OXY RADICALS AND CONTROL OF INFLAMMATION

A thesis submitted for the degree of

Doctor of Medicine

University of Adelaide

By Leslie G. Cleland M.B., B.S.

From the Departments of Medicine and Pathology,

University of Adelaide.

July, 1984.

awarded 30-4-85

INDEX

CHAPTER 2: CHEMILUMINESCENCE OF POLYMORPHONUCLEAR LEUCOCYTES FROM RHEUMATOID JOINTS

CHAPTER 3: PRODUCTION OF OXY RADICALS AND EFFECT OF METAL CHELATORS, ANTI-OXIDANTS AND ANTI-INFLAMMATORY AGENTS I: HYDROXYLATION OF SALICYLATE

CHAPTER 4: PRODUCTION OF OXY RADICALS AND EFFECT OF METAL CHELATORS, ANTI-OXIDANTS AND ANTI-INFLAMMATORY AGENTS II: DEGRADATION OF HYALURONATE

CHAPTER 5 GENTISATE: A SALICYLATE METABOLITE WITH ANTI-OXIDANT PROPERTIES

CHAPTER 6: MACROMOLECULAR CONJUGATES OF SUPEROXIDE DISMUTASE (SOD): STUDIES OF CLEARANCE ANTI-INFLAMMATORY EFFECT AND IMMUNOLOGICAL REACTIVITY

CHAPTER 7: CORTISOL PALMITATE LIPOSOMES FOR SYSTEMIC THERAPY OF INFLAMMATION

STATEMENT OF ORIGINALITY

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

Leslie G. Cleland

ACKNOWLEDGEMENTS

The technical assistance of the following is gratefully acknowledged: David James (assays of leucocyte chemiluminescence), Julie Bielicki (preparation of macromolecular conjugates, salicylate hydroxylation assay), Henry Betts (viscometric studies of hyaluronate degradation), Debbie Imhoff (HPLC assay for salicylates and metabolites), Ken Wong (preparation of conjugates), Shelley Hay and Ross Garrett (anti-inflammatory studies in animal models of inflammation), and Margaret Norton (manuscript preparation).

I thank Mark Poznansky and Michael Whitehouse who made valuable suggestions, and Ian Forbes who provided supervision and encouragement during my initial research training.

I especially wish to thank Professor Barrie Vernon-Roberts, my supervisor, and Henry Betts for invaluable advice and support.

ABSTRACT

Inflammation is a response to injury mediatd through a complex of interacting systems. Clinical disorders may result when inflammation is persistent, uncontrolled or inappropriately triggered.

Oxy radicals are reactive oxygen-derived molecules. Their generation by activated polymorhonuclear (PMN) leucocytes and macrophages constitutes part of the normal defense against microbial invasion. The high reactivity of these molecules makes them likely to be (a) contributors to tissue damage occurring in chronic inflammatory disorders (e.g. damage to joints of patients with rheumatoid arthritis), and (b) a major influence on the metabolism of cells adjacent to their sites of production. Studies were undertaken in order to (i) obtain a better understanding of the ways in which oxy radicals may contribute to chronic inflammation and (ii) establish a basis for new approaches to treatment of inflammatory diseases.

Leucocytes obtained from inflamed joints of patients with rheumatoid arthritis were shown to produce a burst of chemiluminescence following appropriate stimulation, suggesting an intact mechanism for oxy radical production in these cells.

Studies of the mechanisms of production and inhibition of effects of oxyradicals were undertaken. Production of radicals was acheived (a) enzymatically and (b) non enzymatically. Oxy radical effects were monitored by (a) hydroxylation of salicylate measured spectrophotometrically and (b) degradation of hyaluronic acid measured viscometrically. The need for iron in a suitably chelated form for the production of the most potent oxy radical, hydroxyl radical, was established. The inhibitory activity on oxy radical effects of (i) anti-inflammatory agents, (ii) oxy radical eliminating enzymes (superoxide dismutase (SOD), catalase) and (iii) anti-oxidants was established in the above systems. Of particular interest was an observed enhancement of non-enzymatic hydoxyl radical production seen with some concentrations of penicillamine, a thiol containing anti-rheumatic agent.

SOD has been claimed to possess anti-inflammatory effect and this agent was studied. Intravenously administered SOD was rapidly cleared from plasma ($t_{1/2}$ 4-6 minutes) and lacked anti-inflammatory activity. Glutaraldehyde-crosslinked conjugates were formed comprising SOD and albumin or SOD alone. These conjugates retained 60-70% of enzymatic activity of constituent SOD. Plasma clearance of SOD activity following intravenous injection

of conjugates was greatly prolonged ($t_{1/2}$ up to 12 hours). SOD-homologous albumin and SOD-SOD conjugates injected intravenously had potent anti-inflammatory effects in rats. These effects may not necessarily be attributable to their SOD activity because an anti-inflammatory action was also seen with conjugates of albumin alone formed by the same method in absence of SOD.

Arachidonate metabolism, which leads to the production of oxylipids with potent pro-inflammatory properties, may be enhanced indirectly by oxy radical production. Arachidonate metabolism is most comprehensively inhibited by anti-inflammatory corticosteroids, but the use of these agents for therapy is limited by unacceptable unwanted effects. A drug carrier system comprising cortisol palmitate in liposomes, designed to enhance delivery of cortisol to phagocytic leucocytes, was found have 10-fold greater potency than a soluble cortisol preparation when given by intravenous injection.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION: Biology of Inflammation

Inflammation involves a series of responses which may be invoked by tissue damage, microbial invasion, or the presence of agents perceived as foreign (antigens). The inflammatory response must be sensitive to appropriate stimuli yet be neither excessively responsive nor inappropriately triggered. As if to accommodate these requirements, inflammation is mediated by networks of cellular responses and a series of interacting self-regulating humoral (extra-cellular) cascades.

Cellular responses involve multiple cell types and are subject to inter-cellular communication and feedback control. Both peptide factors (1,2,3) and oxylipids (e.g. prostaglandins, leucotrienes) (4,5) are involved in the orchestration of cellular responses. Similarly, sub-populations of cells with both suppressive and enhancing actions have been demonstrated, a phenomenon best characterized in the lymphocyte subsets (6).

Examples of humoral networks involved in inflammation include the complement system, protein mediators, e.g.

plasmin (requiring activation by proteases and subject to inhibition by peptide inhibitors), the kinin system and the clotting/anticoagulant networks (7).

Antibodies play an important role as specific inducers of inflammation in the presence of their matching antigens. Antibodies may also be important as suppressors of inflammation as seen with feedback inhibition of antibody production through actions of anti-idiotypic antibodies (directed against specific antigen-binding sites of immunoglobulins) (8). Rheumatoid factors (antibodies with specificity for Fc portions of immunoglobulins) may be self-aggregating and complement fixing thus acting as non-specific amplifiers or even perpetuators of inflammation (9).

Chronic inflammatory disorders may involve specific organs/tissues or affect multiple systems. These disorders are characterized by persistent active inflammation, damaging to the host and for which no persisting microbial agent can be found. Examples of such disorders include rheumatoid arthritis, inflammatory bowel disease and some forms of lower respiratory tract inflammation. The notion of chronic inflammatory disease outlined above excludes those chronic infections, such as leprosy and tuberculosis, for which specific agents have been identified and for which

appropriate chemotherapy is now available.

1.2 RHEUMATOID DISEASE AND ITS TREATMENT

1.2.1 General aspects

Rheumatoid arthritis poses a particularly important problem because it is (a) relatively common (10) and (b) may result in serious, progressive joint damage leading to joint failure, severe discomfort and loss of independence in severely afflicted individuals.

The central lesion in rheumatoid arthritis is chronic uncontrolled inflammation, primarily localized to joints but with evidence of systemic inflammation and variable inflammation at non-articular sites (11). The inflammation of joints in rheumatoid arthritis is relatively superficial and can thus be observed, and to an extent, quantified (12,13). Moreover, haematological and serological changes accompanying rheumatoid disease can be monitored sequentially and normalization of abnormalities is one possible outcome of anti-inflammatory therapy. Most agents currently used in the treatment of rheumatoid arthritis exert beneficial effects as assessed by studies comparing groups of patients receiving different treatments (including placebo therapy). However, none of the available agents is beneficial in all patients, and none is able to entirely

suppress rheumatoid disease in more than an occasional patient.

1.2.2 Anti-inflammatory agents

Anti-inflammatory agents currently used in clinical practice may be subdivided into the following groups:

1. Anti-inflammatory cortico-steroids.
2. Nonsteroidal anti-inflammatory agents (NSAID'S).
3. Slow-acting disease remitting anti-inflammatory drugs.
4. Cytotoxic agents.

1. Anti-inflammatory steroids are potent inhibitors of inflammation. Their use as systemic agents is associated with serious dose-related unwanted effects, which generally render their use unacceptable, except in low doses (14). Even at low doses (less than 10 mg per day) adverse effects are reduced rather than eliminated. Also steroids, while suppressing inflammation (at least on a short-term basis) may not retard the destructive effects of rheumatoid inflammation. (Intermittent intra-articular injections of depot steroid preparations has a place in the treatment of localised joint inflammation.) An advantage would accrue for the treatment of generalised polyarthritis, however, if it were possible to target

anti-inflammatory steroids toward tissues/cells upon which their effects were wanted and to direct them away from tissues/cells upon which effects of these agents is undesirable.

2. Nonsteroidal anti-inflammatory drugs provide pain relief and reduce the swelling of rheumatoid disease. They have not been shown to prevent destruction of joint structures in rheumatoid disease. Unfortunely, their use is associated with a high incidence of upper gastro-intestinal intolerance (15,16). Both the anti-inflammatory/analgesic effects and the gastric irritancy of non-steroidal anti-inflammatory analgesics appears attributable to their action as inhibitors of prostaglandin H_2 synthetase (17).

3. Slow-acting disease-remitting agents, i.e. anti-malarial agents (18), gold complexes (19,20), penicillamine (20,21), have striking beneficial effects in a minority of patients and are of some benefit to approximately 50% of patients receiving any given agent. Unfortunately, some patients receive no benefit from these agents and many patients receiving gold and penicillamine suffer unwanted effects dictating the withdrawal of therapy. Anti-malarials are associated with infrequent but potentially serious ocular

toxicity. Gold and penicillamine frequently cause rashes and mouth ulcers, not infrequently cause nephrotoxicty (usually reversible) and may cause potentially life-threatening marrow suppression. A further disadvantage of these agents is a requirement of three to six months of treatment before a response can be discerned.

4. Cytotoxic agents, e.g. azathioprine, cyclophosphamide, have been shown to suppress rheumatoid inflammation (23). Intolerance is common at doses required to achieve an anti-inflammatory effect. The possible effects of these agents on reproductive function and reports of malignancies in some patients receiving these agents are serious concerns militating against their use, particularly in younger patients, except in cases of severe progressive disease not responsive to other therapies.

1.3 NEW APPROACHES TO THE TREATMENT OF INFLAMMATION

It is apparent from the above that currently available chemotherapy for rheumatoid arthritis has considerable short-comings. In attempting to achieve more effective treatment of rheumatoid arthritis (or other chronic inflammatory disorders) a number of general approaches may

be taken.

1. Isolation of causative agents, allowing (a) prevention through vaccination or other measures or (b) treatment with a specific microbiocidal agent. To date this approach has met limited success.

2. To identify defects in the modulation or control of inflammation, either intrinsic or triggered by an acquired agent, which may be amenable to remedial action. A most promising area for this approach is offered by two recently described (and inter-related) pro-inflammatory systems of activated leucocytes: (a) oxy radical production (24,25,26) and (b) lipoxygenase pathways of arachidonate metabolism (4). A better understanding of these systems should lead to the development of new therapeutic agents with more potent or novel anti-inflammatory effects.

3. To improve the performance of existing therapies through improvement of the extent of beneficial effects relative to unwanted effects. This general approach may be undertaken in a number of ways:

   (a) Chemical modification of existing agents. The availability of multiple NSAID's for clinical use

reflects the application of this approach. Regrettably, it has proven difficult to dissociate wanted anti-inflammatory effects from unwanted effects, particularly gastric irritancy (15,16).

(b) Formation of pro-drugs which are inactive prior to absorption (thus minimizing upper gastro-intestinal toxicity) and are subsequently converted to an active agent *in vivo* (27).

(c) Utilization of specific targetting systems designed to carry drugs to select cells or sites where drug action is wanted, or to better retain drugs at these sites (28,29).

1.4 OXY RADICALS AND INFLAMMATION

1.4.1 Definition of oxy radicals

The term oxy radical is used to encompass several reactive oxygen-centred species (30). The oxy radicals include superoxide anion, $O_2^{-\bullet}$, and hydroxyl radical, $OH^{\bullet}$, which are true radicals, i.e. have an unpaired outer orbital electron. Also included are hydrogen peroxide, H_2O_2, and singlet oxygen, $^1O_2$, which, although not true radicals, are potent oxygen-centred oxidants often produced contemporarily

with the other oxy radicals. In biological systems oxy radicals are often produced through sequential reactions (to be described), and are likely to produce composite or additive effects. Accordingly "oxy radicals" provides a valuable generic term with which to describe this group of reactive oxygen-centred species. Ground state molecular oxygen, $^3O_2$, has two unpaired outer orbital electrons, and is thus a di-radical, but is less reactive than the oxy radicals and is not encompassed by the term.

1.4.2 Oxy radicals and cell metabolism

Cells capable of living in an aerobic environment, including facultative anaerobes, are endowed with cytoplasmic enzymes which eliminate superoxide (superoxide dismutases (SOD)) and hydrogen peroxide (catalase, peroxidases), whereas strict anaerobes lack these enzymes (31). Three families of SOD have been defined: (a) copper-zinc SOD found in the cytoplasm of eukaryotic cells (32), (b) manganese-containing SOD found in mitochondria and procaryotes (33) and (c) iron containing SOD found in certain bacteria (34). Within each family of SOD a high degree of sequence homology is seen between species (35). The extent of this homology seen with SOD from cells as widely separate phylogenetically as mammalian cells and bacteria, highlights the importance of SOD for survival in oxygen-metabolising cells. Growth of some bacteria in a

hyperoxic environment induces SOD activity (36). It has been concluded on the basis of these observations that the intracellular production of toxic oxy radicals is an inevitable accompaniment of metabolism of oxygen, and that enzymes are required for the elimination of oxy radicals produced within cells (31).

1.4.3 Leucocyte oxidative burst

Phagocytic cells (polymorphonuclear (PMN) leucocytes and monocytes/macrophages) are distinctive in that they have the capacity to secrete superoxide extracellularly and into phagocytic vesicles through the action of an externally-oriented surface membrane oxidase (24,25,26). Intact function of this enzyme is important in defence against certain bacterial infections, as shown by the susceptibilities to infection of children with chronic granulomatous disease who lack leucocyte surface membrane oxidase activity (37).

In vitro studies have shown that PMN leucocytes and mononuclear phagocytes secrete superoxide ions in response to a number of stimuli, and that superoxide production is but one of several inter-related metabolic events. The term "oxidative burst" has been used to encompass these events comprising (a) increased oxygen uptake, (b) secretion of superoxide ions by the surface membrane oxidase which

utilises NADPH as co-factor, (c) increased hexose monophosphate shunt activity (which replenishes NADPH), (d) hydrogen peroxide production (either directly or through spontaneous dismutation of superoxide) (see below) and (e) chemiluminescence (CL) (24,25,26). Luminol-amplified CL generated by stimulated PMN leucocytes may be used as a convenient measure of oxidative burst activity (38). Factors shown to stimulate the oxidative burst <u>in vitro</u> include opsonized particles, particulate and soluble immune complexes, the complement fragment C5a, the synthetic chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP), and certain lectins and chemicals (39).

PMN leucocytes reach the synovial fluid in large numbers where they constitute the dominant cell type (40). Oxy radicals, which are produced during the oxidative burst, have been shown to degrade hyaluronic acid and reduce the viscosity of synovial fluid (41), depolymerize DNA (42) and cause peroxidation of phospholipid membranes (43) and may thus play an important role in the tissue damage occurring in inflamed joints and at other sites of inflammation. In general, agents which stimulate the oxidative burst also stimulate phagocytosis and degranulation of lysosomes (24). Thus oxidative burst activity at inflamed sites is likely to be associated with lysosomal enzyme release. Several degradative enzymes, e.g. plasminogen/plasmin, collagenase,

require activation extracellularly (44,45). A possible effect of oxy radical production on mechanisms of activation of these pro-enzymes in synovial fluid has not been subject to systematic study. The presence of effective protease inhibitors in inflamed synovial fluid (46), and other body fluids may restrict the activity of these enzymes to secondary phagocytic vacuoles and other "privileged" sites where these inhibitors are excluded. However, oxy radicals may be responsible for inactivation of certain protease inhibitors, for example, through irreversible oxidation of alpha-1 antiprotease (47,48,49).

1.4.4 Mechanisms of production of oxy radicals

NADPH-oxidase is a trans-membrane protein utilizing intracellular NADPH (supplied by hexose monophosphate shunt activity) to provide reducing equivalents to effect monovalent reduction of oxygen (24,25,26). The first product, superoxide ion, is released either into the extracellular space or into phagocytic vesicles.

$$O_2 + NADPH \longrightarrow O_2^{-\cdot} + NADP + H^+$$

Superoxide disproportionates to hydrogen peroxide and oxygen, either spontaneously or more rapidly by enzymatic dismutation with one of the family of superoxide dismutases (SOD).

$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \longrightarrow O_2 + H_2O_2$$

In vitro hydrogen peroxide reacts with ferrous ions to

generate the very reactive hydroxyl radical (Fenton reaction).

$$Fe^{2+} + H_2O_2 \quad \text{----->} \quad OH^- + OH^{\bullet} + Fe^{3+}$$

In the presence of a superoxide flux, ferric ions produced in the Fenton reaction are reduced to ferrous ions which can then again react with peroxide to complete a catalytic cycle.

$$Fe^{3+} + O_2^- \quad \text{----->} \quad Fe^{2+} + O_2$$

Thus microconcentrations of iron (1 uM or less) in a suitably available form are sufficient to generate hydroxyl radicals in the presence of a superoxide ion flux. A variety of iron chelators including endogenous agents (e.g. ADP, ferritin) may support this reaction (50,51).

Non-enzymatic autoxidation of iron has also been shown to generate $OH^{\bullet}$ or a similar highly reactive oxy radical (52,53,54,55), and this reaction is supported by iron-containing proteins such as ferritin, lactoferrin and transferrin (51,56). Such reactions could occur <u>in vivo</u> and under conditions of generalised or locally increased iron deposition (e.g. the inflamed joints of patients with rheumatoid arthritis) (57,58), and may be further promoted by the presence of physiologically available reducing agents (e.g. reduced glutathione and ascorbic acid), which are capable of reducing iron from the ferric to the ferrous

form, followed by the subsequent autoxidation of Fe^{2+} with concomitant oxy radical production (59).

The central roles of iron availability/sequestration in allowing/preventing the production of the most potent oxidant, hydroxyl radical, dictates that iron be effectively separated from superoxide ions in organisms living in an aerobic environment. In the early 1960s, Muirden (57) documented increased iron deposition as ferritin in synovium from rheumatoid joints and speculated on the possible role of iron in perpetuating rheumatoid inflammation. Blake and co-workers (60) have recently renewed these speculations in the light of current theories about the role of iron in catalyzing generation of oxy radicals in biological systems.

1.4.5 Oxy radicals and hyaluronate depolymerisation

McCord first demonstrated a reduction in viscosity of (a) bovine synovial fluid and (b) hyaluronic acid (HA) solutions following exposure to an enzymatically generated superoxide flux *in vitro* (61). Using a simple tube viscometer, he observed that viscosity reduction was inhibited by superoxide dismutase, catalase and mannitol, and suggested that hydroxyl radicals ($OH^{\bullet}$) depolymerize the polysaccharide components of the synovial fluid. Halliwell confirmed these findings using HA solutions and demonstrated

the dependence of the observed reduction of viscosity upon the presence of iron salts (62). Greenwald and Moy (41) recently published more detailed studies in which they showed that exposure of HA solutions to an enzymatically generated superoxide flux not only reduced the viscosity of the solutions but also rendered the HA susceptible to further degradation by lysosomal enzymes. They emphasised the importance of this finding by stressing the lack of convincing evidence of the existence of a hyaluronidase enzyme in pathological joint fluids, in spite of the frequent finding of reduced synovial fluid viscosity in inflammatory joint diseases (40,63). They further emphasised the relevance of their in vitro findings to changes occurring in inflamed joints by documenting a reduction of viscosity of HA solutions following exposure to activated PMN leucocytes, and demonstrating impairment of collagen gelation following exposure of collagen solutions to an oxy radical flux in vitro (64).

1.4.6 Oxy radicals and anti-inflammatory agents

It has been proposed that some anti-inflammatory drugs may exert their therapeutic effects by inhibiting the production of oxy radicals or by abrogating the effects of oxy radicals on tissue components. Studies designed to test this hypothesis have been largely concerned with oxy radicals produced by intact cells (65,66,67,68,69,70) or

produced enzymatically by the action of xanthine oxidase (XO) on its substrate hypoxanthine (HX) or xanthine (66,71). The XO/HX system is similar to the surface membrane oxidase responsible for the secretion of oxy radicals by PMN leucocytes in that they are both flavoproteins and produce superoxide ions as a primary product. This enzymatic system accordingly provides a useful experimental model in which superoxide production, by mechanisms broadly similar to the superoxide production of PMN leucocytes, can be studied in isolation from other cellular events. In spite of the potential biological relevance of non-enzymatic oxy radical production (outlined above in 1.4.4), the influence of anti-inflammatory drugs on HA degradation by this mechanism had not been systematically studied prior to the experiments described in this thesis.

1.4.7 Salicylate, gentisate and oxy radicals

Acetylsalicylic acid (aspirin) and its prime metabolite, sodium salicylate, are among the first agents to be shown to possess a clinically useful anti-inflammatory action. Following oral administration, aspirin is rapidly deacetylated to salicylate (72), the major metabolite of which is salicylurate (73). Smaller proportions (less than 2%) can be identified in plasma and urine as gentisate (2,5 dihydroxybenzoate)(74).

Oxidation of salicylate (2-hydroxybenzoate) following production of hydroxyl radicals *in vitro* results in a number of products depending upon the site of the benzene ring attacked by hydroxyl radicals (75). Gentisate and 2,3-dihydroxybenzoate (but not 2,4- or 2,6-dihydroxybenzoates) have been detected using thin layer chromatography) following exposure of salicylate *in vitro* to an oxy radical flux generated by ascorbate and either oxygen or hydrogen peroxide in the presence of Fe(III)-EDTA (75). Diphenol products of salicylate oxidation with an *ortho* configuration (presumably 2,3-dihydroxybenzoate) can be detected spectrophotometrically following chelation with tungsten (76). This latter assay has been used in the present studies to examine the effects of anti-inflammatory agents, anti-oxidants and other reagents of interest on hydroxyl radical formation and reactivity.

A high performance liquid chromatographic (HPLC) method has been developed (77) which allows simultaneous assay of salicylate, salicylurate and gentisate in plasma samples from patients receiving salicylate therapy. Synovial fluid gentisate levels have not been reported and studies were undertaken to determine the degree of correlation between plasma and synovial fluid gentisate in patients taking anti-inflammatory doses of aspirin/salicylate. These studies sought (a) evidence for peripheral conversion of

salicylate to gentisate which might reflect oxidation of salicylate by oxy radicals generated in inflamed joints and (b) to compare plasma and synovial fluid concentrations of gentisate with concentrations of gentisate shown to possess an anti-oxidant effect as determined by inhibition of oxy radical associated chemiluminescence (78).

1.4.8 Effects of oxy radicals on cell metabolism

The way in which endogenous oxidants may influence the metabolism of host tissues is poorly understood. Hydrogen peroxide is cytotoxic to cells in tissue culture (79) and has been implicated in leucocyte-mediated destruction of cultured cells (80,81), although similar conditions may not necessarily prevail *in vivo* where catalase and other potential anti-oxidants are very widely distributed. Superoxide ions show similar *in vitro* cytotoxicity, but this effect can be attributed to derived hydrogen peroxide (being inhibited by catalase but not by superoxide dismutase)(82). Degradation of DNA by oxy radicals generated enzymatically *in vitro* has been demonstrated (83) but the biological significance of this observation is not clear. The pathophysiological effects (e.g. on cartilage metabolism) of hyaluronic acid degradation in inflamed joints, which may be effected by oxy radicals, remains to be established. Also of interest is the recent observation of covalent bonding through dityrosine linkages of immunoglobulin molecules

isolated from rheumatoid joints (84). Similiar covalently bonded IgG aggregates can be formed in vitro using a myelo-peroxidase/hydrogen peroxide system (85). Thus rheumatoid inflammation may be amplified or perpetuated by oxidant-induced formation of immunoglobulin complexes, which may induce rheumatoid factor production and/or fix complement, or activate leucocytes (in turn promoting generation of more oxidants) (85).

Unsaturated fatty acids are particularly susceptible to oxidation and are oxidised to peroxides and their breakdown products which may have potent biological properties. Lipid peroxides and derived lipid alcohols may significantly alter the physico-chemical properties of cell membranes and may thus significantly alter a variety of essential cell functions. Lipid peroxides also significantly influence arachidonate metabolism (these effects are discussed in the next section).

1.5 ARACHIDONATE METABOLISM AND INFLAMMATION

Arachidonate derivatives, e.g. prostaglandins (PGs) and leukotrienes (LTs) are established as important mediators of inflammation. Pathways leading to the formation of PGs and LTs and their relevant pharmacological actions will be reviewed, with particular reference to possible effects of leucocyte surface membrane NADPH-oxidase activity on PG and

LT production.

1.5.1 Synthesis of Prostaglandins

Arachidonate release results from the action of the enzyme phospholipase A_2 upon phosphatidyl choline (86). Arachidonate so released may be further metabolised by one of two pathways, the pivotal enzymes of which are (a) cyclo-oxygenase (PGH_2 synthetase), and (b) several specific lipoxygenases.

Cyclo-oxygenase converts arachidonate to the endoperoxide PGH_2 from which (a) the stable prostaglandins, (b) thromboxane and (c) prostacyclin, are derived. This synthetase appears to have two enzymatic sites exhibiting two distinct enzymatic activities in the one discrete protein (87,88,89). The two activities are cyclo-oxygenase activity and a peroxidase activity. The cyclo-oxygenase activity results in formation from arachidonate of the hydroperoxy-endoperoxide derivative, PGG_2. The peroxidase activity reduces lipid hydroperoxides (ROOH) to lipid alcohols (ROH). This enzyme thus converts PGG_2 to the hydroxyendoperoxide PGH_2, an essential precursor of the stable prostaglandins. The cyclo-oxygenase enzyme requires the presence of small quantities (10^{-8} M) of a lipid peroxide for its activation and to maintain its activity, whereas higher concentrations of lipid hydroperoxides may

inhibit enzyme activity. Lands has put forward the concept of "peroxide tone" based on this small, but critical requirement by prostaglandin synthetase (and lipoxygenase) for lipid peroxide (90,91,92). Under normal physiological conditions peroxidase activity (which may include glutathione peroxidase) suppresses peroxide levels below this critical level and prostaglandin synthesis does not proceed. Following activation of leucocyte NADPH-oxidase, hydrogen peroxide is generated and thus favours the formation of lipid peroxides. Accordingly, activation of NADPH-oxidase may initiate arachidonate metabolism. Furthermore, by depleting co-substrates (e.g. glutathione) for hydroperoxide eliminating peroxidases (92), NADPH-oxidase may contribute to maintenance of lipid peroxide levels that are required for arachidonate metabolism. Furthermore, hydrogen peroxide which is a relatively stable uncharged oxidant and not ionised at physiological pH, may diffuse to other cells, initiate lipid peroxidation and thereby act as a chemical messenger effectively inducing arachidonate metabolism in adjacent cells.

1.5.2 Prostaglandins and inflammation

The importance of prostaglandins, produced through cyclo-oxygenase action, in inflammation was established in the early 1970s through the classic studies of Vane who

correlated the anti-inflammatory, antipyretic and analgesic effects of aspirin with prostaglandin synthetase (cyclo-oxygenase) inhibition (17). Of the first identified biologically active prostaglandins (e.g. PGE_2, $PGF_{2-alpha}$), PGE_2 seemed especially likely to be an important mediator of inflammation through vasodilatory effects and potentiation of nocioceptive and oedema-inducing effects of kinins and histamine (5). PGE_2 also has important effects in mediating bone resorption (93) and influences remodelling of connective tissues (94). The presence of PGE_2 in rheumatoid joints (95) and synthesis of PGE_2 by phagocytic cells (96) and synovial explant cultures (45) has been demonstrated. More recently, two arachidonate derivatives with important and antagonistic roles in the maintenance of vascular integrity have been described : (a) thromboxane A_2 produced by platelets and a promoter of platelet aggregation and vasoconstriction; (b) prostacyclin (PGI_2), produced by endothelial cells, an inhibitor of platelet aggregation, (and disaggregator of aggregated platelets), and a potent vasodilator. Both thromboxane and prostacyclin are produced by cells found outside the vascular compartment at sites of inflammation e.g. macrophages, and effects on inflammation additional to their effects on platelets and vascular smooth muscle are possible (97).

1.5.3 Synthesis of leukotrienes

The lipoxygase enzymes oxygenate arachidonic acid to form hydroperoxides at positions dictated by their specific activities (4). Specific hydroperoxides may either (i) be reduced (spontaneously or through peroxidase action) to mono-hydroxy-eicosatetraenoic acids (mono-HETEs), or (ii) be metabolized enzymatically to an epoxide. In PMN leucocytes the dominant lipoxygenase is a 5-lipoxygenase and its product 5-hydroperoxy eicosatetraenoic acid, (5-HPETE), is converted to 5-HETE and a 5,6 epoxide derivative, leukotriene A_4 (LTA_4). In PMN leucocytes, LTA_4 is converted by a hydrolase to 5(S),12(R) diHETE (LTB_4). In some cells (e.g. PMN leucocytes, mononuclear phagocytes, mast cells), LTA_4 is converted by glutathione-S-transferase to the sulphido-peptide leukotriene LTC_4 which may be further metabolised to LTD_4 and LTE_4. Significantly, in activated PMN leucocytes the 5-lipoxygenase pathway is considerably more active than the cyclo-oxygenase pathway, and mono-HETEs and leukotrienes are the dominant arachidonate derivatives (98).

Soybean 15-lipoxygenase like cyclo-oxygenase has been shown to require the presence of lipid hydroperoxides for activity (99). If this requirement for lipid hydroperoxides exists for the cytosolic lipoxygenases of leucocytes, a functional link may also exist between these enzymes and the

oxyradical-producing leucocyte surface membrane oxidase whose action may result in the formation of (lipid) peroxides.

1.5.4 Leukotrienes and inflammation

The leukotrienes are potent pro-inflammatory substances with activities at concentrations as low as 10^{-10} M. LTB_4 is a potent PMN leucocyte chemotaxin and also mediates PMN leucocyte aggregation and to a lesser extent degranulation (100). LTC_4 and LTD_4 (a) induce constriction of smooth muscle and are potent bronchial constrictors and (b) cause oedema through a direct effect on post-capillary venular permeability (i.e. possess slow reacting substance, SRS-A, activity) (101).

Important synergism may exist between effects of cyclo-oxygenase and lipoxygenase products at sites of inflammation. For example, the potent vasodilatory effects of PGI_2 and PGE_2 may potentiate oedema mediated by increased vascular permeability effected by LTC_4 and LTD_4 (102). This potentiation may be particularly important as these leukotrienes have vasoconstrictor effects, albeit at higher concentrations than required for their effects on vascular permeability.

