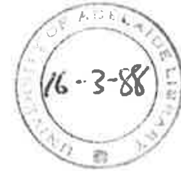
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OXYGEN RADICALS AND TISSUE DAMAGE: THE EFFECT OF OXYGEN RADICALS ON THE STRUCTURE AND SYNTHESIS OF HYALURONIC ACID

by

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A Thesis Submitted for the Degree of Doctor of Philosophy in The University of Adelaide.

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December, 1986.

Awarded : June 29, 1987.

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DECLARATION

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

The work described herein has been the subject of the following publications:-

McNEIL, J.D., WIEBKIN, O.W., BETTS, W.H. and CLELAND, L.G. (1985) Depolymerization products of hyaluronic acid after exposure to oxygen derived free radicals. Annals of the Rheumatic Diseases 44, 780-789.

McNEIL, J.D., WIEBKIN, O.W., CLELAND, L.G. and VERNON-ROBERTS, B. (1986). The generation of reducing ends by exposure of hyaluronic acid to oxygen derived free radicals. Agents and Actions Supplements 18, 95-101.

and abstracts:-

McNEIL, J.D., WIEBKIN, O.W., CLELAND, L.G. and BETTS, W.H. (1983) Analysis of hyaluronic acid degradation products after exposure to an oxygen derived free radical flux. Connective Tissue Research 11, 258.

McNEIL, J.D., WIEBKIN, O.W., CLELAND, L.G. and BETTS, W.H. (1983) Mechanisms of hyaluronic acid degradation by oxygen derived free radicals. Aust. N. Z. J. Med. 13, 211.

McNEIL, J.D., CLELAND, L.G. and BETTS, W.H. (1983) The cytotoxicity of oxygen derived free radicals. Aust. N. Z. J. Med. 13, 225.

McNEIL, J.D., WIEBKIN, O.W., and CLELAND, L.G. (1984) Analysis of the molecular weights of hyaluronic acid from inflamed and non-inflamed synovial fluids. Aust. N. Z. J. Med. 14 (suppl. 1), 343.

McNEIL, J.D., WIEBKIN, O.W., and CLELAND, L.G. (1985) The cytotoxic effect of oxygen derived free radicals on bovine synovial cells in culture. Aust. N. Z. J. Med. 14 (suppl. 1), 357.

Signed:

J. D. McNeil

December, 1986

ACKNOWLEDGMENTS

The work described in this thesis was supported by grants from the National Health and Medical Research Council of Australia and from an E. R. Dawes fellowship from the Royal Adelaide Hospital.

I hereby wish to thank my supervisors Dr. Ole Wiebkin, Dr. Leslie Cleland and Professor Barrie Vernon-Roberts. Dr. Ole Wiebkin has helped me with day to day teaching, guidance, enthusiasm and friendship. Dr. Leslie Cleland has influenced me towards a career involving laboratory research and has provided support, valuable counsel and friendship. Professor Barrie Vernon-Roberts has provided overall supervision and advice and has provided all the facilities of his department for this work. I also wish to thank Professor J. L. Skosey who provided valuable supervision and editorial assistance during the writing up phase.

Other people who have helped me are Dr. W. Henry Betts who provided teaching help and assistance with the chemistry of oxygen radicals, Dr. Stephen Milazzo who provided valuable interest, support and clinical teaching, Mrs. S. C. Wiebkin who provided assistance in technical matters, Mrs. Genevieve LaPinska who provided willing help in typing this manuscript, Mr. David Haynes for help with the macrophage Fc receptor binding assay, and Mr. Dale Caville who contributed of his photographic talents. I have also had valuable discussions with Dr. Michael Whitehouse for which I am grateful.

I am also indebted to the South Australian Meat Corporation for their gift of bovine hocks and Pharmacia, South Seas, for their gift of Healon.

I would like to make a special tribute to my late father and my mother, both of whom have provided me, over many years, with an inexhaustible supply of encouragement and support. Finally, I would like to thank my wife, Jane, who has patiently endured so much so that I could undertake this project.

SUMMARY

In order to examine the role of oxygen radicals in inflammatory joint damage, a study of oxygen radical induced hyaluronic acid depolymerization was undertaken. Hyaluronic acid was used because it is a major extracellular component of synovial fluid and the synovial membrane and a prime secretory product of synovial cells. Also it is known to be sensitive to oxygen radical induced depolymerization and is altered in inflammatory joint disease.

The methods of approach used were:

a. Analysis of the products of oxygen radical induced hyaluronic acid depolymeriztion *in vitro* to determine change in molecular weight, generation of reducing ends and, to a limited extent, change in biological properties.

b. Synovial fluid hyaluronic acid from inflamed synovial fluids was isolated and the spectrum of molecular weights identified in order to make comparisons with those obtained *in vitro*.

c. The effect of an oxygen radical flux on synovial cells in culture was determined and the effect on hyaluronic acid produced by these cells was examined.

Analysis by gel chromatography of oxygen radical induced hyaluronic acid depolymerization products revealed polydispersity in size. The smallest molecules detected had a molecular weight of 10^4, which was not reduced further by exposure to a second oxygen radical flux. Consistently a relatively rapid transition was seen from large to small material suggesting an ordered element to the breakdown process.

Analysis by analytical ultracentrifugation confirmed the decrease in size of hyaluronic acid due to exposure to oxygen radicals; however, this procedure revealed that those samples of

lowest viscosity did not exhibit the lowest sedimentation values, possibly reflecting oxygen radical induced repolymerization.

Oxygen radical induced hyaluronic acid depolymerization is accompanied by the generation of reducing ends as measured by both colorimetric assays and cyanide labeling. There is, however, no detectable increase in Morgan-Elson reactivity thus implying that the reducing ends released are present on the D-glucuronic acid component of the hyaluronic acid product. This is therefore evidence that it is the β1-3 glycosidic linkage that is susceptible to cleavage by oxygen radicals.

Fractionation of synovial fluid hyaluronic acid from patients with both osteoarthritis and inflammatory arthropathies revealed uronic acid containing material that was retarded on Sepharose 4B-CL chromatography in contrast to non-arthritic cadaver subjects in which case all synovial fluid hyaluronic acid was excluded on Sepharose 4B-CL. In the patient group no material was present below a molecular weight of 2×10^4 and no correlations could be made between the amount of uronate retarded and clinical indices of inflammatory activity. Extracted cadaver synovial fluid hyaluronic acid was susceptible to oxygen radical induced depolymerization *in vitro*.

Oxygen radical exposed hyaluronic acid had the same effect on Fc receptor binding as non-oxygen radical exposed hyaluronic acid of the same molecular weight. Thus, at least in this context, no alteration in the properties of hyaluronic acid due to oxygen radical exposure was demonstrable beyond that due to decreasing molecular weight.

Application of an oxygen radical flux, produced by the action of xanthine oxidase on hypoxanthine, is cytotoxic to cultured bovine

synovial cells. Administration of catalase, but not superoxide dismutase, is protective thus indicating H_2O_2 is the agent necessary for toxicity. Sublethal oxygen radical fluxes will result in the cessation of hyaluronic acid synthesis before the cessation of synthesis of other glucosamine-containing products. No low molecular weight hyaluronic acid products could be detected in the supernatants of oxygen radical exposed cells. These findings imply that the lowered molecular weight hyaluronic acid seen in inflammatory joint fluids is due to extracellular depolymerization.

These results provide further evidence for the role of oxygen radicals as agents of hyaluronic acid depolymerization *in vivo*.

GLOSSARY

ADP -	adenosine diphosphate
ATP -	adenosine triphosphate
BPS -	bathophenanthroline sulphonate
Con A -	concanavalin A
CTAB -	cetyl trimethyl ammonium bromide
C5b -	the fifth component of complement (activated)
DETAPAC -	diethylenetriamine pentaacetic acid
EDTA -	ethylaminediamine tetraacetic acid
Fe (11) -	ferrous ion
Fe (111) -	ferric ion
FMLP -	formyl methionyl leucyl phenylalanine
GAG -	glycosaminoglycan
GSH -	reduced glutathione
GSSG -	oxidized glutathione
HA -	hyaluronic acid
HO_2 -	peroxyl radical
Kav -	partition coefficient described by the formula:- $Kav = \frac{Ve-Vo}{Vt - Vo}$
M_{sD}-	molecular weight measured by sedimentation-diffusion
NAD -	nicotine adenine dinucleotide
NADPH -	nicotine adenine dinucleotide phosphate
Nr -	relative viscosity
Nsp -	specific viscosity
ORD -	oxygen radical depolymerization
O_2^- -	superoxide anion
OH· -	hydroxyl radical
OCL^--	hypochlorous ion
ΔO_2-	delta singlet oxygen
ΣO_2 -	sigma singlet oxygen
PHA -	phytohaemagglutinin
PMA -	phorbol myristate acetate
PPD -	purified protein derivative
RPMI -	tissue culture medium [IWAKATA and GRACE, 1964]
SOD -	superoxide dismutase
UDP-GlcA -	uridinedephospho-D-glucuronic acid
UDP-GlcNAc-	uridinedephospho-N-acetyglucosamine
UV -	ultraviolet
Ve -	elution volume
Vo-	void volume
Vt-	total volume
XO/HX -	xanthine oxidase/hypoxanthine
XO/X -	xanthine oxidase/xanthine

CHAPTER I

INTRODUCTION AND REVIEW

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A. GENERAL INTRODUCTION

Diarthrodal joints are specialized connective tissue structures that have developed to allow mobility for an organism with a rigid skeleton. The surfaces of the articulating bones are covered with hyaline cartilage and the bones are united both by a fibrous capsule and by ligaments. In addition, overlying muscles and tendons contribute to the stability of the joint. The inner surface of the joint capsule is lined by the vascular synovial membrane, thus forming the joint space which is totally enclosed by synovial membrane and articular cartilage. The joint space contains a small amount of joint fluid which is broardly similar in composition to blood plasma to which has been added hyaluronic acid [McCARTY, 1985]. Hyaluronic acid is a secretory product of the synovial lining cell. It imparts to synovial fluid a distinctive high viscosity and contributes to the biological functions of synovial fluid.

Joint inflammation results in characteristic changes in all of the above-mentioned anatomical structures, leading to damage to the various components of the joint and ultimately to joint deformity and loss of joint function. Early inflammatory changes are seen in the synovial membrane and are very soon reflected in the synovial fluid, which becomes both increased in volume and is often much less viscous than normal fluid.

A wide variety of effector agents have been examined to determine the biochemical mechanisms involved in the loss of structural integrity of joint tissues. Those examined most closely have been enzyme systems, principally acid proteases and neutral proteases [BARRETT and SAKLATVALA, 1985]. It has become clear from these studies that within the inflammatory process there are a multitude of effector systems (including effectors, their precursors,

activators and antagonists) that can contribute to inflammatory joint destruction.

One such system that has come to light in the last 15 years involves the production and release of oxygen radicals by activated inflammatory cells. Although the existence of oxygen radicals in chemical systems has been appreciated for over 100 years, until recently they were thought to be too active to play a role in biological processes. It is now clear that stimulated phagocytes, in particular polymorphonuclear leukocytes, undergo a "respiratory burst" which is characterized by increased oxygen uptake, chemiluminescence, increased hexose monophosphate shunt activity and production of the superoxide anion. Superoxide anions dismutate either spontaneously or catalysed by the enzyme superoxide dismutase to form hydrogen peroxide. Superoxide anions may react with hydrogen peroxide to produce singlet oxygen and the hydroxyl radical. These radicals are secreted into the phagosome and are utilized in microbicidal killing. Hydrogen peroxide is a substrate for phagosomal myeloperoxidase which produces hypochlorite ions, which also have potent oxidant activity. Oxygen radicals also escape into the extracellular space where, in the absence of complete scavenging, adjacent host components may be exposed to their effects.

The intra-articular space of inflamed joints provides an important site to examine the effects of oxygen radicals for reasons outlined originally by McCORD [1974]. Firstly, levels of endogenous scavengers of oxygen radicals in synovial fluid are low. Superoxide dismutase, catalase and the glutathione/glutathione peroxidase system are predominantly intracellular and their levels in extracellular fluids in general, and synovial fluid in particular, are several orders of magnitude less than intracellular levels [McCORD, 1974; BLAKE *et al.*,

1981]. Secondly, hyaluronic acid has been shown to be susceptible to the effects of oxygen radicals in ways that parallel the changes seen in inflammatory joint disease, that is, lowered viscosity and a decrease in the average molecular weight.

In order to elucidate the role of oxygen radicals in inflammatory joint disease, I have chosen to examine in detail the effect of oxygen radicals on the structure, properties and cellular production of hyaluronic acid *in vitro*, and to determine whether any of these changes can be correlated with the changes found in inflamed synovial fluid hyaluronic acid. The results of these studies form the basis of this thesis.

B. OXYGEN RADICALS AND JOINT BIOLOGY

1. Introduction.

Oxygen has been present in significant quantities in the earth's atmosphere for an estimated 2.5 billion years, its appearance corresponds to the emergence of the first photosynthetic organisms [BERKNER and MARSHALL, 1964]. Since that time the atmospheric oxygen concentration has gradually increased and life forms have developed that are dependent on aerobic metabolism. Concurrently there has been an evolutionary pressure for these organisms to develop effective defenses against the toxic intermediates that are formed during the reduction of oxygen to water [FRANK and MASSARO, 1980]. Most of the oxygen consumed by respiring cells is accounted for by the mitochondrial cytochrome C oxidase system [ANTONINI *et al.*, 1970]. This system effects the tetravalent reduction of oxygen to water without the release of either superoxide or peroxide [FRIDOVICH, 1978]. However there are biological processes that produce superoxide anion, and effective cellular antioxidant defense mechanisms have evolved to protect the cell from these reduced

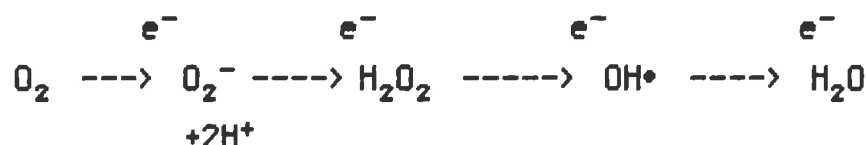
forms of oxygen [SIES and CADENAS, 1983]. Ironically, it was the elucidation of the function of one of these antioxidant enzymes, superoxide dismutase [McCORD and FRIDOVICH, 1968], that led to a wide appreciation of the role of partially reduced forms of oxygen (as oxygen radicals) in biology and their possible pathogenetic role in a number of disease processes.

2. Definitions and chemistry

A free radical may be defined as any species capable of independent existence that has one or more unpaired electrons [HALLIWELL and GUTTERIDGE, 1985]. In general, the presence of an unpaired electron accounts for the high reactivity of radicals. As stated, this definition includes the hydrogen atom and most transition metals. Radicals may be formed if a covalent bond, consisting of two shared electrons, is split such that one electron stays with each atom (homolytic fission). This may be considered an "initiation" reaction. Individual radicals may react with non-radicals with the formation of another radical species (propagation reactions) or two radicals may react together with the formation of a non-radical product (termination reaction).

The ground state oxygen molecule itself may be considered a di-radical since it has two unpaired electrons each located in a different π antibonding orbital. Both of these electrons have the same spin quantum number. Thus, if oxygen attempts to oxidize another atom or molecule by accepting a pair of electrons from it, both new electrons must be of parallel spin so as to enter the vacancies in the unpaired π orbitals [HALLIWELL and GUTTERIDGE, 1984a]. This restriction on oxidations of oxygen dictates that oxygen will tend to accept electrons one at a time, i.e., univalent

reduction, rather than to be directly reduced to water by tetravalent reduction.



$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow[+2H^+]{e^-} H_2O_2 \xrightarrow{e^-} OH\bullet \xrightarrow{e^-} H_2O$$

The univalent reduction of oxygen leads to the production of three major oxygen-centred radical intermediates which are discussed below:

a. Superoxide Anion

When oxygen accepts a single electron it is converted to the superoxide anion (O_2^-) or its protonated form, the hydroperoxyl radical ($HO_2\bullet$). In aqueous solution at a pH of 4.8 the two forms are present in a ratio of 1:1 and at physiological pH only 1% is present in the pronated form [FEE and VALENTINE, 1977]. Superoxide anion however disappears from aqueous solution via a dismutation reaction, which proceeds.

$$HO_2\bullet + O_2^- + H^+ \longrightarrow H_2O_2 + O_2$$

This reaction may occur spontaneously, in which case it will occur most rapidly at an acidic pH (due to the higher concentration of $HO_2\bullet$). It may also be catalyzed by the enzyme superoxide dismutase [McCORD and FRIDOVICH, 1968], which is active over a wide pH range.

b. Hydrogen Peroxide

Addition of two electrons to molecular oxygen will lead to the formation of the peroxide ion (O_2^{2-}). The pK_a of the reaction describing the equilibrium between O_2^{2-} and H_2O_2 is so high that at physiological pH virtually all is present as H_2O_2. Several enzymes, e.g., glucose oxidase, are capable of the direct divalent reduction of O_2 independent of the dismutation reaction [FRIDOVICH, 1976].

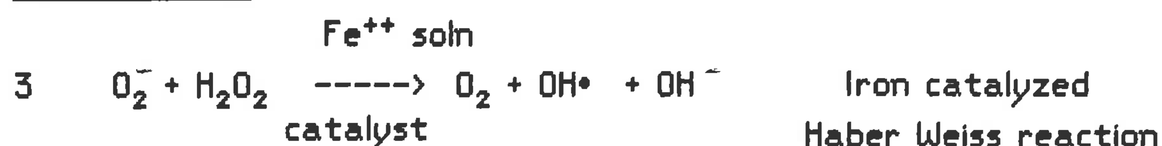
c. Hydroxyl Radical

A mechanism whereby a three electron reduction product of oxygen could be formed from H_2O_2 was first proposed by HABER and WEISS [1934]. However the rate constant for this reaction in aqueous solution has been shown to be virtually zero [HALLIWELL, 1981]. This is not the case however if traces of a transition metal are present, in which case a Fenton reaction is possible, i.e.,

1 $Fe(III) + O_2^- \longrightarrow Fe(II) + O_2$

2 $Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH\bullet + OH^-$ Fenton reaction

Nett Reaction



3 $O_2^- + H_2O_2 \xrightarrow[\text{catalyst}]{Fe^{++}\text{ soln}} O_2 + OH\bullet + OH^-$ Iron catalyzed Haber Weiss reaction

Since the sum of reactions 1 & 2 is the same as the Haber Weiss reaction this reaction is also known as the Iron-catalyzed Haber Weiss reaction. This reaction will be promoted if the iron is appropriately chelated, e.g., by EDTA [McCORD and DAY, 1978; BETTS and CLELAND, 1982]. On the other hand chelators with a very strong affinity for Fe(III) do not allow Fe to shuttle between Fe(III) and Fe(II) and therefore inhibit the reaction [GUTTERIDGE, RICHMOND and HALLIWELL, 1979]. Physiological chelators such as ATP or ADP can act as iron chelators and support the superoxide-driven hydroxyl radical production [FLOYD, 1983] at physiological iron concentrations in the absence of EDTA [FLITTER, ROWLEY and HALLIWELL, 1983]. The hydroxyl radical is extremely reactive and will react with any molecule within the immediate vicinity of its production in contrast to O_2^- and H_2O_2 which are relatively stable and can diffuse away from their site of production [ANBAR and NETA, 1967].

d. Singlet Oxygen

Singlet oxygen is a highly reactive form of oxygen that is formed when molecular oxygen absorbs sufficient energy to cause a shift of one of its two unpaired electrons of similar spin to an orbital position of higher energy. This is associated with the inversion of spin of one of the electrons [KASHA and BRABHAM, 1979]. If the excited electrons form a pair then it is designated $^1\Delta gO_2$ (therefore, by definition not a radical) and if the electrons are not in the same orbital $^3\Sigma gO_2$. Singlet oxygen has been reported to be produced as a result of the non-catalyzed dismutation reaction and during the Haber Weiss reaction [KELLOGG and FRIDOVICH, 1975]. In addition, it has been reported to be produced by the myeloperoxide-H_2O_2-halide system of neutrophils [ROSEN and KLEBANOFF, 1977], where it is believed to be produced by the interaction of the hypochlorite ion and H_2O_2.

3. Possible Sources of Oxygen Radicals within an Inflamed Joint

In addition to the general exposure to oxygen radicals that any cell existing in an oxygen containing environment must endure, (for reviews see; HALLIWELL and GUTTERIDGE, 1985; FRIDOVICH, 1978), cells and extra-cellular components of the inflamed joint are exposed to oxygen radicals as a result of inflammation. As a basis for dicussion of these events, it is appropriate to consider briefly some inflammatory mechanisms that will lead to joint inflammation.

In some situations the inciting agent is obvious, for example, a bacterium in the case of septic arthritis or an immune complex in association with a serum sickness reaction. More commonly, however, the nature of the inciting agent is unknown. Nevertheless it is likely that the train of events similar to that outlined by ZVAIFLER [1973] for rheumatoid arthritis is set in motion. He proposed that the

inciting antigen becomes localized in the articular cavity and stimulates the production of antibody. These combine in the synovial fluid or the synovial membrane and activate the complement pathway thus generating chemotactic agents, particularly C5b. Polymorphonuclear neutrophils will then accumulate, particularly within the synovial fluid, and along with type A synoviocytes, will ingest the immune complexes. At the time that this theory was first proposed it was believed that it was the hydrolytic enzymes that were released from lysosomes during the act of phagocytosis that accounted for the proliferative and destructive changes characteristic of rheumatoid arthritis [WEISSMANN, ZURIER and HOFFSTEIN, 1972]. It has now become apparent that oxygen radicals may also be released at this stage [WEISSMANN _et al_., 1979] and may contribute significantly in the genesis of these changes.

a. _The release of oxygen radicals from activated phagocytes_.

Phagocytes become activated on contact with particulate stimuli, such as immune complexes or bacteria that have been opsonized (i.e. coated with host-derived proteins) [ROOT and METCALF, 1977]. Several soluble factors may also lead to phagocyte activation. Activation involves the "switching on" of a number of metabolic processes, the end result being that the particle is engulfed into an organelle called a phagosome. Granules present in the cytoplasm of the phagocyte fuse with the phagosome and discharge their contents into it (degranulation) to effect bacterial killing and/or degradation of the ingested material. BABIOR, KIPNES and CARNUTTE [1973] were the first to show that included in the process of activation is the release of superoxide anion. This is formed as a component of the "respiratory burst" of the neutrophil which involves :

1) increased O_2 uptake which is independent of mitochondrial respiration [SBARRA and KARNOVSKY, 1959].

2) Increased glucose oxidation *via* the hexose monophosphate shunt with the resulting production of NADPH [IYER, ISLAM and QUASTEL, 1961].

3) Activation of an NADPH oxidase [BABIOR, 1978], which converts molecular oxygen to superoxide anion according to the reaction :

$$2\ NADPH + 2\ O_2 \dashrightarrow 2\ NADP^+ + 2\ O_2^- + H_2O$$

This enzyme is situated on the inner surface of the plasma membrane and accepts NADPH from the cell cytoplasm; it also accepts oxygen and releases superoxide anion from the outer surface of the plasma membrane [BABIOR et al., 1981]. This mechanism ensures that superoxide anion will be generated and deposited on the inside of the phagosome as the plasma membrane invaginates during the cellular ingestion of a particle. However it is clear that not all the superoxide produced in this way is contained by the phagosome [WEISSMANN et al., 1979], possibly because the enzyme is activated before the phagosome has been sealed, but also because enzyme on contiguous areas of membrane is also activated. Also, within the joint, it is clear that immune complexes may adhere to structural components, e.g., collagen, leading to a situation of "frustrated phagocytosis" whereby phagosomal contents, including superoxide anion, can escape freely into the extracellular milieu [ZVAIFLER, 1973]. We have recently obtained some evidence that this may occur with particulate stimuli in the presence of highly polymerized hyaluronic acid [McNEIL, CHOW and SKOSEY, 1986].

The ability of an organic system to be able to generate as highly reactive an agent as the hydroxyl radical was first inferred by BEAUCHAMP and FRIDOVICH [1970], who exposed the thioether

methional to the xanthine oxidase/xanthine oxygen radical generating system and detected ethylene. Since this could not be accomplished by the action of either superoxide anion or hydrogen peroxide alone, they postulated that hydroxyl radicals were produced. Hydroxyl radicals have been detected in suspensions of activated phagocytes, also by the production of ethylene from methional [TAUBER and BABIOR, 1977]. The most likely sequence of events is that superoxide anions produced by the phagocytes undergo dismutation to form H_2O_2 and, in the presence of iron or another transition metal as a catalyst, hydroxyl radicals are formed by the iron-catalyzed Haber Weiss reaction.

Evidence for the extracellular generation of this agent comes from the experiments of SALIN and McCORD [1975] who showed that the auto-inactivation of stimulated phagocytes can be averted by addition of superoxide dismutase, catalase or OH• scavengers to the suspension medium. This suggests that the components necessary for hydroxyl radical production are released into the extracellular milieu by activated phagocytes where they can mediate cytotoxic and other effects.

Chronic granulomatous disease which is due to a non-functional NADPH oxidase, provides insights into the importance of oxygen radicals in microbial killing. At least two inherited forms of chronic granulomatous disease exist. Biochemically, the two forms of the disease demonstrate an inability of neutrophils to effect a respiratory burst. This manifests clinically as persistent infections with several non-peroxide producing bacteria, typically staphlococcus aureus. Without the production of superoxide anions, hydrogen peroxide is not available to the phagocyte and both hydroxyl radical production and hypochlorous ion production via the myeloperoxidase

An abnormality in iron utilization has long been appreciated in active rheumatoid arthritis. This manifests clinically as anaemia, which is associated with a low serum iron and a concomitant raised serum ferritin [MASCIOLI and BLACKBURN, 1985]. The mechanism appears to be related to an inadequate release of iron from reticuloendothelial cells [BENNETT, 1977].

MUIRDEN and SENATOR [1966] have demonstrated that iron deposits were a constant feature of the histopathology of the rheumatoid synovium. Electron microscopy of the synovial membranes of a number of their patients showed that the iron was present both as ferritin and haemosiderin [MUIRDEN, 1966]. Speculation exists as to whether the accumulation of this iron is due to local factors, i.e. repeated episodes of microbleeding consequent upon an abnormal microvasculature, or whether there is a generalized disturbance of iron storage due to chronic systemic disease [OGILVIE-HARRIS and FORNASIER, 1980].

After absorption from the gut, iron, as Fe(III), is transported in the plasma bound avidly to the carrier protein transferrin. Lactoferrin, present in several body fluids, is a protein similar to tranferrin in that two moles of Fe(III) are bound per mole of protein. Transferrin binds to receptors on the cell surface and can be internalized. When the cytoplasmic vacuole so formed becomes acidified, the iron is released and may become bound to various cellular constituents such as citrate, ATP and GTP forming a small pool of non protein-bound iron. Within the cell, iron is stored in the protein ferritin, which comprises a high molecular weight protein shell surrounding an hydrated Fe(III) oxide-phosphate complex. Iron enters ferritin as Fe(II) becoming oxidized by the protein to Fe(III) and deposited in the interior; conversely, in order to be removed,

Fe(III) needs to be reduced to Fe(II), which is believed to be accomplished by biological reducing agents such as ascorbate, cysteine or reduced flavins. Lysosomal degradation of ferritin leaves the insoluble product, haemosiderin. Ferritin has a high storage capacity for iron and is normally only 1/3 saturated.

The nett effect of this system is to keep the intracellular pool of non protein-bound iron low. HALLIWELL and GUTTERIDGE [1984] have suggested that this may be required in order to restrict the ability of non protein-bound iron, (chelated with, say, ATP, GTP or citrate) from reacting with H_2O_2 in a Fenton reaction thus leading to the production of hydroxyl radicals. GUTTERIDGE, ROWLEY and HALLIWELL [1981] have developed an assay for unbound iron salts, which utilizes the requirement of Bleomycin for unbound iron as a catalyst in Bleomycin-induced DNA degradation. Their results indicate no such "catalytic" iron in serum (consistent with the iron being tightly bound by transferrin); however, a level of 2.8 ± 1.2 µmol/L non-protein bound iron salts in rheumatoid synovial fluid. These data show that if H_2O_2 becomes available within the rheumatoid joint, then a Fenton reaction is possible [GUTTERIDGE, ROWLEY and HALLIWELL, 1982].

Other evidence for the role of iron in joint inflammation is less direct. The administration of iron to a rheumatoid patient, particularly by the parenteral route is often associated with an exacerbation of articular inflammation [REDDY and LEWIS, 1969]. Conversely, BLAKE <u>et al</u>. [1983(b)] have claimed that administration of a chelating agent to guinea pigs with antigen-induced chronic arthritis is associated with a transient exacerbation of their disease but is followed by a long term improvement. The same group [BLAKE

et al., 1984] claim a correlation between synovial iron deposits and outcome of disease in a group of patients with rheumatoid arthritis.

Studies of the pathology of iron overload syndromes have indicated radical-mediated mechanisms [YOUNG and AISEN, 1982 ; HEYS and DORMANDY, 1981]. In addition, in the treatment of these conditions, ascorbate administration will enhance the effect of the chelating agent desferrioxamine (consistent with its ability to retrieve Fe(III) from its storage sites). If given alone, however, ascorbate will have a deleterious, sometimes fatal, effect [NIENHUIS, 1981]. This has been linked to its ability to interact with iron to produce hydroxyl radicals, leading to increased lipid peroxidation. Finally, in vitro evidence shows that unsaturated lactoferrin will inhibit hydroxyl radical formation [GUTTERIDGE et al., 1981]. However, if fully saturated, lactoferrin will enhance hydroxyl radical production and lipid peroxidation [AMBRUSO and JOHNSON, 1981]. In summary, inflamed synovium is iron laden and this is associated with an increase in "biologically available" iron that could interact with H_2O_2 in vivo to produce hydroxyl radicals via a Fenton reaction.

c. Could synovial ischaemia lead to oxygen radical production in vivo?

This third mechanism of oxygen radical production is much more speculative and is reviewed as an area warranting further investigation.

Xanthine oxidase has been widely used in vitro to produce a flux of oxygen radicals because it will react with molecular oxygen and one of a variety of substrates (e.g. hypoxanthine) to produce $O_2^{\cdot}$ and H_2O_2. However it appears that in vivo "xanthine oxidase" is a xanthine dehydrogenase which transfers electrons to NAD^+ rather than oxygen. Attack by proteolytic enzymes or oxidation of some

thiol groups during purification is believed to account for its conversion to an oxidase [HALLIWELL and GUTTERIDGE, 1985]. During ischaemia xanthine dehydrogenase is also converted to an oxidase, presumably also by proteolytic attack. Hypoxia also results in the accumulation of one of its substrates, hypoxanthine, from the degradation of ATP in hypoxic cells. PARKS et al. [1981] and others have suggested that this may account for tissue damage occurring during reperfusion of ischaemic tissues. As oxygen supply is restored to hypoxic tissue superoxide anion may be produced by the damaged enzyme.

GRANGER, RUTILI and McCORD [1981] showed that reperfusion injury of cat small intestine, a tissue rich in this enzyme, is decreased if superoxide dismutase is added to the reperfusion fluid. This mechanism has direct relevance in situations of coronary ischaemia [GARDNER et al., 1983] and stroke [FLAMM et al., 1978].

Despite increased blood flow to the synovium as a result of inflammation, there exists a state of relative hypoxia within the synovial fluid and presumably also within the synovium [LUND-OLESEN, 1970; TREUHAFT and McCARTY, 1971]. In patients with severe active rheumatoid arthritis a synovial fluid pO_2 as low as 9 mm Hg has been reported [FALCHUK, GOETZL and KULKA, 1970]. The microvascular lesions of rheumatoid arthritis associated with increased demand of inflamed synovial tissue for oxygen and glucose may lead to areas of unstable oxygen supply which might in turn lead to areas of reperfusion injury. Indeed focal infarction and necrosis have been used to explain the appearance of synovial fluid detritus known as "rice bodies" and also the clinical finding of a low temperature effusion fluid found in severe rheumatoid arthritis has been attributed to an ischaemic synovium [HARRIS, 1985].

Initial studies on the distribution of xanthine oxidase have indicated that it is extremely widely distributed between species [RICHERT and WESTERFIELD, 1951, AL-KHALIDI and CHAGLASSIAN, 1965]. It is not, however found in the parenchyma of many human tissues (apart from liver and small intestine) but is present in human endothelial cells. Little is known of the xanthine oxidase content of synovial tissues.

The potential relevance of this mechanism warrants further study, since the concept of enzymatic denaturation of an enzyme due to hypoxic or proteolytic damage may apply to other flavoprotein dehydrogenases, e.g. aldehyde dehydrogenase, and could lead to oxidase activity with superoxide production [LANDS, personal communication].

4. Protective Mechanisms

Appreciation that biological systems can generate highly reactive partial reduction products of oxygen has largely come about by the demonstration of the cellular defense mechanisms that protect against them. The primary defense is provided by a system of enzymes. The superoxide dismutases will eliminate superoxide anion by catalyzing its conversion to hydrogen peroxide. Hydrogen peroxide is removed by catalase which it will convert to water plus oxygen and by peroxidases which also reduce hydrogen peroxide to water using a variety of reductants available to the cell. Efficient removal of one of the first two intermediates of oxygen reduction prevents the formation of the third, the hydroxyl radical, which is so reactive that enzymatic scavenging would be impossible.

a. Superoxide dismutases.

The existence of haemocuprein, a copper containing protein component of bovine blood with a molecular weight of 34,000 was

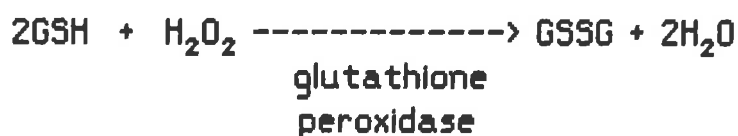
discovered in 1938 by MANN and KEILIN. However, it was not until 30 years later that McCORD and FRIDOVICH [1968] identified its function. They demonstrated that haemocuprein, now known as superoxide dismutase, was able to catalytically remove superoxide anion. No other substrate has been identified for this enzyme which is ubiquitous among aerobic organisms. Three distinct classes of superoxide dismutase, each with a different metal at their active site, have now been recognized [FRIDOVICH, 1975]. Enzymes with iron or manganese at their active sites are generally characteristic for prokaryocytes and mitochondria. They share amino acid sequence homologies suggesting a common evolutionary origin. The copper containing enzyme studied by McCord and Fridovich has been shown to contain zinc as well, although this is believed to fulfill a structural role and is not essential for enzymatic activity [BEEM, RICHARDSON and RAJAGOPALAN, 1977]. Its amino acid sequence is quite unrelated to that of the other two enzymes and it is found in the cytosol of eukaryotic cells [KEELE, McCORD and FRIDOVICH, 1970]. The copper containing enzyme is inhibited by cyanide but is stable to ethanol and chloroform whereas with the manganese and iron containing enzymes the reverse is the case [FRIDOVICH, 1982].

The mechanism of enzyme catalysed dismutation of superoxide anion appears to involve alternate reduction and oxidation of the transition metal ion at the active site [ROTILIO, BRAY and FIELDEN, 1972]. The three dimensional structure of the protein subunits of bovine copper containing superoxide dismutase suggests a positively charged track that leads to the active site and a similar mechanism is likely for the manganese and iron containing enzymes [HALLIWELL and GUTTERIDGE, 1985].

The superoxide dismutases are essentially intracellular enzymes. Levels found in extracellular fluids have been very low [McCORD, 1974]. More recently a high molecular weight form of copper containing superoxide dismutase has been reported [MARKLUND, 1982]. This form is present in the extracellular space, predominantly of the lung. It's exact role is yet to be elucidated.

b. <u>Glutathione (Glutathione peroxidase)</u>.

Glutathione peroxidase detoxifies hydrogen peroxide through the oxidation of reduced glutathione according to the reaction:



$$2GSH + H_2O_2 \xrightarrow[\text{peroxidase}]{\text{glutathione}} GSSG + 2H_2O$$

The oxidized glutathione is then reduced by a second enzyme glutathione reductase using NADPH (generated by the hexose mono-phosphate shunt) as the reducing agent. Other peroxides can act as substrates for glutathione peroxidase e.g., lipid hydroperoxides which will be metabolized to hydroxy fatty acids.

Glutathione peroxidase (MW 85,000) is a selenium dependent enzyme found in the cytosol. It is effective at low concentrations of hydrogen peroxide and therefore probably constitutes the cell's first line of defense against this agent. Further evidence of this comes from the fact that glutathione peroxidase deficient individuals suffer a disease similar to chronic granulomatous disease [HOLMES *et al.*, 1970].

c. <u>Catalase.</u>

Catalase catalyzes the divalent reduction of hydrogen peroxide to water according to the reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

It has a very high V_{max} for hydrogen peroxide i.e. reaction rates approach the diffusion controlled limit and thus it is very difficult to saturate. On the other hand, however, it is not efficient at removing low concentrations of hydrogen peroxide [SCHONBAUM and CHANCE, 1976]. Most purified catalases consist of four protein subunits, each of which has a molecular weight of 60,000 and contains a haem group bound to its active site [VAINSHTEIN et al., 1981]. Catalases are intracellular enzymes found in most aerobic cells and in animals, particularly in liver and red blood cells. Acatalasemic individuals are relatively symptom free [MATSUDA et al., 1976], thus implying that catalase is not the cell's primrary defence against oxidant stress. Nevertheless it will complement the glutathione/glutathione peroxidase system particularly at high levels of hydrogen peroxide.

d. Other Defenses.

