Reactive oxygen species produced by activated neutrophils and monocytes are thought to be involved in mediating the loss of collagen and other matrix proteins at sites of inflammation. To evaluate their potential to oxidize the pyridinoline (Pyd) cross-links found in collagen types I and II, we reacted hydrogen peroxide (H$_2$O$_2$), hypochlorous acid/hypochlorite (HOCl/OCl$^-$), and singlet oxygen (O$_2$(1$\Delta$g)) with the Pyd substitutes, pyridoxamine dihydrochloride and vitamin B$_6$, which share the same chemical structure and spectral properties of Pyd cross-links. Neither H$_2$O$_2$ (125–500 $\mu$M) nor O$_2$(1$\Delta$g) (10–25 $\mu$M) significantly changed the spectral properties of pyridoxamine or vitamin B$_6$. Reaction of HOCl/OCl$^-$ (12.5–50 $\mu$M) with pyridoxamine at pH 7.2 resulted in a concentration-dependent appearance of two new absorbance peaks and a decrease in fluorescence at 400 nm (excitation 325 nm). The new absorbance peaks correlated with the formation of an N-chloramine and the product of its subsequent reaction with pyridoxamine. In contrast, the extent to which HOCl reacted with vitamin B$_6$ which lacks a primary amine group, was variable at this pH. At lysosomal pH 5.5, Cl$_2$/HOCl/OCl$^-$ reacted with both pyridoxamine and vitamin B$_6$. Four of the chlorinated products of this reaction were identified by gas chromatography-mass spectrometry and included 3-chloropyridinium, an aldehyde, and several chlorinated products with disrupted rings. To evaluate the effects of Cl$_2$/HOCl/OCl$^-$ on Pyd cross-links in collagen, we exposed bone collagen type I and articular cartilage type II to HOCl. Treatment of either collagen type with HOCl at pH 5.0 or 7.2 resulted in the oxidation of amine groups and, for collagen type II, the specific decrease in Pyd cross-link fluorescence, suggesting that during inflammation both oxidations may be used by neutrophils and monocytes to promote the loss of matrix integrity.

Inflammation associated with articular cartilage, bone, and dentin surfaces is characterized by accumulation, adhesion, and activation of neutrophils and monocytes, which results in the destruction of cartilage and the loss of bone at these sites (1–4). These effects are thought to be mediated in part by the production of reactive oxygen species (ROS),$^1$ a group of reactants that includes superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the highly reactive species hypochlorous acid (HOCl) (5–8). ROS are produced by neutrophils and monocytes after they are recruited from the circulation to extravascular spaces. Once outside of the circulation, they adhere to extracellular matrix proteins (ECM) and undergo activation, which results in the production of ROS and release of proteolytic enzymes directly onto the matrix surface (4, 9–13). In inflammation associated with arthritic joints, the accumulation and activation of neutrophils and monocytes and increased synovial cell formation result in the loss of synovial membrane integrity and eventually to irreversible damage and destruction of articular cartilage in the afflicted joints (1, 4). Similarly, in periodontitis or inflammation associated with the tooth-supporting periodontal ligament, the recruitment of neutrophils and monocytes from the circulation and the subsequent activation and release of ROS and proteolytic enzymes can eventually result in a significant and irreversible loss of underlying bone matrix at these sites (2, 3). Despite the known participation of ROS in the inflammatory-mediated loss of underlying ECM, the mechanism(s) by which this occurs is not completely understood. This is particularly true for collagen, which is the major ECM protein in cartilage and bone.

Neutrophils and monocytes contain two enzymes that are responsible for producing ROS. The first is NADPH oxidase, which catalyzes the formation of O$_2^-$ by the transfer of electrons from NADPH to oxygen via cytochrome b$_558$ (14–16). Superoxide is rapidly dismutated to H$_2$O$_2$ either spontaneously or via the enzyme superoxide dismutase (17). Neither O$_2^-$ nor H$_2$O$_2$ exhibit significant reactivity with biologic compounds (18). The second enzyme is myeloperoxidase (MPO), which catalyzes the formation of HOCl from H$_2$O$_2$ and Cl$^-$ (19). MPO has been localized to the primary granules of resting neutrophils and the extracellular space and phagolysosomes of phagocytically stimulated neutrophils (20, 21). MPO is also released from cytoplasmic granules of monocytes and some macrophages (22). The cationic nature of MPO allows it to adhere to cell and matrix surfaces and localize to sites of inflammation (23). It is at these sites that MPO produces HOCl, a highly reactive oxidant that readily reacts with primary amines to generate long lived

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$^1$ The abbreviations used are: ROS, reactive oxygen species; O$_2^-$, superoxide anion; H$_2$O$_2$, hydrogen peroxide; OCl$^-$, hypochlorite/hypochlorous acid; O$_2$(1$\Delta$g), singlet oxygen; MPO, myeloperoxidase; ECM, extracellular matrix; Pyd, pyridinoline cross-links; ECM, extracellular matrix; AAF, anthracene-9,10-dipropionic acid; RT, room temperature; DNE, 1,4-dimethyl-1,4-naphthale endoperoxide; PAG, polyacrylamide gel electrophoresis; GC-MS, gas chromatography-mass spectrometry; chloramime-T, N-chloro-p-toluene-sulfonamide sodium salt; BSA, bovine serum albumin.
N-chloramines (24–26). Although N-chloramines exhibit a lower oxidizing potential than HOCl, their much longer effective lifetime (~18 h) would enable them to cause damage at more distant sites than HOCl (26). Under acidic conditions, similar to the environment found in phagolysosomes, MPO generates Cl₂ (27). MPO is also one of the pathways by which neutrophils generate OCl⁻ (Δg) (28).

Determining the ability of HOCl to contribute to the pathogenesis of inflammatory processes associated with rheumatoid arthritis and periodontitis is highly dependent on determining the relevant target(s) at these sites. The most likely protein target for neutrophil oxidants, including N-chloramines, is the ECM. ECM components found in or associated with articular cartilage or bone include hyaluronate, proteoglycans, fibronecin, several tissue-specific and nonspecific protein components, and collagen, the major component of these tissues. It has been reported that neutrophil-mediated ROS mediate the degradation of hyaluronate (29), modify proteoglycan structure and/or synthesis, and alter the structure of fibronecin (29–31). Vissers and Winterbourn (32) reported an increase in proteolytic degradation of glomerular basement membrane collagen by elastase in response to myeloperoxidase/HOCl/Cl⁻. Davies et al. (33) reported that >1.0 mM HOCl was required to cause extensive fragmentation of collagen type II isolated from bovine articular cartilage or collagen type I isolated from bovine tendon. Davies et al. (33) also reported that N-chloramines did not cause direct fragmentation but greatly increased the degradation of collagen by collagenase and elastase. In general, there are very few studies available as to the susceptibility of collagen to oxidation by HOCl.