1.5.5 Corticosteroids and arachidonate metabolism

Anti-inflammatory corticosteroids induce the synthesis by PMN leucocytes and macrophages of a peptide inhibitor (macrocortin, lipomodulin) which inhibits the action of phospholipase A_2 (103,104). This enzyme catalyses the release of arachidonate (and other polyenoic acids) located at the 2-position of phosphatidyl choline and is thus pivotal in providing precursor molecules for the prostaglandin and leukotriene producing pathways (86,105). The anti-inflammatory effects of corticosteroids may thus be explained by inhibition of both prostaglandin and leukotriene production through inhibition of phospholipase A_2 activity. In addition to limitations resulting from unwanted effects of steroids, the value of steroids as anti-inflammatory agents for rheumatoid disease is also mitigated by return of disease activity after several months of therapy at a constant steroid dose or severe exacerbation of disease upon attempted dose reduction. The basis for these effects is not well understood but may be related to alterations in steroid receptor expression (106,107) and/or increased activity of other enzymatic pathways leading to arachidonate release (108,109).

1.5.6 Actions for novel agents

The development of novel therapeutic agents with actions as (a) inhibitors of synthesis of pro-inflammatory

leukotrienes or (b) leukotriene antagonists, should provide an advance in the treatment of chronic inflammatory disorders, provided that beneficial effects can be acheived without unacceptable unwanted effects. The potential advantages are highlighted by animal studies showing enhancement of leucocyte migration to inflamed sites by therapy with indomethacin and other NSAIDs at doses sufficient to suppress PG synthesis by more than 90% (110). This increase in leucocyte numbers was inhibitable by the lipoxygenase inhibitor BW755C, implying that production of chemotaxins by lipoxygenase pathways may be increased in the presence of certain concentrations of NSAIDs.

Agents which act to reduce ambient levels of lipid peroxides (e.g. by scavenging free radicals) could conceivably inhibit both PG and LT synthesizing pathways if delivered to inflamed sites in sufficient concentrations. A better understanding of mechanisms of production and reactivity of oxy radicals in inflamed tissues is one step in the development of this approach.

1.6 SUPEROXIDE DISMUTASE (SOD)

1.6.1 Anti-oxidant and anti-inflammatory effects of superoxide dismutase.

Cells living aerobically possess intracytoplasmic enzymes (e.g. superoxide dismustase, catalase) which eliminate oxy radicals (31) (see 1.4.2). However, extra cellular fluids possess relatively little SOD or catalase activity (111). Thus superoxide secreted by phagocytic cells may result in damage to connective tissue macromolecules and surrounding cells. Accordingly, SOD in a suitably available form, might be able to abrogate the damaging (pro-inflammatory) effects of leucocyte-derived oxy radicals. The effectiveness of this approach depends upon the extent to which oxy radical-induced damage is dependent on the presence of superoxide ions acting either as direct toxins or indirectly through production of hydroxyl radicals by a superoxide-driven Fenton reaction. By contrast, damage mediated by hydrogen peroxide (a product of superoxide dismutation) or oxidant products of the hydrogen peroxide-myeloperoxide system (e.g. hypochlorite ions) should not be influenced by this approach (24,25).

1.6.2 Therapeutic administration of SOD

SOD has a very short plasma half time (4-6 minutes) and exogenous SOD has little anti-inflammatory effect unless

given in multiple doses over short periods (112). Attempts to improve the bio-availability of SOD have involved conjugation of SOD to macro-molecular carriers, e.g. ficoll, dextran, polyethylene glycol (113). McCord has shown that SOD in these conjugated forms has prolonged plasma retention and enhanced anti-inflammatory effects. Catalase conjugated to these carriers had negligible anti-inflammatory effects. Effects of enzyme-free carriers were not reported.

1.6.3 Orgotein

An independent line of investigation has described the use of a protein isolated from bovine liver, orgotein, now known to be copper zinc superoxide dismustase, in the treatment of a variety of human disorders. Diseases for which beneficial effects of orgotein have been claimed include osteoarthritis (114,115), rheumatoid arthritis (116), multiple sclerosis (115) and radiation cystitis (117,118). Unfortunately these studies, in general, have been poorly designed and do not allow a meaningful evaluation of the claimed therapeutic effects. However, these studies and studies in animals (119) do seem to indicate that orgotein is well tolerated when given by repeated injection intramuscularly, intravenously, intra-articularly and even intrathecally.

1.6.4 Soluble macromolecular protein polymers

Remy and Poznansky have developed a system in which heteroenzymes may be conjugated to homologous albumin in excess to form soluble crosslinked polymers with prolonged retention of enzymatic activity and negligible immunogenicity (120). Adaptation of this method to prepare bovine liver SOD and rat albumin conjugates for studies in rats will be described in this thesis. Studies included assessment of retention of conjugates and free enzyme in plasma and pleural spaces, anti-inflammatory effects and immunogenicity.

1.7 LIPOSOMES

1.7.1 General properties

Liposomes are lipid spherules formed by dispersion of phospholipids in aqueous media (121). These multilamellar bodies, which vary in size upto several microns in diameter, are reduced by high energy sonication to unilamellar vesicles of relatively uniform diameter (20-50 nm) (122). The vesicles comprise an outer lipid bilayer surrounding an inner aqueous space. Drugs can be incorporated into the vesicles either as solutes in the inner aqueous space or, in the case of lipophilic substances, as components of the lipid bilayer (29,123). Incorporation of water soluble drugs is relatively inefficient since less than 2% of a solution in which liposomes are dispersed becomes entrapped

within vesicles. Capture of lipophilic substances within the bilayer is far more efficient (100% for highly lipophilic substances) and there is little tendency for lipophilic drugs to leak from vesicles during storage (a problem occuring with liposomes entrapping water soluble and amphipathic compounds).

1.7.2 Liposomes as drug carriers

Liposomes are biodegradeable and readily metabolised and accordingly have attracted much attention as potential carriers of drugs for systemic use (29,123). Because liposomes are particulate in nature, they become concentrated in cells with well developed phagocytic function, such as the mononuclear phagocytes of the liver and spleen (124,125,126). The use of liposomes as a drug delivery system accordingly seems most promising in the treatment of those diseases in which the affected tissues have a highly developed phagocytic function. This contention is supported by *in vitro* studies of Weissman et al (127) showing markedly enhanced delivery of enzymes to enzyme-deficient phagocytes following encapsulation of enzymes in immunoglobulin-coated liposomes. *In vivo* therapeutic trials, comparing free and liposome-encapsulated antimony salts in the treatment of experimental leishmaniasis have also confirmed this expectation (128,129,130). Inflamed tissues are also rich in phagocytic

cells (e.g. macrophages and polymorphs). Therefore liposome encapsulation of anti-inflammatory drugs might improve their localisation in inflamed tissues, thereby enhancing their therapeutic effects.

1.7.3 Cortisol palmitate liposomes

Cortisol and related steroids have potent anti-inflammatory activity. Unfortunately, these effects are accompanied by serious dose-related toxic effects which essentially restrict the use of steroids as anti-inflammatory agents to potentially life-threatening diseases, characterised by inflammation without infection. Shaw et al (131) have shown that the palmitate ester of cortisol forms stable liposomes with dipalmitoyl phosphatidyl choline (DPPC). These liposomes were shown to have enhanced anti-inflammatory effects, compared to free cortisol, when injected directly into the knees of rabbits with an experimentally-induced arthritis (132). Prior to the studies described in this thesis the anti-inflammatory effects of cortisol palmitate liposomes given systemically had not been studied.

1.8 ANIMAL MODELS OF INFLAMMATION

1.8.1 General aspects

Although rheumatoid arthritis is sufficiently quantifiable to allow meaningful assessment of anti-inflammatory effects of chemotherapeutic agents, animal models of inflammation are required for:

a) In depth studies of mechanisms of inflammation.

b) Evaluation of effects of agents on specific aspects of inflammatory responses.

c) Preliminary assessment of efficacy and toxicity of novel agents.

Also animal studies allow control of variables such as genetic background, nutrition, stage of disease, initial severity of disease, which diminish the power of clinical studies.

Several animal models of inflammation are available. No single model closely mimics rheumatoid disease. Individual models are suited for assessment of certain aspects of inflammatory responses and most information is achieved by using several models to yield complementary information. A brief description of some models and their advantages and shortcomings is outlined below.

1.8.2 Paw oedema in rats

Paw oedema is useful for assessing inhibition of swelling associated with acute inflammation (133). This model allows short-term observation of swelling, typically upto 4 hours after the injection of an irritant, e.g. carageenan. This model is sensitive to PG-inhibiting nonsteroidal anti-inflammatory drugs and anti-inflammatory steroids.

1.8.3 Subcutaneous sponge implants in rats

Tissue responses can be induced using a number of irritants taken up into sponges prior to implantation (e.g. tuberculin, carrageenan)(134). The sponge provides a framework into which fluid exudate accumulates and inflammatory cells migrate, and allows quantitation of the fluid and cellular component of an inflammatory response. Inflammatory responses can be studied at different times, from an acute response at 1 or 2 days through to a reparative stage at 7 days or more. Generally the sponges are removed 4 to 5 days after implantation at a late acute inflammatory, early reparative stage.

1.8.4 Irritant-induced pleurisy in rats

Pleural responses can be induced by injection of irritant e.g. carrageenan (135). This model has been used to assess the cellular component of acute inflammatory

responses, particularly the early phase (first few hours) of acute inflammation. (In our experience variability of cell migration within experimental groups limits the utility of this model).

1.8.5 Adjuvant disease in rats

Adjuvant disease is a polyarthritis with systemic features induced in rats by intradermal injection of a heat-killed arthritogenic bacterium (e.g. M tuberculosis) dispersed in a suitable oily vehicle (i.e. a Freund's Complete Adjuvant) (136). Evaluation includes assessment of overall disease activity (joint score) and measurement of blood values, which reflect systemic effects of inflammatory disease. The polyarthritis develops approximately 10 days after the inducing injection. The time of onset of the disease, its severity, and the late (chronic) less inflammatory stage can be examined. The major shortcomings of this model are variability of disease severity within treatment groups and striking differences between rat strains as regards both disease severity and responses to treatment (which may limit the generality of findings to the rat strains studied) (136,137).

1.8.6 Knee joint synovitis in rabbits

Synovitis is induced by injection of an irritant (e.g. carrageenan (138)), or an antigen (e.g fibrin (139),

ovalbumin (140)), to which the rabbit has previously been sensitised. A synovitis, resembling rheumatoid synovitis histologically can be induced but responses vary from animal to animal and quantitation is difficult.

1.9 OUTLINE OF STUDIES

The studies to be described were designed to (a) acheive a better understanding of the role of oxy radicals in inflammation and (b) explore novel methods designed to achieve better control of inflammation. The following studies were undertaken:

1. Demonstration of oxy radical producing oxidative burst activity of leucocytes isolated from inflamed joints of patients with rheumatoid arthritis (and other inflammatory arthropathies).

2. Studies examining the mechanisms of production of (i) enzymatically-derived and (ii) non enzymatically-derived oxy radicals using (a) depolymerisation of hyaluronate and (b) hydroxylation of salicylate.

3. Studies of the effects of established anti-inflammatory agents and of potential therapeutic (anti-oxidant) agents on oxy radical production and reactivity in the

above systems.

4. Studies of synovial fluid concentrations of gentisate, a minor salicylate metabolite with potent anti-oxidant properties in some *in vitro* systems.

5. Studies of the anti-inflammatory effects of SOD (which eliminates the prime oxy radical product of activated leucocytes). These studies include (a) assessment of the anti-inflammatory action of SOD, (b) attempts to improve the bio-availability of SOD through formation of macro-molecular SOD conjugates, and (c) assessment of the immunoreactivity of these conjugates.

6. Studies of a drug carrier system (liposomes) incorporating a long chain fatty acid ester of cortisol designed to better deliver cortisol to phagocytic cells (leucocytes) at an inflammatory focus.

CHAPTER 2

CHEMILUMINESCENCE OF POLYMORPHONUCLEAR LEUCOCYTES FROM RHEUMATOID JOINTS

2.1 INTRODUCTION

The oxidative burst of stimulated PMN leucocytes comprises increased oxygen uptake, generation and secretion of superoxide ions and hydrogen peroxide, increased hexosemonophosphate shunt activity and chemiluminescence (CL) (24,25,26). Luminol-amplified CL generated by stimulated PMN leucocytes can be used as a convenient measure of oxidative burst activity (38). Studies were undertaken to examine chemiluminescent responses of peripheral blood and synovial fluid PMN leucocytes following stimulation with complement opsonized zymosan.

2.2 PATIENTS AND METHODS

2.2.1 Patients

Blood and synovial fluid samples were obtained contemporaneously from each of twenty-two patients with active synovitis and joint effusions. Eighteen patients had RA, one monoarthritis of the knee, one psoriatic arthritis, one juvenile onset polyarthritis with sacro-illiitis and one

Reiter's disease. Blood samples were also obtained from four healthy laboratory personnel, and non-inflammatory synovial fluid from three patients with osteoarthritis.

2.2.2 Isolation of PMN leucocytes

Synovial fluid and blood samples were collected simultaneously into 10 ml plastic tubes containing lithium heparin (125 international units). Peripheral blood PMN leucocytes were purified using a single-step Ficoll-Hypaque technique (141). Briefly, whole blood was layered onto a Ficoll-Hypaque medium of density 1.114 grams per litre in 10 ml plastic tubes and centrifuged for 30 minutes at 200g. The PMN leucocyte band was removed and suspended in calcium- and magnesium-free phosphate buffered saline (PBS), (Dulbecco's formula, Flow Laboratories, Stanmore, N.S.W., Australia). Synovial fluid was diluted 1:1 in PBS and centrifuged at 200g. The leucocyte-containing pellet was resuspended in PBS. Undiluted cell-free supernatants were retained for studies on the effects of autologous synovial fluids upon PMN leucocyte CL.

Following these initial isolation steps, either the synovial fluid or peripheral blood leucocyte preparations were then washed 3 times in PBS. When hypotonic lysis was required for one of the preparations, the same procedure was carried out on the other, whether necessary or not.

Differential counts performed with Turk's stain showed that greater than 95% of the peripheral blood leucocyte preparations were PMN leucocytes and over 70% of the synovial fluid leucocytes were PMN leucocytes.

Final suspensions of synovial fluid and peripheral blood PMN leucocytes were made in Dulbecco's complete buffer (DCB) (Dulbecco's phosphate buffered saline, complete formula, Commonwealth Serum Laboratories, Melbourne, Australia) supplemented with 1% foetal calf serum (FCS) (Flow Laboratories). Leucocyte counts were performed using a Coulter S counter and the stock suspensions adjusted to 1 x 10^6 cells per ml.

2.2.3 Preparation of opsonized zymosan and zymosan activated plasma

Zymosan A (Sigma Chemical Company, St. Louis, MO.) 20 mg was added to 2 ml normal saline, heated for 10 minutes in a boiling water bath and centrifuged for 7 minutes at 200g. The pellet was resuspended in 5 ml of 100% autologous plasma and incubated for 30 minutes at 37^{o}C. Zymosan was then sedimented and washed in DCB. A final suspension containing 10 mg per ml of opsonized zymosan, was made in 2 ml of DCB.

2.2.4 Preparation of chemiluminescence medium

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Sigma Chemical Co.) 17.7 mg was dissolved in 0.5 ml of 1M KOH, then diluted with 9.5 ml distilled water to achieve a stock solution of 10 mM. 100 ul of glucose (200g per litre) and 20 ul of the 10 mM luminol solution were added to 1 ml of medium-199 (10 x concentrate, Flow Laboratories). Following dilution with distilled water to a volume of 10 ml, the pH was adjusted to 7.3 by addition of 0.1 M KOH.

2.2.5 Chemiluminescence assay

CL was measured in a thermostatically controlled luminometer (Packard Pico-lite) at 37°C for up to 1 hour. For each measurement, 125 ul of synovial fluid or peripheral blood PMN leucocyte suspension was placed in a 6 x 50 millimeter disposable borosilicate glass culture tube (Kimble) to which 125 ul of CL medium was added. The maximum CL value obtained from these additions is referred to as the basal CL, and the maximum CL value resulting from the addition of opsonized zymosan is referred to as CL_{max}.

2.3 RESULTS

A typical trace showing basal CL and the CL response of PMN leucocytes to opsonised zymosan is shown in Figure 2.2 The basal CL generated by peripheral blood PMN leucocytes was significantly less than that generated by synovial fluid PMN leucocytes (Figure2.1). The CL_{max} obtained with peripheral blood PMN leucocytes was slightly greater than the CL_{max} of synovial fluid PMN leucocytes (Figure2.3). As there was potentially a difference of up to 25% in the PMN leucocyte content of synovial fluid and peripheral cell suspensions, correction for the PMN leucocyte content of synovial fluid cell suspensions would have served to exaggerate the observed differences in basal CL and reduce the observed differences in the CL_{max}.

In order to determine whether exposure of peripheral blood PMN leucocytes to Ficoll-Hypaque gradient centrifugation accounted for these differences, synovial fluid PMN leucocytes were subjected to this procedure. No significant change in basal CL was observed (paired t test, $p>0.2$, $n=6$). In all subsequent studies, synovial fluid PMN leucocytes were separated by dilution and centrifugation as described above.

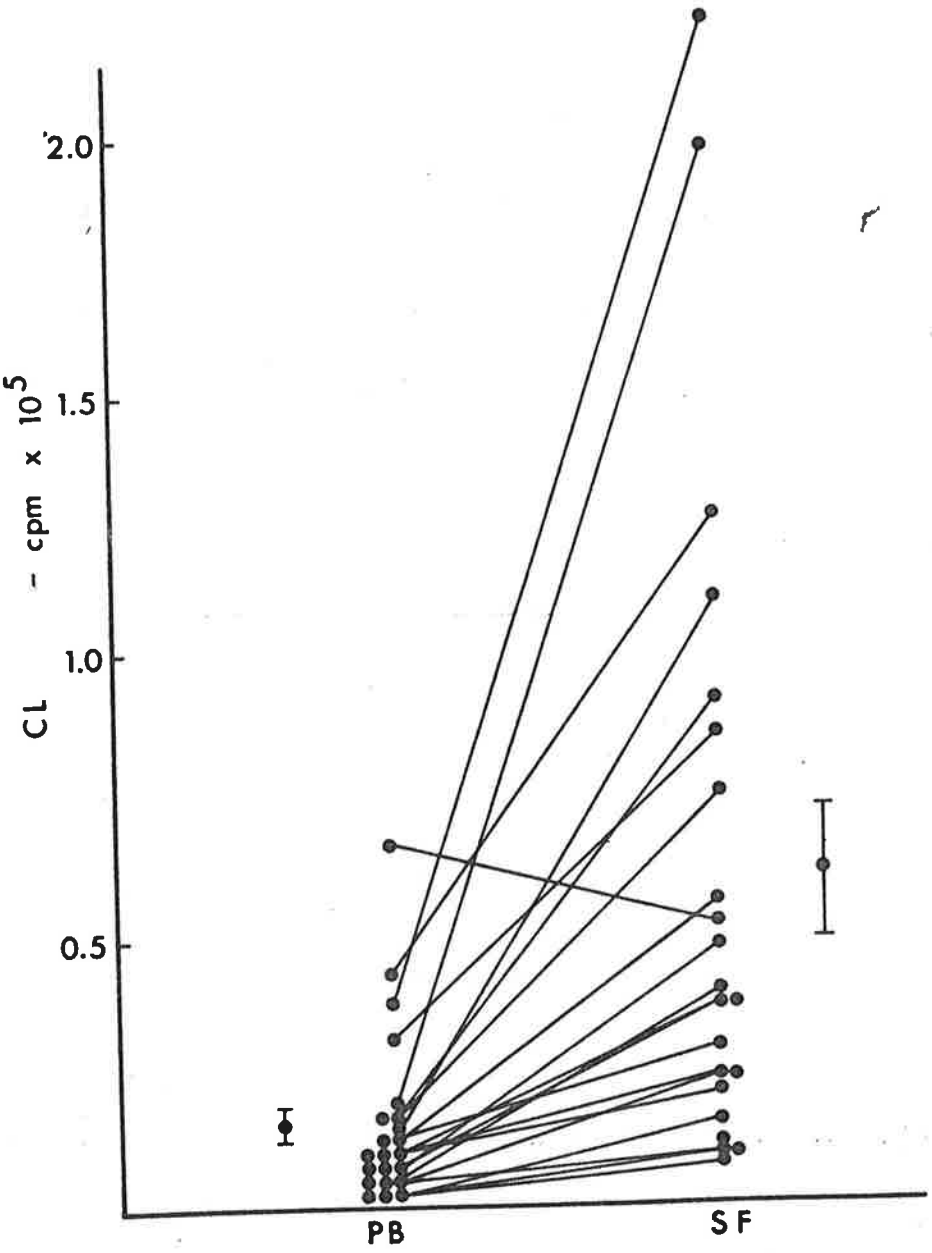

Figure 2.1. The unstimulated baseline CL (counts/min) of peripheral blood and synovial fluid PMN leucocytes from 22 patients. Lines join data points obtained with paired samples from individual patients. Means from each group are shown, error bars indicate $\pm$ SEM. Statistics by paired t test, $p < 0.001$.

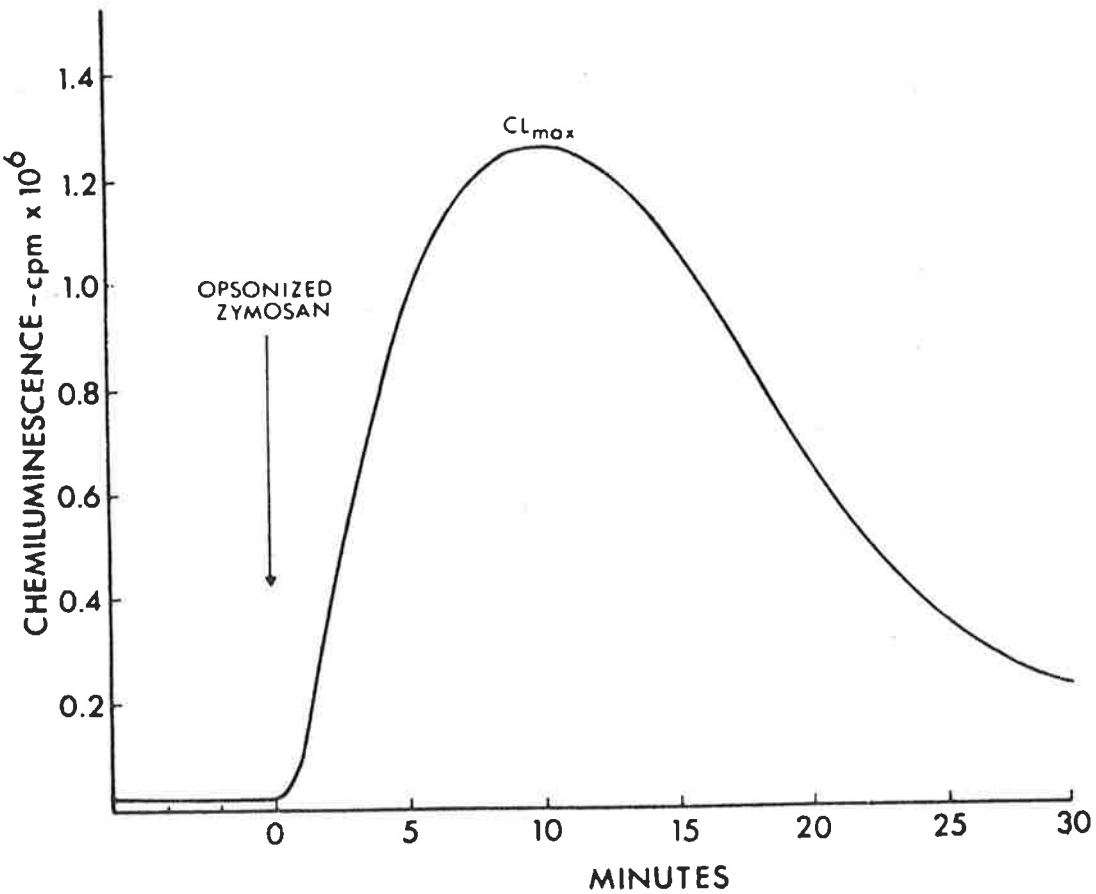

Figure 2.2. A typical CL response of peripheral blood PMN leucocytes following stimulation with opsonised zymosan added at time 0. The peak CL (CL_{max})(counts/min) occurred at 10min. Similiar responses were seen with synovial fluid PMN leucocytes.

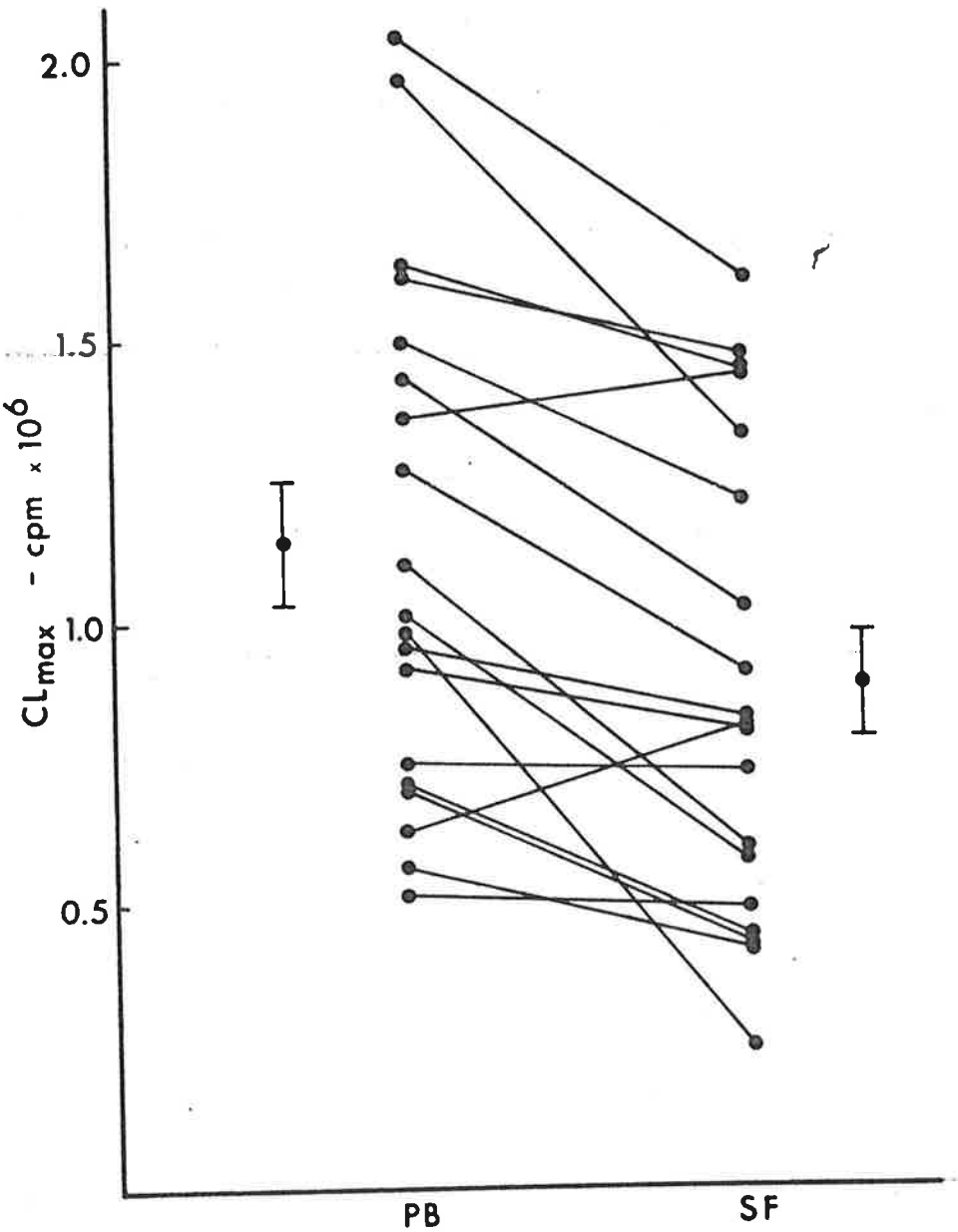

Figure 2.3. The CL_{max} (counts/min) of peripheral blood and synovial fluid PMN leucocytes from 19 patients following stimulation with opsonised zymosan. Lines join data points obtained with paired samples from individual patients. Means for each group are shown, error bars indicate $\pm$ SEM. Statistics by paired t test, $p < 0.001$.

To analyse the possible influence of synovial fluid in causing the differences observed between the responsiveness of peripheral blood and synovial fluid leucocytes, PMN leucocytes isolated from peripheral blood of (a) a patient with RA and (b) a healthy subject, were incubated with the synovial fluid of the patient. In both instances, there was a marked increase in the basal CL (Table 1).

Non-inflammatory (osteoarthritic) synovial fluid displayed no stimulatory activity when tested with peripheral blood PMN leucocytes with RA or healthy subjects. These non-inflammatory fluids did not yield sufficient cells to allow the function of their cellular constituents to be studied.

TABLE 2.1

Effect of inflammatory synovial fluid on CL of peripheral blood PMN leucocytes from a healthy subject and a patient with rheumatoid arthritis

	Healthy subject		RA subject	
	Before	After	Before	After
Baseline CL ($cpm \times 10^3$)	1.7	40.2	3.7	45.0
CL_{max} ($cpm \times 10^6$) (with opsonised zymosan)	1.1	0.9	0.6	0.7

Leucocyte CL was measured before and after incubation with synovial fluid for 1 hour. After incubation the cells were washed and resuspended in Dulbecco's + 1% FCS at 1×10^6 cells/ml.

2.4 DISCUSSION

This study demonstrates the competence of leucocytes isolated from inflamed joints to mount an oxidative burst as determined by CL generation in response to stimulation with serum opsonised zymosan particles. Leucocyte CL responses have been correlated with surface membrane oxidase activity (38) but are only partly inhibitable (approx. 50%) by SOD (142,143). CL generation is also seen in association with activity sub-cellular isolates of arachidonate metabolising enzymes (144) and arachidonate metabolism may contribute to CL responses of activated leucocytes (145).

Basal CL was found to be greater with synovial fluid leucocytes than for peripheral blood leucocytes. The preliminary studies undertaken show enhancement of basal CL following incubation with inflammatory synovial fluid but not with non-inflammatory synovial fluids. Subsequently, these studies have been confirmed and extended (146). These later studies show greater CL generation by synovial fluid PMN leucocytes than peripheral blood PMN leucocytes upon incubation with heat aggregated immunoglobulin G (a model system for soluble immune complexes). This increased responsiveness to aggregated IgG could be induced in peripheral blood leucocytes by preincubation with the

synthetic chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP), at concentrations of 10^{-10} to 10^{-8} M. These concentrations are within the window of concentrations of FMLP associated with chemotactic activity (147) and are lower than concentrations of FMLP required to stimulate PMN leucocyte oxidative burst activity directly (146,148). These later findings suggest that chemotaxins or other membrane perturbating agents present at critical concentrations within synovial fluid could act synergistically with immune complexes to stimulate leucocyte oxidative burst activity *in vivo*.

2.5 SUMMARY

These studies show (a) competence of synovial fluid leucocytes to generate an oxidative burst, (b) increased basal CL of synovial fluid leucocytes compared to peripheral blood PMN leucocytes, possibly resulting from effects on leucocytes of factors present in inflammatory synovial fluid.

