Action of Hypochlorous Acid on Polymeric Components of Cartilage. Use of ^{13}C NMR Spectroscopy

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Z. Naturforsch. **50c,** 721–728 (1995); received May 29/June 21, 1995

Hypochlorous Acid, Cartilage, Chondroitinsulphate, Carbon NMR Spectroscopy

It is a well known fact that neutrophil-derived hypochlorous acid plays an important role in cartilage destruction during rheumatoid arthritis. It has been shown by ^1H NMR spectroscopy in a previous paper (Schiller *et al.* (1994), Biol. Chem. Hoppe-Seyler **375**, 167–172) that sodium hypochlorite affects primarily the N-acetyl side chains of polymeric carbohydrates of cartilage like chondroitinsulphate and hyaluronic acid. An instable intermediate, likely to be a chloramine, is involved in these processes. The present paper deals with the application of carbon NMR spectroscopy for the study of these degradation processes, because carbon NMR gives the opportunity to detect changes on the single sugar ring carbons. Although it was not possible to prove the involvement of an intermediate, because of its fast hydrolysis, we were able to show that the reaction between sodium hypochlorite and N-acetylglucosamine affects mainly the side chain, accompanied by the formation of acetate. The application of a large excess of sodium hypochlorite leads to a breakdown of the carbohydrate ring under the formation of formiate.

Introduction

Rheumatic diseases are characterized by a massive damage of components of the extracellular matrix and an enhancement of the number of polymorphonuclear leukocytes (PMNs) in the inflamed joint (Zvaifler, 1973; Brown, 1988). These cells are able to release the enzyme myeloperoxidase (MPO, E. C. 1.11.1.7), which catalyses the formation of hypochlorous acid from hydrogen peroxide and chloride anions (Thomas, 1979; Albrich *et al.*, 1981):

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

Neutrophil derived hypochlorous acid seems to be involved in cartilage degradation during rheumatoid arthritis. Elevated activities of myeloperoxidase in synovial fluids from patients with rheumatoid arthritis were observed (Edwards *et al.*, 1988; Nurcombe *et al.*, 1991). Moreover, neutrophils from synovial fluids of patient material were characterized by enhanced values in native chemiluminescence (Arnhold *et al.*, 1994).

An action of hypochlorous acid on polymeric components of cartilage has been examined in model experiments (Baker *et al.*, 1988; Kowanko *et al.*, 1989). Whereas a loss of viscosity of hyaluronic acid solutions is observed with small amounts of hypochlorous acid, higher concentrations lead to a breakdown of the polymer chains (Baker *et al.*, 1988).

Because primary reactions of $HOCl/ClO^{\ominus}$ on carbohydrate polymers have been unknown, we investigated its action on different monomers and polymers using ^1H NMR (nuclear magnetic resonance) spectroscopy (Schiller *et al.*, 1994). It was found that N-acetyl side chains in N-acetylglucosamine, N-acetylgalactosamine, chondroitinsulphate and hyaluronic acid are the main target for hypochlorous acid. They are depleted to acetate via the formation of an unstable intermediate, which is probably a chlorinated product of the N-acetyl side chain (Schiller *et al.*, 1994). A breakdown of polymer chains of hyaluronic acid and a disruption of single sugar rings with formation of formiate occured only using higher amounts of hypochlorous acid in these experiments.

This investigation gave only insufficient answers about the relation between N-acetyl side chain degradation, the breakdown of the ring structure, and the oxidation of functional groups of the sugar ring upon the action of $HOCl/ClO^{\ominus}$. Therefore, the higher potential of carbon NMR spectroscopy

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0939–5075/95/0900–0721 $ 06.00

for the detection of changes at single positions of the ring system is used in the present investigation. Recently degradation processes of polymeric components and synovial fluids induced by hyaluronidase from bovine testes were studied by use of ^{13}C NMR (Albert *et al.*, 1993). We present ^{13}C NMR data indicating that only two main processes occur upon the action of $HOCl/ClO^{\ominus}$ on carbohydrate polymers, the degradation of N-acetyl side chains and the breakdown of the single sugar rings.

