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Reactivity of Cartilage and Selected Carbohydrates with Hydroxyl Radicals

An NMR Study to Detect Degradation Products

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It was investigated to what extent isolated, monomeric and polymeric carbohydrates as well as cartilage specimens are affected by hydroxyl radicals generated by γ-irradiation or Fenton reaction and what products can be detected by means of NMR spectroscopy. Resonances of all protons in glucose and other monosaccharides as well as carbon resonances in ^{13}C-enriched glucose were continuously diminished upon γ-irradiation. Formate and malondialdehyde were found as NMR detectable products in irradiated glucose solutions under physiologically relevant (aerated) conditions. In polysaccharide solutions (e.g. hyaluronic acid) γ-irradiation and also treatment with the Fenton reagent caused first an enhancement of resonances according to mobile N-acetyl groups at 2.02 ppm. This indicates a breakdown of glycosidic bonds in polysaccharides. Using higher radiation doses or higher concentrations of the Fenton reagent formate was also detected. The same sequence of events was observed upon treatment of bovine nasal cartilage with the Fenton reagent. First, glycosidic linkages in cartilage polysaccharides were cleaved and subsequently formate was formed. In contrast, collagen of cartilage was affected only to a very low extent. Thus, HO-radicals caused the same action on cartilage as on isolated polymer solutions, inducing a fragmentation of polysaccharides and the formation of formate.

Keywords: Hydroxyl radicals, Fenton chemistry, Cartilage, Carbohydrates, NMR spectroscopy, γ-radiolysis

INTRODUCTION

Although etiology and pathogenesis of rheumatoid arthritis is yet unknown, a progressive destruction of polymeric carbohydrates of cartilage is observable in the course of rheumatic diseases.[1] Cartilage consists of a complex network of collagen (mainly type II) and different carbohydrates including hyaluronic acid, chondroitin sulphate, and keratan sulphate. These negatively

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charged polysaccharides form with the core protein and a special link protein the high molecular mass proteoglycan aggregates of extracellular matrix.[2] Reactive oxygen species (hydrogen peroxide, hypochlorous acid, and hydroxyl radicals) are massively involved in carbohydrate degradation processes.[3,4] We focused our recent interest mainly on the reaction of enzymatically formed hypochlorous acid with carbohydrates of cartilage.[5,6] This powerful oxidant is formed in a myeloperoxidase catalyzed reaction between hydrogen peroxide and chloride anions:[7,8]

$$H_2O_2 + Cl^- \rightarrow HOCl + HO^-$$

Myeloperoxidase (MPO, E.C. 1.11.1.7.) is a unique enzyme of neutrophils and monocytes and released upon cell stimulation.[9,10] Since the number of neutrophils is massively enhanced in the synovial fluid of the inflamed joint,[11,12] high MPO activities are detectable in these fluids.[13]

It has been shown recently that a close correlation exists between the myeloperoxidase activity in synovial fluids from patients suffering from rheumatoid arthritis and the content of NMR-detectable degradation products like highly mobile N-acetyl groups and acetate.[5] This emphasizes the predominant role of hypochlorous acid, because HOCl induces a breakdown of glycosidic linkages in cartilage carbohydrates leading to an enhanced integral intensity of N-acetyl groups in the NMR spectrum.[6] Additionally, HOCl reacts with N-acetyl groups of monomeric and polymeric carbohydrates to give acetate as final product via a transient, chlorinated product.[14]

However, besides hypochlorous acid, other reactive oxygen species may also be involved in cartilage degradation processes. Since superoxide anion radicals as well as hydrogen peroxide are only poor oxidants,[15] having a low reactivity towards most biologically relevant molecules, hydroxyl radicals are widely implicated as a major damaging species in free radical pathology.[16]

Hydroxyl radicals are formed under pathological conditions from hydrogen peroxide and ferrous ions via Fenton chemistry[17] or as a result of the reaction between superoxide anion radicals and hypochlorous acid.[18] Thus, ESR methods have unequivocally shown that stimulated neutrophils release hydroxyl radicals.[19]

Although hydroxyl radicals react rather unspecifically and diffusion-controlled with most biological targets,[20] they are especially known to cleave glycosidic bonds in polysaccharides like hyaluronic acid[21,22] and to degrade monosaccharides like glucose to formate as NMR-detectable final product.[23] These facts have not only been shown using pure aqueous solutions of carbohydrates and their subsequent analysis by GC/MS,[24] but also upon the exposition of synovial fluids to γ-radiolysis[22,23] followed by NMR analysis. Unfortunately, different results were obtained. Radiolysis experiments of synovial fluids gave mainly formate as NMR-detectable product,[22] whereas monosaccharide solutions yielded an extremely large variety of oxidation products.[25] However, the product yield depends on reaction conditions: Whereas only traces of formate are formed in the absence of oxygen, formate is a major product of carbohydrate oxidation in the presence of oxygen.[20]

In the present study, hydroxyl radicals were generated by water radiolysis as well as by the iron-catalyzed decomposition of hydrogen peroxide (Fenton reaction) to obtain information on the action of HO radicals under simplified and also under more physiologically relevant conditions.[26] Water radiolysis yields hydrated electrons, hydrogen atoms and hydroxyl radicals.[27] Whereas hydrogen atoms react about two orders of magnitude slower than hydroxyl radicals with carbohydrates,[20,28] the solvated electron reacts with oxygen to yield superoxide anion radicals which are only poorly reactive oxygen species.[29] Unfortunately, chemical processes in Fenton chemistry are by far less understood than processes in water radiolysis.[15,17,26]

The aim of the present paper was to investigate changes in NMR spectra of monomeric and polymeric carbohydrate solutions, as well as in

supernatants of cartilage upon the action of hydroxyl radicals. Since NMR spectroscopy is a promising tool for the *in vivo* detection of cartilage degradation products in synovial fluids it is used as the main analytical method.

MATERIALS AND METHODS

Materials

Carbohydrates were obtained in highest commercially available purity from Fluka Feinchemikalien (Neu Ulm, Germany). High molecular weight hyaluronic acid (potassium salt) from human umbilicial cords (MW ~ 1×10^6 Da), low molecular weight hyaluronic acid from rooster comb (MW ~ 1×10^5 Da), and chondroitin sulphate sodium salt (MW ~ 5×10^4 Da) from bovine trachea were used.

