Oxygen free-radicals mediate an inhibition of proteoglycan synthesis in cultured articular cartilage

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SUMMARY Superoxide radical, generated enzymatically by the action of xanthine oxidase on hypoxanthine, significantly inhibited proteoglycan synthesis by cultured bovine articular cartilage. This inhibition was not due to the generation of uric acid or to the generation of superoxide per se. It was immediate in onset and still evident after six days in culture. The inhibition was similar for both ^{35}S -sulphate and ^3H -acetate incorporation into glycosaminoglycans and could not be reversed by addition of β -D-benzyl xyloside. Protein synthesis was also inhibited.

Both antigen- and carrageenin-induced arthritis in rabbits^{1,2} are used in this laboratory as experimental models for human rheumatoid arthritis. In the acute phase of both the human disease and animal models vastly increased numbers of inflammatory cells (mainly polymorphonuclear leucocytes) are seen in the joint fluid and synovial tissue.^{1,3} In human rheumatoid arthritis there is a progressive destruction of cartilage which could result from an imbalance between the rate of degradation of the tissue components compared with their rate of renewal. In both animal models there is a loss of proteoglycan from the cartilage^{1,2} which is accompanied by a dramatic decrease in proteoglycan synthesis.^{2,4,5}

It is well known that phagocytic leucocytes (particularly polymorphonuclear leucocytes) reduce oxygen to the superoxide radical when exposed to both phagocytosable and non-phagocytosable stimuli, and this is released into the extracellular space.⁶⁻⁸ Superoxide is believed to be involved in intracellular microbial killing⁹ and in the generation of chemotactic factors,^{10,11} causing the influx of more polymorphonuclear leucocytes to the site of inflammation.

Superoxide in aqueous solvents undergoes a spontaneous reaction with itself known as dismutation, producing hydrogen peroxide. This reaction can also be catalysed by the intracellular enzyme superoxide dismutase. Superoxide may also react with this hydrogen peroxide to form the very reactive hydroxyl

radical and singlet oxygen (reviewed in Del Maestro¹²). The ability of enzymatically generated superoxide (via the production of hydroxyl radicals) to reduce the viscosity of synovial fluid has been taken to indicate that oxygen radical generation may be involved in vivo in the deterioration of synovial fluid characteristic of inflammatory types of arthritis.¹³

The aim of this work was to investigate in vitro whether enzymatically generated superoxide could alter the synthesis of proteoglycan by cartilage cultures in a manner which would indicate that this might be a mechanism involved in arthritis in vivo.

Materials and methods

Enzymes and chemicals were from Sigma Chemical Co., St Louis, Missouri. Chromatographic materials were from Pharmacia, Uppsala, Sweden. Radioactively labelled precursors were from the Radiochemical Centre, Amersham, UK (^{35}S -sodium sulphate and ^3H -sodium acetate) and New England Nuclear, Boston, Massachusetts, USA (^3H -leucine and ^3H -proline). β -D-benzyl xyloside was a gift from Dr H. C. Robinson, Department of Biochemistry, Monash University. Culture medium was Dulbecco's modified Eagle's medium containing 1 g/l glucose (Gibco, New York, USA) and supplemented with organic buffers, amino acids,¹⁴ and 20% (v/v) adult bovine serum. The medium was changed daily and cultures maintained at 37°C.

Xanthine oxidase purification and enzyme assays. Xanthine oxidase was further purified to remove proteolytic contaminants.¹⁵ A 30-fold purification was

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achieved with less than 0.75 ng trypsin equivalent remaining per mU enzyme activity. Xanthine oxidase activity and its ability to generate superoxide from hypoxanthine were determined after each treatment of cartilage. Xanthine oxidase was measured as described by McCord and Fridovich¹⁶ using 50 μ M xanthine as substrate. Superoxide was estimated by following the reduction of cytochrome c (250 μ M) at 37°C in 1 ml of treatment medium.¹⁶ The initial rate of superoxide generation was equivalent to 10–26 nmol cytochrome c reduced per min.

Superoxide dismutase and catalase were dissolved at 4 mg/ml in buffer A consisting of 138 μ M NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4, plus 1 mM EDTA (ethylenediamine tetra-acetic acid). All enzymes were dialysed against 1000 vol of buffer A before use. Superoxide dismutase activity was determined after treatment.¹⁶ In control treatments enzymes were replaced by equal volumes of dialysis buffer.