Material and Methods

Chemicals

All chemicals (NaCl, Na_2HPO_4 and KH_2PO_4) for buffer preparation and the starting materials (glucose, glucuronic acid, N-acetylglucosamine, N-acetylgalactosamine, heparin from pig intestine, chondroitinsulphate from bovine nasal trachea and hyaluronic acid from human umbilicial cords) were purchased from Fluka Feinchemikalien GmbH, Neu-Ulm (Germany). They were used without further purification. The purity of these polymeric components was tested by size exclusion chromatography. All polymers used for this study were chromatographically pure and did not show impurities of other glycosaminoglycans.

Incubation conditions

Sodium hypochlorite (as source for hypochlorous acid) from Sigma Chemie (Deisenhofen) was commercially available. A stock solution of NaOCl was prepared in water. Its concentration was determined spectrophotometrically immediately prior to use at pH 12 (ε_{290nm} = 350 M^{-1} cm^{-1}) (Morris, 1966). Solutions of polymeric and monomeric components were freshly prepared in 50 mmol/l phosphate buffer, 0.14 mol/l NaCl, pH 7.4 and incubated with the corresponding concentrations of NaOCl at 37 °C for different times. The incubation conditions are indicated in each figure. For UV-determinations a Hitachi U-2000 photometer was used.

Concentrations of polymers were related to the sum of the molecular weights of their corresponding repeating units (e.g. for hyaluronic acid the molecular mass of glucuronic acid and N-acetylglucosamine).

NMR-measurements

Proton NMR measurements were conducted on a Bruker AMX-300 spectrometer operating at 300.13 MHz for ^1H. All spectra were recorded at ambient temperature (293 K). Typically 0.40 ml of the corresponding incubation solution was placed in a 5 mm diameter NMR tube and 50 µl of D_2O was added to provide a field frequency lock. The intense water signal and the broad resonances arising from polymeric carbohydrates were suppressed by a combination of the Hahn spin-echo sequence (Bell *et al.*, 1987) and the application of continuous secondary irradiation at the water resonance frequency. The Hahn spin-echo sequence [90°-τ-180°-τ-collect] was usually repeated 128 times with τ = 60 ms and a total delay between two pulses of 5 seconds to allow full spin-lattice (T_1) relaxation of the protons in the sample. All spectra were recorded with a spectral width of 4000 Hz, according to approximately 13 ppm. A line-broadening of 0.5 Hz was used to improve the signal to noise ratio of the spectra. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-propane-1-sulphonate in a final concentration of 500 µmol/l.

^{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 4µs) with a pulse repetition time of 2 s (SW 15600Hz/16 K). Usually 16 K transients were accumulated under WALTZ-16 decoupling overnight. All free induction decays were processed with a 10 Hz line-broadening.

Results

Monomer degradation

Chemical components, containing one or more sugar rings are relatively complex substances, because they contain many different carbon atoms, each of them only slightly different from the others (Yamada *et al.*, 1992). N-acetylglucosamine (N-acetylgalactosamine shows an analogous behaviour) exists in three different forms in aqueous solution. Two ring forms ("anomers") and an open-chain form with a free aldehyde group. Because the aldehyde form is energetically unfavourable (Bunn *et al.*, 1981), signals of both anomeric forms predominate in ^1H and ^{13}C NMR spectra

of N-acetylglucosamine. Examples of ^{1}H and ^{13}C NMR spectra of solutions of N-acetylglucosamine are given in Fig. 1.

Protons (a) of the methyl group of the N-acetyl side chain give an intense singlet at 2.04 ppm, whereas protons of C(1)–H of both anomeric forms of N-acetylglucosamine show two well resolved doublets at 5.188 ppm [$^{3}J(^{1}H^{1}H)$ = 3.4 Hz] for the α(*cis*)-form and at 4.698 ppm [$^{3}J(^{1}H^{1}H)$ = 8.4 Hz] for the β(*trans*)-form (Livant *et al.*, 1992). All other C–H protons yield coupled resonances between about 3.3 and 3.9 ppm. Protons from O–H and N–H are invisible in the spectrum because of their fast exchange with water molecules (Scott *et al.*, 1984).