Polysaccharides were dialysed prior to use against 50 mmol/l isotonic phosphate buffer pH 7.4 to remove traces of low-molecular components.[22] Ferrous chloride, hydrogen peroxide and all chemicals for NMR spectroscopy (sodium 3-(trimethylsilyl)-propane-1-sulphonate (TSP), ^{13}C-enriched glucose (98% ^{13}C) and deuterated water (99.95% ^{2}H) were also purchased from Fluka, just like the chemicals for MALDI analysis (acetonitrile, sinapinic acid and trifluoroacetic acid) and for chemiluminescence (luminol). Malondialdehyde was prepared immediately prior to use from 1,1,3,3-tetramethoxypropane by acidic hydrolysis with hydrochloric acid.[30] The concentration of hydrogen peroxide was determined immediately prior to use photometrically using $\varepsilon_{230} = 74\ \mathrm{l\,mol^{-1}cm^{-1}}$.[31]

Cartilage Preparation

Fresh bovine nasal cartilage was obtained from juvenile animals (about 12 months old and without any traces of disease) within a few hours after slaughter. After removing the surrounding soft tissue (the lipid containing perichondrium), cartilage was cut into small pieces, frozen in liquid nitrogen and finely minced in a portland mortar to minimize biological deviations.[6] Bovine nasal cartilage consists of about 40% collagen, 12% non-collagenous protein, 48% sulphated glycosaminoglycans (chondroitin and keratan sulphate) and small quantities (about 1%) of hyaluronic acid. All values are related to the dry weight of cartilage.[32]

Incubation of Cartilage with the Fenton Reagent

0.5 g (wet weight) of powdered cartilage and 2.0 ml buffer (50 mmol/l phosphate, pH 7.4) containing 10 mmol/l hydrogen peroxide were mixed in plastic vessels and the corresponding amount of ferrous chloride was added.[6]

Ferrous ion solutions were prepared in double-distilled water under an atmosphere of argon. This procedure was necessary to avoid the formation of oxidation products like $Fe(OH)_3$ and $FePO_4$ under alkaline conditions and in the presence of oxygen. The formation of these products would markedly decrease the actual concentration of ferrous ions.[33]

Samples were incubated at 37°C in a water bath. After incubation, the samples were centrifuged for about 10 minutes to remove insoluble material which was not further analyzed. Using monomeric and polymeric carbohydrate solutions, iron ions were precipitated by the addition of $(NH_4)_2S$ and samples centrifuged again.

The resulting clear supernatants were analyzed by ^{1}H NMR spectroscopy and in some cases also by further biochemical methods. All experiments were repeated at least three times. Detailed incubation conditions are given in each figure.

Radiation Experiments

Stationary radiolysis experiments were carried out using the irradiation plant PANORAMA at the Institute for surface modification, Leipzig. The applied γ dose rate from a ^{60}Co source was 10.3 kGy per hour. Doses were determined by

Fricke dosimetry and applied in the range from 5 kGy up to 20 kGy. All carbohydrate samples were irradiated in the presence of atmospheric oxygen in standard laboratory glass tubes.

Although these conditions do not allow an exact control over the oxygen content during radiolysis, these conditions were used as a model for the decreased oxygen content in pathologically changed synovial fluids.[34]

NMR-measurements

High resolution proton NMR measurements were conducted on a Bruker AMX-300 spectrometer operating at 300.13 MHz for ^1H. Spectra were recorded at ambient temperature (293K). Typically 0.40 ml radiated solution was placed in a 5 mm diameter NMR tube and 50 µl of D_2O was added to provide a field frequency lock. Water signal suppression was performed by presaturation, i.e. a 2s pulse was applied on the water resonance frequency before data acquisition. 128 accumulations were acquired with a total delay between two pulses of 8 seconds to allow full spin-lattice relaxation (T_1) of the protons in the sample. In the experiments with cartilage, the Hahn spin-echo sequence (90°-τ-180°-τ-collect) with $\tau = 60$ ms was used to suppress broad resonances arising from proteins and polymeric carbohydrates of the extracellular matrix.[5,6]

All spectra were recorded with a spectral width of 4000 Hz, according to approximately 13 ppm. No line-broadening or Gauss-broadening was applied to the free induction decay (FID). Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-propane-1-sulphonate in a final concentration of 500 µmol/l, which was added after irradiation.

Resonances were identified by their known chemical shifts[35] and by their subsequent enhancement after addition of a small amount of the corresponding pure compound.

Partially relaxed ^{13}C NMR spectra at 75.47 MHz were obtained on the same spectrometer as described above. Spectra were recorded with a flip angle of 45° (90° flip angle: 4 µs) with a pulse repetition time of 2s (SW 15600 Hz/16 K). Usually 1024 transients were accumulated under WALTZ-16 decoupling. All free induction decays were processed with a 5 Hz line-broadening to enhance signal to noise ratio.[36]

Mass Spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) was performed using the Voyager biospectrometry workstation (PerSeptive Biosystems, Framingham). All spectra were acquired in a matrix of sinapinic acid without low-mass gate using standard software. To enhance the signal to noise ratio 128 single shots from the nitrogen laser (337 nm) were averaged for each mass spectrum.

Determination of the Uronic Acid Content

Uronic acid contents were determined after cleavage of the glycosidic linkages in polysaccharides by treatment with concentrated sulphuric acid by the use of carbazole.[36] Optical absorptions were determined at 530 nm on a Hitachi U-2000 spectrophotometer using polypropylene cuvettes. Samples were diluted to obtain uronic acid concentrations within the linear region of the assay.

Chemiluminescence Measurements

Luminescence measurements were performed using a luminometer AutoLumat LB 953 (Fa. Berthold, Wildbad, Germany) and disposable polypropylene tubes with a 1.0 ml reaction mixture. To reveal effects of the stoichiometric ratio, luminol (final concentration 1×10^{-5} mol/l) and then freshly prepared $FeCl_2$ (final concentration 2.5×10^{-6} mol/l) were injected to a hydrogen peroxide solution in varying concentrations. Photons were counted for 10 s. Experiments have been repeated at least three times.

RESULTS

Degradation of Monosaccharides

Figure 1 shows four different ^{1}H NMR spectra of a glucose solution (10 mmol/l in phosphate buffer) after irradiation with a ^{60}Co source at different doses. The up-field (2.7–5.4 ppm) and the down-field regions (8.0–9.0 ppm) of the spectra are separated in the figure for clarity. Trace a shows the spectrum of pure glucose for means of comparison. There are only three well-resolved resonances: a virtual triplet at 3.22 ppm for the H-2 of the β-anomer, a doublet at 4.65 ppm (H1-β) and an additional doublet at 5.24 ppm (H1-α).[38] All other resonances show complex coupling patterns. No resonances were found in the down-field spectrum prior to radiolysis.

However, upon γ-irradiation, there was a steadily decrease (**1c-d**) of all resonances in the up-field region, accompanied by the formation of two additional resonances in the down-field region. Whereas the resonance at 8.44 ppm clearly contributes to the formate proton,[22] which is an indicator for monosaccharide degradation[22,23] the resonance at 8.62 ppm is more difficult to interpret. This resonance is most likely caused by malondialdehyde,[39] which is known to be formed in irradiated carbohydrate solutions.[40] Freshly prepared malondialdehyde resonates at 8.62 ppm (data not shown). Moreover, this resonance changes with time due to the oligomerization of the initially formed malondialdehyde.[41] In the corresponding mass spectrum there are several peaks (m/z = 198.7, 396.3 and 593.8) which correlate well with the masses of oligomerization products of malondialdehyde. Thus, a quantitative analysis of this resonance is rather difficult and was not performed in the present study.