Culture conditions. The removal and preparation of bovine articular cartilage under sterile conditions from the metacarpal-phalangeal joints of freshly slaughtered adult animals has been described previously.¹⁷ Cartilage from one joint (approximately 3.5 g) was used per experiment and maintained in 30 ml of culture medium for 4–5 days before distribution of between 50–150 mg wet weight of tissue into culture vials containing 4 ml of fresh culture medium. The following day the tissue was washed with 2 ml of buffer A and then treated with the superoxide generating system. The tissue was then washed twice with 2 ml of culture medium without serum, and subsequently maintained in 4 ml culture medium for 24 hours (except Fig. 2) before proteoglycan or protein synthesis was determined.

Superoxide generating system. Tissue was incubated for two successive periods of 90 min at 37°C with hypoxanthine (1 mM) and xanthine oxidase (20–60 mU) in 2 ml of buffer A supplemented with 5.8 mM glucose, 0.2 mM EDTA, and 10 μ M FeSO₄ unless indicated otherwise. The medium was replaced after 90 min and the incubation repeated. Media from both incubations were retained and pooled for hexuronic acid analysis. Treatments were performed in triplicate within each experiment.

Measurement of proteoglycan synthesis. Proteoglycan synthesis was measured by the incorporation of ³⁵S-sulphate or ³H-acetate into proteoglycan or glycosaminoglycan chains. The tissue was preincubated for 1 hour at 37°C in a shaking water bath (30 cycle/min) with 2 ml of fresh culture medium. This was replaced with 2 ml of culture medium supplemented with ³⁵S-sulphate (35 μ Ci/incubation, final specific activity 22 μ Ci/ μ mole) or ³H-acetate (20 μ Ci/incubation, final specific activity 13

μ Ci/ μ mole) and incubated for a further 2 hours. The medium was removed and retained. In some experiments ³⁵S-labelled glycosaminoglycans in the tissue were extracted with 2 ml of 0.5 M NaOH for 36 hours at room temperature. In other experiments ³⁵S-labelled proteoglycans in the tissue were first extracted with 2 ml of 4 M guanidine-HCl containing inhibitors¹⁸ for 48 hours at 4°C, and then glycosaminoglycans in the tissue residue were extracted with 2 ml of 0.5 M NaOH for 24 hours at room temperature. The amount of radioactivity incorporated into glycosaminoglycans (NaOH extracts) and proteoglycans (guanidine extracts) was determined by applying 250 μ l aliquots of extracts to Sephadex G-25 PD-10 columns¹⁹ and counting the eluent. Samples of labelling medium were similarly eluted and counted.

To measure the amount of ³H-acetate incorporated into the glycosaminoglycan fraction the tissue was digested with papain and glycosaminoglycans separated by ion-exchange chromatography on diethyl aminoethyl (DEAE) cellulose.¹⁴

Measurement of glycosaminoglycan synthesis in the presence of β -D-benzyl xyloside. The tissue was preincubated with fresh culture medium containing 1 mM β -D-benzyl xyloside. This was replaced with 2 ml of the same xyloside containing medium supplemented with ³⁵S-sulphate for 2 hours. 0.3 ml of 4 M NaOH was added to the whole incubation (i.e., both tissue and medium) to terminate the reaction, as the majority of xyloside initiated glycosaminoglycans are not retained within the extracellular matrix of the tissue unlike newly synthesised proteoglycans. Incubations in the absence of xyloside were treated similarly. After 36 hours' extraction aliquots were applied to Sephadex G-25 PD-10 columns.

Measurement of protein synthesis. Protein synthesis was measured by the incorporation of ³H-leucine or ³H-proline into trichloroacetic acid insoluble protein. After preincubation with fresh culture medium the tissue was incubated for 2 hours with 2 ml of culture medium supplemented with ³H-leucine (20 μ Ci/incubation, final specific activity 16 μ Ci/ μ mole) or ³H-proline (20 μ Ci/incubation, final specific activity 125 μ Ci/ μ mole). The tissue was washed extensively at 4°C¹⁷ and after digestion with papain counted for radioactivity.

Chromatography of labelled proteoglycan. In some experiments 0.5 ml aliquots of guanidine-HCl extracts were chromatographed on a Sepharose CL-2B column (1.6 cm $\times$ 70 cm) eluted at 7 ml/h with 4 M guanidine-HCl, 0.2 M Na₂SO₄, 0.1 M sodium acetate, 0.2% (v/v) Triton X-100, pH 6.1. Fractions (1.5 ml) were collected and counted for radioactivity.