A completely different spectrum is found for an aqueous solution of N-acetylglucosamine using carbon NMR. Resonances from carbon atoms are much better separated from each other (1b). The two resonances at 24.9 and 24.7 ppm at the right end of the spectrum are assigned to the carbon atom of the methyl group of the N-acetyl side chain. The ratio of intensities of these two lines is about 2:3, confirming that the α-anomeric form (shifted to higher field compared to the β-form) of N-acetylglucosamine is preferred compared to the β-anomeric form under our experimental conditions (pH 7.4; 298 K). The resonances of the carbonyl group of the N-acetyl side chain at 177.5 and 177.3 ppm show an analogous behaviour. The signals of the remaining carbon atoms of the sugar ring are distributed over a relatively wide range of chemical shifts from about 57 ppm to 98 ppm. The resonances at lower field (97.6 and 93.5 ppm) correspond to the C-1 atom (containing anomeric protons) and the resonances at higher field (62.9 and 62.7 ppm) to the C-6 atom of N-acetylglucosamine. The remaining eight resonances are assigned to the remaining ring carbons in Fig. 1 (Kalinowski *et al.*, 1984).

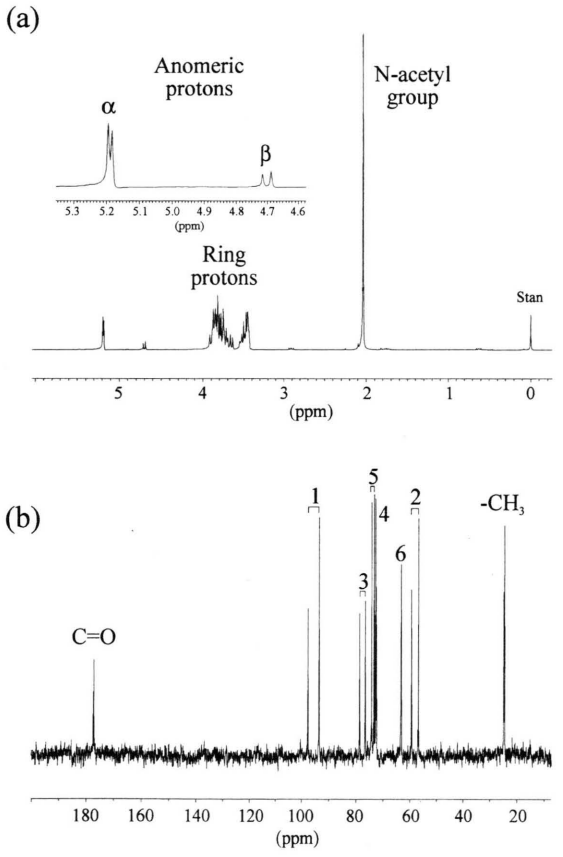


Fig. 1. Comparative NMR spectra of an aqueous solution of N-acetylglucosamine (50 mmol/l). The resonances appearing in the proton NMR (a) and in the carbon NMR (b) are indicated in the figures.

Upon the action of the powerful oxidizing reagent hypochlorous acid characteristic changes in the ^{13}C NMR spectrum of N-acetylglucosamine occur, highly depending on the NaOCl concentration (Fig. 2).

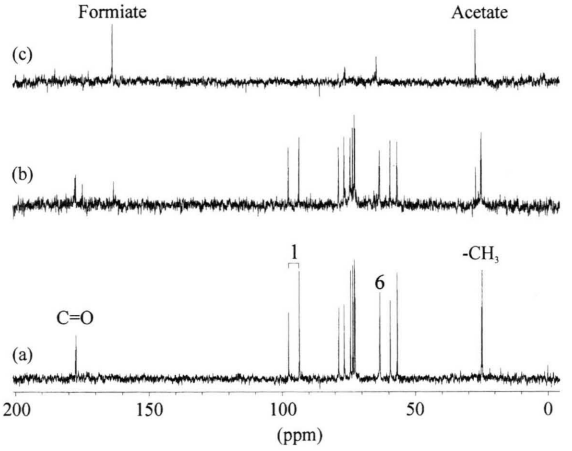


Fig. 2. ^{13}C NMR spectra of a solution of N-acetylglucosamine (50 mmol/l) treated for four hours with different amounts of sodium hypochlorite. The following molar ratios of NaOCl to N-acetylglucosamine were used: 1:5 (a), 1:1 (b), 5:1 (c). Incubations were performed for 4 hours at 37 °C.

