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HYALURONIC ACID DEGRADATION BY ASCORBIC ACID AND INFLUENCE OF IRON

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The effects of ascorbic acid, iron and ADP on hyaluronic acid, a compound present in inflamed joints, were investigated in an *in vitro* system. Ascorbic acid induces degradation of hyaluronic acid which increased in the presence of $FeCl_3$ and which is additionally stimulated by ADP chelated ferric ions. The hyaluronic acid degrading reactions induced by the Fe-III/ADP/ascorbic acid system were inhibited by catalase and formate to various extents whereas the presence of superoxide dismutase did not exert any inhibitory effect. Desferrioxamine, a specific iron chelator, completely inhibited hyaluronic acid depolymerisation by ascorbic acid as well as in combination with $FeCl_3$ or $FeCl_3$/ADP, respectively. We suggest that the ultimate hyaluronic acid degrading species is $OH^{\cdot}$, generated via the Fe-III/ADP catalysed Haber Weiss reaction. There is also an indication for the involvement of perferryl or/and ferryl species in the degradation process.

KEY WORDS: Hyaluronic acid, ascorbic acid, iron/ADP complex, model reactions, reactive oxygen species.

INTRODUCTION

The most important macromolecular components of connective tissue and the extracellular matrix are collagen and glycosaminoglycans. The glycosaminoglycan hyaluronic acid (HA), an alternate copolymer of N-acetyl-β-D-glucosamine and β-D-glucuronic acid, differs from the other glycosaminoglycans in three important properties:[1]

- HA is not covalently bound to protein
- it is the only non-sulphated glycosaminoglycan
- HA has the highest molecular weight

High concentrations of HA are found in certain gel-like tissues such as the vitrous humor of the eye, Wharton's jelly in the umbilical cord and the synovial fluid of joints. In cartilage a great number of proteoglycans interacts with a single chain of HA forming large proteoglycan aggregates. This aggregation determines the water content and the hydrodynamic properties of this tissue. The decrease in synovial fluid viscosity and the erosion of joint cartilage are characteristic features in the pathogenesis of inflammatory arthritis of various etiologies.

It has been shown[2,3] that in synovial fluid of inflamed joints the HA concentration is reduced and the distribution of the molecular weight of HA macromolecules is also shifted to lower values. It has been reported[4–9] that oxygen-derived free radicals, especially the highly reactive hydroxyl radical, may play an important role in the degradation process of hyaluronic acid. There is general agreement that hydroxyl radicals are produced in systems containing reduced iron chelates. In this regard it is