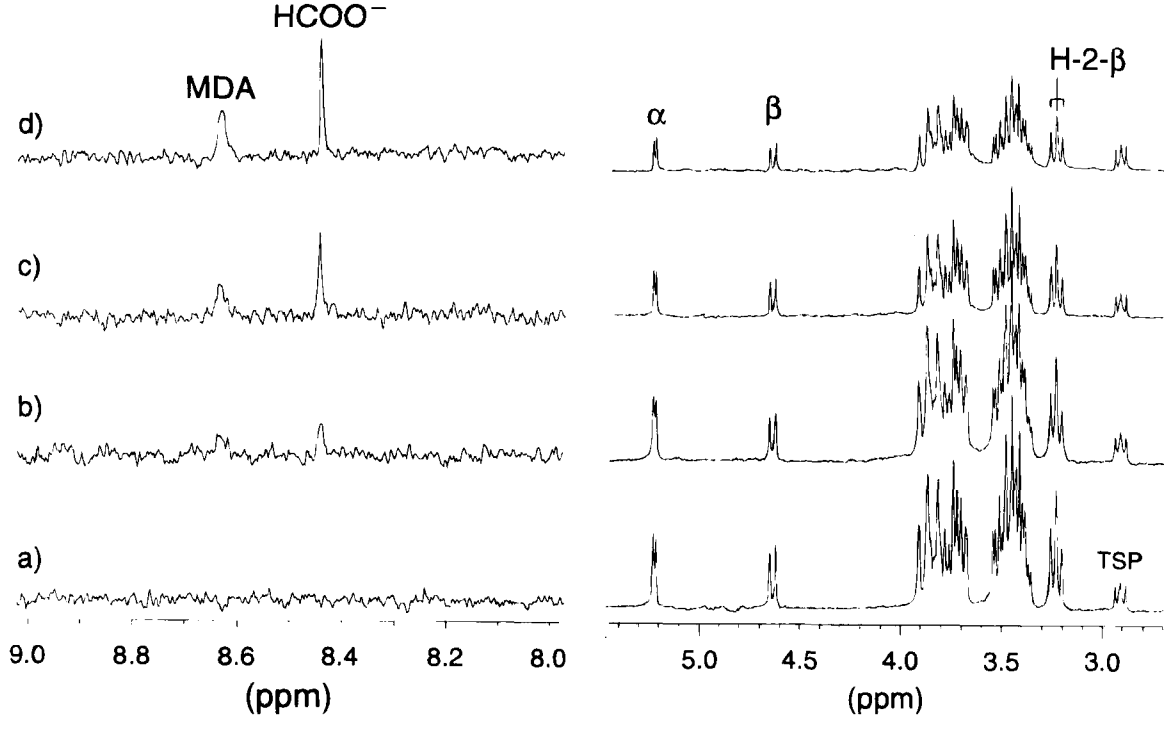

FIGURE 1 High-resolution proton NMR spectra of aqueous solutions of glucose (10 mmol/l) after irradiation with a ^{60}Co source. The following doses have been applied: **(a)** no irradiation, **(b)** 5.15 kGy, **(c)** 10.3 kGy and **(d)** 20.6 kGy. Spectra have been separated into the down-field (left) and the up-field region (right) for clarity. Abbreviations used in peak assignments: TSP, internal standard; MDA, malondialdehyde.

Interestingly, the integration of the different resonances yielded a marked discrepancy between the amount of formate, which is formed upon irradiation and the theoretical value (five molecules per molecule glucose) for formate which would be expected from the diminution of the glucose resonances: Whereas upon irradiation with 20.6 kGy 4.22 mmol glucose are consumed (G = 2.04), only about 0.27 mmol formate are formed (G = 0.13).

These G-values are similar to the values obtained by von Sonntag,[20] who found 5.6 for the consumption of glucose and 0.6 for the formation of formic acid in N_2O/O_2-saturated solutions of glucose. However, we have to mention that the determination of the glucose consumption by NMR spectroscopy from the intensity of the β-anomeric resonances at 4.65 ppm is not accurately if oligomers or polymers of glucose are formed.[40] We have some evidence from MALDI and HPLC analysis that upon γ-irradiation of glucose oligomeric species of glucose are formed. These species must be formed upon the recombination of radicals which arise upon hydrogen abstraction from the glucose molecule. Since this process is random and all hydrogen atoms are abstracted with nearly the same probability,[20] six different radicals are formed. The recombination of these species with each other gives a large variety of products. A mixture of such components should not yield distinct and easily detectable resonances for the anomeric protons, which would make identification of these components very difficult.

However, it remains astonishing that not a single component like D-gluconic acid or β- and γ-lactones, which are known to be formed upon γ-radiolysis of aqueous, oxygen containing glucose samples as main products[20] can be detected under these experimental conditions by means of NMR-spectroscopy. Nevertheless, under our experimental conditions it is likely that most oxygen in solution is consumed and, thus, reactions leading to the formation of oligomeric products get possible.

The same results are obtained by ^{13}C-NMR spectroscopy using 1-^{13}C-enriched glucose. However, only two resonances at 96.0 (β-anomer) and 92.2 ppm (α-anomer) are detectable equally if radiation was applied or not (data not shown). In accordance with the ^1H-NMR spectra, these two resonances were also simultaneously diminished upon irradiation.

However, formate formation could not be monitored by the less sensitive carbon NMR spectroscopy.[36] Additionally, glucose was ^{13}C-enriched only in 1-position and, thus, formate should only be detectable if it is derived from this position.

Analogous reactions were found with all monomeric carbohydrates in this investigation (glucuronic acid, N-acetylglucosamine, N-acetylgalactosamine and others). It is clearly evident that differences in chemical structures do not play a significant role.

DEGRADATION OF POLYSACCHARIDES

Marked differences in NMR spectra were found, when aqueous solutions of polysaccharides were exposed to γ-irradiation (Fig. 2). 2a shows a reference spectrum of an aqueous hyaluronic acid sample, whereas spectra 2b-d were recorded after irradiation with different doses. 2a differs from the spectra of monosaccharides, showing besides the resonances of the TSP standard (2.91 and 1.76 ppm) only one very weak resonance at about 2.0 ppm and a trace of acetate (1.90 ppm) in the up-field and no resonances in the down-field region. Hyaluronic acid from human umbilicial cords possesses a molecular weight of about one million Daltons.[2] The protons of such high molecular polysaccharides are embedded in a rigid structure and are less mobile.[22,23] This conferres broad, low intense resonances (2a) due to the short spin-spin relaxation (T_2) times of these protons. Only the methyl protons of the N-acetyl side chain are mobile enough to give a detectable resonance at about 2.0 ppm.[5,6,22]