A number of other mechanisms exist which appear to function, at least in part, as antioxidants. Caeruloplasmin, which is present in serum, has the ability to act as an oxygen radical scavenger. GOLDSTEIN et al. [1979] have claimed that in combination with copper ions it has the ability to act as a superoxide dismutase, however this has been disputed [BANNISTER et al., 1980]. Ascorbic acid may act as a reductant, in which case it forms the semi-dehydroascorbate radical which is relatively unreactive and usually undergoes disproportionation to ascorbate and dehydroascorbate. In this manner ascorbate may react with superoxide anion acting to detoxify it and its subsequent more reactive radical products. Ascorbate is found in high concentrations in the lens of the eye, which has low superoxide dismutase levels. Its function there is believed to be an antioxidant. Also uric acid, at levels found in

the blood (0.12-0.45 mmol/l), can act as a powerful scavenger of singlet oxygen and of hydroxyl radicals [AMES et al., 1981].

e. Oxidant defenses within the joint.

McCORD [1974] first reported that bovine synovial fluid contains a barely detectable amount of endogenous catalase (i.e., 0.05 μgm/ml or less) and very low concentrations of endogenous superoxide dismutase (1 μgm/ml). These levels are at least 2-3 orders of magnitude less than the intracellular concentrations of these enzymes. He also showed that the activity of the superoxide dismutase present could be inhibited by cyanide, thus suggesting that it was the copper containing enzyme. The endogenous superoxide dismutase present in fresh bovine synovial fluid in McCord's experiments was enough to effect only partial protection of synovial fluid hyaluronic acid from an exogenously applied oxygen radical flux. The magnitude of the flux that he applied was equivalent to that produced by $5x10^6$ activated neutrophils/ml, in vitro. Some inflammatory synovial fluids contain a number as much as twentyfold greater than this. Extracellular fluids other than synovial fluid, including human serum, human cerebro-spinal fluid and bovine aqueous humour, have similar low levels of both enzymes. BLAKE et al., [1981] examined the synovial fluid from eight human patients with rheumatoid arthritis and found no detectable superoxide dismutase activity as assessed by ability to inhibit reduction of nitroblue tetrazolium. They also found low catalase activity (0-55 units catalase/ml). IGARI et al. [1982], using inhibition of reduction of ferricytochrome C, reported that levels of superoxide dismutase in the synovial fluid of patients with rheumatoid arthritis was increased compared to that found in osteoarthritis. Nevertheless the absolute levels that they report are several orders of

magnitude less than intracellular levels [FRIDOVICH, 1975]. Synovial fluid ceruloplasmin levels were found to be increased in rheumatoid arthritis [SCUDDER et al., 1978]. However, the results reported by BLAKE et al. [1981] would suggest this is not associated with significant (i.e., detectable) superoxide dismutase activity. Total ascorbate levels in rheumatoid synovial fluid were found by BLAKE et al. [1981] to be at the low end of the normal range. Reduced ascorbate was not measured.

5. Targets for Oxy Radical Attack

A large number of biological molecules have been reported to be denatured in some way by reactions with oxygen radicals. It is my intention to draw attention to those more relevant to inflammatory joint disease.

a. Proteins

Molecules that are unsaturated or contain a sulphur moiety are susceptible to free radical attack [PRYOR, 1976]. These include the unsaturated amino acids, tryptophan, tyrosine, phenylalanine, and histidine, and sulphur containing amino acids, methionine and cysteine. Thus, enzymes which depend on these amino acids for their reactivity are likely to be inactivated by oxygen radicals [LIN and ARMSTRONG, 1978; BUCHANAN and ARMSTRONG, 1978]. Two examples of this are, firstly, the inactivation of serum α1-proteinase inhibitor upon exposure to an oxygen radical flux generated by stimulated neutrophils [CARP and JANOFF, 1979]. Inactivation is due to oxidation to a sulphoxide of the methionine residue at the active site of this enzyme inhibitor [JOHNSON and TRAVIS, 1979]. This has been proposed as a mechanism of lung damage in cigarette smoke-induced emphysema, since cigarette smoke is a potent source of oxygen radicals [CARP and JANOFF, 1978]. Secondly, JASIN [1982] has

reported the generation of IgG aggregates, with the properties of immune complexes, upon exposure of IgG to the myeloperoxide-H_2O_2-halide system. Further work on this phenomenon by MULLINAX and MULLINAX [1983] has shown the formation of dityrosine crosslinks (i.e., interprotein disulphide bond formation). This could represent an antigen-independent means of formation of "immune complexes", which could lead to the production of rheumatoid factor.

b. Nucleic acid and DNA.

A considerable literature exists which describes cell mutation and death from ionizing radiation [MYERS, 1980]. These effects are primrarily due to free radical reactions with DNA. A number of enzymatic radical generating systems have been tested, including the XO/HX system [BRAWN and FRIDOVICH, 1981], that have shown DNA strand scission. Hydroxyl radical scavengers have been protective, thus implicating this agent.

c. Membrane lipids.

The unsaturated bonds of membrane cholesterol and fatty acids can readily react with free radicals leading to lipid peroxidation [FRANKEL, 1980]. After initiation with an appropriately reactive agent, e.g. the hydroxyl radical, this process will become autocatalytic, yielding a variety of products including lipid peroxides, lipid alcohols, aldehydes. An increase in the serum and synovial fluid content of lipid peroxidation products in active rheumatoid arthritis has been reported [LUNEC et al., 1981]. Lipid peroxidation has been proposed as a cytotoxic event in a number of conditions. However, as HALLIWELL and GUTTERIDGE [1984(b)] have pointed out, lipid peroxidation is a common pathway in the dissolution of a cell and the presence of lipid peroxidation products per se, does not implicate lipid peroxidation as the primary cytotoxic event.

d. Extracellular macromolecules other than hyaluronic acid.

The depolymerization of several macromolecules of biological importance by exposure to a variety of oxygen radical generating systems has been demonstrated. Periodate oxidation, for example, will induce a loss of viscosity in solutions of proteoglycans and glycosaminoglycans [SCOTT, TIGWELL and SAJDERA, 1972], alginate [PAINTER and LARSEN, 1970] and methylcellulose [SCOTT and TIGWELL, 1973]. SCOTT and PAGE-THOMAS [1976] have presented evidence for the production of hydroxyl radicals in aqueous periodate solutions and in general the depolymerizations described could be inhibited by the hydroxyl radical scavenger, propan-l-ol.

A decrease in viscosity in solutions of isolated proteoglycan subunit from bovine nasal cartilage occurs upon exposure to the XO/HX oxygen radical generating system *in vitro* [GREENWALD, MOY and LAZARUS, 1976]. BARTOLD, WIEBKIN and THONARD [1984(a)] have demonstrated a decrease in hydrodynamic size of a gingival proteoglycan upon exposure to oxygen radicals. These authors suggested that glycosaminoglycan side chains were cleaved from the core protein.

GREENWALD and MOY [1979] have shown that soluble collagen after exposure to an oxygen radical flux (generated by the XO/HX) does not gel normally when heated. These authors and VENKATASUBRAMANIAN and JOSEPH [1977] have postulated that this` effect is due to cleavage of the telopeptides of the non-helical region. MONBOISSE *et al*. [1983; 1984], however, have reported that exposure of soluble collagen to the XO/HX oxygen radical generating system leads to multiple cleavages of the collagen microfibrils including the triple helical region. It is not apparent from these

studies, however, how susceptible fully aggregated collagen fibres are to oxygen radicals.

e. Cellular targets

1) Red cells.

Both stimulated phagocytes [SIMCHOWITZ and SPILBERG, 1979; WEISS, 1980] and non-cellular oxygen radical generating systems [KELLOGG and FRIDOVICH 1977] are capable of mediating red blood cell lysis. The biochemical mechanisms leading to lysis appear to be complex and not necessarily applicable to other cell types. The first consideration is the presence of oxyhaemoglobin within the red cell. This is believed to be present as superoxy-ferrihaeme compound [COLLMAN *et al*., 1976] which may autoxidize providing a continuous source of superoxide anion within the cell, which is eliminated by endogenous superoxide dismutase unless this autoxidation is accelerated, e.g., by haemolytic drugs such as phenylhydrazine or menadione. Reaction of superoxide anion with haemoglobin leads to the formation of haemoglobin breakdown products (including methaemoglobin) which bind to the red cell membrane and cause increased osmotic fragility of the cell [GOLDBERG and STERN, 1977]. LYNCH and FRIDOVICH [1978] have demonstrated the existence of anionic membrane channels within the erythrocyte membrane by which superoxide anion can gain free access to the cell interior and thus react with oxyhaemoglobin. In addition, WEISS [1980] has postulated that phagocyte generated hydrogen peroxide, which can diffuse freely across the cell membrane, can react with methaemoglobin to form a cytotoxic complex capable of haemolysing the erythrocyte. This has stemmed from experiments that have shown accelerated phagocyte-induced red cell lysis with exogenous superoxide dismutase, or inhibition of endogenous catalase or

glutathione peroxidase and protection with exogenous catalase [WEISS, 1980]. Other workers have demonstrated damage to membrane lipids and proteins with the use of red cell ghosts, where the effects of oxyhaemoglobin have been eliminated. Thus, primary lipid peroxidation is an alternative mechanism for red cell lysis [KELLOGG and FRIDOVICH, 1977; FLYNN et al., 1983; DAS and NAIR, 1980; ROSEN, BARBER and RAUCKMAN, 1983].

2) Other cells

Given the nonspecific reactivity oxygen radicals, it is not surprising that enzymatic and chemical superoxide generating systems have been shown to be cytotoxic to cells in culture. Cells lines studied have included human glial cells [DEL MAESTRO et al., 1980], human lymphocytes [McNEIL, CLELAND and BETTS, 1983], lung cells [FOX and AUTOR, 1978] and human fibroblasts [SIMON, SCOGGIN and PATTERSON, 1981]. Also, sublethal exposure to oxygen radicals causes a wide range of alterations to cell function. For example, KRAUT and SAGONE [1981] have demonstrated that exposure of lymphocyte cultures to the XO/X system reduces the ability of these cells to form rosettes with sheep red blood cells, thus indicating cell membrane receptor damage. BATES, LOWTHER and HANDLEY [1984] have demonstrated that cultured bovine articular chondrocytes respond to exposure to the XO/HX oxygen radical generating system with reduced proteoglycan and protein synthesis that persists for at least 5 days following exposure. In general these effects have been completely preventable by catalase [BATES, LOWTHER and HANDLEY, 1984; KRAUT and SAGONE, 1982; SIMON, SCOGGIN and PATTERSON, 1981; DEL MAESTRO et al., 1980], thus implicating hydrogen peroxide as a key intermediate. It is appropriate to note that in these cases the oxygen radical generating system is applied

externally to the cell and the non-charged nature of hydrogen peroxide may allow it to penetrate hydrophobic regions of the cell membrane. Indeed the cellular alterations and cytotoxic events may depend upon the site of secondary oxygen radical generation, induced by hydrogen peroxide. The factors likely to be involved here are complex, e.g., availability of transitiion metals and/or chelator, cellular antioxidant defence status and target susceptibility.

Systems utilizing neutrophils as cellular sources of oxygen radicals have also been described. Autocytotoxicity of stimulated neutrophils can be inhibited by superoxide dismutase [SALIN and McCORD, 1975]. This effect and the presence of the myeloperoxide-H_2O_2-halide system create difficulties in interpreting studies of the effects of oxygen radicals generated by stimulated neutrophils. WEISS et al. [1981] demonstrated that granulocyte-induced injury to cultured endothelial cells, as measured by ^{51}Cr release, was inhibited by catalase but not by superoxide dismutase. Additional myeloperoxidase did not enhance ^{51}Cr release and myeloperoxidase deficient neutrophils showed similar effects to normal neutrophils. This suggests a myeloperoxidase independent, hydrogen peroxide mediated cytotoxicity.

There are, however, tissue systems where superoxide dismutase appears protective. Superoxide dismutase will protect against the increased microvasculature permeability observed in the hamster cheek pouch when it is perfused with XO/X [DEL MAESTRO et al., 1980]. It will also ameliorate reperfusion injury in the cat small intestine [PARKS et al., 1982]. Clearly, as the systems studied become more complex, it becomes more difficult to interpret the results of scavenger studies in a way that leads to a clear understanding of the important initial cellular events leading to

toxicity and to determine the radicals involved.

It also appears that oxygen radical dependent cytotoxicity may mediate such immunological process as neutrophil-mediated antibody-dependent cytotoxicity [BORREGAARD and KRAGBALLE, 1980] and possibly natural killer cell cytotoxicity [SUTHANTHIRAN _et al._, 1984] although the latter contention is far from established.

C. HYALURONIC ACID

1. Introduction

The term "synovia" was coined by Paracelcus to describe joint fluid because he likened its viscous nature to that of an egg [RODNAN, BENEDEK and PANETTA, 1966]. The component responsible for the high viscosity of synovial fluid is hyaluronic acid. This widespread polysaccharide is classified as a glycosaminoglycan although many of its characteristics mark it as being unique. In the following paragraphs I shall review the physiology of this macromolecule, in particular, its structure, likely functions, biosynthesis, turnover and its interactions with cells particularly in relation to its presence in the synovial fluid of diathrodal joints.

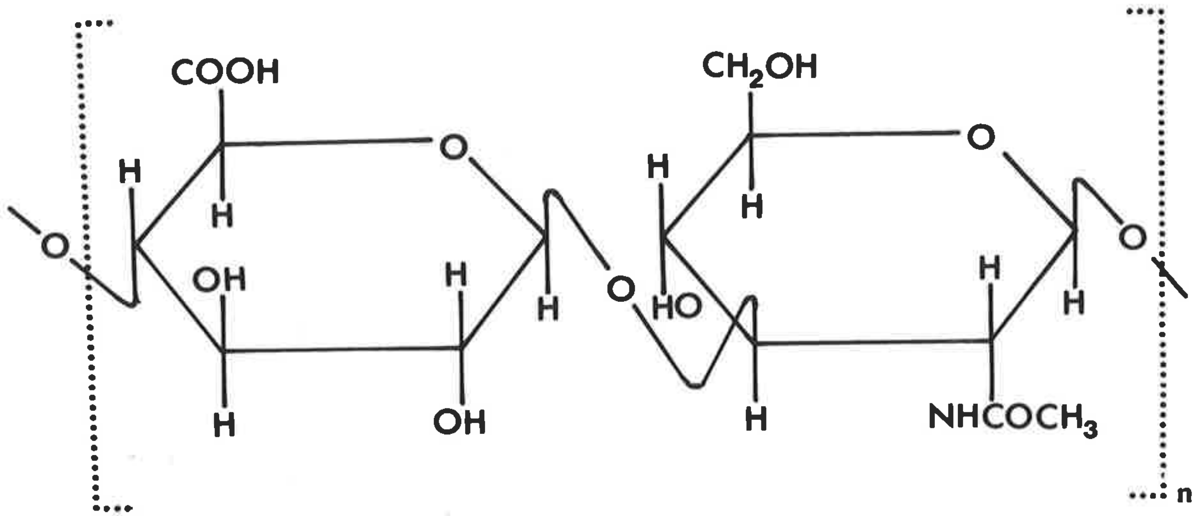
2. Chemical Structure

Hyaluronic acid was discovered by Meyer and Palmer in 1934 in bovine vitreous humour. It has since been isolated from numerous tissues including human vitreous, skin, umbilical cord, blood vessel walls, and is also present in low concentration in the serum. Indeed it is a ubiquitous component of extracellular matrices. Initial chemical studies by MEYER and PALMER [1936] showed that it was a large molecular weight, non-sulphated, acidic polysaccharide. It consists of equimolar amounts of D-glucuronic acid and N-acetyl-glucosamine (2-acetamido-2-deoxy-D-glucose) both in the pyrannose form [MEYER, 1947].

Studies by JEANLOZ and FORCHELLI [1951], more recently confirmed by LONGAS and MEYER [1981], demonstrated that the polymer is unbranched and that the two constituent sugars occur alternately all the way along the molecule (Figure 1-1). The nature of the glycosidic linkages were shown, with the use of specific hyaluronidases, to be alternating β1-3 and β1-4 glycosidic linkages. Both testicular and bacterial hyaluronidases cleave at the β1-4 linkages and leech hyaluronidase cleaves at the β1-3 linkage [LAURENT, 1970]. Hyaluronic acid polymers show considerable variation in length (i.e., polydispersity) and therefore total molecular weight, but apart from this no chemical differences have been demonstrated among hyaluronic acids from various biological sources including bacteria [BRIMACOMBE and WEBBER, 1964]. The isolation methods that have been used to separate hyaluronic acid have produced a product that contains variable amounts of protein. Using mild isolation techniques OGSTON and STANIER [1950] separated a hyaluronate-protein complex from ox synovial fluid that contained 25-30% protein. Because this sedimented as a single peak on ultracentrifugation they suggested that combination with protein may be an integral part of the structure of hyaluronic acid and necessary for some of its functions. The finding of a variation in the amount of protein bound with change in pH has led CURTAIN [1955] and others to suggest that the bulk of this interaction is ionic. Alternatively, however, protein may be adsorbed (trapped) into micelles of the randomly coiled hyaluronic acid molecules [OGSTON and STANIER, 1951]. Using more rigorous techniques hyaluronic acid can be isolated with as little as 0.35% protein [SWANN, 1968]. Whether this protein is covalently bound is unclear [LAURENT, 1970; MIKUNI-TAKAGAKI and POOLE, 1981; TSIGANOS, VYNIOS and KALPAXIS, 1986].

Figure 1-1 STRUCTURE OF HYALURONIC ACID

The structure of hyaluronic acid showing the two component sugars D-glucuronic acid and N-acetylglucosamine joined at the centre by a ß1-3 glycosidic linkage. At the opposite end of each sugar are the components of a ß1-4 glycosidic linkage. These two sugars alternate along the whole length of this unbranched molecule.


COOH
H
H
OH
H
OH
H
O
H
O
CH2OH
H
H
HO
H
O
H
H
NHCOCH3
O
n

indeed removal of protein by trypsin digestion does not affect the intrinsic viscosity of hyaluronic acid solutions [BALAZS and SUNDBLAD, 1959].

3. Hyaluronic Acid in Aqueous Solution - The Formation of a Matrix

Hyaluronic acid has a high affinity for water. When hydrated it swells to occupy a volume one thousand times greater than its anhydrous volume [OGSTON and STANIER, 1951]. Thus a single molecule of sodium hyaluronate (MW = 5×10^6) will occupy a spheroidal domain with a diameter of 0.5 μ or one gram dissolved in physiological saline will completely occupy 3 liters of solvent. Therefore, in concentrations above 0.33 mg/ml there will be overlapping of the molecular domains of macromolecular hyaluronate and the formation of a polymer matrix [BALAZS, 1974]. A coarse network of branching fibrillar strands has been visualized by HADLER et al. [1982] using electron microscopy of freeze etch replicas of hyaluronate solutions.

Initial studies based on viscosity, sedimentation and light scattering data suggested that the molecule adopts a random coil conformation in solution [OGSTON and STANIER, 1951; LAURENT, 1955; BLUMBERG and OGSTON, 1957]. More recent studies using xray crystallography have demonstrated that individual chains of hyaluronic acid adopt a stable left handed helix - most commonly with 3 disaccharide units per complete helical turn [WINTER, SMITH and ARNOTT, 1975]. The chains tend to be fully extended and are highly stable regardless of degree of hydration or ionic strength in the preparations used for crystallography and therefore likely to be found as such in free solution. In addition, structural considerations using the fact that the pyrannose rings of hyaluronic acid are both in the chair form, indicate that intramolecular hydrogen bonding across each glycosidic linkage can occur [ATKINS, MEADER and SCOTT,

1980; WINTER, SMITH and ARNOTT, 1975]. Evidence for a secondary structure stabilized in this manner has been found by SCOTT et al. [1981] using ^{1}H n.m.r., although that study was done using dimethyl sulphoxide rather than water as the solvent. In addition, intermolecular interaction between hyaluronic acid molecules mediated by water molecules, forming bridges between hyaluronate cation chains, has also been postulated [SHEEHAN, ATKINS and NIEDUSZYNSKI, 1975]. These interchain interactions, although transient, would tend to lead to a degree of order through chain packing within the matrix, albeit temporarily. A full discussion of these points is provided by HADLER and NAPIER [1977].

4. Implications for Synovial Fluid Function

a. Lubrication

An appreciation of all the functions of hyaluronic acid in synovial fluid is far from complete. The viscous nature that hyaluronate imparts to synovial fluid has led to the suggestion that it acts as a cartilage-cartilage lubricant. However, RADIN, SWANN and WEISSER [1970] clearly demonstrated that the cartilage lubricating activity of whole synovial fluid is present in the protein fraction and is undiminished by hyaluronidase treatment. SWANN and coworkers have isolated two high molecular weight glycoproteins (lubricin 1 and 2) which account for this property [SWANN and RADIN, 1972; SWANN et al., 1981; SWANN et al., 1984]. Nevertheless, a role for hyaluronic acid in providing lubrication between the soft tissues of the joint has also been postulated. In vitro evidence for this has been provided by RADIN et al., [1971] who showed that, compared with buffer, the coefficient of friction between a flat surface and an appropriately loaded section of synovial tissue was decreased equivalently by synovial fluid and a solution of hyaluronic acid alone.

b. Viscoelastic Properties

Hyaluronic acid imparts to synovial fluid its characteristic viscoelastic properties. The rheological behavior of synovial fluid and of protein-free sodium hyaluronate solutions are virtually identical [GIBBS, MERRILL and SMITH,1968] and this behavior may be considered as having a viscous component (dynamic loss module) and an elastic component (dynamic storage module). These parameters can be measured over a range of strain frequencies corresponding to walking and running. Predominantly viscous behavior will result in energy being dissipated to surrounding tissues as heat and elastic behavior results in energy being stored in the deformation of the hyaluronic acid molecules [for full discussion, see BALAZS, 1974]. When these moduli are measured in synovial fluids from normal (young) knee joints an elastoviscous transformation is observed, i.e., a transformation from predominantly viscous behavior (at low strain frequencies) to predominantly elastic behavior (at strain frequencies corresponding to a fast walk). This conversion to elastic behavior is less marked in synovial fluids from older subjects and was not observed at all in synovial fluids from patients with osteoarthritis and inflammatory joint disease even at strain frequencies corresponding to running [BALAZS, 1974]. Elastic behavior may serve the important function of protecting synovial membrane and articular cartilage from mechanical shock and deformation (i.e., acting as a shock absorber).

c. A Conduit for Nutrients

The second main function of synovial fluid is that of nutrition. Indeed the nutrients required by the surface layers of cartilage are derived from the synovial vasculature and therefore must pass through the synovial fluid hyaluronate matrix. The possible

influence of hyaluronate on the transfer of nutrients has been the subject of considerable investigation. For example, HADLER [1981] claimed that transport of glucose through an hyaluronate matrix is facilitated (i.e. it occurs faster than through water alone). This finding has, however, been disputed [NORTON, URBAN and MAROUDAS, 1982]. The synovial membrane has a rich blood supply [ROPES and BAUER, 1953] and the capillaries have a fenestrated structure [SUTER and MAJNO, 1964]. Thus, although synovial lining cells have intercellular junctions [GROTH, 1975], they do not form a discrete layer [GHADIALLY, 1978]. The result is that, in certain areas, the "ground substance" of the synovial membrane has free access to the joint cavity. These morphological features suggest that the extracellular matrix of the synovial membrane plays an important role in regulating the outflow of fluid and soluble blood components from the capillaries of the synovium. Indirect evidence which supports this concept was obtained by NETTELBLADT, SUNDBLAD and JONSSON [1963], who showed that sedimentation of plasma through a solution of hyaluronic acid produced an ultrafiltrate with composition similar to that found in normal synovial fluid.

5. Biosynthesis

In recent years our understanding of the biosynthesis of hyaluronic acid has increased considerably. PREHM [1983 and 1984] and PHILIPSON and SCHWARTZ [1984] have shown that hyaluronate synthetase is situated on the interior surface of the plasma membrane and that as the molecule is synthesized it extends out into the extracellular space. It is synthesized at the reducing end by alternate transfer of the substrates UDP-GlcNAc and UDP-GlcA to the hyaluronate chain. The substrates can initiate chain formation and no protein primers are required. This is in contrast to the

synthesis of other mammalian glycosaminoglycans which are synthesized at the Golgi apparatus and undergo post-synthetic modification. The mechanism of chain termination for hyaluronic acid synthesis remains unclear.

6. Turnover

Whilst it has been established for many years that hyaluronic acid is synthesized in the tissues where it is found [GROSSFIELD et al., 1958], knowledge concerning its turnover has developed only recently with the availability of radiolabelled high molecular weight hyaluronic acid produced in cell culture [BAXTER, FRASER and CLARRIS, 1973]. The initial study in this area was from ANTONAS, FRASER and MUIRDEN [1973], who followed the fate of radiolabelled high molecular weight hyaluronic acid after injection into rabbit knees. They detected radioactivity in the synovium, cartilage, regional lymph nodes and blood plasma. Appearance within regional lymph nodes was observed within 15 minutes of injection. Hyaluronic acid enters the general circulation most probably from the lymph [LAURENT and LAURENT, 1981]. The normal serum concentration is of the order of 200 ng/ml [LAURENT and TENGBLAD, 1980] and labelled hyaluronic acid is cleared very rapidly from the blood by the liver where it is taken up by the endothelial cells [ERIKSSON et al., 1983] by a receptor mediated mechanism [SMEDSROD et al., 1984]. There it is degraded rapidly and completely [FRASER et al., 1981] in lysosomes [SMEDSROD et al., 1984]. The major products of hyaluronic acid catabolism by endothelial cell in culture are acetate and lactate [SMEDSROD, 1984], although in the presence of hepatocytes and in *in vivo* experiments, these are further metabolised to CO_2 and water.

7. Hyaluronic acid-Cell Interactions

Initial demonstration of an hyaluronic acid-cell interaction related to the demonstration that a variety of cell types have a pericellular coat of hyaluronate [GOLDBERG and TOOLE, 1984] which has the ability to exclude particles. It has been suggested that this cell surface hyaluronate may be important in such diverse cellular properties as aggregation, resistance to viral infection and tumourigenesis. WIEBKIN and MUIR [1975] demonstrated that hyaluronic acid was able to inhibit the synthesis of proteoglycans by chondrocytes in culture and therefore may have a regulatory role in this chondrocyte function. The nature of the interaction is very specific since no inhibition was seen with the other glycosaminoglycans or indeed with chondroitin, the desulphated form of chondroitin sulphate, which differs chemically from hyaluronic acid only in the orientation of the hydroxyl group at C4 on the N-acetylglucosamine.

Further evidence suggesting that hyaluronic acid influences cellular activity was advanced by TOOLE [1982], who demonstrated that during chick embryo development, cell movement takes place within extracellular matrices rich in hyaluronate. For example, invasion of the chick embryo cornea by mesenchymal cells coincides with increased hydration and swelling of the acellular stroma at which stage a major component of the extracellular matrix is hyaluronate. Subsequently, however, migration ceases, the stroma loses water, hyaluronidase activity increases and hyaluronate content of the extracellular stroma falls. It is at this stage that cellular differentiation starts. Toole suggested that hyaluronate probably inhibits cell-cell interactions, thereby permitting migration and proliferation but delaying the onset of differentiation until a

correct sequence of tissue organization could ensue. UNDERHILL and TOOLE [1979] have demonstrated a cell receptor for hyaluronate, in simian virus transformed 3T3 cells. This receptor has a relatively high affinity. They calculated that approximately 3×10^3 molecules could bind to each cell and that hyaluronic acid was probably bound at several sites.

The functions that these receptors are likely to subserve are, firstly, the attachment of the pericellular hyaluronate layer. Secondly, they are believed to mediate the endocytosis of hyaluronate before degradation by lysosomal hyaluronidases. They are also likely to modulate metabolic functions within the cell, for example, the aforementioned inhibition of chondrocyte proteoglycan synthesis.

Hyaluronate has also been shown to affect a number of inflammatory cell functions. Studies in this area have generally been conducted at hyaluronate concentrations similar to those found in the extracellular space, i.e., 1-4 mg/ml. However more recently important observations have been made using concentrations likely to be found in plasma, i.e., 200 ng/ml.

BRANDT [1974] reported that in solutions of isolated synovial fluid hyaluronate, the ingestion of monosodium urate crystals by peripheral blood polymorphonuclear leukocytes was inhibited proportional to hyaluronate concentration over the range 0.2 - 1.0 mg/ml. This effect was diminished by decreasing the molecular weight of the hyaluronic acid to below 10^6 with mild hyaluronidase treatment. However at a molecular weight of 5×10^5, he noted an enhanced uptake of crystals. FORRESTER and BALAZS [1980] have extended these studies. Using elicited mouse peritoneal macrophage phagocytosis of latex spheres, they demonstrated that viscous

solutions of high molecular weight hyaluronic acid (4.6×10^5 -2.8×10^6) caused dose-dependent inhibition of phagocytosis over the concentrations 0.1 - 2.5 mg/ml but, again, at the molecular weight of 9×10^4, a paradoxical stimulation of phagocytosis was seen.

The inhibition of phagocytosis was not present if the cells were pre-incubated with hyaluronic acid which was then removed by washing. Nor was it seen if other large polyanions such as heparin or chondroitin sulphate were substituted for the hyaluronic acid, thus indicating that it was not a charge effect. They postulated that the inhibitory effect was due to molecular interaction between large coils of polymeric polysaccharide preventing the attachment phase of phagocytosis.

Neutrophils have also been studied in this regard by FORRESTER and WILKINSON [1981] who reported that hyaluronic acid, in the same concentration range, inhibited both directed and random locomotion of neutrophils in a dose-dependent and molecular-weight dependent manner. Inhibition was more pronounced with higher molecular weight chemoattractants such as casein, than for small chemoattractant peptides such as FMLP. In addition, hyaluronic acid inhibited the binding of the chemotactic factor, denatured human serum albumin, to the neutrophil surface. This effect was reversible. It appears, therefore, that at these concentrations the physical effects such as molecular exclusion provide the best explanation for the results obtained.

BALAZS and DARZYNKIEWICZ [1973] have examined the effects of hyaluronic acid on various lymphocyte properties. They showed, using a simple migration inhibition assay, that concentrations of hyaluronic acid similar to those found in synovial fluid inhibited lymphocyte mobility. As well, a number of other lymphocyte functions are

depressed, including the response to mitogens such as PHA, lymphocyte dependent cytotoxicity and experimental graft versus host disease. Again, each of these activities is inhibited most effectively by high molecular weight hyaluronic acid and was proportional to the concentration of hyaluronic acid. They found that the effect of hyaluronic acid was similar to that observed by decreasing the cell density. For example, hyaluronic acid was more effective in suppressing stimulation of lymphocytes using pokeweed mitogen and PPD, which don't cause significant leukoagglutination, than it was in suppressing PHA induced stimulation, where leukoagglutination is seen. Other manoeuvres that increase cell density also tended to decrease the effectiveness of hyaluronic acid in inhibiting the stimulation of these cells. Other parallels between the effect of hyaluronic acid and decreasing cell-cell distance were seen such as delayed onset of DNA synthesis, delayed appearance of blasts and the effects of both were additive. They felt that in this situation, hyaluronic acid may act to decrease cell-cell interaction, although the possibility of interference with the diffusability of an intercellular messenger such as interleukin-2 could be an alternate explanation.

HAKANSSON, HALLGREN and VENGE [1980] have reported that if low concentrations of hyaluronic acid (between 5-500 µgm/liter) were incubated with whole blood, a stimulation of various neutrophil functions was seen including phagocytosis, adherence, random migration and chemiluminescence. A similar effect is seen after a subcutaneous injection of hyaluronic acid. They have subsequently determined that the serum cofactor necessary to see this stimulation is fibronectin [HAKANSSON and VENGE, 1985]. This effect is apparently specific for neutrophils since ANASTASSIADES and

ROBERTSON [1984] have shown that at similar low concentrations of hyaluronic acid, mitogen induced lymphocyte stimulation is inhibited, although these authors separated their mononuclear cell fraction before incubation with hyaluronic acid thus not allowing the hyaluronic acid to interact with serum fibronectin.

In summary, therefore, there are a number of important ways in which hyaluronate is likely to interact with cells. At concentrations likely to be found in extracellular matrixes (up to 2 mg/ml) undegraded hyaluronate causes inhibition of a number of inflammatory cell functions including random and directed locomotion, phagocytosis and lymphocyte stimulation. The likely cause of these effects is the entanglement of hyaluronic acid molecules forming a matrix. This will act to increase cell-cell distance and will alter the ability of various factors to interact with cells. At much lower concentrations, analogous to those found in plasma, however, hyaluronic acid, in concert with fibronectin, appears to cause neutrophil stimulation or priming.

8. Synovial fluid hyaluronic acid

Hyaluronic acid isolated from any given source is polydisperse and substantial differences in the average molecular weight of hyaluronic acid occur in fluids and tissues of the same species [CLELAND,1970]. Studies of the molecular weight of normal synovial fluid hyaluronic acid are well represented by BALAZS et al. [1967] who studied pooled fluids from 268 normal adult male volunteers. Using analytical ultracentrifugation, intrinsic viscosity measurements and light scattering, they estimated an average molecular weight of 6.5-10.9 x 10^6 which is essentially in agreement with others who have looked at synovial fluid obtained from fresh cadavers [JOHNSTON, 1955; DAHL et al., 1985].

RAGAN and MEYER [1949] were the first to report a decrease in the average molecular size of synovial fluid hyaluronic acid from patients with rheumatoid arthritis. It has been long appreciated clinically that whole synovial fluid from patients with inflammatory arthropathies has a decreased viscosity. However the hyaluronate concentration is also decreased (see Table 1) thus no inference about the size of the hyaluronic acid present can be made from this observation alone. RAGAN and MEYER determined a quotient (log viscosity/hyaluronic acid concentration) which was linear over the concentrations measured and was dependent upon the degree of polymerization. Thus they were able to demonstrate that the degree of polymerization was decreased in a series of patients with rheumatoid arthritis. Other workers have used other techniques including intrinsic viscosity, analytical ultracentrifugation and light scattering to confirm this observation. There has, however, been some variation in the estimates of average molecular weight for inflamed synovial fluid hyaluronic acid. These studies are summarized in Table 1. Initial reports suggested that the decrease in average molecular weight of hyaluronic acid correlated with the degree of inflammation present [RAGAN and MEYER, 1949]. This, however, has not been confirmed by other authors [SUNDBLAD, 1953; DAHL <u>et al</u>., 1985].

Also apparent from these studies was an increase in the polydispersity of the hyaluronic acid present. It is only in relatively recent years that reports have appeared that show chromatographic profiles of synovial fluid hyaluronic acid. BALAZS, BRILLER and DENLINGER [1981] fractionated synovial fluid from patients with arthritis on glyceralized controlled pore glass columns. They showed the presence of a considerable amount of material in

Table 1-1 AVERAGE MOLECULAR WEIGHT ESTIMATIONS OF NORMAL AND PATHOLOGICAL SYNOVIAL FLUID

	Reference	Method* mg/ml	NORMAL SYNOVIAL FLUID HA Conc.	HA MW mg/ml	PATHOLOGICAL SYNOVIAL FLUID HA Conc.	HA MW
1	RAGAN & MEYER [1948]	ν	1.36	---	0.08-0.630	decreased**
2	SUNDBLAD [1953]	Iν.	2.97	2.85×10^6	0.5-3	1.73×10^6
3	FESSLER, OGSTON & STANIER [1954]	Iν,AU	1.4-10.0	$1-4 \times 10^6$	---	1.7×10^6
4	JOHNSTON 1955	AU	---	$8.4-21 \times 10^6$	---	$1.2-1.5 \times 10^6$
5	BARKER _et al._ [1963]	TC	2.91-3.51	---	1.97-8.0	decreased**
6	CASTOR [1966]	Iν	1.64-2.25	$2.75-4.0 \times 10^6$	1.03-1.45	$1.8-2.15 \times 10^6$
7	BALAZS _et al._ [1967]	Iν, AU, LS	1.45-3.12	$6.5-10.9 \times 10^6$	<1.9	$1.2-2.2 \times 10^6$
8	DAHL _et al._ [1985]	GC	---	$6.3-7.6 \times 10^6$ (ave=7.0)	0.17-1.32	$3.2-6.8 \times 10^6$ (ave=4.8)

*ν = viscosity; Iν = intrinsic viscosity; TC = turbidity curve; AU = analytical ultracentrifugation; LS = light scattering; GC = gel chromatography;
** These methods show decreased parameters, correlating to molecular weight, in the diseased fluids.

the molecular weight fractions 0.5 x 10^5 - 1.3 x 10^6. In addition they showed at least two characteristic profiles of uronate-containing material, one unimodal with skewing into the lower molecular weight fractions and the other bimodal with the presence of a lower molecular weight peak. BJELLE, ANDERSSON and GRANATH [1982] also demonstrated considerable heterogeneity in molecular weight profiles of 17 patients with various forms of inflammatory arthropathy. More recently DAHL et al. [1985] utilizing a specific radioassay for hyaluronic acid and therefore doing away with the necessity for potentially degradative isolation procedures obtained similar results. These latter studies also failed to find a correlation between degree of inflammation and the amount of small molecular weight hyaluronic acid present.