If low concentrations of sodium hypochlorite are used (e.g. in a 1:5 molar ratio between hypochlorous acid and N-acetylglucosamine in Fig. 2a) reaction products do not occur in the spectrum. This is in good agreement to the results obtained by proton NMR spectroscopy (Schiller *et al.*, 1994), where only very low amounts of acetate at 1.90 ppm could be detected under identical experimental conditions. Probably, these low concentrations of acetate cannot be observed because of the very low sensitivity of carbon NMR spectroscopy in comparison to proton NMR.

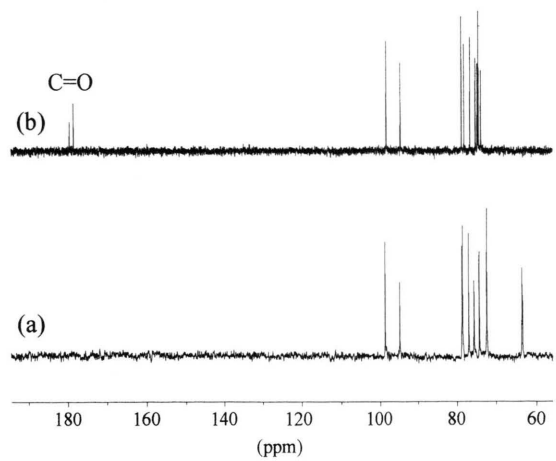
At higher NaOCl concentrations (1:1 molar ratio, 2b) three new resonances appear at 174.8, 163.3 and 27.2 ppm, whereas all other signals are slightly diminished. These resonances originate from formiate (163.3 ppm) and the methyl carbon of acetate (27.2 ppm). The C=O signal of acetate is not visible in the spectrum because of the strongly diminshed T_1 values of quaternary carbon atoms and perhaps because of a diminished nuclear Overhauser enhancement. Acetate results from degradation of N-acetyl side chains of N-acetylglucosamine (Schiller *et al.*, 1994), whereas formiate is the final product of the breakdown of the carbohydrate ring system (Beyer *et al.*, 1984).

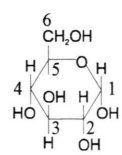
The acetate to formiate ratio is a measure of the contribution of side chain and ring degradation in N-acetylglucosamine. At a molar ratio of 1:1 between hypochlorous acid and N-acetylglucosamine the degradation of the N-acetyl side chain dominates over the ring breakdown. Although both resonances differ only slightly in their intensities the acetate concentration is higher than the formiate one, because both substances differ considerably in their T_1-behaviour. At equal concentrations formiate yields a more intense resonance than acetate (data not shown). Moreover, the complete oxidation of the sugar ring would result in five molecules of formiate.

The remaining signal at 174.8 ppm is not unambiguously fully assigned. It might result from an unstable intermediate of N-acetyl side chain degradation, which was recently found by ^{1}H NMR spectroscopy (Schiller *et al.*, 1994). This conclusion is supported by the fact, that this signal vanishes if very high sodium hypochlorite concentrations are used.

Increasing the NaOCl concentration to a molar excess of 5:1 (2c) strongly reduced signals of the starting material are observed, whereas the resonances arising from acetate and formiate gain higher intensity. Using a very large excess of NaOCl (10:1) the signals from acetate and formiate dominate in the spectrum, showing that the sugar rings are completely destroyed (data not shown).

These experiments do not give indications for the oxidation of the $-CH_2OH$ group of N-acetylglucosamine by hypochlorous acid. If glucose is treated with NaOCl the formation of glucuronic acid is accompanied by characteristic changes in the ^{13}C-NMR spectrum (Fig. 3). Although 12 resonances are expected for the α- and β-anomomeric forms of glucose, only 10 resonances can be observed in the spectrum. Unfortunately, the resonances for the C-4 and C-6 carbon atom cannot be separated under our experimental conditions (Kalinowski *et al.*, 1984). A complete loss of the resonance at 64.2 ppm for the carbon atom of the CH_2OH group occurs after treatment of glucose with NaOCl. At the same time two new resonances at 179.8 and 179.5 ppm for the quaternary





C-1: 97.1/93.3
C-2: 75.6/73.1
C-3: 77.3/74.4
C-4: 71.2
C-5: 77.3/72.9
C-6: 62.4

Fig. 3. ^{13}C NMR spectra of a solution of pure glucose (50 mmol/l) (a) and after treatment with sodium hypochlorite in a molar ratio 1:1 (b) for four hours at 37 °C. Resonances for glucose are assigned to the corresponding formula [in ppm].

carbon of the carboxylate group appear. All other resonances are only slightly shifted (indicating that the ring system is inaffected). Such changes do not occur upon treatment of N-acetylglucosamine with NaOCl.