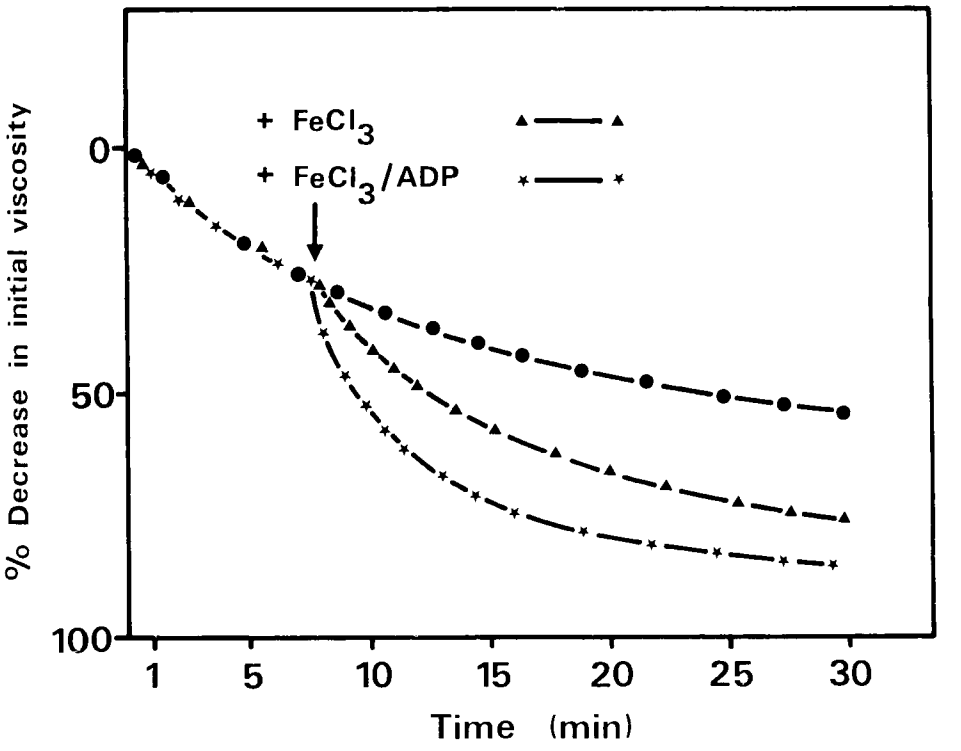

FIGURE 1 Hyaluronic acid degradation by ascorbic acid: Influence of $FeCl_3$ and $FeCl_3$/ADP. The reaction mixture contained in 3 ml: phosphate buffer 66 mM, pH 7.4; hyaluronic acid 2 mg; ascorbic acid (ASC) 0.4 mM; $FeCl_3$ 0.2 mM or $FeCl_3$/ADP 0.2: 2.0 mM. The arrow indicates the time of addition of the marked substance. (HA = hyaluronic acid/buffer solution). (●——●) HA + ASC; (▲——▲) HA + ASC + $FeCl_3$ (↓); (★——★) HA + ASC + $FeCl_3$/ADP (↓).

important that the synovium of inflamed joints shows an increase in the iron containing proteins ferritin and haemosiderin.[3] Additionally it has been demonstrated that in patients with rheumatoid arthritis synovial fluid contains about 80% of the total ascorbate in the oxidised state.[10]

In the present study, in vitro model systems containing ascorbic acid and ADP chelated ferric ions were used to investigate their ability to produce hyaluronic acid degrading species. In contrast to a previous study by[7] we applied hyaluronic acid concentrations as found in synovial fluid of rheumatic patients in the range of about 1.0 mg/ml.[11] A further physiological relevance of the applied model systems should be achieved by using ADP as potential biogenic chelator of ferric ions instead of artificial chelators like EDTA.

MATERIALS AND METHODS

Hyaluronic acid (potassium salt from human umbilical cord) and adenosine-diphosphate (ADP) were obtained from Serva, Heidelberg, ascorbic acid from Sigma, Munich, formate from Merck, Darmstadt, catalase from Boehringer, Mannheim;