Measurement of hexuronic acid. 3 ml aliquots of treatment media were mixed with 3 vol of absolute

Table 1 *Proteoglycan synthesis after treatment of cartilage in culture with the superoxide generating system*

Superoxide generating system	Incorporation of ^{35}S -sulphate into total tissue glycosaminoglycans (p mol/h/mg wet weight tissue)	
	No FeSO_4 added, n=4	FeSO_4 added, n=8
Absent	40.0 $\pm$ 8.1	38.5 $\pm$ 4.1
Incomplete (HX only)	39.9 $\pm$ 9.0 (98.8 $\pm$ 5.2)	38.6 $\pm$ 5.3 (96.9 $\pm$ 5.1)
Incomplete (XO only)	35.6 $\pm$ 9.2	34.1 $\pm$ 3.5
Complete (HX+XO)	22.1 $\pm$ 6.6 (62.3 $\pm$ 5.9*†)	21.1 $\pm$ 3.3 (60.2 $\pm$ 5.1*†)

Values are shown as mean $\pm$ SEM. Numbers in parentheses indicate the percent of activity in cultures to which HX was not added.

*Indicates $p<0.001$ for the effect of HX in the presence of XO.

††Indicates $p<0.05$, 0.001, respectively for the difference between the effect of HX in the absence and presence of XO.

ethanol overnight at 4°C, centrifuged at 2000 g_v for 10 min, and precipitates resuspended in 0.3 ml of 0.4 M guanidine-HCl before hexuronic acid estimation by an automated procedure.¹⁹

2 ml aliquots of culture media collected 24 hours after treatment were incubated at 60°C for 1 hour with 2 ml of 2 M NaOH, and 3 ml samples were then precipitated and analysed as described above.

Radioactive counting. 1 ml samples were counted on a Philips Liquid Scintillation Analyser in 8 ml of toluene : Triton X-100 (1:1, v/v) containing PPO (8 g/l).

Statistical analysis. Analysis of data was performed by a paired sample *t* test.

Results

The level of superoxide used in our experiments is of the same order of magnitude as that generated by 10^8 polymorphonuclear leucocytes,^{7,8,20} the number likely to be present in the arthritic joint.^{1,3}

Hypoxanthine (HX) alone produced a small, variable decrease in proteoglycan synthesis (Table 1). The addition of xanthine oxidase (XO) in the absence of hypoxanthine, resulted in an inhibition of proteo-

glycan synthesis ($17.1 \pm 5.0\%$, $n=9$), which may have resulted from the presence of endogenous substrates for the enzyme in cartilage or the presence of an inhibitory contaminant in the enzyme preparation. By comparing these treatments with the effect of the addition of hypoxanthine in the presence of xanthine oxidase it was possible to show that the complete superoxide generating system further decreased proteoglycan synthesis (Table 1). This inhibition was significant and occurred both in the presence and absence of added FeSO_4 . Statistical analysis was performed on data expressed as a percentage of control in order to overcome the variation observed in the net rate of proteoglycan synthesis between different batches of tissue. Normal laboratory reagents and distilled water contain variable amounts of iron salts,^{21,22} and the inclusion of 10 μM FeSO_4 was to ensure adequate catalytic ferrous ion concentrations for hydroxyl radical generation.²³

The data in Table 1 thus indicate that oxygen free-radicals can inhibit proteoglycan synthesis in cultured articular cartilage. Control experiments (Table 2) showed that the generation of uric acid by the xanthine oxidase/hypoxanthine system was not responsible for this inhibition.

The ability of various scavengers of oxygen free-radicals to protect the tissue against the effects of the superoxide generating system was investigated (Table 3). Superoxide dismutase did not prevent the inhibition, indicating that the superoxide radical per se was not responsible. Catalase, however, in both the presence and absence of superoxide dismutase, prevented inhibition to a considerable extent, indicating that either hydrogen peroxide or hydroxyl radical generation was involved. Mannitol, a hydroxyl radical scavenger,²⁴ was unable to prevent inhibition, though there was considerable variation between experiments. It is likely that both hydrogen peroxide and hydroxyl radicals are responsible. This

Table 2 *Effect of uric acid on proteoglycan synthesis*

Additions	Incorporation of ^{35}S -sulphate into total tissue glycosaminoglycans (pmol/h/mg wet weight tissue)
None	46.5 $\pm$ 8.2
Plus uric acid	47.9 $\pm$ 6.6 (105.8 $\pm$ 6.9)
Plus XO	39.8 $\pm$ 5.3
Plus uric acid and XO	44.0 $\pm$ 9.9 (108.0 $\pm$ 12.5)

Cartilage was treated in the presence and absence of 1 mM uric acid. Values are expressed as mean $\pm$ SEM of four separate experiments. Numbers in parentheses indicate percent of activity in cultures to which uric acid was not added.

