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# Oxidative Damage Due to Copper Ion and Hydrogen Peroxide Induces GlcNAc-Specific Cleavage of an Asn-Linked Oligosaccharide[1]

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**Cleavage of an asparagine-linked sugar chain by hydrogen peroxide ($H_2O_2$) and a copper salt was investigated. Incubation of a 2-aminopyridine (PA)–labeled biantennary sugar chain, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA, with $H_2O_2$ and $Cu^{2+}$ led to formation of four major degradation products. Reversed phase high performance liquid chromatographic analysis coupled with glycosidase digestion indicated that the sugar chain is not randomly degraded but specifically degraded at a GlcNAc residue. Treatment with either of $H_2O_2$ or copper alone did not cleave nor degrade the sugar chain to any extent. Electron spin resonance (ESR) spectra obtained using a spin trap reagent were consistent with the generation of $OH^\cdot$ or an $OH^\cdot$-like radical by the $H_2O_2$/copper salt mixture. The addition of ascorbic acid enhanced this radical generation as well as the degradation of the sugar chain. It was also found that $H_2O_2$/$Cu^{2+}$ destroys the *N*-acetyl group of the monosaccharide GlcNAc, as judged by a decrease in the ultraviolet absorption spectrum of this group. On the other hand, replacement of copper by $Fe^{2+}$ caused no cleavage of the sugar chain, although comparable levels of the same radical species were generated. Furthermore, spectrophotometric analysis showed that a GlcNAc-containing sugar chain coordinates to copper but not to iron, and, thus, the coordination appears to play an essential role in the degradation of the sugar chain. These findings suggest that coordination of copper ions to GlcNAc residues localizes the generation of a radical, which cleaves the glycosidic linkage, possibly involving alteration of the *N*-acetyl group, thereby allowing the GlcNAc-specific cleavage.**

**Key words: hydrogen peroxide, *N*-acetyl group, reactive oxygen species, sugar chain degradation, transition metal.**

Reactive oxygen species (ROS) are produced in normal biological metabolism and also generated in increased amounts under various pathological conditions such as inflammation and ischemia-reperfusion (*1*). In addition, therapeutic treatments with radiation and chemical agents give rise to the generation of ROS. In general, ROS, such as $OH^\cdot$, are highly reactive and non-specific in the damage they cause, making most biological molecules likely targets. It is well known that ROS damage the cell membrane *via* lipid peroxidation in the lipid bilayer (*1, 2*) and also cause oxidative damage to proteins such as Cu,Zn-superoxide dismutase and collagen (*3–5*). It has been suggested that ROS degrade glycosaminoglycans, which are important components in extracellular matrices, and thus cause the subsequent impairment of proteoglycan functions (*6, 7*). Hyaluronic acid, another major component of the extracellular matrix, can also be degraded by ROS, which has implications for rheumatoid arthritis (*8–14*). Thus, ROS have been considered to be important contributors to the progression of arthritis as well as other diseases (*1*).

A previous study found that the treatment of human umbilical vein endothelial cells with a hypoxanthine-xanthine oxidase system facilitates adhesion of intact neutrophils and colon cancer cells without any *de novo* protein synthesis (*15*). Since cell adhesion molecules, such as P-selectin, E-selectin, and ICAM-1, were not induced by ROS treatment in this study, this enhancement of adhesion may have resulted from the modification of glycocalyx of the endothelial cell surface by ROS. In fact, it has been reported that ischemia-reperfusion causes disruption of the endothelial glycocalyx (*16–18*), and this damage can be prevented by the addition of superoxide dismutase (*16*). Therefore, the alteration of cell adhesion by ROS would be associated with a loss of the shielding effect of glycocalyx. These findings are consistent with the suggestion that carbohydrate is an important biological target for ROS damage.

Oligosaccharide structures such as sialyl $Le^x$ and sialyl $Le^a$ serve as ligands for members of the selectin family (*19–*

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Abbreviations: ROS, reactive oxygen species; PA, 2-aminopyridine; DMPO, 5,5-dimetyl-1-pyrroline *N*-oxide; DTPA, diethylenetriamine pentaacetic acid.

*21*), and, in addition, carbohydrate moieties of membrane glycoproteins, such as receptors for epidermal growth factor and nerve growth factor, play critical roles in conferring the functions of these glycoproteins (*22–24*). Since these important carbohydrates can be potential targets for damage by ROS, it is possible that some of the biological actions of ROS are mediated by the oxidative damage to the carbohydrates. Effects of ROS on biological carbohydrates, however, have not been intensively investigated, and the detailed chemistry involved in ROS-mediated degradation is not fully understood.

In this study, we investigated a mechanism of ROS-mediated carbohydrate degradation by using an asparagine-linked (*N*-linked) sugar chain, a major class of cell surface glycoprotein oligosaccharides. A variety of structures of this type of sugar chain are available, but agalacto-biantennary sugar was used as a simple model oligosaccharide because this structure contains a core common to the complex type of sugar chains. The results obtained using this model will therefore be applicable to all complex-type sugar chains, in which the extent of elongation of the antennae and branch formation are variable. The study described here, involving a combination of copper salt and hydrogen peroxide, revealed that the carbohydrate is cleaved in an *N*-acetyl-amino-sugar–specific manner, and further suggest that coordination to a transition metal is an important factor in this specificity, as shown by ESR and spectrophotometric analyses. These findings provide an insight into the mechanisms by which ROS mediate the specific degradation of carbohydrates.