Polymer degradation

Sulphated glycosaminoglycans are important components of cartilage (Hardingham, 1990). Therefore we have also studied the degradation of these polymers.

Unfortunately, polymeric components are characterized by shortened transverse relaxation times in comparison to smaller oligomer units (Grootveld *et al.*, 1991). This property is the reason for the different behaviour of the ^{1}H NMR spectra of chondroitinsulphate and hyaluronic acid. Whereas a proton NMR spectrum with well resolved resonances for the N-acetyl side chain can be obtained from a solution of chondroitinsulphate, an aqueous solution of hyaluronic acid does not show any resonances neither in the proton (Schiller *et al.*, 1994) nor in the carbon NMR spectrum. This fact may be caused by the different molecular weights of these two carbohydrate polymers. Chondroitinsulphate has a molecular weight not higher than 50 kDa but the molecular weight of hyaluronic acid is higher than 1000 kDa, which results in very short T_2 values and low solubility in water. These constraints in mind we focused our interest on chondroitinsulphate to study the influence of sodium hypochlorite on polymeric components of cartilage.

The spectrum of pure chondroitinsulphate looks similiar to the spectrum of N-acetylglucosamine or N-acetylgalactosamine (data not shown). However the signal to noise ratio is not as well under these experimental conditions, because only a part of the carbon atoms is mobile enough to be detected by NMR spectroscopy. There are again three well separated regions. At high field two resonances occur at 26.2 and 25.4 ppm for the methyl group of the N-acetyl side chain. This splitting is not caused by anomeric forms as observed for N-acetylglucosamine (this is not possible, if the single sugar rings build a polymer chain) but results from a mixture of chondroitin-4- and chondroitin-6-sulphate (Torchia *et al.*, 1977). At low field there are two resonances at 177.8 and 177.3 ppm, according to the quaternary carbon of the carbonyl group present in the N-acetyl side chain of the polymer. The C–H groups from the sugar rings show resonances between about 110 ppm and 50 ppm.

Using the same concentrations for the NaOCl treatment as with N-acetylglucosamine similar changes take place (Fig. 4). Already at a chondroitinsulphate to NaOCl ratio of 5:1 there are small traces of acetate at 27.2 ppm present in the spectrum, but not at all formiate (Parkes *et al.*, 1991). This behaviour shows, that a degradation of the N-acetyl side chains takes place at low concentrations of NaOCl (a). Increasing the concentration of NaOCl to a 1:1 molar ratio (b) the resonances from the starting material are markedly diminished under an enhancement of the concentration of acetate. Using a very high excess of NaOCl (c) the resulting spectrum is nearly the same as obtained with N-acetylglucosamine, showing two clear resonances for formiate and acetate.

From the experiments the question arises, to what extent different kinds of monomeric and polymeric carbohydrates are affected by sodium hypochlorite. To answer this question carbohydrate solutions were incubated over a period of one hour with 10^{-3} mol/l NaOCl. The consumption of hypochlorous acid was monitored by the decrease of the absorbance at 290 nm. The carbohydrate concentration was 10^{-3} mol/l for monomeric substances. It was related to monomeric ("repeating") units for polymeric substances. The

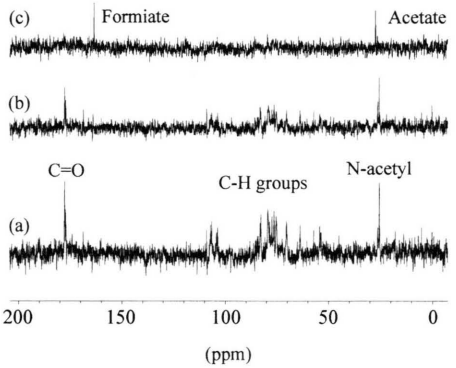


Fig. 4. ^{13}C NMR spectra of a solution of chondroitinsulphate (50 mmol/l = 33.3 g/l) treated for four hours with different amounts of sodium hypochlorite. The following molar ratios of NaOCl to monomer units were used over an incubation period of 4 hours: 1:5 (a), 1:1 (b) and 5:1 (c).

