils with zymosan generates LTB_4. Similarly, FMLP stimulates the generation and release of LTB_4 and PAF release by neutrophils (Palmer and Salmon, 1983; Braquet and Rola-Pleszczynski, 1987, respectively). It would be of great interest to determine whether SAMe treatment has any inhibitory effect on the FMLP stimulated generation of these potent inflammatory mediators. SAMe treatment had no significant effect on FMLP elicited neutrophil chemotaxis *in vivo* into 6-day air pouches. This cell function is mediated by high affinity FMLP receptors (Snyderman et al., 1983). High intracellular levels of SAHc are reported

to inhibit the process *in vitro*. The lack of effect of SAMe was probably due to its rapid metabolism and subsequent low plasma concentration during the main period of neutrophil mobilisation from the bone marrow and marginal pool. At the time of cell harvesting 6 hours after FMLP inoculation, plasma SAMe levels were probably back to normal.
In vivo studies by Gualano et al., (1985) and Stramentinoli (1978) have demonstrated that SAMe has antiinflammatory and analgesic activity in carrageenan and nystatin induced hind paw inflammation and phenylquinone and acetic acid induced writhing in mice. In agreement with the results of these workers, intraperitoneal administration of SAMe exerted a significant antiinflammatory effect at a dose of 50mg/kg. However this dosage appears to be critical since the dose response study showed that 25 mg/kg had no antiinflammatory activity at all and 100mg/kg was significantly less effective. In contrast, Stramentinoli (1987) could only achieve significant inhibition of carrageenan induced inflammation at the higher dose when administered subcutaneously. In this investigation SAMe had no significant antinflammatory activity within the first hour whereas at the same interval Stramentinoli (1987) reports a 43% suppression of oedema. At the time of carrageenan inoculation , 30 minutes after dosing with 50 mg/kg SAMe , the plasma concentration of SAMe would be at its peak of about 130 μM SAMe. However it must be stressed that the maximum inflammation in the control group was only about 8.5% thirty minutes post inoculation. After 1 hour this had increased to 19%. At this stage the paw was obviously red but there was little perceptible oedema. After 90 minutes the paw volume had increased to 28 % in the control group but the mean paw oedema in the SAMe treated group was significantly decreased to half of this value. At this interval plasma SAMe concentration would be about 40 μM. The data shows that the antiinflammatory effect of SAMe is apparant after 90 minutes and is sustained up to 5 hours. Although inhibition was less at this interval it was significant and corresponded to a plasma SAMe value of 1.4 μM barely above the basal plasma SAMe level of 1.1 μM. Maximum inhibitory effect was obtained after 3 hours which corresponds to 15 μM. In contrast, Stramentinoli (1987) found maximum suppression of oedema by 43% at 2 hours after administering the same dose by the intraduodenal route. It is clear that the antiinflammtory effect of SAMe does not correlate with plasma SAMe which suggests that the active antiinflammatory ingredient is a metabolite of SAMe.

Methylthioadenosine formed by the polyamine pathway and adenosine formed via the hydrolysis of SAHc are metabolites of SAMe credited with antiinflammatory activity (DiPadova et al., 1985; Neilson and Vestal, 1989, respectively). These metabolites (50 mg/kg) were found to be antiinflammatory in the carrageenan rat hind paw model at the three hour interval but only MTA sustained the effect to 5 hours. In contrast to SAMe doses of 25 and 100 mg/kg were both equally effective at the 3 hour interval but the antiinflammatory effect at 5 hours seemed to be less than that of SAMe. The lack of effect of adenosine may reflect a lack of influence of the drug on the relevant inflammatory mediators or rapid metabolism and clearance.

The main question in this *in vivo* study was since polyamines have numerous reported direct and potential antiinflammatory effects (Kafy and Lewis, 1983,1987; Oyanagui, 1984; Bird et al., 1983) and as SAMe is intimately involved in polyamine synthesis, could it be that the antiinflammatory effect of SAMe is manifested in some way through polyamines ?. The conclusion reached through the evidence cited in this study suggests that it is not .

