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The reaction of hyaluronic acid and its monomers, glucuronic acid and *N*-acetylglucosamine, with reactive oxygen species

Michael Jahn [a], John W. Baynes [b], Gerhard Spiteller [a,*]

[a] *Lehrstuhl Organische Chemie I, Universität Bayreuth, Universitätsstraße 30, D-95440 Bayreuth, Germany*
[b] *Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208, USA*

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Abstract

Synovial fluid is a ~0.15 % (w/v) aqueous solution of hyaluronic acid (HA), a polysaccharide consisting of alternating units of GlcA and GlcNAc. In synovial fluid of patients suffering from rheumatoid arthritis, HA is thought to be degraded either by radicals generated by Fenton chemistry (Fe^{2+}/H_2O_2) or by NaOCl generated by myeloperoxidase. We investigated the course of model reactions of these two reactants in physiological buffer with HA, and with the corresponding monomers GlcA and GlcNAc. *meso*-Tartaric acid, arabinuronic acid, arabinaric acid and glucaric acid were identified by GC–MS as oxidation products of glucuronic acid. When GlcNAc was oxidised, erythronic acid, arabinonic acid, 2-acetamido-2-deoxy-gluconic acid, glyceric acid, erythrose and arabinose were formed. NaOCl oxidation of HA yielded *meso*-tartaric acid; in addition, arabinaric acid and glucaric acid were obtained by oxidation with Fe^{2+}/H_2O_2. These results indicate that oxidative degradation of HA proceeds primarily at glucuronic acid residues. *meso*-Tartaric acid may be a useful biomarker of hyaluronate oxidation since it is produced by both NaOCl and Fenton chemistry. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Hyaluronic acid; Reactive oxygen species; *meso*-Tartaric acid; Arabinaric acid; Glucaric acid

1. Introduction

Hyaluronic acid (HA) is a linear acidic polysaccharide consisting of alternating units of D-GlcA and D-GlcNAc: [→4)-β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→]$_n$. It is a major component of the extracellular matrix of connective tissue and is found in high concentrations in umbilical cord, vitreous body of the eye, and in synovial fluid. Usually, HA has a high molecular weight (10^5–10^7 g/mol), and the aqueous solutions have gel-like properties and show molecular-weight-dependent viscosity [1].

In synovial fluid of patients with rheumatoid arthritis, the viscosity is reduced in comparison with healthy subjects, due to a reduction in molecular weight of HA [2]. The alteration of the molecular-weight distribution is assumed to be a result of the action of reactive oxygen species (ROS) on HA. Polymorphonuclear leukocytes invade inflamed joints [3] and, upon stimulation, release myeloperoxidase, which catalyses the formation of the powerful oxidant OCl^- from H_2O_2 and Cl^- [4]. H_2O_2 is produced by stimulated leukocytes, and the concentration of

* Corresponding author. Tel.: +49-921-552-680; fax: +49-921-552-671.
E-mail address: gerhard.spiteller@uni-bayreuth.de (G. Spiteller)

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bleomycin-detectable iron in the synovial fluid of patients with rheumatoid arthritis is elevated in comparison with healthy controls [5]. The reaction of Fe^{2+} with H_2O_2 leads to the formation of hydroxyl radicals, which react with and damage biomolecules [6].

Based on size-exclusion chromatography, HA is oxidatively depolymerised by Fenton reagent Fe^{2+}/H_2O_2 [7]. Frati et al. [8] demonstrated, using viscosity measurements, that photosensitive molecules are able to induce the degradation of HA by UV irradiation. Chromogenic assays indicated that the number of reducing GlcA residues was increased after exposure of HA to oxygen-derived free radicals [9]. Electron spin resonance was used as a tool to get insight into the reaction mechanism of OH-radicals with HA, and the intermediates were characterised [10]. In a detailed investigation of the products obtained by reaction of ROS with HA, Uchiyama et al. [11] detected arabinuronic acid and 2-acetamido-2-deoxy-gluconic acid. Schiller et al. [12] conducted ^1H NMR studies on the reaction mixtures of HA and Fe^{2+}/H_2O_2 and found mobile, i.e., not polymer-bound, *N*-acetyl groups and formiate.

In this work, we studied the products of the action of Fe^{2+}/H_2O_2 and NaOCl on HA under physiological conditions, and have applied GC–MS for their characterisation. Our results indicate that these reagents cleave HA primarily at GlcA residues.