CHAPTER 3

PRODUCTION OF OXY RADICALS AND EFFECT OF METAL CHELATORS, ANTI-OXIDANTS AND ANTI-INFLAMMATORY AGENTS I: HYDROXYLATION OF SALICYLATE

3.1 INTRODUCTION

This chapter describes the influence of (i) anti-inflammatory drugs (ii) iron concentration and valency, and (iii) metal chelators, on systems monitoring oxy radical reactions in the presence of oxy radicals produced (a) enzymatically by the xanthine oxidase/hypoxanthine (XO/HX) system and (b) chemically by the autoxidation of ferrous iron. The role of $OH^{\bullet}$ as the proximate reactive species in these two systems was determined using known $OH^{\bullet}$ scavengers, and the possible role of superoxide anions and hydrogen peroxide as intermediate species in the formation of $OH^{\bullet}$ was determined using superoxide dismutase and catalase. Hydroxylation of salicylate to 2,3 dihydroxybenzoic acid was used as an $OH^{\bullet}$-detecting system.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Xanthine oxidase (Grade III from butter milk), superoxide dismutase (SOD) (type I from bovine blood), catalase (from bovine liver) and hyaluronic acid (Grade III from human umbilical cord) were obtained from Sigma Chemical Corp., St. Louis, MO. The following compounds were generously donated: gold sodium thiomalate (May & Baker Aust. Pty. Ltd., Footscray, N.S.W.), indomethacin (Merck Sharp & Dohme (Aust) Pty. Ltd., South Granville, N.S.W.) and penicillamine (Lilly Industries Pty. Ltd., West Ryde, N.S.W.). Heparin was obtained from Allen and Hanburys (Glaxo Aust. Pty. Ltd.), Boronia, Victoria. All other chemicals were of the highest purity available from Sigma, or Ajax Chemicals, Sydney, Australia.

3.2.2 Methods

Superoxide dismutase was assayed by the inhibition of ferricytochrome C reduction by O_2^- generated enzymatically (32).

The salicylate hydroxylation experiments were performed using a modification of the method of Halliwell (76). In the autoxidation system, a 1 ml reaction mixture contained sodium salicylate (concentration range 0 to 5 mM), 5 mM

ferrous sulphate and 20 mM EDTA (unless otherwise stated) and test reagents at specified concentrations, in 0.1 M K_2HPO_4 at pH 7.4. The reaction was initiated by the addition of the Fe^{2+}/EDTA solution. With the enzymatic system the 1 ml reaction mixture contained 2.5 mM sodium salicylate, 100 uM $FeSO_4$, 300 uM EDTA, 10 mM hypoxanthine in 0.1 M K_2HPO_4 at pH 7.4. The reaction was initiated by the addition of 10 ul of 30 units/ml xanthine oxidase. In both systems, the reaction mixtures were incubated for 60 minutes at 25°C and the reactions were terminated by the addition of 10 ul conc. HCl. The reaction products were extracted into 3 ml diethyl ether. The recoveries from the ether extraction were greater than 90%, and 5% of the original total salicylate was hydroxylated to 2,3 dihydroxybenzoic acid. An aliquot of the upper layer was removed and evaporated on a water bath at 50°C, and the residue dissolved in 1 ml water. This solution was then acidified with 0.5 ml of 0.5M HCl containing 80 mg/ml trichloroacetic acid, followed by 1.0 ml of 10% sodium tungstate and 1.0 ml of 0.5% $NaNO_2$. After 5 minutes, 2 ml of 0.5M KOH was added and the absorbance at 510 nm was measured. Increased absorbance is proportional to increased formation of 2,3 dihydroxybenzoic acid which complexes with tungstate.

3.3 RESULTS

3.3.1 Hydroxylation of salicylate by the autoxidation of Fe^{2+}/EDTA

Hydroxylation of salicylate was found to be dependent on the concentration of ferrous ions in the range 0.63 - 5.0 mM, at a fixed Fe^{2+}/EDTA ratio of 1:4, and to be dependent on the salicylate concentration in the range 0-10 mM (Figure 3.1). There was no significant dependence on Fe^{2+} concentration below 0.63 mM. A ratio of Fe:EDTA of 1:4 produced maximal hydroxylation and was therefore chosen for all subsequent experiments. As the degree of hydroxylation decreased with time after preparation of the Fe^{2+}/EDTA stock solution, freshly prepared solutions were always used.

Partial deoxygenation of solutions by displacement with nitrogen resulted in approximately 50% inhibition of hydroxylation. When Fe^{3+} (as $FeCl_3$) was substituted for Fe^{2+}, there was only partial hydroxylation (9%) as shown in Table 3.1. Superoxide dismutase was without effect, and catalase produced minimal inhibition of hydroxylation (Table 3.2).

3.3.2 Hydroxylation of salicylate by the xanthine oxidase/hypoxanthine system

Table 3.1 shows that the enzymatic system only hydroxylates salicylate efficiently in the presence of trace amounts of Fe^{2+}/EDTA chelates. Increasing the Fe^{2+}/EDTA concentrations to 300 uM/900 uM had no significant effect on hydroxylation. When Fe^{2+} was replaced by 100 uM Fe^{3+} (as $FeCl_3$), hydroxylation was similar to the control value, indicating that although hydroxylation by the enzymatic system requires the presence of iron, both the ferrous or ferric oxidation states are active. This suggests a catalyic function for iron in this system. Hydroxylation was markedly inhibited by SOD and catalase (Table 3.2) indicating that both superoxide ions and hydrogen peroxide or their secondary oxidation products are the hydroxylating species in this system.

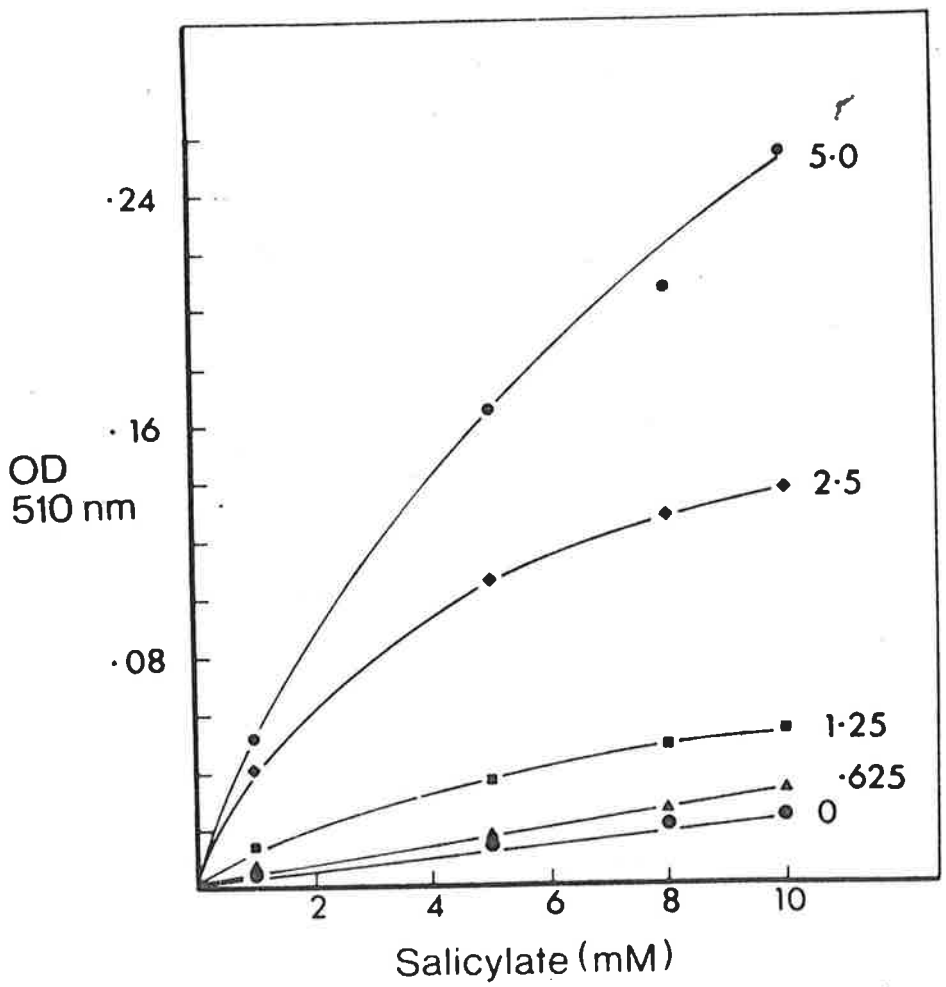

Figure 3.1. Hydroxylation of salicylate by autoxidation system (measured spectrophotometrically by increase in OD at 510nm) with increasing concentrations of Fe^{2+} (indicated in mM for each curve) at a fixed molar ratio of Fe^{2+}:EDTA of 1:4.

TABLE 3.1

Effect of iron salts on salicylate hydroxylation

Experimental conditions	Hydroxylation (% of control)	
	- XO/HX	+ XO/HX
Autoxidation system		
5 mM Fe^{2+}/20 mM EDTA	100	-
5 mM Fe^{3+}/20 mM EDTA	9	-
Enzymatic system		
100 uM Fe^{2+}/300 uM EDTA	3	100
300 uM Fe^{2+}/900 uM EDTA	3	92
100 uM Fe^{3+}/300 uM EDTA	3	95
No Fe/EDTA	-	17

In autoxidation experiments, hydroxylation of 5 mM salicylate measured in the presence of 5 mM Fe^{2+} and 20 mM EDTA is taken as the control value. In enzymatic system, hydroxylation of 2.5 mM salicylate in the presence of 100 uM Fe^{2+}, 300 uM EDTA, 10 mM hypoxanthine and 0.3 units/ml xanthine oxidase is taken as the control value.

TABLE 3.2

Effect of SOD and catalase on salicylate hydroxylation

Enzyme	Concentration (units/ml)	Percentage inhibition of hydroxylation	
		Fe^{2+}/EDTA	XO/HX
SOD	5	0	46
	10	1	69
	20	4	77
	400	3	82
	1000*	2	-
Catalase	10	8	43
	20	10	61
	400#	12	72
	1000	20	-

* = 0.3 mg/ml protein

\# = 0.03 mg/ml protein

Inhibition of hydroxylation was computed by subtraction of experimental values (obtained in the presence of specified quantities of SOD or catalase) from the control values (obtained in the absence of these enzymes), expressed as a percentage of the control values.

3.3.3 Hydroxylation time course

Figure 3.2 shows that after sixty minutes incubation, the final hydroxylation was of the same order of magnitude in both the autoxidation and enzymatic systems, although the initial rate of hydroxylation in the autoxidation system was much greater. Hence the different sensitivities of hydroxylation to catalase and other quenchers and inhibitors (see below) may be attributed to differences in the initial rates of hydroxylation where the flux of the hydroxylating species may be greater in the autoxidation system.

3.3.4 Effect of hydrogen peroxide

Addition of H_2O_2 to the autoxidation system caused an initial stimulation of hydroxylation which was maximal at 10-20 mM H_2O_2 (Table 3.3). However, at higher concentrations (100 mM), the stimulation had dropped to 140% of the control value. A similar observation was made with the enzymatic system, where the decreased stimulation at high concentrations of H_2O_2 may have been due to an inhibitory effect of H_2O_2 on the xanthine oxidase (149) and/or failure of Fe^{3+} reduction under strongly oxidising conditions.

TABLE 3.3

Effect of hydrogen peroxide on hydroxylation of salicylate

H_2O_2 Concentration (mM)	Hydroxylation (% of control)	
	Fe^{2+}/EDTA	XO/HX
0	100	100
1.0	137	155
2.0	179	168
10	350	136
20	363	125
60	122	116
100	140	106

Hydroxylation expressed as a percentage of a control obtained in the absence of H_2O_2 for each of the autoxidation and enzymatic systems.

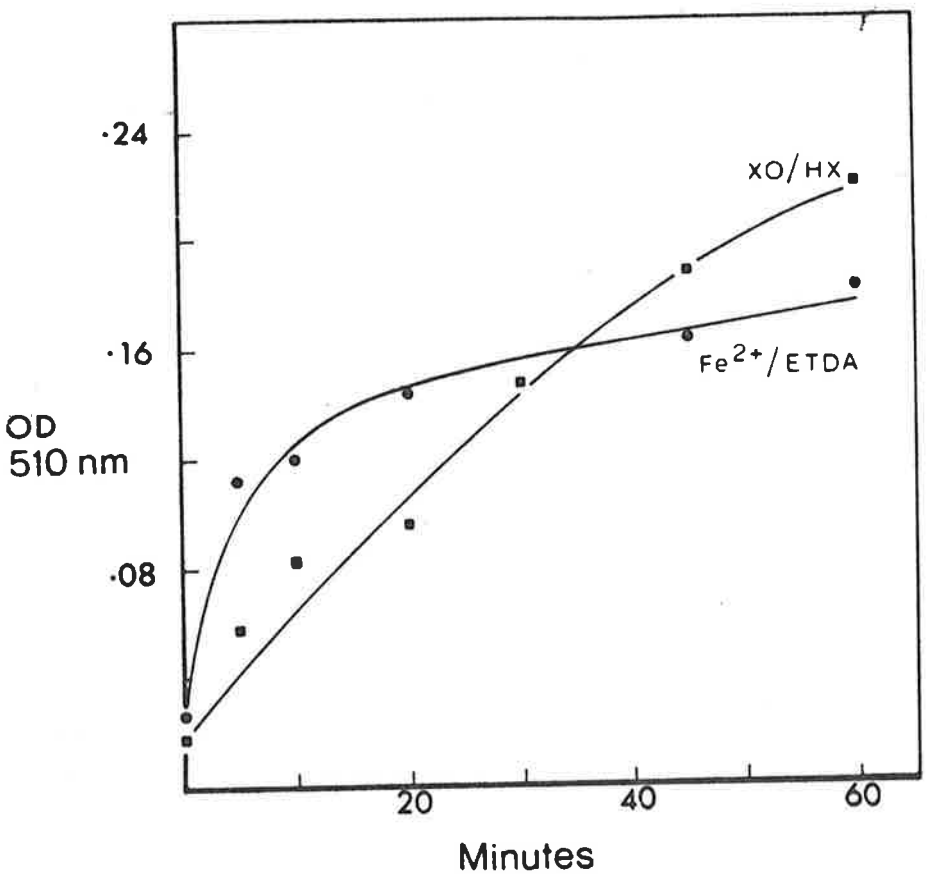

Figure 3.2. Time courses for salicylate hydroxylation by XO/HX system and the autoxidation system, in presence of 2.5 mM salicylate.

3.3.5 Effect of radical scavengers, chelators and therapeutic agents

Scavengers of hydroxyl radicals - mannitol, DMSO (dimethyl sulphoxide), benzoic acid, ethanol, sodium formate and thiourea - produced marked inhibition of hydroxylation in both systems at concentrations in the range 5 to 100 mM (Table 3.4). Generally, hydroxylation produced by the enzymatic system was more sensitive to inhibition than the autoxidation system. Putative singlet oxygen quenchers - sodium azide and histidine - produced significant inhibition in both systems; again, inhibition was more potent in the enzymatic system.

Potent inhibitory effects were seen with the chelators, BPS (bathophenanthroline sulphonate) and DETAPAC (diethylenetriamine pentaacetic acid), at concentrations as low as 10 mM. Similarly, when the ratio of Fe^{2+}/EDTA was low due to increasing EDTA concentrations, there was a marked inhibition of hydroxylation. Penicillamine inhibited the enzymatic system at all concentrations studied, and also inhibited the autoxidation system at concentrations greater than 10 mM; however, at the lower concentrations, hydroxylation by the autoxidation system was enhanced.

The anti-rheumatic drugs, gold sodium thiomalate and chloroquine, both produced inhibition of hydroxylation,

which was significant at less than 10 mM. Indomethacin (0.5 mM) was found to have a modest but definite inhibitory effect on hydroxylation (20% in the enzymatic system and and 11% in the autoxidation system). Detailed study of the effects of higher concentrations of indomethacin and other anti-inflammatory drugs with low solubility in aqueous solutions was not possible, as the inhibitory effects of organic solvents (e.g. ethanol, DMSO) precluded their use as solubilising agents.

High molecular weight mucopolysaccharides - hyaluronic acid and heparin - inhibited significantly at both 0.1 and 1.0 mg/ml. Glucose also inhibited at concentrations approximate to the physiological range. Potassium chloride and urea, both compounds previously shown to lack activity in quenching oxygen free radicals (76), also lacked inhibitory activity in these test systems.

Albumin in the concentration range 0.1 to 1 mg/ml provided a small inhibition of hydroxylation in the enzymatic system and a greater inhibition in the autoxidation system. These protein concentrations far exceeded protein concentrations of SOD and catalase required to produce inhibition of hydroxylation in the enzymatic system indicating that inhibition by SOD and catalase in this system could not be attributed to non-specific

quenching by protein. By contrast, protein concentrations required to produce inhibition of hydroxylation in the autoxidation system were similar for albumin, SOD and catalase. Accordingly the modest inhibition observed in this system in the presence of high concentrations of SOD and catalase may be attributed to non-enzymatic quenching by protein.

TABLE 3.4

Effect of various groups of compounds on salicylate hydroxylation

COMPOUND	Fe^{2+}/EDTA				XO/HO			
Concentration (mM)	1	10	50	100	1	10	50	100
$OH^{\bullet}$ scavengers								
Mannitol	–	15	49	64	26	45	78	86
DMSO	12	26*	75*	80*	41	81	94	96
Benzoate	–	55	94	–	–	71	95	100
Ethanol	–	16	53	57	–	–	46	74
Formate	–	31	61	74	–	–	83	88
Thiourea	–	55	82	96	–	–	89	91
Singlet oxygen quenchers								
Azide	17	55	83	86	–	81	92	93
Histidine	–	14	54	–	–	50*	85*	–
Chelating agents								
BPS	8	58	91	90	24	78	93	93
DETAPAC	0	26	61	75	31	64	88	92
Anti-rheumatic drugs								
Penicillamine	20##	22##	45	65	17	63	89	93
gold sodium thiomalate	10	33	66	75	19	62	91	94
Chloroquine	–	42*	75*	–	–	70*	89*	–
Naturally occuring polysaccharides								
Glucose	14	31	57	72	17	44	78	87
Hyaluronic acid**	15	–	–	–	39	–	–	–
Heparin**	11	23#	–	–	16	34#	–	–
Miscellaneous								
KCl	–	0	–	0	–	0	–	0
Urea	–	0	0	–	–	0	0	–
Albumin (mg/ml)	24	–	–	–	12	–	–	–

Inhibition of hydroxylation determined as in Table 3.2. *Values estimated from a dose response curve. **Quantities in mg/ml. #Value tabled for 2 mg/ml heparin. ##Values represent stimulation not inhibition of hydroxylation (see text).

3.4 DISCUSSION

The production of hydroxyl radicals by both oxy radical-producing systems used in these experiments was detected by the hydroxylation of salicylate to 2,3 dihydroxybenzoic acid. The different sensitivities of these systems to inhibition by SOD suggests a difference in the mechanisms of $OH^{\bullet}$ production in the two systems. The production of $OH^{\bullet}$ by the autoxidation system, which is not inhibited by SOD, has been ascribed to the following series of reactions (150):

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^- \quad (1)$$

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^- \quad (3)$$

Reaction 3 - the Fenton Reaction - should be inhibited by catalase. However, salicylate hydroxylation by the autoxidation system was found to be relatively insensitive to catalase (Table 3.2), and this was consistently observed in replicate experiments. In studies described in chapter 4 and other studies (62,151) using the degradation of hyaluronic acid to detect $OH^{\bullet}$ production by autoxidation of the Fe^{2+}/EDTA, degradation was inhibited by catalase. The marked stimulation of hydroxylation by added H_2O_2 provides further support for reaction 3 in this system (see Table 3.3).

In the enzymatic system, which is inhibited by both catalase and SOD, $OH^{\bullet}$ production can be explained by the following sequence of reactions:

$$\text{Hypoxanthine} + O_2 \longrightarrow \text{urate} + O_2^- \quad (4)$$

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$

$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \quad (5)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^- \quad (3)$$

Nett reaction

$$H_2O_2 + O_2^- \longrightarrow OH^{\bullet} + OH^- + O_2 \quad (6)$$

In this series of reactions, iron plays a catalytic role, being alternatively oxidized and reduced. The reducing agent (for reaction 5) in this case is the superoxide ion, produced by reaction 4, and this explains why $OH^{\bullet}$ production by this system is sensitive to SOD. Equation 3 (and 6) should be sensitive to catalase, as observed. These observations and the sensitivity of the enzymatic system to $OH^{\bullet}$ scavengers provides evidence that hydroxyl radicals are produced from superoxide via an iron-catalysed Haber Weiss Reaction (reaction 6).

Although singlet oxygen quenchers inhibited hydroxylation, suggesting a role for singlet oxygen, the inhibitory effects of these quenchers may not be restricted

to reactions with singlet oxygen, because by virtue of their low ionization potential, they also react with other strongly oxidising species such as hydroxyl radicals (152).

Salicylate hydroxylation by both the autoxidation and the enzymatic systems was shown to be inhibited by high concentrations of the chelating agents DETAPAC, BPS and EDTA (autoxidation system only), in agreement with the findings of Halliwell (76). Studies described in chapter 4 and those of Halliwell (62) have shown that DETAPAC and BPS also inhibit the degradation of hyaluronic acid by both of these radical producing systems.

Gutteridge et al found that EDTA, DETAPAC and BPS at low concentrations (greater than 100 uM) significantly stimulated peroxidation of phospholipid (from bovine brain), whereas higher concentrations(greater than 0.5 mM) inhibited peroxidation (153). They therefore suggested that there was a critical ratio of chelator:Fe^{2+} at which there was a change from a pro-oxidant to an anti-oxidant effect. Buettner and Oberley, using light activated riboflavin to produce oxy radicals and spin trapping to identify the radical species, concluded that iron sequestered by DETAPAC is no longer active, and thus the reactions which lead to $OH^{\bullet}$ formation are inhibited (154). The inhibition of hydroxylation by other chelators (including penicillamine)

could be attributable to similar mechanisms.

Penicillamine produced an inhibition of hydroxylation at all concentrations tested in the enzymatic system and at high concentrations in the autoxidation system. However, in the autoxidation system, low concentrations of penicillamine (1 and 10 mM) stimulated hydroxylation. We have also observed an enhancing effect of penicillamine, at these concentrations upon degradation of hyaluronic acid (62). Gutteridge et al found that penicillamine in the range 14 - 1000 uM produced a stimulation of Fe^{2+}-induced lipid peroxidation (153). This stimulation of oxy radical production by critical concentrations of penicillamine may be due in part to the ability of penicillamine to act as a reducing agent. Under the conditions of excess Fe^{3+}, penicillamine could reduce Fe^{3+} to Fe^{2+}, which could then autoxidise again to produce $OH^{\bullet}$. It is possible that the incompletely explained anti-inflammatory and anti-rheumatic actions of penicillamine may in some way be related to the reduction and chelation of iron in inflamed joints, where increased iron deposition has been demonstrated (57,58,155). Chelates of copper and penicillamine may also be implicated in the therapeutic action of penicillamine because chelates of copper and penicillamine are known to have a superoxide dismutase-like action (156,157).

The inhibition of hydroxylation by hyaluronic acid, heparin and glucose is consistent with the observation that many biological/organic compounds, particularly macromolecules and compounds with hydroxyl groups, react at rapid rates with hydroxyl radicals (158). Hyaluronic acid and salicylate probably react with hydroxyl radicals in a competitive manner and in the process hyaluronic acid is depolymerised (62, chapter 4 of this thesis).

The degree of inhibition of hydroxylation produced by chloroquine, gold sodium thiomalate and penicillamine in oxy radical-producing systems was at least as potent as that of recognised $OH^{\bullet}$ scavengers and singlet oxygen quenchers. Although we have not eliminated the possibility that in the enzymatic system these drugs may affect the enzymatic activity of xanthine oxidase, it should be noted that these drugs show a similar potency in the autoxidation system. Concentrations required for inhibition (10 mM or less) may be achieved at important micro-locations in inflammatory exudates. For example, <u>in vivo</u>, both chloroquine derivatives and gold are selectively concentrated in lysosomal vesicles (159,160), and therefore in secondary lysosomes in which generation of superoxide ions and secondary oxy radicals is known to occur (161,162,163).

These studies, using two *in vitro* models of oxy radical production designed to mimic mechanisms of oxy radical production likely to be seen *in vivo* at sites of inflammation, indicate that some anti-inflammatory drugs (e.g. penicillamine) alter oxy radical generation by non-enzymatic mechanisms, and that these and other anti-inflammatory drugs may competitively inhibit oxidation of tissue components by oxy radicals produced enzymatically or non-enzymatically. Further studies, designed to (a) determine the composite effects of physiological and pharmacological agents likely to effect oxy radical reactivity at sites of inflammation and (b) the relative sensitivities of tissue components and functions to oxy radical induced damage, are required to determine the relevance of the observed effects to the therapeutic action of anti-inflammatory drugs.

3.5 SUMMARY

The production of a potent hydroxylating species, presumed to be hydroxyl radical, was studied using hydroxylation of salicylate as a detector system. Oxygen-derived free radicals (ODFR) were generated by (a) autoxidation of ferrous-EDTA chelates and (b) enzymatically (xanthine oxidase/hypoxanthine). Hydroxylation by these oxy radical-generating systems was inhibited by superoxide dismutase, hydroxyl radical scavengers and singlet oxygen quenchers. Low concentrations (1 mM - 10 mM) of penicillamine stimulated hydroxylation by the autoxidation system, although higher concentrations were inhibitory; all concentrations were inhibitory in the enzymatic system. The chelating agents, DETAPAC and BPS, were inhibitory in both systems, as were the long acting anti-rheumatic drugs, gold sodium thiomalate and chloroquine. The mechanisms of hydroxyl radical generation described may have relevance to the mechanisms of oxy radical generation occuring *in vivo* at sites of inflammation.

CHAPTER 4

PRODUCTION OF OXY RADICALS AND EFFECT OF METAL CHELATORS, ANTI-OXIDANTS AND ANTI-INFLAMMATORY AGENTS II: DEGRADATION OF HYALURONATE

4.1 INTRODUCTION

This chapter describes studies designed (a) to determine the oxy radical species directly or indirectly responsible for degradation of hyaluronate solutions by (i) the xanthine oxidase/hypoxanthine (XO/HX) system and (ii) autoxidation of ferrous ions in phosphate buffer, (b) to establish the role of iron in oxy radical production in the two systems, (c) to re-evaluate earlier studies demonstrating inhibition by iron chelators and anti-inflammatory drugs, of reactivity of oxy radicals produced by enzymatic mechanisms (62,66,71,76) and (d) to extend studies of action of chelators and anti-inflammatory drugs to oxyradicals generated by the non-enzymatic (autoxidation) system, and to compare the relative activities of these drugs in the two oxy radical-producing systems.

The systems used to produce oxy radicals and the agents whose effects are under investigation in these studies are

similar to those employed in the previous chapter. Thus, these studies of hyaluronate degradation and those employing hydroxylation of salicylate to detect oxy radicals (Chapter 3) provide complementary information regarding factors influencing production and effects of oxy radicals.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Hyaluronic acid (Grade III from human umbilical cord), xanthine oxidase (Grade III from butter milk), SOD (type I from bovine blood), and catalase (from bovine liver) were obtained from Sigma Chemical Corp., St. Louis, MO. The following compounds were generously donated: gold sodium thiomalate (May & Baker Aust. Pty. Ltd., Footscray, N.S.W.), indomethacin (Merck Sharp & Dohm (Aust). Pty. Ltd., South Granville, N.S.W.) and penicillamine (Lilly Industries Pty. Ltd., West Ryde, N.S.W.). All other chemicals were of the highest purity available from Sigma, or Ajax Chemicals, Sydney, Australia.

4.2.2 Methods

Hyaluronate stock solutions (4 mg/ml) were prepared by dissolving hyaluronic acid in sterile distilled water. This slightly opaque solution was then cleared by filtration through a 1.2 micron millipore filter, and then stored

either frozen or at 4°C until required. Before use this solution was diluted 1:1 with 100 mM phosphate buffer to give a working solution of 2 mg/ml. For the viscosity measurements, a 1.7 ml solution at 25°C contained (a) for the enzymatic system, 6 mM hypoxanthine, 12 x 10^{-3} units/ml xanthine oxidase, 1 mg/ml HA in phosphate buffer; (b) for the Fe^{2+}/EDTA system, 100 uM ferrous sulphate, 100 uM EDTA, 1 mg/ml HA in phosphate buffer, unless otherwise stated.

In both systems, the buffer used was 50 mM potassium phosphate containing 0.1 mM EDTA at pH 7.4, unless otherwise indicated. Stock solutions of all inhibitors, xanthine oxidase and hypoxanthine were prepared in buffer and added to the experimental solutions in the required amounts. A stock solution of 50 mM ferrous sulphate and 50 mM EDTA in distilled water was freshly prepared before each Fe^{2+}/EDTA experiment and 3.4 ul added to the experimental solutions to initiate the oxy radical flux.

Concentrations of microcontaminant iron in phosphate buffer, HA solutions and distilled water were determined spectrophotometrically using ferrozine (164) and in all cases, total iron was less than 1 uM.

The tube viscometer consisted of a 1 ml tuberculin syringe fitted with a 20 G stainless steel disposable needle

(0.90 x 38 mm). A reservoir consisting of a 10 x 75 mm polystyrene tube was placed under the syringe assembly and the experimental solution drawn into the syringe with care being taken to avoid bubbles. The time for 0.8 ml to drain from the syringe assembly was measured and the relative viscosity (η_R) of the HA solution calculated using the equation:

$$\eta_R = \frac{\text{time for hyaluronate solution}}{\text{time for buffer solution}}$$

The specific viscosity (η_{sp}) is defined as $\eta_R - 1$ and is a measure of the contribution to the total solution viscosity of the solute, in this case HA. The concentration of HA (1 mg/ml) was chosen so that a typical elution time for 0.8 ml using the above system was between 40 and 50 seconds, whereas the elution time for the buffer was approximately 16 seconds, thus giving adequate sensitivity and reproducibility of η_{sp}. The average shear stress applied to the solutions during a typical measurement was approximately 25 dynes/cm^2 while the shear rate at the capillary wall varied from 800 sec^{-1} for the control HA solutions to 3500 sec^{-1} for the buffer. The viscosity of 1 mg/ml HA solutions prior to exposure to oxy radicals at this shear stress was approximately 3.0 cPoise. Over this range a 1 mg/ml HA solution behaves approximately as a Newtonian fluid (165).

4.3 RESULTS

4.3.1 Time course of viscosity change

The time courses for the reduction of viscosity of the HA solutions were noticeably different in the XO/HX system and the Fe^{2+}/EDTA system (Figure 4.1). With the XO/HX system, the viscosity (η_{sp}) was reduced to approximately 20% of the initial value in 40 minutes after the initiation of the oxy radical flux, whereas in the Fe^{2+}/EDTA system, new equilibrium values (20% of control) were reached within 2 minutes. Hence in subsequent experiments, viscosity was measured after 60 minutes in the enzymatic system and after 10 minutes in the Fe^{2+}/EDTA system. The reduction in viscosity has been shown to be a result of the degradation or depolymerization of the HA moiety (53,61).