Table 3 Effect of oxygen free-radical scavengers on the inhibition of proteoglycan synthesis

Scavenger	Incorporation of ^{35}S -sulphate into total tissue glycosaminoglycans (pmol/h/mg wet weight tissue)	
	HX absent	HX present
None	36.8 $\pm$ 5.3	26.1 $\pm$ 5.5 (70.0 $\pm$ 6.7)
Superoxide dismutase	36.0 $\pm$ 6.3	23.8 $\pm$ 6.0 (64.4 $\pm$ 5.1)
Superoxide dismutase plus catalase	36.0 $\pm$ 6.3	33.3 $\pm$ 4.7 (95.5 $\pm$ 9.3)
Catalase	37.4 $\pm$ 4.2	34.2 $\pm$ 7.0 (89.7 $\pm$ 8.1)
Mannitol	41.0 $\pm$ 4.3	26.9 $\pm$ 10.1 (63.6 $\pm$ 20.1)

Treatments of cartilage were performed in the presence of the indicated scavengers. Superoxide dismutase and catalase were each used at 250 units per incubation and mannitol at 50 mM.

Values are expressed as mean $\pm$ SEM for three experiments except when mannitol was used in which case the range of values is given. Numbers in parentheses as for Table 1.

is difficult to prove experimentally due to the non-specific properties of currently available scavengers.

The experiments described above may reflect only chain sulphation and not the synthesis of the glycosaminoglycan chain component of the proteoglycan. The incorporation of ^{35}S -sulphate and ^3H -acetate into glycosaminoglycans by the tissue (Table 4) showed that the incorporation of sulphate was accompanied by a similar inhibition of acetate incorporation. Inhibition thus involved a decreased ability of the tissue to synthesise glycosaminoglycan chains and was not specifically an inhibition of sulphation.

The glycosaminoglycan side chains of proteoglycans are formed by the sequential addition of monosaccharide units to the xylosylated protein core. The observed inhibition of glycosaminoglycan synthesis may be specifically due to decreased cellular synthesis of xylosylated protein core. β -D-xylosides can act as exogenous initiators of the synthesis of chondroitin sulphate containing glycosaminoglycans²⁵ and can be used to measure the capacity of the tissue to synthesise these glycosaminoglycans independently of the availability of endogenous protein core acceptor. Table 5 shows that the addition of 1 mM β -D-benzyl xyloside resulted in a stimulation of

glycosaminoglycan synthesis in both control tissue (XO only) and tissue treated with the complete superoxide generating system. This suggests that the availability of xylosylated protein core was rate limited in both cases. In the presence of xyloside the rate of synthesis in cartilage treated with the complete superoxide generating system was not the same as that observed in control cartilage, indicating that superoxide treatment had probably resulted in a decrease in the availability or activity of the glycosyltransferases responsible for glycosaminoglycan synthesis. This finding strongly suggests that a suppression of glycosaminoglycan chain elongation had occurred and not merely a decrease in the synthesis of xylosylated protein core.

Table 6 shows that the incorporation of ^3H -labelled amino acids into protein by cartilage was also inhibited after treatment. The decrease in glycosyltransferase activity mentioned above may be a reflection of this. In addition part of this inhibition may be due to an effect on protein core synthesis.

The biosynthesis of proteoglycan by chondrocytes involves intracellular synthesis and then secretion of the product into the extracellular space. The ability of the cells to secrete proteoglycan can be assessed by determining the rate of incorporation of isotope into

Table 4 Incorporation of ^3H -acetate and ^{35}S -sulphate into glycosaminoglycans

Superoxide generating system	Incorporation of radioactive precursor into total tissue glycosaminoglycans (pmol/h/mg wet weight tissue)	
	^{35}S -sulphate	^3H -acetate
Absent	30.1 $\pm$ 1.3	3.7 $\pm$ 1.1
Incomplete (HX only)	27.4 $\pm$ 2.5 (91.4 $\pm$ 12.1)	3.3 $\pm$ 0.6 (92.4 $\pm$ 11.5)
Incomplete (XO only)	26.7 $\pm$ 5.0	4.0 $\pm$ 0.6
Complete (HX+XO)	12.7 $\pm$ 1.7 (48.1 $\pm$ 2.7)	2.1 $\pm$ 0.5 (51.4 $\pm$ 4.3)

Values are expressed as mean $\pm$ range of values for two separate experiments. Numbers in parentheses as for Table 1.

