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IR and NMR Studies on the Action of Hypochlorous Acid on Chondroitin Sulfate and Taurine

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Although it has been recently shown by the use of ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy that enzymatically formed hypochlorous acid ($HOCl/Cl^{\ominus}$) reacts with carbohydrates containing *N*-acetyl groups under the formation of acetate and presumably chloramines, mechanistical aspects of this reaction remain unknown. Since NMR spectroscopy suffers a lot from low sensitivity especially toward polymeric components giving less marked resonances, we used here infrared (IR) spectroscopy to characterize the products of the reaction between hypochlorite and chondroitin sulfate. NMR is used for means of comparison. The reaction of $HOCl/Cl^{\ominus}$ with chondroitin sulfate does not change most regions of the IR spectrum of the polysaccharide markedly. Whereas most vibrational bands remain nearly unchanged toward their position and their relative intensities, an additional band at 975 cm^{-1} is clearly detectable. This band is in good accordance with the proposed formation of a N-Cl-bond in chondroitin sulfate upon HOCl treatment. Additionally, NMR experiments clearly indicate that chloramine formation is accompanied by the formation of acetate. For comparison, taurine (2-amino-1-ethanesulfonic acid) was also investigated toward its reactivity with $HOCl/Cl^{\ominus}$. An analogous band at 975 cm^{-1} was found which unequivocally indicates the formation of a chloramine. NMR also detects changes upon this reaction, but these changes cannot be exactly assigned to chloramine formation. Thus, we conclude that IR spectroscopy is most suitable for the detection of chloramines due to its relatively high sensitivity and the possibility to detect directly N-Cl-groups.

Key Words: hypochlorous acid; chondroitin sulfate; taurine; NMR spectroscopy; IR spectroscopy.

INTRODUCTION

An accumulation of large numbers of polymorphonuclear leukocytes (PMNs) occurs in synovial fluids of patients suffering from rheumatoid arthritis (*1*, *2*). It is a widely accepted fact that PMNs are involved in the damage of articular cartilage in chronic inflammatory diseases.

Neutrophils generate potent reactive oxygen species including $O_2^{\cdot -}$, H_2O_2, $HO^{\cdot}$, HOCl, and $^1O_2$ and release different enzymes (e.g., collagenase, elastase, and mye-

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loperoxidase) upon stimulation (*3*, *4*). Although the contribution of neutrophils to these pathologies is well established, detailed mechanisms of damage, regulations, and defense reactions remain widely unknown.

We have focused our recent interest mainly on neutrophil-derived hypochlorous acid due to its strongly oxidizing properties. HOCl is generated in a myeloperoxidase (MPO)-catalyzed reaction between hydrogen peroxide and chloride anions (*5*, *6*):

$$H_2O_2 + Cl^{\ominus} \rightarrow HOCl + HO^{\ominus}.$$

Myeloperoxidase is present in high concentration in azurophilic granules of neutrophils comprising about 5% of the total protein content in these cells (*7*). Thus, high amounts of HOCl are formed under *in vivo* conditions and concentrations up to 340 μM are discussed to be of physiological relevance (*8*).

The predominant role of myeloperoxidase and its product, hypochlorous acid, in cartilage degradation is clearly reflected by two different experimental results: On the one hand, neutrophils isolated from synovial fluids of patients with rheumatoid arthritis are characterized by a markedly increased native chemiluminescence (*9*), which correlates well with MPO activity, i.e., with HOCl concentration (*9*). On the other hand, cell-free synovial fluids from patients with rheumatoid arthritis show intense ^{1}H NMR signals arising from degradation products of cartilage (oligosaccharides with *N*-acetyl groups and acetate). The integral intensities of these resonances correlate extremely well with the myeloperoxidase activity (*10*).

Unfortunately, detailed studies on the reaction of cartilage polysaccharides with hypochlorous acid and on the detailed chemical mechanisms were only scarcely performed. For example, it has been shown by viscosimetry and HPLC (*11*) that a loss of viscosity of hyaluronic acid solutions is observed at relatively small amounts of hypochlorous acid, whereas higher concentrations lead to a cleavage of the polysaccharide chain under the formation of low-molecular-mass breakdown products. Both effects indicate that complex reaction patterns are involved in these oxidation processes (*11*).