D. STUDIES ON OXYGEN RADICAL INDUCED HYALURONIC ACID DEPOLYMERIZATION

1. Initial Studies

Shortly after the enzymatic depolymerization of hyaluronic acid was described [CHAIN and DUTHIE, 1939; MEYER, DUBOS and SMYTH, 1937] a number of reports appeared describing the non-enzymatic degradation of hyaluronic acid containing substances. ROBERTSON, ROPES and BAUER [1939] described a spontaneous loss of viscosity in fresh cattle vitreous upon standing. Because this tissue has a high ascorbic acid content they reasoned that this may be the "mucolytic agent" and subsequently showed that both synovial fluid hyaluronic acid and extracted hyaluronic acid lost viscosity upon addition of ascorbic acid in the presence of phosphate buffer [ROBERTSON, ROPES and BAUER, 1939; 1941]. These workers were quick to appreciate that the process was irreversible. It was soon shown that a

number of other polysaccharides including pectin and starch could be depolymerized in this fashion [ROBERTSON, ROPES and BAUER, 1941] and that other organic reductants such as pyrogallol and hydrogen sulphide could replace ascorbic acid. McCLEAN and HALE [1941] reported that ascorbic acid-induced hyaluronic acid depolymerization was inhibited by catalase, which was important evidence that hydrogen peroxide played an intermediate role.

The next major step in the elucidation of the mechanism of this means of depolymerization was taken by SKANSE and SUNDBLAD [1943] who showed that ascorbic acid-induced depolymerization of hyaluronic acid did not occur in an anaerobic environment, but proceeded as soon as oxygen was added. They also reported that the addition of copper greatly enhanced the effect. In 1949 JENSEN reported that ferrous ions alone or ferric ions in the presence of a reducing agent (hydrazine sulphate) could degrade hyaluronic acid. There was considerable debate in these early papers as to the role of hydrogen peroxide and ascorbic acid. Indeed part of this confusion has subsequently been resolved because we now realize that the phosphate buffers used in these experiments contain iron in sufficient quantity to catalyze a Fenton reaction [WONG et al., 1981].

A major concern at this time was the unexplained degradation of hyaluronic acid associated with various extraction procedures and "preservatives". Cysteine, for example, was used to activate papain which was utilized in the extraction of hyaluronic acid from various organic sources and merthiolate was used as a preservative. Both could act as reductants [PIGMAN and RIZVI, 1959; OGSTON and SHERMAN, 1959]. Perhaps of more interest was the depolymerization seen upon lyophilization. This was noted by PIGMAN and RIZVI [1959] to be accelerated by the presence of phosphate ions. Subsequent

work by WEDLOCK et al. [1983] has shown that the lyophilization procedure is associated with the appearance of radicals as detected by electron spin resonance spectroscopy.

2. Radiation Induced Hyaluronic Acid Degradation

RAGAN et al. [1947] were the first to report that the viscosity of both human synovial fluids and solutions of hyaluronic acid were diminished after exposure to x-irradiation. BALAZS and LAURENT [1951] demonstrated a similar effect seen upon exposure to UV light. Neither UV nor x-irradiation were associated with any change in the polyanionic character of hyaluronic acid, thus indicating that the carboxylic acid groups were not affected.

BALAZS et al. [1959] reported that the molecular weight of a hyaluronic acid preparation, calculated from sedimentation and diffusion constants, decreased from 80,000 to 19,700 after irradiation with UV light. He has also reported the appearance of a dialyzable fraction after x-irradiation suggesting a somewhat smaller end product. SUNDBLAD and BALAZS [1966] have suggested however that the viscosity lowering effect of low doses of x-irradiation may not be solely due to depolymerization of the macromolecule but also may be due to changes in shape, deformability or internal structure.

BALAZS et al. [1959] have reported a decrease in total hexosamine and in total glucuronic acid of hyaluronic acid exposed to 0.5-4 x 10^6 rads, thus suggesting a degree of destruction of these sugars due to irradiation with electrons. Reducing substances, measured by the ferricyanide method, increased after UV irradiation.

Degradation of hyaluronic acid by x-irradiation could be prevented by the addition of sodium thiosulphate, an effect believed to be mediated by the scavenging of hydroxyl radicals.[BRINKMAN et al., 1961]. BALAZS et al. [1967] reported the presence of transient

radical intermediates after the pulse radiolysis of aqueous solutions of hyaluronic acid as detected by electron paramagnetic resonance. They postulated that the radical intermediates observed could be best explained by cleavage of the glycosidic linkage and also hydrogen abstraction from the C_5 carbon of the glucuronic acid moiety with subsequent resonance stabilization with C_4.

3. Oxidation-reduction Depolymerization (ORD Reaction)

This term was formulated by PIGMAN's group who published a number of papers on the non-enzymatic depolymerization of hyaluronic acid in the 1960's. These workers were among the first to postulate that ascorbate-induced hyaluronic acid depolymerization was due to an oxygen radical reaction. Several lines of evidence led to this conclusion. Firstly, they cited the fact that x-irradiation of aqueous solutions of hyaluronic acid leads to depolymerization due to the generation of radicals (the hydroxyl radical is generated along with hydrated electrons). The requirement for oxygen and the presence of a reducing agent suggested that oxygen radicals may be found with the ORD reaction. Secondly a number of hydroxyl radical scavengers including ethanol, toluene and D-glucose were inhibitors of the ORD reaction. Finally electron spin resonance studies demonstrated the presence of free radicals upon the oxidation of ascorbic acid in the presence of molecular oxygen [LAGERCRANTZ, 1964].

HARRIS, HERP and PIGMAN [1971] demonstrated that Fe(II)-EDTA complex could be regenerated from Fe(III)-EDTA at a platinum electrode (cathode) and this would depolymerize hyaluronic acid and alginic acid. This provides further support for cyclic reduction with subsequent autoxidation of Fe(II) hyaluronic acid depolymerization induced by ascorbate and other reductants.

4. Enzymatically Produced Oxygen Radicals and Inhibitors

McCORD [1974] was the first to report the effect of an enzymatic oxygen radical generating system (XO/HX) on synovial fluid and hyaluronic acid solutions which demonstrated loss of viscosity. Using this system he showed that both superoxide dismutase and catalase prevented the loss of viscosity at catalytic concentrations (superoxide dismutase 1 µg/ml; catalase 0.05 µg/ml). He reasoned that since both superoxide dismutase and catalase alone inhibit this reaction then neither the superoxide anion nor hydrogen peroxide could be the depolymerizing agent but must be present simultaneously for the process to occur. The implication being that in this sytem the actual depolymerizing species is generated secondarily by a reaction between superoxide anion and hydrogen peroxide. The most likely candidate being the hydroxyl radical generated by a Haber-Weiss reaction (see page 7). Mannitol, a scavenger of the hydroxyl radical, was also found to prevent loss of viscosity in his system. HALLIWELL [1978] confirmed these findings with XO/HX using the hyaluronic acid solutions and demonstrated the dependence of the observed reduction in viscosity upon the presence of iron salts, albeit in trace quantities.

5. Importance of Metal Chelators and Antiinflammatory Drugs

HALLIWELL [1978] and subsequently BETTS and CLELAND [1982] have shown that the way the metal ions are chelated is of vital importance in both the XO/HX induced depolymerization and other systems dependent on the oxidation of ferrous ions (e.g., ascorbic acid induced hyaluronic acid depolymerization in phosphate buffer). If the chelator binds the Fe(III) tightly (as with the chelators BPS, DETAPAC or desferrioxamine) then this militates against the reduction of Fe(III) and reductant-driven iron-dependent hydroxyl radical

production is inhibited. On the other hand iron chelators which allow iron to shuttle between Fe(III) and Fe(II) by transfer of electrons will facilitate the reaction. The concentration of metal and chelator are of importance in determining this effect for example in the XO/HX system. BETTS and CLELAND [1982] have shown that the antirheumatic agent D-penicillamine can act as a chelator and will inhibit hyaluronic depolymerization in concentrations greater than 5mM. On the other hand in the ferrous ion autoxidation system at concentrations of less than 5mM D-penicillamine is stimulatory but at higher concentrations is inhibitory. Other antirheumatic drugs including salicylate, chloroquine, gold sodium aurothiomalate and indomethacin were in inhibitory in both systems.

6. Effect of superoxide dismutase

Hydroxyl radical production appears to proceed by different pathways in the XO/HX system and in systems dependent on the autoxidation of ferrous ions. The clearest evidence for this is that XO/HX induced hyaluronic acid depolymerization is inhibitable by superoxide dismutase but ferrous ion autoxidation and ascorbic acid induced hyaluronic acid depolymerization are not [HOFMANN and SCHMUT, 1980]. This is also the case with ascorbate induced DNA depolymerization [MORGAN, CONE and ELGERT, 1976]. WINTERBOURNE [1979] has suggested that iron-EDTA catalyzed hydroxyl radical production from hydrogen peroxide and ascorbate could proceed by a mechanism largely independent of superoxide, whereas the reduction of Fe(III) to Fe(II) in the XO/HX system is effected by superoxide anion [BETTS and CLELAND 1982]. Other differences exist between these two oxygen radical generating systems for example XO can effect the divalent reduction of oxygen as well as the univalent reduction and the relative amount of superoxide anion and hydrogen

peroxide produced directly are dependent on pH, pO_2 and substrate concentration [FRIDOVICH, 1970].

7. Degradation of Hyaluronic Acid by Neutrophils

GREENWALD and MOY [1980] reported that phorbol myristate acetate (PMA) stimulated neutrophils induced a progressive decrease in viscosity that was abolished by addition of superoxide dismutase or mannitol. These cells do not contain hyaluronidase [SODER, 1970]. Further work from this group [GREENWALD and MOAK, 1984] has demonstrated that Fe(II) or Fe(III) are required, and that stimulants other than PMA (including zymosan, Con A or FMLP) require added hydrogen peroxide. They also demonstrated that neutrophil lysates do not induce loss of viscosity such as would be expected if this were due to a simple enzyme effect.

8. Molecular Weight Changes

PIGMAN and RIZVI [1959] did not detect dialysable material after treatment of hyaluronic acid with various oxidation-reduction systems yet they did, however, detect a broadening of the hyaluronic acid peak on ultracentrifugal and electrophoretic analysis, similar to mild treatment with hyaluronidase. These results indicate that a true depolymerization occurs. This conclusion is also in keeping with the observation that the changes in intrinsic viscosity were irreversible [PIGMAN, RIZVI and HOLLEY, 1961]. GREENWALD and MOY [1980] were the first to publish gel chromatograms of hyaluronic acid exposed to any of these oxygen radical generating systems. They showed that their initial preparation of (human umbilical cord) hyaluronic acid was large enough to be excluded from Sepharose 2B-CL. After exposure to XO/HX or to PMA stimulated neutrophils, material had a reduced hydrodynamic size being retarded throughout the column. No such information was available until this

current study [McNEIL *et al*., 1985] for the ferrous ion autoxidation system. GREENWALD and MOY [1980] showed the presence of uronic acid containing material virtually to the V_t on Sepharose 2B-CL (K_{av} = 0.8) corresponding to a molecular weight of approximately 2-3 x 10^5. This was achieved using a level of superoxide anion generation that they had calculated could be attained within an inflamed joint. They reported that part of their reaction product became dialyzable but the limiting pore size of their dialysis tubing was not given.

Studies of the generation of reducing ends from hyaluronic acid after exposure to oxygen radicals have provided conflicting results. Early studies with ascorbate induced hyaluronic acid depolymerization suggested that reducing ends were not generated [McLEAN and HALE, 1941]. However, CLELAND *et al*. [1969] subsequently detected reducing ends using sodium borohydride labelling in hyaluronic acid depolymerized by ascorbic acid. GREENWALD and MOY [1980] did not detect reducing ends in XO/HX exposed hyaluronic acid.

9. Conclusion

Most investigations into the oxygen radical induced hyaluronic acid depolymerization systems have used change in viscosity as an index of depolymerization and have centered upon mechanisms of oxygen radical production. Relatively little information exists as to the molecular weight changes and conflicting information exists as to the generation of reducing ends in the reaction product.

The molecular weight of hyaluronic acid is however altered in inflamed synovial fluid hyaluronic acid. In this investigation I have therefore posed the following questions:

1. What are the molecular weight changes induced in solutions of hyaluronic acid by *in vitro* oxygen radical generating

systems that resemble those in the inflamed joint? (i.e., ferrous ion autoxidation, XO/HX, activated neutrophils)

2. What are the molecular weights of hyaluronic acid extracted from inflamed joints? Could any reduction in size be as a result of oxygen radical generating systems?

3. Are reducing ends generated by exposure of hyaluronic acid to oxygen radicals?

4. Is the biological activity of hyaluronic acid altered if it is depolymerized by hyaluronidase or an oxygen radical system?

5. What happens to synovial cells when they are exposed to oxygen radicals and in particular to the hyaluronic acid that they produce?

CHAPTER II

THE DEPOLYMERIZATION PRODUCTS OF HYALURONIC ACID AFTER EXPOSURE TO ODFR *IN VITRO*

A. INTRODUCTION

Much information about the molecular weight changes in hyaluronic acid exposed to oxygen radical generating systems have been inferred from changes in intrinsic viscosity. An exception to this is represented by the two Sepharose 2B-CL chromatograms published by GREENWALD and MOY [1980]. Their data clearly demonstrate an increase in polydispersity but they do not show sequential changes, nor do they demonstrate the effect of a maximal oxygen radical flux. Several studies [PIGMAN and RIZVI, 1959; GREENWALD and MOY, 1980] suggest that products as small as mono- and disaccharides are not obtained following oxygen radical induced depolymerization. However the size of the smallest products obtainable has not been established.

This investigation was therefore undertaken to define by gel chromatography:-

1. the sequence of molecular weight changes seen after exposure of hyaluronic acid to increasing levels of oxygen radical flux.
2. to determine the molecular weight of the smallest degradation products thus obtained.
3. to determine the effect of re-exposure to a second oxygen radical flux.
4. to determine changes in an umbilical cord hyaluronate preparation with a characterized starting molecular weight.

In order to obtain a wide range of relative hydrodynamic size resolutions three gels were used:- Sepharose 2B-CL (included volume for polysaccharides 10^5 - 20×10^6), Sepharose 4B-CL (3×10^4 - 5×10^6) and Sephadex G100 (10^3 - 10^5) [PHARMACIA - PRODUCT INFORMATION].

Three oxygen radical generating systems, each of possible biological relevance were studied i.e. the ferrous ion autoxidation system, the XO/HX system and PMA stimulated neutrophils. The generation of OH· is thought to proceed by different mechanisms in each of these systems, as discussed in Chapter I.

Molecular weight was also determined by analytical ultra-centrifugation as an independent confirmation of the results of gel chromatography in the ferrous ion autoxidation system.

In addition, a purified sample of hyaluronate was prepared from the commercially available human umbilical hyaluronic acid in order to better reflect the molecular weight spectrum of normal synovial fluid hyaluronic acid.

B. MATERIALS AND METHODS

Hyaluronic acid (grade III from human umbilical cord) and xanthine oxidase (grade III from buttermilk) were obtained from the Sigma Chemical Co., St. Louis, Mo. Sepharose CL-2B and CL-4B, Sephadex G-100 and Sephacryl S-400 were obtained from Pharmacia, Uppsala, Sweden. All other chemicals were of the highest purity available from Sigma or Ajax Chemicals, Sydney, Australia.

1. Hyaluronic acid purification

Hyaluronic acid was further purified by passage through a Sephacryl S-400 preparative column (volume 280 mls) using 0.5 M sodium acetate as the buffer. Fractions with a Kav between 0.1 and 0.25 were pooled. This represented a hydrodynamic size of between $2x10^6$ and $5x10^5$. Protein contamination was reduced to less than 1% w/v by predigestion with pronase, a procedure shown by BARTOLD, WIEBKIN and THONARD [1984(b)] to have no detectable effect on the hydrodynamic size of hyaluronic acid.

2. Oxygen radical generation

a. Ferrous ion autoxidation

The reaction mixture comprised:

(1) hyaluronic acid, 1 mg/ml

(2) potassium phosphate buffer, 50 mM, pH 7.4

(3) ferrous sulphate at concentrations varying between 5 µM and 1000 µM

(4) EDTA in a molar ratio to ferrous ions of 1:1.

Stock solutions of hyaluronic acid were prepared at an initial concentration of 4 mg/ml in sterile distilled water. This slightly cloudy solution was cleared by passage through a 1.2 µ Millipore filter and was then stored either frozen or at 4°C until required. Prior to use, a solution of 2 mg/ml hyaluronic acid in 50 mM phosphate buffer was prepared by the addition of an equal volume of 100 mM potassium phosphate buffer to the initial 4 mg/ml hyaluronic acid stock solution. Stock solutions of 10 mM EDTA were prepared in phosphate buffer and stock solutions of 10 mM and 1 mM ferrous sulphate were prepared in water. The reaction volume in each case was 1.7 ml. Under these conditions and in the presence of atmospheric oxygen, autoxidation of ferrous ions proceeds with the production of the hydroxyl radical (OH·), the agent reported to be directly effecting hyaluronic acid depolymerization [HALLIWELL, 1978; BETTS and CLELAND, 1982]. The reaction proceeds to completion in less than 5 minutes [BETTS and CLELAND, 1982].

b. XO/HX

For the enzymatic system the reaction mixture comprised:

(1) hyaluronic acid, 1 mg/ml

(2) potassium phosphate buffer, 50 mM, pH 7.4

(3) HX, 6 mM

(4) XO at concentrations varying between 5×10^{-3} and 1 U/ml

The action of XO on HX under aerobic conditions *in vitro* leads to the production of superoxide anion and hydrogen peroxide in addition to xanthine and subsequently uric acid. Trace quantities of iron, present in the phosphate buffer used, allow further reactions leading to the production of hydroxyl radicals [BETTS and CLELAND, 1982]. The reaction was allowed to proceed to completion (greater than twenty minutes) [BETTS and CLELAND, 1982].

c. Stimulated polymorphonuclear leucocytes

For the PMA stimulated polymorphonuclear leucocyte system the reaction mixture comprised:

(1) hyaluronic acid, 1 mg/ml

(2) Dulbecco's phosphate buffered saline, pH 7.4

(3) EDTA, 60 µM

(4) ferric chloride, 10 µM

(5) phorbol myristate acetate (PMA), 200 ng/ml

Blood was obtained by venipuncture from healthy laboratory personnel and was anticoagulated with EDTA. Polymorphonuclear leucocytes were separated by low speed centrifugation over Ficoll-Hypaque in a single step procedure as outlined by FERRANTE and THONG [1978]. The cells were then washed three times in Dulbecco's phosphate buffered saline. Immediately prior to the experiment, viability was assessed by trypan blue exclusion. Only cell preparations exhibiting greater than 95% exclusion were used. PMA, 200 ng/ml, was used as the stimulant and superoxide production was confirmed by observing the reduction of nitroblue tetrazolium [SEGAL, 1974]. Graded fluxes were obtained by varying the number of cells

added to the reaction mixture between 0.6×10^6/ml and 12×10^6/ml. The cells were incubated at 37°C for 3 hrs before the cells were sedimented by centrifugation for 10 minutes at 1000 g. The viscosity of the supernatant was then measured.

3. <u>Viscometry</u>

The viscosities of solutions of undegraded and degraded hyaluronate were measured simply and reproducibly by using a 1 ml tuberculin syringe and a 20 gauge needle at 2°C according to the method of BETTS and CLELAND [1982]. Relative viscosities were calculated by dividing the time taken for 0.8 ml of test soution to drain from the syringe by the time taken for the same volume of buffer to drain. Specific viscosity (Nsp) was then calculated by subtracting 1 from all the relative viscosities (i.e., subtracting the contribution of the buffer). Changes in Nsp were expressed as the percentage ratio of degraded material to undegraded material. The viscosities of solutions of oxygen radical exposed material were determined at 2 hrs for solutions exposed to the ferrous ion autoxidation or XO/HX systems, and at 3 hrs for solutions exposed to the cellular system.

4. <u>Gel chromatography</u>

Aliquots of 0.25 ml of the reaction products were fractionated on Sepharose 2B or 4B and Sephadex G-100 (30 cm x 0.7 cm). Fractions of 0.7 ml were eluted at 7 ml/hr with 0.5 M sodium acetate at pH 5.6. Characterization of the columns was determined with Dextran Blue, S^{35}-sulphate and glucuronic acid. In addition, in order to verify the resolution of these small columns a series of standards was run through both large (i.e., 1.5 x 90 cm) and small columns and the K_{av}'s of the profile peaks compared.

5. Uronic acid analysis

Uronic acid was estimated by the meta-hydroxydiphenyl method of BLUMENKRANTZ and ASBOE-HANSEN [1973] (see Appendix 1) or a modification of the automated method of ROSENTHAL, BENTLEY and ALBIN [1976].

6. Analytical ultracentrifugation

The sedimentation coefficients of intact and depolymerized hyaluronic acid samples were determined on a Beckman Model E analytical ultracentrifuge. Samples (0.5 ml in 50 mM phosphate buffer) were centrifuged at 59780 r.p.m. (259,000 g at the cell centre) in a 12 mm single sector cell with plane quartz windows in an AnD rotor at 18°C for up to 2 hrs. Photographs of Schlieren patterns were taken at 8 minute intervals and images measured on a Nikon profile analyser with a magnification constant of 2.5. The average molecular weights of the peak material were calculated by reference to standard preparations of hyaluronic acid kindly provided by Dr. M. Mathews of Chicago and to the values derived from the data of CLELAND and WANG [1970] as well as a standard preparation of Healon analysed by BARTOLD, WEIBKIN and THONARD [1984(b)].

C. RESULTS

1. Viscosity

A substantial decrease in Nsp was observed upon exposure of hyaluronic acid to oxygen radicals whether generated by the autoxidation of ferrous ions, enzymatically with XO/HX or by incubation with PMA activated polymorphonuclear leucocytes (Table 2-1). The decreases in viscosity seen were proportional to the intensities of the fluxes generated within each system. Following exposure to fluxes of maximal intensity the viscosity of hyaluronic

Table 2-1 LOSS OF VISCOSITY INDUCED BY EACH OXYRADICAL GENERATING SYSTEM

Autoxidation of ferrous ions		XO/HX		Activated neutrophils	
Fe ion conc. (μM)	Nsp (%)	XO (U/ml)	Nsp (%)	Cell conc. (cells/ml)	Nsp (%)
0	100	0	100	0	100
25	47	0.05	23	0.6×10^6	63
50	31	0.1	22	6×10^6	60
100	19	1.0	12	12×10^6	56
200	11			12×10^6 (+SOD)	67
500	4			12×10^6 (no PMA)	65
				12×10^6 (4°C)	98

In the ferrous ion autoxidation hyaluronic acid, 1 mg/ml, was exposed to ferrous ions in the presence of 50 mM potassium phosphate buffer, pH 7.4. EDTA was present in a ratio to ferrous ions of 1:1. In the XO/HX system, hyaluronic acid, 1 mg/ml, was exposed to XO in the presence of potassium buffer, pH 7.4, and HX, 6 mM. In the cellular system hyaluronic acid, 1 mg/ml was exposed to cells in the presence of Dulbecco's phosphate buffered saline to which had been added $FeCl_3$ 10 μM, EDTA 60 μM and PMA 200 ng/ml. SOD was used at a concentration of 60 units/ml.

acid approached that of the buffer alone. The decrease in Nsp using the cellular system was partly inhibited by superoxide dismutase thus implying that superoxide ions and/or derived oxygen radicals were, at least in part, responsible.

2. Molecular exclusion gel chromatography

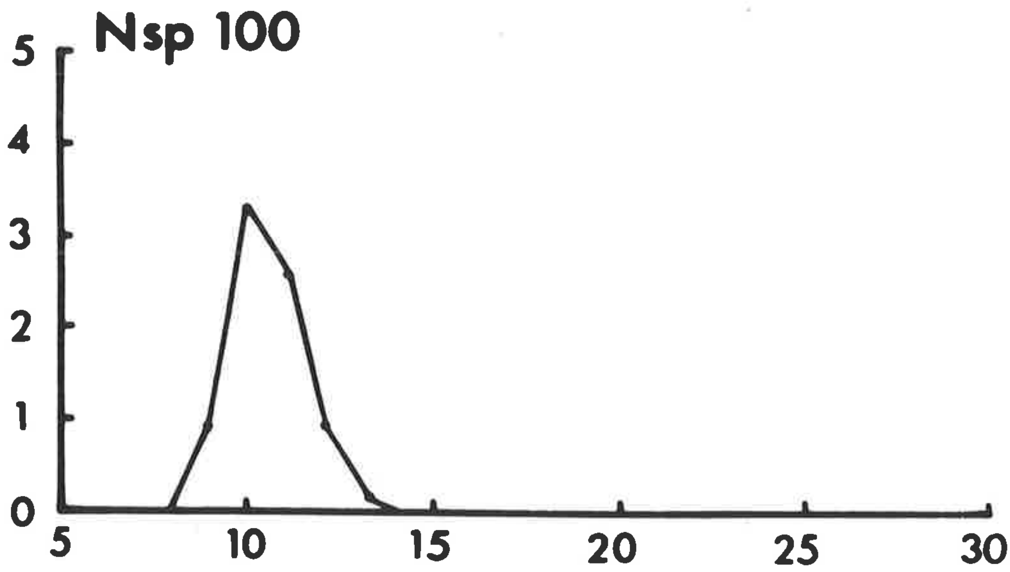
a. Sephadex G-100

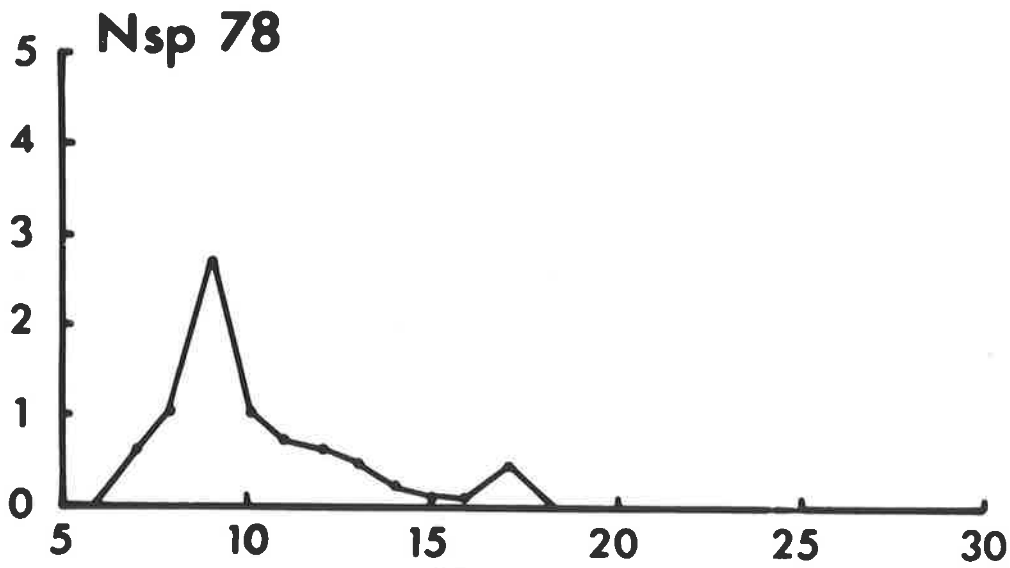
Samples of hyaluronic acid which had been exposed to different oxygen radical generating systems were applied to Sephadex G-100 columns. On elution with 0.5 M sodium acetate, hyaluronic acid from preparations with Nsp's of 10% of control, or greater, were largely excluded from the gel, while a major proportion of the hyaluronic acid from lower viscosity samples had a hydrodynamic size sufficient to be retarded by this gel. The smallest products were seen at a K_{av} of 0.66 which corresponded to a molecular weight of 10^4 (Fig. 2-1).

A further experiment was performed to determine whether the exposure of hyaluronic acid to two sequential oxygen radical fluxes would reduce the hydrodynamic size yet further. Hyaluronic acid was first exposed to the XO/HX system and an aliquot was then chromatographed (Fig. 2-2). The remainder was then exposed to the ferrous ion autoxidation system using 1 mM ferrous sulphate. After this double exposure to an oxygen radical flux no material was observed past a K_{av} of 0.66 on Sephadex G-100 although the overall change in shape of the profile seen on Sepharose 4B with a characteristic shift towards the total column volume (V_t) indicated that some further depolymerization of larger molecules had occurred.

FIGURE 2-1 SEPHADEX G-100 CHROMATOGRAPHY OF HYALURONIC ACID EXPOSED TO THE FERROUS ION AUTOXIDATION SYSTEM

Hyaluronic acid, 1 mg/ml was exposed to ferrous ions at a concentration of 50 μM (Nsp = 78%) and 1 mM (Nsp = 8%) in the presence of 50 mM potassium phosphate buffer, pH 7.4. EDTA was present in a ratio to ferrous ions of 1:1.


Nsp 100
5
4
3
2
1
0
5
10
15
20
25
30


URONIC ACID CONCENTRATION mg/ml x10^{-2}
Nsp 78
5
4
3
2
1
0
5
10
15
20
25
30

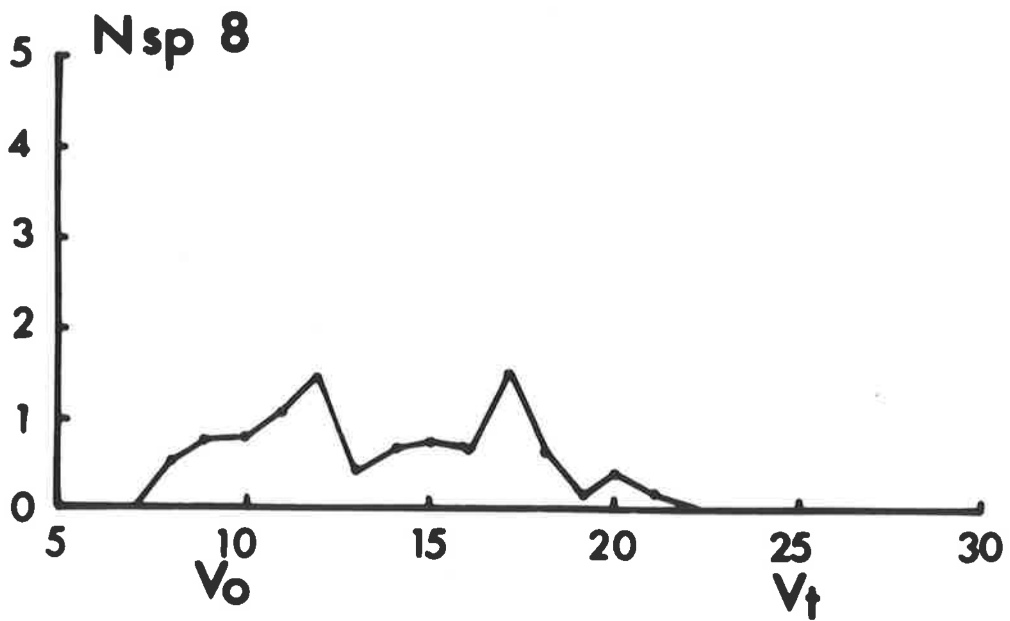

Nsp 8
5
4
3
2
1
0
5
10
15
20
25
30
Vo
Vt

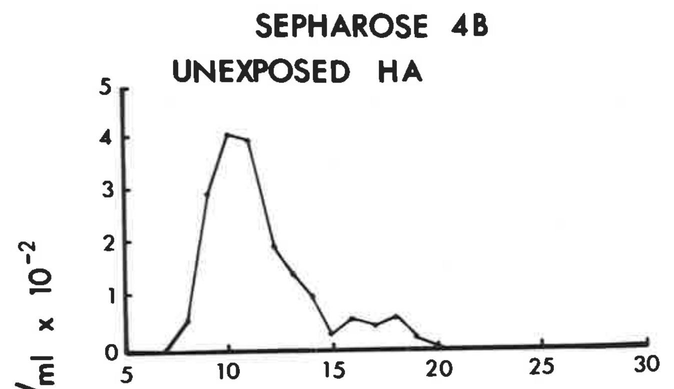
FIGURE 2-2 HYALURONIC ACID EXPOSED TO TWO SEQUENTIAL OXYGEN RADICAL FLUXES.

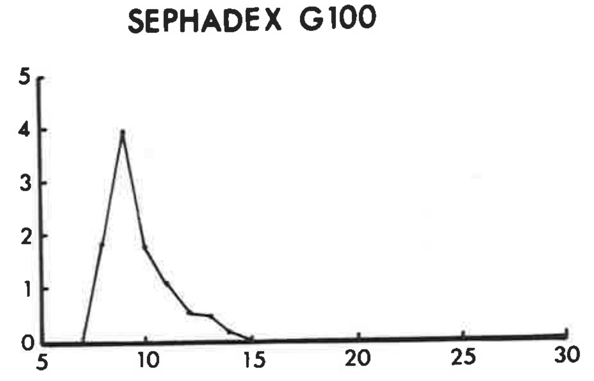
Hyaluronic acid was exposed to 0.3 units/ml XO and 6 mM HX in 50 mM phosphate buffer, pH 7.4. An aliquot was then fractionated on Sepharose 4B and another was fractionated on Sephadex G-100. These profiles are shown in the middle panel. Hyaluronic acid containing fractions were then pooled, dialyzed against water, then dried *in vacuo* over phosphorus pentoxide before exposure to the ferrous ion autoxidation using a concentration of ferrous ions of 1 mM. Profiles obtained after fractionation of this material on the same gels is shown in the bottom panel.

SEPHAROSE 4B

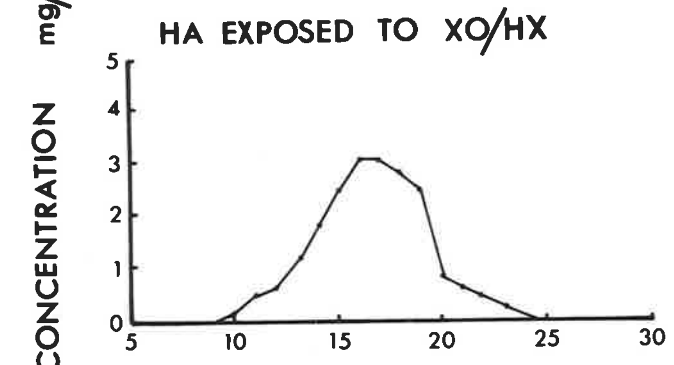
SEPHADEX G100

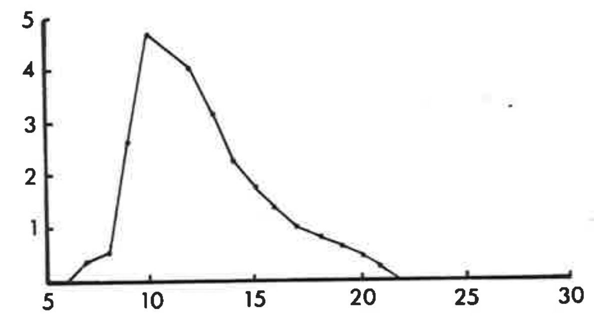
UNEXPOSED HA



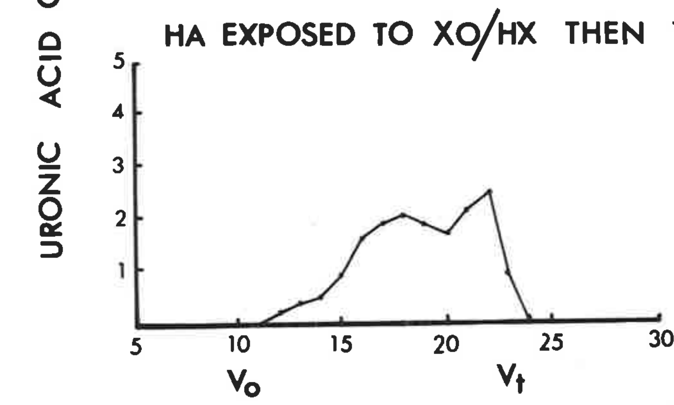


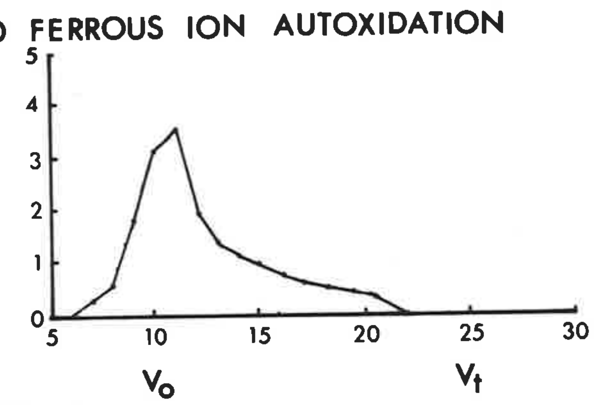
HA EXPOSED TO XO/HX





HA EXPOSED TO XO/HX THEN TO FERROUS ION AUTOXIDATION





URONIC ACID CONCENTRATION mg/ml x 10^{-2}

FRACTION

b. Sepharose 4B

Fractionation of hyaluronic acid (from human umbilical cord, Sigma type III) on Sephacryl S-400 provided a nominally high molecular weight starting material; however there was still some retarded material present on Sepharose 4B chromatography. Using this material, a series of chromatographic profiles was obtained for graded fluxes generated by the ferrous ion autoxidation, the XO/HX and the stimulated polymorphonuclear leucocyte systems. They appear as Fig. 2-3.

The effect of an increasing oxygen radical flux on these high molecular weight hyaluronic acid preparations in each of the oxygen radical generating systems, was to shift the amount of uronic acid containing material from the void volume (V_o) to the included volume, i.e., to the right. Any shift of the chromatographic profile to the right was accompanied by a decrease in Nsp in each of the generating systems. The greatest decrease in viscosity was seen in the ferrous ion autoxidation system. Here an Nsp of 5% of control was obtained and at this viscosity no uronic acid containing material was monitored at V_o. A significant amount of material was, however retarded by the gel (i.e., K_{av} = 0.47 corresponding to a molecular weight of 2×10^5). Also, with an Nsp as low as 32% of control, uronic acid containing material remained present at V_o indicating the presence of material with a hydrodynamic size of 10^6 still present in this sample.

