



HAL
open science

Chlorination of guanosine and other nucleosides by Hypochlorous Acid and Myeloperoxidase of Activated Human Neutrophils

Mitsuharu Masuda, Toshinori Suzuki, Marlin Friesen, Jean-Luc Ravanat, Jean Cadet, Brigitte Pignatelli, Hoyoku Nishino, Hiroshi Ohshima

► **To cite this version:**

Mitsuharu Masuda, Toshinori Suzuki, Marlin Friesen, Jean-Luc Ravanat, Jean Cadet, et al.. Chlorination of guanosine and other nucleosides by Hypochlorous Acid and Myeloperoxidase of Activated Human Neutrophils: Catalysis by nicotine and trimethylamine. *Journal of Biological Chemistry*, 2001, 276 (44), pp.40486-40496. 10.1074/jbc.M102700200 . hal-03391030

HAL Id: hal-03391030

<https://hal.science/hal-03391030>

Submitted on 22 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

Chlorination of Guanosine and Other Nucleosides by Hypochlorous Acid and Myeloperoxidase of Activated Human Neutrophils

CATALYSIS BY NICOTINE AND TRIMETHYLAMINE*

Received for publication, March 27, 2001, and in revised form, August 27, 2001
Published, JBC Papers in Press, August 31, 2001, DOI 10.1074/jbc.M102700200

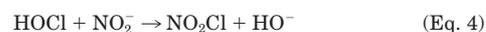
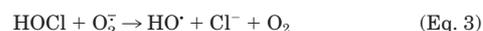
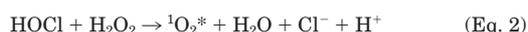
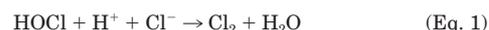
Mitsuharu Masuda‡§, Toshinori Suzuki‡, Marlin D. Friesen¶, Jean-Luc Ravanat||, Jean Cadet||, Brigitte Pignatelli‡, Hoyoku Nishino§, and Hiroshi Ohshima‡***

From the ‡Unit of Endogenous Cancer Risk Factors, International Agency for Research on Cancer, 150 Cours Albert Thomas, F 69372 Lyon Cedex 8, France, the ¶Unit of Nutrition and Cancer, International Agency for Research on Cancer, Lyon, France, the ||Laboratoire "Lésions des Acides Nucléiques," Service de Chimie Inorganique et Biologique, UMR 5046, Département de Recherche Fondamentale sur la Matière Condensée, CEA Grenoble, F 38054 Grenoble Cedex 9, France, and the §Department of Biochemistry, Kyoto Prefectural University of Medicine, Kyoto, 602-8566, Japan

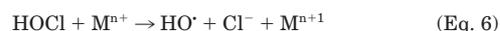
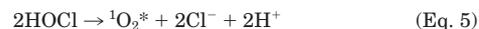
Activated human neutrophils secrete myeloperoxidase, which generates HOCl from H₂O₂ and Cl⁻. We have found that various (2'-deoxy)nucleosides react with HOCl to form chlorinated (2'-deoxy)nucleosides, including novel 8-chloro(2'-deoxy)guanosine, 5-chloro(2'-deoxy)cytidine, and 8-chloro(2'-deoxy)adenosine formed in yields of 1.6, 1.6, and 0.2%, respectively, when 0.5 mM nucleoside reacted with 0.5 mM HOCl at pH 7.4. The relative chlorination, oxidation, and nitration activities of HOCl, myeloperoxidase, and activated human neutrophils in the presence and absence of nitrite were studied by analyzing 8-chloro-, 8-oxo-7,8-dihydro-, and 8-nitroguanosine, respectively, using guanosine as a probe. 8-Chloroguanosine was always more easily formed than 8-oxo-7,8-dihydro- or 8-nitroguanosine. Using electrospray ionization tandem mass spectrometry, we show that several chlorinated nucleosides including 8-chloro(2'-deoxy)guanosine are formed following exposure of isolated DNA or RNA to HOCl. Micromolar concentrations of tertiary amines such as nicotine and trimethylamine dramatically enhanced chlorination of free (2'-deoxy)nucleosides and nucleosides in RNA by HOCl. As the G-463A polymorphism of the *MPO* gene, which strongly reduces myeloperoxidase mRNA expression, is associated with a reduced risk of lung cancer, chlorination damage of DNA/RNA and nucleosides by myeloperoxidase and its enhancement by nicotine may be important in the pathophysiology of human diseases associated with tobacco habits.