2. Materials and methods

Materials.—*N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey and Nagel, Chelex 100 was from Sigma, HA sodium salt from human umbilical cords and all other chemicals were obtained from Fluka. 2-Acetamido-2-deoxy-gluconic acid was synthesised according to Hoffmann et al. [13] and Pravdic et al. [14]. Arabinuronic acid was synthesised according to Wu and Serianni [15], arabinaric and arabinonic acid according to Hardegger et al. [16].

Oxidation with Fe^{2+}/H_2O_2.—D-GlcA or D-GlcNAc (0.8 mg/mL) or HA (1.5 mg/mL) was dissolved in phosphate-buffered saline (pH 7.4) and incubated with $FeSO_4 \cdot 7H_2O$ (40 μM) [5] and H_2O_2 (500 μM) for 6 h at 37 °C. Chelex 100 (1 g dry weight/mL solution) was added for removing iron, vortexed for 2 min, centrifuged and the supernatant was taken to dryness by centrifugal evaporation. Alternatively, HA or oxidised HA was precipitated with 4 volumes of EtOH, the solution stored for 12 h at −4 °C, the precipitate centrifuged, washed with 80% (v/v) aq EtOH, with EtOH and with Et_2O. After drying by centrifugal evaporation, methanolic HCl (2 M, 1 mL/mg carbohydrate) containing MeOAc (2 M) [17] was added, oxygen was removed under a mild flow of argon for 20 s and the vial was stoppered with a Teflon-coated cap and placed in an oven at 100 °C for 24 h. After adding 2 volumes of 4:1 *tert*-BuOH–MeOH [17], the mixture was taken to dryness by centrifugal evaporation. MSTFA (50 μL/mg carbohydrate) and TFA (5 μL/mg carbohydrate) [18] were added, and the turbid solution was heated for 2 h at 35 °C. The centrifuged, clear solution (1 μL) was analysed by GC–MS. For comparison, the same work-up and derivatisation procedure was done without incubation with Fe^{2+}/H_2O_2.

Oxidation with NaOCl.—D-GlcA or D-GlcNAc (0.8 mg/mL) or HA (1.5 mg/mL) was dissolved in phosphate-buffered saline (pH 7.4) and incubated with NaOCl (500 μM) for 6 h at 37 °C. One volume of *iso*-propanol was added [19], and the solvent was evaporated. Alternatively, HA or oxidised HA were isolated from the solutions by EtOH precipitation as described above. Methanolysis and derivatisation were performed as described above. Again, controls without oxidising agent were done.

Viscosimetry.—The kinetics of decrease in viscosity of HA solutions (mixtures as described above) were monitored with a Physica, Rheolab MC10-viscosimeter (concentric cylinders, double-gap-measuring system according to DIN 54453 with a cylinder MS-Z1 DIN) at 2000 rpm and 37 °C.

GC–MS.—GC separation was performed on a HP 5890 Series II gas-chromatograph (30 m DB-5MS column, 0.25 mm ID, temperature program: from 100 to 300 °C with 3 °C/min); MS analysis was done by a Finnigan MAT 95,

equipped with an ICIS data system. EI mass spectra were recorded at an ionisation energy of 70 eV.

For determination of R_i values, an appropriate amount of a mixture of saturated, non-branched hydrocarbons was coinjected and the values were calculated by linear interpolation [20].