An important determinant for stability of the ECM is the degree of cross-linking. One important type of cross-link is pyridoline (Pyd), which was first described by Fujimoto et al. (34) and later confirmed to be a non-reducible intermolecular cross-link of mature fibrillar collagen type I of bone (35). These cross-links were also found to be especially abundant in mature fibrillar collagen type II of articular cartilage (36), where they covalently link collagen type II to other type II helical regions, collagen type IX to the surface of type II, and bind collagen type IX to other molecules of collagen type IX. Their function is to stabilize the collagen fibrillar superstructure, or arrays, and make them more resistant to collagenolysis or proteolytic degradation (37).

The present study focuses on the susceptibility of Pyd cross-links of collagen to reaction with HOCl. Pyd cross-links were chosen as potential oxidation targets because of their importance in maintaining the collagen superstructure and because their chemical structure suggested they would be targets of oxidative modification by OCl⁻/Cl₂. Our findings indicate that these cross-links react with OCl⁻, Cl₂, N-chloramines, and suggest that Pyd would be a site for ROS modification of collagen type I and II in bone and cartilage, respectively.

**Experimental Procedures**

Pepsin-solubilized collagen types I and II isolated from human bone and articular cartilage, respectively, were kindly provided by Dr. David Eyre (University of Washington, Seattle). Pyridoxamine dihydrochloride, pyridoxine hydrochloride, pyridoxine hydrochloride, 30% hydrogen peroxide, chloramines-T, 1,4-dimethylaminophenale, methylene blue, and all other salts and buffer components were purchased from Sigma. Glacial acetic acid and dichromethane were purchased from Fisher. Potassium iodide was purchased from Acros Organics (Fairlawn, NJ). Disodium anthracene-dichloromethane were purchased from Fisher. Potassium iodide was purchased from Molecular Probes (Eugene, OR). Sodium hypochlorite (NaOCl) (4–5% available chlorine) was purchased from Sigma, Acros Organics, Fisher, Alfa (Ward Hill, MA), and Cole-Palmer (Vernon Hills, IL); see comments about NaOCl below.

**Preparation of Pyridoxamine and Vitamin B₆**—10 mM stock solutions of pyridoxamine dihydrochloride (pyridoxamine) and pyridoxine hydrochloride (vitamin B₆) were made fresh weekly and stored at 4 °C with protection from light. Stock solutions were made in one of the following buffers: 0.5 mM glacial acetic acid buffer, pH 3, or 0.5 mM sodium phosphate buffer, pH 5.5 ± 0.2. pH 7.2 ± 0.2, or pH 8.0 ± 0.2. Where appropriate, 0.1 M NaCl was added to the buffers as a source of chloride ions.

**Chlorination Control and Reactive Spectrum with Pyridoxamine and Vitamin B₆**—On the day of use, stock solutions of pyridoxamine and vitamin B₆ were diluted in appropriate buffer to 20 μM. 7.5 mM of the 20 μM solutions of pyridoxamine or vitamin B₆ were then added to control and treated sample tubes so the final concentration of pyridoxamine or vitamin B₆ in all samples was 15 μM in a total volume of 10 ml. A 50 mM solution of NaOCl was brought to a total volume of 10 ml by the addition of 2.5 ml of appropriate buffer to control samples or 1.25 ml to hydrogen peroxide (H₂O₂) and sodium hypochlorite (NaOCl) samples. No buffer was added to samples treated with the combination of H₂O₂ and NaOCl (H₂O₂/NaOCl). H₂O₂ and NaOCl treatment solutions were prepared by diluting the stock solution of H₂O₂ to 1, 2, or 4 mM and diluting the stock solution of NaOCl to 0.1, 0.2, or 0.4 mM in appropriate buffer immediately before use. 1.25 ml of a diluted solution of H₂O₂ or NaOCl or 1.25 ml each of diluted solutions of H₂O₂ and NaOCl were added to appropriate tubes. The final concentrations of H₂O₂ were 125, 250, or 500 μM, and the final concentrations of NaOCl were 12.5, 25, or 50 μM. H₂O₂ was always added first to either the H₂O₂ or H₂O₂/NaOCl samples, and NaOCl was added immediately after, where appropriate. Samples were mixed after each addition. At pH 12, the NaOCl stock solution exists predominantly as the conjugate base, hypochlorite (OCl⁻) (38–40). Therefore, caution should be exercised when using NaOCl, since we found that even new solutions made by different companies showed signs of decomposition.

**Absorbance and Fluorescence**—After the addition of buffer, H₂O₂, NaOCl, or H₂O₂/NaOCl control and treated samples were incubated for 15 min at RT (approximately 25 °C) in a Precision Scientific Low Temperature Incubator 815 (Chicago, IL). At the end of each incubation, the UV absorbance of a 50-μl aliquot of each sample was scanned from 400 to 200 nm in a Beckman DU-640 Spectrophotometer (Fullerton, CA), and the fluorescence intensity of a 3-ml aliquot of each sample was read in a Perkin Elmer Life Sciences Spectrofluorimeter. Optimal fluorescence excitation and wavelengths were determined by referring to UV absorbance peaks and by prescanning samples for maximal excitation and peaks. The known excitation and peak wavelengths for both pyridoxamine and vitamin B₆ are 324 nm excitation and 400 nm emission. A 200-μl aliquot of each control and treated sample was also stored at 4 °C after incubation for later N-chloramine analysis.

**N-Chloramine Assay**—The presence of N-chloramines was determined by the method of Witko et al. (41). This method is based on the colorimetric measurement of triiodide ions formed by the oxidation of potassium iodide (KI) in solution. Chloramine-T (N-chloro-p-toluene-sulfonylamide sodium salt), a commercially available source of N-chloramine, was used to calibrate the assay. A 100 μM stock solution of chloramine-T was made fresh weekly in distilled H₂O and stored at 4 °C with protection from light. The 100 mM chloramine-T solution was then diluted in appropriate buffer to final concentrations of 25, 50, 75, or 100 μM immediately before use. We extended the RT incubation from 2 (41) to 5 min and found no significant difference in results. The direct oxidation of KI by H₂O₂, NaOCl, or H₂O₂/NaOCl was also determined, and these values were subtracted as background from the correspondingly treated samples. The resulting difference represented the amount of N-chloramine present in each sample.