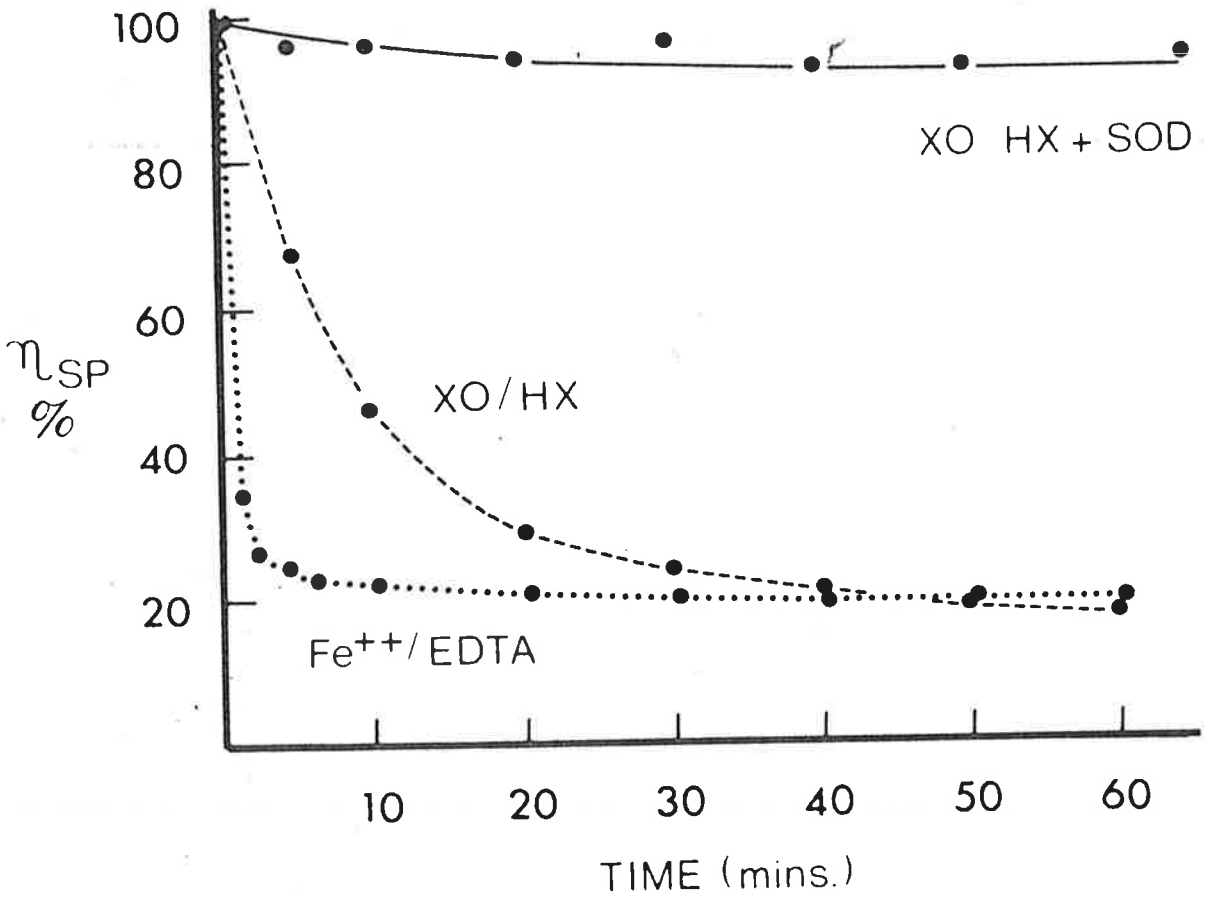

Figure 4.1. Time course for viscosity change of an hyaluronate solution (1 mg/ml) in the presence of (a) 200 uM Fe^{2+}/EDTA (●.....●) and (b) hypoxanthine and xanthine oxidase (●------●). Changes in specific viscosity (η_{sp}) are expressed as a percentage of control values measured before the addition of (a) Fe^{2+}/EDTA and (b) xanthine oxidase, respectively. SOD 150 units/ml inhibited viscosity change induced by the XO/HX system (● —— ●), but had no effect in the Fe^{2+}/EDTA system (not shown).

4.3.2 Requirement for EDTA and iron in XO/HX system

Little degradation of HA was caused by the XO/HX system in the absence of EDTA or added iron (Table 4.1). Substantial HA degradation occurred in the presence of 0.1 mM EDTA, and significant degradation was also observed in the presence of added ferric ions without EDTA. Optimal degradation was observed in the presence of 0.1 mM iron and 0.1 mM EDTA. Since the experimental solutions were shown to contain iron as a microcontaminant, these results indicate a requirement of suitably chelated iron for optimal degradation of HA by the XO/HX system.

Table 4.1

Effect of EDTA and iron on hyaluronate degradation by the XO/HX system

Experimental conditions	Specific viscosity (% of controls)
no XO (control)	100
+ XO	93
+ XO + 0.1 mM EDTA	32
+ XO + 0.1 mM $FeCl_3$	80
+ XO + 0.1 mM $FeCl_3$ + 0.1 mM EDTA	12

All experiments in 50 mM phosphate buffer at pH 7.4 with 1.2 mM hypoxanthine with or without 0.012 units/ml xanthine oxidase (XO) as indicated.

4.3.3 Dependence on concentration and oxidation state of iron

In the Fe^{2+}/EDTA system, the degree of viscosity reduction was shown to be dependent on the Fe^{2+} concentration (Figure 4.2). This was the case in the presence and absence of EDTA. In the latter case, the Fe^{2+} in the stock solution did not completely dissolve but remained in suspension until added to the experimental solutions to initiate the degradation. Despite this difficulty, the results demonstrate degradation of HA by Fe^{2+} without EDTA, suggesting that Fe^{2+} does autoxidize in the absence of EDTA to produce an oxy radical flux. For greatest sensitivity it was decided to use 100 uM Fe^{2+} and 100 uM EDTA in the inhibition studies. Fe^{3+} (as $FeCl_3$) in the presence of EDTA, had no effect on the degradation of HA.

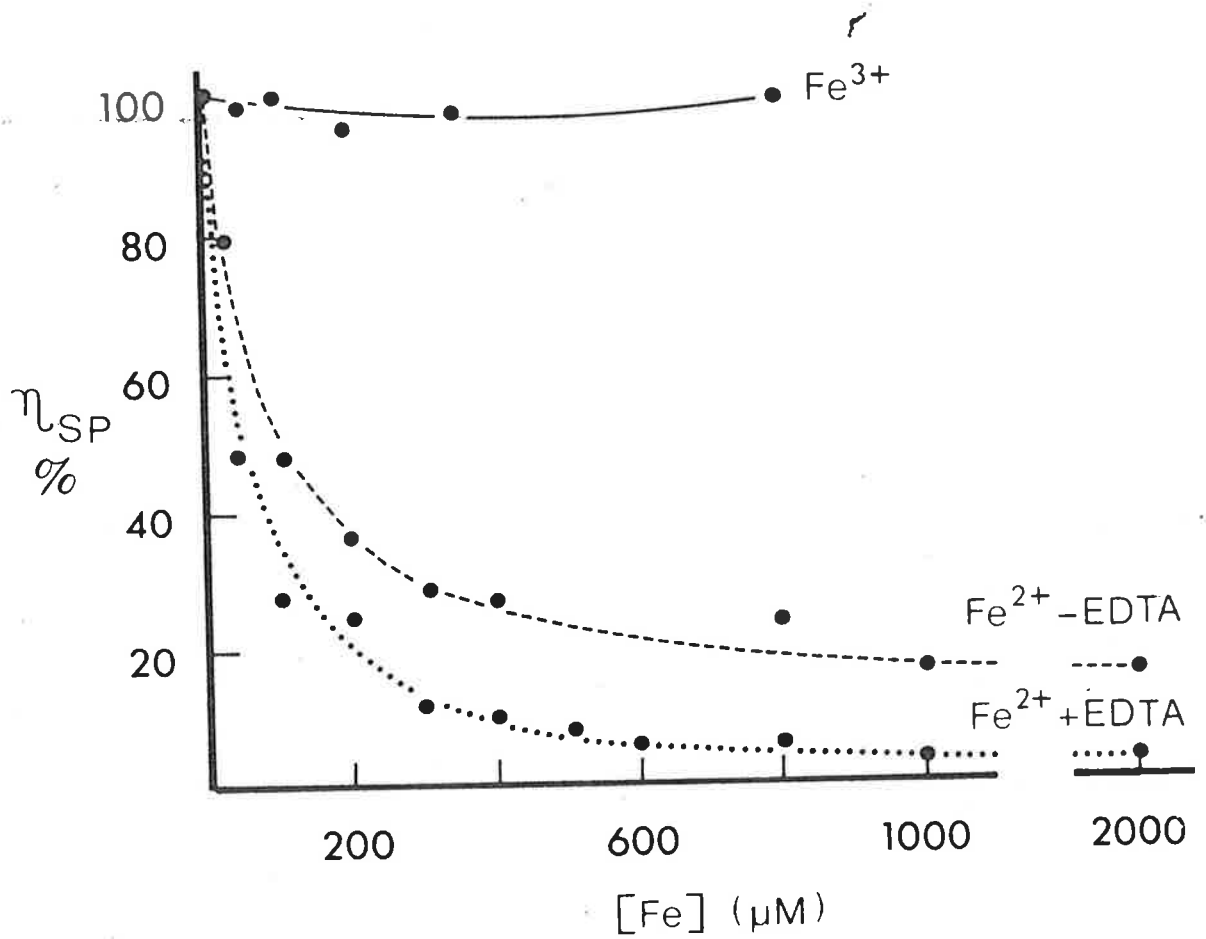

Figure 4.2. Effect on specific viscosity (η_{sp}) of hyaluronate (1 mg/ml) of varying concentrations of Fe^{2+} in the presence (●.....●) and absence (●-----●) of 500 uM EDTA, or Fe^{3+} (●———●) in the presence of EDTA. Viscosity measurements were made 10 minutes after the addition of iron.

4.3.4 Additive effects of Fe^{2+}/EDTA and XO/HX systems

Figure 4.4 shows the additive effects of Fe^{2+}/EDTA (25 uM) and the XO/HX systems in producing HA degradation. In this experiment, 25 uM Fe^{2+}/EDTA was added to HA solution containing all components except the xanthine oxidase. Ten minutes later, when a new stable value had been reached, xanthine oxidase was added to initiate the additional free radical flux. A further reduction in viscosity was seen which was of similar magnitude to that obtained with the enzymatic system alone, and the final viscosity indicated an additive effect of the Fe^{2+}/EDTA and the enzyme system on HA degradation.

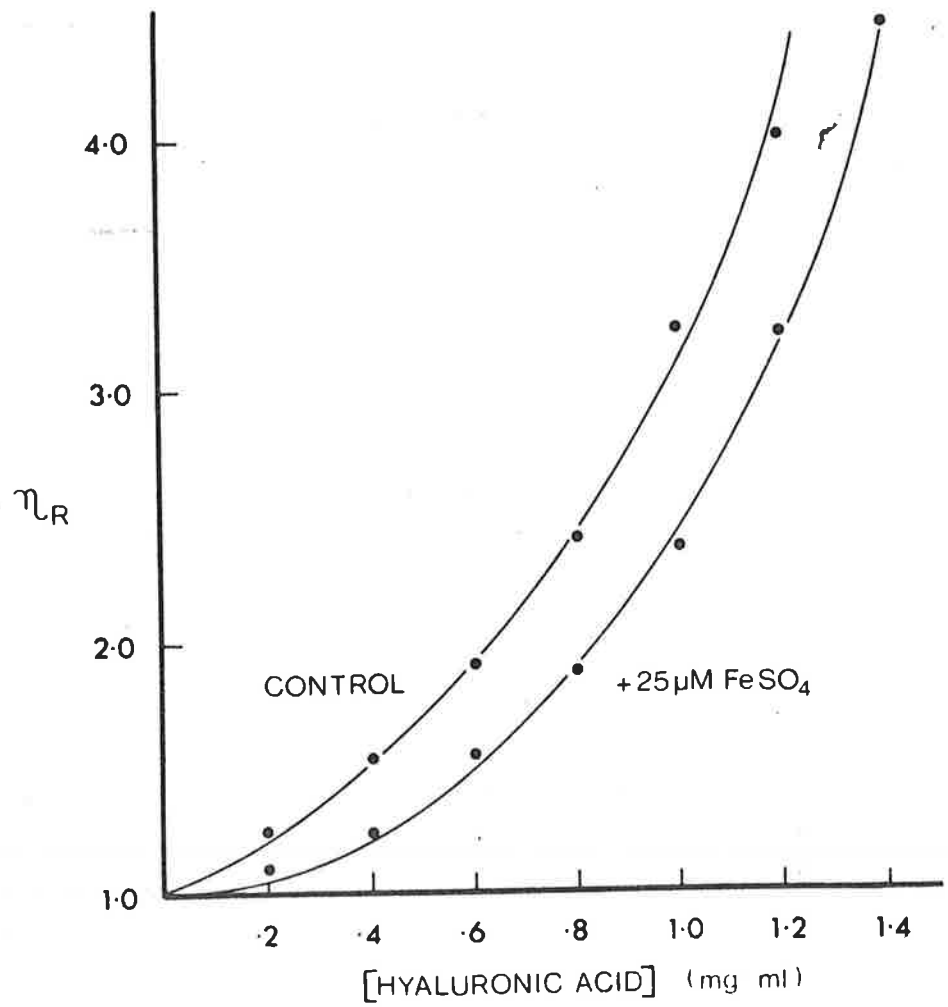

Figure 4.3. Reduction of specific viscosity (η_{sp}) of hyaluronate (1 mg/ml) following addition of xanthine oxidase at time 0, preceded by addition of either $FeSO_4$/EDTA to give final concentration of 25 uM Fe^{2+} (♦.....♦), or an equal volume of EDTA (•———•), 10 minutes earlier.

4.3.5 Effect of Fe^{2+} at different hyaluronic acid concentrations

An increase in viscosity with increasing concentrations of HA is illustrated in Figure 4.3. The relative viscosity (η_R) increased up to 4 as the HA concentration increased to 1.4 mg/ml. The effect of the addition of 25 uM Fe^{2+}/EDTA at each concentration was to displace the curve to the right by the equivalent of 0.2 mg/ml of HA. This indicated (a) that 25 uM Fe^{2+} reduces the viscosity of HA by an amount equivalent to that achieved by reducing the HA concentration by 0.2 mg/ml, and (b) that this reduction is not dependent on the initial HA concentration.

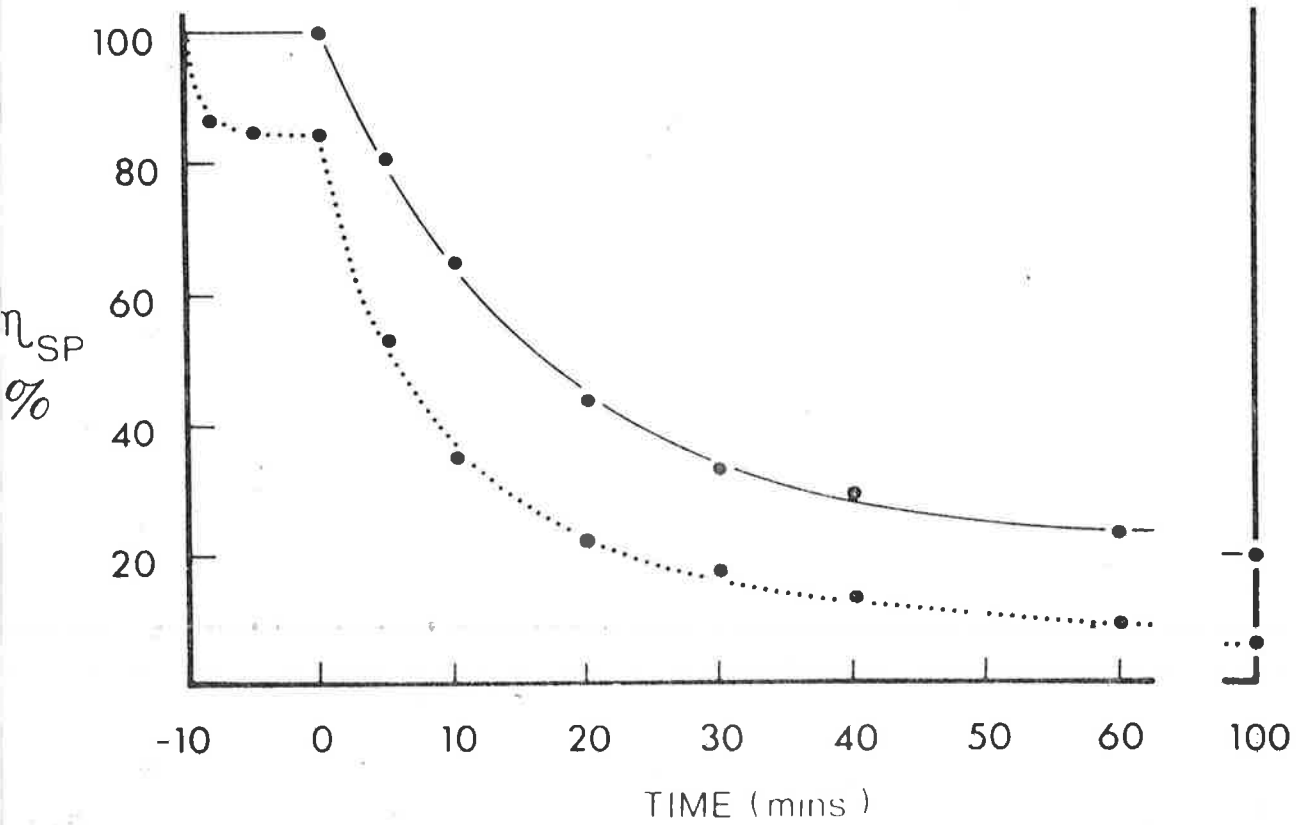

Figure 4.4. Relationship between relative viscosity (η_R) and hyaluronate concentration in the presence and absence (control) of 25 uM $FeSO_4$/EDTA.

4.3.6 Effect of superoxide dismutase and catalase

SOD at an activity of 20 units/ml inhibited the HA degradation by 84% in the XO/HX system (Table 4.2). In the Fe^{2+}/EDTA system, this activity had no effect and increasing the SOD activity up to 2000 units/ml (protein conc. 0.7 mg/ml) produced only a 6% inhibition. This small inhibition can be attributed to non-enzymatic quenching of oxy radicals, because albumin produced a similar degree of inhibition at a concentration approximate to that required to achieve this enzymatic activity.

Catalase, 200 units/ml, inhibited the degradation by the XO/HX system to a greater extent (89%) than the degradation caused by the Fe^{2+}/EDTA system (16%). However, at 2000 units/ml, catalase inhibited the HA degradation by 59% in the Fe^{2+}/EDTA system. At this activity the protein concentration was 0.7 mg/ml, and since 2 mg/ml albumin produced only a 13% inhibition of degradation (Table 4.3), the inhibition by catalase at these higher activities (2000 units/ml) cannot be attributed to non-specific protein quenching of oxy radicals.

TABLE 4.2

Effect of superoxide dismutase and catalase on hyaluronate degradation

Inhibitor units/ml	Percentage inhibition of HA degradation	
	Fe^{3+}/EDTA	XO/HX
SOD		
20	1	84
100	0	94
1000	1	100
2000	6	-
Catalase		
20	6	34
100	3	72
200	16	89
1000	41	112
2000	50	110

Percentage inhibition of hyaluronate degradation is calculated using the equation

$$I(\%) = (\eta_{sp} - \eta_{sp}^{min})/(\eta_{sp}^{max} - \eta_{sp}^{min}) \times 100,$$

where η_{sp} is the specific viscosity of the experimental solutions (containing the enzymes) and η_{sp}^{min} is the specific viscosity of the control solutions (without the enzymes), both measured after exposure to an oxy radical flux. η_{sp}^{max} is the specific viscosity of the the control hyaluronate solutions not exposed to an oxy radical flux.

4.3.7 Effect of radical scavengers, chelators and therapeutic agents

The effects of specific groups of compounds on the degradation of HA by the Fe^{2+}/EDTA system and the XO/HX system are shown in Table 4.3 (Fe^{2+}/EDTA) and Table 4.4 (XO/HX) respectively.

The $OH^{\bullet}$ scavengers, benzoic acid and mannitol both produced significant inhibition of HA degradation in both systems in the millimolar range. Similarly the solvents, ethanol and DMSO - both known $OH^{\bullet}$ scavengers - inhibited HA degradation by the Fe^{2+}/EDTA system. Singlet oxygen quenchers (sodium azide and histidine) produced a 50% (or greater) inhibition of degradation at 10 mM in both systems.

The results with chelating agents differed not only between the Fe^{2+}/EDTA and the XO/HX systems, but also between the agents themselves. DETAPAC 1 mM and penicillamine 1-5 mM (a chelating and anti-rheumatic agent) produced significant increases in HA degradation in the Fe^{2+}/EDTA system, whereas the same concentrations produced significant inhibition in the XO/HX systems. BPS (bathophenanthroline sulphonate), on the other hand, produced significant inhibition of degradation at 1 mM or

greater in both systems. However, at a concentration of 50 mM, all the chelating drugs produced a significant inhibition of the HA degradation, regardless of the system used to induce the degradation.

All of the anti-rheumatic drugs tested, with the exception of penicillamine (as discussed above), showed inhibitory activity at all concentrations and at each concentration the degree of inhibition was similar to that obtained with $OH^{\bullet}$ scavengers and singlet oxygen quenchers at similar concentrations. Indomethacin, which because of its low water solubility was tested only at a concentration of 1 mM, showed a modest but reproducible inhibition of HA degradation.

Gentisic acid, which is a hydroxylated form of salicylate (5-hydroxy-salicylate) was equipotent with salicylate in inhibiting HA degradation in both oxy radical-generating systems. Urea, a compound known to have no activity as a scavenger or quencher (76), had no inhibitory effect on degradation.

Table 4.3

Effects of various groups of compounds on hyaluronate degradation by Fe^{2+}/EDTA.

COMPOUND	Percentage inhibition of HA degradation						
Concentration (mM)	1	2	5	10	20	50	100
$OH^{\bullet}$ scavengers							
Mannitol	5	17	33	57	59	-	-
Benzoic acid	25	36	49	68	71	-	-
DMSO	22	-	52	65	86	93	100
Ethanol	-	-	-	38	55	71	84
Singlet oxygen quenchers							
Azide	39	55	70	80	86	-	-
Histidine	22	28	47	65	76	-	-
Chelating agents							
DETAPAC	9*	1*	19	48	51	-	-
BPS	19	52	88	100	100	-	-
Anti-rheumatic drugs							
Penicillamine	16*	16*	5*	8	23	63	91
Salicylate	28	-	-	72	80	84	91
Chloroquine	20	35	49	60	68	81	91
Gold sodium thiomalate	11	21	35	48	62	75	83
Indomethacin	18	-	-	-	-	-	-
Miscellaneous							
Gentisic acid	27	45	71	77	89	89	95
Urea	-	-	-	1*	0	3*	1*
Albumin (mg/ml)	13#	13#	-				

Inhibition calculated as in Table 4.2. *Values represent stimulation of hyaluronate degradation not inhibition. #Values for albumin concentration are in mg/ml.

Table 4.4

Effects of various groups of compounds on hyaluronate degradation by XO/HX.

COMPOUND	Percentage inhibition of HA degradation						
Concentration (mM)	1	2	5	10	20	50	100
$OH^{\bullet}$ scavengers							
Mannitol	6	-	11	64	70	92	-
Benzoic acid	6	-	16	30	42	73	91
Singlet oxygen quenchers							
Azide	24	39	57	69	80	84	84
Histidine	9	16	34	49	65	82	93
Chelating agents							
DETAPAC	15	22	36	53	67	76	-
BPS	41	77	95	97	94	-	-
Anti-rheumatic drugs							
Penicillamine	13	23	42	63	80	99	-
Salicylate	15	27	51	62	75	89	93
Chloroquine	39	46	-	69	75	100	-
Indomethacin	13	-					
Miscellaneous							
Gentisic acid	22	31	60	94	106	115	113
Urea	-	-	-	0	2	2	3
Albumin (mg/ml)	11#	28#					

Inhibition calculated as in Table 4.2.
#Values for albumin concentration are in mg/ml.

4.4 DISCUSSION

Viscometric analysis of HA degradation caused by oxy radicals, provides a convenient *in vitro* model system, with which to study factors potentially influencing synovial fluid degradation in diseased joints. The reproducibility of the viscosity changes had allowed detailed examination of the sequences of events leading to the formation of the active radical species in different oxy radical-generating systems.

The rapid degradation of HA observed in the Fe^{2+}/EDTA system (Figure 4.1) is due to the rapid autoxidation of Fe^{2+} to Fe^{3+} in the presence of phosphate buffer with subsequent $OH^{\bullet}$ production (150). The slower degradation in the enzymatic system is in agreement with that observed by other workers (41,61) and is due to the slower rate of production of oxy radicals by this system. The degree of degradation was much greater than that observed by Hofmann & Schmut (166) whose solutions did not contain EDTA which enhances HA degradation (see Table 4.1).

The inhibition of HA degradation by $OH^{\bullet}$ scavengers suggests that $OH^{\bullet}$ is the oxy radical directly responsible for degradation in both the Fe^{2+}/EDTA and enzymatic systems.

However, based on differences in sensitivities to inhibition by SOD and catalase, the mechanisms by which this radical is generated appear to be different in the two systems. In the Fe^{2+}/EDTA system which is sensitive to catalase but not SOD (see also 62, 166), the following equations describe the reaction sequence leading to $OH^{\bullet}$ formation.

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^- \quad (1)$$

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+} + O_2^- + 2H^+ \longrightarrow Fe^{3+} + H_2O_2 \quad (7)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^- \quad (3)$$

Reactions (2) and (7) result in the formation of H_2O_2 which participates in the Fenton reaction (reaction 3), which is sensitive to catalase.

In the enzymatic system which is sensitive to both catalase and SOD, the formation of $OH^{\bullet}$ may proceed as follows:-

Hypoxanthine + O_2	----> urate + O_2^-	(4)
O_2^- + O_2^- + $2H^+$	----> H_2O_2 + O_2	(2)
O_2^- + Fe^{3+}	----> Fe^{2+} + O_2	(5)
Fe^{2+} + H_2O_2	----> Fe^{3+} + $OH^{\bullet}$ + OH^-	(3)

Nett reaction

H_2O_2 + O_2^-	----> $OH^{\bullet}$ + OH^- + O_2	(6)

At pH 7.4, and in the absence of SOD, reaction (2) proceeds at a slow rate and sufficient O_2^- is available for reaction (5). However, in the presence of SOD, reaction (2) proceeds so rapidly that reaction (5) is inhibited by depletion of O_2^-. In the absence of added iron, the Fe^{2+}/Fe^{3+} required for the Haber-Weiss Reaction (6) may be provided by impurities in the buffer system. Similarly, in the synovial fluid of patients with rheumatoid arthritis, there should be adequate levels of iron (57,58,155) to catalyse such a reaction in the presence of O_2^- generated by activated leucocytes.

The effect of iron chelation on the degradation of HA was shown to be important. The observed stimulation of HA

degradation by penicillamine, EDTA and DETAPAC in the autoxidation system is congruent with studies using hydroxylation of salicylate to detect $OH^{\bullet}$ radicals (Chapter 3), and those on Fe^{2+}-induced lipid peroxidation (153). In the XO/HX system, all concentrations of penicillamine, DETAPAC and BPS studied, inhibited HA degradation. Similar observations with DETAPAC and BPS were made by Halliwell (76), who suggested that this effect is due to formation of chelates which sequester iron required for the Haber-Weiss reaction (see also 154). The differences in HA degradation achieved in the presence of comparable concentrations of different chelating agents suggests that important differences exist in the chelating action of different agents. The ratio of chelator to Fe^{2+} concentration also appears to influence Fe^{2+} autoxidation as shown by differences in HA degradation observed at varying concentrations of individual chelators.

The degree of inhibition of HA degradation by chloroquine, gold sodium thiomalate and salicylate was similar in both the oxy radical-producing systems and was comparable to the inhibition produced by $OH^{\bullet}$ scavengers and singlet oxygen quenchers at equivalent concentrations. Similar results were obtained with these drugs in experiments using hydroxylation of salicylate (Chapter 3). Salicylate and gentisic acid showed similar inhibitory

effects, suggesting that salicylate retains its ability to scavenge $OH^{\bullet}$ after metabolic hydroxylation to gentisic acid.

In *in vitro* experiments, some anti-rheumatic drugs also show significant inhibition of oxy radical-mediated reactions at micromolar concentrations. Herzer and Lemmel (69) showed that 150 uM indomethacin produced a 50% inhibition of NBT reduction by dextran sulphate activated leucocytes, and Van Dyke and workers (70) found that 20 uM indomethacin and 30 uM salicylate both produced a 50% inhibition of chemiluminescence produced by zymosan-activated leucocytes. Similarly, indomethacin (100 uM) inhibited by 50% the cytochrome C reduction produced by tetrapeptide-activated leucocytes (67), and 80 uM inhibited bovine synovial fluid degradation by 96% (71). However, in some of these experiments, ethanol and DMSO were used in the preparation of solutions and as is shown in Table 4.2, these solvents are potent $OH^{\bullet}$ scavengers. In our experiments, 1 mM indomethacin only produced a 13% inhibition of HA degradation.

These examples underline the differences in reported findings on inhibition of oxy radical reactions by scavengers, quenchers, chelating agents and anti-rheumatic drugs in *in vitro* experiments. These differences are

undoubtedly influenced by differences in methods of production and detection of oxy radicals and other experimental conditions (167). The superiority of one method over another particularly in predicting the therapeutic activity of test compounds is yet to be established. However, all studies to date, including the present study, are compatible with the concept that the therapeutic activity of some anti-inflammatory drugs may to some extent be attributed to their ability to intercept oxy radicals. In this context it is worth noting that, although the concentrations of the anti-rheumatic drugs required for inhibition of oxy radical effects *in vitro* have generally been higher than those achieved in plasma and synovial fluid *in vivo* at therapeutic doses, higher concentrations may be achieved at important micro-locations, such as secondary lysosomal vesicles in the case of chloroquine and gold (159,160), where oxy radicals are generated (161,162,163).

Because oxy radicals, in particular the hydroxyl radical, may be important mediators of inflammation, the development of new therapeutic agents capable of abrogating the formation of oxy radicals, may offer a new approach to the treatment of inflammation. Iron chelators, by sequestering iron so as to preclude its participation in $OH^{\bullet}$ generating reactions, provide an example of this approach.

4.5 SUMMARY

Degradation of hyaluronic acid (measured viscometrically) by oxy radicals generated by (a) autoxidation of ferrous EDTA chelates and (b) enzymatically by xanthine oxidase and hypoxanthine (XO/HX), was used as an *in vitro* model of oxy radical-mediated tissue damage. Degradation of HA by XO/HX was strongly inhibited by superoxide dismutase and catalase, whereas degradation of HA by autoxidation of ferrous ions was weakly inhibited by catalase, and unaffected by SOD. Both oxy radical producing systems were inhibited by hydroxyl radical scavengers suggesting that hydroxyl radical was the proximate damaging species in both systems. Penicillamine at concentrations 1-5 mM stimulated HA degradation by ferrous EDTA chelates but inhibited degradation by the XO/HX system. Higher concentrations of penicillamine, and all concentrations studied (1-100 mM) of other anti-inflammatory drugs (chloroquine, gold sodium thiomalate, salicylate) inhibited HA degradation by both the autoxidation and enzymatic oxy radical producing systems, with inhibitory potency similar to that seen with known hydroxyl radical scavengers.