Table 5 Effect of β -D-benzyl xyloside on glycosaminoglycan synthesis

Superoxide generating system	Incorporation of ^{35}S -sulphate into total tissue glycosaminoglycans (pmol/h/mg wet weight tissue)	
	Minus xyloside	Plus xyloside
Incomplete (XO only)	25.1 $\pm$ 2.8	37.2 $\pm$ 0.9 (149.5 $\pm$ 13.3)
Complete (XO+HX)	12.9 $\pm$ 1.1	20.7 $\pm$ 1.9 (160.9 $\pm$ 1.6)

Values are expressed as mean $\pm$ range of values for 2 separate experiments. Numbers in parentheses indicate the percent of synthesis in cultures incubated in the presence of xyloside compared to cultures incubated in the absence of xyloside.

guanidine-extractable and non-extractable fractions of the tissue,²⁶ assuming extracellular proteoglycans are extracted, by guanidine, whereas intracellular glycosaminoglycans are obtained after subsequent extraction with alkali. Such an experiment (results not shown) indicated that there was no significant change in the distribution of the newly synthesised proteoglycan between the intracellular and matrix compartments of cartilage or the medium after treatment with the complete superoxide generating system. The release of ^{35}S -labelled proteoglycan into the medium represented only $2.1 \pm 0.3\%$ (n=6) of the total proteoglycan synthesised by these cartilage cultures.

Similarly, the release into the medium of hexuronic acid, which represents the endogenous proteoglycan already present in the cartilage matrix, was not affected by the complete superoxide system compared with control (XO only) treated tissue (Table 7). However, it is apparent that the addition of xanthine oxidase to the cultures did cause an increase in the release of hexuronic acid into the medium. This occurred both during treatment with the superoxide generating system and during the 24 hours after treatment (Table 7). These results may indicate that the xanthine oxidase has some residual proteolytic activity, which can cause degradation of proteoglycans and thus hexuronic acid release into the medium.

The molecular size distribution of the proteoglycans synthesised was determined by gel filtration on Sepharose CL-2B under dissociative conditions (Fig. 1). Two distinct populations of proteoglycans were apparent as previously described,¹⁷ and neither the size nor the relative amounts of these populations differed in any way after treatment of the tissue.

In the experiments described above proteoglycan synthesis was determined 24 hours after treatment of the cartilage. The results shown in Fig. 2 indicate that synthesis was severely inhibited immediately after treatment. Some recovery occurred during the six-day period after treatment but was incomplete. Measurement of lactate dehydrogenase, as an index of cellular integrity, in incubation media after treatment indicated that no loss of viability had occurred during treatment (results not shown).

Discussion

Extensive loss of proteoglycan has been described in cartilage biopsies taken from rheumatoid arthritic patients as early as 18 days after the onset of symptoms.²⁷ This may occur either by an accelerated breakdown of proteoglycan or by an inhibition of its renewal through loss by normal turnover. Here we have shown evidence that the generation of oxygen free-radicals *in vitro* can inhibit the synthesis of proteoglycan by articular cartilage (Table 1). The effect

Table 6 Protein synthesis after treatment of cartilage in culture with the superoxide generating system

Superoxide generating system	Incorporation of radioactive precursor (pmol/h/mg wet weight tissue)	
	^3H -leucine	^3H -proline
Absent	36.1 $\pm$ 12.2	4.3 $\pm$ 1.3 (27.3 $\pm$ 0.9)
Incomplete (HX only)	47.8 $\pm$ 18.9	5.0 $\pm$ 1.8 (26.4 $\pm$ 0.5)
Incomplete (XO only)	31.7 $\pm$ 6.6	3.8 $\pm$ 0.7 (26.5 $\pm$ 1.7)
Complete (HX+XO)	22.1 $\pm$ 6.3	2.7 $\pm$ 0.2 (13.5 $\pm$ 0.2)

Values are expressed as mean $\pm$ range of values for two separate experiments. Numbers in parentheses indicate the incorporation of ^{35}S -sulphate (pmol/h/mg wet weight tissue) into total tissue glycosaminoglycans in these experiments.