Hyaluronic acid exposed to the stimulated polymorphonuclear leucocytes remained predominantly excluded by the gel over the range of viscosities achieved, however there was an increase in the amount of material seen in the included volume and in the samples with Nsps of 63% and 56% of control, a small amount of material was

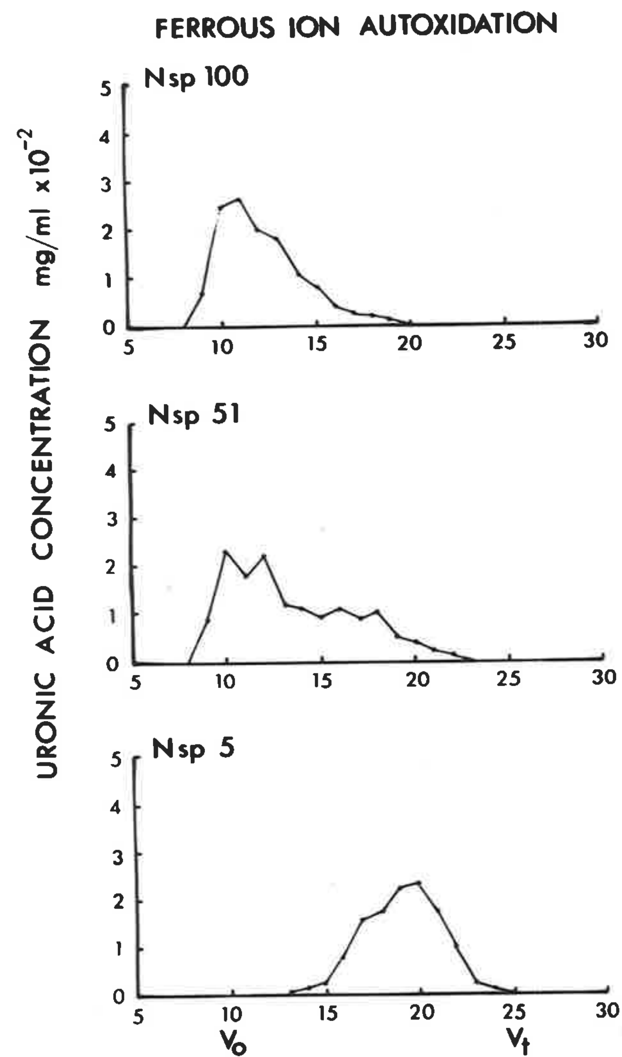
FIGURE 2-3 SEPHAROSE 4B CHROMATOGRAPHY OF HYALURONIC ACID EXPOSED TO EACH OF THE OXYGEN RADICAL GENERATING SYSTEMS

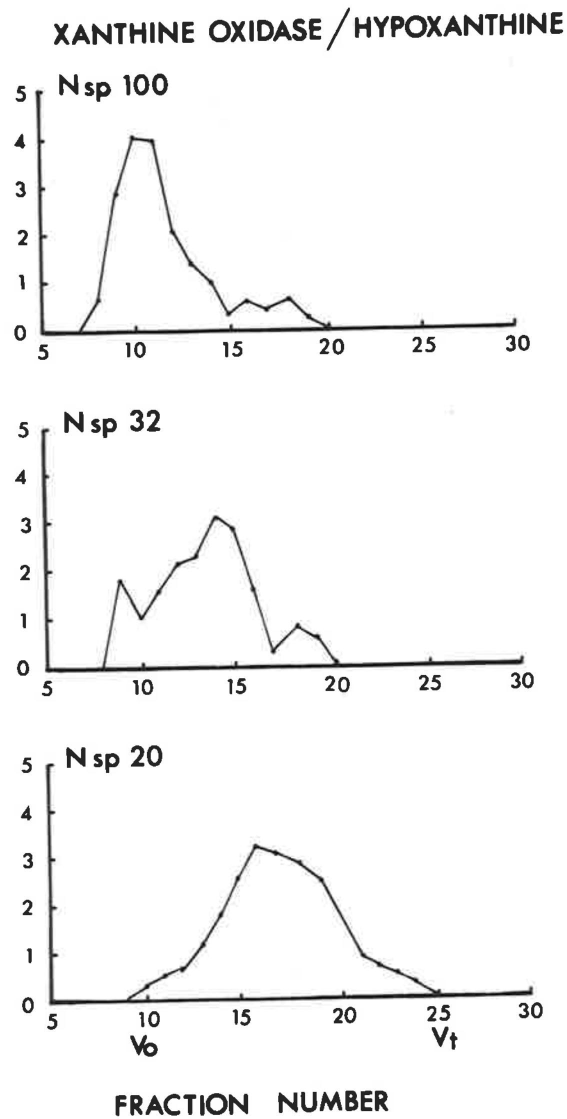
In the ferrous ion autoxidation system hyaluronic acid, 1 mg/ml, was exposed to ferrous ions at concentrations of 100 µM (Nsp = 51%) and 1 mM (Nsp = 5%) in the presence of 50 mM potassium phosphate buffer, pH 7.4. EDTA was present in a ratio to ferrous ions of 1:1.

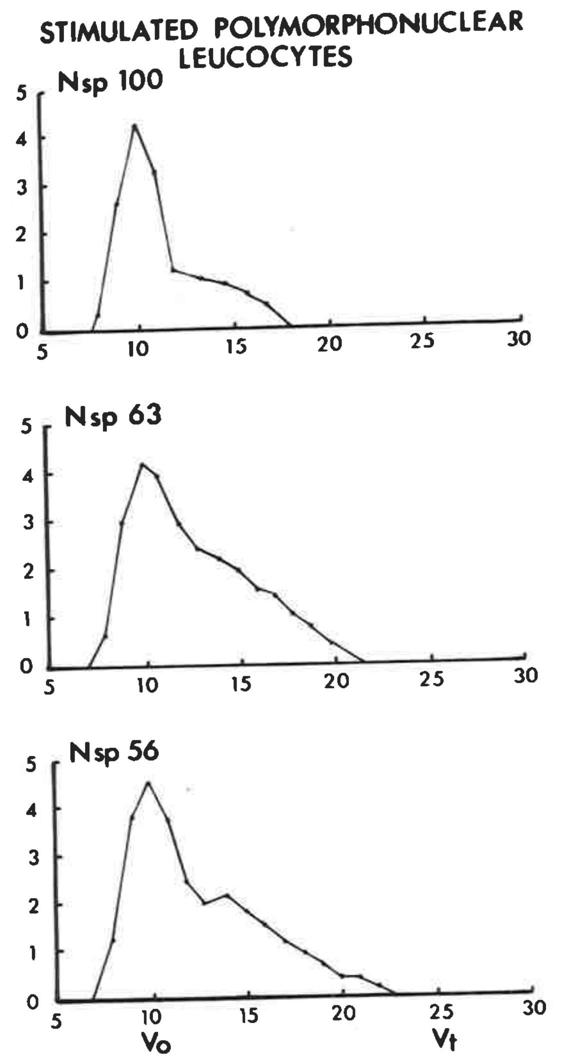
In the XO/HX system, hyaluronic acid, 1 mg/ml, was exposed to XO at concentrations of 0.1 unit/ml (Nsp = 32%) and 1 unit/ml (Nsp = 20%) in the presence of 6 mM HX and 50 mM potassium phosphate buffer, pH 7.4.

In the cellular system hyaluronic acid, 1 mg/ml, was exposed to concentrations of polymorphonuclear leukocytes of 0.6×10^6 cells/ml (Nsp = 65%) and 12×10^6 cells/ml (Nsp = 56%) in the presence of Dulbecco's phosphate buffered saline to which had been added $FeCl_3$ 10 µM, EDTA 60 µM and PMA 200 ng/ml.

URONIC ACID CONCENTRATION mg/ml x10^{-2}







FRACTION NUMBER

present at a K_{av} = 0.66 which was not present in the starting material.

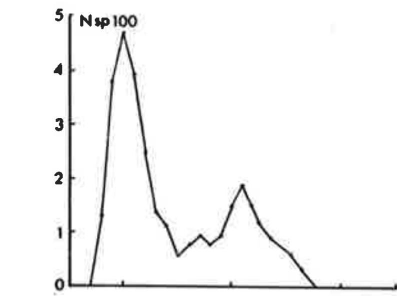
c. Sepharose 2B

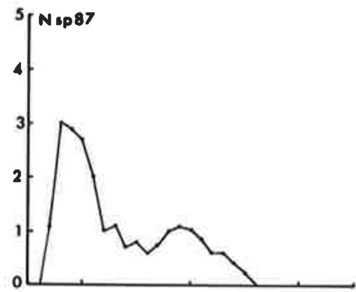
A large series of hyaluronic acid samples were exposed to the ferrous ion autoxidation system in order to resolve changes in the molecular weight spectra induced by graded oxygen radical fluxes. Small increments in ferrous ion concentration (and therefore oxygen radical flux) were used and chromatographic profiles were obtained on Sepharose 2B. A representative sample of these is shown in Fig. 2-4. Examination of these profiles indicated that there were two major peaks, one toward V_o and the other approaching V_t. As degradation progressed there was a rapid transition of material from the V_o to the V_t rather than a progression across the profile. For example in the fractionation of the sample with an Nsp of 87% of control the majority of uronic acid containing material was excluded whilst a profile obtained for a sample with an Nsp of 8% of control shows a peak of uronic acid containing material approaching V_t. In fact, between the relatively intact hyaluronic acid and the low molecular weight degradation product, intermediate sized material does not appear as a discrete peak. When the area under the curve in the first, middle and final thirds of chromatographic profiles from Sepharose 2B separation are plotted against % Nsp (Fig. 2-5), the area in the middle third does not increase above 33% of the total area.

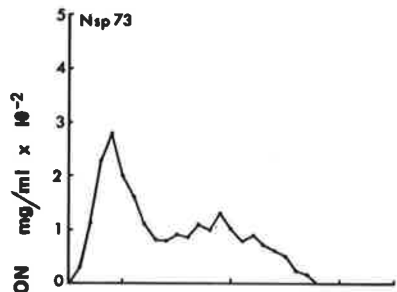
Using a high molecular weight fraction of hyaluronic acid, obtained from a preparative Sephacryl S-400 column and exposing it to the autoxidation system a similar computation was applied to the first, middle and final thirds of a series of Sepharose 4B

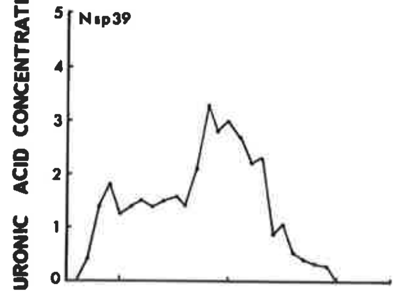
FIGURE 2-4 A SELECTION OF SEPHAROSE 2B CHROMATOGRAPHY PROFILES AFTER EXPOSURE OF HYALURONIC ACID TO THE FERROUS ION AUTOXIDATION SYSTEM USING SMALL INCREMENTS IN FERROUS ION CONCENTRATION

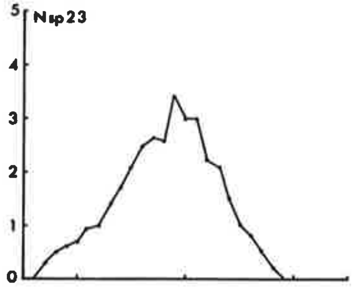
The experimental conditions were the same as outlined in Fig. 1. The concentrations of ferrous ions used in the profiles shown were 20 µM (Nsp = 87%), 50 µM (Nsp = 73%), 100 µM (Nsp = 39%), 200 µM (Nsp = 23%) and 1000 µM (Nsp = 8%).


Nsp100
5
4
3
2
1
0


Nsp87
5
4
3
2
1
0


Nsp73
5
4
3
2
1
0


Nsp39
5
4
3
2
1
0


Nsp23
5
4
3
2
1
0

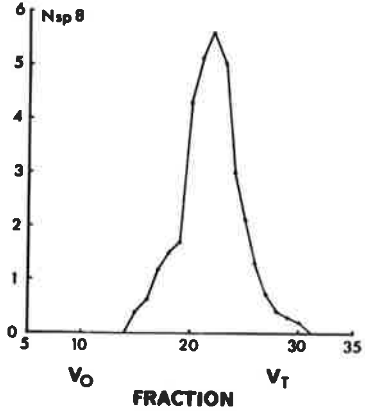
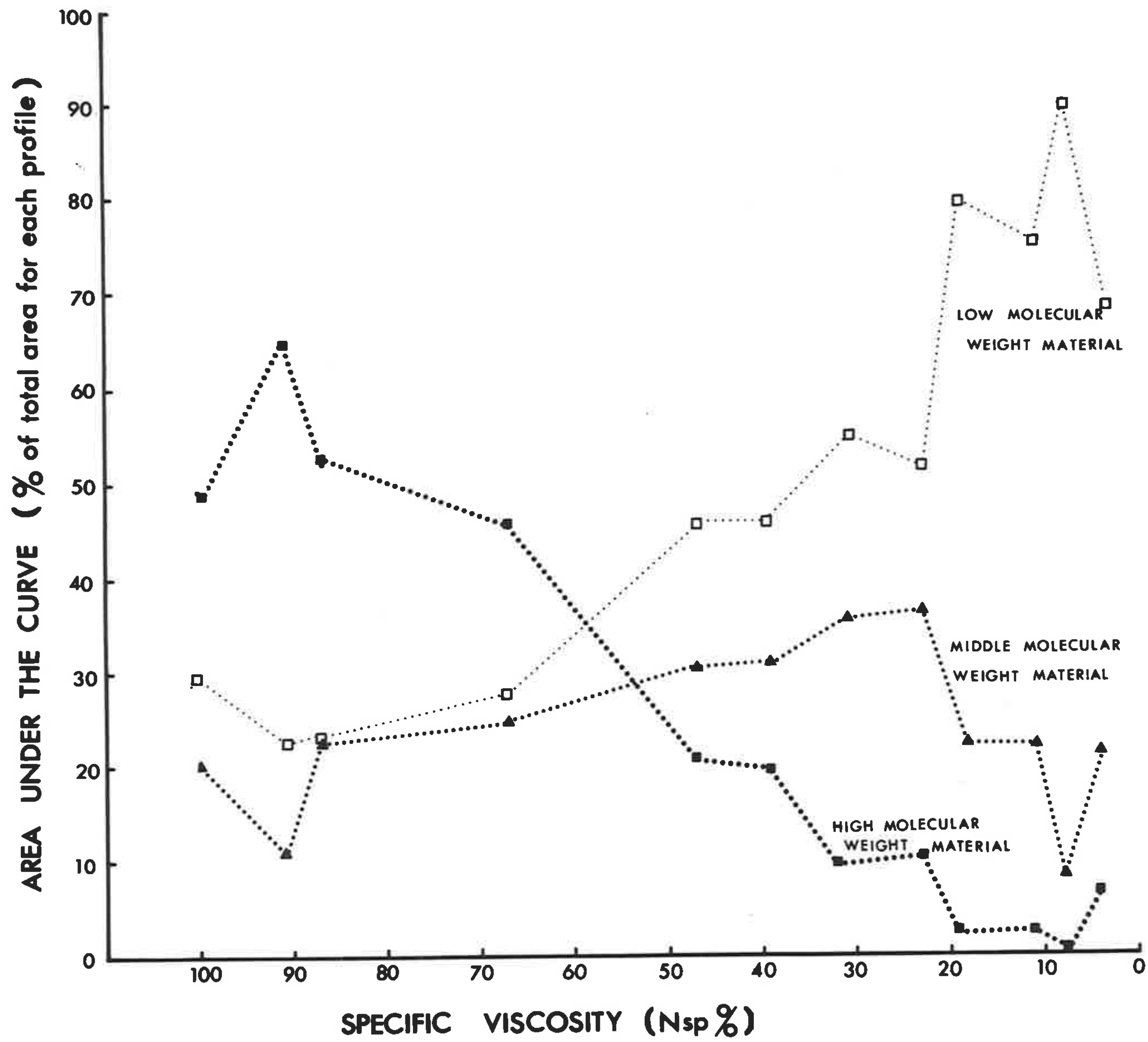

Nsp8
6
5
4
3
2
1
0
5
10
20
30
35
V_O
V_T
FRACTION
URONIC ACID CONCENTRATION mg/ml x 10^{-2}

FIGURE 2-5 AREA UNDER THE CURVE AS A FUNCTION OF % Nsp FOR HYALURONIC ACID EXPOSED TO THE FERROUS ION AUTOXIDATION SYSTEM AND THEN FRACTIONATED ON SEPHAROSE 2B-CL

A large series of hyaluronic acid samples were exposed to the ferrous ion autoxidation system and then fractionated on Sepharose 2B. In each profile obtained, the area under the curve in each third was measured. This figure shows those measurements plotted as a function of % Nsp.

The area for the first third of each profile (■·····■) therefore represents high molecular weight material. The area under the curve for the middle third of each profile (▲·······▲) represents middle molecular weight material and the last third of each profile (□·······□) represents small molecular weight material.



AREA UNDER THE CURVE (% of total area for each profile)
100
90
80
70
60
50
40
30
20
10
0
LOW MOLECULAR
WEIGHT MATERIAL
MIDDLE MOLECULAR
WEIGHT MATERIAL
HIGH MOLECULAR
WEIGHT MATERIAL
100
90
80
70
60
50
40
30
20
10
0
SPECIFIC VISCOSITY (Nsp %)

fractionations. Again no more than 33% of the uronic acid appeared in the transition portion of the profiles (Fig. 2-6).

3. Analytical ultracentrifugation

Sedimentation of hyaluronic acid and its oxygen radical induced degradation products was achieved on the analytical ultracentrifuge. The reciprocals of the sedimentation coefficients (So^{-1}) and the extrapolations to derived M_{sD} values [CLELAND and WANG, 1970; BARTOLD, WIEBKIN and THONARD, 1984(b)] are shown in Table 2-2. Three samples of hyaluronic acid were used, each of a different starting molecular weight. They were all exposed to the ferrous ion autoxidation system. Again a lower molecular weight limit of approximately 10^4 was obtained. The calculated molecular weight values (M_{sD}) followed a predictable overall drop with decreased viscosity. However in Experiment 1 the sample exposed to 20 µM ferrous ions (Nsp = 87% of control) had an M_{sD} of 60,000, whereas in the sample exposed to 100 µM ferrous ions (Nsp = 39% of control) the M_{sD} value was 88,000. Similarly in Experiment 3, where more samples were analyzed, the lowest M_{sD} value calculated was 9,000 in the sample that had been exposed to 125 µM ferrous ions, whereas samples exposed to 150 and 200 µM ferrous ions showed M_{sD} values to 39,000 and 42,000 respectively prior to a further drop occurring.

D. DISCUSSION

The commercial hyaluronic acid used in this study was supplied as a lyophilysate and Sepharose 4B chromatography shows this to be polydisperse as regards hydrodynamic size. Attempts to obtain a high molecular weight hyaluronic acid preparation by fractionation on Sephacryl S-400 chromatography with subsequent dialysis and lyophilization of the fractions reduced the amount of material

FIGURE 2-6 AREA UNDER THE CURVE AS A FUNCTION OF % Nsp FOR PURIFIED HYALURONIC ACID EXPOSED TO THE FERROUS ION AUTOXIDATION SYSTEM THEN FRACTIONATED ON SEPHAROSE 4B-CL

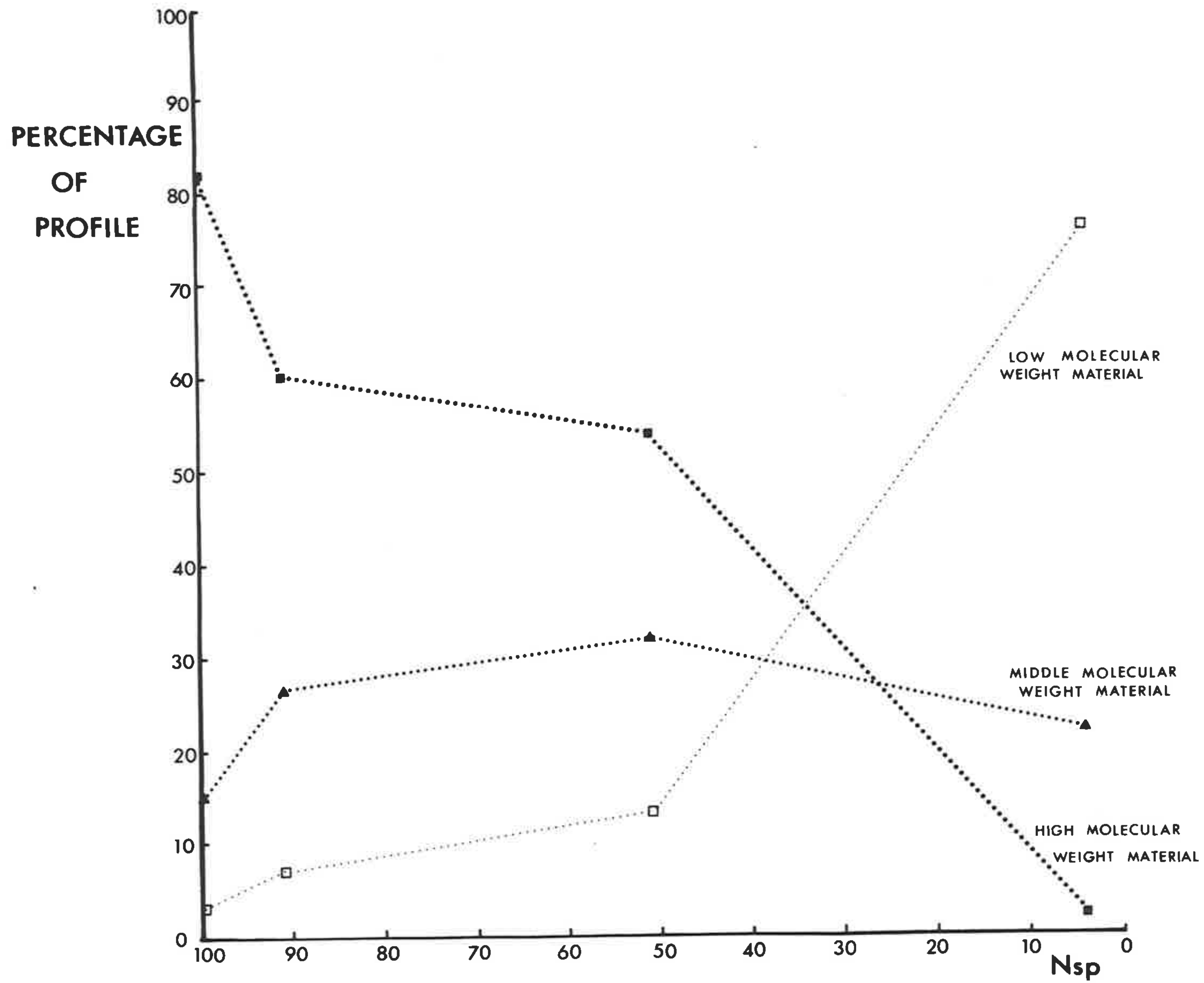

PERCENTAGE
OF
PROFILE
100
90
80
70
60
50
40
30
20
10
0
100
90
80
70
60
50
40
30
20
10
0
Nsp
LOW MOLECULAR
WEIGHT MATERIAL
MIDDLE MOLECULAR
WEIGHT MATERIAL
HIGH MOLECULAR
WEIGHT MATERIAL

Table 2-2 MOLECULAR WEIGHT ESTIMATION OF HYALURONIC ACID EXPOSED TO THE FERROUS ION AUTOXIDATION SYSTEM USING THE ANALYTICAL ULTRACENTRIFUGE

Exp.	Ferrous ion concentration micromolar	Specific viscosity Nsp (%)	Reciprocal of the sedimentation coefficient at zero concentration ($1/So^{-1}$)	Calculated molecular weight (MsD)
1	0	100	0.060	1,450,000
	10	91	0.045	84,000
	20	87	0.485	60,000
	100	39	0.418	88,000
2	0	100	0.297	232,000
	20	89	0.302	213,000
	100	41	0.422	95,000
	1000	10	0.583	43,000
3	0	100	0.428	92,000
	25	86	0.417	98,000
	50	72	0.495	65,000
	75	56	0.518	58,000
	100	52	0.897	15,000
	125	46	1.106	9,000
	150	35	0.610	39,000
	200	24	0.589	42,000
	400	13	0.906	15,000

For each experiment, the reaction concentration of hyaluronic acid was 1 mg/ml, EDTA was present in a ratio to ferrous ions of 1:1 and the buffer used was 50 mM phosphate buffer. The sedimentation coefficients were then determined on concentrations of hyaluronic acid of 1 gm/ml, 0.5 mg/ml and 0.25 mg/ml, although at the latter concentration in some of the more depolymerized samples no peak was identified and therefore no value for the sedimentation constant could be used. Since the hyaluronic acid concentrations used were relatively low, a linear regression of the reciprocal of the sedimentation coefficient could be used to determine So^{-1} [BARTOLD, WIEBKIN and THONARD, 1984].

retarded on Sepharose 4B chromatography but did not eliminate it entirely. This preparation was performed before the publication of the work of WEDLOCK et al. [1983]. They showed that lyophylization is accompanied by radical generation and depolymerization. Nevertheless a large amount of material in the commercial product that was retarded by Sepharose 4B-CL was able to be eliminated. All subsequent preparations were made without lyophylization.

The controlled exposure of hyaluronic acid to oxygen radicals is accompanied by a predicted decrease in viscosity and the appearance of a polydisperse population of breakdown products. This phenomenon is noted whether the radicals are generated by the autoxidation of ferrous ions, the action of xanthine oxidase on hypoxanthine or by stimulated peripheral blood polymorphonuclear leucocytes. GREENWALD and MOY [1980] have reported two Sepharose 2B-CL chromatographic profiles which adequately demonstrate a generalized reduction of hydrodynamic size of hyaluronic acid exposed to the XO/HX system and to PMA stimulated neutrophils. We have endeavoured to define the progress of oxygen radical induced degradation by increasing the number of profiles analyzed. Of the oxygen radical producing systems studied, the ferrous ion autoxidation system could be most readily manipulated to provide a comprehensive series of degraded hyaluronic acid samples. Less extensive studies using the XO/HX system produced congruent findings compatible with a similar form of hydroxyl radical attack on hyaluronic acid in both systems. The relatively modest degradation achieved by PMA stimulated polymorphonculear leucocytes could be partly explained by: (a) lesser fluxes of superoxide

(not quantitated) and/or (b) less uniform production of oxygen radicals by the cellular system.

The effects of oxygen radicals on hyaluronic acid observed by concurrently monitoring several parameters (i.e., viscosity, change in hydrodynamic profile on several gels and M_{SD}) indicated that change in viscosity is a sensitive indicator of early damage to hyaluronic acid. For example, a sample with an Nsp = 38% of control (Fig. 2-3) still contains a significant amount of material that is excluded on Sepharose 4B chromatography. An explanation of this may be evident from the concept expounded by BALAZS [1974] and others that hyaluronic acid in solution forms an interlocking molecular matrix. If several large chains of hyaluronic acid in this molecular matrix are depolymerized, the matrix would become less ordered, despite the persistence of some intact molecules. Indeed fractionation of hyaluronic acid on Sepharose 4B after exposure to a mild oxygen radical flux showed some excluded material, representing the intact chains together with some material in the included volume representing the depolymerized chains. Yet as a whole the solution was dramatically altered in viscosity. In turn these changes are likely to result in alterations in physiological properties.

In the present study the smallest degradation products obtained had a hydrodynamic size of the order of 10^4. This lower limit was not shifted by subsequent exposure to a second oxygen radical flux.

Analysis of the large series of chromatographic profiles obtained with the ferrous ion autoxidation system suggested that the depolymerization of hyaluronic acid by hydroxyl radicals may be at least partly ordered with rapid progression from large to small

material, such as may occur with a chain reaction of cleavage events.

The observed increase in sedimentation coefficient of lower viscosity hyaluronic acid samples on analytical ultracentrifugation may reflect some form of molecular interaction (aggregation) or conformational change due to crosslinking. BALAZS et al. [1967] have postulated that, during pulse radiolysis of hyaluronic acid, in addition to direct oxidative cleavage of the glycosidic linkage between glucuronic acid and N-acetylglucosamine, uronate free radicals may be formed by elimination of the hydrogen at C5. This radical would then be resonance stabilized by interaction with the carboxyl group. These radicals could interact to form cross-linked hyaluronate degradation products. Indeed the identification of these branched products in synovial fluid could be used to more directly implicate radical-induced damage to macromolecules in inflamed joints. Certainly such structural alterations could have important consequences in terms of the altered physiological properties of the hyaluronate matrix.

E. SUMMARY

Preparative chromatographic fractions of human umbilical cord hyaluronic acid of a molecular weight of 10^6 were subjected to graded oxygen radical fluxes produced by (a) the autoxidation of ferrous ions, (b) the action of xanthine oxidase on hypoxanthine and (c) by peripheral blood polymorphonuclear leucocytes which had been stimulated by PMA. Analysis by gel chromatography of the products obtained with each of the oxygen radical generating systems revealed polydispersity in size. The smallest molecules detected had a molecular weight of 10^4. This limiting size was not reduced further

by exposure to a second oxygen radical flux. The relative proportions of large, medium and small degradation products were established for various levels of oxygen radical flux. Consistently a relatively rapid transition from large to small material was seen on Sepharose 2B chromatography suggesting an ordered element to the breakdown process. Although the decrease in molecular weight, after oxygen radical exposure, was confirmed by analytical ultracentrifugation, this procedure revealed that samples of lower viscosity did not necessarily exhibit lower sedimentation values, possibly reflecting oxygen radical-induced repolymerization. By altering size and possibly the conformational characteristics of hyaluronic acid, oxygen radical exposure may be expected to alter its biological properties.

CHAPTER III

CHANGES IN MOLECULAR WEIGHT SPECTRA OF SYNOVIAL FLUID HYALURONIC ACID SEEN IN INFLAMMATORY JOINT DISEASE

A. INTRODUCTION.

The changes in several parameters of synovial fluid hyaluronic acid in response to inflammation have been well characterized. These include changes in concentration, average molecular weight and intrinsic viscosity and are summarized in Table 1-1, page 41. However, sparse and conflicting information exists concerning the effect of inflammation upon the polydispersity of molecular weights of hyaluronic acid from inflamed synovial fluids. One means of implicating or discounting the possibility of oxygen radical induced hyaluronic acid depolymerization in inflamed joints is comparison of the molecular weight profiles of hyaluronic acid isolated from inflammatory synovial effusions to profiles seen after exposure of hyaluronic acid to radical fluxes _in vitro_. The study described in Chapter II provided a large range of profiles demonstrating the changes noted in the molecular weight of hyaluronic acid as the size of the oxygen radical flux was increased. However no suitable data obtained from inflamed and non-inflamed synovial fluid hyaluronic acid was available in the literature to enable a valid comparison.

GREENWALD [1980] has reported in abstract that in 21 patients with rheumatoid arthritis none of the synovial fluid hyaluronic acid was of sufficiently small hydrodynamic size to be retarded on Sepharose 2B-CL chromatography. In another study by BALAZS, BRILLER and DENLINGER [1981] who analysed molecular weight profiles of synovial fluid hyaluronic acid from 15 horses with traumatic arthritis and 20 humans with osteoarthritis, a large amount of hyaluronic acid was identified within the included volume of glyceralized controlled pore glass columns calibrated between 6×10^6 and 5×10^4. Their results therefore indicate the presence of material which would be included upon Sepharose 2B and Sepharose 4B-CL chromatography. More recently BJELLE,

ANDERSSON and GRANATH [1982] have reported gel profiles of 17 "arthritic" patients, predominantly rheumatoid arthritis, using a composite gel column packed with consecutive portions of Sepharose 2B, Sepharose 6B and agarose 0.5%, cross-linked with divinyl-sulphone. This composite gel required the treatment of their samples with 2.5 M sodium hydroxide (pH < 12) and their column was eluted using a buffer with a pH 11.6. This strong alkaline treatment leaves this technique open to criticism because of the possibility of alkaline hydrolysis of the hyaluronic acid. However the authors claim this would have been minimal due to the rapidity of analysis (< 12 hrs), yet no internal standard was shown to demonstrate this. Nevertheless they showed a wide variety of molecular weight distributions with material still present at a Kav corresponding to a molecular weight of 2×10^4 on their column.

None of the above studies have included a control group of "normal" fluids. The results of these latter two studies suggested that a portion of hyaluronic acid from inflamed synovial fluids would elute within the included volume after fractionation on Sepharose 4B-CL. The following study was therefore undertaken with the following aims:-

1. To determine the molecular weight profiles of synovial fluid hyaluronic acid from a series of patients with inflammatory arthropathies.

2. To determine similar molecular weight profiles for a group of non-inflamed synovial fluids.

3. To determine whether any correlation exists between the amount of material retarded by these gels and clinical parameters of inflammation (erythrocyte sedimentation rate and synovial fluid cell count).

4. To determine whether extracted synovial fluid hyaluronic acid was susceptible to an in vitro oxygen radical flux in the same way as commercially available human umbilical hyaluronic acid used in the previous study.

Non-inflamed synovial fluid samples were taken from fresh cadavers. In order to be included in the study, there was to be no history of sepsis, malignancy or arthropathy. In addition there was to be no macroscopic evidence of osteoarthritis or other arthropathy at autopsy.

B. METHODS

Sepharose 4B-CL, and Sephadex G100 were obtained from Pharmacia, Uppsala, Sweden. Cellogel cellulose acetate membranes were obtained from Chemetron, Milan, Italy. Alcian blue was obtained from Difco, Surrey, UK. Glycosaminoglycan standards were a gift from Dr. M.B. Matthews, Chicago. All other chemicals were of the highest purity available from Sigma Chemical Company, St. Louis, Missouri or Ajax Chemicals, Sydney, Australia.

1. Synovial Fluid

Details of patients are given in Table 3-1. The erythrocyte sedimentation rate was determined by the Westergren method and synovial fluid white cells were counted using a haemocytometer. After aseptic aspiration of the knee joint in each case the synovial fluid was placed into a sterile tube (without anti-coagulant) and diluted with an equal volume of normal saline. Details of the cadaver subjects are given in Table 3-2. Synovial fluid from these cadavers was obtained within 6 hours of death by closed irrigation of the joint with 10 ml of normal saline, after which the joint was opened to establish the lack of macroscopic evidence of joint pathology. The cellular content of the fluids thus obtained was removed by

Table 3-1 CLINICAL DETAILS FROM PATIENTS WITH ARTHROPATHY

Patient (yrs)	Sex	Age	Duration of disease (yrs)	ESR (mm/hr)	Synovial fluid white cell count (cells/mm^3)	% of total uronic acid retarded by Sepharose 4B-CL
Seropositive rheumatoid arthritis						
P1	F	63	12	109	NA*	20
P2	F	71	3	108	NA	29
P3	M	66	13	105	900	16
P4	M	43	11	68	NA	10
P5	F	47	25	50	4,500	21
P6	F	79	16	39	31,900	27
P7	M	60	12	32	100	12
P8	F	52	18	24	NA	14
P9	M	67	24	20	NA	13
P10	F	48	19	19	4,500	14
P11	F	36	14	12	NA	19
Seronegative rheumatoid arthritis						
P12	F	68	16	81	1,400	27
P13	F	67	19	70	4,500	22
P14	F	74	11	51	9,400	18
P15	F	23	2 mo.	15	400	9
P16	M	80	2	8	1,600	15
Psoriatic arthritis						
P17	M	61	6	8	6,200	11
P18	M	45	2	4	1,700	11
Reiter's syndrome						
P19	M	19	2 mo.	23	100	35
Ankylosing spondylitis with peripheral arthropathy						
P20	F	75	25	45	2,800	17
Osteoarthritis						
P21	F	59	25	13	NA	31
P22	F	79	25	14	NA	6

*NA = not available

Table 3-2 DETAILS FROM THE CADAVER SUBJECTS

Subject	Sex	Age	Autopsy diagnosis	% Material retarded by Sepharose 4B-CL
C1	M	55	Myocardial infarction	7
C2	M	57	Myocardial infarction	10
C3	F	77	Ruptured aorta	10
C4	M	58	Subarachnoid haemorrhage	14

centrifugation at 1000 x g for 15 min. The supernatant was collected and frozen until assayed.

2. Hyaluronic acid separation

Synovial fluid hyaluronic acid was isolated by precipitation with cetyl trimethyl ammonium bromide (CTAB) according to the method of SCOTT [1960]. A 1% solution of CTAB in 0.3 M sodium chloride was added to synovial fluid in 0.3 M sodium chloride in a ratio of 3:1. This was then centrifuged for 30 min at 14,000 x g to remove any precipitate. Water was then added dropwise to the synovial fluid: CTAB mixture until the sodium chloride concentration approached 0.075 M at which stage a heavy precipitate formed. This contained the hyaluronic acid-CTAB complexes. The sodium chloride concentration was then decreased to 0.047 M to ensure that all the hyaluronic acid present was in the precipitate. This was then centrifuged (5,000 x g for 10 min) before removal of the supernatant (S2). The precipitate was then redissolved in 1 M sodium chloride at which stage the hyaluronic acid CTAB complexes dissociate. This solution was then reprecipitated with 4 volumes of ethanol at 3°C and recentrifuged. This procedure was repeated twice, after which the final precipitate was redissolved in water (there being enough salt present in the pellet to allow this to occur). It was then ethanol precipitated, centrifuged and dried *in vacuo* over phosphorus pentoxide. The removal of the protein from the synovial fluids was monitored by optical scanning of the supernatants between 190-370 nm on a Perkin-Elmer spectrophotometer. It was determined that material that absorbed at 280 nm was removed with supernatant S2 and the final product had negligible absorption at this wavelength.