**Chloramine-T Reactivity**—On the day of use, 10 mM stock solutions of pyridoxamine or vitamin B₆ were diluted to a concentration of 1 mM, and a 100 mM stock solution of chloramine-T was diluted to final concentrations of 25, 50, 75, or 100 μM in appropriate buffer. Control and chloramine-T-treated samples contained 15 μl of the diluted 1 mM pyridoxamine or vitamin B₆ solution plus 1 ml of appropriate buffer (control) or 1 ml of 25, 50, 75, or 100 μM chloramine-T solution. The final concentration of pyridoxamine or vitamin B₆ for all control and chloramine-T-treated samples was 15 μM. Following preparation, samples were incubated for 15 min at RT. At the end of each incubation, the UV absorbance of each sample was scanned from 400 to 200 nm to look for any changes in the absorbance of pyridoxamine or vitamin B₆ due to a
reaction with chloramine-T and to identify the absorbance peaks for N-chloramines and pyridoxine-chloramine or vitamin B6-chloramine reaction products. In addition, the N-chloramine assay of Witko et al. (41) was performed to determine if a reaction of chloramine-T with pyridoxamine or vitamin B6 had occurred as indicated by a decrease in the absorbance of chloramine-T available and reacted.

1,4-Dimethyl-1,4-naphthalene Endoperoxide (DNE) Synthesis and Release of O2(1 D)—DNE, a pure chemical source of O2(1 D) that thermally releases O2(1 D) at 37 °C, was synthesized by the method of Wasserman and Larsen (42, 43). A duplicate set of coverslips was coated with either dichloromethane or a solution of DNE in dichloromethane (3.6 mg/100 μl) by surface evaporation at 4 °C. 200 μl of either a 15 μM pyridoxamine or vitamin B6 solution in appropriate buffer was added to a duplicate set of dichloromethane- (control) and DNE-coated coverslips. One set of control and DNE-coated coverslips was incubated overnight at 4 °C and another at 37 °C. The release of O2(1 D) from DNE was confirmed after overnight incubation at 37 °C by following the decrease in absorbance at 400 nm of anthracene-9,10-dipropionic acid (AAP) (1 × 10–5 M) in 0.5 mM sodium phosphate buffer, pH 7.2, according to the method of Deby-Dupont et al. (44).

Gas Chromatography-Mass Spectrometry—Gas chromatography-mass spectrometry (GC-MS) using electron ionization-mass spectrometry were performed by M-Scan, Inc., West Chester, PA. In brief, reagent samples containing vitamin B6 alone or vitamin B6 in a 1:1 ratio with pyridoxamine, dissolved in 95% (v/v) formamide, and sialylated derivatives prepared by the addition of 100 μl of N,O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (Supelco, PA 16823) followed by heating to 35 °C for 5 min. Derivatized products were concentrated to ~50 μl under anhydrous N2 and analyzed on capillary column (PerkinElmer Life Sciences PE-5MS, 30 m × 0.25 mm × 25 μm) by GC-MS (PerkinElmer Life Sciences Auto System XL, gas chromatograph with Turbomass Quadrupole mass spectrometer) in the positive electron ionization mode. Electron ionization-mass spectrometry was used to identify the structure of individual compounds in each GC peak. The source and interface temperatures were both 200 °C. The injector temperature was maintained at 280 °C, and the initial GC oven temperature was 70 °C for 2 min followed by an increase to 140 °C/min to 300 °C.

Preparation of Pepsin-solubilized Collagen—The lyophilized collagen samples were dissolved in 0.5 M acetic acid at a concentration of 1.2 mg/ml overnight at 4 °C with gentle stirring and protection from light. Samples were then dialyzed against 0.02 M acetic acid at 4 °C for 48 h using a dialysis cassette made by Pierce Slide-A-lyzer (Pierce) according to manufacturer's instructions. Both assays were performed on aliquots of each collagen sample, acetone precipitates of each sample, or sample supernatants of acetic precipitates after ROS and/or protease treatments. Triplicates of each sample (200 μl) were placed in a 96-well plate (CFCPN9610, Millipore Corp.; Bedford, MA), and the fluorescence was read at an excitation wavelength of 340 ± 20 nm and an emission wavelength of 400 ± 20 nm (Cytofluor 2350, Perceptive Biosystems, Inc.; Cambridge, MA).

RESULTS

UV Absorbance and Fluorescence Emission of Pyridoxamine and Vitamin B6—Pyridoxamine dihydrochloride (Fig. 1B) and vitamin B6 (Fig. 1C) share the pyridinium ring structure and spectral properties of the pyridoxil (Pyd) trifunctional cross-links (Fig. 1A) of collagens, including collagen types I, II, III, IX, and XI. The spectral properties of pyridoxamine, vitamin B6, and Pyd include their characteristic UV absorbance and excitation maximum at 325 nm, pH 7.2 (Fig. 1B). The absorbance characteristics of pyridoxamine and vitamin B6 include three peaks at 217–219, 251–252, and 321–325 nm. At pH 5.5, a hydrogen ion binds to the nitrogen group (52) of the pyridinium ring of vitamin B6, resulting in an additional peak at 292 nm (Fig. 1C).

Oxidative Modification of Pyridoxamine and Vitamin B6 after Exposure to the ROS, H2O2, HOCl/OCl(–), or O2(1 D)—To evaluate the oxidation of pyridoxamine or vitamin B6 by H2O2, HOCl/OCl(–) (which will be referred to as HOCI), or O2(1 D), a 15 μM solution of pyridoxamine or vitamin B6 dissolved in 0.5 mM sodium phosphate buffer, pH 7.2 ± 0.2, was exposed for 15 min at RT to 125, 250, or 500 μM of H2O2, 12.5, 25, or 50 μM of HOCI, or a combination of H2O2/HOCI in a ratio of 10:1. The absorbance characteristics of HOCI and H2O2 are within the predicted range generated by activated neutrophils or monocytes at sites.
of inflammation (6). Concentrations of H$_2$O$_2$ and HOCl in this range are possible within specialized microenvironments, such as phagocytic vacuoles or the neutrophil or macrophage attachment sites, because the aqueous volumes in these microenvironments are thought to be nanoliters or less, resulting in pH 6.0, peaking at pH 5.25) in the presence of Cl$^-$ (56), and acidification of HOCl (pH below 6.0) (56). No O$_2$(1$^g$) generated in the reaction of H$_2$O$_2$ with HOCl is pH-dependent (56), with the greatest amount of O$_2$(1$^g$) being produced at alkaline pH, intermediate amounts at neutral pH, and essentially non-measurable amounts at acidic pH due to assay limitations and interference by chlorine (Cl$_2$). No O$_2$(1$^g$) is produced by HOCl in the absence of Cl$^-$ or at pH < 4.2 or = 8.0 (55).