results are given in Fig. 5. It is possible to differentiate between different classes of components. Whereas glucose and glucuronic acid show only a very small consumption for NaOCl (about 5% during 1 hour), N-acetylglucosamine and N-acetylgalactosamine react more vigorously with NaOCl under these experimental conditions. They consume about 80% of the offered NaOCl. If solutions of polymers are compared with each other, a rather complicated behaviour is found. Whereas heparin shows only low reactivity against sodium hypochlorite, chondroitinsulphate is much more affected; hyaluronic acid shows a reactivity between heparin and chondroitinsulphate. This might be explained by the different degree of sulphatation in these polymers and their different molecular weights. Thus, heparin shows low reactivity, because it is the most sulphated polymer (containing two sulphate groups per repeating unit) conferring a very high negative charge density. The repulsion of the negatively charged $ClO^{\ominus}$ reduces its concentration at the polymer surface. Hyaluronic acid does not contain any $-SO_3^-$-groups but it has a very high molecular weight, leading to a low mobility of the chain, which decreases the probability that a NaOCl molecule hits a polymer chain. Although chondroitinsulphate contains one sulphate group per repeating unit it might react more vigorously than hyaluronic acid due to its lower molecular weight, which favours the reaction.

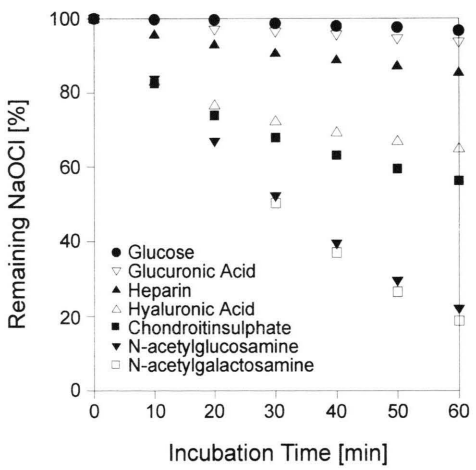


Fig. 5. A comparison of the reactivities of different monomeric and polymeric carbohydrates present in cartilage towards sodium hypochlorite. The carbohydrate concentration was 10^{-3} mol/l for monomeric substances. It was related to monomeric units for polymeric substances. At the beginning (t = 0 min) NaOCl was added in a 1:1 molar ratio. The decrease in NaOCl concentration was related to the starting value (100%) and recorded over an incubation period of one hour.

Discussion

Results of the present and previous paper (Schiller *et al.*, 1994) indicate that $HOCl/ClO^{\ominus}$ causes two main processes in degradation of monomeric and polymeric carbohydrates. At low concentrations of HOCl the N-acetyl side chains are predominately changed. They are depleted to acetate as the final product. An instable intermediate appears which is likely a chloramine of the N-acetyl groups. Here the ^1H NMR provides more informations to follow these reactions compared to ^{13}C NMR because of its higher sensitivity. The intermediate is either invisible or overlaps with other resonances in the carbon NMR spectra or it is completely depleted after the incubation period of 4 hours. A new resonance at 174.8 ppm could not be assigned. It can result from the quaternary carbon atom neighbouring to the $-N(Cl)$-group.

Higher amounts of $HOCl/ClO^{\ominus}$ lead to a complete breakdown of sugar rings with formiate as final product. Any evidence for a selective oxidation of functional groups at the C-1 and C-6 atom of sugar rings in N-acetylglucosamine, N-acetylgalactosamine and chondroitinsulphate could not be found. This result is in contrast to the action of $HOCl/ClO^{\ominus}$ on glucose. Here, a preferential oxidation of glucose to glucuronic acid occurs.

Our data from ^{13}C NMR show that formiate appears simultaneously to the disappearance of all resonances of the six carbon atoms of sugar rings in both, monomers and chondroitinsulphate.

Fig. 6 summarizes all pathways discussed in degradation of N-acetylglucosamine by hypochlorous acid. Pathway **1** (ring oxidation) and **4** (chlorination of N-acetyl side chains) are consistent with our NMR data.