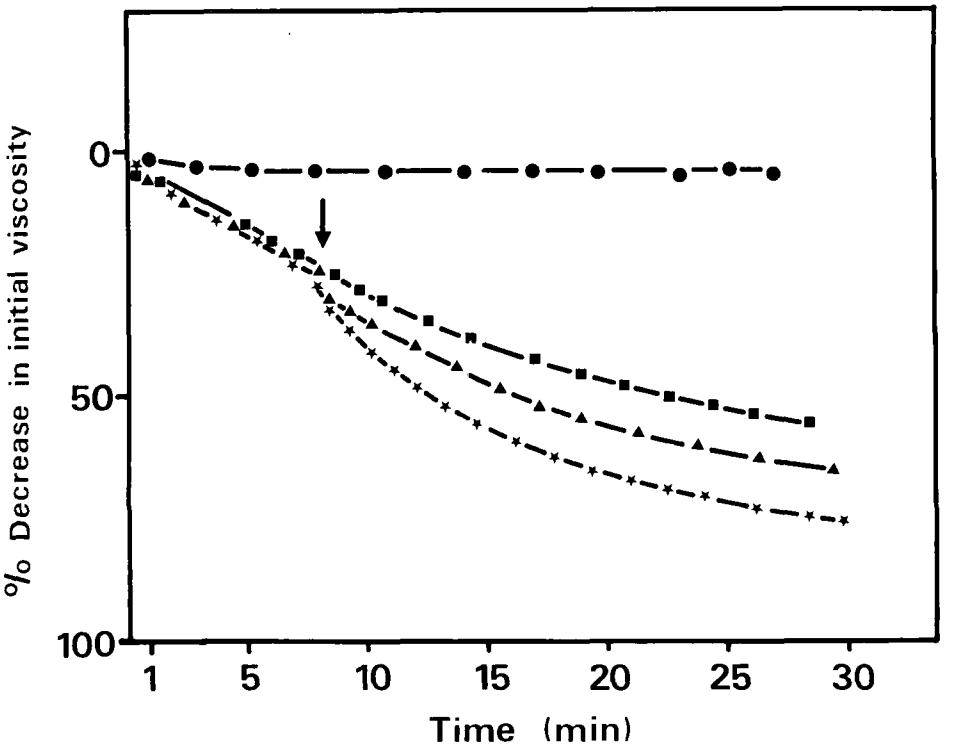

FIGURE 2 Effect of ascorbic acid concentration on HA degradation in combination with $FeCl_3$. The reaction mixture contained in 3 ml: phosphate buffer 66 mM, pH 7.4; hyaluronic acid 2 mg; where indicated, the hyaluronic acid/buffer solution was supplemented with desferrioxamine (DF) 2 mg; ascorbic acid 0.1 or 0.4 mM; $FeCl_3$ 0.2 mM. (●——●) HA/DF + 0.1 mM ASC; (■——■) HA + 0.1 mM ASC; (▲——▲) HA + 0.1 mM ASC + $FeCl_3$ (↓); (★——★) HA + 0.4 mM ASC + $FeCl_3$ (↓).

superoxide dismutase from Diagnostic Data, California, and desferrioxamine (= desferal) from CIBA-Geigy, Basel.

The basic reaction mixture contained 1 mg/ml in 66 mM sodium phosphate buffer pH 7.4. The buffer was purified by Chelex 100 treatment. Where indicated, the hyaluronic acid solutions additionally contained 1 mg desferrioxamine per ml. Catalase and superoxide dismutase were dissolved in 66 mM sodium phosphate buffer pH 7.4. All solutions including the buffer were prepared in double-distilled and additionally pyrolized water. Degradation of hyaluronic acid was determined viscometrically using a Schott Micro Ubbelohde precision viscometer in a temperature controlled water bath (37 ± 0.05°C). The results are expressed as percentage of decrease in initial HA viscosity due to a particular substance or reaction. During the viscometer measurements reaction mixtures were continually gassed with oxygen.

RESULTS

As shown in Fig. 1, in presence of ascorbic acid (0.4 mM) we observed a substantial yield of hyaluronic acid degradation. Addition of $FeCl_3$ (0.2 mM) to hyaluronic acid