At pH 7.2, HOCl would exist in almost equal concentrations with OCl$^-$ (pK$_a$ = 7.4) (56), and acidification of HOCl (pH below 6.0, peaking at pH 5.25) in the presence of Cl$^-$ results in the evolution of chlorine (Cl$_2$), Reaction 3 (38, 54, 56).

\[
\text{HOCl} + \text{Cl}^- + \text{H}^+ \rightarrow \text{HOCl} + \text{Cl}_2 \quad \text{REACTION 3}
\]

The amount of O$_2$(1$^g$) generated in the reaction of H$_2$O$_2$ with HOCl is pH-dependent (56), with the greatest amount of O$_2$(1$^g$) occurring at pH 7.2 (56). The amount of O$_2$(1$^g$) was measured in the present study by using H$_2$O$_2$ generated by the same cells stimulated under the same conditions (6).

O$_2$(1$^g$) is generated when both HOCl and H$_2$O$_2$ are added together (Reaction 1) (54) or when HOCl is added in buffer containing Cl$^-$ pH 7.2 (Reaction 2) (55).

\[
\text{H}_2\text{O}_2 + \text{HOCl} \rightarrow \text{O}_2(1^g) + \text{HOCl}^- + \text{Cl}^- + \text{H}^+ \quad \text{REACTION 1}
\]

\[
2\text{HOCl} \rightarrow \text{O}_2(1^g) + 2\text{Cl}^- + 2\text{H}^+ \quad \text{REACTION 2}
\]

The combination treatment of H$_2$O$_2$/HOCl also shifted the absorbance (Fig. 2A) and decreased the fluorescence (Fig. 3A) of pyridoxamine. The absorbance and fluorescence changes in response to H$_2$O$_2$/HOCl were similar to the changes observed when pyridoxamine was treated with the corresponding concentrations of HOCl alone. When 0.1 M NaCl was omitted from the buffer system, only minor effects on the absorbance and fluorescence were observed in response to either HOCl (Fig. 2B and Fig. 3B) or H$_2$O$_2$/HOCl (Fig. 2D and Fig. 3B). H$_2$O$_2$ alone had no effect on the UV absorbance or fluorescence of pyridoxamine (Fig. 2C and Fig. 3A) in either buffer system, although the absorbance of H$_2$O$_2$ around 219 nm contributed to the slight increase in absorbance at this wavelength in samples treated with this ROS.

As shown in Fig. 1, vitamin B$_6$ has essentially the same chemical structure as pyridoxamine, except for a –CH$_2$OH group in the para position of the pyridinium ring instead of the –CH$_2$NH$_2$ group. This difference is important because a reaction of HOCl with the primary amine, –CH$_2$NH$_2$, of pyridoxamine is favored at pH 7.2 (58) over a reaction with the nitrogen of the pyridinium ring. The reaction of HOCl with the primary amine of pyridoxamine would produce an N-chloropyridoxamine group/N-chloropyridoxamine. In contrast to HOCl, the reactivity of O$_2$(1$^g$) with the nitrogen of the pyridinium ring has been reported (59). Despite the potential of O$_2$(1$^g$), and possibly HOCl, to react with the ring nitrogen, there was essentially no reaction of H$_2$O$_2$ or H$_2$O$_2$/HOCl or little reaction of HOCl with vitamin B$_6$ in the presence or
absence of NaCl, although the degree to which HOCl reacted with vitamin B6 varied (data not shown).

Oxidation of Pyridoxamine and Vitamin B6 at pH 5.5 ± 0.2—The pH of the phagolysosomes of neutrophils during the first 15 min following the ingestion of opsonized particles is 7.4–7.8 (60, 61), but after 15 min, the pH within the phagolysosomes, and presumably the underlying extracellular attachment site, decreases to pH 5.5–6.0. pH 5.5 is also the optimal pH for the generation of Cl2 and O2(1g) by HOCl in solutions containing 0.1 M NaCl (53–54). To evaluate the reactivity of HOCl, H2O2, or H2O2/HOCl with pyridoxamine and vitamin B6 at pH 5.5 ± 0.2, ROS were added to 15 μM pyridoxamine in 0.5 M sodium phosphate buffer ± 0.1 M NaCl. Pyridoxamine or vitamin B6 control and treated samples were then incubated at RT for 15 min. The UV absorbance data for a typical pyridoxamine experiment is presented in Fig. 4. At pH 5.5 the absorbance of pyridoxamine at 217 and 325 nm, and to a lesser extent the absorption at 252 nm, decreased after treatment with HOCl (Fig. 4, A and B), and a new absorbance peak at 228–229 nm, which is within the range of known N-chloramine absorbance peaks, was formed (27).

At pH 5.5, HOCl also reacted with vitamin B6, which resulted in a decrease in the absorbance in the range of 292 and 325 nm (Fig. 4C). In the absence of NaCl, vitamin B6 samples treated with HOCl showed only a slight decrease in absorbance in response to 50 μM HOCl (Fig. 4D), suggesting that either Cl2 or O2(1g) are involved in this reaction.

The fluorescence data for all samples are presented in Fig. 3. C and D, as the mean ± S.E. (n = 3, *p < 0.01, **p < 0.05). The fluorescence of pyridoxamine (Fig. 3C) and vitamin B6 (Fig. 3D) at 400 nm was decreased in response to either HOCl or H2O2/HOCl treatments. The decrease in absorbance (Fig. 4) and fluorescence (Fig. 3) of both pyridoxamine and vitamin B6 suggests that the ring structure is disrupted by this reaction.

N-Chloramine Formation in HOCl-treated Samples—To determine whether the reaction of HOCl with pyridoxamine or vitamin B6 leads to the formation of N-chloramines, 15 μM pyridoxamine or vitamin B6 in 0.5 M sodium phosphate ± 0.1 M NaCl, pH 7.2, was reacted with increasing concentrations of HOCl. The oxidation of potassium iodide (KI) to triiodide by HOCl or by N-chloramines formed in the reaction of HOCl with pyridoxamine was then determined after incubation for 15 min at RT.