The administration of SAMe to rats in the presence and absence of inflammation had no effect on the polyamine status of blood but did stimulate putrescine synthesis in the liver presumably through the direct stimulation of ornithine decarboxylase by SAMe or decarboxylated-SAMe or by induction by a second messenger such as cAMP (Russell, 1976). It is well documented that the 'biosynthetic' decarboxylases are rapidly induced by inflammatory stimuli and that polyamine synthesis precedes the production of acute phase proteins as part of the acute phase response to infection or injury (Scalabrino et al., 1987). Spermidine synthesis was stimulated in the presence of inflammation only and spermine levels were not significantly affected by any treatment. Adenosine treatment caused the same degree of putrescine stimulation as SAMe but was the strongest stimulator of spermidine synthesis. Methylthioadenosine strongly stimulated putrescine synthesis but had less effect than SAMe on spermidine production. Both methylthioadenosine and adenosine have been reported to elevate cAMP levels (Wolberg et al., 1982; Cronstein et al., 1985, respectively) and this may be sufficient to stimulate or potentiate the activation of ODC. The product of ODC putrescine stimulates the decarboxylation of SAMe (J änne and Williams-Ashman, 1971) which donates an aminopropyl group to form spermidine or

spermine. The extra spermidine stimulated by adenosine could have originated from the synthesis of endogenous putrescine which could only have been aminopropylated by endogenous decaSAMe or from spermine via polyamine interconversion (although spermine levels were not significantly reduced). In contrast , in MTA treated animals SAMe can be recycled by the MTA salvage pathway to form methionine which can be adenosylated to form SAMe. Consequently, it might be expected that MTA would form more spermidine than adenosine but not as much as SAMe, since exogenous SAMe would be a direct source for deca SAMe. Preliminary evidence showed a circumstantial link between the stimulation of putrescine and spermidine synthesis and the antiinflammatory effect of SAMe. However, pretreatment of the animals for two days with specific polyamine inhibitors had no significant effect on the antiinflammatory activity of the drug. Pretreatment with DFMO massively reduced putrescine levels and reduced spermidine levels to normal but the antiinflammatory action of SAMe was sustained and even enhanced at the 5 h interval. Similarly, pretreatment with DFMO and MBGB had no significant effect on basal spermidine or spermine but putrescine was restored to normal levels. Such an effect can be explained by studying the effect of the two drugs in isolation. Treatment with DFMO abolished putrescine and significantly reduced spermidine levels. Conversely MBGB nearly tripled putrescine levels and increased spermidine to the same extent as MTA. MBGB was not antiinflammatory in this model. Elevation of putrescine can be explained by the inhibition of the catabolic enzyme diamine oxidase by MBGB (Pegg, and McGill, 1978) and the inhibition of SAMe decarboxylase. The elevation of spermidine is hard to explain since inhibition of SAMe decarboxylase would be expected to reduce the availability of deca SAMe and thus spermidine and spermine levels. However, Pegg (1986) has reported that MBGB also induces spermidine / spermine -N^1-acetyltransferase activity which may raise both spermidine and putrescine levels via acetylase / oxidase mediated polyamine interconversion (Seiler et al., 1981). The resistance of spermine to any treatment may be due to the high affinity of spermine synthase for decaSAMe (Pajula , 1983) which would successfully compete for depleted levels of deca SAMe against spermidine synthase. However, one would also predict that spermine levels would also be significantly increased by SAMe treatment. That this was not the case may tentatively be attributed to inhibition of spermine synthase by SAHc (Hibasami and Pegg, 1978). Although this only occurs at high

concentrations of SAHc. In normal rat liver only 1% of endogenous SAMe is used for polyamine synthesis (Eloranta and Kajander, 1984). But in times of SAMe depletion SAMe decarboxylation and polyamine synthesis take priority over transmethylation reactions indicating the critical need of these compounds for the viability of the cell (Kramer et al., 1988).

The evidence suggests that the relative aniinflammatory efficacy of each drug was not related to any changes in the polyamine status of the liver (although other organs / cell systems can not be discounted) during the inflammatory episode. Thus although SAMe stimulates liver polyamine metabolism its antiinflammatory activity is not mediated directly through this pathway.