The second pathway (**2**) concerns the possible oxidation of the $-CH_2OH$ group in monomers investigated and polymeric units of carbohydrates. ^{13}C-NMR data did not indicate this pathway. In contrast to this, glucose is converted to glucuronic acid by $HOCl/ClO^{\ominus}$. Whereas the $-CH_2OH$ group is presumably protected by the secondary

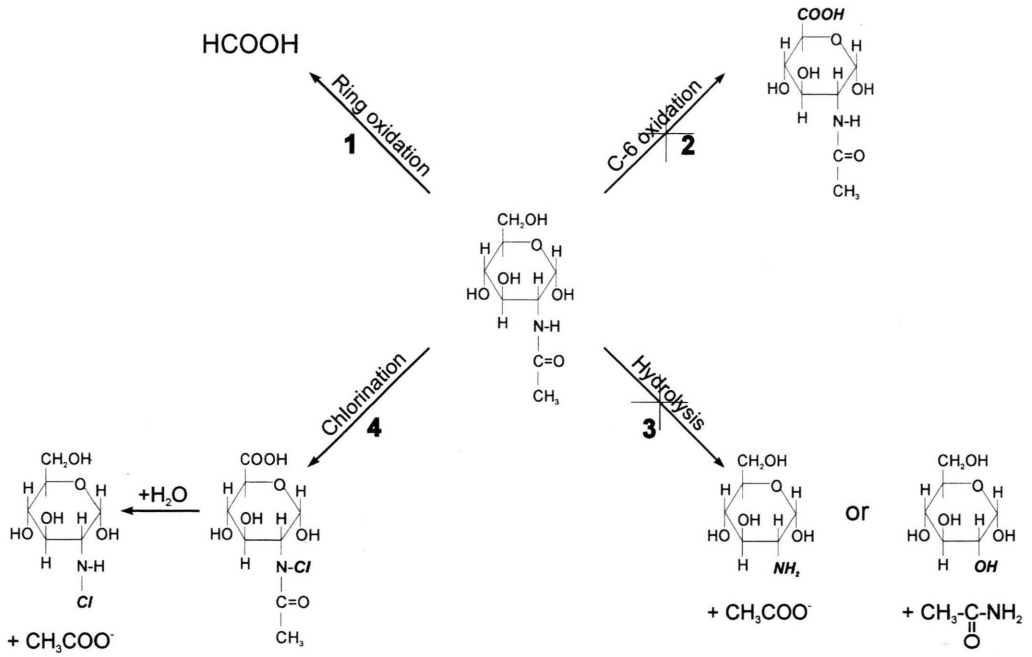


Fig. 6. Scheme of the different reaction pathways of N-acetylglucosamine with sodium hypochlorite. The different reaction sites and reaction products are emphasized in the figure.

structure in polysaccharides (Scott *et al.*, 1983) the absence of this pathway is not understood in N-acetylglucosamine.

A hydrolysis (**3**) of N-acetyl side chains of N-acetylglucosamine upon the action of sodium hypochlorite is also incompatible with results of ^{1}H- and ^{13}C-NMR spectroscopy. Either glucosamine and acetate or glucose and acetylamide should be expected.

Therefore, polysaccharides were degraded by $HOCl/ClO^{\ominus}$ mainly on their N-acetyl side chains. A progredient destruction of cartilage components occurs in inflamed joints in rheumatoid arthritis. An accumulation of large amounts of neutrophils (Zvaifler, 1973; Brown, 1988) was provided elevated values for myeloperoxidase activities (Edwards *et al.*, 1988; Nurcombe *et al.*, 1991) and increased amounts of acetate (Grootveld *et al.*, 1991; Parkes *et al.*, 1991) are other characteristic features of synovial fluids of patients with rheumatoid arthritis. In own experiments up to 2.3 units myeloperoxidase have been detected in 1 ml cell-free synovial fluids of such patients (Sonntag *et al.*, 1994) as determined by oxidation of guaiacol to tetrahydroguaiacol (Klebanoff *et al.*, 1984). By them one unit MPO was determined as utilization of 1 μmol H_2O_2 per minute. Over long periods sufficient high amounts of hypochlorous acid can be produced in inflamed joints.

Acknowledgements

This work was supported by the German Ministry of Research and Technology (Grant 01 ZZ 9103/9-R-6) and a grant of the Graduiertenkolleg (Molekular- und Zellbiologie des Bindegewebes, Universität Leipzig) for one of the authors (Jürgen Schiller) was provided by the Deutsche Forschungsgemeinschaft.

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