CHAPTER 5

GENTISATE:
A SALICYLATE METABOLITE WITH ANTI-OXIDANT PROPERTIES

5.1 INTRODUCTION

Gentisate may be formed by the action of hydroxyl radicals on salicylate (75). Synovial fluid gentisate levels had not been reported prior to these studies which were undertaken to determine the degree of correlation between plasma and synovial fluid gentisate in patients taking anti-inflammatory doses of aspirin/salicylate in order to determine (a) whether there was likely to be ready equilibration between gentisate in plasma and synovial fluid, (b) whether there was any significant peripheral conversion of salicylate to gentisate (possibly reflecting oxidation of salicylate by oxy radicals generated in inflamed joints), and (c) whether gentisate levels acheived in inflamed joints were comparable to concentrations causing anti-oxidant effects in some *in vitro* systems.

5.2 METHODS

5.2.1 Patients

Patients with knee effusions attending the Royal Adelaide Hospital-The Queen Elizabeth Hospital Rheumatology Unit were invited to participate in the study. Patients already taking aspirin in anti-inflammatory doses were assessed to obtain clinical disease activity scores (see below) and synovial fluid and blood samples were taken contemporaneously. Patients not taking aspirin and for whom no contraindications to aspirin therapy existed, were given sustained release aspirin (a gift from the Boots Co. Australia Pty. Ltd.) six to eight 650 mg tablets daily in divided doses for one week. They were then subjected to disease activity assessment, aspiration of knee effusion and venepuncture. Fourteen patients had rheumatoid arthritis. Six additional patients had other diagnoses; psoriatic arthritis (2), osteoarthrosis (2), ankylosing spondylitis (1) and post-traumatic synovitis (1).

5.2.2 Assessment of disease activity

Disease activity was assessed as follows:-

1. Tenderness or pain on motion was scored as present (1) or absent (0) on a total of 21 joints or groups of joints (see below - maximum possible score 21).
2. Joint swelling was scored as present (1) or absent (0)

in 16 joints/groups of joints
(maximum possible score = 16).

3. Duration of morning accentuation of stiffness.

4. Erythrocyte sedimentation rate (Westergren).

The following joints on each side were assessed for (a) joint tenderness or pain on movement and (b) swelling: proximal interphalangeal joints of hands, metacarpophalangeal joints, metatarsophalangeal joints (each of these groups of joints contributed to the score as if a single unit based on relevant positive findings in one or more joints of a group), wrists, elbows, shoulders, ankles/hindfeet, knees. The hips and temporomandibular joints on each side, and the neck were assessed for pain on movement or tenderness (but not swelling).

A total score was obtained as follows:-
Joint tenderness/pain on movement score plus joint swelling score plus morning stiffness in hours plus ESR in cms/hour (i.e. mm/hour divided by 10).

5.2.3 Collection of samples and assays

Blood and synovial fluid samples for assay of salicylate and metabolites were placed directly into tubes containing lithium heparin. Salicylate, salicylurate and gentisate concentrations in plasma and synovial fluid were

measured using a modified HPLC assay (77). Following protein precipitation with an equal volume of acetonitrile containing the internal standard o-methoxybenzoic acid (20 mg/ml), samples were chromatographed using a Waters uBondapak C_{18} column and detected at 323 nm. The mobile phase, methanol/glacial acetic acid/water in the ratio 20/4/76, was run at 2 ml/minute.

5.2.4 In vitro studies of conversion of salicylate to gentisate by an oxy radical flux

Ferrous sulphate (100 mM) was freshly prepared in 100 mM EDTA (pH 7.4). To initiate the hydroxyl radical flux, this was then added (in amounts required to give final concentrations ranging from 0.1 to 5.0 mM $FeSO_4$/EDTA) to 1.0 mM sodium salicylate in 50 mM potassium phosphate, pH 7.4. After incubation for one hour at 25^{o}C, these solutions were frozen until assayed by HPLC for salicylate and gentisate, as above.

5.3 RESULTS

Results of plasma and synovial fluid salicylate analyses are shown in Figure 5.1. It can be seen that there was a close correlation between plasma and synovial fluid salicylate levels ($r = 0.94$, $n = 20$) suggesting ready equilibration of salicylate between the plasma and synovial spaces. Similar degrees of correlation were observed between plasma and synovial fluid levels of salicylurate (Figure 5.2, $r = 0.90$, $n = 20$) and gentisate (Figure 5.3, $r = 0.93$, $n = 16$).

In order to determine whether there was any correlation between the degree of conversion of salicylate to gentisate and disease activity scores, ratios of gentisate and salicylate in plasma and synovial fluid were computed. Patients with rheumatoid arthritis were grouped according to severity of disease activity. Although numbers in each group are small there were no discernible trends in ratios of gentisate to salicylate between patients with mild or more severe disease activity or between patients with rheumatoid arthritis and those with other diagnoses (Figure 5.4).

The *in vitro* conversion of salicylate to gentisate in the presence of a hydroxyl radical flux is shown in Figure 5.5. Concomitant disappearance of salicylate was observed with the appearance of gentisate, which accounted for approximately 35% of the salicylate loss. The remaining salicylate oxidation products were not detectable using the HPLC method described above. In particular, a 2,3-dihydroxybenzoate standard was not satisfactorily resolved.

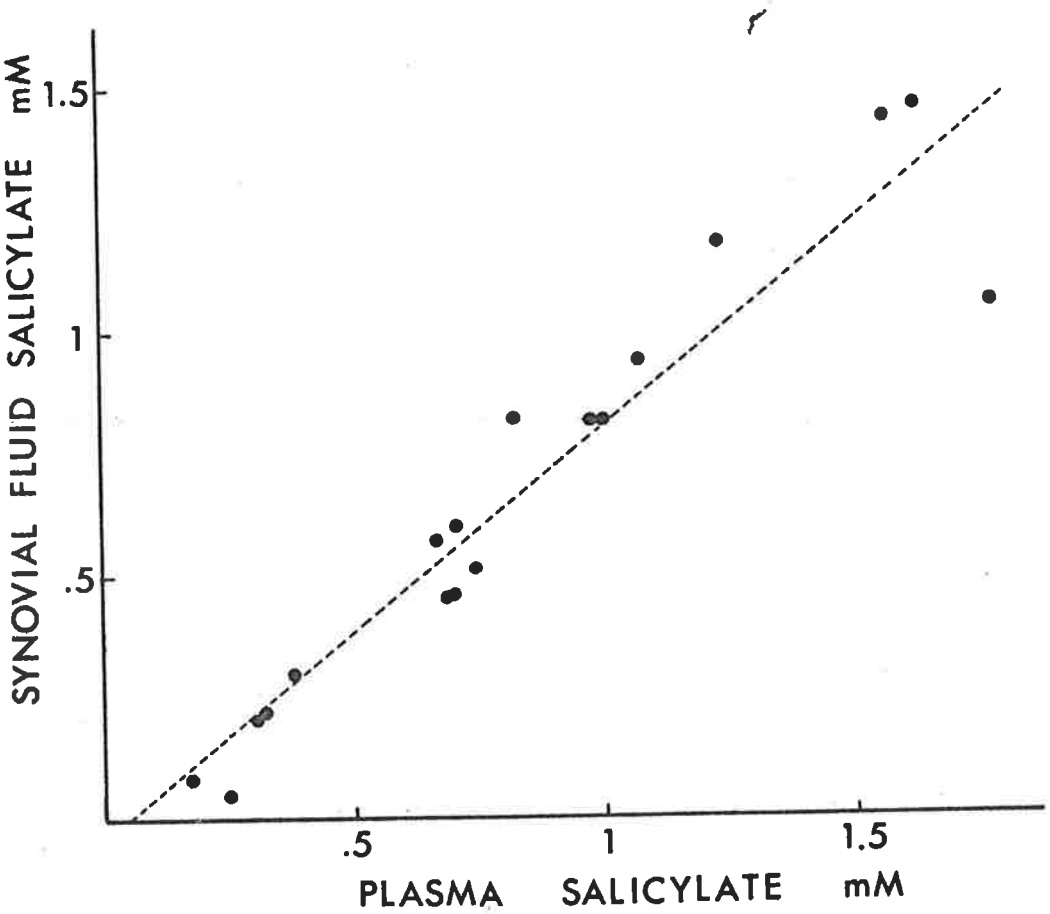

Figure 5.1. Correlation between plasma and synovial fluid salicylate levels in 18 patients taking between 3.9 and 5.2 grams total dose of sustained release aspirin daily.

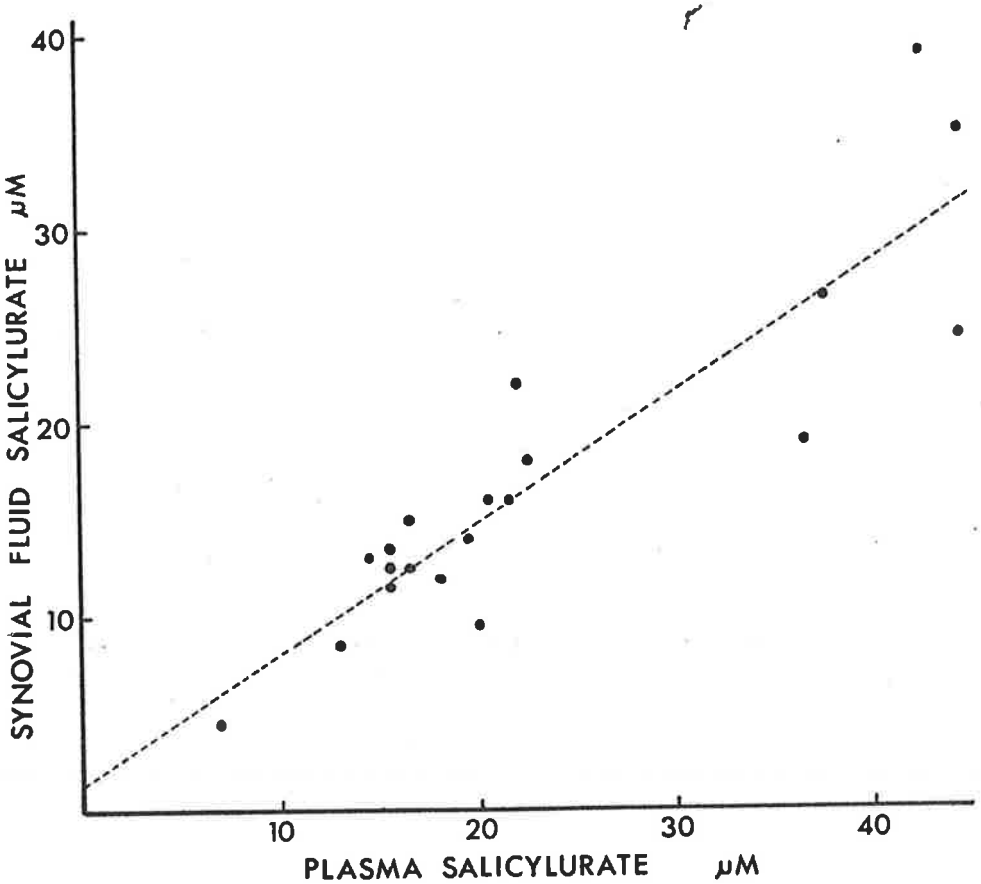

Figure 5.2. Correlation between plasma and synovial fluid salicylurate levels in 20 patients taking between 3.9 and 5.2 grams total dose of sustained release aspirin daily.

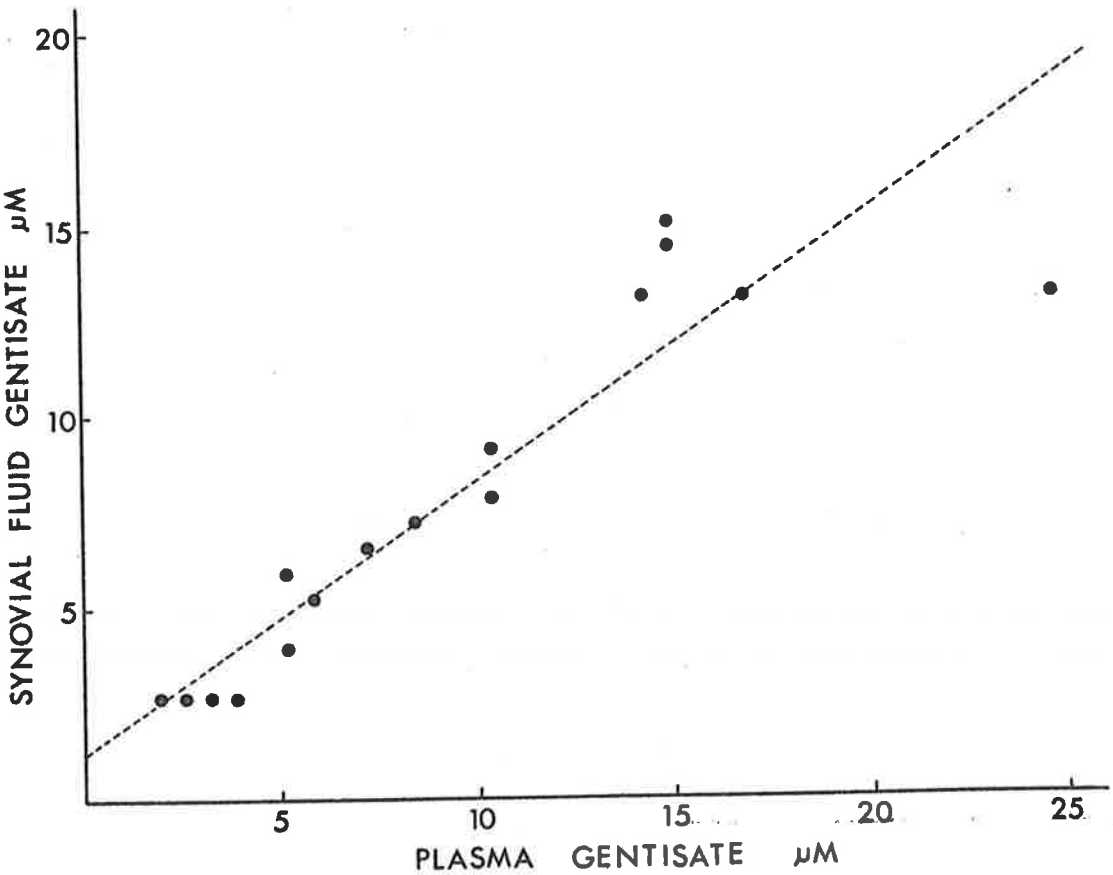

Figure 5.3. Correlation between plasma and synovial fluid gentisate levels in 16 patients taking between 3.9 and 5.2 grams total dose of sustained release aspirin daily.

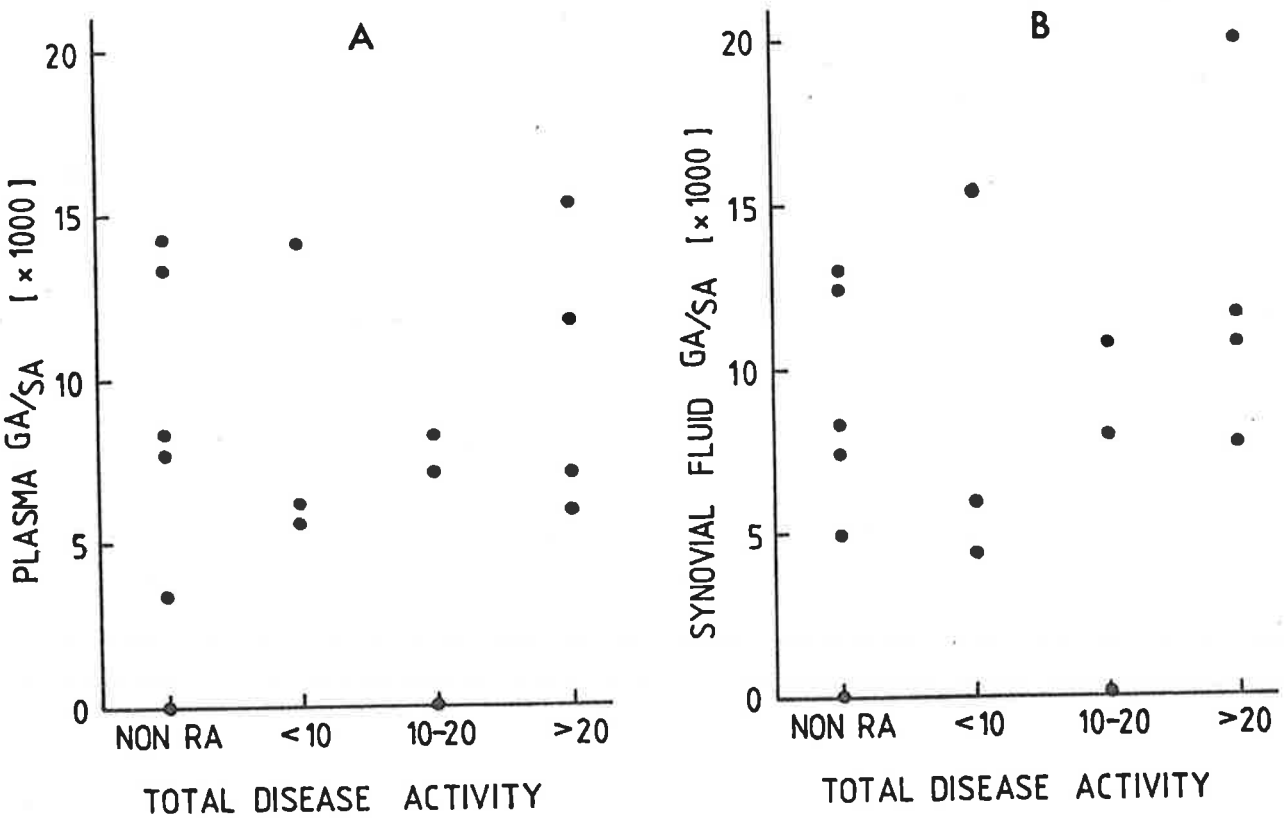

Figure 5.4. Ratio of gentisate to salicylate in both plasma and synovial fluid computed for non-rheumatoid subjects, and rheumatoid subjects stratified according to disease activity. No differences between groups by Rank analysis.

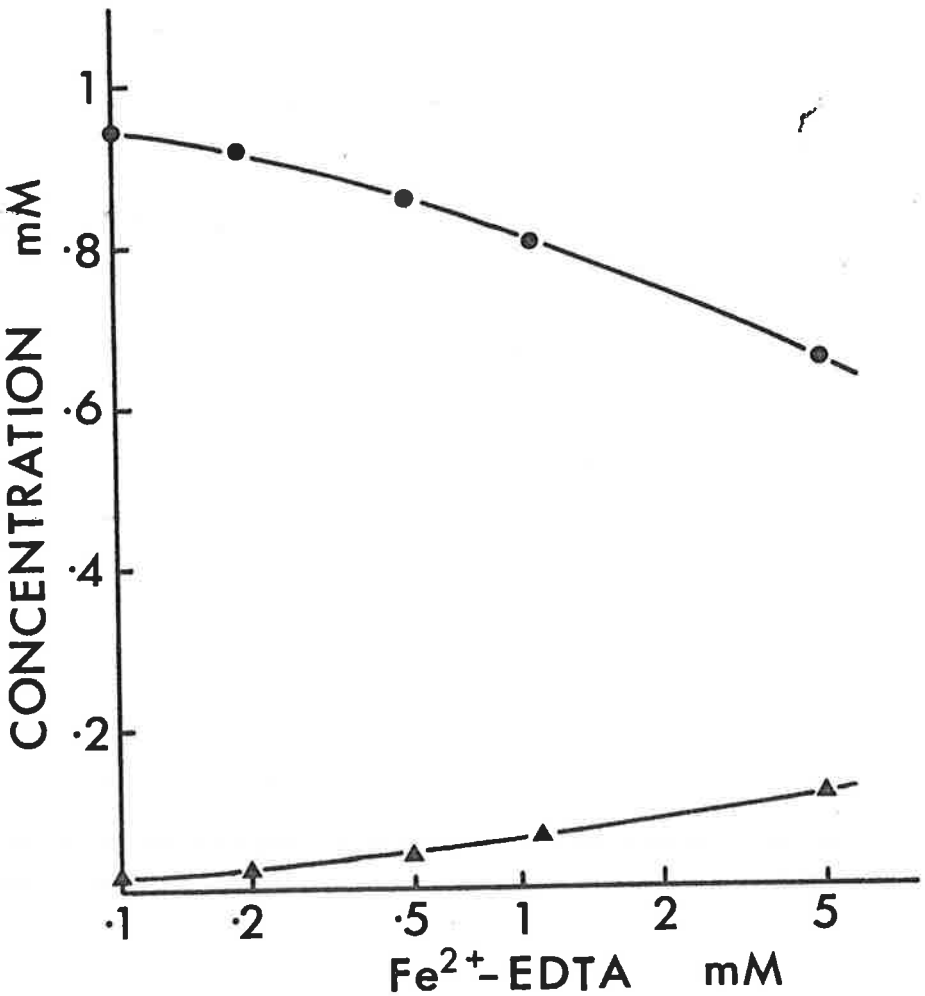

Figure 5.5. In vitro oxidation (loss) of salicylate (● ——— ●) and conversion to gentisate (▲ ——— ▲) in 50 mM potassium phosphate buffer at pH 7.4, in the presence of an oxy radical flux produced by 0.1 to 5.0 mM $FeSO_4$-EDTA. Samples were incubated for one hour at 25°C.

5.4 DISCUSSION

A close concordance between steady state plasma (total and unbound) and synovial fluid levels of salicylate has been shown previously (168). The present study confirms this work and demonstrates a similar concordance between levels of salicylurate and gentisate. It has been shown (169), that salicylate can be measured in synovial fluid within 10-30 minutes of single dose salicylate ingestion. Accordingly, local production of gentisate in inflamed joints may have occurred but have been masked by rapid equilibration of the gentisate into the plasma space.

There was no discernible difference in salicylate to gentisate ratios in plasma or synovial fluid between the rheumatoid and non-rheumatoid groups nor were there any significant differences (as assessed by rank analysis) between the rheumatoid patients with mild, moderate or severe disease. It could be argued that if conversion of salicylate to gentisate by oxygen radicals in inflamed joints were the dominant mechanism for bioconversion of salicylate to gentisate then one would expect higher ratios of gentisate to salicylate in plasma and synovial fluid samples of patients with active disease than in samples from patients with less active disease or non-inflammatory joint

disorders. However, to date a valid measure of overall leucocyte-mediated oxy radical production in inflammatory disorders has not been established, and any correlation between oxy radical production and clinical measures of disease activity remains speculative.

If an oxygen radical-mediated conversion of salicylate to dihydroxybenzoates were operative, then dihydroxybenzoates with hydroxyl groups in an Ortho-configuration (e.g. 2,3-dihydroxybenzoate) should be formed in addition to gentisate (hydroxyl groups in Para-configuration). In the present study, it was not possible to confirm or exclude the presence of 2,3-dihydroxybenzoate in plasma and synovial fluid samples because this compound was not detected by the HPLC assay used. Under in vivo conditions, conversion of Para- and Ortho- dihydroxybenzoates to corresponding quinones may be expected resulting in reduction of measurable gentisate and 2,3-dihydroxybenzoate (if present). Futhermore, since 2,3-dihydroxybenzoate is more susceptible to oxidation to its (Ortho)quinone than gentisate (to a Para-quinone), its presence is likely to be more transient.

While no support for peripheral conversion of salicylate to gentisate or 2,3-dihydroxybenzoate was obtained, on the basis of available evidence, this mechanism

cannot be excluded. The presence of gentisate (and possibly 2,3-dihydroxybenzoate) in synovial fluid may have significance because quinone-forming diphenols, including gentisate, are anti-oxidants (170). Furthermore, these agents may modify the effects of oxidants such as those secreted by leucocytes at sites of inflammation at concentrations similar to those found in these patient samples (78). Also their quinone derivatives may have important biological effects through covalent bonding with proteins.

5.5 SUMMARY

A study was undertaken to determine levels of gentisate, an antioxidant metabolite of salicylate, in plasma and synovial fluid samples from patients taking anti-inflammatory doses of aspirin. A close correlation between plasma and synovial fluid concentrations was found for (i) salicylate, (ii) salicylurate, and (iii) gentisate, in 20 patients studied. These data suggest ready equilibration of these compounds between the plasma and synovial spaces. *In vitro* experiments confirmed that in the presence of an oxy radical flux, salicylate is oxidised to gentisate. However, no evidence was obtained to implicate peripheral conversion of salicylate to gentisate in inflamed joints where oxy radicals may be produced.

CHAPTER 6

MACROMOLECULAR CONJUGATES OF SUPEROXIDE DISMUTASE (SOD): STUDIES OF CLEARANCE, ANTI-INFLAMMATORY EFFECT AND IMMUNOLOGICAL REACTIVITY

6.1 INTRODUCTION

This chapter describes studies using three different crosslinked protein macromolecular conjugates comprising respectively (a) SOD and albumin, (b) SOD-SOD without albumin and (c) albumin-albumin without SOD. Studies of clearance (from blood and pleural spaces), anti-inflammatory effect and immunological reactivity are described.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of conjugates

Cu-Zn superoxide dismutase (SOD) was isolated from bovine liver or human erythrocytes according to the method of McCord and Fridovich (32). Bovine liver SOD was also obtained from Sigma Chemicals, St, Louis, MO. SOD activity was assayed by the inhibition of ferricytochrome C reduction by enzymatically generated superoxide (32). Rabbit and rat albumins (Sigma Chemicals, St. Louis, MO.) were crosslinked to SOD with glutaraldehyde using a

modification of a previously described method (120). The procedure was as follows (Method A): SOD 1 mg and albumin 50 mg were added to 0.02 M sodium phosphate buffer, pH 7.4, 1 ml followed by glutaraldehyde 25% in distilled water 10 ul. The reaction was allowed to proceed at 4°C for 4 to 5 h.. The reaction was terminated by the addition of glycine 12 mg. and the mixture dialyzed against several changes of 0.13 M NaCl-0.16 M glycine, followed by gel filtration on a Bio-Gel A-0.5 m agarose column with an exclusion limit of 500,000 daltons (Bio-Rad, Richmond, Ca.).

In later studies the crosslinking method was modified as follows (Method B): 30 mg SOD and 300 mg albumin were dissolved in 3.0 ml of 0.02 M sodium phosphate buffer, pH 7.4, followed by 30 ul glutaraldehyde 25% in water, with termination at 5 hours by adding 36 mg glycine. The mixture was dialysed overnight against 0.13 M NaCl + 0.16 M glycine, followed by gel filtration on Sepharose 4B.

The columns were equilibrated and material eluted with 0.02 M sodium phosphate buffer, pH 7.4, containing 0.13M NaCl. Calibration was acheived using as standards cytochrome C, SOD, bovine serum albumin, B-galactosidase, myosin, thyroglobulin and xanthine oxidase. The conjugates were concentrated using an Amicon ultrafiltration cell with a PM 10 filter, and then stored at 4°C with 10 units/ml

penicillin.

The protein concentration of the conjugate solutions was determined by the method of Bradford (171) using Coomassie brilliant blue G-250 (Sigma Chemicals). The conjugates were concentrated using an Amicon ultrafiltration cell with a PM 10 filter and stored at 4°C with penicillin 10 U/ml, or snap frozen in dry ice-ethanol, lyophilized, stored at 4°C and reconstituted with phosphate-buffered saline, prior to use.

6.2.2 Clearance studies in rats

Lewis rats (150-300 g) were used throughout for *in vivo* studies. Plasma clearance of SOD enzymatic activity was determined following intravenous injection of rat albumin-SOD conjugates of specified molecular weight ranges. Rats were bled from the tail vein. In intrapleural clearance studies, the test preparations were injected into the right pleural cavity. At specified times the rats were sacrificed, the cavity exposed and washed out with 5.0 ml isotonic saline. The washings were then centrifuged to remove cells, and the supernatant assayed for SOD activity.

6.2.3 Assessment of anti-inflammatory effect in rats

Anti-inflammatory activity in rats was assessed by inhibition of carrageenan-induced paw swelling (133). Test preparations were injected into the right saphenous vein of unanaesthetized rats immediately prior to injection of carrageenan 1% in saline 0.05 ml into the left paw. Each treatment group comprised 8 to 10 rats matched for weight. Paw volume was determined blind by displacement of water before and 4 h after injection. Percent inhibition was calculated by comparison with a saline (intravenous) and carrageenan (paw) injected control group included in each experiment.

6.2.4 Immunoreactivity of conjugates in rabbits

To test the immunogenicity and antigenicity of bovine liver SOD-albumin conjugates prepared by Method A, groups of three, 2 month old male New Zealand rabbits were given repeated injections of the following: (a) free SOD, (b) rabbit albumin, (c) conjugates of rabbit albumin alone (prepared by the glutaraldehyde-crosslinkng reaction in the absence of SOD), (d) rabbit albumin-SOD conjugates, (e) rat albumin, (f) conjugates of rat albumin alone, (g) rat albumin-SOD conjugates. All conjugates for injection were obtained from included fractions collected by Bio-Gel A-0.5 m agarose chromatography. All injections were given

according to the following schedule. On days 1,2 and 3, 2 ml protein (2 mg/ml) homogenized with 1 ml complete Freund's adjuvant was injected intramuscularly into the hindquarter of the rabbit. On days 10, 11 and 12 the injections were repeated without the adjuvant. On day 19 the rabbits were given booster injections of 1 ml protein, and 7 days later bled from the ear vein. The anti-sera obtained were tested for precipitating antibodies by Ouchterlony double diffusion in agar gel (172). Approximately 12 ul of each antiserum was tested against 0.1 to 50 ug of antigen protein. In addition, up to 300 ug of the albumin-SOD conjugates were used in order to compensate for the relatively low contribution of SOD to the total protein content of the conjugates.

6.3 RESULTS

6.3.1 Characteristics of macromolecular SOD conjugates

Under the conditions of preparation described, the glutaraldehyde mediated crosslinking reaction provided more than 50% retention of the original enzymatic activity of (bovine liver) SOD in (a) rat albumin-SOD conjugates (methods A and B), (b) rabbit albumin-SOD (method A), (c) human albumin-SOD (methods A and B), (d) horse albumin-SOD (method B), and (e) SOD-SOD conjugates. Similiar findings were obtained with human erythrocyte SOD conjugated to either rat albumin or human albumin (method B). Fractionation of conjugation products by gel filtration yielded conjugates with apparent molecular weights ranging upto 2×10^6. Figure 6.1 shows a representative profile of elution of SOD activity and protein (absorbance at 254 nm) obtained with a rat albumin-SOD preparation (Method B). A small proportion (up to 5%) of SOD remained unconjugated in some preparations (molecular weight of native CuZn SOD 3.2×10^4). These findings are congruent with chromatographic profiles of conjugates prepared using SOD and ^{125}I-labelled rat albumin (Method A) (173).

Individual and pooled fractions stored at 4°C in the presence of 10 units of pencillin suffered no detectable loss of enzymatic activity over a period of one month. Fractions quick frozen in a dry ice-ethanol bath and lyophilized for long term storage, exhibited virtually complete retention of activity upon reconstitution. Albumin-SOD complexes subjected to slow freezing (placed directly into a -20°C freezer) and thawing formed visible aggregates with removal of SOD activity from solution and accordingly, this procedure was avoided.