GC–MS data for standard compounds are indicated below. The five most prominent MS fragments are listed with relative intensity in parentheses.—Dimethyl *meso*-tartrate (2TMS) (**1a**): R_i 1420. MS 73(100), 89(35), 147(65), 234(45), 307(20). Methyl trimethylsilyl *meso*-tartrate (2TMS) (**1b**): R_i 1500. MS 73(100), 147(70), 219(35), 234(40), 247(35). Methyl (methyl arabinofuranosid)-uronate (2TMS) (**2a**): R_i 1540, 1555. MS 73(60), 75(50), 89(20), 159(15), 217(100). Methyl (trimethylsilyl arabinofuranosid)uronate (2TMS) (**2b**): R_i 1615, 1625. MS 73(45), 75(40), 147(20), 191(10), 217(100). Dimethyl arabinarate (3TMS) (**3**): R_i 1690. MS 73(100), 75(50), 103(70), 147(50), 234(85). Methyl glucofuranosidurono-6,3-lactone (2TMS) (**4a**): R_i 1750, 1770. MS 73(100), 75(40), 147(20), 217(30), 230(65). Methyl (methyl glucofuranosid)uronate (3TMS) (**4b**): R_i 1785, 1795. MS 73(50), 75(30), 133(20), 217(100), 277(15). Trimethylsilyl glucofuranosidurono-6,3-lactone (2TMS) (**4c**): R_i 1815, 1830. MS 73(60), 75(50), 147(20), 217(15), 230(100). Methyl (methyl glucopyranosid)uronate (3TMS) 4d: *R*: 1905, 1920. MS 73(70), 75(50), 147(30), 204(70), 217(100). Methyl (trimethylsilyl glucopyranosid)uronate (3TMS) (**4e**): R_i 1935, 1945. MS 73(45), 75(25), 147(30), 204(80), 217(100). Trimethylsilyl (methyl glucopyranosid)uronate (3TMS) (**4f**): R_i 1955, 1960. MS 73(50), 147(35), 204(70), 217(100), 292(20). Glucaric acid lactone methyl ester (3TMS) (**5a,c**): R_i 1850, 1880. MS 73(100), 75(35), 147(40), 217(35), 407(20). Dimethyl glucarate (4TMS) (**5b**): R_i 1865. MS 73(95), 75(35), 103(40), 147(35), 275(100). Methyl glycerate (2TMS) (**6**): R_i 1225. MS 73(100), 89(20), 103(25), 117(25), 147(35). Methyl erythrofuranoside (2TMS) (**7**): R_i 1300, 1305. MS 73(60), 75(35), 147(100), 191(20), 217(30). Methyl erythronate (3TMS) (**8**): R_i 1460. MS 44(100), 73(80), 75(60), 147(50), 234(45). Methyl arabinopyranoside (3TMS) (**9**): R_i 1545, 1560. MS 73(100), 75(50), 147(30), 204(35), 217(100). Arabinonic acid γ-lactone (3TMS) (**10a**): R_i 1630. MS 73(100), 75(85), 117(10), 147(25), 217(10). Methyl arabinonate (4TMS) (**10b**): R_i 1710. MS 73(100), 75(50), 103(50), 147(50), 217(45). Methyl 2-amino-2-deoxy-glucopyranoside (2TMS) (**11a**): R_i 1830, 1835. MS 73(50), 75(45), 131(100), 147(20), 217(10). Methyl 2-deoxy-2-trimethylsilylaminoglucofuranoside (3TMS) (**11b**): R_i 1845, 1865. MS 73(35), 75(40), 147(20), 216(100), 217(40). Methyl 2-deoxy-2-trimethylsilylamino-glucopyranoside (3TMS) (**11c**): R_i 1900. MS 73(40), 75(20), 147(15), 203(100), 204(25). Trimethylsilyl 2-deoxy-2-trimethylsilylamino-glucopyranoside (3TMS) (**11d**): R_i 1915, 1960. MS 73(30), 75(50), 147(15), 203(100), 204(25). Methyl 2-acetamido-2-deoxy-glucopyranoside (3TMS) (**11e**): R_i 2085. MS 73(60), 75(100), 147(30), 173(100), 217(25). Methyl 2-deoxy-2-trimethylsilylamino-gluconate (4TMS) (**12**): R_i 1960. MS 73(100), 75(50), 147(65), 205(70), 319(50).

3. Results

Oxidation of GlcA.—A comparison of the chromatograms of a GlcA solution (A) incubated with Fe^{2+}/H_2O_2 (B) or with NaOCl (C) is given in Fig. 1 (the numbers of the peaks in the chromatograms correspond to the numbers of the compounds). The methanolysis products of GlcA are methyl glucofuranosidurono-6,3-lactone (**4a**), methyl (methyl glucofuranosid)uronate (**4b**), trimethylsilyl glucofuranosidurono-6,3-lactone (**4c**), methyl (methyl glucopyranosid)uronate (**4d**), methyl (trimethylsilyl glucopyranosid)uronate (**4e**) and trimethylsilyl (methyl glucopyranosid)-uronate (**4f**) (all as pertrimethylsilylated compounds; α and β anomers for each glycoside). Oxidation products with both oxidising agents are *meso*-tartaric acid as dimethyl ester (**1a**) and mixed methyl trimethylsilyl ester (**1b**), methyl (methyl arabinofuranosid)uronate (**2a**), methyl (trimethylsilyl arabinofuranosid)-uronate (**2b**), dimethyl arabinarate (**3**), glucaric acid lactone methyl ester (**5a,c**), and dimethyl glucarate (**5b**). As above, the gly-

cosides give two peaks corresponding to α and β anomers.