Table 7 Effect of the superoxide generating system on the release of hexuronic acid from cartilage

Superoxide generating system	Hexuronic acid released ($\mu\text{g}/100 \text{ mg wet weight tissue}$)	
	During treatment	During 24 h after treatment
Absent	3.2 ± 0.4	7.0 ± 2.6
Incomplete (HX only)	2.1 ± 0.3	10.4 ± 2.5
Incomplete (XO only)	7.0 ± 1.1	13.6 ± 4.7
Complete (HX+XO)	$7.6 \pm 0.7 (115 \pm 12)$	$13.2 \pm 3.2 (114 \pm 22)$

Values are expressed as mean $\pm$ SEM for six separate experiments. Numbers in parentheses as for Table 1.

was rapid and prolonged (Fig. 2), and of the same order of magnitude as we have reported for antigen- and carrageenin-induced arthritis.^{4,5} The incorporation of ^{35}S -sulphate or ^3H -acetate precursor into glycosaminoglycans was inhibited (Table 4), suggesting a general dysfunction in glycosaminoglycan synthesis rather than a specific inhibition of sulphation. A similar response is seen in arthritic models.^{4,5} It is possible that the inhibition reflects a generalised

effect of oxygen free-radicals on cellular metabolism and the inhibition of protein synthesis (Table 6) is consistent with this possibility.

The use of oxygen free-radical scavengers indicated that superoxide radicals per se were not causing this inhibition (Table 3). Studies of tissue damage in other systems have shown that the secondary formation of other oxygen free-radicals is often of prime importance. Although superoxide radicals have been implicated in red blood cell lysis,^{28,29} hydroxyl radicals and hydrogen peroxide are thought to be involved in increased microvascular permeability in

Fig. 1 Chromatography of proteoglycans synthesised by cartilage on Sepharose CL-2B. Guanidine-extractable products after incubation with ^{35}S -sulphate were chromatographed. Values are for one experiment and represent the radioactivity incorporated in fractions per mg wet wt. tissue. Open and closed symbols indicate treatment in the absence and presence of XO and circles and squares indicate treatment in the absence and presence of HX.

Fig. 2 Time course of proteoglycan synthesis after treatment with the superoxide generating system. Cartilage was treated in the presence of XO and in the absence (●) and presence (○) of HX. Proteoglycan synthesis was measured at the indicated times after treatment. Values shown are as mean $\pm$ range of values for two separate experiments.

the hamster cheek pouch,³⁰ cell damage in cultures of human endothelial³¹ and glial cells,³⁰ and human fibroblasts,³² peroxidation of lipids in membranes³³ and suppression of serum antiprotease activity.⁶ There have been no studies, however, on the effect of oxygen free-radicals on intact articular cartilage, although many laboratories have studied synovial fluid, hyaluronic acid, collagen, and proteoglycan degradation.^{13 21 24 34}

There are recent reports of increased superoxide dismutase activity in the synovial fluid of patients with rheumatoid arthritis,³⁵ and that polymorphonuclear leucocytes from children with rheumatoid arthritis can release more than normal amounts of superoxide radical.³⁶ These changes, together with the greater numbers of inflammatory cells,³⁷ may allow the generation of large amounts of hydrogen peroxide and hydroxyl radicals. Although there is a controversy in the literature regarding in-vivo evidence for hydroxyl radical generation,³⁸ reports that rheumatoid synovial fluid contains more iron than normal fluid²¹ and that there is sufficient present as free iron to allow hydroxyl radical formation²² argue in favour of in vivo generation. Large amounts of oxygen free-radicals may therefore be generated in synovial fluid in vivo. Indeed, free radical oxidation products have been found in the synovial fluid of patients with rheumatoid arthritis.³⁹

Oxygen free-radicals did not cause release of hexuronic acid from cartilage (Table 7), suggesting that little degradation of hyaluronic acid or proteoglycan had occurred, although it is known that both these molecules are degraded, in solution, by oxygen free-radicals.^{24 34} It is possible that, through a combination of both oxygen free-radical and proteolytic attack by enzymes released from inflammatory cells,^{37 40 41} the cartilage may be damaged in a manner characteristic of rheumatoid arthritis.

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