## EXPERIMENTAL PROCEDURES

*Materials*—Di-*N*-acetylchitobiose (chitobiose) and cellobiose were purchased from Seikagaku Corporation (Tokyo). Asialo-agalacto-biantennary sugar chain was prepared from hen egg sialoglycopeptide, as described by Seko *et al.* (*25*). Pyridylaminated (PA)-chitobiose and cellobiose were prepared using a Glyco TAG (Takara, Kyoto), according to the manufacturer's instructions. All other PA-sugar chains were enzymatically synthesized, as described previously (*26*). Hydrogen peroxide ($H_2O_2$), catalase and *N*-acetylglucosamine (GlcNAc) were purchased from Wako Pure Chemicals Industries (Osaka). 5,5-Dimetyl-1-pyrroline *N*-oxide (DMPO) was purchased from Dojindo Laboratories (Tokyo).

*Treatment of Sugar Chains with $H_2O_2$/$Cu^{2+}$ and Analysis of Degradation Products by High Performance Liquid Chromatography (HPLC)*—PA-biantennary sugar chain, PA-chitobiose, and PA-cellobiose were incubated with $H_2O_2$ (4 mM) and $CuSO_4$ (40 μM) in PBS (pH 7.4), and the reaction products were then analyzed by reversed phase HPLC. The samples were applied to an HPLC system (LC10A VP, Shimadzu) equipped with a TSK-gel ODS 80TM column (4.6 × 150 mm, Tosoh), and column temperature was 55°C. When the products from the reaction of the PA-biantennary were analyzed, elution was carried out with 20 mM ammonium acetate buffer (pH 4.0) containing 0.1% 1-butanol at a flow rate of 1 ml/min. In the case of the PA-disaccharides, samples were eluted with the same buffer without 1-butanol at a flow rate of 0.5 ml/min. Fluorescence was monitored at excitation and emission wavelengths of 320 and 400 nm, respectively.

*Glycosidase Digestion*—To identify the product sugar chains from the reaction with $H_2O_2$ and $CuSO_4$, the product sugar chain peaks were subjected to glycosidase digestion as well as comparison of the retention time with those of standard PA-sugar chains. The samples separated by the reversed phase HPLC were collected and dried using a centrifugal vacuum concentrator. These samples were dissolved in 15 μl of 100 mM citrate-phosphate buffer (pH 6.0) and then incubated with 100 mU of β-*N*-acetylhexosaminidase at 37°C for 12 h. Samples were boiled for 2 min and centrifuged at 12,000 rpm for 2 min. Supernatants were analyzed in the reversed phase HPLC, as described above, and it was confirmed that the digested sugar chains were identical to Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-PA or GlcNAc-PA.

*Characterization of Free Radical Generated by $H_2O_2$ and $CuSO_4$*—Electron spin resonance (ESR) analysis was performed using a spin trap reagent, DMPO, in order to characterize free radical species produced by $H_2O_2$ and $Cu^{2+}$. The DMPO-containing solutions were covered to prevent any light-induced degradation and kept at –20°C. $H_2O_2$ (4 mM) and $CuSO_4$ (40 μM) were incubated in 25 mM sodium bicarbonate buffer (pH 7.5), and DMPO was added immediately to this solution. ESR spectra were recorded at room temperature using an ESP 300E (Bruker) operated at a modulation frequency of 100 kHz, modulation amplitude of 5.01 G, and microwave power of 4.76 mW.

*Low Temperature ESR Analysis*—Low temperature ESR analyses were carried out according to Shinar *et al.* (*27*) to examine the interaction of $Cu^{2+}$ with sugar chains. A 400-μl portion of 1 mM chitobiose, 1 mM cellobiose, 1 mM ADP or 1 mM EDTA was mixed with 400 μl of 0.6 mM $CuSO_4$, and the mixture was frozen in liquid nitrogen. ESR spectra were obtained at 160 K using an ESP 300E (Bruker) operated at a modulation frequency of 100 kHz, modulation amplitude of 15.8 G and microwave power of 2.39 mW.

*UV-Visible Spectroscopic Analysis*—$FeCl_2$ or $CuSO_4$ was dissolved at a concentration of 0.5 mM in PBS, and UV-visible spectra of the metal ions were recorded using a U-2000A spectrophotometer (Hitachi). To examine the interaction of the metal ion with chelating agents and sugars, these compounds were added at various concentrations, and the spectra were recorded within 1 min after mixing.