Oxidation of GlcNAc.—The course of oxidation of GlcNAc (A) with Fe^{2+}/H_2O_2 (B) or with NaOCl (C) is shown in Fig. 2 (the numbers of the peaks in the chromatograms correspond to the numbers of the compounds). Methanolysis and derivatisation of GlcNAc yields methyl 2-amino-2-deoxy-glucopyranoside (**11a**), methyl 2-deoxy-2-trimethylsilylamino-glucofuranoside (**11b**), methyl 2-deoxy-2-trimethylsilylamino-glucopyranoside (**11c**), trimethylsilyl 2-deoxy-2-trimethylsilylamino-glucopyranoside (**11d**) and methyl 2-acetamido-2-deoxy-glucopyranoside (**11e**). The identified products of oxidation with Fe^{2+}/H_2O_2 are methyl erythronate (**8**), arabinonic acid γ-lactone (**10a**), methyl arabinonate (**10b**) and methyl 2-deoxy-2-trimethylsilylamino-gluconate (**12**). The same products are obtained when NaOCl is the oxidising agent, and in addition, methyl glycerate **6**, methyl erythrofuranoside **7** and methyl arabinopyranoside **9** are formed.

Oxidation of HA.—The time-course of the oxidation of HA with Fe^{2+}/H_2O_2 and NaOCl is illustrated in Fig. 3 by means of viscosimetry. Within 14 h, the viscosity of the pure HA solution is reduced by ~15%, the oxidised solution with NaOCl by ~35% and by oxidation with Fe^{2+}/H_2O_2 by ~75%. The degradation process seems to be completed within 6 h. The GC–MS chromatograms of a methanolised and derivatised HA solution (A), oxidised with Fe^{2+}/H_2O_2 (B) or NaOCl (C) are shown in Fig. 4 (the numbers of the peaks in the chromatograms correspond to the numbers of the compounds). When HA is methanolised and trimethylsilylated, the derivatives of GlcA (**4a**–**4f**) and of GlcNAc (**11b**,**c**,**d**,**e**) can be observed. Mixtures incubated with Fe^{2+}/H_2O_2 contain, in addition,

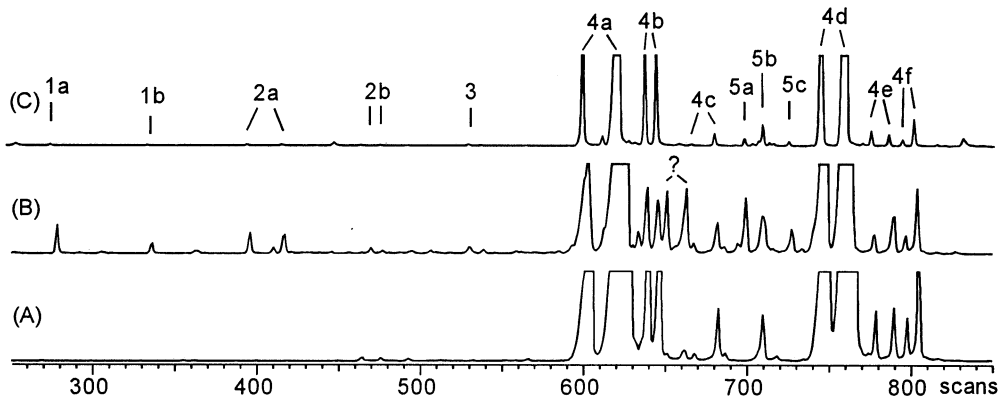

Fig. 1. Reconstructed ion current of a 0.08% (w/v) D-GlcA solution (A) either incubated with 40 μM Fe^{2+} and 500 μM H_2O_2 (B) or with 500 μM NaOCl (C) at 37 °C for 6 h. Preparation of samples for GC–MS is described in Section 2. Designation of peaks is given in the text.