Chronic infection by bacteria, parasites, or viruses and tissue inflammation such as gastritis and colitis are recognized risk factors for human cancers at various sites (1, 2). In tissues with inflammation or infection, various cells in the host immune system are activated to produce various enzymes, including inducible nitric-oxide synthase, NAD(P)H oxidase, and myeloperoxidase, which generate reactive species such as nitric oxide, superoxide anion (O₂⁻), and HOCl, respectively (3). These reactive species can react with each other to form new and more reactive species. HOCl produced by myeloperoxidase re-

acts with Cl⁻, H₂O₂, O₂⁻, and nitrite to form Cl₂, ¹O₂^{*}, HO^{*}, and NO₂Cl, respectively (4–6).



It has been also reported that ¹O₂^{*} is generated when HOCl is added to buffer containing Cl⁻ at pH 7.2 (Equation 5) (7) and that HO^{*} is produced from HOCl in the presence of metallic ions (Equation 6) (8).



These oxidants can chlorinate, oxidize, and nitrate many biological molecules such as proteins and lipids. Recent studies have demonstrated that HOCl reacts with DNA and RNA to form chlorinated bases, including 8-chloroadenine (9, 10) and 5-chlorocytosine (4, 11, 12). In the presence of nitrite, HOCl, myeloperoxidase, and activated human neutrophils can also nitrate guanosine to form 8-nitroguanosine (8-nitro-Guo)¹ (13). HOCl, myeloperoxidase, and human neutrophils can chlorinate, oxidize, and nitrate tyrosine to form 3-chlorotyrosine, di-tyrosine, and 3-nitrotyrosine in the presence and the absence of nitrite (14–16). The relative chlorinating, oxidizing, and nitrating capabilities of the myeloperoxidase/neutrophil system have been studied, using phagocytosable fluorescein-conjugated particles and soluble phenolic compounds as probes (17). This study demonstrated that chlorination but not nitration occurred extensively in phagocytosed probes, whereas both nitration and chlorination were catalyzed by secreted myeloperoxidase under extracellular conditions.

In the present study, we have found that various nucleosides react with HOCl to form chlorinated nucleosides, including the novel 8-chloroguanosine (8-Cl-Guo). To compare chlorination, oxidation, and nitration activities of HOCl in the presence or

* This work was supported in part by a Special Training Award from the International Agency for Research on Cancer (to T. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*** To whom correspondence should be addressed. Fax: 33-4-72-73-80-88; E-mail: ohshima@iarc.fr.

¹ The abbreviations used are: 8-nitro-Guo, 8-nitroguanosine; 8-Cl-dAdo, 8-chloro-2'-deoxyadenosine; 5-Cl-dCyd, 5-chloro-2'-deoxycytidine; 8-Cl-dGuo, 8-chloro-2'-deoxyguanosine; 8-Cl-Guo, 8-chloroguanosine; ESI-MS, electrospray ionization mass spectrometry; HPLC-MS/MS, HPLC associated with tandem mass spectrometry; 8-oxo-dGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxo-Guo, 8-oxo-7,8-dihydroguanosine; TMA, trimethylamine.

absence of nitrite, we carried out the reactions with guanosine and analyzed the products 8-Cl-Guo, 8-oxo-7,8-dihydroguanosine (8-oxo-Guo), and 8-nitro-Guo by HPLC. We found that chlorination is a major reaction mediated by HOCl, even in the presence of nitrite. Tertiary amines such as trimethylamine (TMA) and nicotine at physiologically relevant concentrations were found to enhance the chlorination dramatically. These catalytic effects of tertiary amines could be important in host defense as well as in various pathophysiological conditions associated with chronic inflammation, especially those caused by tobacco habits.