*$H_2O_2$/$Cu^{2+}$-Mediated Degradation of GlcNAc*—GlcNAc (83 μM) were incubated with 4 mM $H_2O_2$ and 0–160 μM $CuSO_4$ in PBS (pH 7.4). The reactions were stopped by the addition of catalase. The reaction mixtures were centrifuged, and the supernatants were applied to normal phase HPLC with a Shodex Asahipak $NH_2$P-50 column (4.6 mm × 250 mm, Showa Denko). Elution was performed at 50°C with 70% acetonitrile/$H_2O$ at a flow rate of 0.8 ml/min. Absorbance at 210 nm was monitored for the *N*-acetyl group.

## RESULTS

To investigate the oxidative damage caused to a sugar chain by ROS, we used asialo-agalacto-biantennary sugar chain (Fig. 1) as a model oligosaccharide, because this sugar chain is common to the complex type *N*-linked oligosaccharide (*28*), which is a major class of carbohydrates found in the cell surface-localizing and secretory glycoproteins. The pyridylamine (PA)-labeled sugar chain was incubated with several agents which are known to generate ROS, and the resulting degradation of the sugar chain was

monitored by reversed phase HPLC using a fluorescence detector. As previously reported for *p*-aminobenzoic ethyl ester–labeled disialo-biantennary sugar chain (*29*), incubation of the PA-labeled sugar chain with $H_2O_2$ and $Cu^{2+}$ degraded the sugar chain. This degradation yielded five major peaks, as found in the HPLC elution profile (Fig. 2), and was further enhanced in the presence of ascorbic acid. However, no degradation was observed when $Cu^{2+}$ was replaced by $Fe^{2+}$ (Fig. 2F) or $Fe^{3+}$ (data not shown), or when the sugar chain was exposed to a hypoxanthine-xanthine oxidase system, which is known to generate superoxide anion and hydrogen peroxide (data not shown).

The peaks of the degradation products produced by $H_2O_2$/$Cu^{2+}$ indicate reducing end fragments of degradation products, because the sugar chain had been labeled with the fluorescent reagent at the most proximal (reducing end) GlcNAc. Fragmentation was not observed when incubation was carried out with either $H_2O_2$ or $CuSO_4$ alone (Fig. 2, B and C), and thus the degradation involves a mechanism requiring both $H_2O_2$ and $Cu^{2+}$. If the oligosaccharide had been cleaved at all glycosidic linkages in a random manner and to a similar extent, a total of 11 fragments, ranging from intact PA-sugar chain to PA-GlcNAc, would have been observed. However, fewer peaks were actually detected, suggesting that the cleavage by a combination of $H_2O_2$ and $Cu^{2+}$ occurs at specific sites rather than randomly.

To examine the nature of the $H_2O_2$ and $Cu^{2+}$-mediated cleavage of the carbohydrate in more detail, the reaction products were collected and analyzed by glycosidase digestion. Since digestion of the products with retention times of 5 min and 11 min by β-*N*-acetylhexosaminidase did not affect their retention times in the HPLC profile, it is indicated that these products do not contain any GlcNAc residues at their non-reducing ends. On the other hand, when the products with retention times of 15 and 17 min as well as the intact PA-biantennary sugar chain were digested with the same glycosidase, their peaks were shifted to the position of the digestion-resistant product with the retention time of 11 min. By comparison with a standard PA-sugar chain, this digestion-resistant product was found to be Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA. In addition, a similar assignment identified the product eluted at 5 min as PA-GlcNAc. Thus, these analyses show that the products with retention times of 15 and 17 min resulted from a loss of either one of the two β1-2GlcNAc residues from the non-reducing termini. On the other hand, the peak with a retention time of 7.5 min was not shifted by β-*N*-acetylhexosaminidase digestion, and hydrolysis of this species with trifluoroacetic acid, followed by re-*N*-acetylation, did not yield PA-GlcNAc. Therefore, it seemed unlikely that this species is a PA-oligosaccharide, although its formation was increased by the addition of ascorbic acid. These re-sults suggest that cleavage by the combination of $H_2O_2$/$Cu^{2+}$ occurs predominantly at the glycosidic linkages of a se-quence GlcNAcβ1-X in the sugar chain.

Since either $H_2O_2$ or $Cu^{2+}$ alone did not degrade the sugar chain, it appears that their cooperation produces a radical which reacts with the sugar chain. To identify this possible radical species, ESR analysis was carried out using DMPO as a spin trap. An adduct was obtained 1 min after mixing hydrogen peroxide and $CuSO_4$. As shown in Fig. 3, a spectral profile with a height ratio of 1:2:2:1 was observed, and this signal strength was decreased by the addition of ethanol, while ascorbic acid stimulated radical production. The properties of this adduct were consistent with the generation of a hydroxyl radical or hydroxyl radical–like species (*30*), and this radical appears to be involved in the

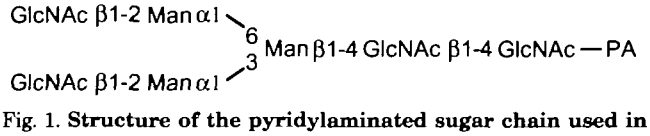


Fig. 1. **Structure of the pyridylaminated sugar chain used in this study.** PA represents 2-aminopyridine, which is conjugated to the reducing end *via* reductive amination.