Hind paw inflammation induced by carrageenan consists of a number of phases mediated by a succession of mediators (Vinegar et al., 1976). Di Rosa et al., (1971) have divided the response into three phases ; an early phase of 0-1.5 h duration initiated by the release of histamine and 5-HT, a second phase from 1.5-2.5 h mediated by kinins and a third phase after 2.5 h mediated by prostaglandins and infiltrating neutrophils. Since then Holsapple and Yim (1984) have suggested a fourth phase which involves the synthesis of leukotrienes and Hwang et al., (1986) have presented evidence that PAF may also be involved in the early phase.

In many animals in both control and SAMe treated groups, there was little inflammation during the first 30 minutes while there was a response up to 25% in some individuals, which may have been the result of injury during inoculation rather than the inflammatory effect of carrageenan. Hence the mean response of each group showed great variation in the very early stages.

Histamine and 5-HT are both released by rat mast cells during the first phase of carrageenan induced oedema (Crunkhorn and Meacock, 1971) but the mechanism is obscure since carrageenan does not degranulate mast cells *in vitro* (Garcia-Leme et al., 1971). It has been suggested that degranulation may be elicited via anaphylotoxins generated by the alternative complement pathway which is activated by carrageenan *in vitro* (DiRosa et al., 1971) but this has been difficult to demonstrate *in vivo* (Vinegar et al.,1976). Histamine was approximately 20 fold less potent than 5-HT in inducing hind paw oedema but although SAMe treatment had no effect on the initial 5 minute phase of the

response, recovery from the oedema was accelerated. Mepyramine inhibited both the initial response and accelerated recovery from the oedema. In this model SAMe significantly inhibited histamine induced oedema at a dose as low as 10 mg/kg. Methythioadenosine had a similar effect to mepyramine , suppressing the initial response by about 2/3 and maintaining this throughout the inflammatory episode. Neither SAMe nor MTA inhibited inflammation induced by compound 48/80, and SAMe had no inhibitory effect at any phase during inflammation elicited by dextran, zymosan and hydrogen peroxide from glucose oxidase. These results are consistent with the finding that SAMe had little effect on inflammation induced by 5-HT. All of these compounds cause mast cell degranulation but through different mechanisms. Dextran through crosslinking mast cell membrane receptors (Nishida et al., 1979), zymosan through anaphylotoxin receptors (Hugli and Muller-Eberhard, 1978) and compound 48/80 and hydrogen peroxide through direct mast cell degranulation (DiRosa et al., 1971; Ohmuri et al., 1979). Mast cell amines have a major role in zymosan and dextran induced inflammation (Damas and Remacle-Velon, 1984; Nishida et al., 1979), the development of which can be antagonised by histamine and 5-HT blockers mepyramine and methysergide, respectively. These blockers have little effect on the development of carrageenan induced oedema as does depletion of mast cell amine by pretreatment with compound 48/80 (Damas and Remacle-Velon, 1984). Such results suggest that histamine and 5-HT have a minor role in carrageenan inflammation and would explain why although SAMe inhibits the inflammatory response to histamine , it has little significant inhibitory effect during the first 60 minutes of the oedema.

Hwang et al., (1986) report that 50% of the initial inflammatory response is attributable to the synthesis and effect of endogenous PAF. The time course of PAF production reached a maximum 1 hour after inoculation and subsided to basal levels after 2 hours. SAMe and MTA both inhibited PAF induced hind paw inflammation but MTA had greater efficacy and potency. Although SAMe reduced the maximal response it had no significant effect on the rate of recovery from the oedema. Further, SAMe was only effective at a dose of 50 mg/kg ie the optimum dose for the inhibition of carrageenan oedema. In contrast, MTA strongly suppressed the maximal response, increased the speed of recovery and was effective at 25 mg/kg. This is also consistent with the results obtained for the effect of MTA on the carrageenan model where inflammation was significantly inhibited at the 3 hour interval.