EXPERIMENTAL PROCEDURES

Materials—8-Nitro-Guo and several chlorinated nucleosides, including 8-Cl-Guo, 8-chloro-2'-deoxyguanosine (8-Cl-dGuo), 8-chloro-2'-deoxyadenosine (8-Cl-dAdo), and 5-chlorocytidine (5-Cl-Cyd) were purchased from Biolog Life Science Institute (Bremen, Germany). 5-Chloro-2'-deoxycytidine (5-Cl-dCyd) was obtained from Sigma. 8-Oxo-Guo and 8-oxo-dGuo were from Cayman (Ann Arbor, MI). Human myeloperoxidase (EC 1.11.1.7) and Polymorphprep™ were obtained from Alexis (San Diego, CA) and Nycomed (Torshov, Oslo, Norway), respectively. Hank's buffered salt solution was provided by Life Technologies, Inc. All other chemicals were commercially available and were obtained from Aldrich, Sigma, Merck, and Fluka (Buchs, Switzerland).

Chemical Reaction of Nucleosides with Hypochlorous Acid—Chloride ion-free sodium hypochlorite was prepared, and its concentration was determined spectrophotometrically ($\epsilon_{292} = 350 \text{ M}^{-1} \text{ cm}^{-1}$) as described (18). The reaction was carried out in 1 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 0.5 mM guanosine or other nucleosides, 0.5 mM HOCl with or without 0.5 mM nitrite, and/or 0.05 mM nicotine at room temperature for 15 min. The reactions were started by adding HOCl to the reaction mixture and terminated by adding 0.1 ml of 50 mM *N*-acetylcysteine or methionine.

Enzymatic Reaction of Nucleosides with Human Myeloperoxidase—Reaction mixture (0.5 ml) contained 50 mM sodium phosphate buffer (final pH, 7.4–7.5), 0.5 mM guanosine, 0.1 mM H_2O_2 , and 100 mM sodium chloride with or without 0.1 mM nitrite and/or 0.05 mM nicotine. The reaction was initiated by adding 20–80 nM human myeloperoxidase and, after incubation at 37 °C for 30 min, was terminated with 0.05 ml of 50 mM *N*-acetylcysteine or methionine. The concentration of myeloperoxidase was determined spectrophotometrically ($\epsilon_{430} = 178 \text{ mM}^{-1} \text{ cm}^{-1}$) (4, 19).

Reaction of Nucleosides with Human Neutrophils—Neutrophils were isolated from the fresh blood of healthy volunteers by density gradient centrifugation with Polymorphprep™ (20). Residual red blood cells were removed by hypotonic lysis at 4 °C. The cells were resuspended in Hank's buffered salt solution and counted. Neutrophils (5×10^5 cells/ml) activated with 200 nM β -phorbol myristate acetate were incubated in 0.5 ml of Hank's buffered salt solution containing 0.5 mM guanosine with or without 0.1 mM nitrite and/or 0.05 mM nicotine at 37 °C for 1 h (final pH, 7.6). After the reaction, the cells were pelleted by centrifugation, and the supernatant was transferred to a new tube containing $\frac{1}{10}$ volume of 50 mM *N*-acetylcysteine or methionine.