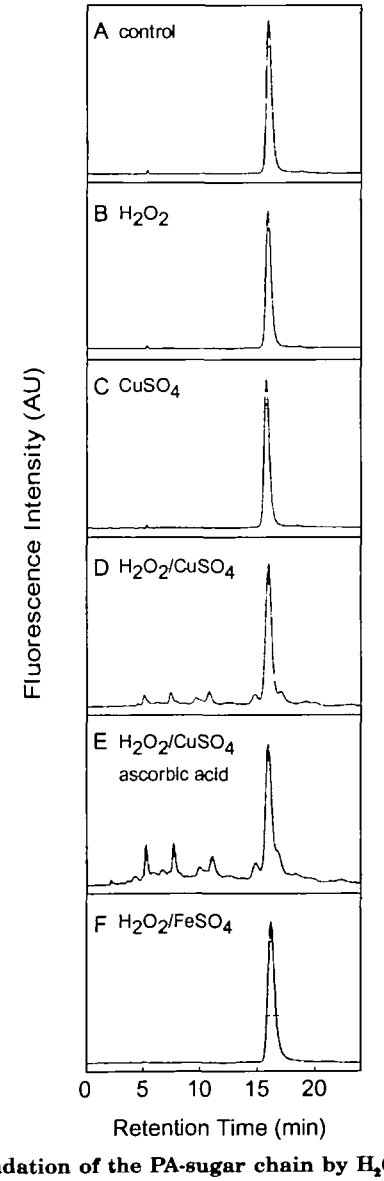


Fig. 2. **Degradation of the PA-sugar chain by $H_2O_2$ and $Cu^{2+}$.** The PA-sugar chain was incubated with various reagents at 37°C for 5 min, and the products were analyzed by reversed phase HPLC. Fluorescence was monitored at excitation and emission wavelengths of 320 and 400 nm, respectively. The reagents were: (A) none, (B) 4 mM $H_2O_2$, (C) 40 μM $CuSO_4$, (D) 4 mM $H_2O_2$ and 40 μM $CuSO_4$, (E) 4 mM $H_2O_2$, 40 μM $CuSO_4$ and 100 μM ascorbic acid, and (F) 4 mM $H_2O_2$ and 40 μM $FeSO_4$.

degradation of the sugar chain when a mixture of $H_2O_2$ and $Cu^{2+}$ is added. Nevertheless, comparable generation of the radical was found with the combination of $H_2O_2$ and $Fe^{2+}$, although this combination did not break the sugar chain (Fig. 2). In addition, a hypoxanthine-xanthine oxidase system, which was also incapable of degrading the sugars, produced the radical to a lesser extent (data not shown). Therefore, it is likely that generation of the radical alone is not sufficient for degradation of the sugar chain, and that an additional factor(s) is involved.

It is conceivable that a reactive radical is produced on, or in proximity to, a transition metal such as copper, which can transfer or provide an electron. It is most probable that there is a specific association of copper ions with GlcNAc which enables the limited degradation of the sugar chain through localized radical generation. In fact, it is well known that copper is capable of binding to many biomolecules such as nucleic acids and proteins (*31, 32*), probably by coordinating to their functional amide groups. It seems that an amide is a potential site for the interaction with the metal, and this structural feature is contained in the *N*-

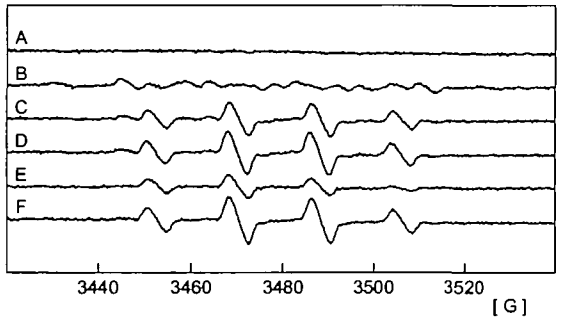


Fig. 3. **Detection of free radical by ESR.** DMPO was used as a spin trap. ESR spectra of the adducts are shown for (A) 4 mM $H_2O_2$, (B) 40 μM $CuSO_4$, (C) 4 mM $H_2O_2$ and 40 μM $CuSO_4$, (D) 4 mM $H_2O_2$, 40 μM $CuSO_4$, and 100 μM ascorbic acid, (E) 4 mM $H_2O_2$, 40 μM $CuSO_4$, and 0.4 M ethanol, and (F) 4 mM $H_2O_2$ and 40 μM $FeSO_4$. Experimental conditions are detailed under "EXPERIMENTAL PROCEDURES."