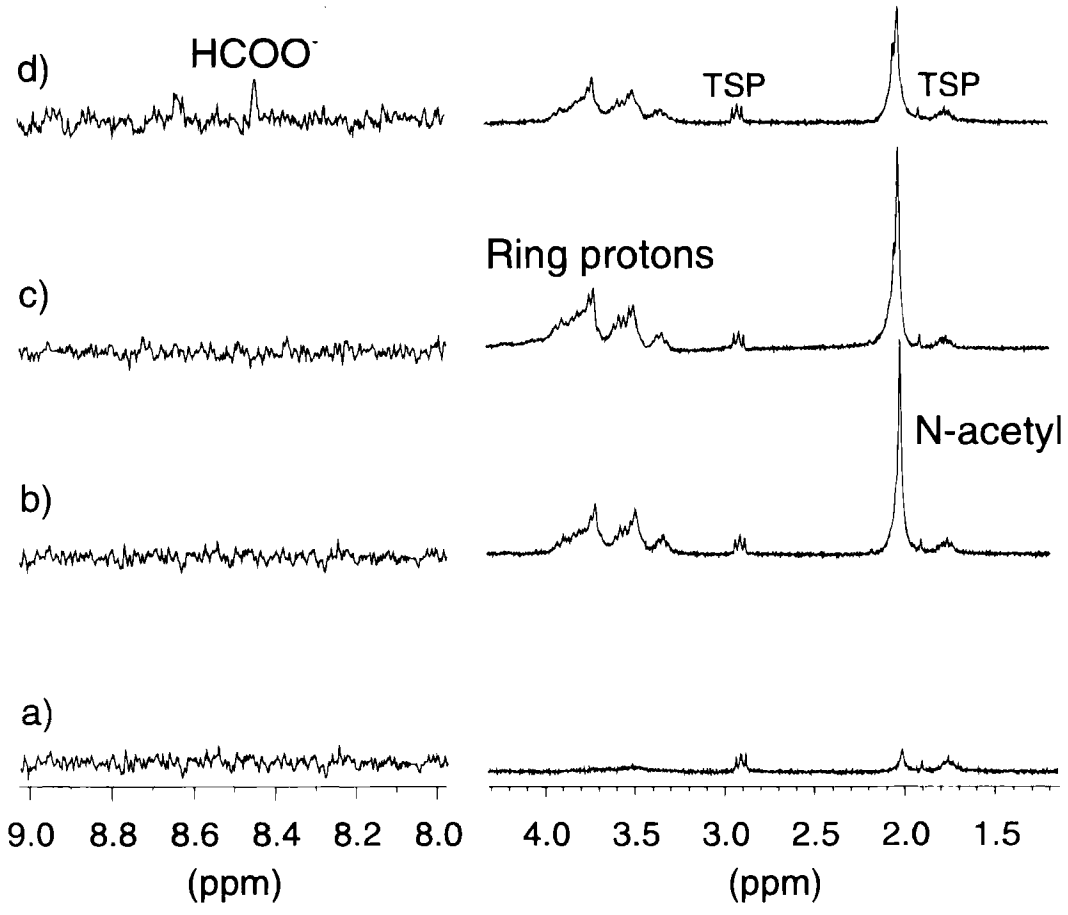

FIGURE 2 ^{1}H-NMR spectra of aqueous solutions of hyaluronic acid (4.17 g/ml according to 10 mmol/l per disaccharide unit) from human umbilicial cords after exposition to a ^{60}Co source. The following doses have been used: **(a)** no irradiation, **(b)** 5.15 kGy, **(c)** 10.3 kGy and **(d)** 20.6 kGy. Abbreviations used in peak assignments: TSP, internal standard; N-acetyl, mobile N-acetyl groups.

With increasing radiation doses (2b,c) N-acetyl groups at about 2.0 ppm were markedly enhanced and resonances of carbohydrate ring protons became detectable (between 3.3 and 3.9 ppm). These effects are caused by a cleavage of the glycosidic linkages leading to prolonged T_2-relaxation times and easier detectable, less broader resonances.[22] In contrast to the monosaccharides, no formate is detectable at these doses.

However, the N-acetyl resonance was diminished again at the highest radiation dose (20.6 kGy, 2d) accompanied by formate formation. Additionally, the formate resonance appeared at 8.44 ppm. This indicates that NMR detectable low molecular mass products compete with intact hyaluronic strands for hydroxyl radicals and are depleted in secondary reactions to formate.

An analogous behaviour was also found with analogous carbohydrates like chondroitinsulphate, heparin or low molecular weight hyaluronic acid. However resonances of their N-acetyl groups were already more expressed without irradiation due to their lower molecular weight.[2] Formate was found already at lower radiation doses (data not shown).

Effects of HO radicals were also investigated by the determination of the uronic acid content with the carbazole assay[37] in the corresponding

samples. Since each polysaccharide contains one uronic acid per disaccharide unit,[2] a decrease in uronic acid content can be used as a measure of carbohydrate degradation. As shown in Figure 3 the uronic acid content was markedly diminished upon exposition to ionizing radiation. This agrees with the data obtained by NMR spectroscopy. However, one important difference between two methods exists: The uronic acid determination showed already at small radiation doses a considerable decrease in uronic acid content, whereas still no formate was detectable in the NMR spectra.

For means of comparison with physiologically more relevant conditions, Fenton reaction was also used. Figure 4 shows the proton NMR spectra of a hyaluronic acid solution (4.17 mg/ml, 10 mmol/l with respect to the sum of the molecular weight of one repeating unit) incubated for two hours at 37°C with pure 50 mmol/l phosphate buffer, containing 10 mmol/l hydrogen peroxide (a). No differences are detectable in comparison with the spectrum in buffer (**2a**). This indicates that hydrogen peroxide alone (in the absence of transition metals) is unable to cleave glycosidic linkages.[15] However, differences occurred in the presence of iron ions. Ferrous chloride was added to the hyaluronic acid solution to give the following Fe^{2+} concentrations: 1 mmol/l (b), 2.5 mmol/l (c), 6 mmol/l (d), 10 mmol/l (e) and 15 mmol/l (f). In principle, the same effects were obtained as upon γ-irradiation: The N-acetyl resonance at 2.0 ppm increased with increasing concentrations of ferrous ions and formate was formed at higher concentrations of ferrous ions (about 6 mM).

However, using an excess of ferrous ions cleavage of glycosidic linkages and formate formation decreased again. Although this diminution may also be (at least partially) caused by shortened

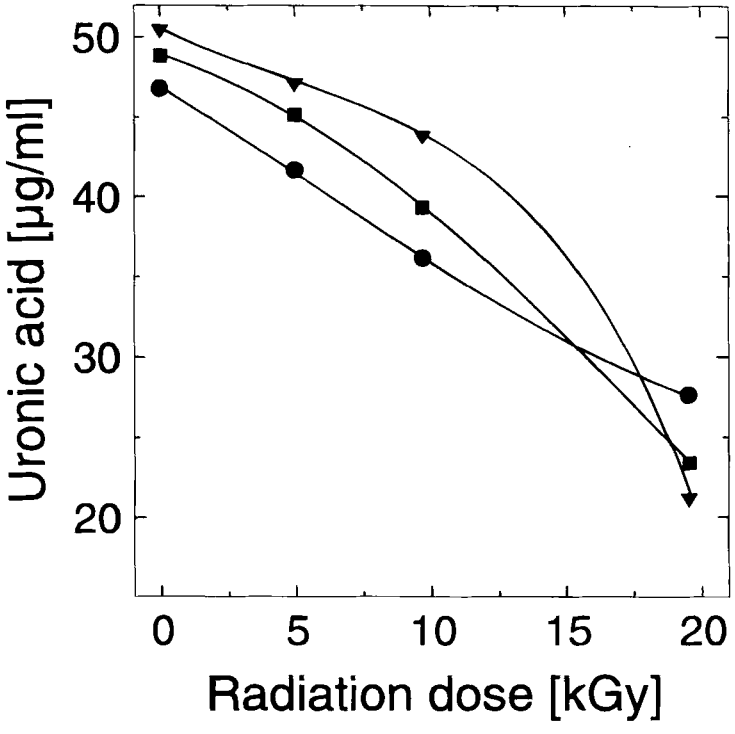

FIGURE 3 Determination of the content of uronic acid in two different hyaluronic acid (● 1×10^6, ■ 5×10^5 Da) preparations and a chondroitinsulphate (▼) solution after exposure to γ-radiation.