Isoprenaline is a potent ß1 and ß2 adrenoceptor agonist with powerful stimulatory effects on the heart. It also dilates bronchial smooth muscle and vasodilates arterioles in skeletal muscle (Foster and Cox, 1985). Isoprenaline inhibits both carrageenan (Mohd-Hidir and Lewis, 1984) and PAF induced hind paw oedema. The drug was highly potent in the latter reducing swelling at a dose as low as 0.5 mg/kg. Combinations of different doses of isoprenaline with SAMe had varying effects. The subthreshold dose of 0.2 mg/kg actually blocked the inhibitory action of SAMe (50 mg/kg). SAMe in combination with 2 mg/kg enhanced the inhibitory effect of isoprenaline alone. Whereas, it had the opposite effect in combination with 4mg/kg isoprenaline. Combination of 0.5 mg/kg isoprenaline with MTA (50 mg/kg) had no significant effect on the efficacy of either drug. Similarly, the inhibitory effect of verapamil, a calcium channel blocker, was unaltered in combination with SAMe but the recovery from the oedema was significantly prevented when in combination with MTA. The inhibition of PAF induced oedema by isoprenaline and verapamil is consistent with the fact that drugs that interfere with intracellular calcium either directly (eg calcium channel antagonists, calmodulin inhibitors, local anaesthetics) or indirectly (via receptor mediated signal transduction mechanisms such as the effect of adrenoceptor agonists on cyclic nucleotides) cause inhibition of the effects of PAF in both in vitro and in vivo systems (Braquet and Godfroid, 1986). It is tempting to speculate that some of the inhibitory activity of SAMe and MTA in the carrageenan model may be due in part to suppressing the PAF mediated component of the oedema by receptor independent means. Neither SAMe nor MTA inhibited the maximal response or time course of inflammation induced by arachidonic acid. DiMartino et al., (1987) state that the oedema is sensitive to inhibition by dual inhibitors of arachidonate metabolism and corticosteroids but not by selective cyclooxygenase blockers. Anti histamine and anti-5HT agents also suppress the response which suggests that mast cell mediator release may contribute to arachidonic acid induced oedema. The potent effect of indomethacin on carrageenan induced oedema at 3h and 5h suggests that lipoxygenase products , which have been postulated as mediators of the 4 th phase of the oedema (Damas and Remacle-Volon, 1986), play a small role in the overall response.

Both SAMe and MTA were most effective in the carrageenan model at the 3 h interval. After this time the rate of swelling was the same as in the control group although the actual

swelling was less. The phase from 1.5 h to 2.5 h is mediated by kinins and thereafter by prostaglandins and PMNLs (DiRosa et al., 1971). It would be of interest to determine whether SAMe has any effect on kinin synthesis, conversion or inflammatory effect since kinins exert inflammatory effect directly through kinin receptors and by stimulating phospholipase A_2 (Wiggins and Cochrane , 1984). So although SAMe has no direct antiprostaglandin action (Stramentinoli, 1987), blockade of kinins would reduce the stimulus for PA_2 activity and thus eicosanoid synthesis. In addition inhibition of kinins would prevent the further stimulation of degranulation from mast cells (Fearon and Austen , 1979) and prevent the pain and odema caused through synergy with prostaglandins (Williams, 1979). It is unlikely that plasma SAMe concentrations would be high enough to influence PMNL function during the later phase(s).

How can SAMe and MTA inhibit acute inflammation induced by carrageenan, histamine and PAF ?. An answer may be provided by the investigation of the intracellular metabolism of SAMe. If high plasma levels of SAMe cause the same degree of elevation of SAHc in cells and tissues *in vivo* as was found *in vitro* , then the consequent effects on cAMP and phosphoinositide metabolism may alter or attenuate the response to certain mediators. Both PAF and histamine (via H1 receptors) signals are transduced by PIP_2 cleavage to DAG and IP_3 (Heure et al., 1991; Lonchampt et al., 1988). Elevation of cAMP activates protein kinase A which activates calcium uptake by the endoplasmic reticulum and thus is involved in the sequestration of intracellular calcium into calcium stores (Lehninger, 1982). Likewise a depletion of released IP_3 would reduce intracellular calcium release for a given stimulus. The overall effect would be depletion of intracellular calcium which could have marked effects on calcium activated enzymes or events mediated by calmodulin. It is tentatively suggested that the inhibitory effect of SAMe and MTA on carrageenan induced inflammation is through nonspecific interference, i.e non-receptor mediated,with the transduction mechanisms of some of the component mediators of the response and thus interference with the responses of those cells (eg mast cells, endothelial cells, macrophages) at the site of inflammation to inflammatory stimuli. At doses of 50 mg/kg this was manifested as an antiinflammatory effect, but at lesser doses SAMe had no significant effect on carrageenan induced inflammation . The same suggested interference