Additionally, the molecular mechanism of the involved reactions has been studied using NMR spectroscopy (*12*, *13*). HOCl clearly damages *N*-acetylglucosamine as well as chondroitin sulfate and hyaluronic acid *in vitro*. Two different effects have been observed: a breakdown of large polymers, such as hyaluronic acid, into smaller oligomeric units, and a simultaneous reaction of HOCl with *N*-acetyl side groups of these carbohydrates to yield acetate as final product via a transient chlorinated product (*12*). Only in the presence of extremely high concentrations of hypochlorous acid are further oxidation products like formate detectable (*13*). On the basis of these investigations, the following model has been proposed (Fig. 1). Carbohydrates with *N*-acetyl side chains are chlorinated in a first step at the N-H-amide group. This transient product is hydrolyzed in a second step to the corresponding chloramine and acetate (*12*, *13*).

Although this mechanism is consistent with experimental results, two important drawbacks of NMR spectroscopy must be considered: First, NMR is not suitable for detection directly of the presence of chlorinated products, and NMR spectroscopy suffers a lot from its relatively low sensitivity. Unfortunately, other spectro-

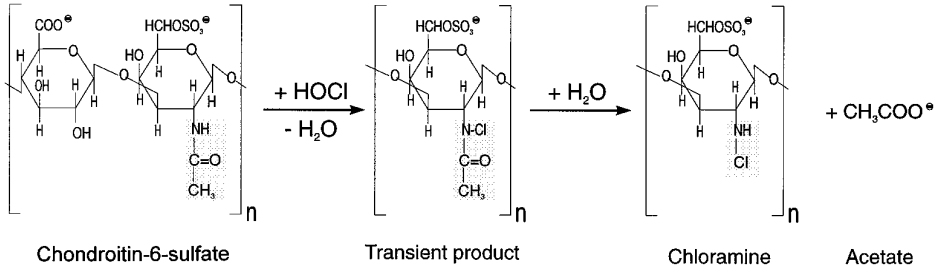

FIG. 1. Proposed mechanism of the reaction between chondroitin sulfate and hypochlorous acid under the formation of a transient product, which is gradually depleted to a chloramine and acetate. Groups of interest are emphasized.

scopic methods such as the detection of chloramines by their strong UV absorption at about 254 nm (*14*) are influenced by the absorption of the carbohydrates (*14*).

On the other hand, infrared spectroscopy should provide a considerably higher sensitivity compared to NMR and should clearly monitor the formation of chloramines due to its high sensitivity toward strongly polar functional groups (*15*).

The present paper deals with the examination of pathways of the reaction of hypochlorite with N-acetylated polysaccharides. These experiments are compared with the reactions of taurine (2-amino-1-ethanesulfonic acid). Taurine, as a simple amino-group-containing component, is well characterized toward its ability to form chloramines (*16*). It is shown that the performed experiments are in good agreement with the previously postulated pathway and that IR spectroscopy is most suitable for the detection of chloramines.

MATERIALS AND METHODS

Chemicals

All chemicals (NaCl, Na_2HPO_4, KH_2PO_4, chondroitin sulfate from bovine trachea, and taurine) were purchased from Fluka Feinchemikalien GmbH (Neu Ulm, Germany) of analytical grade and used without any further purification.

Sodium hypochlorite (as source for HOCl) was from Sigma Chemie (Deisenhofen, Germany). A stock solution of NaOCl was prepared in water. Its concentration was determined spectrophotometrically at pH 12 (ε_{290} = 350 liters mol^{-1} cm^{-1}) each day prior to use (*17*).

Incubation Conditions

All solutions were freshly prepared in 50 mmol/liter phosphate buffer, pH 7.4. Under these conditions, HOCl solutions consist of a nearly 1 : 1 mixture of the acid (HOCl) and the corresponding salt (p*K* 7.53) (*17*). Taurine was used just like the chondroitin sulfate as a 50 mmol/liter solution. In the case of chondroitin sulfate

the molecular mass of one repeating unit (sodium glucuronate and monosulfated *N*-acetylgalactosamine) was used to calculate the molar concentrations (*13*) of the polysaccharide.

Solutions were subsequently incubated in the presence of a 1:1 molar ratio of hypochlorous acid for 2 h at 37°C in a water bath. It has been previously shown by time-dependent NMR spectroscopic investigations that an incubation of 2 h gives most expressed changes (*12*). After this period 400 μl of incubation solutions was used for NMR spectroscopy and the remaining solution was concentrated in a vacuum centrifuge (Jouan, Germany) for infrared spectroscopy.

UV Measurements

UV measurements were performed to obtain information on the extent of chloramine formation ($\lambda \sim 250$–255 nm) (*14*) and the residual content of $HOCl/ClO^{\ominus}$ ($\lambda = 290$ nm) (*17*). These measurements were performed on an Hitachi U-2000 photometer, using quartz cuvettes.