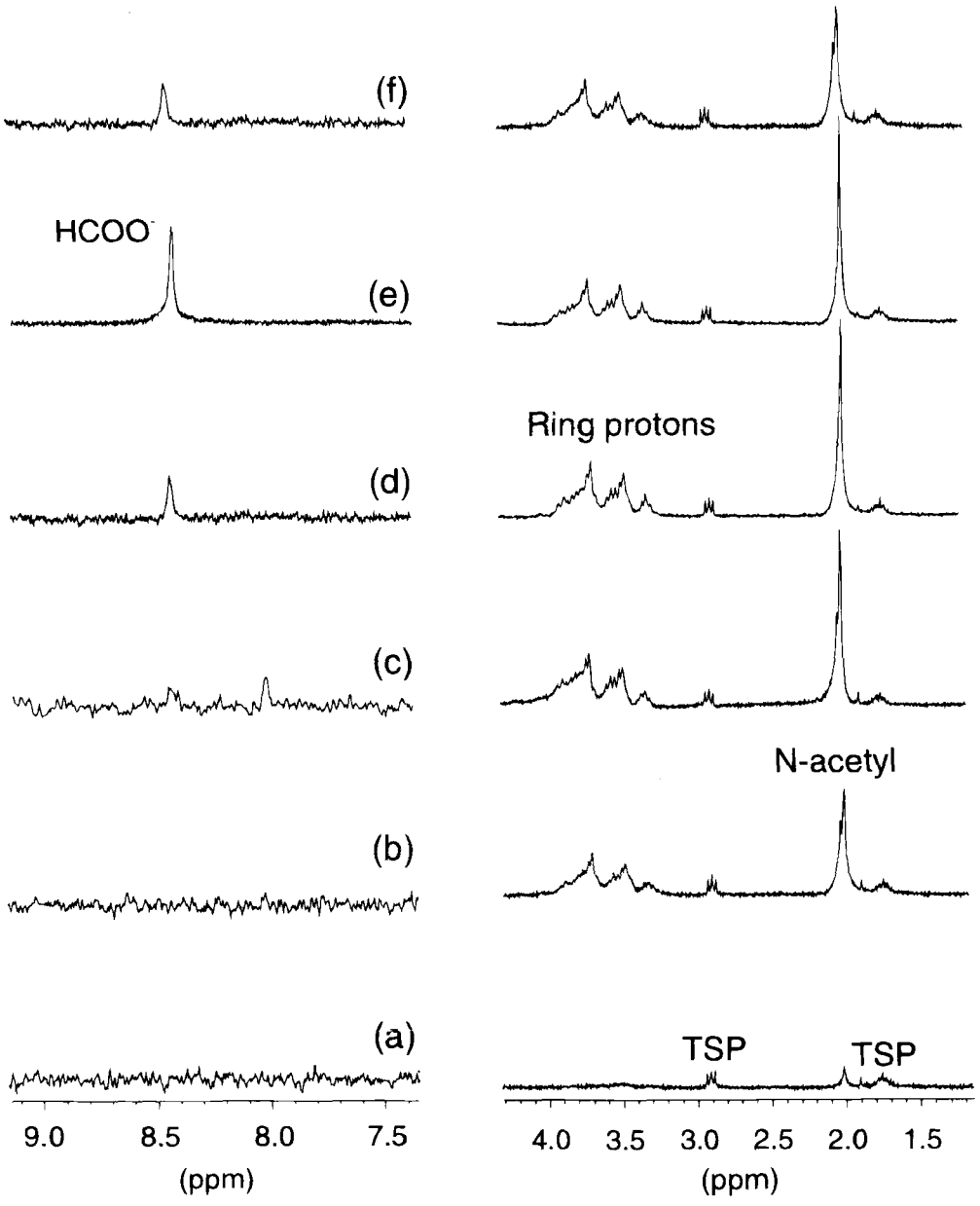

FIGURE 4 Proton NMR spectra of a solution of hyaluronic acid from human umbilicial cords (4.17 mg/ml) incubated for two hours at 37°C with 50 mmol/l phosphate buffer, containing 10 mmol/l hydrogen peroxide **(a)**. The following concentrations of ferrous chloride have been added: 1 mmol/l **(b)**, 2.5 mmol/l **(c)**, 6 mmol/l **(d)**, 10 mmol/l **(e)** and 15 mmol/l **(f)**. Abbreviations used in peak assignments: TSP, internal standard; N-acetyl, mobile N-acetyl groups; Lac, lactate; Coll, collagenous fragments.

relaxation times of the protons in the sample due to an incomplete removal of iron ions, a correlation exists between the concentration of ferrous ions and the yield of hydroxyl radicals. Ferrous ions do not only serve as a catalyst in Fenton chemistry but are also consumed by the reaction with hydrogen peroxide. This has also been shown by luminol amplified chemiluminescence

in the presence of hydrogen peroxide and ferrous ions. Upon the treatment of a luminol solution was treated with the complete Fenton reagent, a flash of light is generated. This light intensity depends on the stoichiometry of both reagents, yielding a maximum if Fe^{2+} and hydrogen peroxide are used in a 1:1 molar ratio (data not shown).

In summary, the NMR experiments on γ-irradiated carbohydrates gave marked differences between monomeric and polymeric carbohydrates: Monomeric carbohydrates were depleted to formate and malondialdehyde, in polymeric carbohydrates like hyaluronic acid, however, a cleavage of the glycosidic linkages predominated.

Degradation of Cartilage

Unfortunately, degradation experiments using solutions of isolated carbohydrates are oversimplified if one wants to obtain a more realistic model for cartilage destruction under pathological conditions: Cartilage contains besides its carbohydrate moiety also varying amounts of proteins, especially collagens,[2] which may compete with the polysaccharides for hydroxyl radicals.

Thus, to obtain information on the reactivity of cartilage with hydroxyl radicals, native cartilage samples were treated with the Fenton reagent. This procedure gives more marked effects than γ-radiolysis and allows also the control over the iron content in the samples. Bovine nasal cartilage was chosen since it contains more carbohydrates[32] than pig articular cartilage and, thus, effects towards polysaccharides should be more expressed.

Figure 5 shows the proton spin-echo (90°-τ-180°-τ-collect) NMR spectra of bovine nasal cartilage incubated for two hours at 37°C with 50 mmol/l phosphate buffer, containing 10 mmol/l hydrogen peroxide (a). The spin-echo sequence eliminates the broad resonances from proteins and polymeric carbohydrates[5,6] and, thus, resonances from low-molecular weight substances are easier detectable.[35] **5a** is dominated by the lactate resonance (1.31 ppm), whereas other metabolic species like creatine ($\sigma = 3.90$ ppm for the CH_2 group and $\sigma = 3.00$ ppm for the CH_3 group), quarternary ammonium salts like choline and betaine (3.19 and 3.25 ppm), and alanine (1.46 ppm) are present only to a less extent. Mobile N-acetyl groups of cartilage polymers ($\sigma \sim 2.0$ ppm)[6,22] and mobile collagen fragments (0.85 ppm) are not detectable.[6]

Whereas the treatment of cartilage with hydrogen peroxide alone gave only small effects, which were mainly caused by small quantities of iron and other transition metal ions like copper in the cartilage matrix, an enhanced degradation of cartilage occurred upon the addition of ferrous ions. The following concentrations of ferrous chloride have been used: 1 mmol/l (b), 2.5 mmol/l (c), 6 mmol/l (d), 10 mmol/l (e) and 15 mmol/l (f).