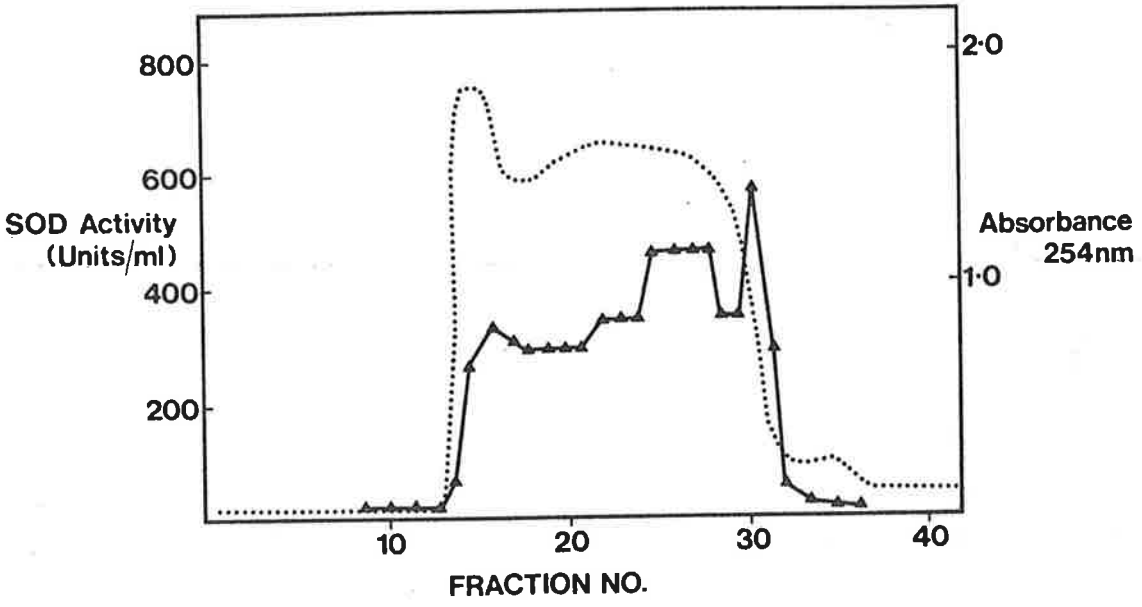

Figure 6.1; A typical elution profile of SOD enzymatic activity (▲——▲) and absorbance at 254 nm (.....) of SOD-albumin conjugates (prepared by Method B) obtained with column chromatography using Sepharose 4B. Approximate molecular weight (daltons) of fraction constituents include: Fraction 14, 1.8×10^6; Fraction 28, 1.3×10^5; Fraction 33, 2.0×10^4.

6.3.2 Clearance studies in rats

Plasma clearance of intravenously injected unconjugated SOD was very rapid ($t_{1/2}$ = 6 mins), whereas the clearance of the conjugated forms of SOD was biphasic and considerably slower (Figure 6.2) with $t_{1/2}$ = 3-5 hrs for phase I, and $t_{1/2}$ = 6-12 hrs for phase II (Table 6.1). Figure 6.2 and Table 6.1 show results obtained using conjugates formed by Method B. Similar findings were obtain with conjugates with a proportionately higher albumin content formed by Method A.

TABLE 6.1

Plasma clearance of SOD activity following intravenous injection into rats

Injection	Plasma half time phase I	phase II
SOD (3000 units/kg)	6 min	-
SOD-SOD conjugates (3000 units/kg)	3 hrs	10 hrs
SOD-Albumin conjugates* (2500 units/kg)	4 hrs	6 hrs
SOD-Albumin conjugates** (2500 units/kg)	5 hrs	12 hrs

The molecular weights were as follows: SOD-SOD conjugates, $(3.5 - 9.0) \times 10^5$; SOD-Albumin conjugates*, $(6.0 - 17) \times 10^5$; SOD-Albumin conjugates**, $(2.5 - 6.0) \times 10^5$.

SOD activity in the pleural cavity diminished with a half-time of 2.5 hours following intrapleural injection of free SOD, whereas the clearance was delayed ($t_{1/2}$ = 7 hrs) when SOD conjugated to albumin was injected (Figure 6.3).

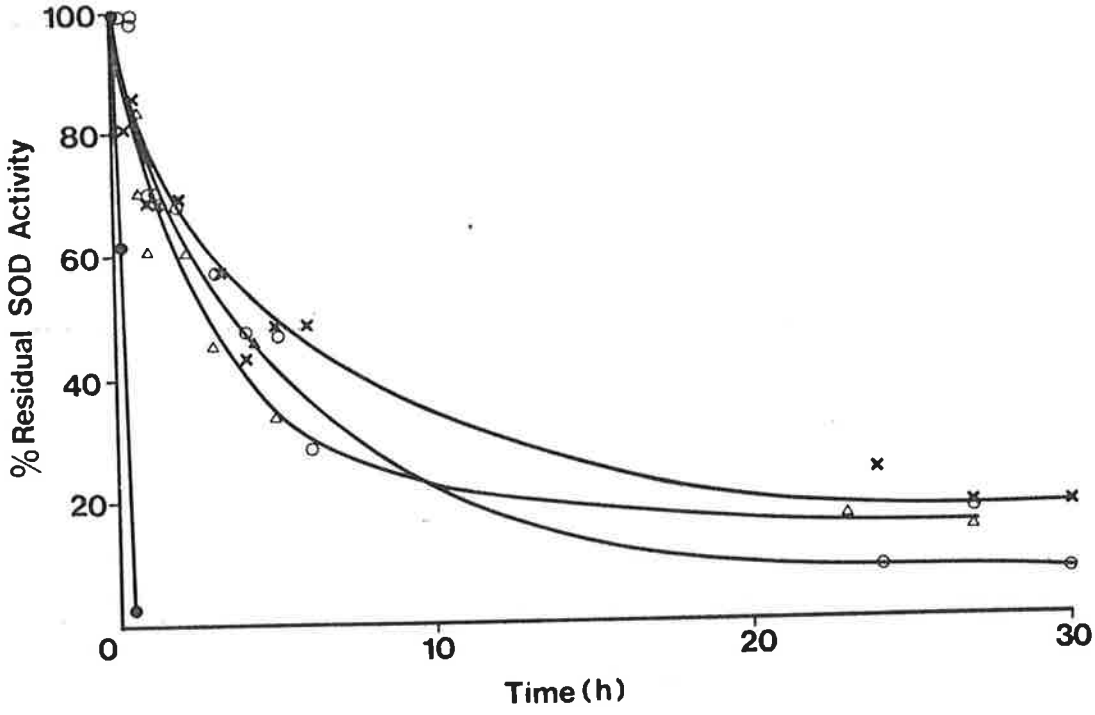

Figure 6.2. Plasma clearance of SOD activity following intravenous injection of SOD preparations into rats.
Free SOD (●——●),
SOD-Albumin conjugates MW (6.0 - 17) x 10^5 (○——○),
SOD-Albumin conjugates MW (2.5 - 6.0) x 10^5 (×——×),
SOD-SOD conjugates MW (3.5 - 9.0) x 10^5 (△——△).
Computations of phase I and II plasma half-times are presented in Table 6.1.

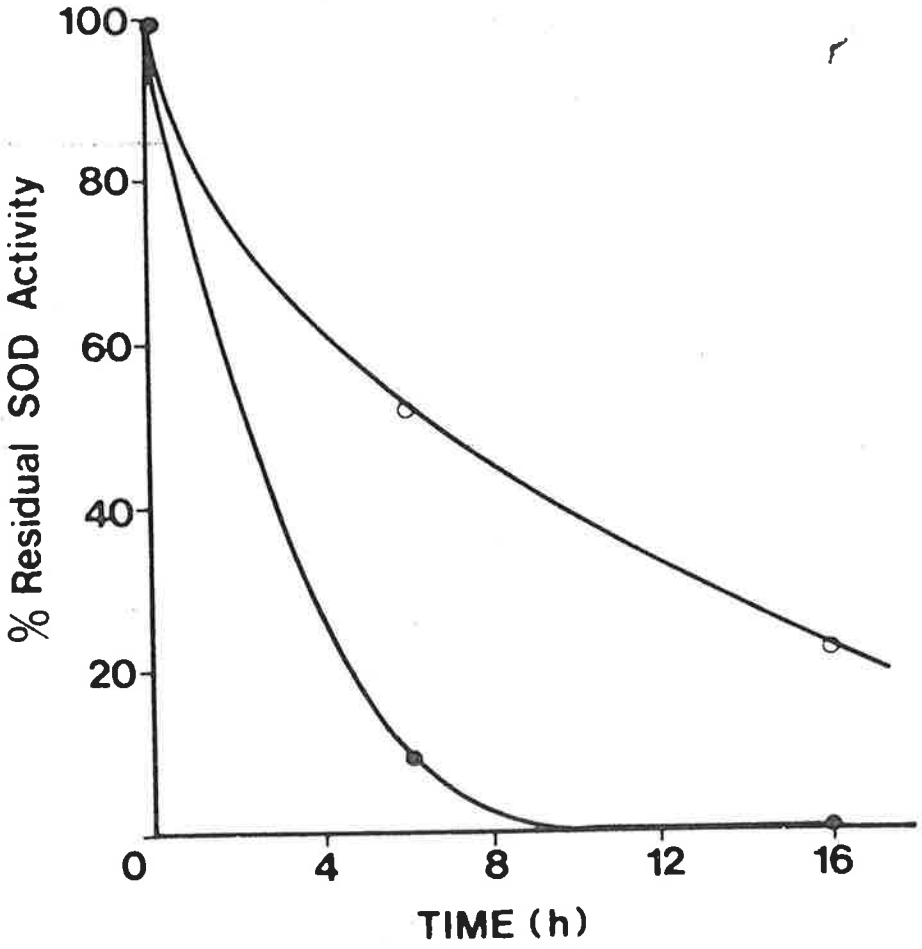

Figure 6.3. Clearance of SOD (●———●) and conjugated SOD-Albumin (○———○) from the pleural cavity, following intrapleural injection.

6.3.3 Anti-inflammatory effects of conjugates in rats

Unconjugated SOD was without detectable anti-inflammatory effect in rats when given as a single intravenous dose of up to 3,000 units/kg body-weight (Table 6.2). Similarly unconjugated albumin, given intravenously at 33 mg/kg, was without detectable effect.

SOD-albumin conjugates had anti-inflammatory activity with maximum inhibition (70 - 80 %) being seen with doses of 3000 units/kg, protein 33 mg/kg (Table 6.2). Albumin-albumin conjugates had a comparable anti-inflammatory effect in similar experiments (Table 6.2). The anti-inflammatory effects of the SOD-albumin and the albumin-albumin conjugates were independent of the molecular weights of the conjugates within the range 30,000 to 2,000,000 Daltons (Table 6.2). Table 6.3 shows that in a single experiment, albumin-albumin conjugates and SOD-albumin conjugates had equipotent anti-inflammatory activity, and that this activity was dose-dependent. Conjugates of SOD alone (prepared by cross-linking SOD in the absence of albumin) were tested for anti-inflammatory activity in three separate experiments (total of 30 rats injected). At a dose of 3000 units/kg, 0.7-2.0 mg protein/kg 41-68% inhibition of paw oedema was seen. Thus the anti-inflammatory action of SOD-SOD was approximately equipotent to that of SOD-albumin conjugates when related to

enzymatic activity, but was considerably more potent than either SOD-albumin or albumin alone conjugates when related to the amount of protein injected.

Table 6.2

Inhibition of carrageenan-induced rat paw oedema by SOD, albumin and their conjugates

Injection (units/ml)	paw swelling (gms)	% inhibition of paw swelling	plasma SOD activity
EXPERIMENT 1			
Saline control	0.98 + 0.2(24)	0	<10
SOD	0.87 + 0.2(16)	11	<10
SOD-albumin conjugates			
Pool I	0.21 + 0.1(8)	80	34 ± 4*
Pool II	0.22 + 0.1(9)	78	56 ± 10
Pool III	0.24 + 0.2(10)	76	50 ± 12
Pool IV	0.22 + 0.2(9)	78	35 ± 12
EXPERIMENT 2			
Saline control	1.12 + 0.2(8)	0	<10
Albumin	1.07 + 0.2(7)	4	<10
Albumin-albumin conjugates			
Pool I	0.53 + 0.1(3)	53	<10
Pool II	0.53 + 0.1(7)	53	<10
Pool III	0.44 + 0.2(8)	61	<10
Pool IV	0.26 + 0.1(4)	77	<10

Bracketed values indicate animal numbers. In experiment 1 - SOD injection iv. of 3000 units/kg, protein approx. 33 mg/kg. In experiment 2 - injection iv. of 33 mg/kg of albumin; molecular weight ranges of pooled fractions were:- Pool I (17 - 20)x 10^5 Daltons, Pool II (6.4 - 17)x 10^5 Daltons, Pool III (2.7 - 6.4)x 10^5 Daltons, Pool IV (0.3 - 2.7)x 10^5 Daltons. *Experiments 1 and 2 were performed on different days. Mean ± SD

Table 6.3

Comparison between inhibition by SOD-albumin and albumin-albumin conjugates of carrageenan-induced rat paw oedema

Injection	paw swelling (gms)	% inhibition of paw swelling
Saline control	1.24 + 0.2	0
Albumin-albumin conjugates		
5 mg protein/kg	0.72 + 0.2	42
20 mg protein/kg	0.40 + 0.2	68
SOD-albumin conjugates		
500 units SOD/kg (5 mg protein/kg)	0.71 + 0.2	43
2000 units SOD/kg (20 mg protein/kg)	0.49 + 0.3	61

Sixteen rats were used in each group. The molecular weights of SOD-albumin and albumin-albumin conjugates were (2.7 - 17) x 10^5 Daltons.

6.3.4 Immunoreactivity of conjugates in rabbits

The results of studies in rabbits of immunogenicity and antigenicity of homologous (rabbit) albumin-SOD conjugates and heterologous (rat) albumin-SOD conjugates and their components are shown in Table 6.3. The immunogenicity (ability to elicit an antibody response) and antigenicity (ability to react with antibody) of bovine liver Cu-Zn SOD was demonstrated by a positive precipitin reaction between native SOD and sera from rabbits injected with native SOD. Native SOD reacted with the serum from one of three rabbits injected with rabbit albumin-SOD conjugates indicating the presence of antibodies against SOD in this animal. The serum of this animal did not react with rabbit (or rat) albumin presented as native albumin or conjugates, indicating that no antibodies were formed against the albumin moiety of the conjugates. Rabbit albumin-SOD conjugates were non-antigenic as evidenced by absent precipitin reactions to all anti-sera including anti-sera to SOD. Predictably, rat albumin was immunogenic and antigenic whether presented as native rat albumin or conjugates. Anti-sera to rat albumin-SOD conjugates reacted with native rat albumin, conjugates of rat albumin and rat albumin-SOD conjugates, but did not react with native SOD. This indicates that the antibody response was restricted to the albumin moiety of the conjugates.

TABLE 6.3

Immunogenicity and antigenicity of crosslinked albumin-SOD conjugates.

	ANTIGENS SOD	Rabbit albumin	Rabbit albumin alone conjug	Rabbit albumin SOD conjug	Rat albumin	Rat albumin alone conjug	Rat alb SOD conj
SOD	+	-	-	-	-	-	-
	+	-	-	-	-	-	-
	+	-	-	-	-	-	-
Rabbit	-	-	-	-	-	-	-
albumin	-	-	-	-	-	-	-
	-	-	-	-	-	-	-
Rab alb	-	-	-	-	-	-	-
alone	-	-	-	-	-	-	-
conjug	-	-	-	-	-	-	-
Rabbit	+	-	-	-	-	-	-
alb-SOD	-	-	-	-	-	-	-
conjug	-	-	-	-	-	-	-
Rat	-	-	-	-	+	+	+
albumin	-	-	-	-	+	+	+
	-	-	-	-	+	+	+
Rat alb	-	-	-	-	+	+	+
alone	-	-	-	-	+	+	+
conjug	-	-	-	-	+	+	+
Rat alb	-	-	-	-	+	+	+
SOD	-	-	-	-	+	+	+
conjug	-	-	-	-	+	+	+

Results are shown for reactions of antisera raised in three separate rabbits for each antigen. Conjugates of albumin alone were formed by glutaraldehyde reaction in absence of SOD.

\+ Indicates a precipitin reaction with the antigen shown.

\- Indicates a negative precipitin reaction.

6.4 DISCUSSION

These studies demonstrate a number of desirable properties of albumin-SOD and SOD-SOD conjugates. The conjugates are easy to prepare and composed of relatively inexpensive and readily available materials (bovine liver Cu Zn SOD and albumin). The conjugates are relatively stable upon storage with respect to SOD activity and *in vivo* anti-inflammatory properties. Lyophilized conjugates, which can be reconstitutd without loss of activity, provide a formulation suitable for prolonged storage prior to use. The plasma clearance of conjugates (half-time of enzymatic activity of up to 15 h) is sufficiently slow to allow use of conjugates in studies designed to examine the actions of superoxide ions (and related oxygen-free radicals) and SOD *in vivo*.

SOD-albumin and SOD-SOD conjugates were found to have potent anti-inflammatory activity in rats with carrageenan-induced paw oedema. The inhibition of carrageenan-induced paw swelling achieved was similar to the maximum effect obtained with non-steroidal anti-inflammatory drugs (NSAIDs). (With NSAIDs this degree of inhibition is invariably associated with acute gastric toxicity (133)).

However, albumin-albumin conjugates were found to have anti-inflammatory activity, so that the anti-inflammatory action of these SOD conjugates could not necessarily be attributed to their enzymatic activity.

The enzyme independent anti-inflammatory action of conjugates may, in part, be related to their macromolecular configuration, because a variety of macromolecules have been shown to possess anti-inflammatory effects (174). Alternatively or additionally, the crosslinking procedure may alter the protein constituents of conjugates in such a way as to make them capable of depleting circulating factors (e.g. complement) required to mount an inflammatory response at the assessment site. The findings dictate caution in interpreting studies demonstrating in vivo effects of macromolecular conjugates of SOD (or other enzymes) and attributing the observed effects to alterations in the availability of the enzyme at the site of the process under assessment.

Conjugation of SOD with albumin resulted in diminished immunogenicity and antigenicity of SOD. Using the rigorous immunization schedule described (involving repeated injection of antigens with and without complete Freund's adjuvant), only one of three rabbits injected with rabbit albumin-SOD conjugates developed a precipitating antibody

against SOD, whereas all three rabbits given native SOD developed antibodies to SOD. Furthermore, albumin-SOD conjugates were shown to be nonantigenic in the presence of antibodies to SOD. These findings are encouraging for the possible therapeutic administration of homologous albumin-SOD conjugates by intravenous injection.

Further studies are required to assess the toxicity and immunogenicity and antigenicity of conjugates given repeatedly by injection intravenously or by other routes (intramuscularly, intra-articularly). Rates of clearance of plasma SOD activity could alter with prolonged dosing. If the circulatory characteristics of albumin-SOD remained unchanged over a period of months, then it could be inferred that no antibodies directed against the conjugate were produced in vivo to accelerate the removal of conjugates. Injections of conjugates given for therapeutic indications are likely to be less immunizing than the regimen of repeated intramuscular injection with complete Freund's adjuvant used in the present study. However, should the broad molecular weight range of conjugates (MW 1.0×10^5 - 17×10^5) tested in this study show significant immunogenicity after repeated intravenous administration, it may prove useful to discard conjugates at the lower end of the range (eg. MW 1×10^5 - 2.5×10^5) and retain conjugates of higher molecular weight (eg. MW 2.5×10^5-5×10^5)

(although this would involve some loss of retention in plasma and other spaces). The lower molecular weight conjugates contain less albumin (conjugates of 1×10^5 would contain one albumin and one SOD molecule) than high molecular weight conjugates and accordingly might be expected to less efficiently mask antigenic sites on heterologous SOD molecules.

Fortunately SOD is a molecule with a high degree of sequence homology between species (35) and relatively low immunoreactivity. Orgotein, a protein product promoted for anti-inflammatory and other claimed therapeutic effects, before its identity with CuZn bovine liver SOD was known, has been given to hundreds of patients (114,115,116,117,118) and has also been used in vetinerary practice (especially in horses). Although it is difficult to accept various therapeutic claims as proven on the basis of published data, these studies at least indicate that SOD is well tolerated when repeatedly injected intravenously, intramuscularly and into joints. Accordingly, bio-availablity could be a more important consideration than immunoreactivity, and SOD-SOD conjugates could conceivably be as well tolerated as SOD-albumin following repeated injections in most subjects.

The development of conjugates of SOD with long plasma half times and low immunogenicity provides new possibilities

for the study of inflammation and its treatment. Other potential applications for these conjugates in biology and medicine may be found in radiation oncology (175), toxicology (e.g. paraquat and oxygen toxicity) (176), and surgery (e.g. protection of autologous and allologous grafts from reperfusion injury)(177,178).

6.5 SUMMARY

Crosslinked polymers of CuZn SOD and albumin from several mammalian sources have been prepared with (a) more than 60% retention of enzymativity, (b) stability on storage, (c) delayed clearance of enzymatic activity from plasma and pleural spaces, (d) anti-inflammatory action (also found with cross-linked albumin and (e) low immuno-reactivity.

CHAPTER 7

CORTISOL PALMITATE LIPOSOMES FOR SYSTEMIC THERAPY OF INFLAMMATION

7.1 INTRODUCTION

This chapter describes the preparation of cortisol palmitate liposomes and documents their anti-inflammatory effects following systemic administration using two separate animal models of inflammation.

7.2 MATERIALS AND METHODS

7.2.1 Materials

Dipalmitoyl chloride, DPPC, and hydrocortisone were obtained from the Sigma Chemical Co. (St. Louis, Mo.). Hydrocortisone succinate was obtained from Eli Lilly, Sydney, Australia. Cortisol palmitate (CP) was obtained from ICI PharmaceCuticals Division, Macclesfield, Cheshire, U.K.. ^{3}H-cortisol palmitate was prepared according to the method of Shaw et al (131). ^{14}C-cholesteryl oleate was obtained from New England Nuclear, Boston, Mass..

7.2.2 Preparation of liposomes

Liposomes were prepared as follows:- DPPC 90mg and cortisol palmitate 10 mg were dissolved in chloroform and dried *in vacuo* on a rotary evaporator. The resulting film of lipid was redissolved in chloroform benzene (1:10, 2 mls) which was frozen in dry ice/ethanol before lyophilization.

The freeze-dried solid was resuspended in phosphate buffered saline 0.15 M, pH 7.4 and sonicated at 52°C for 15 minutes using a probe sonicator (375W Cell Disruptor, Heat Systems Ultrasonics Inc., Plainsville, N.Y.). After standing at 40°C for 30 minutes to allow the liposomes to anneal, the resulting transluscent suspension was centrifuged at 500g for 10 minutes at room temperature to remove any titanium particles shed by the probe during sonication. Liposome preparations for use in animal experiments were then dialysed for 60 minutes. Sonicated suspensions of cortisol palmitate in saline were also prepared without using DPPC. These latter suspensions were not stable, with cortisol palmitate forming a precipitate within a few minutes inspite of sonication for up to 60 minutes at 40°C or room temperature.

In studies designed to assess (a) the amount of cortisol palmitate incorporated into DPPC liposomes and (b)

possible leakage of cortisol palmitate from the liposomes, liposomes were prepared with DPPC 90mg, ^{3}H-cortisol palmitate 10 mg (50 uCi) and ^{14}C-cholesteryl oleate, 10 ug (50 uCi). The latter compound is highly lipophilic and insoluble in water and was used as a liposome constituent marker. After centrifugation to remove titanium particles, the liposomes were applied directly to a Sephadex G50 column (Pharmacia, Fine Chemicals, Uppsala, Sweden). Liposomes eluting in the void volume were pooled and stored at 4^{o}C for 7 and 21 days and then re-chromatographed on Sephadex G50. ^{3}H and ^{14}C content of these purified liposomes were determined in a Beckman L5-230 liquid scintillation counter after adding 100 ul aliquots of eluted liposome fractions to 2 ml of Bray's solution. (New England Nuclear,Boston, Mass.).

7.2.3 Carrageenan-induced paw oedema in rats

Groups of 6-8 rats were injected intravenously with liposome preparations (volume 0.3-0.5 ml) immediately prior to injecting 0.1 ml carrageenan (1%) in saline into the left paw. Paw volume was determined by the weight of water displaced from a 12 x 75 mm test tube by immersion of the paw to the anatomical hairline. Paw volumes were measured before, and 4 hours after, injection of carrageenan. The increases in paw volume over this four hour period were calculated, and differences between groups analysed using

the unpaired t test. Results are presented graphically as percentage inhibition of paw volume increase, by comparison with control groups of rats receiving carrageenan but injected intravenously with saline *in lieu* of liposomes.

7.2.4 Inflammatory response in subcutaneous sponges impregnated with heat-killed M. tuberculosis

Each experimental group contained 7 or 8 rats. Polyurethane sponges (Dunlopillo Ltd., Adelaide, Australia) 40 mg were washed twice in distilled water and then immersed in a suspension of heat-killed M. tuberculosis, 0.5 mg/ml in distilled water prepared as follows: 80 mg of heat-killed, lyophilized M. tuberculosis (Strains C,DT, PN mixed, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey, U.K.) was ground to a paste with 0.5 ml distilled water using a pestle and mortar then diluted to the final concentration, and sonicated for 2 minutes using a 60W Mullard tissue sonicator with 1/4" probe to otain an even suspension. These sponges were then dried overnight at 37°C to constant weight. Each sponge contained approximately 1.5 mg dry *M. tuberculosis*. On day 0, sponges were implanted through a dorsal midline incision, one into each flank of each rat and the wound closed with staples. Animals were then injected with test preparations or saline via the saphenous vein on days 0,1,2,3. On day 4 animals were sacrificed, the sponges were

removed and adherent tissue external to the sponges carefully cut away. The sponges were then dried at 37°C for 3 days prior to weighing. For statistical analysis, using the unpaired t test, the average weight gain of the two sponges from each animal was taken (except for those animals contributing sponges for cell density analysis, in which case only the weight of one dried sponge weight was used for this analysis). Percentage weight inhibition was computed by comparison with the mean increase in sponge weight in control rats, i.e. receiving saline rather than liposome preparations.

One sponge from each of two animals in each treatment group was immediately fixed in formal-saline, without prior excision of adherent tissue. These sponges were later embedded in paraffin wax, and 5 micron sections were cut and stained with haematoxylin and eosin. Three sections from each sponge were examined to quantify the density of cellular infiltration using a Transidyne 2955 scanning densitometer. Cell density data was expressed as the mean of 2 averaged density scores, obtained after examining sponges from 2 animals in each group. The small numbers of samples in these histological studies precluded statistical analysis.

7.3 RESULTS

7.3.1 Incorporation and retention of cortisol palmitate into DPPC liposomes

Liposomal preparations appearing in the void volume contained more than 99% of the ^{3}H-cortisol palmitate and ^{14}C-cholesteryl oleate applied (Figure 7.1). After repeat chromatography at 7 and 21 days all the radioactivity remained in the liposome (void volume) fractions, indicating no measurable leakage of cortisol under the conditions of storage. Attempts to incorporate unesterified ^{3}H-cortisol, 5 mg (50 uCi) into DPPC (90mg) liposomes resulted in very low entrapment of cortisol (<2%). These latter preparations were deemed unsuitable for *in vivo* studies.

7.3.2 Anti-inflammatory effect against carrageenan-induced paw oedema

The results comparing the anti-inflammatory effects of equivalent doses of cortisol given as (i) cortisol palmitate in DPPC liposomes, (ii) cortisol succinate and (iii) cortisol palmitate as aqueous suspensions, in rats with paw oedema are shown in Figure 7.2.

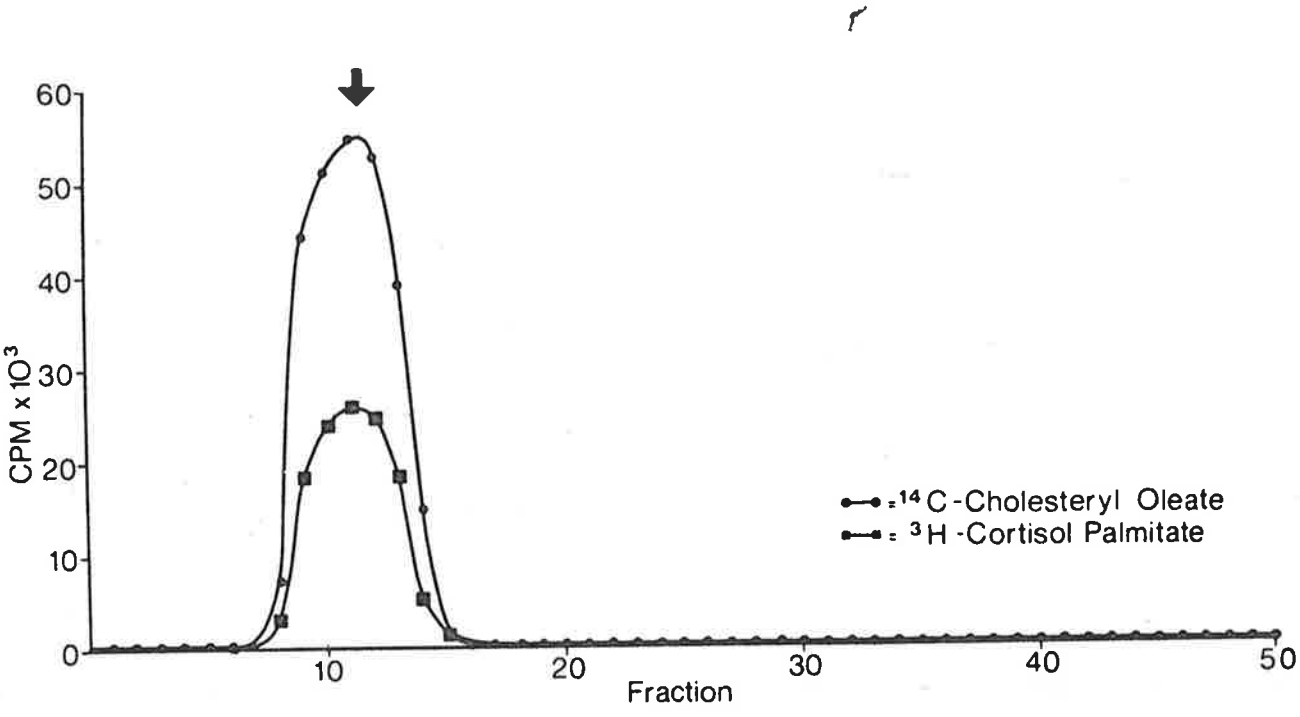

Figure 7.1. Elution profile following Sephadex G50 chromatography of liposomes containing DPPC (90mg), cortisol palmitate (5mg, 50uCi) and cholesteryl oleate (10 ug, 50 uCi). ^{14}C-cholesteryl oleate was used as a liposome marker. Liposomes appear in the void volume. Repeat chromatography after seven and 21 days storage at 4°C gave identical profiles. ^{3}H-cortisol palmitate was also found to elute entirely in the void volume (liposome) fractions both before and after storage. The arrow shown indicates the location of the void volume peak obtained with blue dextran (Pharmacia).