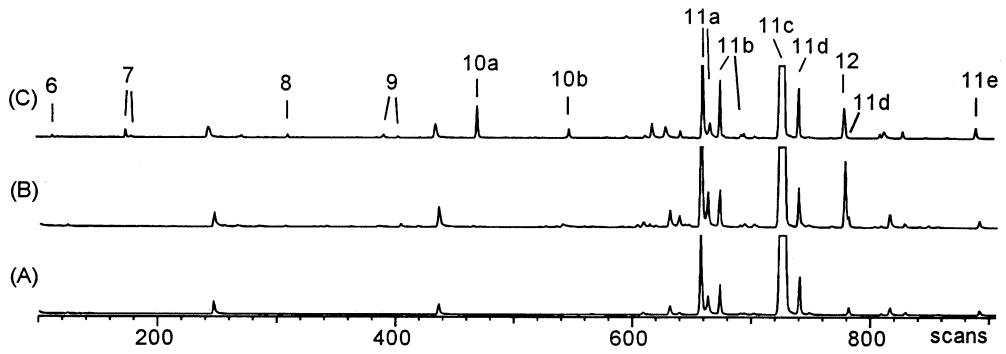

Fig. 2. Reconstructed ion current of a 0.08% (w/v) D-GlcNAc solution (A) either incubated with 40 μM Fe^{2+} and 500 μM H_2O_2 (B) or with 500 μM NaOCl (C) at 37 °C for 6 h. Preparation of samples for GC–MS is described in Section 2. Designation of peaks is given in the text.

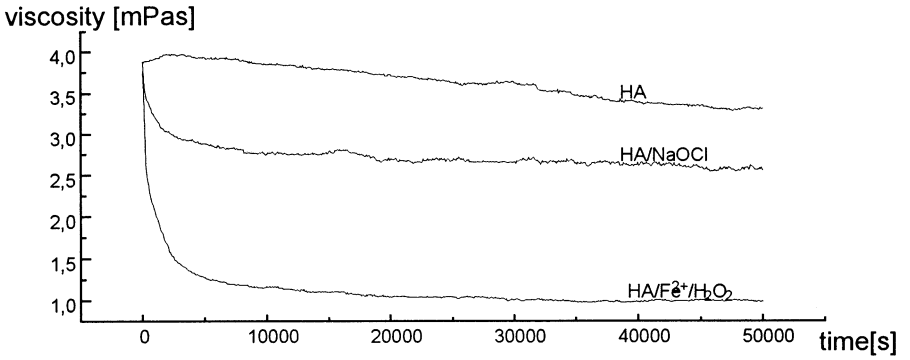

Fig. 3. Kinetics of the decrease in viscosity of a 0.15% (w/v) HA solution either incubated with 40 μM Fe^{2+} and 500 μM H_2O_2 or with 500 μM NaOCl. As a control, the loss of viscosity of a pure HA solution is shown. Conditions are given in Section 2.

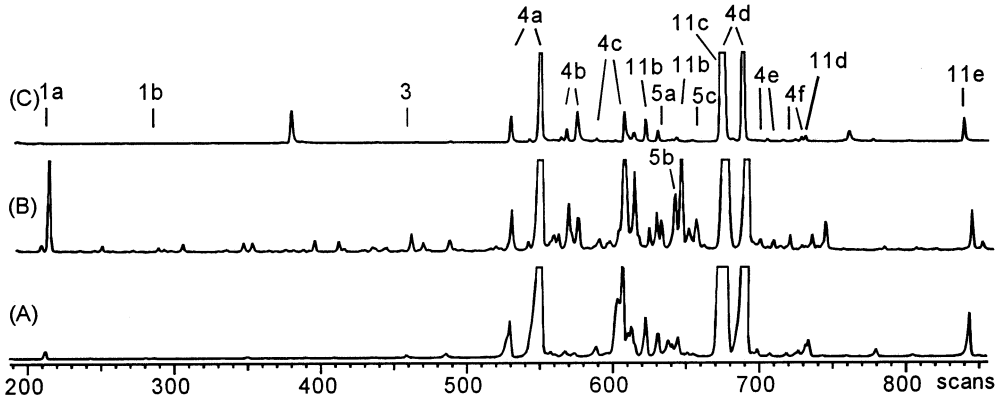

Fig. 4. Reconstructed ion current of a 0.15% (w/v) HA solution (A) either incubated with 40 μM Fe^{2+} and 500 μM H_2O_2 (B) or with 500 μM NaOCl (C) at 37 °C for 6 h. Preparation of samples for GC–MS is described in Section 2. Designation of peaks is given in the text.

the derivatives of *meso*-tartaric acid (**1a**,**b**), arabinaric acid (**3**) and glucaric acid (**5a**,**b**,**c**). When HA was incubated with NaOCl, only *meso*-tartaric (**1a**,**b**) could be identified as an oxidation product. Again, the glycosides show two peaks for α and β anomers. We did not detect any significant differences in the chromatograms when HA or oxidised HA were isolated from the solution by ethanol precipitation. This indicates that the oxidised residues are situated on the polymer.