NMR Measurements

NMR measurements were conducted on a Bruker AMX-300 spectrometer operating at 300.13 MHz for ^1H. All spectra were recorded at ambient temperature (25°C). These conditions were the best compromise between enhanced resolution at higher temperature and diminished denaturation of carbohydrates at lower temperature.

Typically 0.40 ml 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 was suppressed by the application of a 2-s presaturation pulse at the water resonance frequency (*10*).

Thirty-two free induction decays were usually accumulated with a pulse delay of 8 s between two pulses to allow full T_1 relaxation. No line broadening or Gauss broadening was used. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-propane-1-sulfonate (TSP) added in a final concentration of 5 mmol/liter.

Infrared Spectra

Concentrated solutions from incubation experiments with hypochlorous acid were spread on one side of a ZnSe attenuated total reflection (ATR) crystal which was mounted on a commercially available horizontal Benchmark unit (Specac, UK). Infrared spectra were measured using a Bio-Rad FTS-60a Fourier-transform infrared spectrometer equipped with a deuterated triglycine detector. Typically, 128 scans were accumulated. Absorbance spectra of the sample were calculated using the respective single-channel spectra of the empty ATR crystal (without sample) as background. Selected absorption bands are characterized in terms of the wavenumber at maximum absorbance (*18*).

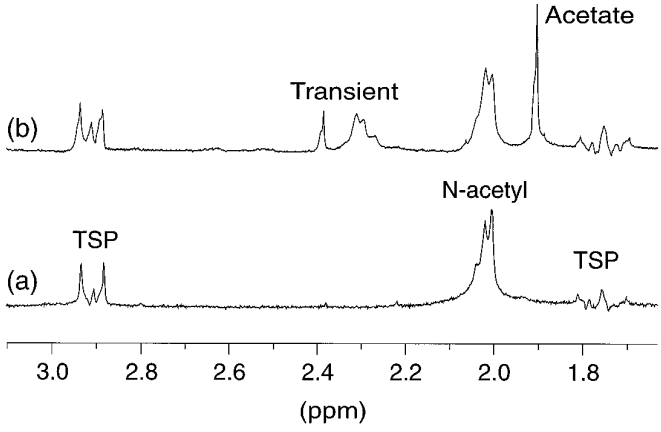

FIG. 2. Proton–NMR spectrum of an aqueous solution of chondroitin sulfate prior (a) and after (b) treatment with an equimolar quantity of sodium hypochlorite (b). The weak resonances at 1.76 and 2.91 ppm result from the standard sodium 3-(trimethylsilyl)-propane-1-sulfonate. Abbreviations used in peak assignment: TSP, standard; *N*-acetyl, mobile *N*-acetyl groups.

RESULTS

Chondroitin Sulfate and Hypochlorous Acid

Since NMR spectroscopy is a powerful tool for the detection of molecular changes in aqueous solutions, chondroitin sulfate samples were analyzed by NMR spectroscopy in the presence (1 : 1 molar ratio) or absence of HOCl (Fig. 2).

The ^{1}H NMR spectrum of an aqueous solution of chondroitin sulfate prior to the addition of hypochlorous acid is given in Fig. 2a. With the exception of the resonances of the TSP standard (1.76 and 2.91 ppm), only a single, broad resonance with two maxima at 2.005 and 2.020 ppm can be detected (*12*). The two maxima are most likely attributable to the isomeric forms of the polysaccharide, chondroitin 4- and chondroitin 6-sulfate. Both isomers are present in nearly equal amounts in commercially available chondroitin sulfate (*19*) resulting in two resonances of comparable intensity for the protons of the *N*-acetyl side group. These resonances are much broader than observed for the monomers, e.g., *N*-acetylglucosamine, because of the high molecular weight of the polymer conferring restricted mobility of the protons in the sample (*12*). Since the *N*-acetyl side chain is by far the most mobile functional group of chondroitin sulfate, C-H protons of carbohydrate rings are only scarcely detectable between 3.3 and 4.0 ppm (data not shown).

The remaining O-H protons from the sugar ring and the N-H proton from the *N*-acetyl side chain are quickly exchanged with the solvent (a H_2O/D_2O mixture) and are not detectable under our experimental conditions using presaturation of the water resonance. Although the use of deuterated dimethyl sulfoxide (*20*) is useful, because it hinders the protons from exchanging, it is not the solvent of choice due to its unphysiological properties.