In the absence of NaCl, HOCl in solution at pH 7.2 was slow to decompose, and the direct oxidation of KI by HOCl remained high even after 24 h incubation at 37 °C (Table I). Due to the high background, the formation of N-chloramines by HOCl in the absence of NaCl could not be determined, although an immediate reaction of HOCl with pyridoxamine could be observed as a decrease in KI oxidation in these samples (data not shown).

In the presence of NaCl, there was an immediate decrease in background oxidation of KI by unreacted HOCl and the production of N-chloramines could be detected, after 15 min, in pyridoxamine samples treated with 50 μM HOCl (Table I). As would be expected from the limited effect of HOCl on the absorbance and fluorescence of vitamin B6, there was no formation of N-chloramines in the reaction of HOCl with vitamin B6 at pH 7.2.

Despite the more rapid decomposition of HOCl or the rapid reaction of HOCl with NaCl at pH 5.5, the formation of N-chloramines could not be determined at this pH. The increased oxidation of KI under these conditions may be due to the production of O2(1g).
Reaction of Chloramine-T with Pyridoxamine and Vitamin B₆—To evaluate further the formation of N-chloropyridoxamine in the reaction of HOCl with pyridoxamine, the absorbance of chloramine-T in 0.5M sodium phosphate buffer, pH 7.2 and 5.5, was determined (Fig. 5A). The absorbance peaks for chloramine-T were 219–224 nm and corresponded with the initial absorbance peaks of HOCl-treated pyridoxamine samples (Fig. 2), indicating the reaction of HOCl with pyridoxamine generates N-chloropyridoxamine. The absorbance of unreacted chloramine-T remained constant at both pH values. To evaluate the reaction of N-chloramines with pyridoxamine or vitamin B₆, increasing concentrations of chloramine-T were added to 15 μM pyridoxamine or vitamin B₆ in 0.5 M sodium phosphate buffer, pH 7.2 or 5.5, and the samples were incubated for 15 min RT, 2 h at 37 °C, or overnight at 37 °C. Chloramine-T reacted with pyridoxamine at pH 7.2 as indicated by the concentration-dependent shifts in the major absorbance peak of pyridoxamine from 325 to 312–318 nm (Fig. 5B and C). There was essentially no reaction of chloramine-T with vitamin B₆.

At pH 7.2, the reaction of chloramine-T with pyridoxamine was verified using the N-chloramine assay as described above. There was a decrease over time in the amount of chloramine-T available to oxidize KI after reaction with pyridoxamine, but not vitamin B₆ at pH 7.2 (data not shown). The reaction of chloramine-T with pyridoxamine was immediate and continued to increase over time.

At pH 5.5, chloramine-T reacted with both pyridoxamine (Fig. 5C) and vitamin B₆ (Fig. 5D), although the vitamin B₆ reaction was not immediate. The reactivity of chloramine-T with vitamin B₆ suggests that the nitrogen in the pyridinium ring is reactive at this pH, although to a lesser degree compared with the primary amine group of pyridoxamine.

Thermal Release of O₂(¹Dg) from DNE—DNE, a pure chemical source of O₂(¹Dg), can provide a reaction system that does not rely on the presence of other ROS that may interfere or react with the target of interest to evaluate more definitively a reaction of O₂(¹Dg) with pyridoxamine or vitamin B₆. We incubated 200 μl of 15 μM pyridoxamine or vitamin B₆ in 0.5 M sodium phosphate containing 0.1 M NaCl, pH 7.2 or 5.5, on a DNE-coated coverslip. Duplicate samples were incubated for 24 h at 4 or 37 °C, and the absorbance readings were compared. Control coverslips were coated with the dichloromethane solvent. There were no changes in the absorbance of either pyridoxamine or vitamin B₆ in response to O₂(¹Dg) (data not shown). The release of O₂(¹Dg) was verified using AAP (44). After 24 h at 4 °C, the absorbance of AAP at 400 nm was 0.715 ± 0.003, and AAP incubated on a DNE-coated coverslip had an absorbance of 0.702 ± 0.005. For duplicate samples...
incubated at 37 °C, the absorbance of AAP after 24 h on control coverslips was 0.718 ± 0.003, and the absorbance of AAP incubated on DNE-coated coverslips was 0.588 ± 0.008. The decrease in absorbance of AAP on DNE-coated coverslips after incubation at 37 °C confirmed that O2(1Dg) was released and reacted with AAP in the aqueous buffer systems used in our study. Taken together these findings eliminate O2(1Dg) in the reaction of HOCl with either pyridoxamine or vitamin B6 at pH 5.5, leaving Cl2 and HOCl as the remaining reactants.

Gas Chromatography and Mass Spectrometry—Samples of vitamin B6 and vitamin B6 reacted with 50 μM HOCl for 15 min were analyzed by GC-MS as described under "Experimental Procedures." Fig. 6 presents the results of these analyses. Electron ionization-mass spectrometry of the vitamin B6-GC peak showed two peaks at 12.73 and 13.42 (Fig. 6A). After reaction with 50 μM HOCl at pH 5.5 (Fig. 6B), four chlorinated products were identified at 11.03, 12.29, 13.17, and 14.19 min as 4-chloro-2-hydroxymethyl-2,4-hexadiene-3-carboxaldehyde, 5-chloromethyl-3-hydroxy-4-hydroxymethyl-1,3,5-hexatriene, N-chloro-3-chloromethyl-4-hydroxy-2-hydroxymethyl-1-imino-2,4-pentadiene, and 3-chloro-4,5-dihydroxymethyl-2-methylpyridine (3-chloropyridinium), respectively. The product at 13.57 min was not chlorinated, and no further characterization was carried out.

Carbonyl (Aldehyde and Ketone) Formation after Exposure of Collagen to H2O2, HOCl, or H2O2/HOCl—To evaluate the oxidation of human collagen types I (bone) and II (articular cartilage) by H2O2, HOCl, or H2O2/HOCl, 50 μg of collagen in 0.5 M sodium phosphate buffer containing 0.1 M NaCl, pH 7.2 or pH 5.0, were exposed for 1 h at 37 °C to 125, 250, or 500 μM H2O2, 12.5, 25, or 50 μM HOCl, or a combination of H2O2/HOCl in a ratio of 10:1. The above concentrations of HOCl and H2O2 are within the predicted range generated by activated neutrophils or monocytes at sites of inflammation (6). A ratio of 10:1 was

![Graphs](image)

**Fig. 4.** The UV absorbance spectra of 15 μM pyridoxamine in 0.5M sodium phosphate buffer ± 0.1 M NaCl, pH 5.5, reacted for 15 min at RT with 0 μM (●), 12.5 μM (△), 25 μM (○), or 50 μM (□) HOCl in the presence or absence of 0.1 M NaCl (A) or 15 μM pyridoxamine reacted with 500 μM H2O2 (△), 0 μM H2O2/HOCl (●), 125 μM, 12.5 μM H2O2/HOCl (△), 250 μM, 25 μM H2O2/HOCl (○), or 500 μM, 50 μM H2O2/HOCl (□) in the presence or absence of 0.1 M NaCl (B). The UV absorbance spectra of 15 μM vitamin B6 in 0.5 M sodium phosphate buffer ± 0.1 M NaCl, pH 5.5, reacted for 15 min at RT with 0 μM (●), 12.5 μM (△), 25 μM (○), or 50 μM HOCl (□) in the presence of 0.1 M NaCl (C) or without NaCl present (D). Graphs present a representative set of data from experiments that were repeated three times.