Analogous effects on cartilage as obtained with polysaccharide solutions were detectable: The concentration of N-acetyl groups and formate increased with increasing concentrations of ferrous ions, but decreased again if an excess of iron is added (**5f**). Unfortunately, a careful control over the iron concentration in biological tissues is difficult to obtain since the natural iron content of cartilage specimens and the interaction between iron ions and other metabolic species is not known.

Increasing amounts of iron obviously diminish the lactate resonance (1.31 ppm). Lactate is most likely complexed by iron ions and gets, thus, more and more invisible in the NMR spectra with increasing iron concentrations. Additionally, also relaxation effects caused by the paramagnetic Fe^{2+} may play an essential role, although no significant broadening of resonances occurred. We assume that iron ions are bound tightly to the negatively charged polysaccharides of cartilage[43] and are removed by centrifugation.

Further changes occur at about 3.25 ppm. Here, compounds with quarternary ammonia groups like choline, which is an important part in the structure of biological membranes[44] yielded intense resonances due to their nine equivalent

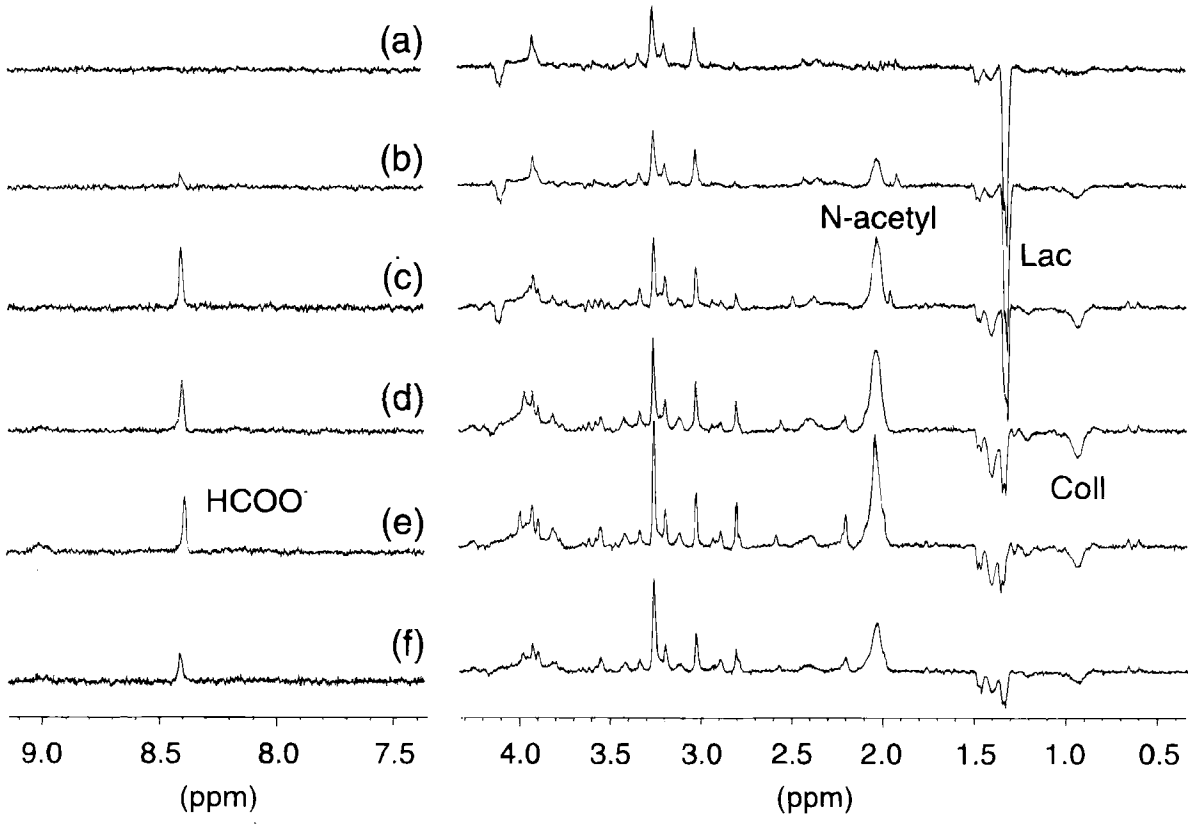

FIGURE 5 Proton spin-echo NMR spectra ($\tau = 60$ ms) of bovine nasal cartilage incubated for two hours at 37°C with 50 mmol/l phosphate buffer, containing 10 mmol/l hydrogen peroxide **(a)**. The following concentrations of ferrous chloride have been added: 1 mmol/l **(b)**, 2.5 mmol/l **(c)**, 6 mmol/l **(d)**, 10 mmol/l **(e)** and 15 mmol/l **(f)**. Abbreviations used in peak assignments: TSP, internal standard; N-acetyl, mobile N-acetyl groups; Lac, lactate; Coll, collagenous fragments.

protons. We assume that their intensity gain is caused by a decreased uronic acid content (Fig. 4) and, thus, by the reduced negative charge density in cartilage specimens. Under these conditions, positively charged components like choline do not more bind to the polysaccharides which enhances signal intensity in NMR. Naturally, the reaction of HO-radicals with lipids of cartilage cells may also lead to enhanced resonances of quarternary ammonia groups.

Only small effects were detectable on the collagen moiety of cartilage. Degradation products of collagen can be easily monitored by a broad resonance at about 0.85 ppm.[45] In this spectral region only a very small intensity enhancement was observed with enhanced iron concentrations. This indicates that the molecular weight of collagen was not significantly changed upon the action of hydroxyl radicals.

In summary, the carbohydrates of cartilage were affected by hydroxyl radicals in an analogous manner as polysaccharides in solution, whereas the collagen moiety of cartilage was not a primary target for HO-radicals.

DISCUSSION

Recently it has been shown by our group that a close correlation exists between myeloperoxidase activity in synovial fluids from patients with rheumatoid arthritis and the content of

NMR-detectable degradation products of polysaccharides (N-acetyl groups and acetate).[5] NMR spectroscopy was used since experiments are easily to perform, the sample is not destroyed, and no time-consuming pretreatment of the samples is required.[46] The data on MPO-activity and cartilage degradation clearly emphasize a prominent role of HOCl in rheumatic diseases. However, the knowledge to what extent other reactive oxygen species, especially hydroxyl radicals, may also contribute to these degradation processes is still relatively scarce.

The present NMR study shows that hydroxyl radicals cause two different effects on isolated polysaccharides and cartilage carbohydrates: First, a cleavage of glycosidic bonds and the formation of formate using more vigorous conditions. Whereas these effects were also observed by other groups,[22] we reported here for the first time a comparison between γ-irradiation and Fenton chemistry. Although the applied doses and the concentrations of the Fenton reagent are unphysiologically high it must be considered that (a) formate formation in synovial fluids was detectable already at lower doses[22] and (b) in the inflamed joint over long periods sufficient high amounts of reactive oxygen species can be produced. Since no one knows the detailed conditions in inflamed joints here very high amounts of HO-radicals were generated to simulate drastic conditions.