The action of sodium hypochlorite leads to pronounced changes in the ^{1}H NMR spectrum of chondroitin sulfate (Fig. 2b). The intensity of the signal at 2.005–2.020 ppm for the *N*-acetyl group is slightly diminished in comparison with the reference chondroitin sulfate sample (Fig. 2a). The reduction of this resonance is accompanied by the formation of an additional, well-resolved resonance at 1.90 ppm, which corresponds to acetate. The nature of this resonance was confirmed by the addition of a small amount of sodium acetate. An enhancement of the resonance at 1.90 ppm clearly proved the presence of acetate (*21*).

Additionally, a broad signal appears at about 2.35 ppm. We assume that this resonance is caused by the chlorination of the *N*-acetyl side chains of the polymer as a transient reaction product. The existence of various chlorinated side chains in the polymer results in different chemical shifts of the reaction product in the ^{1}H NMR spectrum. All these signals interfere with each other leading to a broad NMR signal. The resonance at 2.35 ppm obviously contributes to the methyl group of the transient product since its integral intensity is too high for a single proton of the carbohydrate ring. Additionally, protons of the carbohydrate ring should be strongly coupled with each other and, thus, result in (at least) doublets instead of singlets.

On the other hand, carbohydrate rings are not affected upon the action of NaOCl, since only small amounts of formate (8.44 ppm) were detectable under these experimental conditions (data not shown). Formate is a well-known final product of degradation of carbohydrates (*12*, *13*, *22*, *23*).

Unfortunately, a direct proof that chloramines are really formed upon the action of hypochlorous acid on chondroitin sulfate is not provided by the NMR experiments, since NMR is not able to detect directly N-Cl-groups. NMR is only capable of detecting changes in the chemical shift of characteristic resonances (e.g., the *N*-acetyl group), which may indicate chlorination.

On the other hand, IR spectroscopy should detect most sensitively such highly polar functional groups even in polymeric components. The molecular mass and consequently the reduced mass ($m_1m_2/m_1 + m_2$) of a N-Cl-group are about 10 times higher than the masses of the other remaining functional groups like -OH or -NH. Thus, the chloramine band is expected to be shifted to much lower wave numbers in comparison to the bands of the residual functional groups of chondroitin sulfate (*15*, *24*, *25*).

Figure 3 shows the FT-IR spectra of an aqueous chondroitin sulfate solution without addition (solid line) and after addition of an equimolar ratio of NaOCl (dashed line). Other spectral regions are not influenced by the addition of hypochlorite and have been omitted for clarity. Assignments of individual vibration bands were performed according to (*26*, *27*).

Whereas there are only very slight changes toward the position and the intensities of the valence vibration of the carbohydrate ring at 1100 cm^{-1} [ν (C-C)] and the valence vibration of the sulfate residue [ν (S-O)] in chondroitin sulfate at about 1200 cm^{-1}, an additional band is clearly detectable at 975 cm^{-1} upon the addition of HOCl. This band is most likely attributable to the presence of a N-Cl-bond which is formed by the action of hypochlorous acid on the *N*-acetyl side chain of chondroitin sulfate. The transient product as well as the chloramine as final product may contribute to this resonance.

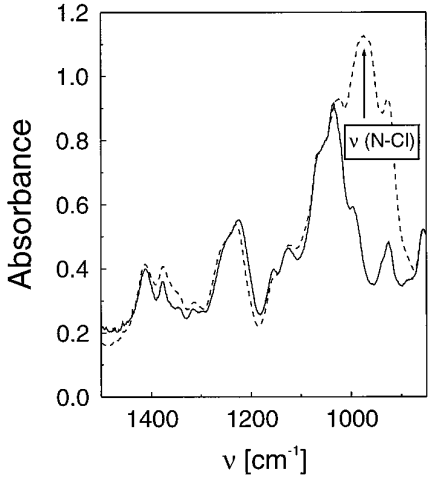

FIG. 3. FT-IR spectrum of an aqueous solution of chondroitin sulfate prior (solid line) and after (dashed line) treatment with an equimolar quantity of sodium hypochlorite.

Indeed, also remaining hypochlorous acid might cause this band because the masses of the -O-Cl-group and -N-Cl-group are very similar. However, it has been shown that HOCl is completely consumed by the reaction with the polysaccharide under our experimental conditions. Therefore, the O-Cl-band cannot contribute to the observed band at 975 cm^{-1} (*13*).