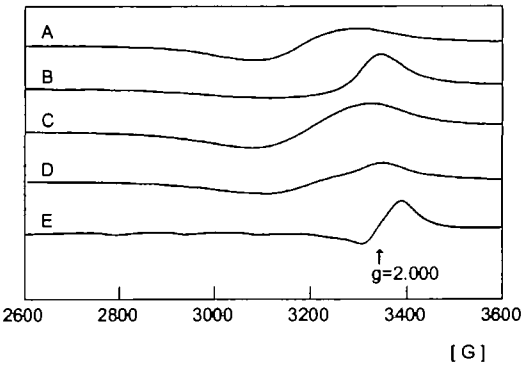


Fig. 4. **ESR analysis for the interaction of $Cu^{2+}$ with carbohydrate.** ESR spectra of $Cu^{2+}$ were recorded in the presence of sugars, (B) chitobiose and (C) cellobiose, or chelating agents, (D) ADP and (E) EDTA. The spectrum obtained without any additive is also shown (A). Concentrations of the sugars and chelating agents were used at 0.5 mM. Details are described under "EXPERIMENTAL PROCEDURES."

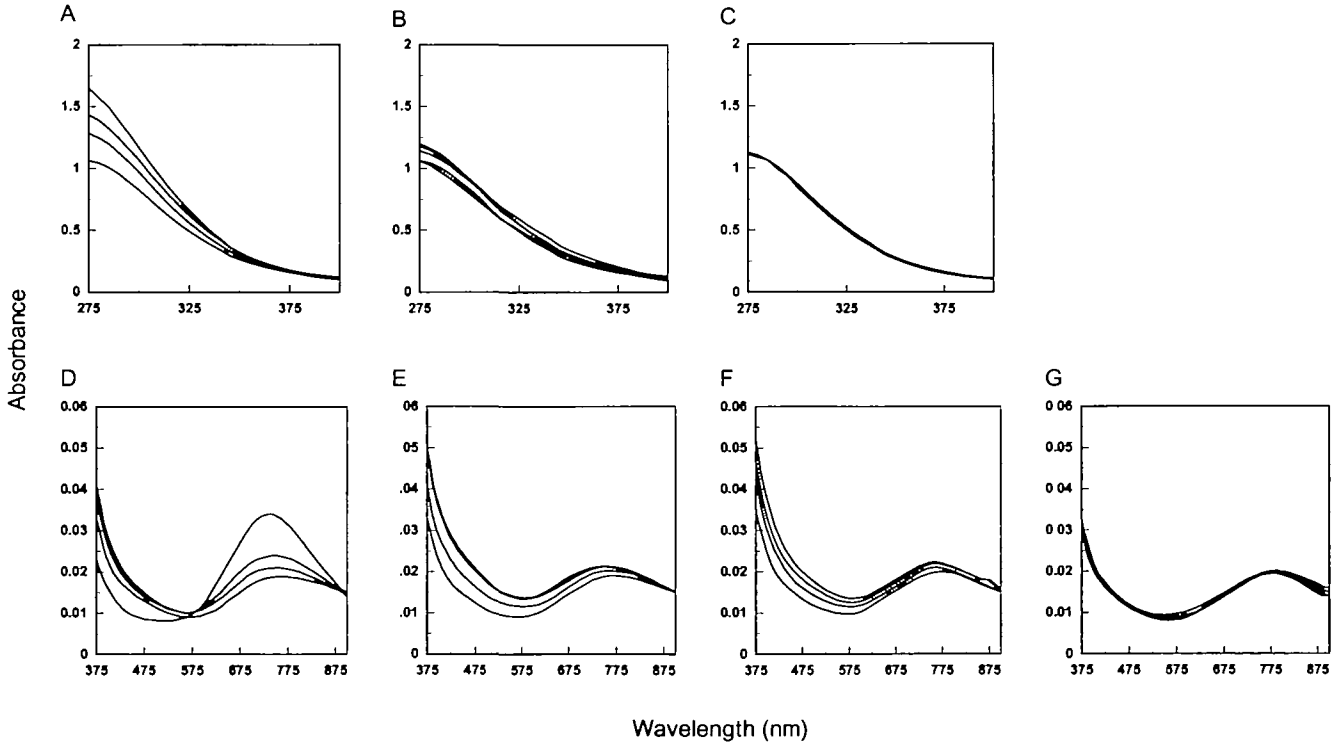


Fig. 5. **Ultraviolet-visible spectroscopic analysis of the interaction of metals and sugars.** Spectra of $Fe^{2+}$ (A–C) and $Cu^{2+}$ (D–G) are shown. The spectra of $Fe^{2+}$ (0.5 mM) were recorded in the presence of (A) EDTA (0, 25, 50, 100 μM), (B) DTPA (0, 0.05, 0.1, 0.25, 0.5 mM), and (C) chitobiose (0, 1, 2.5, 5 mM). The spectra of $Cu^{2+}$ (0.5 mM) were obtained with (D) EDTA (0, 50, 100, 250 μM), (E) ADP (0, 0.1, 0.25, 0.5 mM), (F) chitobiose (0, 1, 2.5, 5 mM), and (G) cellobiose (0, 1, 2.5, 5 mM).

acetyl group of the sugar. Hence, the involvement of the *N*-acetyl group in the binding of copper ions to carbohydrates was examined by ESR analysis using two model disaccharides, namely, chitobiose and cellobiose, the respective structures of which are GlcNAcβ1-4GlcNAc and Glcβ1-4Glc. ESR spectra for $Cu^{2+}$ were obtained in the absence or presence of these disaccharides. In the presence of coordinating agents such as EDTA and ADP (Fig. 4), a shift in the spectrum towards higher magnetic field was observed in the coordinated state. A similar shift was also found in the presence of chitobiose, but not in the presence of cellobiose. These results indicate that chitobiose, but not cellobiose, is capable of coordinating copper ions, suggesting that copper binds to the sugar chain *via* interaction with the *N*-acetyl group.