Carbohydrates are affected by hydroxyl radicals depending on their molecular weight and reaction conditions.[28] On the one hand, high molecular carbohydrates like hyaluronic acid are depolymerized under the influence of γ-irradiation[21–23] as well as upon the addition of the Fenton reagent. On the other hand, monomeric carbohydrates like glucose and oligosaccharides derived from hyaluronic acid are depleted in oxygenated solutions under the formation of a large variety of reaction products.[20] Unfortunately, the number and the yield of reaction products is still under discussion and up to hundred different reaction products are assumed to be formed.[25] Additionally, formation of polymeric oxidation products of glucose occurs, if no sufficient amount of oxygen is present.[47–50]

We have shown in the present investigation that formate is a potent NMR-detectable final product of carbohydrate degradation, despite of its widely unknown formation processes.[22] To realize more physiologically relevant conditions, samples were not continuously purged with oxygen but were only in contact with air, since synovial fluids also decrease in their oxygen content in the course of rheumatic diseases.[34] Thus, malondialdehyde and glucose oligomers were also formed under partially deoxygenated conditions. Additionally, we cannot exclude the formation of volatile products like CO or CO_2 since they are not detectable by NMR. Despite of these drawbacks of NMR spectroscopy and its comparable low sensitivity it is the only analytical methods which can be used for clinical investigations *in vivo*. Thus, the present investigation has been carried out to seek for possible NMR detectable markers in cartilage degradation. In contrast to HOCl, HO-radicals lead mainly to the formation of formate, but not to the formation of acetate. Both substances may serve as clinical markers in rheumatic diseases.

Whereas HOCl is formed only by enzyme catalysis, hydroxyl radicals can be formed under *in vivo* conditions by the reaction between hypochlorous acid and superoxide anion radicals[18] and Fenton like reactions.[26] Whereas HO-radicals are extremely reactive and react in a diffusion-controlled way, hydrogen peroxide is relatively slow-reactive, but is able to diffuse over large distances.[50] Thus, hydrogen peroxide can be formed intracellularly, pass the cell membrane and then produce HO radicals outside the cell upon its reaction with ferrous ions. It has been shown recently[51] that iron is released under pathological conditions from its complexes (e.g. ferritin) and, thus, Fenton chemistry may play a dominant role under *in vivo* conditions.

Although Fenton is still under discussion[17] and also iron in high oxidation degrees (perferryl species) may not be excluded,[15] it is likely

that hydroxyl radicals may here arise at least partially.

We conclude that hypochlorous acid as well as hydroxyl radicals are both able to cleave the polysaccharides of cartilage under the formation of NMR-detectable fragments. However, secondary reactions of the resulting oligosaccharides differ. Whereas hydroxyl radicals produce mainly formate, hypochlorous acid is characterized by the formation of acetate.[14] Because of the high amounts of acetate in pathologically changed synovial fluids[5] we conclude that hypochlorous acid is the species, mainly responsible for cartilage degradation and hydroxyl radicals are not the primary damaging species in these pathologies.

Nevertheless, further investigations are required for an detailed understanding of these reactions and to obtain information about processes of tissue injury under pathological conditions, like rheumatoid arthritis, which are in industrial countries the major cause for early retirements.

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References

[1] Liszt, F., Schnittker-Schulze, K., Stuhlsatz, H. W. and Greiling, H. (1991). Composition of proteoglycan fragments from hyaline cartilage produced by granulocytes in a model of frustrated phagocytosis. *European Journal of Clinical Chemistry and Clinical Biochemistry*, **29**, 123–130.

[2] Ebert, G. (1993). *Biopolymere*. Teubner Studienbücher, Stuttgart.

[3] Fantone, J. C. and Ward, P. A. (1982). Role of oxygen-derived radicals and metabolites in leukocyte-dependent inflammatory reactions. *American Journal of Pathology*, **107**, 397–418.

[4] Allen, R. C. (1987). Phagozyte oxygenation activities: quantitative analysis based on luminescence of hypochlorous acid: Implication for microbicidal mechanisms of leukocyte myeloperoxidase. *Proceedings of the National Academy of Sciences of the USA*, **78**, 210–214.

[5] Schiller, J., Arnhold, J., Sonntag, K. and Arnold, K. (1996). NMR studies on human, pathologically changed synovial fluids: Role of hypochlorous acid. *Magnetic Resonance in Medicine*, **35**, 848–853.

[6] Schiller, J., Arnhold, J. and Arnold, K. (1995). The action of hypochlorous acid on pig articular cartilage. *European Journal of Biochemistry*, **233**, 672–676.

[7] Thomas, E. L. (1979). Myeloperoxidase, hydrogen peroxide, chloride antimicrobial system: nitrogen-chlorine derivatives of bacterial components in their actions against Escherichia coli. *Infection and Immunity*, **25**, 110–116.

[8] Albrich, J. M., Mc Carthy, M. C. and Hurst, J. K. (1981). Biological reactivity of hypochlorous acid: Implications for microbicidal mechanisms of leukocyte myeloperoxidase. *Proceedings of the National Academy of Sciences of the USA*, **78**, 210–214.

[9] Klebanoff, S. J. (1970). Myeloperoxidase: contributions to the microbicidal activity of intact leukocytes. *Science*, **169**, 1095–1097.

[10] Klebanoff, S. F. and Clark, R. A. (1978). *The neutrophil: function and clinical disorders*. North Holland Publishing Company, Amsterdam.

[11] Brown, K. A. (1988). The polymorphonuclear cell in rheumatoid arthritis. *British Journal of Rheumatology*, **27**, 150–155.

[12] Zvaifler, N. J. (1972). The immunopathology of joint inflammation in rheumatoid arthritis. *Advances in Immunology*, **16**, 265–336.

[13] Sonntag, K., Arnhold, J. and Arnold, K. (1994). Influence of myeloperoxidase on the generation of native chemiluminescence. In *Bioluminescence and chemiluminescence* (eds. A.K. Campbell, L.J. Kricka and P.E. Stanley), Wiley, Chichester, pp. 187–190.

[14] Schiller, J., Arnhold, J., Gründer, W. and Arnold, K. (1994). The action of hypochlorous acid on polymeric components of cartilage. *Biological Chemistry Hoppe-Selyer*, **375**, 167–172.

[15] Wardman, P. and Candeias, L. P. (1996). Fenton chemistry: An introduction. *Radiation Research*, **145**, 523–531.

[16] Winterbourn, C. C. (1987). The ability of scavengers to distinguish OH^- production in the iron-catalyzed Haber-Weiss reaction: Comparison of four assays for OH^-. *Free Radical Biology and Medicine*, **3**, 33–39.

[17] Goldstein, S., Meyerstein, D. and Czapski, G. (1993). The Fenton Reagents. *Free Radical Biology and Medicine*, **15**, 435–445.

[18] Long, C. A. and Bielski, B. H. J. (1980). Rate of reaction of superoxide radical with chloride-containing species. *Journal of Physical Chemistry*, **84**, 555–557.