Taurine and Hypochlorous Acid

Unfortunately, carbohydrates and especially polysaccharides are relatively complex molecules and it might be possible that the observed changes in IR spectra may also be caused by other reaction pathways and not absolutely necessary by a chlorination of the *N*-acetyl side chain. Thus, analogous experiments were also performed using a more simple molecule. Taurine (2-amino-1-ethanesulfonic acid) was used because it occurs in neutrophilic granulocytes in high concentrations [about 22 mmol/liter (*14*)] and is, thus, of physiological relevance. Additionally, it is a very simple molecule and possesses with its amino group only one reactive group toward sodium hypochlorite. Interpretations of changes are, thus, much easier since it exhibits only a very simple IR spectrum. An exhaustive description of the vibrational spectrum of taurine is given in (*28*).

In Fig. 4 the FT-IR spectrum (a) and the corresponding UV spectrum (b) of taurine upon treatment with an equimolar ratio of sodium hypochlorite is shown. Analogous changes as observed upon HOCl treatment of chondroitin sulfate are detectable. Whereas the ν (C-C) valence vibration of the ethylene group and the ν (S-O) valence vibration of the sulfate residue in taurine are not influenced by HOCl, an additional vibration at 975 cm^{-1} is clearly detectable.

Since it has been unequivocally shown in the past that taurine is affected under our

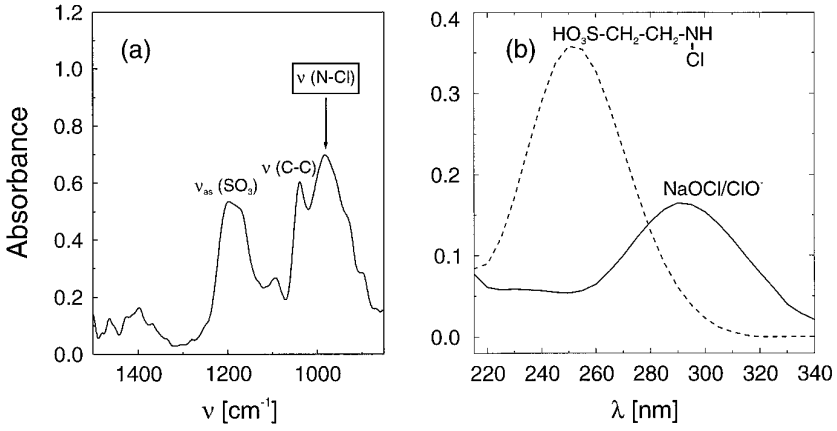

FIG. 4. FT-IR (a) and UV spectrum (b) of an aqueous solution of taurine after treatment with an equimolar quantity of sodium hypochlorite. Functional groups of interest are emphasized in (a). In (b) the dashed line corresponds to the UV spectrum of taurinchloramine, whereas the solid line represents a pure HOCl solution under the same experimental conditions.

experimental conditions by hypochlorous acid under the formation of chlorotaurine (*14*), we conclude that the band at 975 cm^{-1} is caused by the formation of the N-Cl group in chlorotaurine. In accordance with the IR spectrum, the corresponding UV spectrum in Fig. 4b shows a complete consumption of hypochlorous acid which would give a strong absorption at 290 nm (solid line). The loss of the HOCl absorption is accompanied by the formation (dashed line) of an intense absorption band centered at 251.8 nm which clearly contributes to clorotaurine formation (*14*). Taurine itself does not give a significant absorption in the UV range under investigation.

Whereas such strong effects upon the reaction of taurine with hypochlorous acid can be detected by IR spectroscopy, the question arises whether NMR spectroscopy can also be used for monitoring this reaction. Figure 5 shows the corresponding NMR spectra recorded upon the reaction of taurine (50 mmol/liter) with different concentrations of sodium hypochlorite (Fig. 5a, pure taurine; Fig. 5b, 25 mmol/liter NaOCl; Fig. 5c, 50 mmol/liter NaOCl).

It is clearly evident from Fig. 5 that upon the treatment of taurine with hypochlorous acid only very small changes in the corresponding NMR spectrum can be observed. An aqueous solution of taurine exhibits two well-resolved resonances at 3.41 and 3.25 ppm, which are split into two triplets (^3J = 6.5 Hz) caused by the coupling of the protons of both methylene groups (*28*). Unfortunately, formation of chlorotaurine, which is clearly visible in the IR and UV spectra, cannot be detected by NMR.