3. Cellulose acetate gel electrophoresis

In order to demontrate that the glycosaminoglycan in the final product was hyaluronic acid, cellulose acetate gel electrophoresis was performed according to the method of BARTOLD, WIEBKIN and THONARD [1982]. A characteristic "fingered" Alcian blue staining band on electrophoresis corresponding to the migration of a standard sample of hyaluronic acid was required before the sample was further analyzed.

4. Gel chromatography

Aliquots of 0.2 ml of a solution of purified synovial fluid hyaluronic acid (1 mg/ml in buffer) were eluted on Sepharose 4B-CL columns (30 x 0.7 cm) at 7 ml/hr using 0.5 M sodium acetate, pH 5.6, as the buffer. Fractions of 0.7 ml were collected. The columns were characterized using Dextran blue, ^{35}S-sulphate and glucuronic acid. Retarded volume material was defined as uronic acid containing material beyond fraction 12 (void volume at fraction 10). Areas under the curve for each profile were measured on a Hewlett-Packard 987A digitizer.

5. Uronic acid analysis

Uronic acid was determined by the meta-hydroxy diphenyl method of BLUMENKRANTZ and ASBOE-HANSEN [1973] or a modification of the automated method of ROSENTHAL, BENTLEY and ALBIN [1976].

6. Oxygen radical generation

The ferrous ion autoxidation system was used as outlined in Chapter II. A stock solution of 2 mg/ml was made from the extracted desiccated non-arthritic cadaver synovial fluid hyaluronic acid in potassium phosphate buffer, 50 mM, pH 7.4. The reaction mixture consisted of hyaluronic acid, 1 mg/ml, buffer, ferrous sulphate at concentrations of 0, 50 and 1000 μM and EDTA

was present in a molar ratio to ferrous ions of 1:1. Viscosity was measured after 2 hr as described in Chapter I.

C. RESULTS

Fractionation with subsequent analysis of raw synovial fluid resulted in the presence of another material or materials that eluted at least partly within the included volume and which interfered with the uronic acid assay used. Using the CTAB precipitation method, as outlined above, no other glycosaminoglycans were detected by Alcian blue staining of the electrophoretically separated samples (Fig. 3-1) and no interference occurred with the uronic acid assay. It was also established that degradation of control samples of human umbilical cord hyaluronic acid did not occur during the separation procedure.

Fractionation on Sepharose 4B-CL of synovial fluid hyaluronic acid from control subjects demonstrated that the uronic acid containing material eluted at the V_0 of the column. Peaks were either symmetrical (Fig. 3-2, A) or were slightly skewed towards the included volume. The mean % of total uronic acid in the retarded material was 10.3 (SD = 2.9%, SEM = 1.4%) [Fig. 3-3].

For the patients with inflammatory arthropathies the mean % of uronate in the included volume after fractionation on Sepharose 4B-CL was 18.0 (SD = 7.0, SEM = 1.6) [Fig. 3-3]. This was significantly different. In most cases this was present as a skewing of the excluded volume material peak (Fig. 3-2, B), although in some cases there was a separate peak of retarded uronic acid-containing material (Fig. 3-2, C). The Kav's of these peaks varied between 0.27 and 0.75, corresponding to molecular weights between 9×10^5 and 2×10^4. No material was seen with a molecular weight lower than 2×10^4. This finding was confirmed by

FIGURE 3-1 ELECTROPHORESIS OF STANDARD GLYCOSAMINOGLYCANS AND EXTRACTED PATIENT AND CONTROL SAMPLES.

5 microliters of each sample (1 mg/ml) was applied to a 0.5 cm line on cellulose acetate strips and electrophoresed at 50 volts/cm in 0.2 M calcium acetate. The strips were stained with 0.2% Alcian blue.

Top panel: Standards

a. Hyaluronic acid

b. Chondroitin 4 sulphate

c. Chondroitin 6 sulphate

d. Mixture of each of the above

Bottom panel: Sample patients and controls

e. Hyaluronic acid standard

f. C_2

g. P_{14}

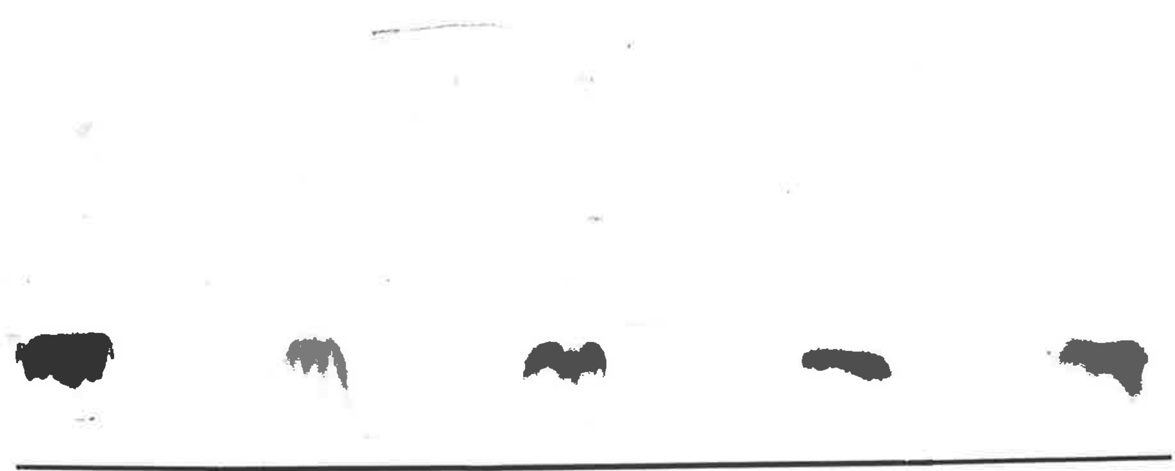
h. P_{19}

i. P_3

+ve

a b c d

−ve



e f g h i

FIGURE 3-2 CHROMATOGRAPHIC PROFILES OF EXTRACTED SYNOVIAL FLUID HYALURONIC ACID AFTER FRACTIONATION ON SEPHAROSE 4B-CL.

(1A) Control subject,

(1B) seronegative rheumatoid arthritis,

(1C) seropositive rheumatoid arthritis,

(1D) psoriatic arthritis,

(1E) Reiter's syndrome,

(1F) patient rejected as a control who died after a prolonged illness that included a lymphoma and a mediastinal abscess complicated by an episode of septicaemia.

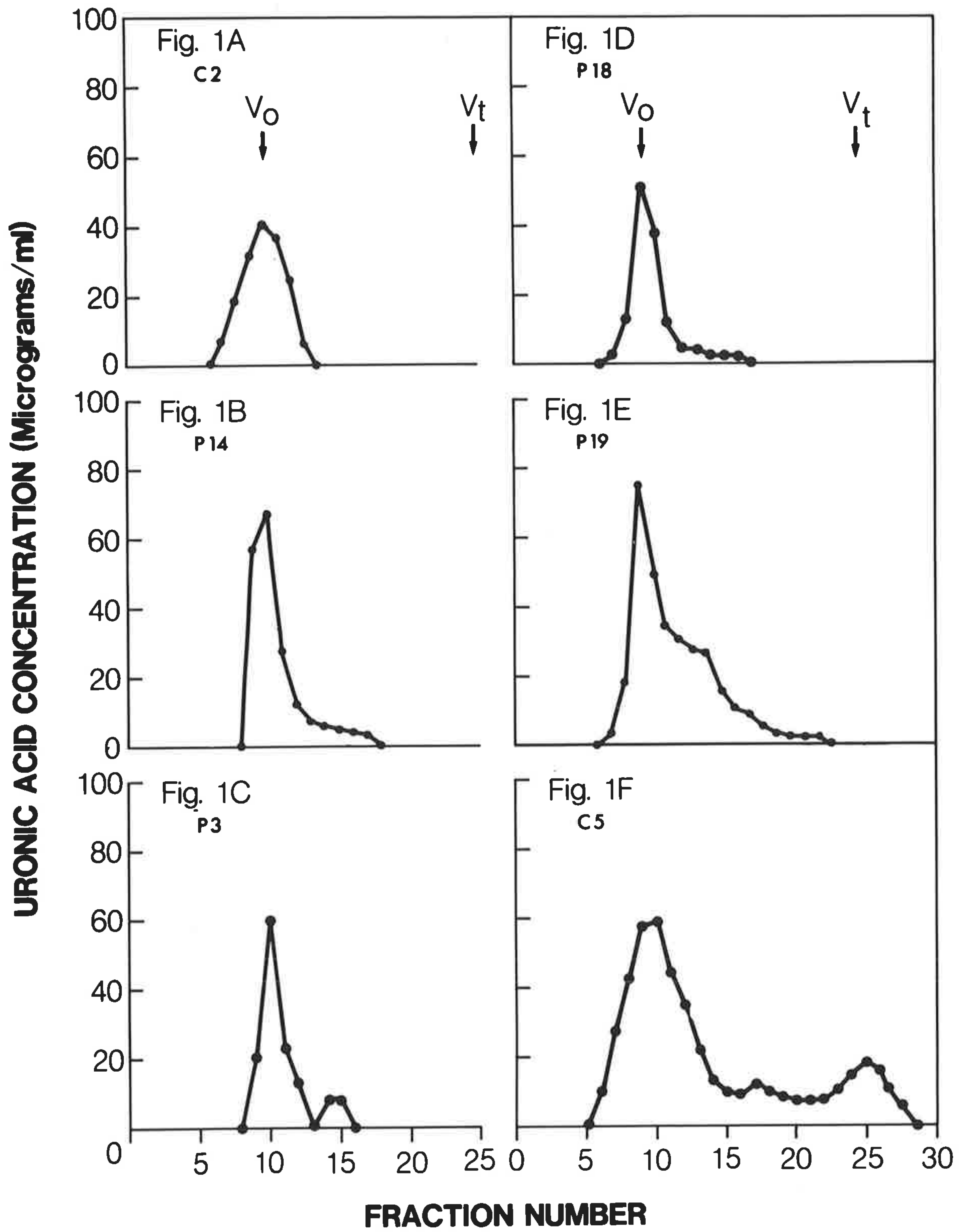
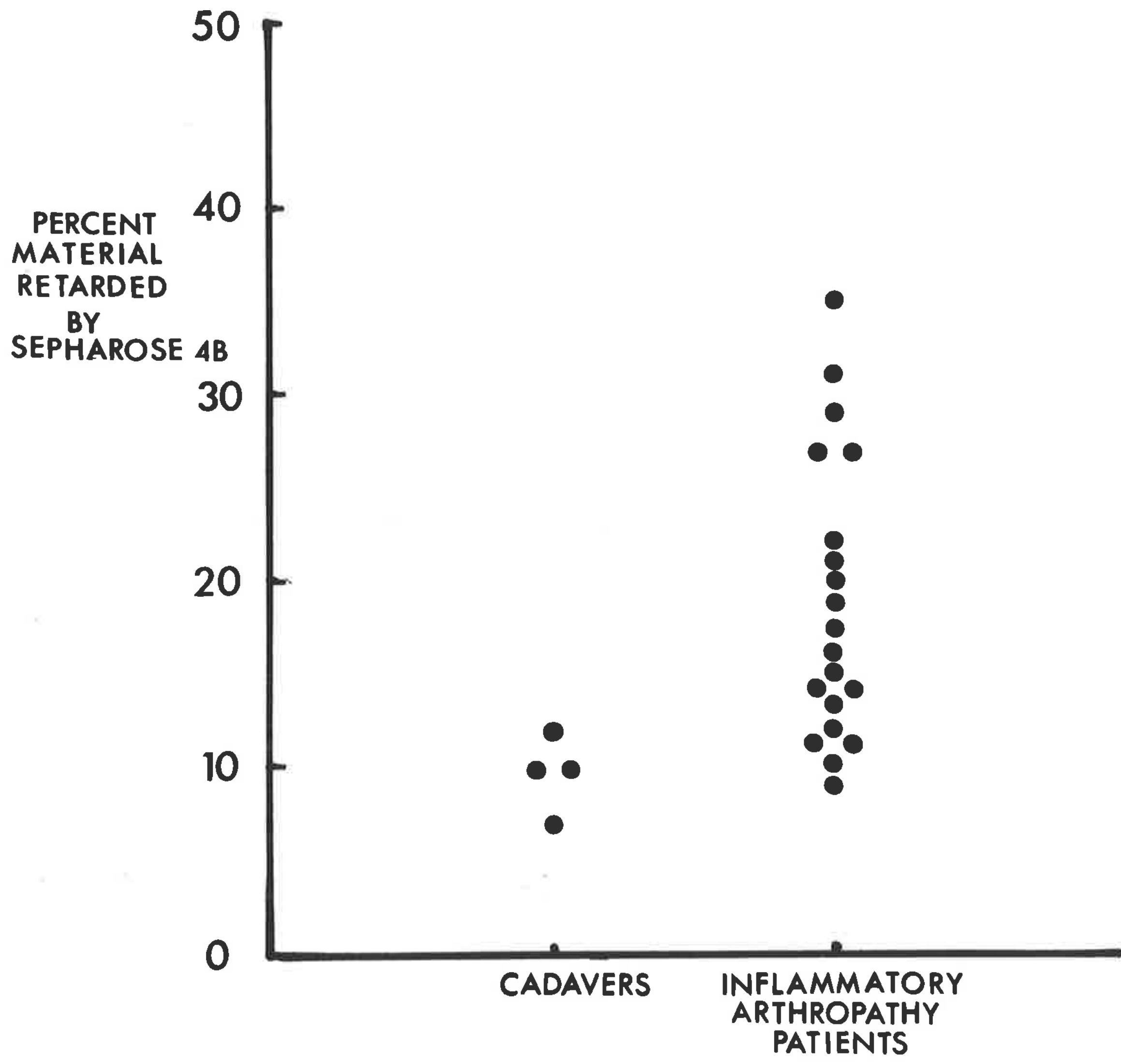


Fig. 1A
C2
Fig. 1B
P14
Fig. 1C
P3
Fig. 1D
P18
Fig. 1E
P19
Fig. 1F
C5
V_o
V_t
URONIC ACID CONCENTRATION (Micrograms/ml)
FRACTION NUMBER

FIGURE 3-3 PERCENTAGES OF MATERIAL RETARDED BY SEPHAROSE 4B-CL IN CADAVER SUBJECTS AND INFLAMMATORY ARTHROPATHY PATIENTS.


50
40
30
20
10
0
PERCENT
MATERIAL
RETARDED
BY
SEPHAROSE 4B
CADAVERS
INFLAMMATORY
ARTHROPATHY
PATIENTS

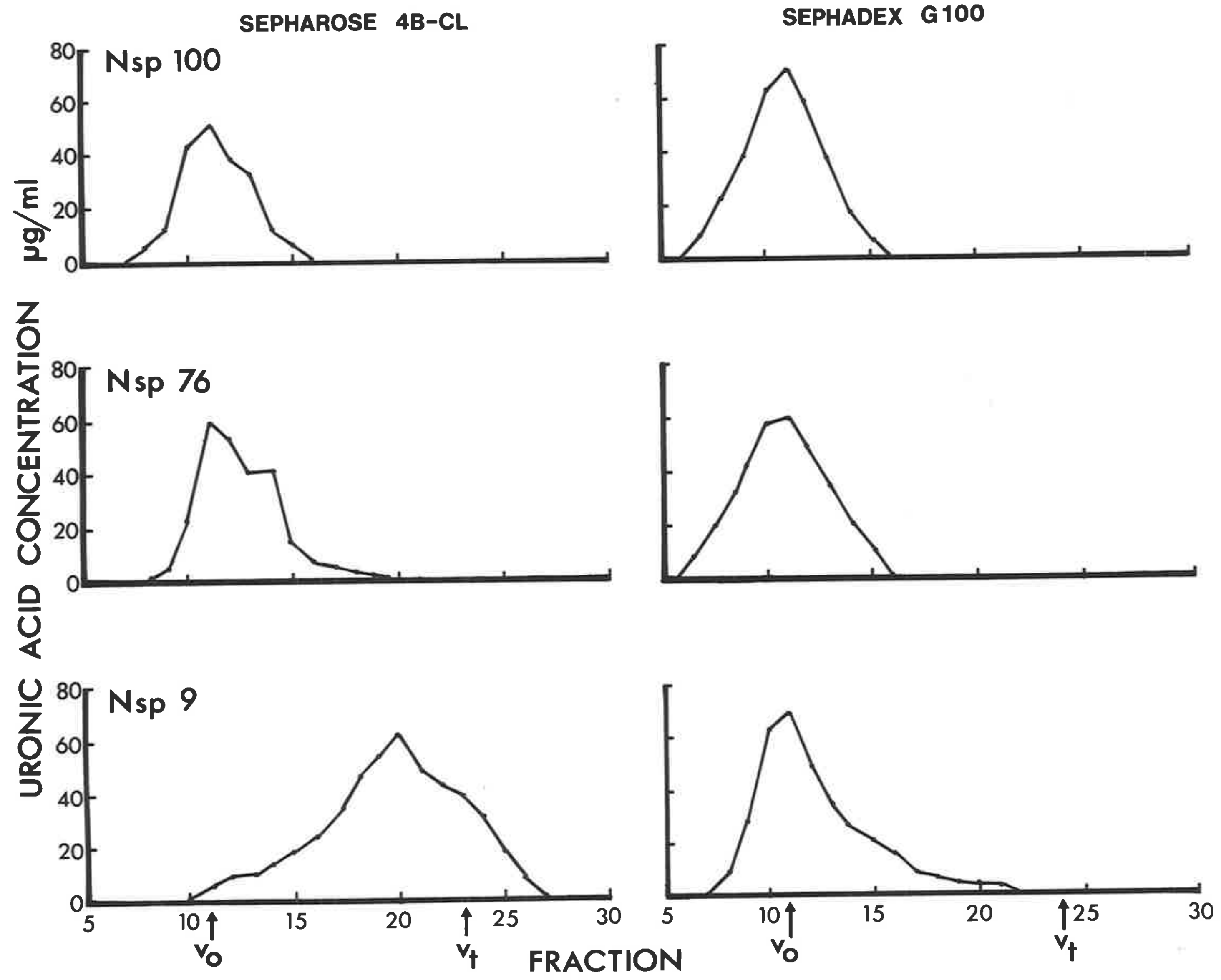
fractionation on Sephadex G100 as all material analysed from the inflammatory arthropathy and control groups was excluded from this gel. Fractionation of several samples on Sepharose 2B-CL (data not shown) resulted in similar profiles and these were not altered by fractionation under dissociative conditions using 4M guanidine HCL as the elution buffer. Smaller molecular weight material was found in all varieties of inflammatory arthropathy studied which included sero-positive and sero-negative rheumatoid arthritis (Fig. 3-2, C & B), psoriatic arthritis (Fig. 3-2, D), Reiter's syndrome (Fig. 3-2, E) and ankylosing spondylitis with peripheral arthropathy. Also in two patients with osteoarthritis retarded material was present, although these patients were not otherwise included in the analysis.

There was no significant correlation between the percentage of uronate retarded on Sepharose 4B-CL from the synovial fluids of patients with inflammatory arthropathies and (a) the erythrocyte sedimentation rate, (b) the synovial fluid white cell count, (c) age, or (d) duration of disease.

Extracted synovial fluid hyaluronic acid from cadaver subject C1 was exposed to the ferrous ion autoxidation system using ferrous ion concentrations of 50 and 1000 uM. The results are illustrated as Fig. 3-4. There is migration of the uronic acid containing peak across the partially included volume in a manner identical to that seen with human umbilical cord hyaluronic acid. No uronic acid-containing material from either the cadaver subjects or the inflammatory arthropathy patients was retarded by Sephadex G100.

FIGURE 3-4 SEPHAROSE 4B-CL AND SEPHADEX G100 CHROMATOGRAPHY OF SYNOVIAL FLUID HYALURONIC ACID EXPOSED TO THE FERROUS ION AUTOXIDATION SYSTEM.

Extracted synovial fluid hyaluronic acid from control subject C3 was exposed to the ferrous ion autoxidation system at ferrous ion concentrations of 50 µM (% Nsp = 76) and 1000 µM (% Nsp = 9) under conditions outlined in the text.


SEPHAROSE 4B-CL
SEPHADEX G100
Nsp 100
Nsp 76
Nsp 9
URONIC ACID CONCENTRATION μg/ml
0
20
40
60
80
5
10
15
20
25
30
V_o
V_t
FRACTION

D. DISCUSSION

The initial studies in this investigation using untreated synovial fluid highlight potential pitfalls in the use of colorimetric assays for uronic acid upon untreated samples from human tissue and body fluids. The detection of a substance that is retarded on Sepharose 4B-CL and produces an interfering colour reaction in the uronic acid assay used makes necessary a purification procedure to isolate the hyaluronic acid before chromatography and uronic acid analysis. DAHL et al. [1985] were able to circumvent this problem by using a specific radioassay (using radiolabeled link protein). Another factor dictating the necessity for a separation procedure is the fact that chondroitin sulphate has been shown to be present in normal synovial fluid [SILPANATNA, DUNSTONE and OGSTON, 1967] and to be present in elevated concentrations in pathological fluids [SEPPALA et al., 1972]. This may be cartilage derived or a secretory product of synovial cells [MARSH et al., 1979; SAARNI et al., 1980]. The fact that after extraction this glycosaminoglycan could not be demonstrated by Alcian blue staining after electrophoretic separation indicates that chondroitin sulphate is unlikely to account for any of the uronic acid containing material retarded by Sepharose 4B-CL.

The selection criteria for donor patients (i.e. lack of infection or cancer in the pre-morbid illness) were imposed due to the possibility of colonization of the synovial fluid and/or synovial membrane with bacteria or metastatic carcinoma cells, both of which may be sources of exogenous hyaluronic acid [STOOLMILLER and DORFMAN, 1970; KIMATA et al., 1984]. These criteria excluded one autopsy subject as a non-arthritic control. This subject (Fig. 3-2, F) demonstrated a large amount of hyaluronic acid (43%) in the included volume on Sepharose 4B-CL chromatography. In this

case no overt synovial inflammation was present at autopsy and her synovial fluid was analyzed before the full clinical details were available. Those clinical details revealed lymphoma, mediastinal abscess and an episode of septicaemia. She was not used as a control in this study due to the possibility of bacterially-derived hyaluronic acid. However, another explanation for the large amount of low molecular weight hyaluronic acid in her case is the effect of a prolonged and severe illness with its associated metabolic derangements upon synovial cell hyaluronate production (see Chapter VI). This is an area that warrants further investigation.

Cadavers cannot be considered an ideal source of control synovial fluid due to the possibility of post-mortem changes. It was, however, considered unethical to expose volunteers (if they could be found!) to the risks of arthrocentesis, however small. Nevertheless, our results show that the molecular weight of synovial fluid hyaluronate from non-arthritic subjects, who died suddenly, was uniformly high, with mainly symmetrical peaks at the V_0 on Sepharose 4B-CL. No more than 14% of the uronic acid containing material was retarded by Sepharose 4B-CL. Thus post-mortem depolymerization does not appear to have been a factor. DAHL <u>et al</u>. [1985] have independently reached the same conclusion.

Molecular weight profiles on Sepharose 4B-CL demonstrate considerable variation in the shape. A striking feature, however, was that the predominant amount of uronic acid-containing material was excluded by Sepharose 4B-CL i.e. it represented a hydrodynamic size of 4×10^6 or greater. Samples run on Sepharose 2B-CL, which can characterize molecules up to a higher limiting molecular weight, indicated that much of the material has

a hydrodynamic size as high as 10^7. Therefore, in inflamed synovial fluid a significant proportion of the hyaluronic acid present is still of a high molecular weight and well able to interact to form a polymer network at concentrations normally found within inflamed joints, i.e., 1 mg/ml [BALAZS, 1974].

The amount of material included on Sepharose 4B-CL ranged between 6% and 35% of the total and the smallest material present had a K_{av} of 0.75 corresponding to a molecular weight of 2×10^4. This is within the region where hyaluronic acid is found to be stimulatory of macrophage phagocytosis [FORRESTER and BALAZS, 1980].

There was no correlation between the relative amount of smaller molecular weight hyaluronic acid and clinical indices of severity of joint inflammation including erythrocyte sedimentation rate and synovial fluid cell count.

Extracted cadaver synovial fluid hyaluronic acid was susceptible to oxygen radical induced depolymerization to the same degree as human umbilical cord hyaluronic acid. Oxygen radical exposure results in depolymerization products that fall within the same molecular weight range as seen in extracted inflamed synovial fluid hyaluronic acid. A mild oxygen radical flux ($[Fe^{++}] = 50\mu M$) applied to extracted cadaver synovial fluid hyaluronic acid produced a Sepharose 4B-CL profile similar in appearance to many extracted inflamed synovial fluid hyaluronic acid's. This finding supports the hypothesis that oxygen radical depolymerization can cause a decrease in average molecular weight (and increased polydispersity) of inflamed synovial fluid hyaluronic acid *in vivo*.

E. SUMMARY

Hyaluronic acid was isolated from the synovial fluid of 20 patients with inflammatory arthropathies, 2 osteoarthritic patients and 4 cadaver subjects. Fractionation of hyaluronic acid from inflammatory arthropathy and osteoarthritis patients on Sepharose 4B-CL revealed that uronic acid containing material was retarded (ave. 17.5%). Synovial fluid hyaluronic acid from cadaver subjects who died suddenly was excluded from Sepharose 4B-CL (i.e., molecular weight $>5\times10^6$). In most patients retarded uronate was present as a skewing of the excluded volume peak towards the included volume, however in some patients small discrete peaks were present. No material was present below a molecular weight of 2×10^4 and most material present had a molecular weight greater than 10^6, i.e., within the region that hyaluronic acid will form a polymer network at concentrations found in the inflamed joint. In patients with inflammatory arthropathies, there was no significant correlation between the percent of uronate retarded on Sepharose 4B-CL and (a) the patient's erythrocyte sedimentation rate, (b) the synovial fluid white cell count, (c) age, or (d) disease duration. Extracted synovial fluid hyaluronic acid was susceptible to oxygen radical induced depolymerization *in vitro* to the same degree as human umbilical cord hyaluronic acid.

CHAPTER IV

MEASUREMENT OF REDUCING END GROUPS GENERATED BY EXPOSURE OF HYALURONIC ACID TO OXYGEN RADICALS

A. INTRODUCTION

Enzymatic depolymerization of hyaluronic acid is accompanied by the release of reducing ends irrespective of the linkage cleaved. Testicular hyaluronidase (EC 3.2.1.35) and hyaluronate lyase from Streptomyces hyaluronolyticus (EC 4.2.2.1) are both endoenzymes that can cleave hyaluronic acid at the β1-4 glycosidic linkage [HOUCK and PEARCE, 1957; LINKER, MEYER and HOFFMAN, 1956]. In the latter case an unsaturated uronide is formed at the non-reducing end. This form of cleavage will leave N-acetylglucosamine at the reducing end and can thus be measured by an increase in Morgan-Elson reactivity. On the other hand leech hyaluronidase (hyaluronic acid-endo-β-glucuronidase), also an endoenzyme, cleaves at the β1-3 glycosidic linkage, thus leaving D-glucuronic acid at the reducing end [YUKI and FISHMAN, 1963]. No increase in Morgan-Elson reactivity can be detected following this form of depolymerization, however an increase in total reducing ends will be detected by less restricted assays of reducing ends, such as that of PARK and JOHNSON [1949].

It is not clear from the literature if reducing ends are detected after depolymerization of hyaluronic acid induced by oxygen radicals. McCLEAN and HALE [1940] did not detect reducing ends after ascorbate induced depolymerization of hyaluronic acid and more recently GREENWALD and MOY [1980] did not detect reducing ends after exposure of hyaluronic acid to the XO/HX oxygen radical generating system. However CLELAND *et al.* [1969] were able to demonstrate that the depolymerization of hyaluronic acid exposed to ascorbic acid at pH 3.3 is accompanied by the release of reducing ends as demonstrable by reaction with sodium borohydride. The resolution of this discrepancy is

of importance in the further elucidation of the mechanism of oxygen radical induced depolymerization.

The aim of this investigation was to determine whether reducing ends were detectable in samples of hyaluronic acid that had been exposed to oxygen radicals generated either by the autoxidation of ferrous ions or by the action of XO upon HX. The assays for reducing sugars were the ferricyanide assay of Park and Johnson and ^{14}C-cyanide labelling of reducing ends, both of which will detect N-acetylglucosamine and glucuronic acid at the reducing end and the Morgan-Elson assay, which is specific for N-acetylglucosamine at the reducing end in this system.

B. METHODS

1. Oxygen radical generation

a. Ferrous ion autoxidation

Hyaluronic acid was exposed to ferrous ions in the presence of 50 mM phosphate buffer pH 7.4 under conditions identical to those outlined in Chapter II. Ferrous ion concentrations between 50-1000 uM were used.

b. XO/HX

Hyaluronic acid was exposed to the XO/HX system under conditions similar to those outlined in Chapter II, except that in this series of experiments a fixed concentration of XO of 5 mU/ml was used. The magnitude of the flux was varied by altering the initial concentration of HX between 10-1000 uM and the reaction was allowed to go to completion (i.e. 1hr).

2. Enzymatic degradation of hyaluronic acid

a. Testicular hyaluronidase

Bovine testicular hyaluronidase (Sigma Type IV-S, EC 3.2.1.35) was added to hyaluronic acid, 1 mg/ml, in 50 mM phosphate buffer, pH 7.4, and incubated at 37°C at enzyme

concentrations between 10 and 500 ug/ml for 2 hrs. The reaction was stopped by changing the pH to 9 with the addition of NaOH and placing the digestion tube on ice.

b. Streptomyces hyaluronidase digestion.

Hyaluronate lyase from Streptomyces hyalurolyticus (EC 4.2.2.1) was obtained from Seikagaku Kogo Co., Ltd., Tokyo, Japan. Solutions of 1 mg/ml hyaluronic acid in 50 mM phosphate buffer, pH 6.0, were incubated with a concentration of hyaluronate lyase of 10 TRU/ml at 60°C for times between 5 and 120 minutes. The reaction was again stopped by raising the pH of the solution to 9 with NaOH and placing the sample on ice.

3. Gel chromatography

The average molecular weights of the hyaluronic acid samples were determined by gel permeation chromatography. Aliquots of 0.2 ml of a 1 mg/ml solution of hyaluronic acid were applied to Sepharose 4B-CL or Sephadex G100 (Pharmacia, Uppsala, Sweden) using perspex columns (30 x 0.7 cm). The columns were eluted at 7 ml/hr using 0.5 M sodium acetate, pH 5.6, as the elution buffer and fractions of 0.7 ml were collected. The columns were calibrated with Dextran blue, [^{35}S]-sulphate and D-glucuronic acid. K_{av}'s were measured and converted to molecular weights by reference to data given by Fisher [1980] for dextrans.

4. Colorimetric assays

Prior to analysis the samples were exhaustively dialyzed against water.

a. Uronic acid

Uronic acids were determined by the metahydroxydiphenyl method of BLUMENKRANTZ and ASBOE-HANSEN [1973].

b. Park-Johnson assay

A modification of the ferricyanide method of Park and Johnson as outlined by ASHWELL [1957] was used. This assay is a very sensitive measure of reducing substances. The modification applied was as outlined by HOUCK and PEARCE [1957] and entails ensuring that the carbonate-cyanide reagent has a pH no higher than 9. At a more alkaline pH, hydrolysis of the hyaluronic acid occurs leading to falsely elevated values of the reducing ends obtained [ROBINSON and HOPWOOD, 1973].

c. Morgan-Elson reaction

The Reissig modification [REISSIG, STROMINGER and LELOIR, 1955] of the Morgan Elson reaction was used. This assay is specific because in order to be positive, in addition to a free reducing end, it requires the presence of an acylamido group and a free hydroxyl at the 4th carbon of the sugar molecule. Therefore, in the case of hyaluronic acid, it will only be positive when N-acetylglucosamine is at the reducing end.

5. Cyanide labelling

The method used was essentially that outlined by SWANN *et al*. [1982]. Degraded samples of hyaluronic acid were dialyzed against 1000 volumes of 0.2 M Na_2CO_3/$NaHCO_3$ buffer, pH 9.1. The uronic acid concentration of the solution was then determined and an equimolar concentration of $K^{14}CN$ (60 µCi/ml) was incubated with the mixture for 36 hrs at 22°C. Then unlabelled KCN was added to achieve a concentration four times that of the uronic acid concentration. After a further 12 hours the reaction was terminated by the addition of acetic acid to give a pH of 4.5. The resultant sample was then fractionated on a Sepharose 4B-CL column (1 x 60 cm) at 10 ml/hr using 0.5 M sodium acetate, pH 5.6, as the elution buffer. 1 ml fractions were collected and

The reason that there is a discrepancy between the degree of Park-Johnson reactivity for a given molecular weight remains unclear, although it may be related to the inherent inaccuracy in using the broad peak, obtained after gel chromatography, to measure molecular weight.

assayed for uronic acid content and radioactivity was counted using a Beckman liquid scintillation counter.

6. Ascending Paper Chromatography.

Hyaluronic acid samples labelled with ^{14}C-CN were hydrolysed by incubation at 100°C with an equal volume of 4N HCl for 48 hrs under nitrogen. Monosaccharides were isolated as a single peak after fractionation on Biogel P2. Ascending chromatography of material from the monosaccharide peak was performed on Whatman No 1 chromatography paper. The solvent used was ethyl acetate / pyridine / water in a ratio of 12:5:4. Samples of D-glucuronic acid, N-acetylglucosamine and glucuronic acid lactone were labelled with ^{14}C-CN as described previously and were run concurrently. After 18 hrs the paper was dried and cut into 1cm strips and the radioactivity counted.

C. RESULTS

The results of the colorimetric assays are shown in Figure 4-1. Hyaluronic acid degraded by either testicular hyaluronidase or hyaluronate lyase from Streptomyces hyaluronolyticus shows an increase in Park-Johnson reactivity. This is closely reflected by a similar increase in Morgan-Elson reactivity. This is in keeping with the known action of these enzymes which are both endoenzymes that cleave hexosaminidic linkages thus leaving N-acetylglucosamine at the reducing end [LAURENT, 1970]. In both oxygen radical generating systems, however, there is a marked divergence between the reducing end to uronic acid ratio using the two assays. There is a rise in reducing ends with decreasing molecular weight as measured by the Park-Johnson assay, but this is not mirrored by a rise in reducing ends as measured by the Reissig modification of the Morgan-Elson assay.

FIGURE 4-1 GENERATION OF REDUCING ENDS AFTER DEPOLYMERIZATION OF HYALURONIC ACID BY EXPOSURE TO ENZYMATIC AND OXYGEN RADICAL GENERATING SYSTEMS.

Reducing ends were detected by the Park-Johnson assay as modified by HOUCK and PEARCE [1957], (●———●) and the modification of the Morgan-Elson assay according to REISSIG, STROMINGER and LELOIR [1957], (▲———▲).

Each point represents the mean of triplicate determinations of the reducing end assay used, expressed as a ratio of the mean of triplicate determinations of the uronic acid value of the sample.

The raw data are shown in Table 4-1.