**Table I**

| Additions                  | 0 μM | 12.5 μM | 25 μM | 50 μM |
|---------------------------|------|---------|-------|-------|
| No NaCl                   | 0    | 13.4 ± 1.2 | 24.3 ± 1.0 | 48.0 ± 3.2 |
| 0.1 M NaCl                | 0    | 8.4 ± 0.5  | 18.2 ± 2.0  | 3.1 ± 0.4 |
| 15 μM pyridoxamine + 0.1 M NaCl | 0.6 ± 1.1 | 0.5 ± 0.5  | 0.4 ± 1.1  | 15.1 ± 2.5 |

*Statistical significance is defined as p < 0.01.

Statistical significance is defined as p < 0.05.
used in the present study because the amounts of HOCl generated by activated neutrophils is 5–20 times less than relative amounts of H₂O₂ generated by the same cells stimulated under the same conditions (6). The pH values were chosen based on the neutral pH of the neutrophil phagolysosomes during the first 15 min following the ingestion of opsonized particles (60, 61) and the acidic pH after 15 min (62).

To determine the oxidation of primary amines or pyridino-line cross-links by H₂O₂ or HOCl, immuno-chemiluminescence analysis (Oxyblot kit) was used to assess the presence of carbonyl groups within the protein of interest. A concentration-dependent increase in the number of reactive carbonyl groups in both collagen types was found after exposure to HOCl, with the amount varying depending on collagen type. The results for collagen reacted with 50 μM vitamin B₆ reacted with 50 μM HOCl for 15 min at pH 5.5 (B). Two peaks at 12.73 and 13.42 min characterized the GC-MS for vitamin B₆ (A), and four chlorinated products were identified at 11.03, 12.29, 13.17, and 14.19 min for vitamin B₆ (B) after reaction with HOCl and were identified as 4-chloro-2-hydroxymethyl-2,4-hexadiene-3-carboxaldehyde, 5-chloromethyl-3-hydroxy-4-hydroxymethyl-1,3,5-hexatriene, N-chloro-3-chloromethyl-4-hydroxy-2-hydroxymethyl-1-imino-2,4-pentadiene, and 3-chloro-4,5-dihydroxymethyl-2-methylpyridine (3-chloropyridinium), respectively. The product at 13.57 min was not chlorinated, and no further characterization was carried out.

**Fig. 5.** The UV absorbance peaks for 0 μM (●), 25 μM (○), 50 μM (□), 75 μM (■), and 100 μM (▲) chloramine-T in 0.5 M sodium phosphate buffer ± 0.1 M NaCl at pH 7.2 (A) or pH 5.5 (C). One major peak at 219–224 nm characterized the absorbance for chloramine-T. The UV absorbance for 15 μM pyridoxamine reacted with increasing concentrations of chloramine-T ± 0.1 M NaCl for 15 min RT at pH 7.2 (B) or pH 5.5 (C). The UV absorbance scans for 15 μM vitamin B₆ reacted with increasing concentrations of chloramine-T in 0.5 M sodium phosphate buffer pH 5.5 ± 0.1 M NaCl for 15 min at RT (D), and then incubated overnight (16–18 h) at 37 °C.

**Fig. 6.** Gas chromatography and electron ionization-mass spectrometry (GC-MS) of vitamin B₆ (A) and vitamin B₆ reacted with 50 μM HOCl for 15 min at pH 5.5 (B). Two peaks at 12.73 and 13.42 min characterized the GC-MS for vitamin B₆ (A), and four chlorinated products were identified at 11.03, 12.29, 13.17, and 14.19 min for vitamin B₆ (B) after reaction with HOCl and were identified as 4-chloro-2-hydroxymethyl-2,4-hexadiene-3-carboxaldehyde, 5-chloromethyl-3-hydroxy-4-hydroxymethyl-1,3,5-hexatriene, N-chloro-3-chloromethyl-4-hydroxy-2-hydroxymethyl-1-imino-2,4-pentadiene, and 3-chloro-4,5-dihydroxymethyl-2-methylpyridine (3-chloropyridinium), respectively. The product at 13.57 min was not chlorinated, and no further characterization was carried out.
suggesting that \( H_2O_2 \) is reacting with HOCl and decreasing the availability of HOCl to react with collagen.

Pyridinoline Cross-link and Aromatic Amino Acid Fluorescence of Collagen before and after Exposure to HOCl—To assess oxidation of the pyridinoline cross-links and aromatic amino acids of collagen types I and II by HOCl, the fluorescence emission of HOCl-treated collagen was measured and compared with untreated control samples subjected to the same conditions. The fluorescence of collagen type I samples was below measurable levels, and no further fluorescence studies were done on this collagen type. Exposure of 0.125 mg/ml collagen type II to HOCl at pH 5.0 and 7.2 for 15 min at 37 °C resulted in a concentration-dependent decrease in fluorescence emission at 400 nm (excitation 325 nm) (Table II). No other changes in fluorescence emission or excitation were observed.