[19] Cheung, K., Lark, J., Robinson, P. J., Pomeny, P. J. and Hunter, S. (1986). The production of hydroxyl radical by human neutrophils stimulated by arachidonic acid—measurement by esr spectroscopy. *Australian Journal of Experimental Biological and Medical Sciences*, **64**, 157–164.

[20] von Sonntag, C. (1985). *The chemical basis of radiation biology*. Taylor & Francis, London.

[21] Deeble, D. J., Bothe, E., Schuchmann, H.-P., Parsons, B. J., Phillips, G. O. and von Sonntag, C. (1990). The kinetics of hydroxyl-radical-induced strand breakage of hyaluronic acid. A pulse radiolysis study using conductometry and laser-light-scattering. *Z. Naturforsch.*, **45c**, 1031–1043.

[22] Grootveld, M., Henderson, E. B., Farrell, A., Blake, D. R., Parkes, H. and Haycock, P. (1991). Oxidative damage to hyaluronate and glucose in synovial fluid during exercise of the inflamed rheumatoid joint. *Biochemical Journal*, **273**, 459–467.

[23] Parkes, H. G., Grootveld, M. C., Henderson, E. B., Farrell, A., Blake, D. R. (1991). Oxidative damage to synovial fluid from the inflamed rheumatoid joint detected by ^{1}H NMR spectroscopy. *Journal of Pharmaceutical & Biomedical Analysis*, **9**, 75–82.

[24] Schuchmann, M. N. and von Sonntag, C. (1977). Radiation chemistry of carbohydrates. Part 14. Hydroxyl radical induced oxidation of D-glucose in oxygenated aqueous solution. *Journal of the Chemical Society Perkin II*, 1958–1963.

[25] von Sonntag C. (1984). Carbohydrate radicals: from ethylene glycol to DNA strand breakage. *International Journal of Radiation Biology*, **46**, 507–519.

[26] Walling, C. (1975). Fenton's reagent revisited. *Accounts of Chemical Research*, **8**, 125–131.

[27] Asmus, K.-D. (1984). Pulse radiolysis methodology. *Methods in Enzymology*, **105**, 167–178.

[28] Kemsley, K. G., Moore, J. S., Phillips, G. O. (1979). The effect of molecular size on the rate constants for reaction of hydrated electrons and hydroxyl radicals with carbohydrates. *International Journal of Radiation Biology*, **36**, 429–432.

[29] Bielski, B. H. J., Cabelli, D. E., Arudi, R. L. and Ross, A. B. (1985). Reactivity of HO_2/O_2 radicals in aqueous solution. *Journal of Physical and Chemical Reference Data*, **14**, 1041–1100.

[30] Gutteridge, J. M. C., Heys, A. D. and Lunec, J. (1977). Fluorescent malondialdehyde polymers from hydrolysed 1,1,3,3-tetramethoxypropane. *Analytica Chimica Acta*, **94**, 209–211.

[31] Beers, R. F. and Sizer, I. W. (1952). A spectrometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry*, **195**, 133–140.

[32] Torchia, D. A., Hasson, M. A., Hascall, V. C. (1977). Investigation of molecular motion of proteoglycans in cartilage by ^{13}C magnetic resonance. *Journal of Biological Chemistry*, **252**, 3617–3625.

[33] Bottu, G. (1989). The effect of quenchers on the chemiluminescence of luminol and lucigenin. *Journal of Bioluminescence and Chemiluminescence*, **3**, 59–65.

[34] Treuhaft, P. S. and McCarthy, D. J. (1971). Synovial fluid pH, lactate, oxygen and carbon dioxide partial pressure in various joint diseases. *Arthritis & Rheumatism*, **14**, 475–484.

[35] Agar, N. S., Rae, C. D., Chapman, B. E. and Kuchel, P. W. (1991). ^{1}H NMR spectroscopic survey of plasma and erythrocytes from selected marsupials and domestic animals of australia. *Comprehensive Physiology*, **99b**, 575–597.

[36] Albert, K., Michele, S., Günther, U., Fial, M., Gall, H. and Saal, J. (1993). ^{13}C NMR investigation of synovial fluids. *Magnetic Resonance in Medicine*, **30**, 236–240.

[37] Bitter, T. and Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry*, **4**, 330–334.

[38] Hobley, P., Howarth, O. and Ibbutt, R. N. (1996). ^{1}H and ^{13}C NMR shifts for aldopyranose and aldofuranose monosaccharides: Conformational analysis and solvent dependence. *Magnetic Resonance in Chemistry*, **34**, 755–60.

[39] Bertz, S. H. and Dabbogh, G. (1990). NMR spectroscopy of malondialdehyde. *Journal of Organic Chemistry*, **55**, 5161–5165.

[40] Scherz, H. (1970). Formation of deoxycompounds and malondialdehyde in irradiated aqueous solutions of carbohydrates and related compounds. *Radiation Research*, **43**, 12–24.

[41] Esterbauer, H., Schaur, R. J. and Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynoneal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine*, **11**, 81–128.

[42] Kalinowski, H.-O., Berger, S. and Braun, S. (1984). *^{13}C-NMR Spektroskopie*. Georg Thieme Verlag, Stuttgart.

[43] Hunter, G. K., Wong, K. S. and Kim, J. J. (1988). Binding of calcium to glycosamino-glycans. *Archives of Biochemistry and Biophysics*. **260**, 161–167.

[44] Stryer, L. (1987). *Biochemie*, Friedrich Vieweg & Sohn, Braunschweig.

[45] Schiller, J., *NMR-Untersuchungen an menschlichen Gelenkflüssigkeiten und Beiträge zur Modellierung der Knorpeldestruktion bei der Rheumatoid Arthritis*. Thesis, University of Leipzig, 1995.

[46] Williamson, M. P., Humm, G. and Crisp, A. J. (1989). ^{1}H nuclear magnetic resonance investigation of synovial fluid components in osteoarthritis, rheumatoid arthritis and traumatic effusions. *British Journal of Rheumatology*, **28**, 23–27.

[47] Scherz, H. and Stehlik, G. (1968). Bildung von Malonaldehyd bei der Bestrahlung wäßriger Glucoselösungen. Monatshefte für Chemie, **99**, 1143–1144.

[48] Heusinger, H. (1991). Gel-permeation chromatography of solutions of D-glucose after irradiation by ultrasound and γ-rays. *Carbohydrate Research*, **209**, 109–118.

[49] Barker, S. A., Grant, P. M., Stacey, M. and Ward, R. B. (1961). Effect of γ-radiation. Part I. Polymer formation from sugars, hydroxy-acids and amino-acids. *Journal of the Chemical Society*. 2648–2654.

[50] Bailey, A. J., Barker, S. A. and Stacey, M. (1961). Polymer production from carbohydrates. *Radiation Research*, **15**, 538–545.

[51] Schubert, J. and Wilmer, J. W. (1991). Does hydrogen peroxide exist "free" in biological systems? *Free Radicals in Biology and Medicine*, **11**, 545–555.

[52] Parkes, H. G., Allen, R. E., Furst, A., Blake, D. R. and Grootveld, M. C. (1991). Speciation of non-transferrin-bound iron ions in synovial fluid from patients with rheumatoid arthritis by proton nuclear amgnetic resonance spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, **9**, 29–32.