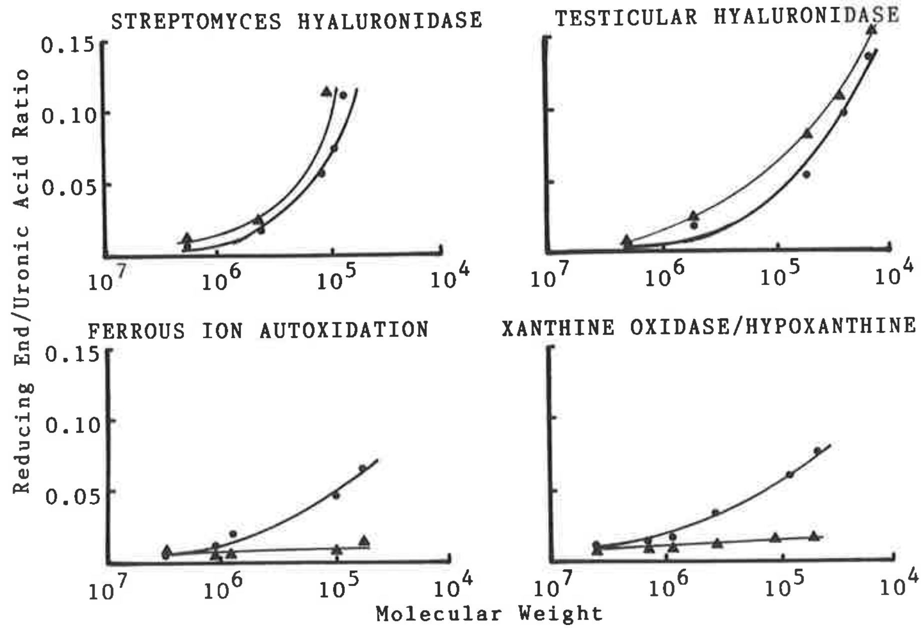

STREPTOMYCES HYALURONIDASE
TESTICULAR HYALURONIDASE
FERROUS ION AUTOXIDATION
XANTHINE OXIDASE/HYPOXANTHINE
Reducing End/Uronic Acid Ratio
0.15
0.10
0.05
10⁷ 10⁶ 10⁵ 10⁴
Molecular Weight

TABLE 4-1 COMPARISON OF REDUCING END TO URONIC ACID RATIOS AS DETERMINED BY THE PARK-JOHNSON ASSAY AND MORGAN-ELSON ASSAY

		Park-Johnson UA ratio			Morgan-Elson UA ratio		
		MW	x	SD	MW	x	SD
Ferrous ion autoxidation							
	Fe(II) μM						
1	0	$3x10^6$	0.0016	0.0002	$3x10^6$	0	
2	10	$1.5x10^6$	0.0018	0.0004	$1.5x10^6$	0	
3	100	$1x10^6$	0.0083	0.0011	$1x10^6$	0.0012	0.0002
4	200	$7x10^5$	0.0154	0.0018	$7x10^5$	0.0028	0.0005
5	500	$1x10^5$	0.0356	0.0042	$1x10^5$	0.0043	0.0010
6	1000	$4x10^4$	0.0451	0.0080	$4x10^4$	0.0115	0.0011
Hypoxanthine/Xanthine oxidase							
	Hx μM						
1	0	$3x10^6$	0.0036	0.0023	$3x10^6$	0.0046	0.0003
2	20	$1.5x10^6$	0.0128	0.0087	$1.5x10^6$	0.0045	
3	100	$1x10^6$	0.0166	0.0031	$1x10^6$	0.0085	0.0003
4	200	$4x10^5$	0.0302	0.0022	$4x10^5$	0.0106	0.0003
5	500	$1x10^5$	0.0534	0.0079	$1x10^5$	0.0149	0.0002
6	1000	$5x10^4$	0.0542	0.0040	$5x10^4$	0.0159	0.0010
Testicular Hyaluronidase							
	Conc(ug/ml)						
1	0	$2x10^6$	0.0006	0.0003	$2x10^6$	0.0017	0.0018
2	10	$3x10^5$	0.0125	0.0010	$3x10^5$	0.026	0.0072
3	50	$3x10^4$	0.0490	0.0040	$3x10^4$	0.0704	0.0182
4	100	$1x10^4$	0.0720	0.0053	$1.5x10^4$	0.1020	0.0504
5	200	$9x10^3$	0.1218	0.0014	$9x10^3$	0.1529	0.0792
6	500	$8x10^3$	0.1790	0.0106	$8x10^3$	0.2325	0.0817
Streptococcal Hyaluronidase							
	Time(mins)						
1	0	$2x10^6$	0.0081	0.0014	$2x10^6$	0.0075	0.0001
2	5	$3x10^5$	0.0094	0.0014	$3x10^5$	0.0297	0.0052
3	15	$9x10^4$	0.0405	0.0006	$9x10^4$	0.1240	0.0072
4	30	$5x10^4$	0.0543	0.0048	$5x10^4$	0.1963	0.0235
5	60	$4x10^4$	0.1168	0.0050	$4x10^4$	0.303	0.0057
6	120	$2x10^4$	0.1815	0.0060	$2x10^4$	0.356	0.0325

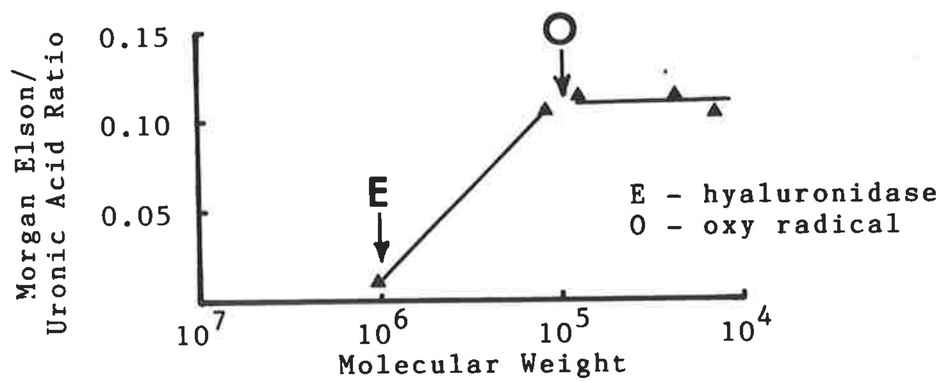
The rise in Park-Johnson activity in hyaluronic acid exposed to the ferrous ion autoxidation system was further tested by measuring the Park-Johnson to uronic acid ratio before and after extensive dialysis against water. Since all the components of this autoxidation system are of low molecular weight they would not be retained by the dialysis bag (molecular weight cut off = 10,000). No change in the Park-Johnson to uronic acid ratio was seen after this manoeuvre (data not shown).

Since it has been suggested that oxygen radical exposure might cause post-cleavage modification of the reducing end [GREENWALD and MOY, 1980], this possibility was tested. A sample of hyaluronic acid was exposed to a limited digestion with hyaluronate lyase and was then retrieved by ethanol precipitation X3 and was then dried _in vacuo_ over phosphorus pentoxide. This was then exposed to the ferrous autoxidation system (Figure 4-2). The Morgan-Elson to uronic acid ratio of enzymatically degraded hyaluronic acid was unchanged by exposure to the oxygen radical generating system. This demonstrates that post-cleavage modification cannot account for the divergence between Park-Johnson and Morgan-Elson reactivities noted in Figure 4-1.

Figure 4-3 shows the profile, after fractionation on Sepharose 4B-CL of hyaluronic acid samples that have been reductively labelled with ^{14}C-CN. Labelling with ^{14}C-CN occurs with hyaluronic acid that has been exposed to both hyaluronidase digestion as well as both the ferrous ion autoxidation and the XO/HX oxygen radical generating systems. The profile for ^{14}C mirrors the uronic acid profile until V_t was reached where free ^{14}C-CN elutes. The rate of increase in the ratio of radioactivity to uronic acid between fractions 20 - 28 was determined for each profile by

FIGURE 4-2 EFFECT OF AN OXYGEN RADICAL FLUX ON THE MORGAN-ELSON REACTIVITY OF AN ENZYMATICALLY DEGRADED SAMPLE OF HYALURONIC ACID.

A series of samples of hyaluronic acid were digested with hyaluronate lyase from Streptomyces hyaluronolyticus for 10 min under the conditions outlined in the text. The hyaluronic acid was then retrieved by precipitation x3 with ethanol, dried <u>in vacuo</u> over phosphorus pentoxide and made up with the appropriate components of the ferrous ion autoxidation system at ferrous ion concentrations of 100, 200 and 1000 uM respectively.



Morgan Elson/
Uronic Acid Ratio
0.15
0.10
0.05
O
E
E - hyaluronidase
O - oxy radical
10^7
10^6
10^5
10^4
Molecular Weight

FIGURE 4-3 SEPHAROSE 4B-CL CHROMATOGRAPHY OF ^{14}C-CYANIDE LABELLED HYALURONIC ACID DEGRADATION PRODUCTS.

The uronic acid value is closely reflected by the level of ^{14}C-cyanide until Vt is reached when unbound ^{14}C-CN elutes.

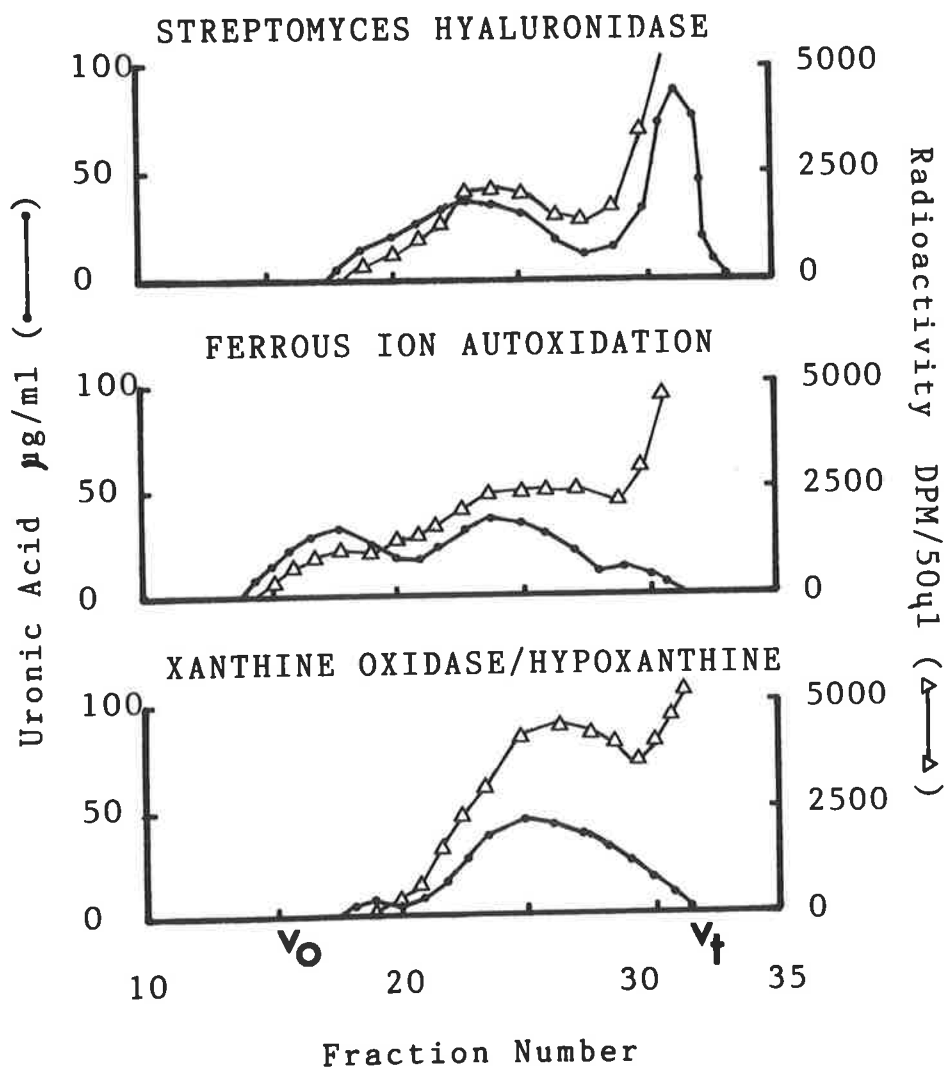
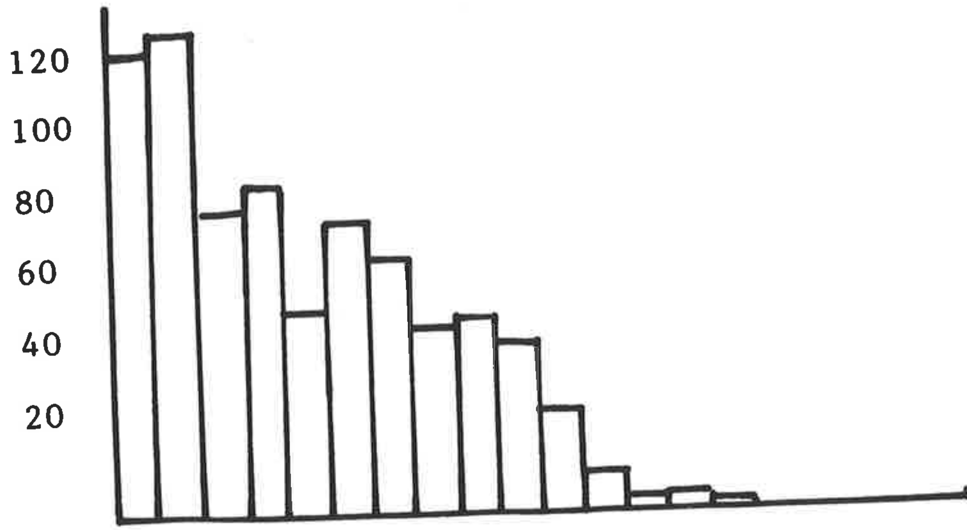

STREPTOMYCES HYALURONIDASE
FERROUS ION AUTOXIDATION
XANTHINE OXIDASE/HYPOXANTHINE
100
50
0
5000
2500
Uronic Acid µg/ml
Radioactivity DPM/50µl
Vo
Vt
10
20
30
35
Fraction Number

FIGURE 4-4 ASCENDING PAPER CHROMATOGRAPHY OF MONOSACCHARIDES ISOLATED AFTER ACID HYDROLYSIS OF ^{14}C-CYANIDE LABELLED HYALURONIC ACID DEGRADATION PRODUCTS.

The top panel shows the results obtained after initial depolymerization with the XO/HX system. The bottom panel shows the results obtained after initial digestion with hyaluronidase from streptomyces hyaluronidase. A ^{14}C-cyanide labelled N-acetylglucosamine standard migrated to 12 cm, ^{14}C-cyanide labelled D-glucuronic acid and D-glucuronolactone standards migrated to between 5 and 7 cms.

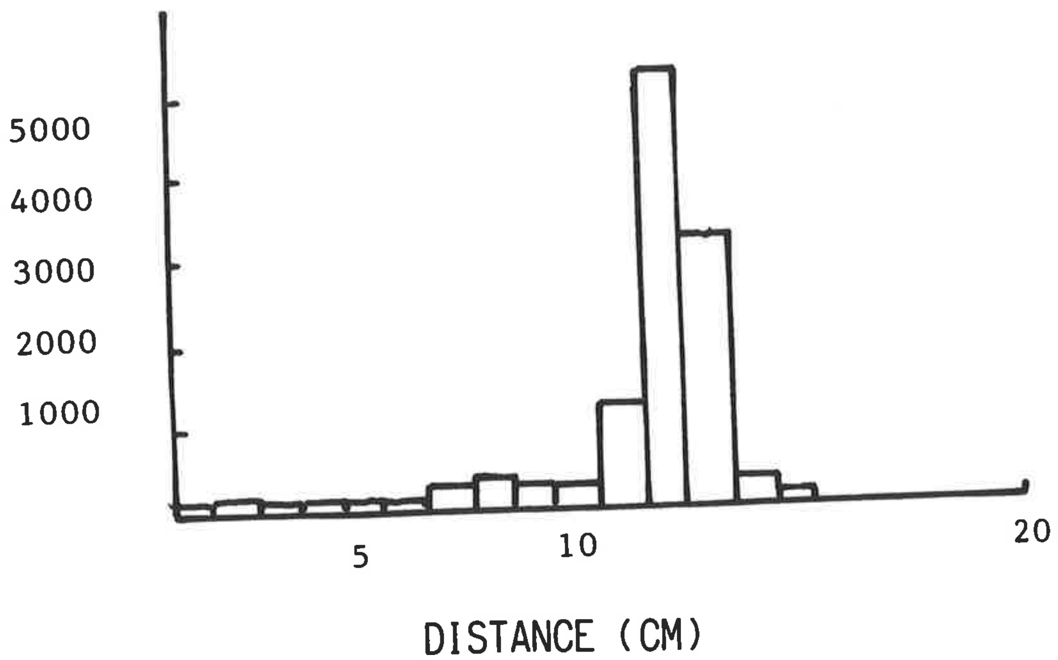
ASCENDING PAPER CHROMATOGRAPHY

XANTHINE OXIDASE/HYPOXANTHINE



DPM/CM

STREPTOCOCCAL HYALURONIDASE



linear regression analysis. The slopes are all comparable (hyaluronidase = 19.6, Fe^{2+} autoxidation = 14.0 and XO/HX = 19.6). Since the only aldehyde that the enzyme will release is at the reducing end, this finding suggests that aldehyde or ketone formation in the oxygen radical systems does not occur significantly other than from the reducing end created at the cleavage site (i.e. each cleavage site = 1 aldehyde group created).

Further efforts were undertaken to identify the sugar to which ^{14}C-CN is binding by hydrolysing the ^{14}C-CN labelled degradation products. Monosaccharides were isolated by fractionation on Biogel P2 and were separated by ascending paper chromatography. Figure 4-4 shows that for hyaluronidase degraded hyaluronic acid the label migrates with a similarly labelled N-acetylglucosamine standard. This is in keeping with the known action of this enzyme. With hyaluronic acid exposed to the XO/HX system ^{14}C-CN does not migrate with the N-acetylglucosamine standard but is spread over the areas of migration of the ^{14}C-CN labelled D-glucuronic acid and a similarly labelled glucuronic acid lactone standard.

D. DISCUSSION

The "reducing end" of a sugar molecule is due to the presence of a free or potentially free aldehyde group [LEHNINGER, 1983]. The structure of six carbon sugars is usually depicted as a ring structure, however with reducing sugars a small portion is present in solution as an open ring structure with an aldehyde group centered on the 1st carbon. The two forms are in an equilibrium that strongly favours the closed ring form.

The colorimetric assay of Park and Johnson relies on the ability of the aldehyde group to act as a reducing agent with a metal ion (Fe^{3+}). Cyanide will also react with the free aldehyde

group of a sugar in a reaction first described by FISCHER [1890] and known as cyanohydrin synthesis. Initially utilized as a means of adding an extra carbon to a sugar molecule, this reaction was subsequently developed by MOYER and ISBELL [1958] as means of reductive labelling of sugars. It is clear from our results with both of these assays, that free aldehyde groups (i.e. reducing sugars) are present in oxygen radical depolymerized hyaluronic acid.

The finding that one does not see Morgan-Elson reactivity after depolymerization of hyaluronic acid by the two oxygen radical generating systems tested indicates that when depolymerization occurs, the reducing sugar generated is not centered on the N-acetylglucosamine. This is contrary to the findings of CLELAND et al. [1969], who reported that for hyaluronic acid depolymerized by an ascorbate driven system, the β1-4 hexosaminidic linkage was preferentially cleaved (thus leaving N-acetylglucosamine at the reducing end). In Chapter I, I have drawn attention to the similarities between the ascorbate system and the ferrous ion autoxidation system (ascorbate reduces Fe^{3+} to Fe^{2+}). However, it may be the differences in these systems that explain the divergent findings c.f. the possibility of a more direct reaction between ascorbate or its oxidized products and hyaluronic acid.

Our findings are consistent with the hypothesis that oxygen radicals cleave at the glucosaminidic linkage thus leaving D-glucuronic acid at the reducing end. Indeed BALAZS et al. [1967] have postulated that it is the this glycosidic linkage that is cleaved during pulse radioylsis of aqueous solutions of hyaluronic acid. Both pulse radiolysis and the two oxygen radical generating

systems used in the present study are believed to cause hyaluronic acid depolymerization by OH• production.

It is also possible to interpret from the colorimetric data that cleavage may occur by lysis of the carbon ring of either sugar with the creation of a terminal aldehyde or ketone group, both of which may also react with cyanide. However this is less likely than β1-3 glucosaminidic linkage rupture because although the labelled monosaccharides do not clearly separate on ascending paper chromatography solely as labelled D-glucuronic acid, probably due to lactone formation during hydrolysis, it is clear that a considerable portion is labelled D-glucuronic acid. Should cleavage occur by lysis of a sugar ring, then no such labelled sugar should be present.

The identification of reducing sugars by radioactive labelling and subsequent separation may prove to be a suitably discriminating method to determine whether hyaluronic acid has been exposed to oxygen radicals _in vivo_.

E. SUMMARY

After depolymerization of hyaluronic acid by exposure to the ferrous ion autoxidation system and to XO/HX there is an increase in reducing end activity as measured by the Park-Johnson assay and by reductive labelling with radiolabelled cyanide. There is however no increase in Morgan-Elson reactivity, nor is there any change in Morgan-Elson reactivity in hyaluronic acid pre-exposed to hyaluronidase and subsequently exposed an oxygen radical generating system. These findings were consistent with our hypothesis that oxygen radical induced depolymerization of hyaluronic acid occurs by preferential cleavage of the β1-3 glucuronidic glycosidic linkage thus leaving D-glucuronic acid at the reducing end.

CHAPTER V

DOES OXYGEN RADICAL EXPOSURE ALTER THE ABILITY OF HYALURONIC ACID TO MODULATE MONOCYTE Fc RECEPTOR BINDING?

A. INTRODUCTION

The indication that exposure to oxygen derived free radicals might lead to cleavage of specific glycosidic linkages and possibly other alterations along the length of the hyaluronic acid molecule leads to the question:- Do the chemical sequelae from exposure to oxygen free radicals lead to an alteration in biological or chemical properties apart from those resulting from decreased molecular weight?

The observation of FORRESTER and BALAZS [1980] that hyaluronic acid would inhibit the phagocytosis of latex beads demonstrated a specific cellular effect that is of considerable potential biological relevance, particularly in relation to phagocytosis occurring within an inflamed joint. They observed that the phagocytosis of latex beads by elicited rat peritoneal macrophages was inhibited by high molecular weight (i.e. MW = 4.6 $\times 10^5$ - 2.8 $\times 10^6$), hyaluronic acid at concentrations between 0.1-0.2 mg/ml. Paradoxically, however hyaluronic acid of molecular weight 9.0 $\times$ 10^4 had a stimulatory effect at concentrations between 0.2 -0.5 mg/ml. Hyaluronic acid of this size can be expected following depolymerization with oxygen radicals (see Chapter II) and is found in the synovial fluid of a considerable proportion of patients with inflammatory arthropathies (see Chapter III).

We therefore sought to verify the findings of Forrester and Balazs by utilizing the Fc receptor binding assay. There were several reasons why this assay was used. Firstly the Fc receptor mediates both binding and internalization of IgG coated particles and IgG aggregates. It is a well characterized membrane receptor [GRIFFIN, BIANCO and SILVERSTEIN, 1975]. Finally, the parameter measured, i.e. Fc receptor binding is more directly

linked to the phagocytosis of immune complexes and is thus is more clearly linked to the pathogenesis of inflammatory joint disease than the phagocytosis of latex beads.

B. METHODS

1. Adherent monocytes

10 ml samples of venous blood, anticoagulated with lithium heparinate, were obtained from healthy Red Cross blood donors. These were then diluted with an equal volume of Hank's balanced salt solution (BSS), placed in a 30 ml siliconized glass centrifuge tube and underlayed with 7.5 ml Ficoll Paque (Pharmacia, South Seas, Sydney). The tubes were then centrifuged at 400g for 35 min at room temperature. The mononuclear cell fraction was then aspirated from the Ficoll Paque-Hank's BSS interface. They were then washed three times in Hank's BSS. The final cell pellet was resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. They were then transferred to 24-well tissue culture plates (16 mm diam) Costar (Cambridge, Mass, U.S.A). At the bottom of each well had been placed a small circular glass coverslip that had previously been washed in 10% sulphuric acid followed by 70% ethanol. Approximately 4 $\times 10^6$ cells were added to each well. The cells were then incubated for 1 hour at 37°C in a 5% CO_2 incubator. Non-adherent cells were then washed off with Hank's BSS.

2. Fc receptor assay

a. Preparation of antibody coated red blood cells

A mixture was made consisting of 50 µl packed washed red blood cells, 200 µl anti-D serum (Commonwealth Serum Laboratories, Sydney, Australia) and 2 ml Hank's BSS. This mixture was placed in a 37°C incubator for 1 hour. During the incubation, the tube was inverted every 20 minutes. The red cells were then

washed three times in sterile normal saline and finally resuspended in 25 ml RPMI.

b. Fc receptor assay

500 ul of the antibody coated erythrocytes were added to each washed monocyte monolayer. This was then incubated for 1 hour after which each well was washed three times with Hank's BSS to remove non-bound erythrocytes. The cells were fixed by the addition of 3% gluteraldehyde in Hank's BSS for 30 min at room temperature (or 12 hrs at 4°C).

c. Staining and counting

The coverslips were stained with Giemsa stain. After 60 seconds the coverslips were washed in water and allowed to stand in distilled H_2O for 30 min, the water being changed every 10 minutes. They were dried and mounted. The microscopic appearance of these preparations showed readily identifiable adherent monocytes to which were attached red blood cells, either touching or partially or completely ingested. Since the end point in this investigation was receptor attachment, no attempt was made to distinguish attachment from complete phagocytosis. For each coverslip five high power fields were counted or enough to guarantee at least 100 monocytes and the corresponding number of erythrocytes. This was then expressed as a binding ratio. The results for each assay were expressed as the Binding Index.

$$\text{Binding Index} = \frac{\text{ratio RBC per monocyte with HA}}{\text{ratio RBC per monocyte without HA}}$$

3. Hyaluronic acid preparation

a. Oxygen radical exposed hyaluronic acid

Purified high molecular weight hyaluronic acid (Healon: MW = $>2\times10^6$) was the gift of Pharmacia, South Seas, Sydney, Australia.

This was exposed to the ferrous ion autoxidation oxygen radical generating system under conditions identical to those outlined in Chapter II. Average molecular weights of the depolymerized samples were determined by fractionating aliquots upon Sepharose 4B-CL columns (10 x 0.5 cm) and the K_{av} of the peak uronic acid value was measured. The corresponding molecular weight was determined by reference to data for polysaccharides given by FISHER [1970]. The ferrous ion concentration and the corresponding average molecular weight of the product are shown in Table 5-1.

b. Enzyme exposed hyaluronic acid

A preparation of Healon was exposed to testicular hyaluronidase (Sigma Type IV-S, EC 3.2.1.35), 0.1 mg/ml for 5 mins at 60°C and the reaction was stopped by decreasing the pH to 6.0 and dropping the temperature to 4°C. This was then fractionated on a Sephacryl S1000 column. Pooled fractions were dialyzed extensively against water, ethanol precipitated and then dried *in vacuo*. The average molecular weights of pooled fraction used was determined by fractionation on a Sepharose 4B-CL column as described previously. Characteristics of these preparations are given in Table 5-1. In this assay only one hyaluronic acid concentration of 0.3 mg/ml was used.

C. RESULTS

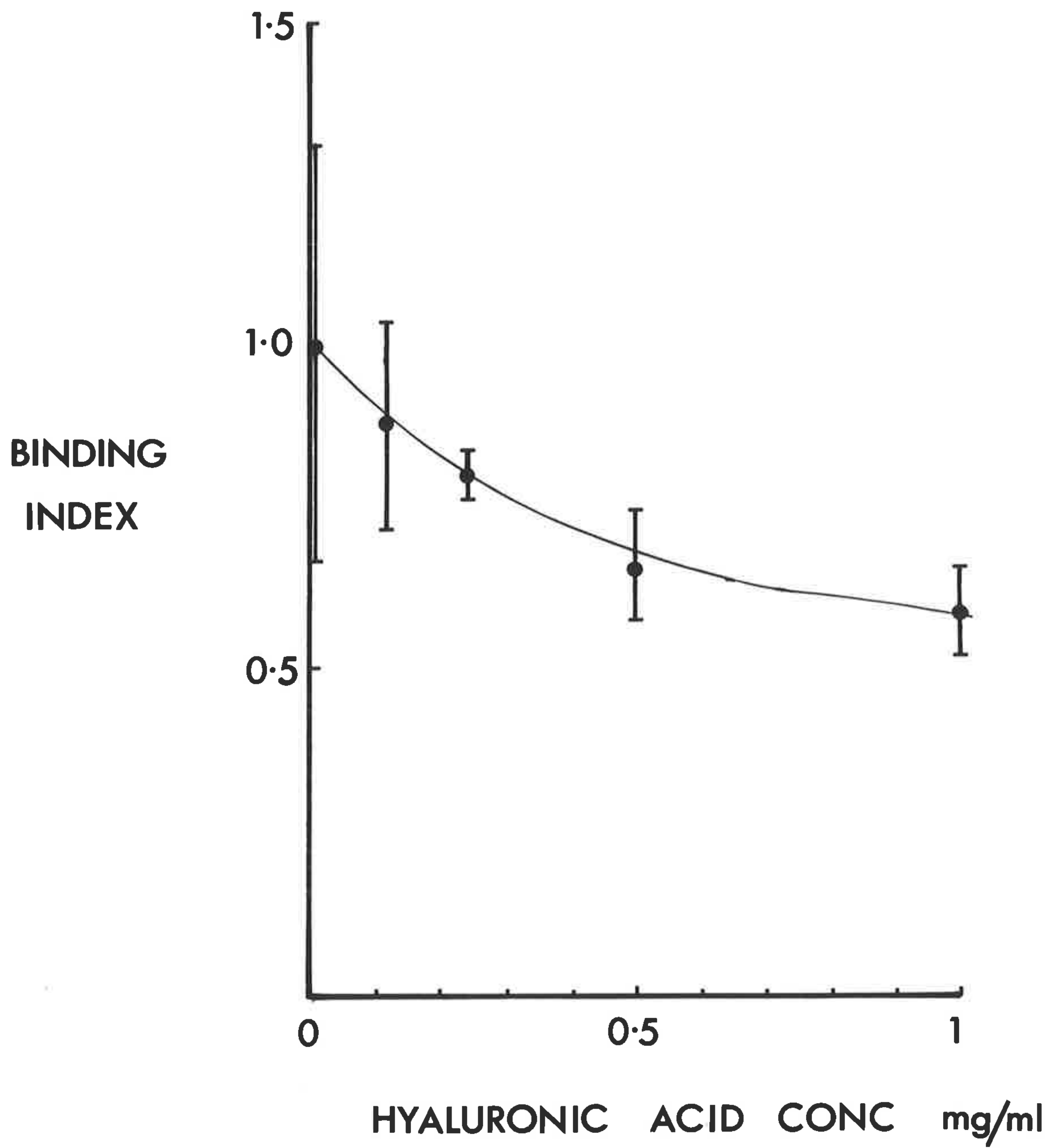
Using undegraded high molecular weight hyaluronic acid there was a dose dependent inhibition of Fc receptor binding over the concentration range 0.2 - 1 mg/ml (Fig. 5-1).

At a concentration of 0.3 mg/ml there was a biphasic effect upon Fc receptor binding dependent upon molecular weight. Inhibition of Fc receptor binding was seen with preparations with an average molecular weight of 1×10^6 and above and stimulation

Table 5-1 MOLECULAR WEIGHTS OF THE HYALURONIC ACID PREPARATIONS USED IN THE Fc RECEPTOR BINDING ASSAYS

Oxygen radical depolymerized HA		Enzyme degraded HA	
Ferrous ion concentration (micromolar)	Average MW (x10^{-6})	Pooled fractions	Average MW (x10^{-6})
0	2	HEALON	2
50	1.5	11- 20	1.5
100	1.0	51- 60	1.0
500	0.5	101-110	0.5
1000	0.2		

FIGURE 5-1 EFEECT OF THE CONCENTRATION OF A HIGH MOLECULAR WEIGHT HYALURONIC ACID PREPARATION (HEALON - PHARMACIA) UPON Fc RECEPTOR BINDING.
Each data point = mean (± S.D.) of triplicate determinations.


1·5
1·0
0·5
BINDING
INDEX
0
0·5
1
HYALURONIC ACID CONC mg/ml

of Fc receptor binding was seen in preparations with an average molecular weight of $5x10^5$ and below (Fig. 5-2). This effect is seen with both oxygen radical exposed and enzyme digested hyaluronic acid.

D. DISCUSSION

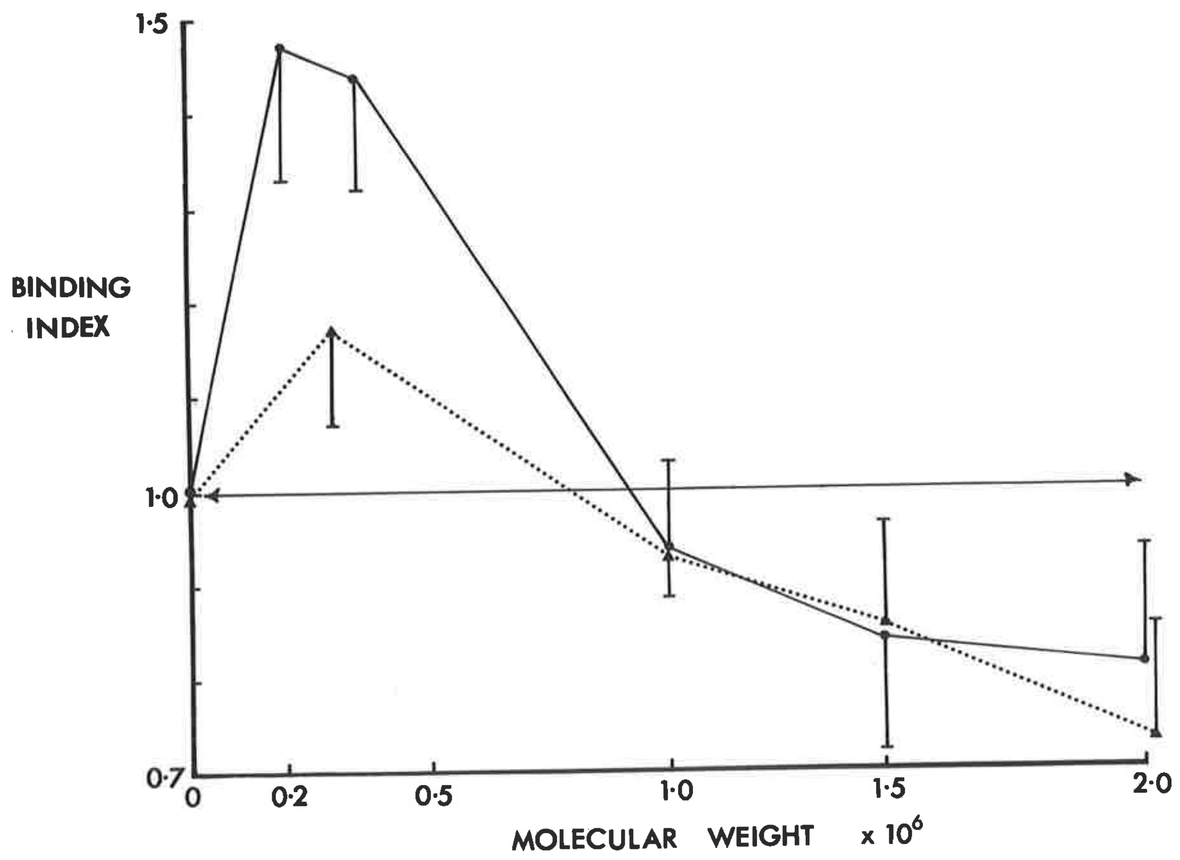
The Fc receptor binding assay system used in this study differed from the latex phagocytosis assay studied by FORRESTER and BALAZS [1980] because a specific receptor for the uptake of polystyrene latex spheres has not been demonstrated. Latex is considered to be engulfed in a non-specific manner possibly related to its hydrophobicity [FROMAGEOT, 1977]. The results of this study indicate that receptor mediated binding is inhibited by high molecular weight hyaluronic acid ($>1x10^6$). However at a low concentration (i.e., 0.3mg/ml) of low molecular weight hyaluronic acid, a paradoxical stimulation of Fc receptor binding is evident. This is in accord with the findings of Forrester and Balazs for latex phagocytosis, however the molecular weight at which this stimulation first becomes evident is somewhat higher in our system (i.e. $5x10^5$ compared with $9x10^4$).

Depolymerization of hyaluronic acid by both enzymatic digestion and oxygen radical exposure resulted in hyaluronic acid degradation products that were inhibitory at high hyaluronic acid molecular weight yet caused paradoxical stimulation at lowered molecular weights. Oxygen radical depolymerized hyaluronic acid caused a greater degree of stimulation of binding index but this did not reach statistical significance.

The hyaluronic acid concentration of 0.3 mg/ml was chosen after consideration of Forrester and Balaz's data to give maximal stimulation at the decreased molecular weight and it is pertinent

FIGURE 5-2 EFFECT OF ENZYMATIC (▲·····▲) AND OXYGEN RADICAL DEPOLYMERIZED (●——●) HYALURONIC ACID UPON Fc RECEPTOR BINDING AT A CONCENTRATION OF 0.3 MG/ML.

Each data point = mean ± S.D. of triplicate determinations.


1·5
BINDING
INDEX
1·0
0·7
0
0·2
0·5
1·0
1·5
2·0
MOLECULAR WEIGHT x 10⁶

to note that at this concentration a matrix of hyaluronic acid is unlikely to form especially at lowered molecular weights.

Clearly more extensive studies are required to probe the mechanisms involved in both the inhibitory and the stimulatory effects demonstrated. However it is pertinent to consider several recent studies. In considering the inhibitory effect, Forrester and Balazs concluded that electrostatic repulsion (latex, IgG, and hyaluronic acid are electronegative) is unlikely to have an effect since sulphated glycosaminoglycans which are more electronegative are without effect. Similarly an hyaluronic acid induced membrane pertubation is unlikely since other membrane modifying agents were without effect. They favored a steric exclusion of latex from the area of the cell surface thus decreasing the proximity of cell and latex. Alternately they speculated that latex beads and indeed IgG and complement coated particles are hydrophobic thus making it easier for the cell to effect phagocytosis. Hyaluronic acid, being hydrophilic would decrease this phagocytic advantage. Both of these explanations could apply to our assay. Similarly, steric exclusion effects have been implicated in the inhibitory effect of hyaluronic acid on mitogen induced lymphocyte stimulation.

E. SUMMARY

High molecular weight hyaluronic acid (Healon-Pharmacia) caused a dose dependent inhibition of macrophage Fc receptor binding between the concentrations of 0.2 - 1 mg/ml. At a concentration of 0.3 mg/ml both oxygen radical depolymerized and enzymatically degraded hyaluronic acid cause an inhibition of Fc receptor binding at molecular weights of 1×10^6 and 1.5×10^6. However when the molecular weight was reduced to 2×10^5 and 5×10^5 by oxygen radical degradation, stimulation of Fc receptor binding occurred. Stimulation was also seen with enzymatically degraded hyaluronic acid with a molecular weight of 2.5×10^5.

CHAPTER VI

EXPOSURE OF CULTURED SYNOVIAL CELLS TO OXYGEN RADICAL FLUXES. CYTOTOXICITY AND EFFECT ON HYALURONIC ACID PRODUCTION

A. INTRODUCTION

The studies described so far have explored the possibility that oxygen radicals, generated in the inflamed joint, can act to depolymerize fully synthesized hyaluronic acid. However, the finding of hyaluronic acid of decreased average molecular weight in inflamed synovial fluids may also be explained by the synthesis of a less polymerized product. The synovial cell represents a potential target for damage by oxidants released by leucocytes as part of the inflammatory process. It is possible that a synoviocyte damaged by oxidants may continue to produce a smaller molecular weight hyaluronic acid product.