with calcium metabolism may also account for part of the effect of SAMe and MTA on neutrophil function.

In conclusion, since SAMe is involved in a multitude of cellular reactions it is not surprising that large exogenous doses of the drug exert profound effects on cell and tissue responses. Attenuation of the respiratory burst by SAMe could have many possible antiinflammatory implications such as the inhibition of myeloperoxidase mediated inactivation of alpha 1 antiproteinase (Borregaard et al., 1987) which regulates the activity of proteases. This would prevent tissue damage by breaking the synergy between myeloperoxidase products and proteolytic enzymes such as elastase. Restriction of the supply of reactive oxidative species may also prevent lipid peroxidation and thus further tissue damage.It is doubtful though whether plasma SAMe levels would reach levels in patients which could influence neutrophil function at pharmacological doses of 1200mg/day.

Although SAMe had antiinflammatory activity in some models of acute inflammation it was no greater than other compounds such as verapamil and isoprenaline which are not considered to be antiinflammatory drugs. Exogenous MTA was found to be a metabolite of SAMe with greater antiinflammatory activity than the parent compound, but it is unlikely that the administration of SAMe elevates the intracellular levels of this substance to a concentration which could exert pharmacological effect since it is rapidly removed by MTA phosphorylase (Pegg and Williams-Ashman, 1987). Further, analysis of plasma samples by HPLC after SAMe treatment showed no increase in the plasma levels of this nucleoside. However, Iizaza et al., (1984) has reported that at high concentrations , the lipophilicity of MTA allows influx at rates greater than those of degradation. Polyamines although credited with antiinflammatory activity in their own right, were not found to mediate the antiinflammatory activity of SAMe in this investigation. It is of interest that inhibition of polyamine synthesis would also restrict the synthesis of endogenous MTA but this had no effect on the inhibitory action of SAMe.

This study has failed to show the definitive pathway for the antiinflammatory effect of SAMe but it may well be that such a pathway does not exist. For SAMe is an important pivotal molecule in cell physiology. It is rapidly metabolised and yet relieves both chronic and acute disease states. Metabolites of SAMe have confirmed direct and indirect

antiinflammatory properties. Therefore the sum of these would be the observed total antiinflammatory effect of SAMe.

Future Experiments

1. Establish the basal levels of SAHc in neutrophils with [^{14} C]- methionine

2. Investigate whether incubation of cells with SAMe treatment elevates intracellular cAMP. Determine cAMP by radioimmunoassay.

3.Effect of the incubation of whole cells with SAMe on phosphoinositide metabolism. Measure PIP_2 content and degradation to IP_3 on stimulation with chemoattractant activators. Is the intracellular elevation of SAHc content sufficient to inhibit PI kinase. Relate this to inhibition of cell function by SAMe.

4. Effect on SAMe treatment on the mobilisation of calcium visualised by quin-2 or fura-2, in response to stimulants ; FMLP, PAF and zymosan.

5. Effect of SAMe and MTA on functions activated by PAF such as platelet aggregation, neutrophil aggregation.

6. Effect of SAMe on neutrophil enzyme degradation activated by membrane receptor stimulants and direct degranulators such as calcium ionophore A23187.

7. Expand the study to include mast cells, macrophages and monocytes. Examine the effect of SAMe on synovial cells from normal and osteoarthritic patients.

8. Since inflammatory microcrystals are implicated in the aetiology of osteoarthritis (Schumacher, 1987), it would be interesting to determine whether cellular responses elicited by these particulate activators are inhibited by SAMe.

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