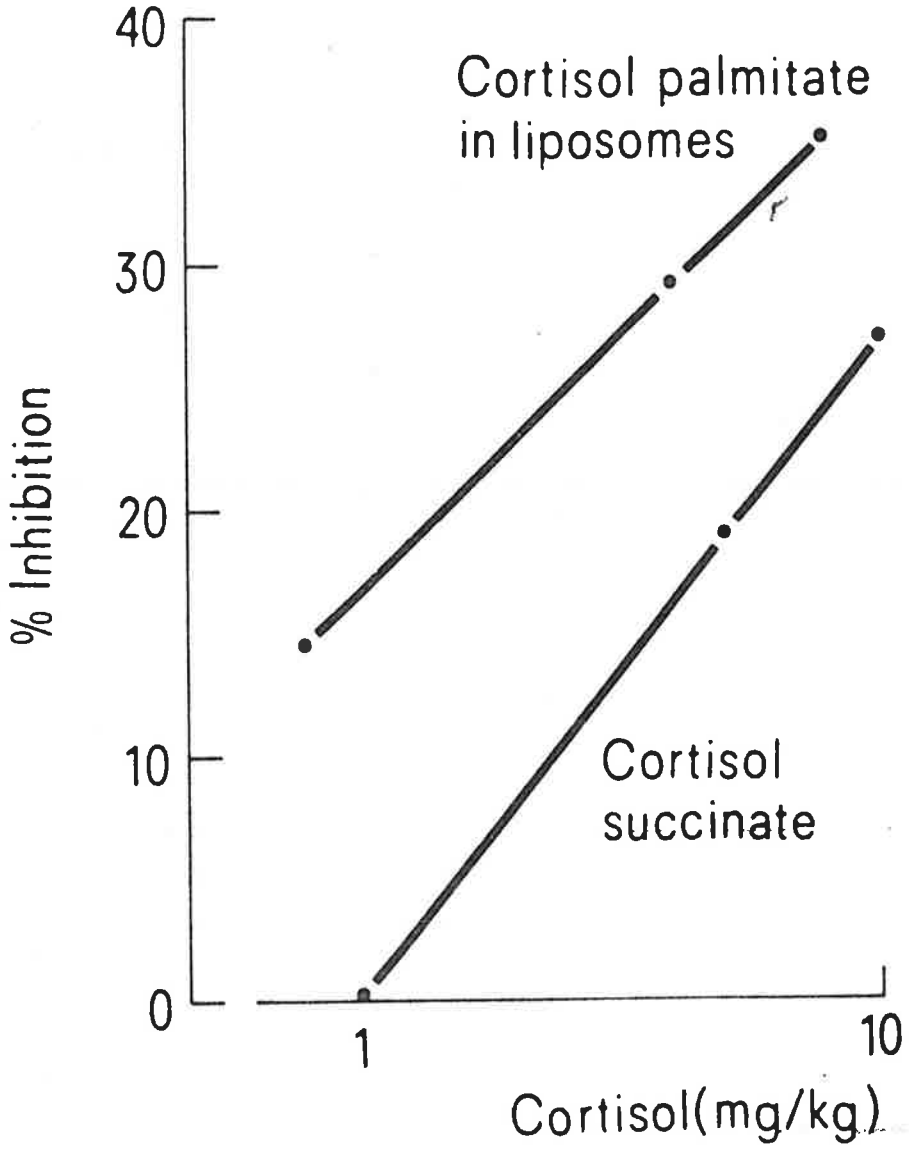

Figure 7.2. Inhibition of carrageenan-induced paw swelling following intravenous injection of test preparations immediately prior to injecting carrageenan (0.1 ml) into the left hind paw of rats. Paw volume was measured before and four hours after injection. Results are expressed as percentage inhibition of paw swelling observed in control rats injected intravenously with saline. Experimental groups comprised 6-8 rats.

The liposome-encapsulated cortisol palmitate preparations were approximately 10 times as potent as cortisol succinate, a water-soluble cortisol preparation. Significant differences in paw swelling were seen at cortisol doses of 4 mg/kg ($t=4.81$, $p<0.001$, d.f. 11) and 40 mg/kg ($t=7.67$, $p<0.001$, d.f. 13). In the absence of phospholipid, cortisol palmitate formed an unstable suspension exhibiting little anti-inflammatory activity *in vivo*, presumably due to its poor bioavailability in this type of preparation.

7.3.3 Anti-inflammatory effect against cellular infiltration of subcutaneous sponges impregnated with heat-killed M. tuberculosis

Results of studies of anti-inflammatory activity using this model are shown in Figure 7.3. Both cortisol palmitate in DPPC liposomes and cortisol succinate showed potent anti-inflammatory activity following intravenous injection, shown by reduction of both the sponge weight and the extent of cellular infiltration into the implanted sponge. Liposome-encapsulated cortisol palmitate, given intravenously was superior to cortisol succinate given by the same route, in suppressing the increase of sponge weight. Significant differences in sponge weight were seen

when liposome- encapsulated cortisol palmitate and cortisol succinate were compared at doses of approximately 1 mg/kg cortisol ($t=2.0$, $p<0.95$, d.f. 14), 5 mg/kg ($t=3.38$, $p<0.0025$, d.f. 15) and 10 mg/kg ($t=2.6$, $p<0.025$, d.f. 14). The amount of cortisol, given as cortisol succinate was actually 1.25 times the amount given as liposome-encapsulated cortisol (as palmitate). The liposome-encapsulated cortisol palmitate and cortisol succinate preparations were approximately equipotent in inhibiting cellular migration into the sponges (Table 7.1).

DPPC vesicles without cortisol palmitate showed no anti-inflammatory effect at all at lipid concentrations used in these experiments. Furthermore, they provided no additional anti-inflammatory effect when given with anti-inflammatory doses of the water-soluble cortisol succinate in either model of inflammation.

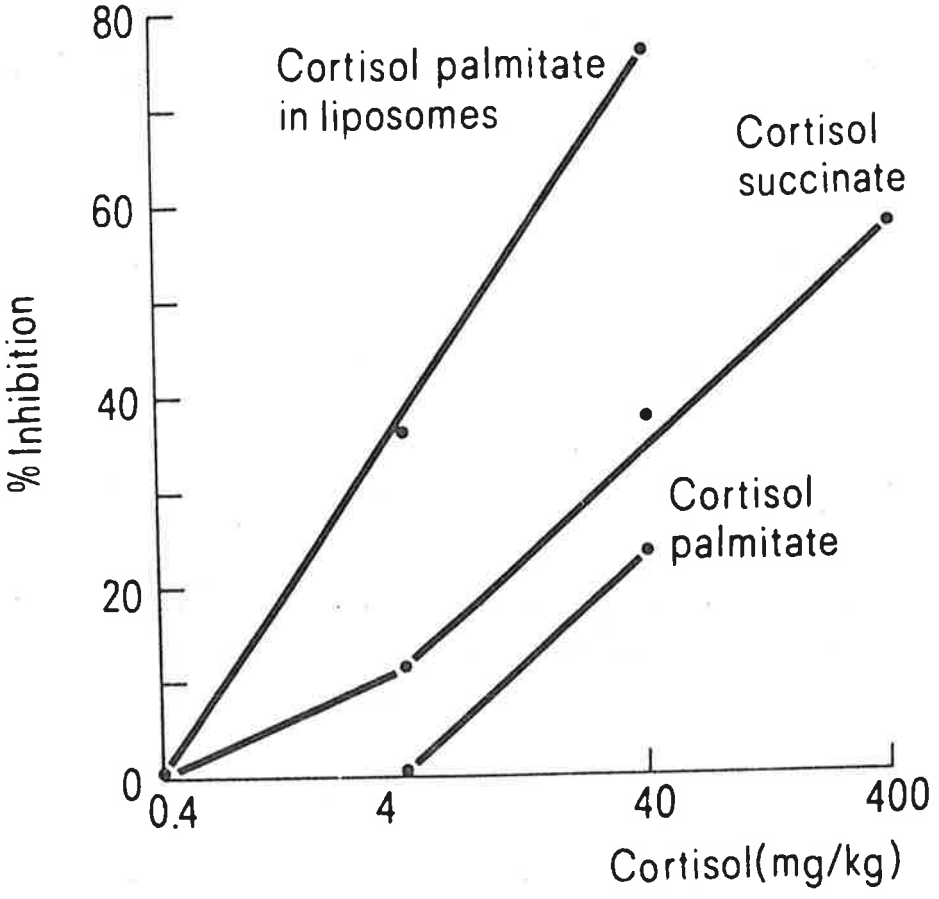

Figure 7.3. Inhibition of weight gain of sponge implants in rats following intravenous administration of test preparations on days 0, 1, 2 and 3. Heat killed M. tuberculosis treated sponges were implanted on day 0. Animals were sacrificed and sponges removed on day 4. Results are expressed as percentage inhibition of weight gain of sponges observed in control rats injected intravenously with saline. Experimental groups comprised 7 or 8 rats.

TABLE 7.1

Effect of cortisol esters on cellular infiltration into implanted sponges in rats

Test preparations[a]	Cortisol[b] mg/kg	Cell density[c]
Saline	0	100
Cortisol palmitate (liposome encapsulated)	0.8	91
	4	55
	8	49
Cortisol succinate	1	83
	5	53
	10	45

a test preparations injected intravenously on days 0, 1, 2 and 3. Rats sacrificed on day 4.

b values refer to daily dose of cortisol given as test preparations.

c cell densities expressed as percentage of mean values obtained for saline injected (control) rats.

7.4 DISCUSSION

Liposomes containing cortisol palmitate and DPPC provided a greater anti-inflammatory effect when compared with equivalent doses of the water-soluble cortisol succinate, in two models of inflammation. The first model, carrageenan-induced paw inflammation, measures oedema forming in the first few hours following an irritant stimulus (133). The second model involving implantation of polyurethane sponges impregnated with heat killed M. tuberculosis, and studied over a 4 day treatment period, provides a model of continuing severe acute inflammation. It is characterised by a predominance of neutrophil polymorphonuclear cells and early infiltration of mononuclear cells together with collagen formation within the sponges at the time of sacrifice (134). This model allows quantitation of both cellular infiltration and increase in sponge weight (contributed by oedema fluid and cellular infiltrate). Liposomal cortisol palmitate was significantly more effective in suppressing the increase in sponge weight and equipotent in suppressing cellular infiltration, when compared with cortisol succinate. Appropriate control experiments failed to show any influence of liposomes, prepared without cortisol succinate or cortisol palmitate, on the inflammatory response in the

presence or absence of cortisol succinate in either inflammatory model. The enhanced affects seen with liposome encapsulation of cortisol palmitate in DPPC thus could not be attributed to any intrinsic anti-inflammatory effects of the DPPC liposomes alone. Similarly, enhancement of inflammatory effect of cortisol by esterification with palmitate was excluded in experiments using sonicated suspensions of cortisol palmitate in saline in the carrageenan-induced paw oedema model. These unstable suspensions which are difficult to inject were not tested in the sponge implant model.

These studies indicate an enhanced anti-inflammatory effect of cortisol following esterification with palmitate and incorporation within DPPC-based liposomes. The degree of enhancement could be of practical therapeutic value in some clinical situations, if unaccompanied by an enhancement of unwanted effects.

7.5 SUMMARY

Liposomes containing cortisol palmitate showed greater anti-inflammatory activity than water soluble cortisol succinate, following intravenous injection into rats, as assessed by inhibition of (a) carrageenan-induced paw swelling, and (b) inflammatory responses in subcutaneous sponge implants impregnated with heat-killed *M. tuberculosis*.

CHAPTER 8

GENERAL DISCUSSION AND DIRECTIONS FOR FURTHER STUDIES

The major contributions of this thesis are outlined and discussed below with comments regarding later studies and directions for future studies.

8.1 PRODUCTION OF OXY RADICALS WITHIN INFLAMED JOINTS

The capacity of synovial fluid leucocytes for generation of an oxidative burst was established by measuring the chemiluminescent responses of these cells to stimulation by serum opsonised zymosan *in vitro*. Observed higher basal chemiluminescence of synovial fluid PMN leucocytes compared to their matched peripheral blood counterparts suggested a degree of *in vivo* activation of cells sampled from inflamed joints. These studies add relevance to considerations of oxy radicals as potential mediators of tissue damage and as possible modifiers of cell metabolism in inflamed joints. More direct evidence for oxy radical production within inflamed joints awaits the identification of stable conversion products specifically attributable to the effects of oxy radicals on an endogenous or exogenous probe substrate.

8.2 IRON CHELATION AND HYDROXYL RADICAL PRODUCTION

Earlier suggestions that iron is important as a catalyst for hydroxyl radical production in the presence of a superoxide flux were confirmed and extended to show the importance of state of iron chelation for catalytic activity. The observed dose dependent inhibition of hydroxyl radical production at the relatively high concentrations of chelators studied (1 mM and greater) led to further studies examining effects of lower concentrations of chelators in the same experimental systems. A biphasic effect on hydroxyl radical production in the presence of a superoxide flux and catalytic amounts of iron has been observed in these studies (179) with concentrations of certain chelators (EDTA, DETAPAC, penicillamine) enhancing hydroxyl radical production at concentrations up to approximately 1mM with dose dependent inhibition again being observed at higher concentrations.

8.3 AUTOXIDATION OF IRON AND REDUCING AGENTS

Observed enhancement of hydroxyl radical production at some concentrations of penicillamine in the iron autoxidation system (Fe^{2+}/EDTA) drew attention to a superoxide independent mechanism for iron catalysed hydroxyl radical production. This mechanism involves reduction of

$$2Fe^{3+} + 2RSH \longrightarrow 2Fe^{2+} + RSSR + 2H^{+} \quad (8)$$

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{-} \quad (1)$$

$$O_2^{-} + O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+} + O_2^{-} + 2H^{+} \longrightarrow Fe^{3+} + H_2O_2 \quad (7)$$

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

Fe^{3+} by thiol agents with thiols thus driving the cyclic oxidation and reduction of suitably chelated iron and associated hydroxyl radical production. The endogenous thiol, glutathione, was one of several agents shown to be active in this system. Using hyaluronate depolymerisation (viscometry) as detector system, hydroxyl radical production was correlated with extent of thiol oxidation (179). Thus the following sequence of reactions can be postulated:

This or a similar mechanism may have relevance at inflammatory sites, e.g. rheumatoid joints where increased iron deposits may provide ample iron (57,58,155) to support such a reaction in the presence of endogenous bioreductants (glutathione, ascorbic acid) (59).

8.4 GENTISATE AND RELATED SUBSTANCES AS ACTIVE METABOLITES

Gentisate was demonstrated in synovial fluid at concentrations (ranging from 2 to 24 uM), at which gentisate causes inhibition of chemiluminescence (amplified by luminol) associated with an enzymatically (xanthine/hypoxanthine) generated superoxide flux, ID_{20} 2.7 uM, ID_{70} 3.7 uM (78). (ID_{20} and ID_{70} refer to concentrations causing 20% and 70% inhibition of chemiluminecence respectively). Gentisate was also found

to have similar potency as an inhibitor of oxy radical associated chemiluminescence in the presence of several oxy radical generators and another lumigenic probe (lucinigenin). These studies, which include examination of several isomers and structural analogues of gentisate have shown that chemiluminesence-inhibiting activity in these systems correlates with a structural configuration allowing oxidation to a quinone (see figure 8.1). For example, a potent inhibitory activity similar to that of gentisate was observed with 2,3 dihydroxybenzoate (both produced by hydroxyl radical attack on salicylate in vitro). Gentisate has hydroxyl groups in a Para- configuration and can be oxidised to a Para-quinone. 2,3 dihydroxybenzoate (with hydroxyl groups in the Ortho- configuration) may be oxidized to an Ortho-quinone. The ability of these diphenols to readily yield to hydrogen abstraction to form stable radical intermediates (hydroquinones) may account for their activity in the oxy radical/chemiluminesence systems. Salicylate, other monohydroxybenzoates, and dihydroxybenzoates with hydroxyl groups in a Meta- configuration (which cannot be oxidised to quinones) have relatively weak activity in these systems (IC_{20} > 200 uM).

Studies of aminosalicylates and aminophenols demonstrate analagous structure function relationships with 5-aminosalicylate and 4-aminophenol (both of which have an

amino group Para- to an hydroxyl group) having ID_{20} of 0.1 uM or less. By contrast, 4-aminosalicylate (amino group Meta- to hydroxyl group) was found to have an ID_{20} > 1 mM. Para- and Ortho- aminophenols, like diphenols in this configuration yield to proton abstraction to form relatively stable hydroquinone radical intermediates. These latter studies achieve relevence because 5-aminosalicylate and sulphasalazine, of which 5-aminosalicylate is a prime metabolite, have been shown to have anti-inflammatory activity against inflammatory bowel disease and, in the scase of sulphasalazine, rheumatoid arthritis.

Studies using a variety of detector systems to monitor hydroxyl radical production (depolymerisation of hyaluronic acid, hydroxylation of salicylate, 1-^{14}C decarboxylation of radiolabelled benzoate or formate) have failed to discriminate between the activities of (a) Ortho- or Para- diphenols/aminophenols and (b) monophenols, and diphenols/aminophenols with a Meta- configuration. Thus the potency of agents such as gentisate and 5-aminosalicylate as inhibitors of chemiluminescence is not simply related to their effects as hydroxyl radical scavengers. The importance of these effects in relation to the anti-inflammatory effects of salicylate and 5-amino salicylate/sulphasalazine remain to be established. Certainly these quinone-forming agents could conceivably,

through their actions as proton donors exert important effects on some metabolic events at inflammatory sites. Gentisate has little if any anti-inflammatory effect when given orally, possibly because its chemical instability and/or susceptibility to bacterial chlorinases in the gut militate against effective absorption. Further studies, using the HPLC method described, designed to document the characteristics of absorption/non-absorption of gentisate following oral administration may help to evaluate the conjecture that gentisate may be an active metabolite of salicylate. Also further studies are required to explore more directly possible anti-inflammatory effects of gentisate, including attempts to correlate structure-function relationships with anti-inflammatory activity of gentisate and its analogues, following local injection into experimentally induced inflammatory lesions in animals.

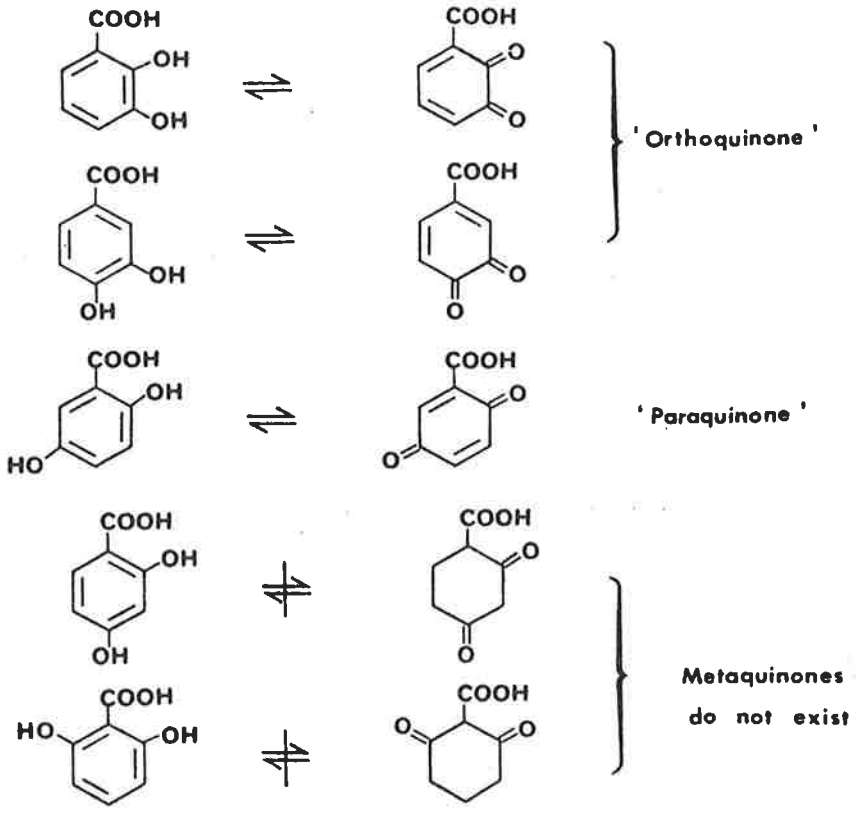

Figure 8.1. Structural configuration of quinone-forming and nonquinone-forming diphenols.

8.5 SOD CONJUGATES

Studies described indicate an anti-inflammatory effect of SOD conjugates which cannot be attributed entirely to their enzymatic activity because albumin-albumin conjugates formed by the same crosslinking method were also anti-inflammtory. The anti-inflammatory effects of albumin-albumin were not shared by unconjugated homologous serum albumin. Subsequent experiments have been undertaken to test whether any of the reagents used in the crosslinking procedure react with albumin to form an anti-inflammatory derivative. These studies have not identified the reagent or step in the reaction sequence reponsible for this effect. In addition, reaction of glutaldehyde and glycine (which may result in glutaldehyde-glycine polymers) also fails to generate an anti-inflammatory agent. It is possible that the macromolecular configuration of crosslinked albumin imparts a pro-inflammatory effect by triggering inflammtory cells/cascades within the intra-vascular compartment and thereby detracting from the inflammatory response induced and measured at the index site (i.e. paw oedema). A similar effect would result if the conjugates were contaminated by endogenous pyrogen. However, native albumin subject to similar procedures including incubation with gluraldehyde (for intervals insufficient to cause extensive crosslinking) and glycine followed by chromatography does not show

anti-inflammatory effect. In the studies described the anti-inflammatory effects of albumin-albumin were consistent and reproducable. Previous studies undertaken at another centre did not show an anti-inflammatory effect with albumin-albumin conjugates (173) but were less detailed than the studies described in this thesis.

Because albumin-albumin conjugates possess anti-inflammatory activity it is desirable that the effects of SOD conjugates be examined using systems in which alterations in tissue structure or function may be more directly attributable to superoxide ions or their derivatives (rather than a nonspecific tissue irritant like carrageenan), in the expectation that these systems may more clearly differentiate between the effects of SOD conjugates and conjugates containing albumin alone. For this purpose it is proposed to explore the possible protective effects of locally applied and intravenously injected SOD conjugates (and native SOD, albumin-albumin and native albumin for comparison) against oxy radical-induced damage to hamster cheek pouch vascular endothelium (monitored by microscopically evident leakage of fluorescent dye from blood vessels within the pouch) (180). Del Maestro has shown that in increased vascular permeability induced by XO/HX can be inhibited by local application of SOD, catalase and hydroxyl radicals scavengers (suggesting mediation

through superoxide-driven hydroxyl radical production) (180). The proposed studies would assess whether SOD conjugates within the vascular compartment could protect against endothelial damage/dysfunction resulting from the extra-vascular superoxide flux, as well as seeking to confirm the protective effect of locally applied (extra-vascular) SOD (in native and conjugated forms).

McCord and co-workers have suggested that reperfusion injury is mediated by superoxide ions generated by xanthine dehydrogenase which has been denatured in ischaemic tissues and has thus acquired xanthine oxidase activity (178). Experiments are planned to assess the activity of SOD conjugates as protectants against necrosis in ischaemic regions of pedicle skin flaps. These studies will utilize an established model in pigs with which it has been shown that agents injected during the operative period become entrapped within the ischaemic zone of the flap (181). Thus SOD conjugates given at operation should be retained within the flap long after SOD activity has been cleared from plasma. SOD conjugates may thereby exert a protective effect against necrosis since superoxide generation may be important in determining the extent of tissue necrosis (e.g through xanthine oxidase action in areas of the flap subject to ischemia then reperfusion). This model will also offer the opportunity to assess the relative activities of other

putative protectants against reperfusion injury, e.g. allopurinol.

8.6 DRUG DELIVERY SYSTEMS

The demonstration of enhanced anti-inflammatory effect of cortisol following esterification and incorporation in liposomes demonstrates an approach of potential value for systemic treatment of severe inflammatory disorders. The current practice of administering intravenously large "bolus" doses of corticosteroids to patients with severe uncontrolled inflammatory disorders (e.g. systemic lupus, rheumatoid vasculitis) suggests a place in which a trial of intermittent intravenous injection of cortisol liposomes could be undertaken.

Before proceding to a clinical application further animal studies are required to ensure that the enhancement of potency of anti-inflammatory effect of cortisol in liposomes is not accompanied by a parallel enhancement of unwanted effects. In particular, dose response relationships for effects at sites other than the inflammatory focus (e.g. thymic involution) need to be established. Correlations should be sought between observed effects (including anti-inflammatory effects) and pharmacokinetic studies. (Plasma clearance and tissue

distribution studies were attempted using ^{3}H-cortisol palmitate liposomes but the specific activity of the radio-labelled preparation was not sufficient to provide meaningful data). A further experimental approach involves assessment of relative potencies of free cortisol (and other anti-inflammatory corticosteroids) and liposomal cortisol upon the hypothalamic- pituitary - adrenal axis after acute and chronic dosing. For these studies to be undertaken in rats an assay for corticosterone is required since this is the dominant glucocorticoid in the rat.

Conversion of a pro-drug to an active form at sites of inflammation could provide an effective drug targetting system in which unwanted effects of an agent (at other sites) could be minimised. In order to achieve this localised conversion it may be necessary to employ a metabolic process unique, or at least largely confined, to the inflamed site. Oxy radicals produced by the surface membrane oxidases of activated leucocytes could provide such a mechanism. However oxy radical production is not restricted to these oxidases but also results from activity of ubiquitous microsomal enzymes, including the cytochrome P_{450} oxidase/reductase system and cyclo-oxygenases. Thus oxidative conversion of pro-drugs by oxy radicals may not be restricted to inflammatory sites sufficiently to acheive desired localisation of drug conversion. Although evidence

for conversion of salicylate to gentisate in inflamed joints was not obtained, rapid equilibration of these compounds between synovial fluid and plasma may have masked this event. It thus remains possible that salicylate may be converted by oxy radicals within inflamed joints to gentisate and (possibly 2,3 dihydroxybenzoic acid). These diphenol derivatives of salicylate may be far more active (e.g. as hydrogen donors) than salicylate in some metabolic systems. If these derivatives have important metabolic effects then salicylate may be seen as an oxy radical-convertible pro-drug for gentisate/ 2,3 dihydroxybenzoate. Further studies are required to examine the effects of gentisate/ 2,3 dihydroxybenzoate upon metabolic pathways (e.g. electron transport systems) where increased activity relative to salicylate might be seen. Also further studies are required to seek enzymatic systems which may account for metabolic conversion of salicylate to gentisate by mechanisms other than putative oxy radical attack.

BIBLIOGRAPHY

1. Diamanstein T., Oppenheim J.J., Unanue E.R., Wood D., Handschumacher R.E., Rosenstreich D.L., Waksman B.H. Nonspecific "lymphocyte activating" factors produced by macrophages.
Clin. Immunol. Immunopathol. 14: 264-67, 1979.

2. Rocklin R.E., Bendtzen K., Greineder D.
Mediators of immunity - lymphokines and monokines.
Adv. Immunol. 29: 55-136, 1980.

3. Waksman B.H.
Immunoglobulins and lymphokines as mediators of inflammatory cell mobilization and target cell killing.
Cell. Immunol. 27: 309-315, 1976.

4. Samuelsson B.
Leukotrienes: A new class of mediators of immediate hypersensitivity reactions and inflammation.
in Advances in Prostaglandin, Thromboxane and Leukotriene Research, Vol 2.
Ed. B. Samuelsson, R. Paoletti, P. Ramwell.
Raven Press, New York. 1983, pp 1-13.

5. Higgs G.A., Palmer R.M.J., Eakins K.E., Moncada S.
Arachidonic acid metabolism as a source of inflammatory mediators and its inhibition as a mechanism of action for anti-inflammatory drugs.
Molec. Aspects Med. 4: 275-301, 1983.

6. Moretta L., Webb S.R., Grossi C.E., Lydyard P.M.L., Cooper M.D.
Functional analysis of two human T cell subpopulations: help and supression of B cell responses by T cells bearing receptors for IgM or IgG.
J. Exp. Med. 146: 184-200, 1977.

7. Fearon D.T., Austen K.F.
Acute inflammatory response
in Arthritis and Allied Conditions, Ninth Edition.
Ed. D.J. McCarty, Lea & Febiger, Philadelphia, 1979, 214-228.

8. Geha R.S.
Regulation of the immune response by idiotypic-antiidiotypic interactions.
N. Engl. J. Med. 305: 25-28, 1981.

9. Zvaifler N.J.
The immunopathology of joint inflammation in rheumatoid arthritis.
Adv. Immunol. 16: 265-336, 1973.

10. National Centre for Health Statistics.
PHS, Publication No. 1000, Series 11, No. 17.
Washington, U.S. Government.
Printing Office, 1966.

11. Hurd E.R.
Extraarticular manifestations of rheumatoid arthritis.
Seminars Arthritis Rheum. 8: 151-176, 1979.

12. De Cevaler K., Carson Dick W.
The Clinical evaluation of antirheumatic drugs.
in Text Book of Rheumatology.
Ed. W.N. Kelley, E.D. Harris, S. Ruddy, C.B. Sledge,
W.B. Saunders.
Philadelphia, 729-739, 1981.

13. Rhind V.M., Bird H.A., Wright V.
A comparison of clinical assessments of disease activity in rheumatoid arthritis.
Ann. Rheum. Dis. 39: 135-137, 1980.

14. Axelrod L.
Steroids.
in Textbook of Rheumatology.
Ed. W.N. Kelley, E.D. Harris, S. Ruddy, C.B. Sledge.
Philadelphia, 822-840, 1981.

15. Pemberton R.E., Strand L.J.
A review of upper-gastrointestinal effects of the new non-steroidal anti-inflammatory agents.
Digestive Diseases Sciences 24: 53-64, 1979.

16. Lanza F.L., Royer G.L., Nelson R.S., Chen T.T., Seckman C.E., Rack M.F.
A comparison of endoscopic evaluation of the damaging effects of nonsteroidal anti-inflammatory agents on the gastric and duodenal mucosa.
Am. J. Gastroenterol. 75: 17-21, 1981.

17. Vane J.R.
Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs.
Nature New Biol. 231: 232-235, 1971.

18. McKenzie A.H., Scherbel A.L.
Chloroquine and hydroxychloroquine in rheumatological therapy.
Clinics Rheum. Dis. 6: 545-566, 1980.

19. Empire Rheumatism Council Subcommittee
Gold therapy in rheumatoid arthritis
Ann. Rheum. Dis. 20: 315-333, 1961.

20. Sigler J.W., Bluhm G.B., Duncan H., Sharp J.T., Ensign D.L., McCrum W.R.
Gold salts in the treatment of rheumatoid arthritis: a double blind study.
Ann. Intern. Med. 80: 21-26, 1974.

21. Multicentre trial group
Controlled trial of D(-)-penicillamine in severe rheumatoid arthritis
Lancet I: 275-280, 1973.

22. Stein H.B., Patterson A.C., Offer R.C., Atkins C.J., Teufel A., Robinson H.S.
Adverse effects of D-penicillamine in rheumatoid arthritis.
Ann. Intern. Med. 92: 24-29, 1980.

23. Currey H.L.F., Harris J., Mason R.M., Woodland J., Beveridge T., Roberts C.J., Vere D.W., Dixon A. St. J., Owen B.
Comparison of azathioprine, cyclophosphamide and gold in treatment of rheumatoid arthritis.
Br. Med. J. 3: 763-766, 1974.

24. Babior B.M.
Oxygen dependent microbial killing by phagocytes.
N. Engl. J. Med. 298: 721-725, 1978.

25. Klebanoff S.J.
Oxygen metabolism and toxic properties of phagocytes.
Ann. Int. Med. 93: 480-489, 1980.

26. De Chatelet L.R.
Initiation of the respiratory burst in human polymorphonuclear neutrophils.
J. Reticuloendothel. Soc. 24: 73-91, 1978.

27. Stella V.J., Mikkelson T.S, Pipkin J.D.
Prodrugs: The control of drug delivery via bioreversible chemical modification.
in Drug Delivery Systems.
Ed. R.L. Juliano.
Oxford University Press, New York, 112-76, 1980.

28. Drug Delivery Systems.
Ed. R.L. Juliano.
Oxford University Press, New York, 1980.

29. Liposomes and their uses in biology and medicine.
Part III. Lipid vesicles as carriers for introducing drugs and other biologically active materials into cells.
Ed. D. Papahadjopoulos.
Ann. N.Y. Acad. Sci. 308: 226-432, 1978.