SDS-PAGE Results of Collagen Types I or II Reacted with \( H_2O_2 \), HOCl, or a Combination of \( H_2O_2 \)/HOCl—After exposure to ROS as described above, 4-\( \mu \)g aliquots of each collagen sample were subjected to SDS-PAGE analysis (4–15% gradient) and stained with silver. Results of a representative gel of an experiment performed three times are presented in Fig. 8. Exposing collagen types I (Fig. 8A, lanes 3 and 7) or II (Fig. 8B, lane 3) to 50 \( \mu \)M HOCl resulted in a 60–80% decrease in the intensity of collagen electrophoretic band staining by silver (arrows indicating form top to bottom, \( \gamma \), \( \beta \), and \( \alpha \)). Except at pH 7.2, the decrease in silver staining for collagen type II reacted with HOCl was much less (Fig. 8B, lane 7). Concentration-dependent decreases in silver staining are shown in Fig. 8, C and D, for collagen reacted with HOCl at pH 5.0 and 7.2. No smearing (fragmentation) or low molecular weight bands were observed after any treatment. A decrease in reactivity of silver with the HOCl-reacted collagen monomers is consistent with the formation of N-chloroamines (33) in the reaction of HOCl with collagen and ultimately the spontaneous deamination and decarboxylation of N-chloroamines (\( \alpha \)-amino groups) to form aldehydes (26, 63), thus suggesting that the collagen monomers are still intact but have been oxidatively modified and no longer react with silver. The oxidative modification of amine groups by HOCl would no longer make them available for reaction with silver.

To determine if the decrease in silver reactivity with collagen after oxidation by HOCl is due to oxidative modification of primary amine groups and not protein fragmentation or both, we reacted \( ^{14}C \)-labeled bovine serum albumin (BSA) with increasing concentrations of HOCl (12.5–50 \( \mu \)M). After exposure to HOCl as described above, 4-\( \mu \)g aliquots of \( ^{14}C \)BSA were subjected to SDS-PAGE analysis (10% acrylamide), stained with silver, then dried and exposed to autoradiographic film. Silver staining and autoradiogram results are presented in Fig. 8, C and D, respectively. A concentration-dependent decrease in silver staining intensity was observed after treatment of \( ^{14}C \)BSA with 12.5–50 \( \mu \)M HOCl, without affecting the autoradiogram band intensity of these same samples. This provided us with some supportive evidence that the decrease in collagen staining after reaction with HOCl is due to oxidative modifica-
tion rather than protein fragmentation or a combination of both processes.

Fluorescamine and o-Phthalaldehyde Primary Amine and Imino Acid Measurements—To determine if the decrease in detectable electrophoretic band staining was the result of complete collagen fragmentation by HOCl, aliquots of each collagen sample, acetone precipitates of each sample, and sample supernatants of acetone precipitates were analyzed by fluorescamine and o-phthalaldehyde assays. Both assays detect primary amines and imino acids, which would increase if collagen were fragmented. However, neither assay detected an increase in free amine or imino acid groups in any of the collagen samples.

DISCUSSION

ROS are produced and released into extracellular spaces and contribute to the development and progression of inflammatory diseases. However, little is known about direct ROS-induced molecular modifications of individual matrix proteins and the consequence of these modifications on the structure, physical properties, or function of the matrix. The present study tested the hypothesis that HOCl participates in the inflammatory-mediated loss of connective tissue collagen by oxidizing the Pyd cross-links found in abundance in adult articular cartilage. HOCl, produced by the enzyme MPO, is the major highly reactive oxidant produced by activated neutrophils and to a lesser extent by monocytes and some macrophages. Based on its reactivity, HOCl has the potential to cause the bulk of the tissue damage at sites of acute inflammation (64). Pyd cross-links are of particular importance because their function is to help maintain the structure of the collagen fibrils and make them more resistant to collagenolysis or proteolytic degradation (37, 65). Our current findings indicate that OCI⁻/Cl₂, and to a lesser extent N-chloramines, chlorinate pyridinium compounds with structures similar to Pyd and that these HOCl species react with both collagen types I and II, resulting in the oxidation of amine groups and Pyd cross-links.

Our findings indicate that HOCl rapidly reacts with and chlorinates pyridinium compounds, but the chlorination reaction and the N-chlorination sites are pH-dependent. At pH 7.2 ± 0.2, HOCl/OCI preferentially reacted with the para-CH₂NH₂ group of pyridoxamine to form N-chloropyridoxamine, which was detected using the KI oxidation assay for N-chloramine and by the appearance of the characteristic N-chloramine absorbance peak at 220–225 nm. In pyridoxamine samples treated with HOCl, a second absorbance peak at 307–320 nm was observed. This peak corresponds to a peak observed for the product that results from the reaction of pyridoxamine with chloramine-T, a commercial N-chloramine standard. Based on the presence of these two peaks, we suggest that as soon as N-chloropyridoxamines are formed in the reaction of pyridoxamine and HOCl, they in turn react with other pyridoxamine...
molecules and initiate the formation of N-chloropyridoxamine-pyridoxamine dimers. The reaction of HOCl/\cdot OCl with the para-CH,NH$_2$ of pyridoxamine is in agreement with the report of Davies et al. (33) stating that the preferred reaction of HOCl/OCl$^-$ with collagen at neutral pH is with the primary amine groups.

At pH 5.5 ± 0.2 and in the presence of Cl$^-$, HOCl, and N-chloramines reacted with both pyridoxamine and vitamin B$_6$. The reactivity of HOCl at this pH with the pyridinium compounds is consistent with the reported increase in reactivity of OCl$^-$ with compounds that possess extensive p electrons (ring nitrogen) (66). The significant loss in absorbance and fluorescence of either compound after reaction with HOCl at pH 5.5 suggests that a percentage of the pyridinium ring structure was disrupted in these reactions. Disruption of the ring structure was confirmed by GC-MS analysis and is consistent with the formation of an aldehyde as a result of the spontaneous deamination and decarboxylation of an N-chloramine (in this case the ring nitrogen) to form an aldehyde (58, 63).

The reactivity of HOCl with vitamin B$_6$ at pH 5.5 in the presence of Cl$^-$ is also consistent with the evolution of Cl$_2$ (55). GC-MS analysis also identified 3-chloropyridinium as one of the products of this reaction, similar to a previous report of 3-chlorotyrosine formation in the reaction of Cl$_2$ with the aromatic amino acid, L-tyrosine (27). The reactivity of Cl$_2$ is also of interest because it has been demonstrated that neutrophils generate Cl$_2$ via the MPO-H$_2$O$_2$-Cl$^-$ system (66).