HPLC Analysis of Modified Nucleosides—Chlorinated nucleosides, including 8-Cl-Guo, 8-Cl-dGuo, 5-Cl-dCyd, and 8-Cl-dAdo, were separated by a Spectraphysic HPLC (model SP 8810) equipped with a reverse-phase column (4.6 \times 250 mm Ultrasphere ODS column, 5 μm ; Beckman, Fullerton, CA) under isocratic conditions. A mobile phase of 20 mM ammonium formate buffer (pH 5.0) containing 5–15% methanol depending on the chlorinated nucleosides was used at a flow rate of 1 ml/min (5% for 5-Cl-dCyd and 5-Cl-Cyd, 10% for 8-Cl-dGuo and 8-Cl-Guo, and 15% for 8-Cl-dAdo and 8-Cl-Ado). The chlorinated nucleosides were detected by a Waters Lambda-Max model 481 UV/vis LC spectrophotometer at 260 nm. On-Line UV spectra were obtained with a Spectra Focus UV-visible photodiode array detector (Spectra Physics, Fremont, CA). The nature of the chlorinated nucleosides was confirmed by the identical chromatographic and spectrometric properties of the reaction products and authentic compounds. Identification of *N*-chloro-2'-deoxycytidine was based on the UV spectrum of the compound, which showed absorbance maxima at 227 and 273 nm as reported previously (4). 8-Oxo-Guo was analyzed by HPLC with electrochemical detection using a Shimadzu HPLC pump (model LC-10AD) equipped with the same reverse-phase column as above under isocratic conditions and a mobile phase of 12% methanol in 20 mM citric acid-sodium acetate buffer (pH 3.75) at a flow rate of 1 ml/min. A Coulochem II electrochemical detector (ESA Inc., Chelmsford, MA) was used with a guard cell (model 5020, +400 mV), a conditioning cell (model 5021, 0 mV), and an

analytical cell (model 5011; electrode 1, +150 mV; electrode 2, +350 mV). 8-Nitro-Guo was converted to 8-amino-guanosine with a small amount of sodium hydrosulfite and analyzed by a HPLC-electrochemical detector under the same conditions as 8-oxo-Guo analysis (21).

Electrospray Ionization-Mass Spectrometry (ESI-MS) and NMR Measurements—To obtain enough product, we carried out a large scale reaction (120 ml) with 0.5 mM guanosine, 0.5 mM HOCl, and 0.05 mM nicotine in 50 mM sodium phosphate buffer (pH 10). The product was purified using Sep-Pak C18 cartridges (10 g; Waters), followed by HPLC with a preparative Nucleosil C18 column (10 \times 250 mm; 7 μm ; Interchim, France) at a flow rate of 3 ml/min (10% methanol in water). ESI-MS measurements were performed on a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK). The purified sample dissolved in 0.1% formic acid was directly infused into the MS system by a syringe pump without a column at a flow rate of 0.3 $\mu\text{l}/\text{min}$. The sample was analyzed by the mass spectrometer in the positive ion mode with an electrospray capillary potential of 3.0 kV and a cone potential of 15 V. Collision-induced dissociation of the protonated molecular ions was carried out with a collision energy of 21 V at an argon collision cell pressure of 4.7×10^{-4} kPa. NMR samples were prepared by dissolving guanosine and its HPLC-purified chlorinated adduct in 100% D_2O . Analyses were performed at 20 °C with a Bruker DRX-500 spectrometer. The chemical shifts (ppm) were referenced to 3-(trimethylsilyl) propionate-2,2,3,3- d_4 in D_2O as an external standard.