Using moderate concentrations of NaOCl, the resonances of remaining taurine overlap with the resonances of the chlorotaurine (Fig. 5b) since the obtained differences in chemical shift are only very small. This becomes more evident if an equimo-

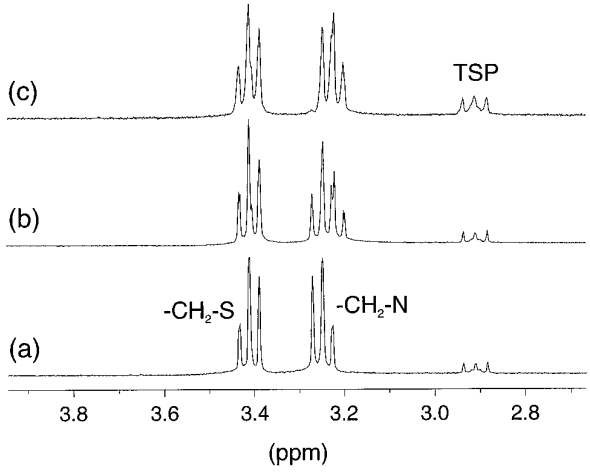

FIG. 5. Proton NMR spectrum of an aqueous solution (50 mmol/liter) of taurine (a) and after treatment with increasing amounts of sodium hypochlorite. The following concentrations of NaOCl have been used: (a) none, (b) 25 mmol/liter, and (c) 50 mmol/liter. The weak resonance at 2.91 ppm is due to the standard sodium 3-(trimethylsilyl)-propane-1-sulfonate (TSP).

lar ratio between HOCl and taurine is used (Fig. 5c). Whereas the resonance of the -CH_2-S-group at 3.41 ppm remains unchanged, the replacement of the proton in the NH_2-group with a chlorine atom changes the position of the protons of the CH_2-N-group only very slightly. This resonance is only shifted for about 0.03 ppm to 3.22 ppm in comparison with pure taurine (δ = 3.25 ppm).

This clearly indicates that by means of NMR it cannot be determined with sufficient certainty that a reaction has taken place under these experimental conditions.

DISCUSSION

Hypochlorous acid plays a prominent role in cartilage degradation in the course of rheumatic diseases, affecting mainly the polysaccharides of cartilage (*10*, *29*). Whereas characteristic breakdown products like acetate or formate can be detected by means of NMR spectroscopy in supernatants of cartilage samples as well as in pathologically changed synovial fluids (*10*, *22*, *29*), for the detection of chlorinated products NMR is not the method of choice. On the other hand, ^{13}C and ^{15}N NMR spectroscopies are still by far less sensitive than proton NMR spectroscopy and cannot be used in a physiologically relevant concentration range.

Chlorinated products are assumed to be formed upon the action of enzymatically formed HOCl on polysaccharides of cartilage (Fig. 1) (*13*). In the present study it is shown that the formation of chloramines can be easily monitored by FT-IR

spectroscopy since the formation of the nitrogen–chlorine bond is accompanied by an intense absorption band at 975 cm^{-1}.

Taurine is used for means of comparison since it is known to give only a sole reaction product with HOCl, whereas carbohydrates are additionally cleaved at the glycosidic linkage under the formation of formate.

The relatively high sensitivity of the IR spectroscopy in comparison to the NMR spectroscopy makes it a reliable tool for the detection of chloramine formation, despite some experimental problems arising from the presence of the intense water absorption which can, however, be overcome by the use of the attenuated total reflection (ATR) technique (*18*).

As shown by the use of taurine and the physiologically relevant polysaccharide chondroitin sulfate, this technique can be used for the analysis of reaction products of different classes of chemical substances. Especially for the investigation of polymers, IR is more suitable than NMR because NMR signals of polymers are dominated by broad, less intense resonances. Additionally, no expansive labeling with isotopes as required for recording ^{13}C NMR spectra under physiologically relevant conditions in needed for recording IR spectra (*29*).

Finally, in the present investigation we have shown for the first time that not only amines, but also carbohydrates with *N*-acetyl side chains are converted by hypochlorous acid to the corresponding chloramines, which may play an important role under *in vivo* conditions.

In conclusion, we propose for further investigations the use of a combination of NMR spectroscopy and FT-IR spectroscopy to obtain more detailed information on cartilage degradation processes and chloramine formation under physiologically relevant conditions.

However, there is still some work required to answer the most important question of to what extent chlorinated products are involved in inflammatory processes. Additionally, the question of what method is most suitable for the detection of characteristic products in cartilage degradation still must be answered.

ACKNOWLEDGMENTS

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