30. Greenwald R.A.
Oxyradicals and connective tissue.
J. Rheumatol. 8: 185-87, 1981.

31. McCord J.M., Keele B.B., Fridovich I.
An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase.
Proc. Natl. Acad. Sci. U.S.A. 68: 1024-1027, 1971.

32. McCord J.M., Fridovich I.
Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein).
J. Biol. Chem. 244: 6049-6055, 1969.

33. Keele B.B. Jr., McCord J.M., Fridovich I.
Superoxide dismutase from Escherichia coli.
J. Biol.Chem. 245: 6176-6181, 1970.

34. Yost F.J. Jr., Fridovich I.
An iron-containing superoxide dismutase from Escherichia coli.
J. Biol. Chem. 248: 4905-4908, 1973.

35. Harris J.I., Steinman H.M.
Amino acid sequences among superoxide dismutases
in Superoxide and Superoxide Dismutases.
Ed. A.M. Michelson, J.M. McCord, I Fridovich.
Academic Press, London 1977, 225-230.

36. Gregory E.M., Fridovich I.
Induction of superoxide dismutase with hyperoxia.
J. Bacteriol. 114: 543-548, 1973.

37. Harvarth L., Anderson B.R.
Defective initiation of oxidative metabolism in polymorphonuclear leukocytes.
N. Engl. J. Med. 300: 1130-1135, 1979.

38. Allen R.C., Stjernholm R.L., Steele R.H.
Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity.
Biochem. Biophys. Res. Commun. 47: 679-684, 1972.

39. Weissman G., Korchak H.M., Perez H.D., Smolen G.E., Goldstein I.M., Hoffstein S.T.
Leucocytes as secretory organs of inflammation.
in Advances in Inflammation Research, Vol 1.
Ed. G. Weissman, B. Samuelsson, R. Paoletti.
Raven Press, New York, 1979, pp 95-112.

40. Ropes M.W., Bauer W.
Synovial Fluid Changes in Joint Disease.
Cambridge Mass., Harvard Univ. Press, 1953, p. 56.

41. Greenwald R.A., Moy W.W.
Effect of oxygen derived free radicals on hyaluronic acid.
Arthritis Rheum. 23: 455-463, 1980.

42. Que B.G., Downey K.M., So A.G.
Degradation of deoxyribonucleic acid by a 1,10-phenanthroline-coper complex: The role of hydroxyl radicals.
Biochem 19: 5987-5991, 1980.

43. Kellogg E.W. III, Fridovich I.
Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by the xanthine oxidase system.
J. Biol. Chem. 250: 8812-8817, 1975.

44. Hamilton J.A.
Plasminogen activitor of rheumatoid and nonrheumatoid synovial fibroblasts.
J. Rheumatol. 9: 834-842, 1982.

45. Dayer J.M., Krane S.M., Russell R.G.G., Robinson D.R.
Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells.
Proc. Nat. Acad. Sci. U.S.A. 73: 945-949, 1976

46. Ohlsson K.
Antitrypsin and Alpha$_2$ macroglublin.
Interactions with human neutrophil collagenase and elastase.
Ann. N.Y. Acad. Sci. 256: 409-419, 1975.

47. Johnson D., Travis J.
The oxidative inactivation of human alpha 1-proteinase inhibitor: further evidence for methionine at the reactive centre.
J. Biol. Chem. 254: 4022-4026, 1979.

48. Carp H., Janoff A.
In vitro suppression of serum elastase inhibitor capacity by reactive oxygen species generated by phagocytosing polymorhonuclear leucocytes.
J. Clin. Invest. 63: 793-797, 1979.

49. Skosey J.L., Chow D.C.
Inactivation of serum elastase inhibitory capacity by products of stimulated neutrophils: protection by gold salts and D-penicillamine.
in Advances in Inflammation Research, Vol. 3
Ed. M. Ziff, G.P. Velo, S. Gorini.
Raven Press, New York, 1979, pp 95-112.

50. Svingen B.A., O'Neal F.O., Aust S.D.
The role of superoxide and singlet oxygen in lipid peroxidation.
Photochem. Photobiol. 28: 803-809, 1978.

51. Whiting R.F., Wei L., Stich H.F.
Chromosome-damaging activity of ferritin and its relation to chelation and reduction of iron.
Cancer Res. 41: 1628-1636, 1981.

52. Wong S.F., Halliwell B., Richmond R., Showroneck W.R.
The role of superoxide and hydroxyl radicals in the degradation of hyaluronic acid induced by metal ions and by ascorbic acid.
J. Inorg. Biochem. 14: 127-134, 1981.

53. Pigman W., Rizvi S., Holley H.L.
Depolymerization of hyaluronic acid by the ORD reaction.
Arthritis Rheum. 4: 240-252, 1961.

54. Matsumura G., Pigman W.
Catalytic role of copper and iron ions in the depolymerization of hyaluronic acid by ascorbic acid.
Arch. Biochem. Biophys. 110: 526-533, 1965.

55. Matsumura G., Herp A., Pigman W.
Depolymerization of hyaluronic acid by autoxidants and radiations.
Radiat. Res. 28: 735-752, 1966.

56. Ambruso D.R., Johnston R.B., Jnr.
Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system.
J. Clin. Invest. 67: 352-360, 1981.

57. Muirden K.D.
Ferritin in synovial cells in patients with rheumatoid arthritis.
Ann. Rheum. Dis. 25: 387-401, 1966.

58. Ogilvie-Harris D.J., Fornasier V.L.
Synovial iron deposition in osteoarthritis and rheumatoid arthritis.
J. Rheumatol. 7: 30-36, 1980.

59. Wright J.R., Colby H.D., Miles P.R.
Cytosolic factors which affect microsomal lipid peroxidation in lung and liver.
Arch. Biochem. Biophys. 206: 296-304, 1981.

60. Blake D.R., Hall N.D., Bacon A., Dieppe P.A., Halliwell B., Gutteridge J.M.C.
The importance of iron in rheumatoid disease.
Lancet II: 1142-1144, 1982.

61. McCord J.M.
Free radicals and inflammation:
Protection of synovial fluid by superoxide dismutase.
Science 185: 529-531, 1974.

62. Halliwell B.
Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts: Its role in degradation of hyaluronic acid by a superoxide generating system.
FEBS Lett 96: 238-242, 1978.

63. Kofoed J.A., Barcelo A.C.
The synovial fluid hyaluronic acid in rheumatoid arthritis.
Experientia 34: 1545-1546, 1978.

64. Greenwald R.A., Moy W.W.
Inhibition of collagen gelation by action of the superoxide radical.
Arthritis Rheum. 22: 251-259, 1979.

65. Miller D.R., Kaplan H.G.
Decreased NBT dye reduction in the phagocytes of patients receiving prednisolone.
Paediatrics 45: 861-865, 1970.

66. Lehmeyer J.E., Johnston R.B.
Effect of anti-inflammatory drugs and agents that elevate intracellular cyclic AMP on the release of toxic oxygen metabolites by phagocytes: Studies in a model of tissue bound IgG.
Clin. Immunol. Immunopath. 9: 482-490, 1978.

67. Simchowitz L., Mehta J., Spilberg I.
Chemotactic factor-induced generation of superoxide radicals by human neutrophils: Effect of metabolic inhibitors and anti-inflammatory drugs.
Arthritis Rheum. 22: 755-63, 1979.

68. Levine P.H., Hardin J.C., Scoon K.L., Krinsky N.I.
Effect of corticosteroids on the production of superoxide and hydrogen peroxide and the apearance of chemiluminescence by phagocytosing PMNL.
Inflammation 5: 19-27, 1981.

69. Herzer P., Lemmel E.M.
Inhibition of granulocyte function by prednisone, and non-steroid anti-inflammatory drugs. Quantitative evaluation with NBT test and its correlation with phagocytosis.
Immunobiol. 157: 78-88, 1980.

70. Van Dyke K., Van Dyke C., Udeinya J., Brister C., Wilson M.
A new screening system for non-steroidal anti-inflammatory drugs based upon inhibition of chemiluminescence produced from human cells (granulocytes).
Clin. Chem. 25: 1655-1661, 1979.

71. Puig-Parellada P., Planas J.M.
Synovial fluid degradation induced by free radicals. In vitro action of several free radical scavengers and anti-inflammatory drugs.
Biochem. Pharmacol. 27: 535-537, 1978.

72. Cham B.E., Ross-Lee L.M., Bochner F., Imhoff D.
Measurement and pharmacokinetics of acetylsalicylic acid by a novel high performance liquid chromatographic assay.
Therapeutic Drug Monitoring 2: 365-372, 1980.

73. Grunsberg M., Bochner F., Graham G.G., Imhoff D., Parsons G., Cham B.E.
The disposition of and clinical response to salicylates in patients with rheumatoid disease.
Clin. Pharmacol. Ther. (in press).

74. Bochner F., Graham G.G., Cham B.E., Haavisto T.M.
Salicylate metabolite kinetics after several salicylates.
Clin. Pharmacol. Ther. 30: 266-275, 1981.

75. Grinstead R.R.
Oxidation of salicylate by a model peroxidase catalyst iron-ethylenediaminetetraacetato-iron(III) acid.
J. Amer. Chem. Soc. 82: 3464-3471, 1960.

76. Halliwell B.
Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates.
FEBS Lett. 92: 321-326, 1978.

77. Cham B.E., Johns D., Bochner F., Imhoff D., Rowland M.
Simultaneous liquid-chromatographic quantitation of salicylic acid, salicyluric acid, and gentisic acid in plasma.
Clin. Chem. 25: 1420-1425, 1979.

78. Whitehouse M.W., Betts W.H., Cleland L.G., Vernon-Roberts B.
Anti-oxidant properties of dihydroxybenzoates, 5-aminosalicylate and 4-aminophenazone; potential biotransformation products of salicylate, sulphasalazine and aminodpyrine.
(submitted for publication).

79. Simon R.H., Scoggin C.H., Paterson D.
Hydrogen peroxide causes fatal injury to human fibroblasts exposed to oxygen radicals.
J. Biol. Chem. 256: 7181-7186, 1981.

80. Nathan C., Silverstein S.C., Brukner L.H., Cohn Z.A.
Extracellular cytolysis by activated macrophages and granulocyte II: Hydrogen peroxide as a mediator of cytotoxicity.
J. Exp. Med. 149: 100-113, 1979.

81. Weiss S.J., Young J., LoBuglio A.F., Slivka A.
Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endophelial cells.
J. Clin. Invest. 68: 714-721, 1981.

82. Simon R.H., Scoggin C.H., Patterson D.
Hydrogen peroxide causes fatal injury to human fibroblasts exposed to oxygen radicals.
J. Biol. Chem. 256: 718-7186, 1981.

83. Brawn K., Fridovich I.
DNA strand scission by enzymatically generated oxygen radicals.
Arch. Biochem. Biophys. 206: 414-419, 1981.

84. Mullinax F., Mullinax G.L.
Tyrosine-tyrosine crosslinks in IgG.
Generation by peroxidation and occurrence in RA synovial fluids.
Arthritis Rheum. 26: S16, 1983.

85. Jasin H.E.
Generation of IgG aggragates by the myeloperoxidase-hydrogen peroxide system.
J. Immunol. 130: 1918-1923, 1983.

86. Bills T.K., Smith J.B., Silver M.J.
Selective release of arachidonic acid from phospholipids of human platelets in response to thrombin.
J. Clin. Invest. 60: 1-6, 1977.

87. Hemler M.E., Smith W.L., Lands W.E.M.
Purification of the cyclo-oxygenase that forms prostaglandins.
J. Biol. Chem. 251: 5575-5579, 1976.

88. Miyamoto T., Ogino N., Yamamoto S., Hayaishi O.
Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes.
J. Biol. Chem. 251: 2629-2636, 1976.

89. Van Der Ouderaa F.J., Buytenhek M., Nugteren D.H., Vand Dorp D.A.
Purification and characterisation of prostaglandin endoperoxide synthetase from sheep vesicular glands.
Biochim. Biophys. Acta: 487, 315-331, 1977.

90. Smith W.L., Lands W.E.M.
Oxygenation of unsaturated fatty acids by soybean lipoxygenase.
J. Biol. Chem. 247: 1038-1047, 1972.

91. Smith W.L., Lands W.E.M.
Oxygenation of polyunsaturated fatty acids during prostaglandin biosynthesis by sheep vesicular gland.
Biochem. 11: 3276-3285, 1972.

92. Cook H.W., Lands W.E.M.
Mechanism for supression of cellular biosynthesis of prostaglandins.
Nature 260: 630-632, 1976.

93. Robinson D.R., Tashjian A.H., Levine L.
Prostaglandin-stiumlated bone resorption by rheumatoid synovia. A possible mechanism for bone destruction in rheumatoid arthritis.
J. Clin. Invest. 1181-88, 1975.

94. Norstrom A.
Effects of endocervical administration of prostaglandin E_2 on cervical dilatation and connective tissue biosynthesis in the first trimester of pregnancy.
in Advances in Prostaglandin, Thromboxane and Leukotriene Research, Vol 12.
Ed. B. Samuelsson, R. Paoletti, P. Ramwell.
Raven Press, New York, 1983, pp 461-464.

95. Tokunga M., Ohuchi K., Yoshizawa S., Tsurufuji S., Rikimaru A., Wakamatsu E.
Change of prostaglandin E level in joint fluids after treatment with flurbiprofen in patients with rheumatoid arthritis and osteoarthritis.
Ann. Rheum. Dis. 40: 462-465, 1981.

96. Kunkel S.L., Kaercher K., Plewa M., Fantone J.C., Ward P.A.
Production of cyclo-oxygenase products and superoxide anion by macrophages in response to chemotactic factors.
Prostaglandins 24: 789-799, 1982

97. Moncada S., Vane J.R.
The role of prostaglandins in platelet vessel wall interactions.
in Pathobiology of the Endothelial Cell.
Ed. H.L. Nossel, H.J. Vogel.
Academic Press, New York, 1982, pp 253-286.

98. Borgeat P., Hamburg M., Samuelsson B.
Transformation of arachidonic acid and homo-gamma-linolenic acid by rabbit polymorphonuclear leucocytes: monohydroxy acids from novel lipoxygenases.
J. Biol. Chem. 251: 7816-7819, 1976.

99. Smith W.L., Lands W.E.M.
Oxygenation of unsaturated fatty acids by soya bean lipoxygenase.
J. Biol. Chem. 247: 1038-1047, 1972.

100. Ford-Hutchinson A.W., Bray M.A., Doig M.V., Shipley M.E., Smith M.J.H.
Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leucocytes.
Nature 286: 265-265, 1980.

101. Sirois P., Borgeat P.
From slow-reacting substance of anaphylaxis (SRS-A) to leukotriene D_4 (LTD_4).
Int. J. Immunopharmac. 2, 281-93, 1980.

102. Williams T.J., Jose P.J., Wedmore C.V., Peck M.J., Forrest M.J.
Mechanisms underlying inflammatory oedema: The importance of synergism between prostaglandins, leukotrienes, and complement-derived peptides.
in Advances in Prostaglandin, Thromboxane and in Leukotriene Research. Vol. 11.
Ed. B. Samuelsson, R. Paoletti, P. Ramwell.
Raven Press, New York, 1983, pp 33-38.

103. Flower R.J., Blackwell G.J.
Anti-inflammatory steroids induce biosynthesis of a phopholipase A_2 inhibitor which prevents prostaglandin generation.
Nature 278: 456-459, 1979.

104. Hirata F, Schiffmann E, Venkatasubranian K, Salomon D., Axelrod J.
A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids.
Proc. Natl. Acad. Sci. U.S.A. 77: 2533-2536, 1980.

105. Hsueh W., Desai V, Gonzalez-Czussi F., Lamb R., Chu A.
Two phospholipase pools for prostaglandin synthesis in macrophages.
Nature 290: 710-713, 1981.

106. Feldman D.
The role of hormone receptors in the action of adrenal steroids.
Ann. Rev. Med. 26: 83-90, 1975.

107. Fahey J.V., Guyre P.M., Munck A.
Mechanisms of antiinflammatory actions of glucocorticoids.
in Advances in Inflammation Research, Vol 2.
Ed. G Weissmann, Raven Press, New York, 1981, pp 21-51.

108. Bell, R.L., Kennerly D.A., Stanford N., Majerus P.W.
Diglyceride lipase: A pathway for arachidonate release from human platelets.
Proc. Natl. Acad. Sci. U.S.A. 76: 3238-3241, 1979.

109. Kennerly D.A., Sullivan T.J., Sylwester P., Parker C.W.
Diacylglycerol metabolism in mast cells: A potential role in membrane fusion and arachidonic acid release.
J. Exp. Med. 150: 1039-1044, 1979.

110. Higgs G.A., Eakins K.E., Mugridge K.G., Moncada S., Vane J.R.
The effects of non-steroidal anti-inflammatory drugs on leukocyte migration in carrageenan-induced paw oedema.
Europ. J. Pharmacol. 66: 81-86, 1980.

111. Marklund S.L., Holme E., Hellner L.
Superoxide dismutase in extracellular fluids.
Clin. Chim. Acta 126: 41-51, 1982.

112. Oyanagui Y.
Participation of superoxide anions at the prostaglandin phase of carrageenan foot-oedema.
Biochem. Pharmacol. 25: 1465-1472, 1976.

113. McCord J.M., Wong K.
Phagocyte-produced free radicals: roles in cytotoxicity and inflammation.
in Oxygen Free Radicals And Tissue Damage.
Ciba Foundation Symposium 65 (new series),
Excerpta Medica, Amsterdam 1979, pp 343-351.

114. Lund-Oleson K., Menander K.B.
Orgotein: a new anti-inflammatory metalloprotein drug: preliminary evaluation of clinical efficacy and safety in degenerative joint disease.
Current Therap. Res. 16: 706-717, 1974.

115. Lund-Olesen K.
Superoxide dismutase therapy in degenerative joint disease
in Pathology of Oxygen.
Ed. A.P. Autor.
Academic Press, New York, 1982, pp. 339-353.

116. Goebel K.M., Storck U., Neurath F.
Intrasynovial orgotein therapy in rheumatoid arthritis.
Lancet I : 1015-1017, 1981.

117. Marberger M.D., Bartsch G., Huber W., Menander K.B., Schulte T.L.
Orgotein : a new drug for the treatment of radiation induced cystitis.
Current Therap. Res. 18: 466-475, 1975.

118. Menander-Huber K.B., Edsmyr F., Huber W.
Orgotein (Superoxide Dismutase): a drug for the amelioration of radiation cystitis.
Urological Res. 6: 255-257, 1978.

119. Carson S., Vogin E.E., Huber W., Schulte T.L.
Safety Tests of Orgotein, an anti-inflammatory protein.
Toxicol. Appl. Pharmacol. 26: 184-202, 1973.

120. Remy M.H., Poznansky M.J.
Immunogenicity and antigenicity of soluble cross-linked enzyme/albumin polmers.
Lancet II, 68-70, 1978.

121. Bagham A.D.
Properties and uses of lipid vesicles.
Ann. N.Y. Acad. Sci. 308: 2-6, 1978.

122. Mason J.T., Huang C.
Hydrodynamic analysis of egg phophatidyl choline vesicles.
Ann. N.Y. Acad. Sci. 308: 29-48, 1978.

123. Gregoriadis G.
The carrier potential of liposomes in biology and medicine.
N. Engl. J. Med. 295: 704-710, 765-770, 1976.

124. Juliano, R.L., Stamp. D.
Pharmacokinetics of liposome encapsulated anti-tumour drugs.
Studies with vinblastine, actinomycin D, cytosine arabinoside, and daunomycin.
Biochem. Pharmacol. 27: 21-28, 1978.

125. Juliano R.L., Stamp, D.
The effect of particle size and charge on the clearance rates of liposomes and liposome encapsulated drugs.
Biochem. Biophys. Res. Commun. 63: 651-658, 1975.

126. Jonah M.M., Cerny C.A., Rahman Y.E.
Tissue distribution of EDTA encapsulated within liposomes of varying surface properties.
Biochim. Biophys. Acta 401: 336-348, 1975.

127. Weissman G., Bloomgarden D., Kaplan R., Cohen C., Hoffstein S., Collins T., Gottlieb A., Nagle D.
A general method for the introduction of enzymes by means of immunoglobulin coated liposomes into lysosomes deficient cells.
Proc. Nat. Acad. Sci. U.S.A. 72: 77-92, 1975.

128. Black C.D.V., Watson G.J., Ward R.J.
The use of pentostam liposomes in the chemotherapy of experimental leishmaniasis.
Trans. Royal. Soc. Trop. Med. Hyg. 71: 550-552, 1977.

129. New R.R.C., Chance M.L., Thomas S.C., Peters W.
Anti-leishmanial activity of antimonials entrapped in liposomes.
Nature 272: 55-56, 1978.

130. Alving C.R., Steck E.A., Hanson W.L., Loizeaux P.S., Chapman W.L.Jr., Waits V.B.
Improved therapy of experimental leishmaniasis by use of a liposome encapsulated antimonial drug.
Life Sci. 22: 1021-1026, 1978.

131. Shaw I.H., Knight C.G., Dingle J.T.
Liposome retention of a modified anti-inflammatory steroid.
Biochem. J. 158: 473-476, 1976.

132. Dingle J.T., Gordon J.C., Hazelman B.L., Knight C.G., Thomas D.P., Phillips N.C., Shaw I.H., Glides F.J.T., Oliver J.E., Jones G., Turner E.A., Lowe J.S.
Novel treatment for joint inflammation.
Nature 271: 327-373, 1978.

133. Swingle K.F.
Evaluation of anti-inflammatory activity.
In Anti-inflammatory Agents Vol II, Chemistry and Pharmacology.
Ed. R.A. Scherrer, M.W. Whitehouse.
New York, Academic Press Inc., 1974, p 33-122.

134. Clarke A.K., Vernon-Roberts B., Currey H.L.F.
Assessment of anti-inflammatory drugs in the rat using subcutaneous implants of polyurethane foam impregnated with dead tubercle bacilli.
Ann. Rheum. Dis. 34: 326-331, 1975.

135. Vinegar R, Traux J.F., Selph J.L., Voelker F.A.
Pathways of onset, development and decay of carrageenan pleurisy in the rat.
Fed. Proc. 41: 2588-2594, 1982.

136. Whitehouse M.W., Orr K.J., Beck F.W.J., Pearson C.M.
Freund's Adjuvants: relationship of arthritogenicity and adjuvanticity to vehicle composition.
Immunology 27: 311-330, 1974.

137. Muir V.Y., Dumonde D.C.
Different strains of rat develop different forms of adjuvant disease.
Ann. Rheum. Dis. 41: 538-543, 1982.

138. Lowther D.A., Gillard G.C.
Carrageenin-induced arthritis II: effect of intraarticular injection of carrageenin on the synthesis of proteoglycans in articular cartilage.
Arthritis Rheum. 19: 918-922, 1976.

139. Dumonde D.C., Glynn L.E.
The production of arthritis in rabbits by an immunological reaction to fibrin.
Br. J. Exper. Path. 43: 373-383, 1962.

140. Consden R., Doble A., Glynn L.E., Nind A.P.
Production of a chronic arthritis with ovalalbumin, its retention in rabbit knee joints.
Ann. Rheum. Dis. 30: 307-315, 1971.

141. Ferrante A. James D.W., Betts W.H., Cleland L.G.
Rapid single step method for purification of polymorphonuclear leucocytes from blood of patients with rheumatoid arthritis.
Clin. Exp. Immunol. 47: 749-752, 1982.

142. Cheson B.D., Christensen R.L., Sperling R., Kohler B.E., Babior B.M.
The origin of the chemiluminescence of phagocytosing granulocytes.
J. Clin. Invest. 58: 789-796, 1976.

143. Webb L.S., Keele B.B. Jr., Johnston R.B. Jr.
Inhibition of phagocytosis-associated chemiluminescence by superoxide dismutase.
Infect. Immun. 9: 1051-1056, 1974.

144. Boveris A., Cadenas E., Chance B.
Low level chemiluminescence of the lipoxygenase reaction.
Photobiochem. Photobiophys. 1: 175-182, 1980.

145. Cheung K., Archibald A.C., Robinson M.F.
The origin of chemiluminescence produced by neutrophils stimulated by opsonized zymosan.
J. Immunol. 130: 2324-2329, 1983.

146. James D.W., Betts W.H., Cleland L.G.
Chemiluminescence of polymorphonuclear leukocytes from rheumatoid joints.
J. Rheumatol. 10: 184-189, 1983.

147. Boxer L.A., Yoder M., Bonsib S., Schmidt M., Ho R., Jersild R., Braehner R.L.
Effects of a chemotactic factor, N-formylmethionyl peptide, on adherence, superoxide anion generation, phagocytosis, and microtubular assembly of human polymorphonuclear leukocytes.
J. Lab. Clin. Med. 93: 506-514, 1979.

148. English D., Roloff J.S., Lukens J.N.
Regulation of human polymorphonuclear leukocyte release by cellular response to chemotactic factors.
J. Immunol. 126: 165-171, 1981.

149. Fried R.
Metabolic role of xanthine oxidase as source of superoxide radicals and hydrogen peroxide.
in "Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase".
Ed. J.V. Bannister, H.O.A Hill, Developments in Biochemistry, Vol 11A, 1980, pp 65-75.

150. Cohen G., Sinet P.M.
Fenton`s reagent - once again revisited.
in Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase".
Ed. J.V. Bannister, H.O.A Hill, Developments in Biochemistry, Vol 11A, 1980, pp 27-37.

151. Schmut O., Hofmann H.
Studies on the generation of hydrogen peroxide during some non-enzymic reactions changing the hyaluronic acid molecule.
Biochim. Biophys. Acta 411: 231-235, 1975.

152. Spikes J.D., Swartz H.M.
International conference on singlet oxygen and related species in chemistry and biology. Review and general discussion.
Photochem. Photobiol. 28: 921-933, 1978.

153. Gutteridge J.M.C., Richmond R., Halliwell B.
Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine.
Biochem. J. 184: 469-472, 1979.

154. Buettner G.R., Oberley L.W.
Considerations in the spin trapping of superoxide and hydroxyl radical in aqueous systems using 5,5-dimethyl-1-pyrroline-1-oxide.
Biochem. Biophys. Res. Commun. 83: 69-74, 1978.

155. Niedermeier W., Creitz E.E., Holley H.L.
Trace metal composition of synovial fluid from patients with rheumatoid arthritis.
Arthritis Rheum. 5: 439-444, 1962.

156. Younes M., Weser U.
Superoxide dismutase activity of copper-penicillamine: possible involvement of Cu(I) stabilized sulphur radical.
Biochem. Biophys. Res. Commun. 78: 1247-1253, 1977.

157. Lengfelder E., Fuchs C., Younes M., Weser U.
Functional aspects of the superoxide dismutase action of Cu-penicillamine.
Biochim. Biophys. Acta 567: 492-502, 1979.

158. Dorfman L.M., Adams G.E.
Reactivity of the hydroxyl radical in aqueous solutions
NSRDS-NBS No 46, US Department of Commerce, National Bureau of Standards, Bethesda, 1973.

159. De Duve C., DeBarsy T., Poole B., Trouet A., Tulkens P., Van Hoof F.
Commentary - lysosomotropic agents.
Biochem. Pharmacol. 23: 2495-2531, 1974.

160. Penneys N.S., McCreary S., Gottlieb N.L.
Intracellular distribution of radiogold.
Localization to large granule membranes.
Arthritis Rheum. 19: 927-932, 1976.

161. Goldstein I.M., Cerqueira M., Lind S., Kaplan H.B.
Evidence that the superoxide-generating system of human leucocytes is associated with the cell surface.
J. Clin. Invest. 59: 249-254, 1977.

162. Root R.K., Metcalf J.

H_2O_2 release from human granulocytes during phagocytosis: relationship to superoxide anion formation and cellular metabolism of H_2O_2: studies with normal and cytochalasin B treated cells.

J. Clin. Invest. 60: 1266-1279, 1977.

163. Tauber A.I., Babior B.M.

O_2^- and host defense: the production and fate of O_2^- in neutophils.

Photochem. Photobiol. 28: 701-709, 1978.

164. Stokey L.

Ferrozine - A new spectrophotometric reagent for iron.

Anal. Chem. 42: 779-781, 1970.

165. Johnson R.S., Niedermeier W., Bobo P.

The pseudoplastic behaviour of aqueous solutions of hyaluronic acid.

Biorheology 7: 177-187, 1971.

166. Hofmann H., Schmut O.
The inability of superoxide dismutase to inhibit the depolymerization of hyaluronic acid by ferrous ions and ascorbate.
Arch. Klin. Ophthalmol. 214: 181-185, 1980.

167. Betts W.H., Cleland L.G., Whitehouse M.W.
In vitro studies of anti-inflammatory copper complexes: some difficulties in their chemical interpretation.
in Inflammatory Diseases and Copper.
Ed. J.R.J. Sorenson, Humana Press Inc, Clifton, NJ, 1982, pp 553-564.

168. Rosenthal R.K., Bayles T.B., Fremont-Smith K.
Simultaneous salicylate concentrations in synovial fluid and plasma in rheumatoid arthritis.
Arthritis Rheum. 7: 103-109, 1965.

169. Soren A.
Kinetics of salicylates in blood and joint fluid.
J. Clin. Pharmacol. 15: 173-177, 1975.

170. Dugan L.R.
Natural antoxidants.
in Autoxidation in Food and Biological Systems.
Ed. M.G. Simic, M. Karel, Plenum Press, New York,
1980, pp 261-282.

171. Bradford M.M.
A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.
Anal. Biochem. 72: 248-254, 1976.

172. Ouchterlony O., Nilsson L.A.
Immunodiffusion and immunoelectrophoresis.
in Handbook of Experimental Immunology, 2nd Edition.
Ed. D.M. Weir, Blackwell Scientific Publications
Oxford 1973, pp 19.1-19.3.

173. Wong K., Cleland L.G., Poznansky M.J.
Enhanced anti-inflammatory effect and reduced immunogenicity of bovine liver superoxide dismutase by conjugation with homologous albumin.
Agents Actions 10: 231-239, 1980.

174. Poznansky M.
Perspectives of soluble cross-linked enzyme/albumin polymers
in Biomedical Applications of Immobilized Enzymes and Proteins, Vol. 2.
Ed. T.M.S. Chang, Plenum Press, New York, 1977, pp 341-345.

175. Petkau A.
Radiation protection by superoxide dismutase.
Photochem. Photobiol. 28: 765-774, 1978.

176. McLennan G., Autor A.
Effect of intraperitoneally administered superoxide dismutase on pulmonary damage resulting from hyperoxia.
in Pathology of Oxygen.
Ed. A. Autor, Academic Press, New York, 1982, pp 85-96.

177. Gardner T.J., Stewart J.R., Casale A.S., Downey J.M., Chambers D.E.
Reduction of myocardial ischaemic injury with oxygen-derived free radical scavengers.
Surgery 94: 423-427, 1983.

178. Granger D.N., Rutili G., McCord J.M.
Superoxide radicals in feline intestinal ischaemia.
Gastroenterology 81: 22-29, 1981.

179. Betts W.H., Cleland L.G., Gee D.J., Whitehouse M.W.
Effects of D-penicillamine on a model of
oxygen-derived free radical mediated tissue damage.
Agents Actions 14: 283-290, 1984.

180. Del Maestro R.F., Bjork J., Arfors K.-E.
Increase in microvascular permeability induced by
enzymatically generated free radicals.
Microvascular Res. 22: 239-254, 1981.

181. Young C.M.A.
The revascularization of pedicle skin flaps in pigs.
A Functional and Morphological Study.
Plastic and Reconst. Surg. 70: 455-464, 1982.