Consistent with the results from the ESR analysis, a significant shift in the UV-visible absorption spectrum of $Cu^{2+}$ was also observed in the presence of chitobiose (Fig. 5). However, when cellobiose was added at the same concentrations as used for chitobiose, essentially no spectral shift was found. These results further support the finding that $Cu^{2+}$ efficiently binds to the oligosaccharides which contain GlcNAc residues. On the other hand, the spectrum for $Fe^{2+}$ was not altered by the presence of chitobiose, whereas EDTA and DTPA shifted this spectrum remarkably. Thus, it appears that $Fe^{2+}$ lacks the ability to bind to the carbohydrate in the way that copper does. These results may account for the observation (Fig. 1) that $Fe^{2+}$ with $H_2O_2$ does not cleave the biantennary sugar chain, although this metal generates the same radical, and to a similar extent that copper does.

To further ascertain whether the coordination of $Cu^{2+}$ to a sugar is associated with the degradation by $H_2O_2/Cu^{2+}$, PA-chitobiose, and PA-cellobiose were incubated with $H_2O_2$ and $Cu^{2+}$. As shown in Fig. 6, only PA-chitobiose was degraded into the corresponding monosaccharide, PA-GlcNAc, whereas PA-cellobiose was resistant. This degradation was also confirmed by the same treatment of non-labeled disaccharides followed by thin layer chromatography analysis (data not shown), indicating that the cleavage does not depend on the PA moiety. The results support the notion that the *N*-acetyl group is essential for the degradation by $H_2O_2/Cu^{2+}$, providing a basis for the GlcNAc-specific cleavage of the sugar chain.

When the monosaccharide GlcNAc was incubated with $H_2O_2/Cu^{2+}$, the UV absorption rapidly decreased, as shown in Fig. 7. The results indicate that the *N*-acetyl group is rapidly destroyed by the action of $H_2O_2/Cu^{2+}$, because the UV absorption of GlcNAc is based on the *N*-acetyl group. No such decrease was observed when incubations were carried out with either $H_2O_2$ or $Cu^{2+}$ alone. The *N*-acetyl group appears to be a primary target for the radical formed by the action of $H_2O_2/Cu^{2+}$, and thus it seems that the cleavage of the sugar chain involves alteration of the *N*-acetyl group of the GlcNAc residue.

To examine whether other types of *N*-glycans are de-

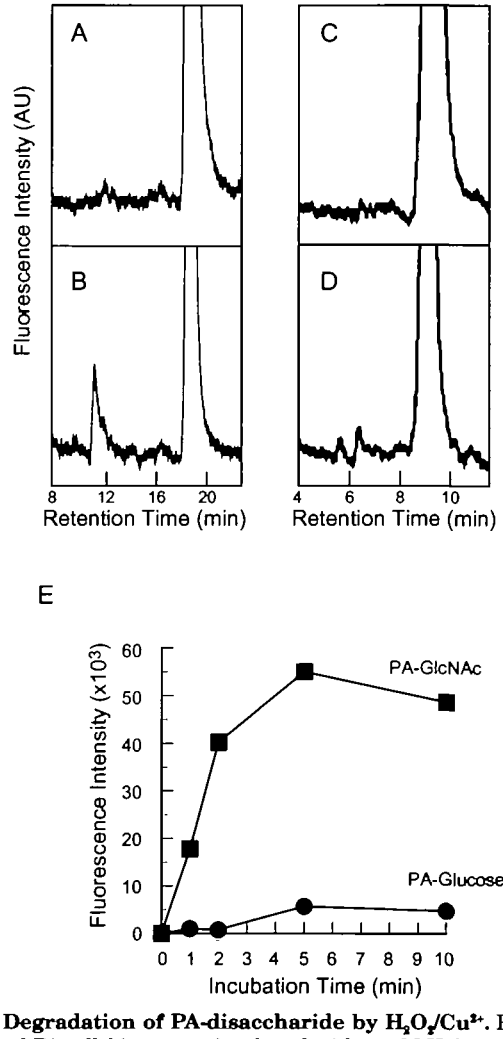


Fig. 6. **Degradation of PA-disaccharide by $H_2O_2/Cu^{2+}$.** PA-chitobiose and PA-cellobiose were incubated with 4 mM $H_2O_2$ and 40 μM $CuSO_4$ at 37°C for 5 min, and then subjected to the reversed phase HPLC analysis. The respective elution profiles are shown in B and D. A and C show the elution profiles for non-treated PA-chitobiose and PA-cellobiose, respectively. (B) The peak at 11.5 min in degradation of PA-chitobiose and (D) peak at 6.8 min in degradation of PA-cellobiose correspond to PA-GlcNAc and PA-glucose, respectively, as indicated by comparison with the standard PA-sugars. (E) Time courses of the formation of these PA-monosaccharides are also shown as the increase in the fluorescence intensity.