Reaction of Calf Thymus DNA and Calf Liver RNA with HOCl and Detection of Chlorinated Nucleosides by HPLC Associated with Tandem Mass Spectrometry (HPLC-MS/MS)—Calf thymus DNA or calf liver RNA (1 mg/ml) was incubated at 37 °C for 15 min in the presence and the absence of 200 μM HOCl with or without 20 μM nicotine in 50 mM phosphate buffer (pH 7.4). The reactions were stopped by the addition of excess *N*-acetylcysteine. The reaction mixtures were dialyzed against water at 4 °C overnight. DNA or RNA samples (100 μg in 60 μl) were hydrolyzed enzymatically with nuclease P1 and alkaline phosphatase. Proteins in the samples were removed by treatment with chloroform. Quantification of chlorinated nucleosides was performed following separation on an octadecylsilyl silica gel column (2.1 \times 250 mm; Alltech, Deerfield, IL) at a flow rate of 0.2 ml/min and linear gradient between 0 and 30% CH_3CN in 2 mM ammonium formate over the course of 35 min. To monitor unmodified nucleosides, the output of the column was connected to a UV detector (UV 4000; Merck) set at 260 nm. The UV detector was connected to the API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray source (SCIEX, Thornhill, Canada). An auxiliary gas (nitrogen) heated to 400 °C was used at a flow rate of 8 liters/min to facilitate the ionization and to improve the sensitivity of the detection. The total eluent was directed to the turbo ion spray source. Chlorinated nucleosides were detected using electrospray ionization tandem mass spectrometry in the multiple reaction monitoring mode (22, 23). Specific transitions used to detect chlorinated nucleosides in the positive ionization mode were those between the molecular cation of the chlorinated nucleosides and the characteristic chlorinated daughter ion formed from the loss of the sugar moiety: 8-Cl-Guo, m/z 318 \rightarrow 186; 8-Cl-dGuo, m/z 302 \rightarrow 186; 8-Cl-Ado, m/z 302 \rightarrow 170; 8-Cl-dAdo, m/z 286 \rightarrow 170; 5-Cl-Cyd, m/z 278 \rightarrow 146, and 5-Cl-dCyd, m/z 262 \rightarrow 146. Linear calibration curves were obtained for all of the studied chlorinated nucleosides with a correlation coefficient of at least 0.99. The limit of detection was found to be around 0.1 pmol for the different nucleosides analyzed. The hydrolyzed DNA or RNA samples (20 μl) were then injected onto the HPLC-MS/MS system. The amounts of dGuo or Guo were determined by external calibration using UV detection. The amounts of chlorinated nucleosides were expressed as the number of modified nucleosides/ 10^5 dGuo or Guo.

RESULTS

Identification of 8-Cl-Guo as a Major Reaction Product of Guanosine with HOCl—Reverse-phase HPLC-UV analyses of products formed by the reaction of guanosine with HOCl in phosphate buffer (pH 7.4) revealed formation of several new compounds. We isolated a major compound, which was eluted at 12 min (Fig. 1A). Because the C-8 position of guanosine is often modified by radicals such as HO^\bullet and NO_2^\bullet to form 8-oxo-Guo and 8-nitro-Guo, respectively (21), we speculated that this unknown compound might be 8-Cl-Guo. We synthesized 8-Cl-Guo (24), and it co-eluted with the unknown compound and also had an identical UV spectrum (Fig. 1A). The mass spectra of the isolated product and standard 8-Cl-Guo, obtained by ESI-MS, showed the same major ion at m/z 318 $[\text{M} + \text{H}]^+$ and one

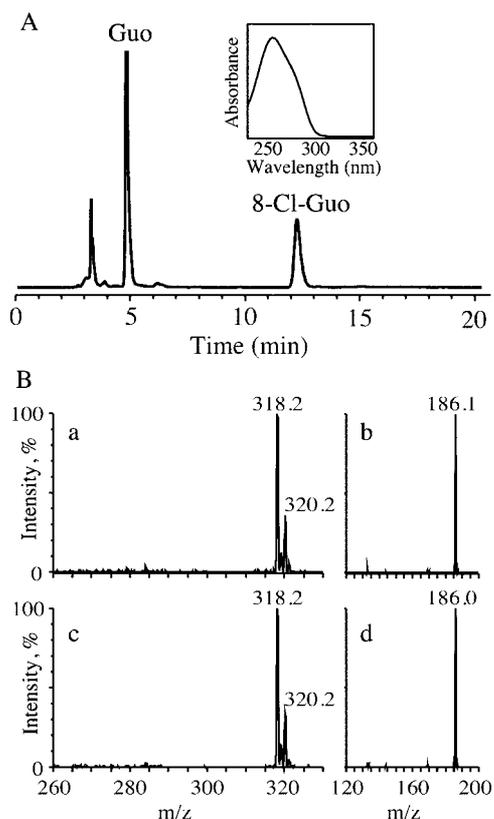


FIG. 1. Identification of a major unknown product formed by reaction of guanosine with HOCl. A, a typical reverse-phase HPLC chromatogram. Guanosine (0.5 mM) was incubated with HOCl (0.5 mM) in 50 mM phosphate buffer, pH 7.4. Inset, UV spectrum of 8-Cl-Guo. B, ESI-MS spectra of authentic 8-Cl-Guo (a) and its daughter ions from m/z 318 (b) and ESI-MS spectra of the isolated product (c) and its daughter ions from m/z 318 (d).