In contrast to HOCl, H$_2$O$_2$ alone was without effect under any condition, which is in keeping with its low reactivity with biological molecules. The third of the nonradical species tested in this study, O$_2$(1$\Delta g$), is a relatively long lived (2 μs) and highly reactive oxidant produced by HOCl at pH 5.5 and to a lesser extent at pH 7.2 in the presence of Cl$^-$ (55). Because O$_2$(1$\Delta g$) would be produced by both of these ROS systems, it would seem likely that O$_2$(1$\Delta g$) would react with the pyridinium ring nitrogen (59). However, O$_2$(1$\Delta g$) did not significantly contribute to the derivatization of pyridoxamine, and at pH 7.2 the production of O$_2$(1$\Delta g$) by the reaction of H$_2$O$_2$ with HOCl actually interfered with the reaction of HOCl and pyridoxamine. Similarly, at pH 5.5 HOCl should generate maximal amounts of O$_2$(1$\Delta g$) in the presence of NaCl (55); however, even at this pH our findings indicate that HOCl and not O$_2$(1$\Delta g$) is the major reactant with either pyridoxamine or vitamin B$_6$. Finally, the thermal release of O$_2$(1$\Delta g$) by DNE, a pure chemical source of O$_2$(1$\Delta g$), did not result in an absorbance or fluorescence change in the pyridoxamine or vitamin B$_6$ spectra. It has been reported that O$_2$(1$\Delta g$) can react with pyridinium compounds, resulting in cleavage of the nitrogen-carbon bond between the nitrogen-containing pyridinium ring and the terminal carbon of a substituted group at this site (59). This reaction leaves the pyridinium ring structure intact and unchanged. Although we did not detect a reaction of O$_2$(1$\Delta g$) with an unsubstituted nitrogen group in the pyridinium rings of pyridoxamine and vitamin B$_6$, it is still possible that a reaction between O$_2$(1$\Delta g$) and the Pyd cross-links could take place in vivo when the nitrogen is covalently linked to the triple helical region of a collagen molecule. This type of reaction would also result in the disruption of the intermolecular bond between two molecules of collagen.

In a previous study by Davies et al. (33) the oxidation of bovine collagen type I isolated from tendon and collagen type II isolated from articular cartilage was assessed as the amount of collagen fragmentation taking place in response to HOCl or N-chloramines. Only at superphysiological concentrations of 1–5 mM did HOCl cause extensive fragmentation (smearing) of collagen. In contrast, the addition of N-chloramines (5–50 μM) did not cause fragmentation but, instead, greatly increased the degradation of collagen by collagenase and elastase. The mechanism by which N-chloramines increased the proteolytic susceptibility of collagen was not specifically determined, although it was assumed that N-chloramines were reacting with amine groups and disrupting the secondary and tertiary structure of the collagen molecules. In many cases, oxidation of proteins appears to result in the partial unfolding of target proteins, exposing hydrophobic regions that are normally shielded and promoting the preferential degradation of these proteins by proteases (67–69). Disruption of the secondary and tertiary structure of proteins by oxidative damage has been reported to expose hydrophobic regions of proteins and promote their preferential degradation by proteases (67–71). We would also suggest that oxidation and disruption of the Pyd cross-links could result in the loss of functional interactions of collagens, destabilization of the structural integrity of collagen fibrils, and/or an increase in the susceptibility of collagen to proteolytic degradation. Any of these changes would result in potentially irreversible damage of the tissue.

Our present findings agree with those of Davies et al. (33) that no fragmentation of either collagen takes place in response to physiological concentrations of HOCl (12.5–50 μM). However, we show that at these concentrations HOCl directly oxidizes human collagen type I from bone and collagen type II from articular cartilage. We were able to demonstrate the direct oxidation of collagen types I and II by taking advantage of the fact that N-chloramines, formed by the reaction of HOCl with α-amino groups, decompose to aldehydes (26, 63) and that the formation of N-chloramines contributes to the decrease in silver staining intensity of HOCl-reacted protein. We were also able to demonstrate the direct oxidation of Pyd cross-links in collagen type II by taking advantage of the fact that aldehydes are also formed in the reaction of HOCl with pyridinium compounds and that the formation of aldehydes results in the disruption of the pyridinium ring and the loss of Py fluorescence (325nm/400nm). We have focused on the oxidation of the Pyd cross-links because their chemical makeup suggests that they should be reactive with HOCl (27) and possibly O$_2$(1$\Delta g$) (72). Using the decrease in fluorescence emission as a measure for the reaction of HOCl with the Pyd cross-links is supported by the fact that other fluorescent groups present or generated by oxidation of collagen do not fluoresce at 400 nm (325 nm excitation) or have increased fluorescence at this wavelength. These groups include tryptophan residues (275nm/334nm) that are not present in these collagens, glycation end products (350nm/430nm), and bityrosines (325nm/400nm), which when formed have an increased fluorescence emission at 400 nm (27, 30).

Although both collagen types I and II were oxidized by HOCl, the reactions differed. Oxidized collagen type II showed a greater amount of carbonyl formation and a decrease in fluorescence (325nm/400nm), as compared with oxidized collagen type I. The increase in carbonyl formation may have resulted from the reaction of HOCl with Pyd cross-links that are more abundant in this collagen type (36). This reaction would also account for, but may not completely explain, the more intense silver staining of collagen type II since a reaction of HOCl with Pyd cross-links would result in a greater amount of carbonyl formation without a corresponding decrease in amine groups.

In support of a reaction of HOCl with the Pyd cross-links, we detected a decrease in fluorescence emission at 400 nm (excitation 325 nm) in the collagen type II samples reacted with HOCl. The formation of aldehydes in the reaction of HOCl with pyridinium compounds is rapid and can be detected after only 15 min of incubation at 37 °C as opposed to the
slower decomposition of N-chloramines to aldehydes, which can take hours (25).

In summary, activated neutrophils and monocytes are present in early inflammatory lesions and in later foci regions of acute inflammation in arthritic joints (1) and periodontitis (3). The presence of activated neutrophils results in the deposition of MPO onto the ECM surface and the production of the reactive oxidant, HOCl. Unlike a reaction with HOCl, the Pyd cross-links need not be on the surface of the fibril to be accessible to attack by Cl₂ or N-chloramines, which are small and less reactive, so they can diffuse from their sites of production. Thus, suggesting that the Pyd cross-links linking collagen types IX–II and II–II in articular cartilage would be assessable to reaction during inflammation associated with articular cartilage. However, the Pyd and deoxyripyridinoline cross-links of bone collagen type I are probably less susceptible to oxidation, because this collagen is more densely packed. First, the mineralization of bone collagen type I reduces the diffusibility of Cl₂ or N-chloramines in this matrix. Second, the collagen fibril itself is more tightly aligned (i.e. lateral packing of the molecules within the fibril and the spacing between collagen molecules is smaller in bone than cartilage (45, 46)) allowing less solvent and small molecule access to inner spaces. Finally, our findings suggest that if formed in vivo, 3-chloropyridinium might serve as a specific marker for the production of HOCl and the involvement of MPO in inflammation of tissues containing significant quantities of Pyd cross-links.

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