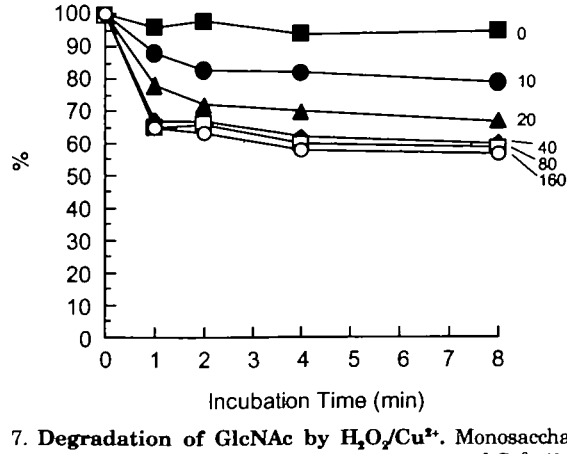


Fig. 7. **Degradation of GlcNAc by $H_2O_2/Cu^{2+}$.** Monosaccharide GlcNAc was incubated with various concentrations of $Cu^{2+}$ (0–160 μM) and 4 mM $H_2O_2$. Samples were analyzed at intervals by normal phase HPLC. Degradation of GlcNAc was monitored at 210 nm, which is based on the absorption of *N*-acetyl group, using a UV detector. The data are shown as percent of GlcNAc remaining. Experiments are detailed under "EXPERIMENTAL PROCEDURES."

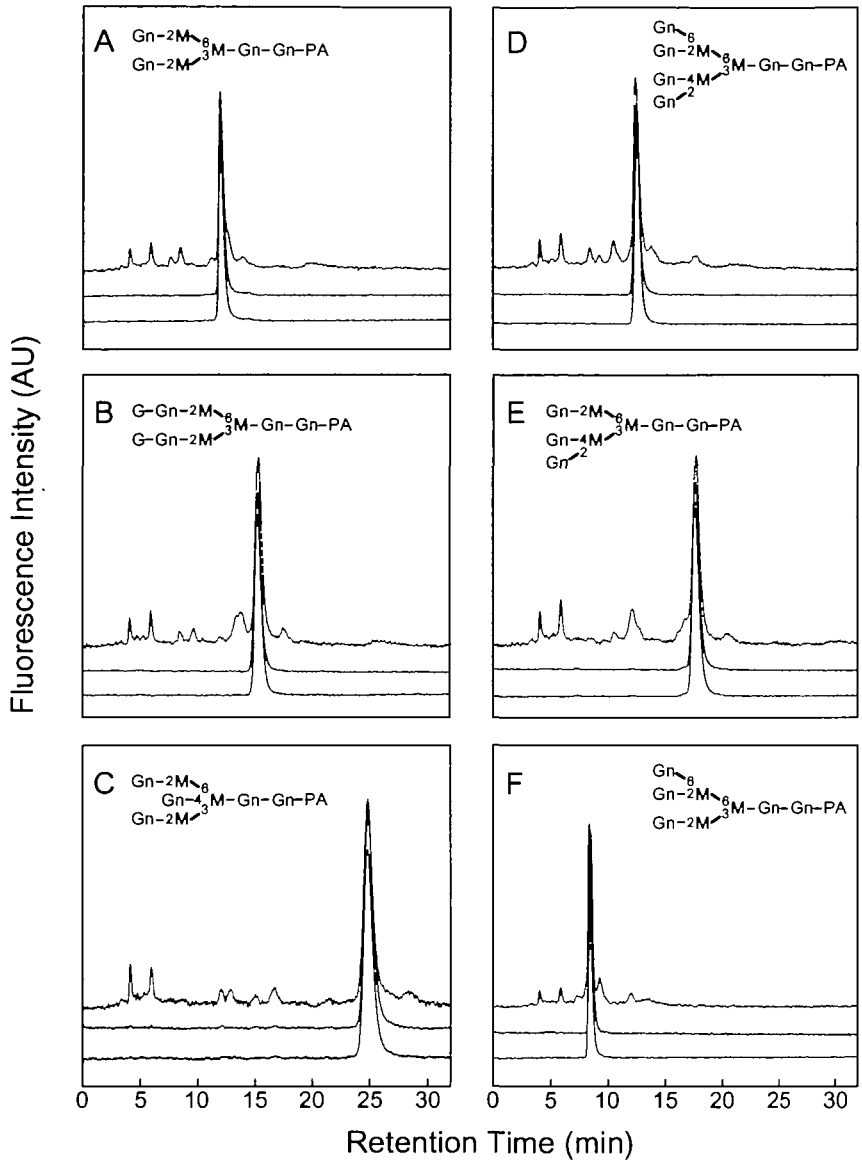


Fig. 8. **Degradation of various PA-sugar chains by $H_2O_2/Cu^{2+}$.** Elution profiles of reversed phase HPLC of various PA-sugar chains are shown. The PA-sugar chains used are (A) agalacto-biantennary PA-sugar chain, (B) digalacto-biantennary PA-sugar chain, (C) bisected biantennary PA-sugar chain, (D) agalacto-tetraantennary PA-sugar chain, (E) agalacto-triantennary PA-sugar chain, and (F) agalacto-tri'antennary PA-sugar chain. The upper trace in each panel indicates the reaction with 4 mM $H_2O_2$ and 40 μM $CuSO_4$ in the presence of 1 mM ascorbic acid. Middle and lower traces show the reaction with $H_2O_2$ and $CuSO_4$, respectively. The structures of the sugar chains are also shown.