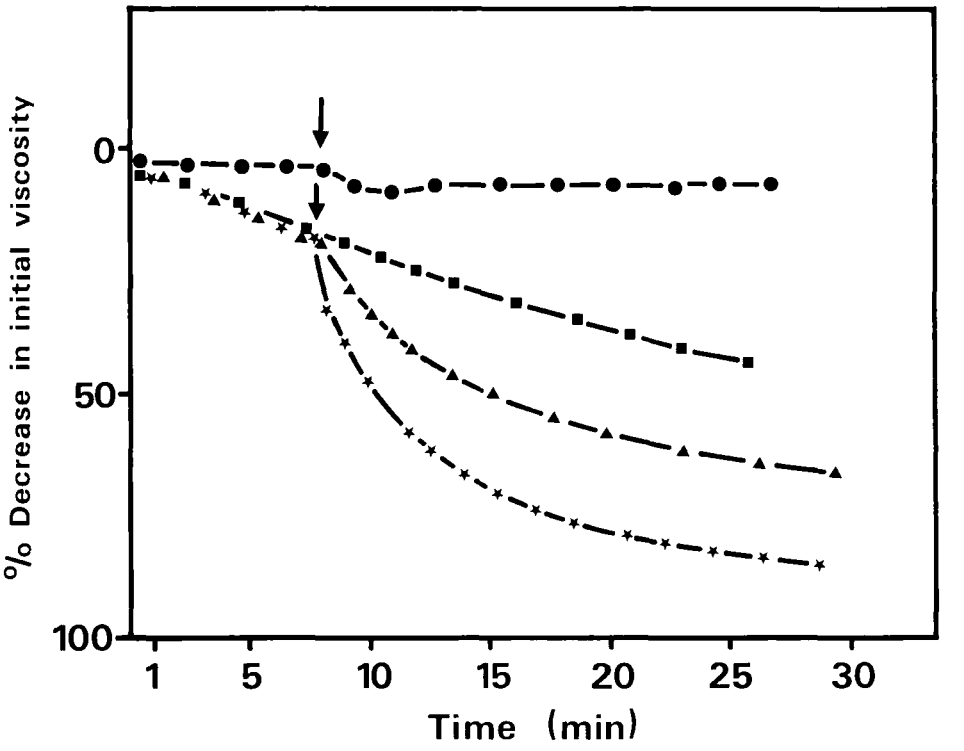

FIGURE 3 Effect of ascorbic acid concentration on HA degradation in combination with $FeCl_3$/ADP. The composition of the reaction mixture was the same as listed in the legend of Fig. 2. Instead of $FeCl_3$ in these experiments $FeCl_3$/ADP 0.2: 2.0 mM was applied. (●——●) HA/DF + 0.1 mM ASC + $FeCl_3$/ADP (↓); (■——■) HA + 0.1 mM ASC; (▲——▲) HA + 0.1 mM ASC + $FeCl_3$/ADP (↓); (★——★) HA + 0.4 mM ASC + $FeCl_3$/ADP 0.1 mM (↓).

solutions containing 0.4 mM ascorbic acid resulted in an increased depolymerisation of hyaluronic acid. When $FeCl_3$ (0.2 mM) chelated by ADP (1.0 mM) was added to hyaluronic acid solutions in presence of ascorbic acid (0.4 mM) there was a markedly additional stimulation of hyaluronic acid degradation.

Figures 2 and 3 illustrate the influence of the applied ascorbic acid concentration on the extent of hyaluronic acid degradation mediated by the addition of either $FeCl_3$ or $FeCl_3$/ADP. The iron salts were added 8 min after starting the HA-degradation reaction by ascorbic acid. In both cases (Figs. 2 and 3) there was a considerable increase in HA-depolymerisation at the higher ascorbic acid concentration. These results also confirmed the greater stimulating effect of ADP chelated $FeCl_3$ during hyaluronic acid degradation compared to $FeCl_3$ alone.

Since the physiological concentration of ascorbic acid normally does not exceed 140 μM in body fluids,[12] ascorbic acid concentration of 100 μM represents a physiologically relevant concentration. As shown in Figs. 2 and 3, hyaluronic acid depolymerisation mediated by ascorbic acid alone or by Fe/ADP was completely inhibited by the presence of desferrioxamine, a specific iron chelator. These results also suggest