The aim of this experiment was to determine the extent of the susceptibility of synovial cells to an enzymatically generated oxygen radical flux _in vitro_, and to determine the molecular weight characteristics of the hyaluronic acid produced by synoviocytes given a sub-lethal exposure to an oxygen radical flux.

B. METHODS

1. Synovial cell culture

Bovine synovial cells were obtained by trypsin lavage of bovine metacarpophalangeal joints according to a modification of the method of FRASER and McCALL [1965]. Fresh bovine hocks were a gift from the South Australian Meat Corporation. Within 4 hours of slaughter they were washed and the skin was dissected free from the metacarpophalangeal joint. An area of subcutaneous tissue was swabbed with 70% alcohol and an 18 gauge needle inserted. The metcarpophalangeal joint was lavaged with two 50 ml volumes of normal saline to remove synovial fluid and synovial fluid cells. 40 ml of 0.25 trypsin (type III from bovine pancreas, Sigma Chemical Co., St. Louis, Missouri) in Ca^{++} and Mg^{++} free phosphate buffered saline (Dulbecco's formula) was then injected.

Approximately 25 ml was then withdrawn into the syringe and the remainder was left *in situ* for 15 minutes. The joint was gently massaged initially to ensure that the trypsin solution entered recesses in the synovial space. The joint was then rewashed with the complete volume of the trypsin solution which was withdrawn and centrifuged at 1000 x g.

The cell pellet obtained was then resuspended in 1° tissue culture medium (see Appendix II) supplemented with 20% foetal calf serum and seeded into a plastic 25 cm^2 tissue culture flask (Grand Island Biological Company, Grand Island, New York). The cells were then grown at 37°C in 5% carbon dioxide. After 2 days scattered adherent cells could be seen with characteristic sharp cytoplasmic processes. The cells were then washed twice with Dulbecco's phosphate buffered saline (PBS) and the 1° tissue culture medium replaced. The medium was changed in this manner twice weekly. The primary culture was maintained until the cells had reached confluence (usually about 10-14 days). They were then subcultured by washing twice in Dulbecco's PBS and then incubating at 37°C for 5 min in 2-3 ml of 0.25% trypsin in PBS. When it was observed that the cells had separated, 10 ml of 2° tissue culture medium (see Appendix II), supplemented with 10% fetal calf serum was added. This was then divided into two or three aliquots which were then added to fresh tissue culture flasks. All subsequent cultures were grown in 2° tissue culture medium unless otherwise specified. Cells were harvested for the chromium release assay between the 2nd and 7th subcultures. In this way a relatively homogenous population of synoviocytes with a fibroblast-like appearance were obtained [FRASER *et al.*, 1979].

2. Chromium release assay

Synovial cells were harvested for the chromium release assay by subculturing in the manner described above. 10^5 cells (in 1.5 ml tissue culture medium) were then added to each well of a 24 well tissue culture plate (Costar, 16 mm diameter wells, Cambridge, Mass.). These were then incubated at 37°C in 5% CO_2 for two days when a confluent layer of cells could be seen at the bottom of each well. Each well was then washed twice with Dulbecco's PBS. Care was taken during washing to point the tip of the pasteur pipette at a single point on the circumference of the well in order to disturb the cell layer as little as possible. One-tenth milliliter of ^{51}Cr (specific activity = 50 μCi/ml, Amersham, Australia) was added to each well and incubated for 1 hour at 37°C. The cell layers were then washed three times in Dulbecco's PBS and the reagents for each experiment were added in a total volume of 1 ml. Experiments were performed in triplicate with +ve controls (20% acetic acid) and -ve controls (buffer) in each plate.

After incubation for the appropriate time, the cell layers were inspected to determine that they were still adherent. Then 0.5 ml of the supernatant (S) was aspirated and placed in a polycarbonate tube for counting. The remainder (R) was then aspirated and the cell layers were removed by trypsinization x2 with 0.5% trypsin in PBS and a final wash with water to hypotonically lyse any residual cells. These washings were then pooled, the volumes equalized and the tubes were counted on a Roche Gammamatic gamma counter.

Percentage ^{51}Cr release was obtained by the formula

$$\% \ ^{51}Cr \text{ release} = \frac{2S}{R+S} \times 100$$

Background %^{51}Cr release was determined from the -ve control and was subtracted from the gross % ^{51}Cr release. The mean and standard deviation of triplicate determinations were then calculated.

3. Oxygen radical generation

The oxygen radical generating system used was the XO/HX system as previously outlined (see Chapter II). Proteolytic contamination of the xanthine oxidase preparations were decreased according to the method of GREENWALD and MOY [1979] by Sephadex G100 chromatography. Because all the protease activity was not completely removed by this method and indeed efforts to completely remove all protease activity will result in loss of xanthine oxidase activity [BATES, LOWTHER and HANDLEY, 1984], the experiments were carried out in the presence of 1% foetal calf serum in order to swamp residual protease activity (see results section).

4. Hyaluronic acid production

Hyaluronic acid production was assessed using synovial cells grown to confluence in 25cm^2 tissue culture flasks. After washing with Dulbecco's PBS, 5 mls Ham's F10 tissue culture medium containing 50 µCi ^{3}H-glucosamine hydrochloride was added. This was then incubated for 48 hours before the supernatant was collected after gentle washing of the cell layer with the supernatant.

The collected supernatant was then concentrated in a Millipore 25 mm stirred cell over a Pellicon PT-GC filter. Approximately 2.5 ml of concentrated supernatant was then applied to a Sepharose 4B-CL column (100 x 1 cm) and eluted with 4 M guanidinium chloride. Since the peak of radioactivity identified as hyaluronic acid did not change significantly under associative or

dissociative conditions subsequent elutions were performed using 0.5 M NaAc pH 5.6. Fractions of 1 ml were collected. 5 µl of each fraction were then added to 2 ml scintillation fluid and counted in a Beckmann liquid scintillation counter.

5. Cellulose acetate gel electrophoresis

The hyaluronic acid containing peaks after fractionation were identified by applying 5 µl of the peak fraction to cellulose acetate strips. Electrophoresis was conducted as described in Chapter III. After electrophoresis, standard hyaluronic acid samples were read by Alcian blue staining whereas radiolabelled samples were read by cutting the cellulose acetate strip into 2 mm sections from the point of origin. Each strip was added to a counting vial with 2 ml of liquid scintillation fluid and counted.

C. RESULTS

1. XO/HX induced cytotoxicity

The initial part of this experiment was to determine the levels of oxygen radical flux that produced a cytotoxic effect using synovial cells as targets. Figure 6-1 shows the results of a time course experiment in which 1 cm^2 wells of confluent monolayers of synoviocytes were exposed to 10 mU/ml xanthine oxidase in the presence of 2 mM hypoxanthine. After 8 hrs a cytotoxic effect was manifested by the release of ^{51}Cr into the medium. Subsequently all measurements in the ^{51}Cr release assay were made at 12 hrs.

Because xanthine oxidase preparations contain some proteolytic activity, in spite of chromatographic purification, foetal calf serum was added to the mixture to swamp this activity. Figure 6-2 shows the results of an experiment using progressively increasing amounts of foetal calf serum up to 50%. Above 10% foetal calf serum, the % ^{51}Cr release is brought back

FIGURE 6-1 KINETICS OF ^{51}Cr RELEASE FROM OXYGEN RADICAL EXPOSED AND UNEXPOSED SYNOVIOCYTES.

(———): XO = 10 mU/ml, HX = 2 mM

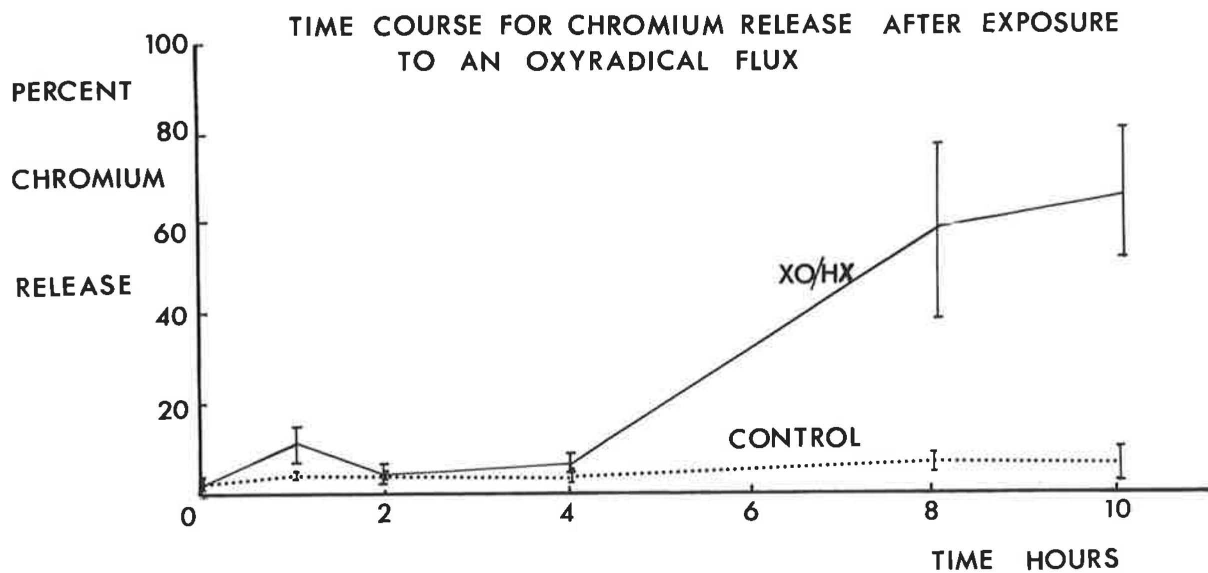
(········): HX (2 mM) alone.

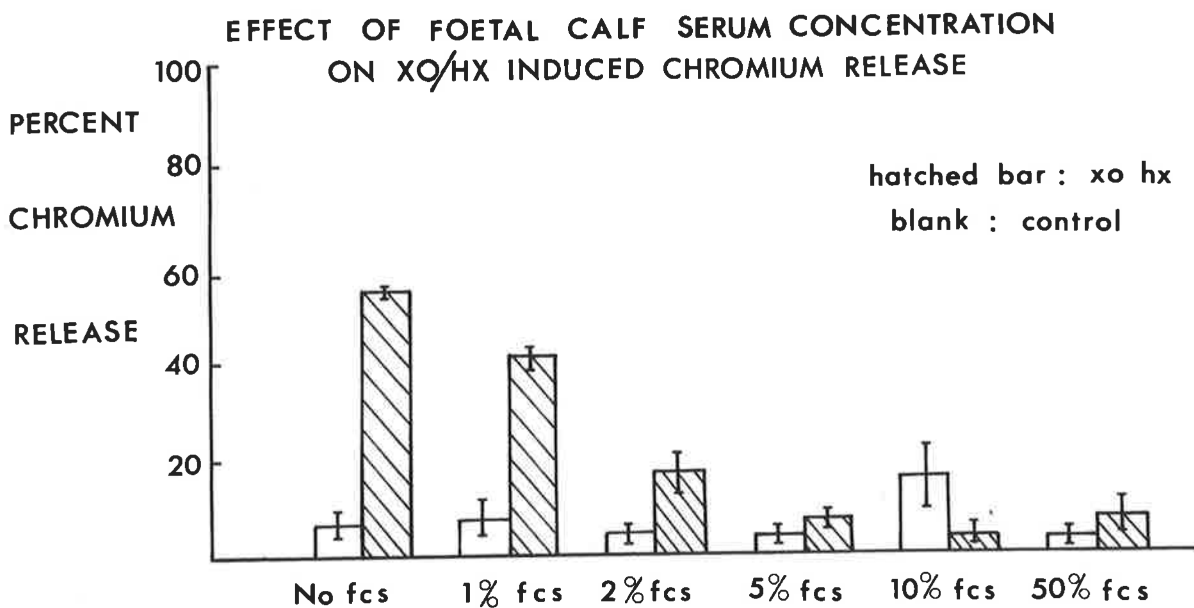
(Data points represent mean ± S.D.)

FIGURE 6-2 EFFECT OF FOETAL CALF SERUM CONCENTRATION ON XO/HX INDUCED ^{51}Cr RELEASE.

Hatched bars: XO = 5 mU/ml, HX = 2 mM

Blank bars: HX only


TIME COURSE FOR CHROMIUM RELEASE AFTER EXPOSURE TO AN OXYRADICAL FLUX
PERCENT
CHROMIUM
RELEASE
100
80
60
40
20
0
2
4
6
8
10
TIME HOURS
XO/HX
CONTROL


EFFECT OF FOETAL CALF SERUM CONCENTRATION ON XO/HX INDUCED CHROMIUM RELEASE
PERCENT
CHROMIUM
RELEASE
100
80
60
40
20
hatched bar : xo hx
blank : control
No fcs
1% fcs
2%fcs
5% fcs
10% fcs
50% fcs

to control levels, using an xanthine oxidase concentration of 5mU/ml. Subsequently 1% foetal calf serum was added to the reaction mixture when the cells were exposed to the XO/HX system.

Figure 6-3 shows the effect of superoxide dismutase on XO/HX induced ^{51}Cr release. At a superoxide dismutase concentration of 75 U/ml, no protective effect was seen over a xanthine oxidase concentration range of 5 to 100 mU/ml. These levels of superoxide dismutase have been shown to be protective in the XO/HX induced hyaluronic acid degradation system and XO/acetaldehyde induced erythrocyte lysis (KELLOGG and FRIDOVICH, 1977). Catalase, on the other hand was protective at a concentration of 50 U/ml (Figure 6-4). Mannitol had a minor protective effect at low xanthine oxidase concentrations (Figure 6-5).

Based on the indication from the above experiments that hydrogen peroxide was the agent necessary for demonstration of a cytotoxic effect, the effect of glucose/glucose oxidase was examined. This enzyme produces the divalent reduction of oxygen to H_2O_2 without production of the superoxide anion [NILSSON, PICK and BRAY, 1969]. A cytotoxic effect was readily demonstrated at a glucose oxidase concentrations of 50 - 500 mU/ml. This was completely inhibited by the addition of catalase at a concentration of 50 U/ml (Figure 6-6).

2. <u>Effect of oxygen radical exposure on hyaluronic acid production</u>

The next phase in this series of experiments was to determine the effect of an exogenously applied oxygen radical flux upon hyaluronic acid production by synovial cells in culture. Non-diseased bovine synovial cells, as used in the cytotoxicity studies, were incubated with ^3H-glucosamine hydrochloride. Initial

FIGURE 6-3 EFFECT OF SUPEROXIDE DISMUTASE ON XO/HX INDUCED ^{51}Cr RELEASE.*

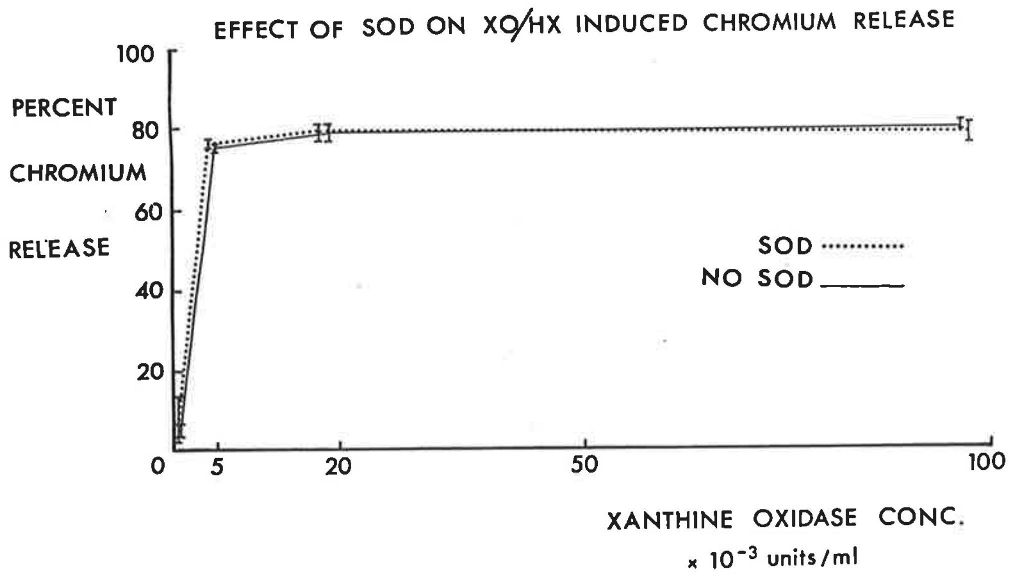
SOD concentration = 75 units/ml

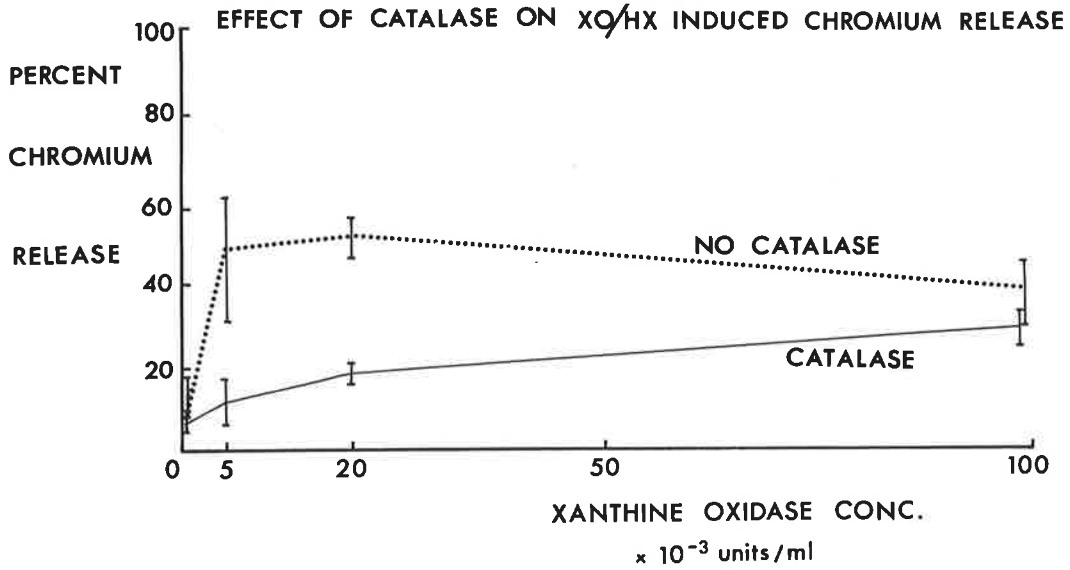
FIGURE 6-4 EFFECT OF CATALASE ON XO/HX INDUCED ^{51}Cr RELEASE.*

Catalase concentration = 50 units/ml

FIGURE 6-5 EFFECT OF MANNITOL ON XO/HX INDUCED ^{51}Cr RELEASE.*

* The error bars represent the mean ± S.D. of triplicate determinations. The % ^{51}Cr release in the +ve controls in these experiments = 94.2 ± 4.1.


EFFECT OF SOD ON XO/HX INDUCED CHROMIUM RELEASE
PERCENT CHROMIUM RELEASE
100
80
60
40
20
0
5
20
50
100
SOD
NO SOD
XANTHINE OXIDASE CONC.
× 10^{-3} units/ml


EFFECT OF CATALASE ON XO/HX INDUCED CHROMIUM RELEASE
PERCENT CHROMIUM RELEASE
100
80
60
40
20
0
5
20
50
100
NO CATALASE
CATALASE
XANTHINE OXIDASE CONC.
× 10^{-3} units/ml

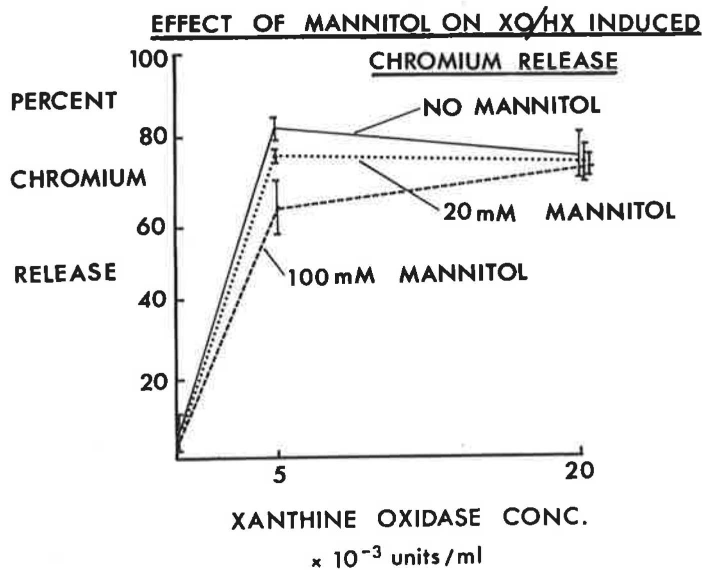
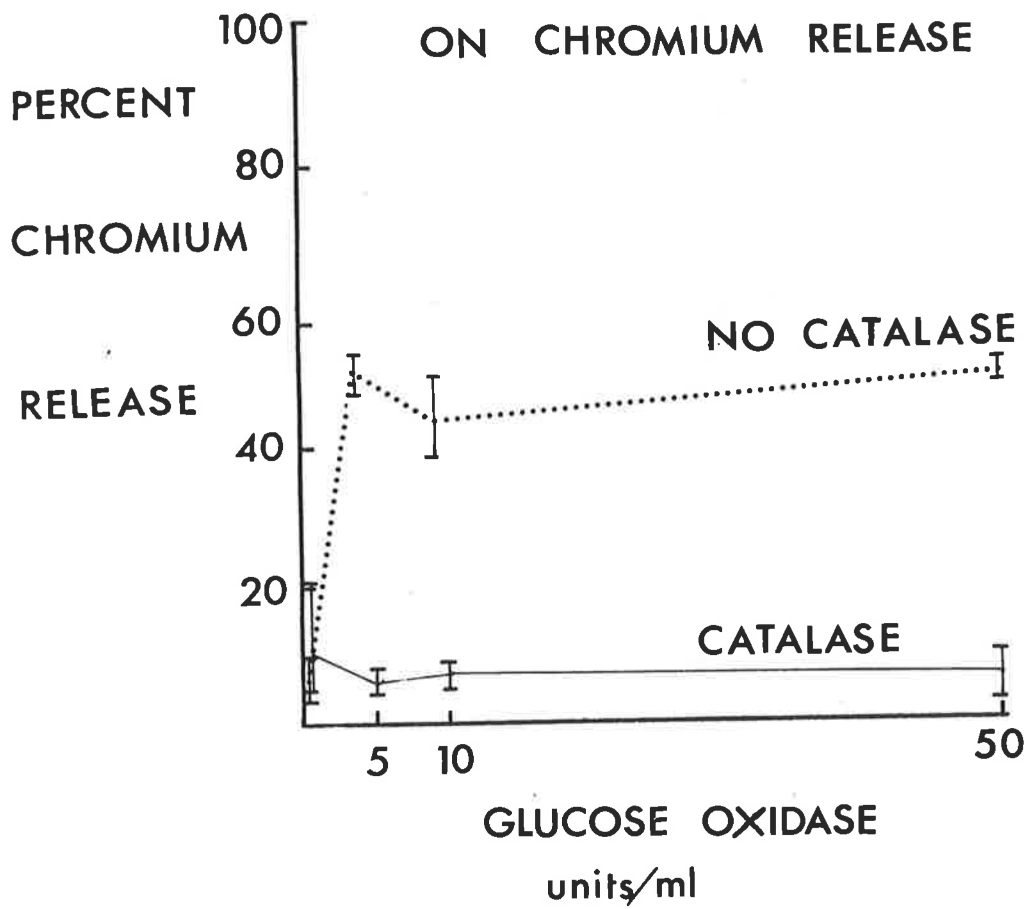

EFFECT OF MANNITOL ON XO/HX INDUCED CHROMIUM RELEASE
PERCENT CHROMIUM RELEASE
100
80
60
40
20
5
20
NO MANNITOL
20 mM MANNITOL
100 mM MANNITOL
XANTHINE OXIDASE CONC.
× 10^{-3} units/ml

FIGURE 6-6 EFFECT OF GLUCOSE/GLUCOSE OXIDASE ON ^{51}Cr RELEASE.

Glucose oxidase = 50-500 mU/ml

Glucose = 0.3 mM

Catalase = 50 units/ml


EFFECT OF GLUCOSE/GLUCOSE OXIDASE
ON CHROMIUM RELEASE
PERCENT
CHROMIUM
RELEASE
100
80
60
40
20
NO CATALASE
CATALASE
5
10
50
GLUCOSE OXIDASE
units/ml

Peak A is also completely susceptible to digestion with hyaluronate lyase from streptomyces hyaluronolyticus (data not shown).

experiments, shown in Figure 6-7 and 6-8, were performed to identify hyaluronic acid in the cell culture medium. The supernatants from two 25 cm^2 tissue culture flasks containing confluent synoviocytes were pooled after a 48 hr labelling period. After concentration, these were fractionated on a Sepharose 4B-CL column under dissociative conditions (i.e. 4 M guanidinium chloride). The chromatographic profile demonstrated 2 peaks of ^{3}H-glucosamine containing material. The higher molecular peak (peak A) eluted near V_0 and a lower molecular weight peak (peak B) eluted in the included volume. Electrophoretic separation of material from each peak was performed on cellulose acetate strips, (Figure 6-8), and hyaluronic acid was identified in peak A but not peak B.

Bovine synovial cells therefore produced relatively small quantities of a high molecular weight hyaluronic acid that elutes at the V_0 of a Sepharose 4B-CL column under dissociative conditions.

We next exposed confluent monolayers of synoviocytes in 25 cm^2 tissue culture flasks to the XO/HX oxygen radical generating system. The xanthine oxidase concentration used was 5 mU/ml in the presence of 2 mM hypoxanthine. This resulted in complete abolition of both peaks and all the radioactivity eluted near V_t (Figure 6-9).

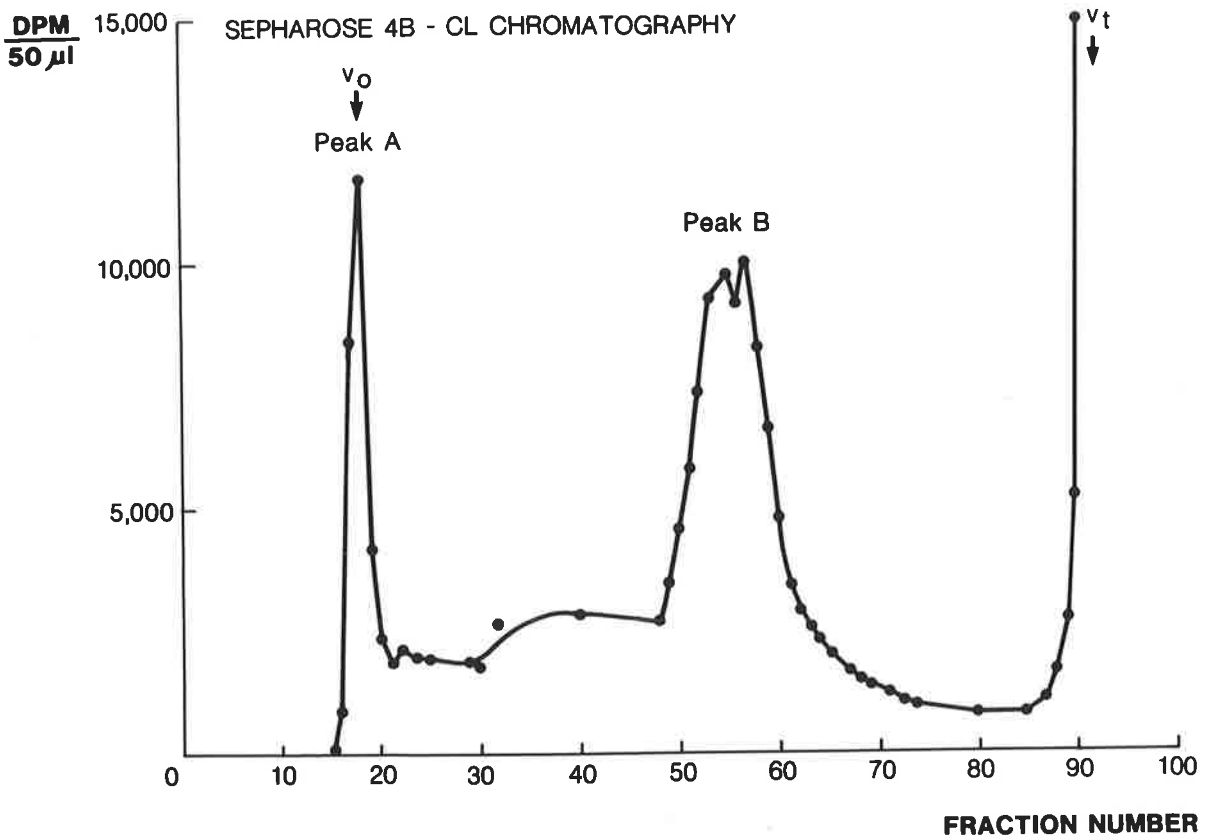
Subsequently confluent monolayers were exposed to a xanthine oxidase concentration of 1 mU/ml. Figure 6-10 shows the results one such experiment. Exposure to xanthine oxidase alone shows the presence of two peaks, as is seen with unexposed cells, however in the presence of 2 mM hypoxanthine the higher molecular weight peak disappears. The profiles shown in Figure 6-10 differ from those shown in Figure 6-7 in that the cell products

FIGURE 6-7 SEPHAROSE 4B-CL CHROMATOGRAPHY OF THE SUPERNATANTS OF ^{3}H-GLUCOSAMINE LABELLED BOVINE SYNOVIAL CELLS.

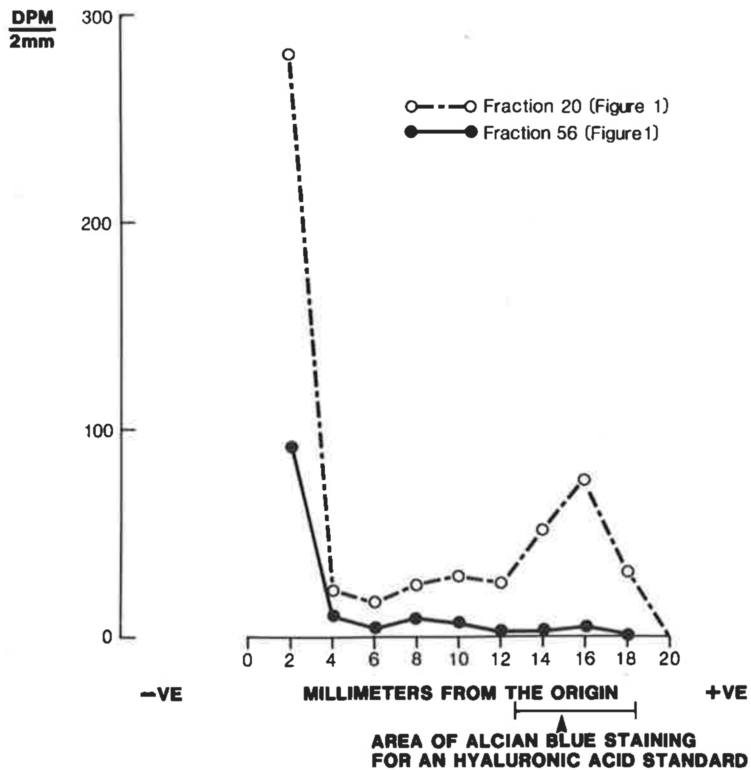
Bovine synovial cells, grown to confluence in two 25 cm^2 tissue culture flasks. They were then cultured in routine tissue culture medium (see Appendix III) containing 50 µCi ^{3}H-glucosamine for 24 hrs. The supernatants were then pooled and concentrated, and the concentrate applied to a Sepharose 4B-CL column which was eluted with 4 M guanidine chloride. Two peaks of glucosamine containing material are apparent, a high molecular weight peak (A) and a lower molecular weight peak (B).

FIGURE 6-8 CELLULOSE ACETATE GEL ELECTROPHORESIS OF MATERIAL FROM PEAKS A AND B.

5 µl of fraction 20 (○- -○) and 5 µl from fraction 56 (●—●) from the material demonstrated in Figure 6-7 were electrophoresed under conditions outlined in Chapter III. A standard of hyaluronic acid was run concurrently and stained with Alcian Blue. The area of Alcian Blue staining is indicated at the bottom of the figure. Radioactive hyaluronic acid was identified in peak A only.



^{3}H- GLUCOSAMINE INCORPORATION BY UNEXPOSED SYNOVIAL CELLS
DPM / 50 µl
15,000
10,000
5,000
0
SEPHAROSE 4B - CL CHROMATOGRAPHY
Vo
Peak A
Peak B
Vt
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER



CELLULOSE ACETATE GEL ELECTROPHORESIS OF MATERIAL FROM PEAKS A&B FROM FIGURE 1
DPM / 2mm
300
200
100
0
Fraction 20 (Figure 1)
Fraction 56 (Figure 1)
0
2
4
6
8
10
12
14
16
18
20
−VE
MILLIMETERS FROM THE ORIGIN
+VE
AREA OF ALCIAN BLUE STAINING FOR AN HYALURONIC ACID STANDARD

FIGURE 6-9 SEPHAROSE 4B-CL CHROMATOGRAPHY OF SYNOVIAL CELL SUPERNATANTS LABELLED AFTER EXPOSURE TO THE XANTHINE OXIDASE/HYPOXANTHINE OXYGEN RADICAL GENERATING SYSTEM.

Bovine synovial cells, grown to confluence in two 25 cm^2 tissue culture flasks were exposed to xanthine oxidase 5 mU/ml XO and hypoxanthine 2 mM in Dulbecco's PBS for 2 hours. They were then washed and cultured in routine tissue culture medium containing 50 µCi ^3H-glucosamine for 24 hrs. The supernatants were then pooled and concentrated, and the concentrate applied to a Sepharose 4B-CL column which was eluted with 4 M guanidine chloride.