graded by a similar mechanism, various PA-sugar chains were treated by $H_2O_2/Cu^{2+}$, and the resulting products were analyzed by reversed phase HPLC. Higher branched sugar chains as well as bisected biantennary and digalacto-biantennary sugar chains were incubated with $H_2O_2/Cu^{2+}$ in the absence or presence of ascorbic acid. It was found that these oligosaccharides are also degraded by $H_2O_2/Cu^{2+}$, and the cleavage of the sugar chains was enhanced in the presence of ascorbic acid. Elution profiles of the degradation products obtained in the presence of 1 mM ascorbic acid are shown in Fig. 8. Thus, it seems likely that complex type *N*-linked oligosaccharides are generally susceptible to the oxidative damage by $H_2O_2/Cu^{2+}$.

## DISCUSSION

This study found that an *N*-linked oligosaccharide, agalacto-biantennary sugar chain, is degraded by treatment with $H_2O_2/Cu^{2+}$. Analysis of the degradation products suggests that the sugar chain is cleaved predominantly at glycosidic linkages involving GlcNAc residues. This specific cleavage is probably due to the binding of copper ions to GlcNAc residues, thereby leading to localized generation of, probably, $OH^{\bullet}$ radicals, as demonstrated by spin trapping experiments. As suggested by many earlier studies, $OH^{\bullet}$ radicals may be generated from $H_2O_2$ by electron transfer involving oxidation of $Cu^{2+}$ to $Cu^{3+}$ or prior reduction of $Cu^{2+}$ by $H_2O_2$ (*33–39*). The *N*-acetyl group of the GlcNAc residue appears to play a role in this specific interaction with $Cu^{2+}$, and, in fact, no copper binding or cleavage was observed in the case of sugars which lack an *N*-acetyl group. These findings suggest that the mechanism of this GlcNAc-specific cleavage of the sugar chain by $H_2O_2/Cu^{2+}$ involves ROS-generation at a site localized by the interaction of the *N*-acetyl group of GlcNAc residues with $Cu^{2+}$. This suggestion is also supported by the observation that $Fe^{2+}$, which does not interact with GlcNAc residues, did not degrade the sugar chain in spite of generating comparable amounts of the radical.

Hawkins and Davies reported that $H_2O_2/Cu^{2+}$ gives rise

to site-specific oxidative damage to collagen (*40*). They suggested that the binding of the metal ion to particular sites on the protein gives rise to localized generation of ROS and, as a result, allows the site-specific damage and cleavage. When the degradation of hyaluronic acid by HOCl/$OCl^-$ was investigated, it was suggested that a nitrogen-centered radical was formed by homolysis of the chloramide species. This was followed by a rearrangement that led to formation of a carbon-centered radical on the *N*-acetyl group with subsequent cleavage of the glycosidic linkage (*12*). In the light of these suggestions, it seems most likely that radical formation on the *N*-acetyl group of the sugar is the basis of the GlcNAc-specific cleavage by $H_2O_2/Cu^{2+}$. Thus, it is probable that the hydroxyl radical, which is derived from hydrogen peroxide by electron transfer involving copper ions, could secondarily yield a carbon-centered or nitrogen-centered radical in the *N*-acetyl group of GlcNAc, due to its close vicinity and reactivity. The resulting secondary radical may then attack, possibly, C-1 of the GlcNAc, leading to the cleavage of the glycosidic linkage. However, the radical species that is directly involved in the cleavage of the glycosidic linkage remains to be identified.

Since most glycoconjugates, such as glycoproteins and proteoglycans, contain GlcNAc residues, they are potentially vulnerable to this type of degradation. In fact, we observed that hyaluronic acid is also degraded under the same conditions used in this study (data not shown). Another *N*-acetyl aminosugar, GalNAc, could also serve as the target and would undergo the same damage, due to the presence of the *N*-acetyl group. For example, fragmentation of proteoglycans would lead to alteration of cell adhesion and impairment of signaling mediated by several types of growth factors such as fibroblast growth factor. On the other hand, it is possible that cleavage or degradation of *N*-linked oligosaccharides of glycoproteins causes dysfunction of glycoproteins such as the EGF receptor, since it is known that many glycoproteins require *N*-linked sugar chains for their function (*41–46*). Therefore, this type of carbohydrate degradation may play a role in the pathogenesis and development of diseases in which pathological changes result in increased levels of "free" transition metal ions and hydrogen peroxide, as was previously suggested to occur during the oxidative damage of proteins and nucleic acids (*47–49*).

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