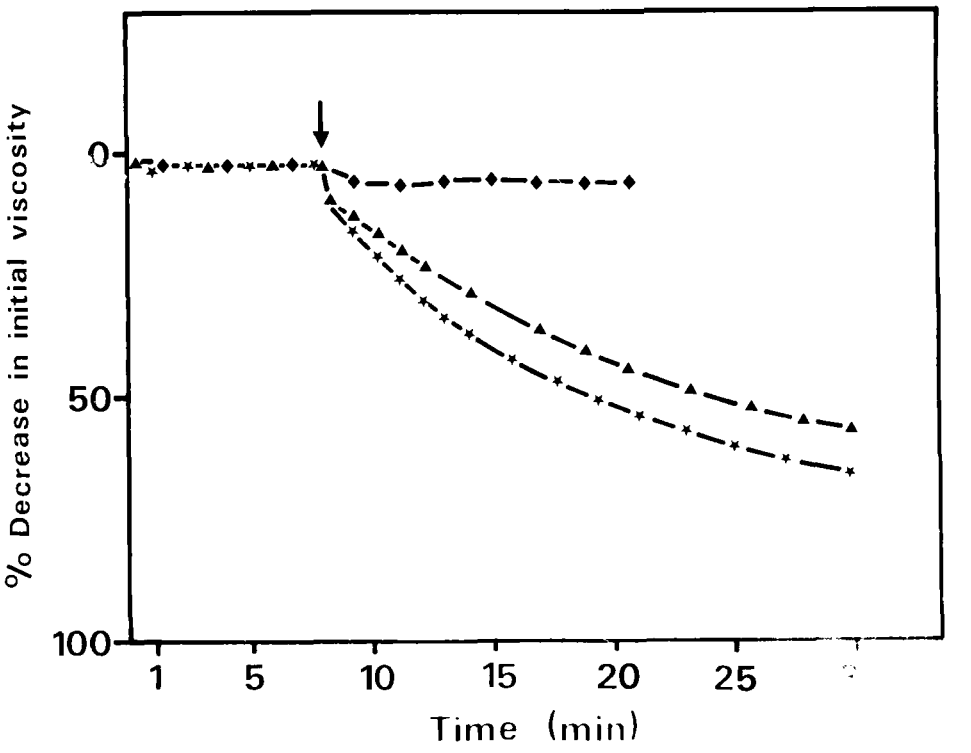

FIGURE 4 Effect of $FeCl_3$ on hyaluronic acid degradation. The components of the reaction mixture and the reaction conditions were the same as described in the legend of Fig. 2. The arrow indicates the time of addition of ascorbic acid. (◆——◆) HA/DF + $FeCl_3$ + 0.1 mM ASC (↓); (▲——▲) HA + $FeCl_3$ + 0.1 mM ASC (↓); (★——★) HA + $FeCl_3$ + 0.4 mM ASC (↓).

that some iron is already present in the test system due to inevitable traces of iron in the hyaluronic acid, the ascorbic acid and the phosphate buffer.

Figures 4 and 5 demonstrate that $FeCl_3$ alone as well as the Fe/ADP-complex were not able to cause any hyaluronic acid degradation in absence of ascorbic acid. The extent of decrease in initial viscosity after addition of ascorbic acid to the $FeCl_3$ or Fe/ADP containing hyaluronic acid solution also depended on the applied ascorbic acid concentration. In a series of experiments shown in Figs. 4 and 5 the presence of desferrioxamine in the HA solution resulted again in a complete prevention of the hyaluronic acid degradation.

Figure 6 shows the effect of superoxide dismutase (SOD), catalase (CAT) and formate on the hyaluronic acid degradation by the $FeCl_3$/ADP/ascorbic acid system. The presence of SOD did not reduce the rate of hyaluronic acid degradation. The addition of catalase, however markedly inhibited the depolymerisation reaction, the most efficient inhibition was obtained by adding formate which scavenges hydroxyl radicals (Table I).