4. Discussion

Preliminary experiments with LC–MS (data not shown) indicated that the fragmentation products formed during oxidation of HA under physiological conditions are present at only trace concentrations in the reaction mixture, in spite of a visible loss in viscosity of the solutions compared with non-oxidised HA solutions. Therefore, we first examined the oxidation behaviour of the monomers.

The product spectrum of GlcA indicates oxidation at C-1 to yield glucaric acid, which is the most prominent product formed with both oxidants; the C-1–C-2-bond is cleaved and C-2 is oxidised to give arabinuronic and arabinaric acid. When C-2–C-3 is cleaved, and C-3 is oxidised, *meso*-tartaric acid is the product. It is not clear why the oxidation does not proceed on C-3 to an aldehydic product (erythruronic acid), or why we could not find products of oxidation at C-4 and C-5. Although the kinetics and yield of products differed, similar products were obtained on oxidation of GlcA by Fe^{2+}/H_2O_2 and NaOCl.

In contrast, the products found by oxidation of GlcNAc differ according to the oxidation reagent: C-1 is oxidised to give 2-amino-2-deoxy-gluconic acid, which is a major product formed by the action of the two oxidants. Experiments where the methanolysis

step was omitted showed that 2-acetamido-2-deoxy-gluconic acid is formed as well; during methanolysis, the amide group is hydrolysed completely. Cleavage of C-1–C-2 and oxidation at C-2 yields arabinonic acid when Fe^{2+}/H_2O_2 is used as oxidant and also arabinose, when NaOCl is used. Arabinonic acid is the other major product that is obtained by oxidation with NaOCl. Cleavage of C-2–C-3 and oxidation of C-3 is observed by application of both oxidants; but again, only NaOCl gives both the aldehyde (erythrose) and the acid (erythronic acid), whereas only the acid is detected by oxidation with Fe^{2+}/H_2O_2. Cleavage of C-4–C-5 with oxidation at C-4 to glyceric acid is observed only with NaOCl. Both monomers seem to be oxidised in a manner, that C-6 remains in the products.

HA oxidation yielded only the products of oxidation of GlcA, but none from the GlcNAc part of the polymer. We propose that Fe^{2+} is bound to the carboxylic groups of GlcA. The Fenton reaction takes place in the proximity of GlcA, and as the reaction of the hydroxyl-radical is almost diffusion controlled, oxidised GlcA residues are formed in high yield. It is not clear why only *meso*-tartaric acid is formed by NaOCl oxidation. Measurement of viscosity during oxidation indicates that the breakdown of HA proceeds more drastically in the case where the Fenton reactant is used as oxidant. We could find the same oxidised products from HA when we isolated the degraded polymer by ethanol precipitation. Therefore, it seems likely that the oxidised residues are situated on the polymer.

The reported products of HA oxidation (~ 30 mM Fe^{2+}, oxygen atmosphere) obtained by Uchiyama et al. [11], arabinuronic acid and 2-acetamido-2-deoxy-gluconic acid, were found in our investigations only by oxidation of the monomers. As ferric ions, which are formed immediately under these conditions, are known to be very strong Lewis acids, we suppose, that degradation of HA in this investigation is a hydrolysis step and not an oxidative cleavage of the polymer. The newly formed reducing ends might then be oxidatively modified by ferric ions. This suggestion is supported by the observation that carbohydrates that are subjected to free radicals yield a broad product spectrum [10]. In contrast, in our investigation the polysaccharide chain is cleaved by hydroxyl radicals formed by the Fenton reaction. Our model reactions have biological relevance, because we obtain a limited degradation of HA under conditions and with reagents that might be encountered in vivo as it is postulated in the literature.

We also examined urine and synovial fluid of patients with rheumatoid arthritis [21]. In spite of a very low detection limit of the oxidised products of HA, *meso*-tartaric acid, arabinaric acid and glucaric acid (~ 100 ng/mg HA), we were unable to detect the expected degradation products. We used spiking experiments and found that we can recover the three aldaric acids when we add oxidised HA to the biological samples. This would mean that the long postulated process of oxidative degradation of HA takes place — if at all — only in a very limited way. Therefore, we consider that HA in the joints of patients with rheumatoid arthritis might suffer degradation by hydrolysis rather then by oxidation.

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