^{3}H - GLUCOSAMINE INCORPORATION BY SYNOVIAL CELLS EXPOSED TO 5 mU/ml XANTHINE OXIDASE

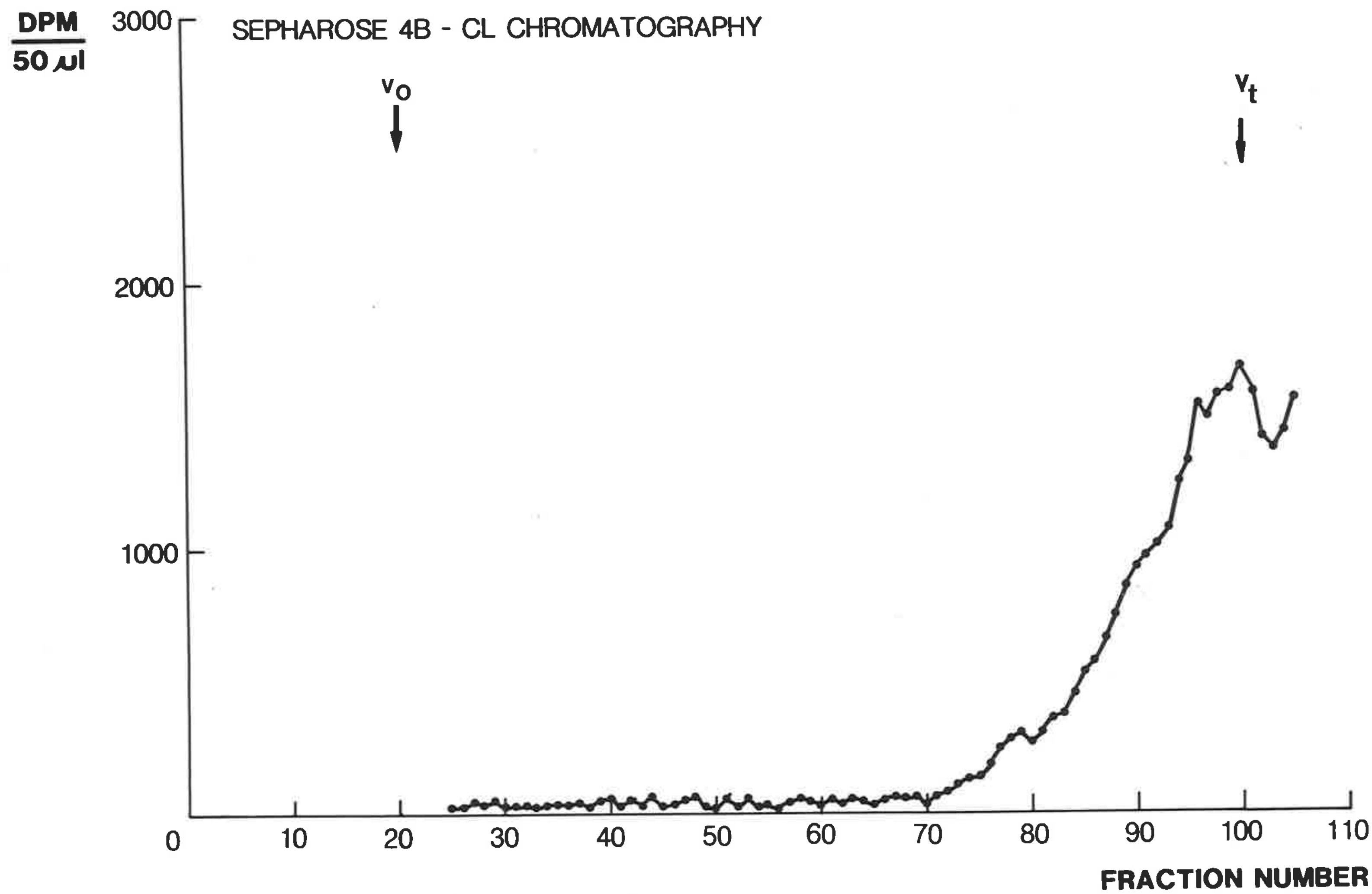
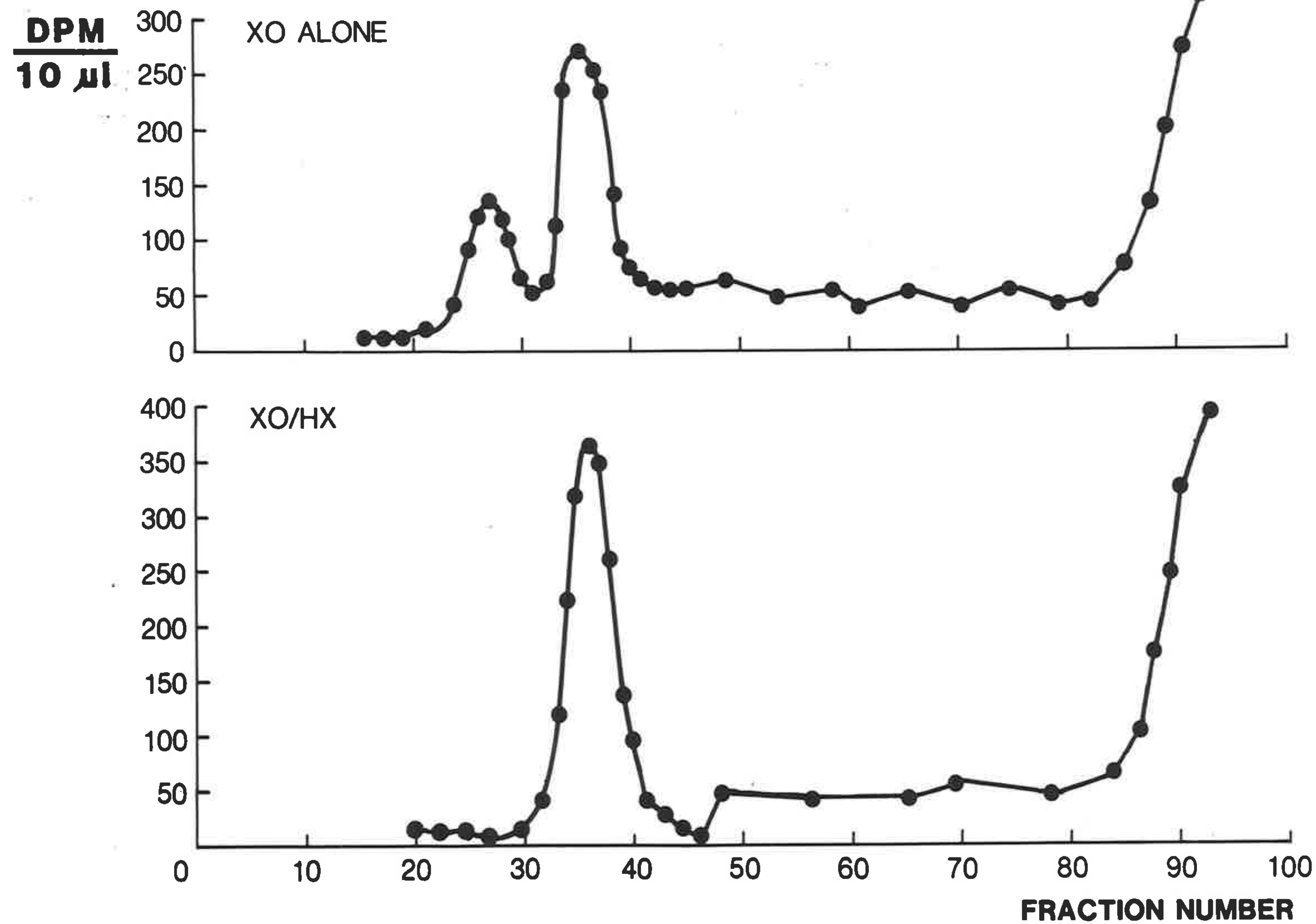


FIGURE 6-10 SEPHAROSE 4B-CL CHROMATOGRAPHY FROM SINGLE 25 CM^2 TISSUE CULTURE FLASKS CONTAINING CONFLUENT SYNOVIAL CELLS AFTER EXPOSURE TO XANTHINE OXIDASE ALONE AND XANTHINE OXIDASE/HYPOXANTHINE.

The panel shows results from a tissue culture flask that was exposed to 1 mU/ml XO in Dulbecco's PBS before a 24 hour labelling period with ^{3}H-glucosamine as outlined in the legend to Fig. 6-8. The bottom panel shows the supernatant from a flask exposed to 1 mU/ml XO in the presence of 2 mM HX. The columns were eluted with 0.5 M NaAc, pH 5.6. The V_o was at fraction 21 and V_t at fraction 93.

Hyaluronic acid was not identified by cellulose acetate gel electrophoresis in peak B in either profile.



^{3}H - GLUCOSAMINE INCORPORATION BY SYNOVIAL CELLS
EXPOSED TO 1 mU /ml XANTHINE OXIDASE
DPM / 10 µl
XO ALONE
0
50
100
150
200
250
300
XO/HX
50
100
150
200
250
300
350
400
0
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

fractionated are the contents of one 25 cm^2 tissue culture flask that has been labelled for 24 hrs. In addition, fractionation was performed under associative conditions (i.e. 0.5M Na Ac, pH 5.8). This has resulted in movement of peak B towards Vo suggesting that the components of peak B have the ability to aggregate and that this is not affected by exposure of the cell to the full XO/HX system in the manner described. Efforts to identify the presence of hyaluronic acid, by gel electrophoresis, in the single peak of the profile seen in the XO/HX exposed cell supernatants were unsuccessful (data not shown).

The final series of experiments, shown in Figure 6-11, were designed to examine the effect of superoxide dismutase and catalase in this system. The conditions were identical to those described above. Superoxide dismutase added at a concentration of 100 U/ml had no effect, however with the addition of 100 U/ml catalase, two peaks were again evident after fractionation.

D. DISCUSSION

The cytotoxicity studies described above demonstrate that catalase protects adherent synoviocytes from the effects of an extracellular oxygen radical generating system. Superoxide dismutase will remove O_2^-, to a degree that will inhibit a Fenton reaction and thus hydroxyl radical production. The finding that superoxide dismutase was not effective in this system would suggest that hydroxyl radical, generated extracellularly, was not necessary for the lethal event to occur. The fact that mannitol, a scavenger of the hydroxyl radical, was minimally effective would support this finding. The toxicity of the glucose/glucose oxidase system and its ablation by catalase, confirms the ability of hydrogen peroxide, generated extracellularly, to act as a cytotoxic agent.

FIGURE 6-11 EFFECT OF SUPEROXIDE DISMUTASE AND CATALASE ON XANTHINE OXIDASE/HYPOXANTHINE INDUCED SUPPRESSION OF HYALURONIC ACID PRODUCTION BY CULTURED BOVINE SYNOVIAL CELLS.

This figure shows a series of profiles, each representing the radioactivity after fractionation of the concentrated supernatants of single 25 cm^2 tissue culture flasks. The elution buffer was 0.5 M NaAc, pH 5.6.

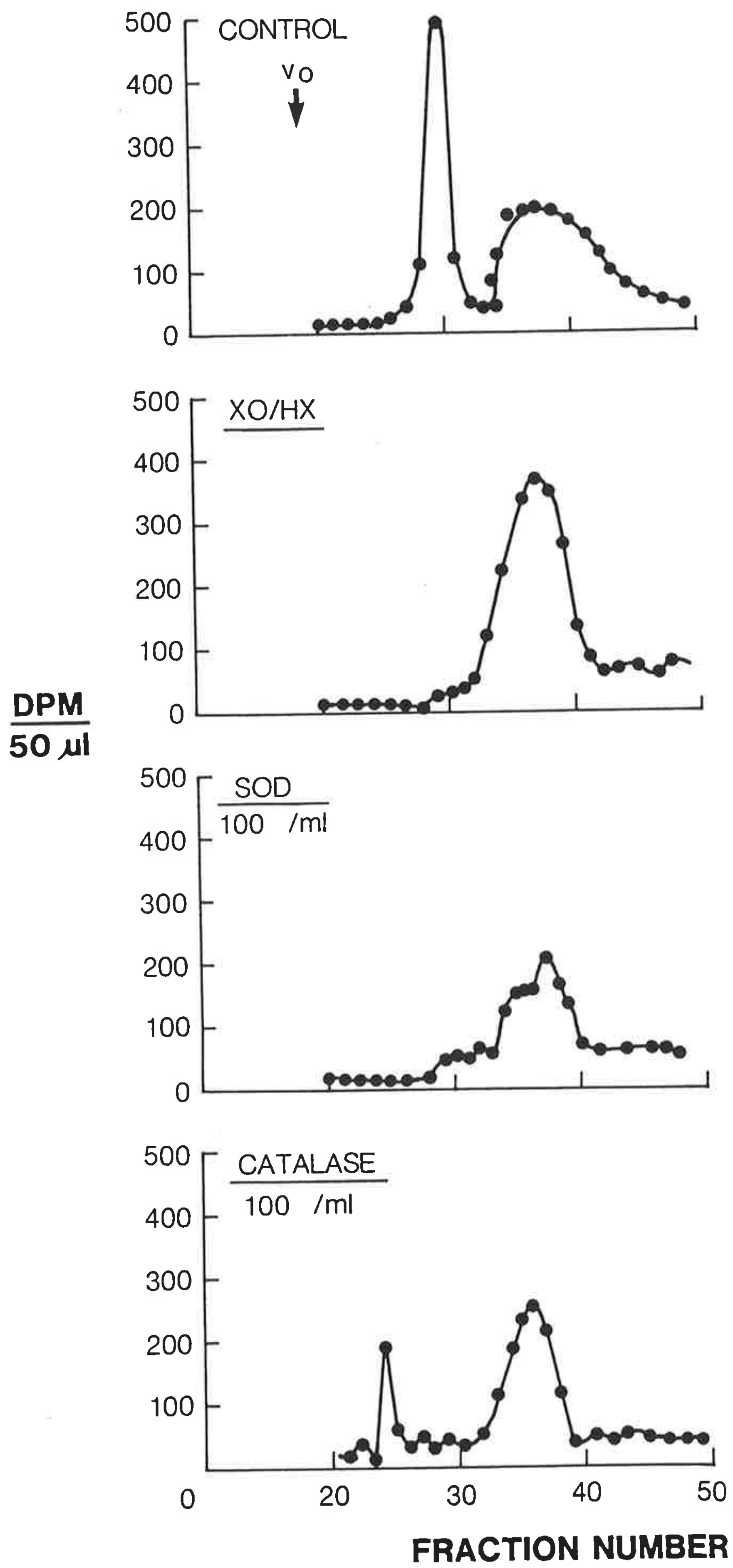
The top panel represents the control i.e. unexposed cells.

The second shows exposure to the XO/HX system (XO, 1 mU/ml; HX, 2 mM).

The third panel shows the effect of the addition of 100 U/ml SOD.

The fourth panel shows the effect of the addition of 100 U/ml catalase.

The two primary peaks as illustrated in Fig. 6-7 are apparent. Exposure to the XO/HX system results in the disappearance of the higher molecular weight HA containing peak which is partially restored by catalase but not by SOD.



CONTROL
Vo
XO/HX
SOD
100 /ml
CATALASE
100 /ml
DPM
50 µl
500
400
300
200
100
0
20
30
40
50
FRACTION NUMBER

Hydrogen peroxide is a small and uncharged molecule and relatively stable when compared to the hydroxyl radical. Hydrogen peroxide may therefore be expected to be able to diffuse away from its extracellular site of production and cross the cell membrane. This will raise intra-cellular hydrogen peroxide levels and if enough hydrogen peroxide is generated, the intra-cellular antioxidant defenses would be overwhelmed. Hydrogen peroxide would then be free, not only to mediate intracellular damage per se, but also to interact with other radical species that are generated as by-products of cell metabolism. Superoxide anion for example, is generated by a number of intracellular processes. If endogenously produced superoxide anion is not adequately scavenged in a situation of oxidative stress, it could interact with hydrogen peroxide to lead to hydroxyl radical production via a Haber-Weiss reaction. The presence of intracellular iron would cátalyse this reaction (i.e. as a Fenton reaction). Evidence that supports this mechanism has recently been produced by SCHRAUFSTATTER et al. [1985] who have recently documented the depletion of intracellular NAD and glutathione in a cell line exposed to hydrogen peroxide. This indicates a depletion of the capacity of the glutathione/glutathione peroxidase system to act as an oxidant defence mechanism.

The addition of superoxide dismutase and catalase to the extracellular medium is unlikely to alter intracellular superoxide dismutase or catalase levels due to the size of these molecules. Mannitol, however is small and uncharged and therefore likely to cross the cell membrane. Its minor protective effect may be due to hydroxyl radical scavenging at intracellular sites. Also however its minor protective effect, seen at high concentrations, may be

due to non-specific scavenging since so-called oxygen radical scavengers are notoriously non-specific.

Chromium release is a manifestation of cell lysis since intracellular chromium is bound to proteins that will only be released with loss of cell membrane integrity [RONAI, 1969]. Cell membranes are particularly susceptible to the deleterious effects of oxidants through the process of lipid peroxidation. Neither of these observations in themselves indicate whether membrane damage is a 1° or 2° event in oxidant induced cell damage. However the sensitivity of hyaluronic acid production to low levels of XO/HX would suggest cell membrane damage was an early event in oxidant damage. PREHM [1984] and PHILIPSON and SCHWARTZ [1984] have demonstrated that hyaluronic acid synthetase is situated on the internal surface of the cell membrane and newly synthesized hyaluronic acid is secreted directly into the extracellular space whereas other glycosaminoglycans are synthesized in the cytoplasm. Therefore the observation that exposure of synoviocytes to 1 mU xanthine oxidase in the presence of hypoxanthine results in the loss of hyaluronate production, but not the production of other N-acetylglucosamine containing products, suggests early membrane damage at a stage when cytoplasmic components are still functional.

VUORIO et al. [1982] have reported that synoviocytes grown from patients with rheumatoid arthritis will produce low molecular weight hyaluronic acid when established in culture. Synthesis of such a product in vivo is an alternative explanation for the finding of decreased average molecular weight hyaluronic acid found in inflammatory arthropathies. Our finding that exposure to sublethal levels of XO/HX results in cessation of hyaluronic acid

production rather than synthesis of a smaller product would suggest that oxidant exposure can be discounted as a cause for such an alteration in synthesis. It is of interest to note that BATES, LOWTHER and HANDLEY [1985] have also reported that chondrocytes exposed to sublethal levels of oxygen radicals will decrease the amount of proteoglycan produced, but will not effect the size of the proteoglycan produced by these cells.

E. SUMMARY

Bovine synoviocytes in culture are susceptible to oxygen radicals produced by XO/HX as measured by ^{51}Cr release. Administration of catalase was protective whereas superoxide dismutase was ineffective and mannitol had a minimal effect. This indicates that hydrogen peroxide is the agent necessary for toxicity. Sublethal oxygen radical fluxes will result in cessation of hyaluronic acid synthesis even when the synthesis of other ^{3}H-glucosamine containing products continues. No low molecular weight hyaluronic acid products could be detected in the supernatants of oxygen radical exposed cells.

CHAPTER VII

DISCUSSION

A. *IN VITRO* OXYGEN RADICAL INDUCED HYALURONIC ACID DEPOLYMERIZATION.

1. Molecular weight studies.

The simple chemical structure of hyaluronic acid belies its complex physico-chemical properties and biological roles. These properties are due to its macromolecular structure which depends upon both inter- and intra- chain interactions and which lead to the formation of a matrix in aqueous solutions of sufficient contentration. It is also apparent from the data presented in this thesis, that a consideration of macromolecular structure is necessary before an hypothesis of hyaluronic acid degradation by oxygen radicals can be formulated.

The findings that emerge from the studies done on the molecular weight changes after oxygen radical induced depolymerization and which must be reconciled into such an hypothesis are:

1. The smallest depolymerization product is 10^4 molecular weight.

2. There is a rapid progression from large molecular weight hyaluronic acid to the smallest size degradation product.

3. A degree of repolymerization of degradation products is probable.

The first finding is the most clear cut and may be accounted for by a number of depolymerization mechanisms. Most obvious are those mechanisms that require that there be a site along the hyaluronic acid molecule that is particularly susceptible to cleavage by oxygen radicals.

Perhaps the most likely property of hyaluronic acid that would lead to a series of susceptible sites along its length is its

tertiary structure in aqueous solution. As outlined in Chapter I, hyaluronate, in aqueous solution, is believed to adopt random coil best characterized as a left-handed helix. The random nature of the coiling of the hyaluronate molecule is one feature that may lead to exposure (or increased accessability) of a glycosidic linkage to the surrounding environment. This in turn could make such a linkage susceptible to oxidative damage. Alternately structural constraints may be imposed upon the tertiary structure of a hyaluronic acid molecule in solution by its interaction with other hyaluronate molecules in the same solution. Structural studies [WINTER, SMITH and ARNOTT, 1975] indicate a degree of stiffness of the hyaluronate molecule in aqueous solution, clearly then the formation of a matrix in solution with so many "stiff" molecules would cause irregularities in tertiary structure to occur. What is clear from our studies is that these susceptible sites must be a considerable distance apart to account for the smallest molecular weight degradation products having a molecular weight of 10^4 (i.e. approximately 50 saccharides). This hypothesis predicts that an hyaluronate chain of lesser length is not long enough to adopt the tertiary structure that leads to the formation of a susceptible site.

The chelation of iron is clearly a critical factor in all the oxygen radical reactions utilized in this study, and many <u>in vivo</u> reactions involving radicals (see Chapter I). It may be that by virtue of its tertiary structure that hyaluronic acid can chelate iron or another metal ion in such a way that the generation of the hydroxyl radical occurs in close proximity to the hyaluronate chain. It is clear now from a number of studies that hydroxyl radical production is necessary for hyaluronic acid

depolymerization [BETTS and CLELAND, 1982; HOFMANN AND SCHMUT, 1980]. The extremely high reactivity of the hydroxyl radical dictates that it must be generated close to its target site since it so indisriminately reactive [FEE and VALENTINE, 1977]. This situation would therefore represent a form of "site-specific Fenton reaction" i.e. the site of chelation of a metal ion capable of catalysing a Fenton reaction determines where hydroxyl radical damage occurs. In the case of hyaluronic acid this would be close to the β1-3 glycosidic linkage as discussed in Chapter IV. This hypothesis would thus reconcile the high and non-specific reactivity of the hydroxyl radical with a specific glycosidic bond cleavage.

An implication of these "susceptible site" hypotheses is that they may provide an explanation for the second stated finding of the molecular weight studies. Depolymerization proceeding from "susceptible site" to "susceptible site", in an inevitable chain reaction would result in the observation of a rapid progresssion of large molecular weight hyaluronic acid to the smallest size degradation product with a relative paucity of intermediate sized material.

Other hypotheses are possible. The generation of lower molecular weight hyaluronic acid may interfere with further oxygen radical production. This may occur if the small molecular weight products act as highly efficient oxygen radical scavengers. Alternately hyaluronic acid depolymerization products may interfere with chelator-metal binding, thus preventing further hydroxyl radical generation. The chelator may be EDTA, or, as suggested previously, hyaluronic acid itself.

Finally the suggestion that oxygen radical damaged hyaluronic acid may repolymerize suggests a further mechanism that would limit the size of the degradation product to 10^4 . An equilibruim may be reached with depolymerization predominating at large molecular weights and repolymerization predominating at small molecular weights.

A number of these hypotheses are testable and lead to the following specific questions being posed.

1. To what extent and how tightly does hyaluronic acid bind (labelled) iron or other metal ions?

2. Can hyaluronic acid degradation products interfere with chelator-metal binding?

3. Are hyaluronic acid degradation products more efficient oxygen radical scavengers on a mole for mole basis that undepolymerized hyaluronate?

4. Can cross linked or branched hyaluronic acid be found in the depolymerization products of oxygen radical exposed hyaluronic acid?

SATO and NIWA (1985) have addressed the question of the radical scavenging capacity of hyaluronic acid (yes) and its constituent sugars, D-glucuronic acid (No) and N-acetylglucosamine (yes). Hyaluronic acid degradation products (i.e. of about 10^4 molecular weight or smaller oligosaccharides) however have not been examined.

We have looked for crosslinked or "branched" hyaluronic acid in oxygen radical exposed hyaluronic acid. This was done by gel chromatography of oxygen radical exposed hyaluronic acid that was exhaustively digested with streptomyces hyaluronidase. However our ability to resolve the oligosaccharides was not

sufficient to allow a conclusion. Newer methods using polyacrylamide gel electrophoresis to separate hyaluronate oligosaccharides may be helpful in this regard [HAMPSON and GALLAGHER, 1985]. The finding of a product, characteristically altered by oxygen radical exposure would provide a means to establish unequivocally whether inflamed synovial fluid hyaluronic acid is depolymerized by oxygen radical exposure *in vivo.*

2. *Reducing Ends.*

The finding in our study that reducing ends are released during the oxygen radical induced depolymerization of hyaluronic acid is clear cut since it has been observed by both colorimetric and radiolabeling techniques. This finding is in contrast to some other early reports [McCLEAN and HALE, 1940; GREENWALD and MOY, 1980]. However CLELAND *et al.* [1969], using the ascorbic acid induced depolymerization of hyaluronic acid were able to detect the release of reducing ends using sodium borohydride labelling. We differ from these authors however in finding that it is the β1-3 glucuronidic linkage that is preferentially cleaved rather than the β1-4 hexosaminidic linkage. We have been careful not to let cleavage by the alkaline conditions required during both the colorimetric and radiolabelling assays lead to misleading results. SWANN *et al.* [1968] have determined that hyaluronic acid is not depolymerized under the conditions used in our study for cyanide labelling. A similar criterion was also established for the colorimetric assay of Park and Johnson (i.e. pH not greater than 9.0). This is of importance since the β1-3 glycosidic linkage of hyaluronic acid is relatively labile in alkaline conditions [ROBINSON and HOPWOOD, 1973].

Two other lines of evidence would also support cleavage at the β1-3 glycosidic linkage. The first comes from the work of

BALAZS et al. [1967] who reported the electron spin resonance spectroscopy findings of hyaluronic acid in aqueous solution exposed to pulse radiolysis. Pulse radiolysis in aqueous solutions leads to the generation of the hydroxyl radical as well as the aqueous electron and superoxide anion. Their findings were best explained by the formation of two transient intermediates that could be produced by either the hydroxyl radical (relevant to our oxygen radical generating systems) or the aqueous electron (not relevant). The first transient intermediate is produced by removal of an electron from the oxygen of the β1-3 glycosidic linkage. This would react further giving rise to cleavage at this linkage. The second intermediate, seen at high doses of radiation, involves labilization of the hydrogen on C_5 of D-glucuronic acid. This could be stabilized by a resonance interaction with the caboxylic acid group of this sugar. This latter intermediate may have significance in any repolymerization reaction seen as a result of interaction of hyaluronic acid with the hydroxyl radical.

Secondly our results allow an explanation of the findings of GREENWALD and MOY [1979], who reported that hyaluronic acid exposed to oxygen radicals is more susceptible to digestion by the neutrophil lysosomal enzyme, N-acetylglucosaminidase. This enzyme will remove N-acetylglucosamine from the non-reducing end of hyaluronic acid [LINKER, MEYER and WEISSMAN, 1955]. Therefore if oxygen radical depolymerization of hyaluronic acid represents a situation where only the β1-3 glycosidic linkage is cleaved, then all cleavages will leave N-acetylglucosamine at the non-reducing end. This will allow maximal substrate for this enzyme and thus a maximal amount of N-acetylglucosamine will be released upon digestion with this enzyme.

B. STUDIES OF SYNOVIAL FLUID HYALURONIC ACID ISOLATED FROM INFLAMED JOINTS. ARE OXYGEN RADICALS RESPONSIBLE?

A large number of studies have reported the changes in synovial fluid that occur as a result of inflammation. Although a number have presented divergent results for the molecular weight changes seen in synovial fluid hyaluronic acid (see BALAZS et al. [1967], DAHL et al. [1985]), a number of characteristic changes are agreed upon. These are:

- increased synovial fluid volume
- decreased synovial fluid hyaluronic acid concentration (and hence the clinical finding of a decreased viscosity of raw synovial fluid).
- increased "total amount" of hyaluronic acid present in the synovial fluid as a result of the increased volume.
- decreased average molecular weight of the hyaluronic acid present.

The molecular weight spectra of inflammatory synovial fluid hyaluronic acid presented in this thesis are consistent with other studies demonstrating that the decrease in average molecular weight, seen during inflammation is small, but definite. In inflamed synovial fluids, most synovial fluid hyaluronic acid remains excluded on Sepharose 4B suggesting a very high molecular weight. Nevertheless, with inflammation a greater proportion elutes in the included volume and material can be indentified down to a molecular weight of 2×10^4. Of additional interest are the shapes of the profiles. Most included volume material is seen as a skewing of the excluded volume peak into the included volume however a number of our inflamed fluids have a separate peak in the included volume on Sepharose 4B. This would be predicted from our findings

in the *in vitro* studies that degradation proceeds rapidly to the smallest degradation product seen (i.e. molecular weight of 10^4). This second peak may represent small molecular weight breakdown products, present in sufficient quantity before they can be cleared from the joint space.

We, like others [SUNDBLAD, 1953; DAHL *et al.* 1985] could not find any significant correlation between the decrease in average molecular weight and other indices of inflammation (erythrocyte sedimentation rate, haemoglobin, synovial fluid white cell counts). Both sides of this attempted correlation however, represent imprecise measures. Systemic measures of inflammation (erythrocyte sedimentation rate and fall in haemoglobin) may be at variance with the degree of synovitis involving the joint sampled. Local measures (synovial fluid cell count) are also imprecise e.g. some very inflamed joints have no effusion at all.

Other factors, such as the production and clearance of various sizes of hyaluronic acid from the synovial fluid are going to effect the molecular weight profile of synovial fluid hyaluronic acid sampled at any particular time. Hyaluronic acid is being sampled at one point in a dynamic process dependent upon the rates of both synthesis of hyaluronic acid and its clearance from the joint space. The peturbations of these latter processes by inflammatory change are not known, indeed little is known of hyaluronic acid turnover. Studies by ANTONAS, FRASER and MUIRDEN [1972] have shown the very rapid appearance (<15 minutes) of labelled hyaluronic acid in the draining lymph node of the rabbit knee after intra-articular injection. This suggests a rapid rate of turnover. Any molecular weight change produced by a depolymerization process *in vivo* is dependent in part upon the

length of time hyaluronic acid is exposed to the depolymerzing agent. A teleological explanation for a high rate of hyaluronic acid turnover is that this will limit the extent of deterioration of the synovial fluid hyaluronate matrix due to a depolymerization process and thus will preserve its biological functions.

C. EFFECT OF OXYGEN RADICALS ON SYNOVIOCYTES AND THEIR PRODUCTION OF HYALURONIC ACID.

Finally there remains the proposition that hyaluronic acid is not depolymerized in vivo - that there are enough oxygen radical scavenging mechanisms available in the synovial fluid to prevent post-synthetic depolymerization. This hypothesis suggests that inflammatory damage to type II synoviocytes causes them to synthesize hyaluronic acid with a decreased average molecular weight. In support of this hypothesis VUORIO et al. [1982] have published the profiles of hyaluronic acid produced in culture by synoviocytes obtained by explant culture of rheumatoid and normal synovium. They reported that synoviocytes obtained from inflamed rheumatoid and joints inflamed for other reasons produced hyaluronic acid of a lower molecular weight than synoviocytes obtained from normal or non-inflamed synovium. The Sepharose 2B profiles for the products of inflamed synoviocytes show minimal or no material in the excluded volume and the peak of the included volume material has a Kav of approximately 0.75 corresponding to a molecular weight of 10^5. Simply stated this material is too small to compare with hyaluronic acid extracted from inflamed synovial fluids. A number of factors will affect N-acetylglucosamine incorporation into hyaluronic acid under culture conditions. These include the growth phase of the cells and the glucose concentration. Also it may be preferable in this circumstance to consider organ culture as suggested by MYERS [1985]. The findings

of VUORIO et al. [1984] therefore are more likely to indicate that the synoviocytes from inflamed joints are more susceptible to in vitro influences that cause an alteration in hyaluronic acid synthesis rather than proving that a smaller size hyaluronic acid molecule is produced by inflamed synoviocytes in vivo.

We approached this question by asking whether cultured synovial cells could be altered by exposure to oxygen radicals in such a way as to produce a lower molecular weight hyaluronic acid. In short we found that cells exposed to sublethal doses of oxygen radicals shut off their production of hyaluronic acid rather than produced a smaller product. Hyaluronic acid production was more sensitive to oxygen radical damage than other cellular synthetic functions. This is in keeping with the work of BATES et al. [1984] who found that upon exposure of chondrocytes to a sublethal oxygen radical flux the rates of synthesis decreased but the size of the product was unaltered. Clearly we have only tested one of a possibly very large number of factors acting on the synoviocyte at an inflammatory site and which includes other neutrophil secretory products, T cell and macrophage products, cytokines and so on. It is possible that one or more of these could lead to an alteration in the size of the hyaluronate produced. Also more understanding of the mechanisms of initiation and cessation of hyaluronate chain synthesis are required before this question can be completely resolved. Hyaluronate synthetase, by virtue of its close association with the cell membrane is likely to be susceptible to membrane disruption. One of these is the production of oxygen radicals due to the propensity of membranes to undergo lipid peroxidation. A logical extension to our studies is therefore to examine other membrane damaging agents for their effect on hyaluronate synthetase function and also to examine the cell

supernatants for the appearance of lipid peroxidation products particularly in relation to the cessation of hyaluronic synthesis.

D. CONCLUDING REMARKS.

The effect of oxidants generated within a biological system have been seen as an interplay between the reactivity of the oxidant generated and the defence or scavenger systems available to protect against that oxidant. It has emerged from the work in this thesis that a third important factor in this interplay is target susceptibility. The evidence in our studies on isolated hyaluronic acid has pointed towards a site specific susceptibility to depolymerization. Specifically β1-3 glycosidic linkages spaced at sites along the molecule are susceptible. Equally with synoviocytes in monolayer culture the loss of cell viability and cessation of hyaluronate synthetase function are mediated by hydrogen peroxide, underlining the importance of access to a target site within the cell.

A study of the effects of oxidants within the context of joint inflammation has been undertaken by examining in detail an important component of joint physiology, namely hyaluronic acid. Such a reductionist tactic has been used since the number of effector agents, modifying factors and structures at risk within the inflamed joint are too numerous to study effectively. Yet this approach has proven useful in exposing target sites susceptibile to oxidant damage

An ultimate aim of research in the field of rheumatology is prevention, by therapeutic intervention, of joint deformity wrought by the inflammatory process. A clear appreciation of the specific type and extent of this damage that can be attributed to oxidants is required so that appropriate therapeutic steps can be taken to prevent this form of joint damage from occurring.

APPENDIX I

URONIC ACID ASSAY

The technique described by BLUMENKRANTZ and ASBOE-HANSEN [1973] was used.

A. REAGENTS

1. Meta-hydroxydiphenyl solution

An 0.15% solution of meta-hydroxydiphenyl (Eastman Kodak Co., Rochester, New York) in 0.5% NaOH was prepared and stored in the refrigerator with an aluminum foil covering. This solution was remade every three months.

2. H_2SO_4/tetraborate solution

An 0.0125 M solution of sodium tetraborate in concentrated sulphuric acid.

B. METHOD

0.2 ml of unknown and standards containing from 2.5 - 100 ug/ml D-glucuronic are added to 13 x100 mm test tubes. 1.2 ml of the sulphuric acid/tetraborate solution is then added and the tubes are refrigerated on crushed ice. The tubes are then shaken in a Vortex mixer and heated in a boiling water bath for 5 minutes. After cooling on crushed ice for 10-15 minutes, 20 µl of the m-hydroxydiphenyl reagent is added. The tubes are shaken and the color allowed to develop for 5 minutes. Absorbance measurements are then read at 520 nM on a Perkin-Elmer (LS 1500) spectrophotometer. Measurements on samples from fractions after gel chromatography are done in duplicate, all other determinations are in triplicate.

FERRICYANIDE METHOD OF PARK AND JOHNSON

This technique is a modification of the assay originally described by PARK and JOHNSON [1947] and subsequently modified by HOUCK and PEARCE [1957] for use with hyaluronic acid.

A. Reagents. Three reagents are prepared :

1) Ferricyanide solution. 0.5 gm of potassium ferricyanide per liter; stored in a brown bottle.

2) Carbonate-cyanide solution; 5.3 gm of sodium carbonate + 0.65 gm of potassium per liter of solution. In our experiments the pH of this reagent was adjusted to a pH of 9.0 [HOUCK and PEARCE, 1957].

3) Ferric ion solution; 1.5 gm of ferric ammonium sulphate + 1 gm of Duponol (sodium monolauryl sulphate, Du Pont Inc., Wilmington, Delaware) in 1 liter of 0.05N sulfuric acid.

B. Method

0.5 ml of the unknowns and standards containing 1 -20 ug/ml glucuronic acid are added to 13 x100 mm pyrex test tubes. 0.5 ml potassium ferricyanide and 0.5 ml carbonate-cyanide solutions are then added. The mixture is then shaken on a Vortex mixer and heated in a boiling water bath for 15 minutes. The tubes are then cooled by immersion in a water bath at room temperature for a further 15 minutes. 2.5 mls of the ferric ion solution are then added and the mixture again shaken on a Vortex mixer. The colour reaction is then read at 690 nM on a Perkin Elmer (LS 1500) spectrophotometer. All measurements are done in triplicate.

MORGAN - ELSON REACTION

This technique is the modification of the Morgan-Elson reaction according to REISSIG, STROMINGER and LELOIR [1955].

A. REAGENTS

1. Potassium tetraborate solution; An 0.8 M potassium tetraborate solution is made and the pH adjusted to 9.1 with potassium hydroxide.

2. P - dimethylaminobenzaldehyde (DMAB) solution; 10 gm DMAB (Eastman Kodak, Rochester, New York.) is dissolved in 100 ml reagent grade glacial acetic acid with 12.5% (volume per volume) 10 N hydrochloric acid. This reagent can be stored at 2°C for up to a month in a dark bottle. Shortly before use the solution is diluted with 9 volumes of reagent grade acetic acid.

B. METHOD

0.25 ml volumes of unknowns and standards containing 2.5 -50 ug/ml N-acetylglucosamine are added to 13 x 100 mm pyrex test tubes. 50 ul of potassium tetraborate solution is then added. The tubes are heated in a boiling water bath for 3 minutes. Then they are cooled in tap water and 1.5 DMAB solution is added and the tubes are shaken on a Vortex mixer. The tubes are then heated in a water bath at 36 - 38°C for 20 minutes. They are then read at 585 nM on a Perkin Elmer (LS 1500) spectrophotometer. All measurements are done im triplicate.

APPENDIX II

TISSUE CULTURE SOLUTIONS

A. PRIMRARY CELL CULTURE MEDIUM.

- Ham's F10 medium [HAM, 1963]
- 20% foetal calf serum.
  (heat inactivated by incubating at 56°C for 30 mins)
- gentamycin 0.2 mg/ml.
- L-glutamine 3 mM added fresh.

B. ROUTINE SYNOVIAL CELL CULTURE MEDIUM.

- Ham's F10 medium.
- 10% foetal calf serum. (heat inactivated)
- gentamycin 0.2 mg/ml.
- L-glutamine 3 mM added fresh.

C. DULBECCO'S PHOSPHATE BUFFERED SALINE.

$NaCl$	- 8.0 g/l	KCl	- 0.2 g/l
$CaCl_2$	- 0.1 g/l	$MgCl_2.6H_2O$	- 0.1 g/l
$Na_2HPO_4.2H_2O$	- 1.15 g/l	KH_2PO_4	- 0.2 g/l

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NOTE:
This publication is included in the print copy
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It is also available online to authorised users at:
http://dx.doi.org/10.1136/ard.44.11.780

McNeil, J. D., Wiebkin, O. W., Cleland, L. G., & Vernon-Roberts, B. (1986). The generation of reducing ends by exposure of hyaluronic acid to oxygen derived free radicals. *Agents and actions. Supplements, 18*, 95-101.

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ERRATA SHEET

P. 7, reaction (3)	$O_2^- + H_2O_2$ ------ $O_2 + OH\cdot + OH^-$
P. 10, reaction line 8	$2NADPH + 2O_2$ ----- $2NADP^+ + 2O_2^- + H_2O$
P. 18, line 7	"Glutatione" should read "Glutathione"
P. 19, line 28	"to be an an antioxidant" should read "to be an antioxidant"
P. 90, line 21	($[Fe^{2+}]$ = 50 µL) should read ($[Fe^{2+}]$ = 50 µM)

P. 97, line 29, Insert after "Morgan-Elson assay"

The reason that there is a discrepancy between the degree of Park-Johnson reactivity for a given molecular weight remains unclear, although it may be related to the inherent inaccuracy in using the broad peak, obtained after gel chromatography, to measure molecular weight.

P. 128, line 13, Insert after "but not Peak B"

Peak A is also completely susceptible to digestion with hyaluronate lyase from streptomyces hyaluronolyticus (data not shown).

P. 139, line 14	insert "in" after "irregularities"
P. 140, Table 5-1	heading factor is "x 10^{-6}"
P. 141, line 2	"repolymerized" should read "repolymerize"
P. 148, line 22	"so" should read "too"