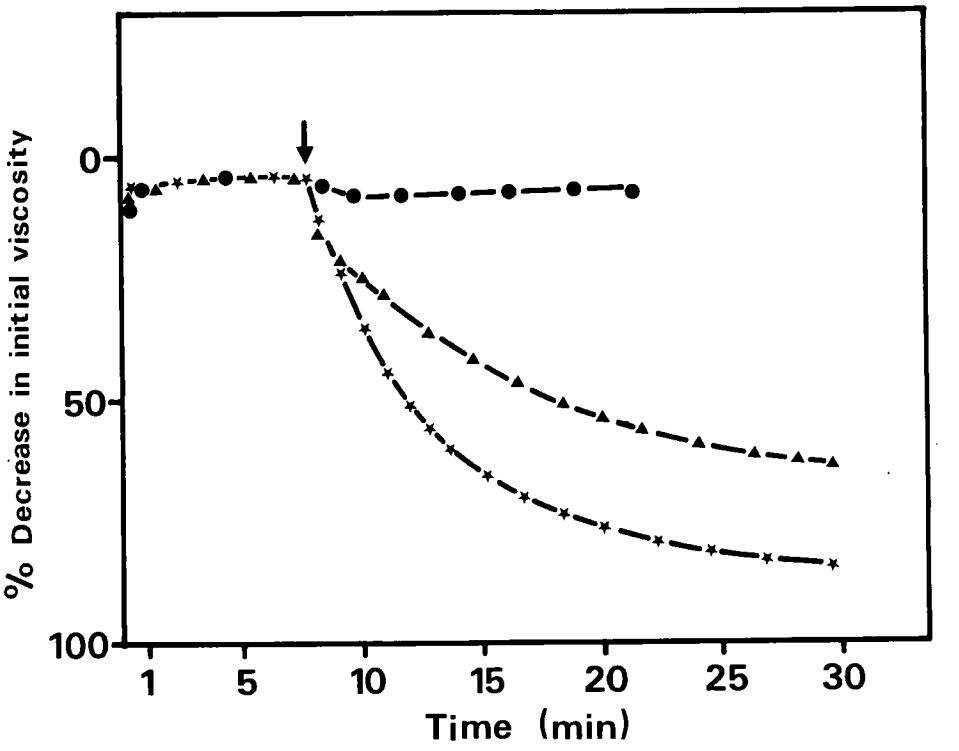

FIGURE 5 Effect of $FeCl_3$/ADP on hyaluronic acid degradation. The components of the reaction mixture and the reaction conditions were the same as described in the legend of Fig. 3. (●——●) HA/DF + $FeCl_3$/ADP + 0.1 mM ASC (↓); (▲——▲) HA + $FeCl_3$/ADP + 0.1 mM ASC (↓); (★——★) HA + $FeCl_3$/ADP + 0.4 mM ASC (↓).

DISCUSSION

Ascorbic acid at physiological concentration can induce hyaluronic acid degradation as has already been reported.[7,13] The extent of the observed depolymerisation by ascorbic acid was increased in presence of ferric ions and additionally enhanced by ADP chelated ferric ions. As illustrated in Fig. 3, the kinetic of the decrease in viscosity is greatly influenced by the available amounts of ascorbic acid which reacts with Fe-III/ADP resulting in the formation of Fe-II/ADP and dehydroascorbate. Autoxidation of the ferrous ADP complex leads to the formation of $OH^{\cdot}$ radicals or similar highly oxidising species which actually attack and degrade HA macromolecules. Reducing species like hydrated electrons or $^{\cdot}COO^{-}$ radicals as well as superoxide radicals have been shown not to be able to degrade hyaluronic acid.[8]

The following reactions are supposed to be involved in hydroxyl radical generation:

$$2Fe^{3+}/ADP + \text{ascorbate} \longrightarrow 2Fe^{2+}/ADP + \text{dehydroascorbate} + 2H^{+}$$

$$Fe^{2+}/ADP + O_2 \longrightarrow Fe^{3+}/ADP + {}^{\cdot}O_2^{-}$$

$$Fe^{2+}/ADP + {}^{\cdot}O_2^{-} + 2\,H^{+} \longrightarrow Fe^{3+}/ADP + H_2O_2$$

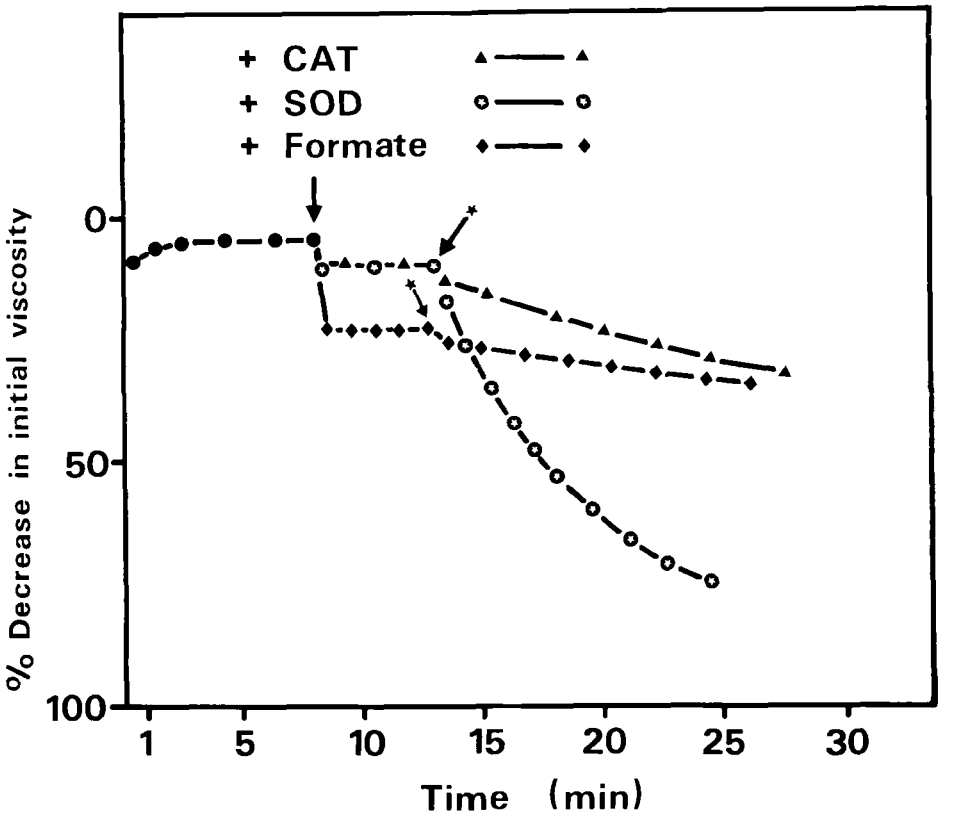

FIGURE 6 Effect of superoxide dismutase, catalase and formate on hyaluronic acid degradation by the $FeCl_3$/ADP/ascorbic acid system. The reaction mixture contained in 3 ml: phosphate buffer 66 mM, pH 7.4; hyaluronic acid 2 mg; ascorbic acid 0.4 mM; $FeCl_3$/ADP 0.2: 2.0 mM; where indicated superoxide dismutase 2 μM; catalase 1300 U; or formate 0.5 M. (◆——◆) HA + $FeCl_3$/ADP + Formate (↓) + ASC (↓★); (▲——▲) HA + $FeCl_3$/ADP + CAT (↓) + ASC (↓★); (✪——✪) HA + $FeCl_3$/ADP + SOD (↓) + ASC (↓★); (●——●) HA + $FeCl_3$/ADP.

$$\cdot O_2^- + \cdot O_2^- + 2\,H^+ \longrightarrow O_2 + H_2O_2$$

$$Fe^{2+}/ADP + H_2O_2 \longrightarrow Fe^{3+}/ADP + OH^{\cdot} + OH^-$$

The phenomenon of hyaluronic acid degradation induced by ascorbic acid alone is probably due to traces of iron in hyaluronic acid preparations. It has been reported that high purity ascorbic acid may also contain 0.02% iron.[7] In this case in absence of other chelators even the phosphate of the buffer system can act as iron chelating compound. The finding that catalase at high concentrations inhibited the depolymerisation of hyaluronic acid by Fe/ADP ascorbate system only to 70%, lets us think of an additional degradation mechanism independent of H_2O_2. As possible reactive species we presume the implication of ferryl ($Fe^{2+}O$) and/or perferryl ($Fe^{2+}O_2 \rightleftharpoons Fe^{3+}O_2^-$) species, formed by interaction of dioxygen with ferrous ions.[14]

TABLE I
Effect of superoxide dismutase, catalase and formate on HA degradation mediated by the Fe/ADP/ascorbic acid system

Additions	Concentrations	% Inhibition of HA degradation†
SOD	2.0 μM	0
CAT	1300 U	70
Formate	0.5 M	85

† Measured 10 min after addition of ascorbic acid.

$$Fe^{2+} + O_2 \longrightarrow [Fe^{2+}O_2 \rightleftharpoons Fe^{3+}O_2^-] \text{ perferryl ion}$$

$$Fe^{2+}O_2 + Fe^{2+} \longrightarrow Fe^{2+}O_2Fe^{2+} \longrightarrow 2\ Fe^{2+}O \text{ ferryl ion}$$

As a result of other experiments in our laboratory, this species seems also to be involved in the degradation of HA by 6,7-dimethyl-5,6,7,8-tetrahydropterin (manuscript in preparation).

The inhibitory effect of desferrioxamine is due to the fact that desferrioxamine forms with Fe^{3+} an extremely stable complex (log K = 31) preventing the reduction to Fe^{2+}. However, if iron is complexed by other chelators or ligands, including those present in the human body like ADP, the redox cycling of iron is possibly leading to the generation of $OH^{\cdot}$ radicals.

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