at m/z 186 $[M - \text{ribose} + 2H]^+$ (Fig. 1B). The ^1H NMR spectrum of 8-Cl-Guo in D_2O showed a set of signals of the intact ribose moiety and a lack of the H-8 aromatic proton signal (Fig. 2). The ^1H NMR spectrum of isolated 8-Cl-Guo was identical to that of authentic 8-Cl-Guo. On the basis of these findings, we concluded that 8-Cl-Guo was the major compound detected by our HPLC-UV method.

Formation of 8-Cl-, 8-Oxo-, and 8-Nitro-Guo by HOCl with or without Nitrite—The reaction between HOCl and guanosine at pH 7.4 was very rapid, being complete within 1 min. However, we routinely terminated the reaction after 15 min of incubation by adding excess *N*-acetylcysteine or methionine, which did not affect the yield of 8-Cl-Guo, compared with no addition (Fig. 3A). Recent studies have demonstrated that myeloperoxidase in the presence of nitrite can nitrate guanosine to form 8-nitro-Guo (13) and that guanosine is easily oxidized to 8-oxo-Guo by oxidants such as HO^\bullet and singlet oxygen (25). We analyzed these guanosine derivatives (8-oxo-Guo and 8-nitro-Guo) after reaction of guanosine with HOCl in the presence or absence of nitrite and compared the levels with that of 8-Cl-Guo. Under our standard conditions (0.5 mM each of guanosine and HOCl at pH 7.4), about 30 and 7% of guanosine disappeared after reaction with HOCl in the absence and the presence of 0.5 mM nitrite, respectively. The yields of 8-Cl-Guo were 1.6 and 0.2%, and those of 8-oxo-Guo were 0.1 and 0.01% in the absence or presence of 0.5 mM nitrite, respectively. The yield of 8-nitro-Guo was 0.01% in the reaction with 0.5 mM each of guanosine, HOCl, and nitrite at pH 7.4. Thus only 5–6% of the loss of guanosine could be explained by formation of the identified products (8-Cl-, 8-oxo-, and 8-nitro-Guo), suggesting that the

majority of compounds were not detected by our HPLC-UV method.

To obtain further information on the reaction of guanosine with HOCl at pH 7.4, the concentrations of guanosine and HOCl were varied. When the guanosine concentration was kept constant at 0.5 mM, the yield of 8-Cl-Guo increased dose-dependently with increasing concentrations of HOCl up to 1 mM but decreased at > 2 mM (data not shown). When the concentrations of guanosine and HOCl were kept constant at 0.5 mM, increasing concentrations of nitrite decreased the yield of 8-Cl-Guo. When the ratio of nitrite concentration to HOCl concentration was fixed at 1.0, the yield of 8-Cl-Guo increased dose-dependently with their increasing concentration up to 2 mM. The yield of 8-oxo-Guo increased dose-dependently with HOCl up to 2 mM but diminished at higher HOCl concentrations in the presence or absence of nitrite. The yield of 8-nitro-Guo increased proportionately with increasing concentrations of HOCl and nitrite, when the ratio of nitrite to HOCl concentration was fixed at 1.0.

Effects of pH and Chloride Ion on the Formation of 8-Cl-Guo by HOCl—Recently Henderson *et al.* (4) reported that the formation of 5-Cl-dCyd from 2'-deoxycytidine and HOCl under acidic conditions is mediated by a Cl_2 -like species formed from HOCl and Cl^- (Equation 1) but not by HOCl itself. We examined the effects of pH and Cl^- on the formation of 8-Cl-Guo, 8-oxo-Guo, and 8-nitro-Guo by HOCl with or without nitrite (Fig. 4). When nitrite was present in the reaction mixture, more 8-Cl-Guo was formed under alkaline conditions (pH > 9) with or without Cl^- (Fig. 4A), but without nitrite an additional peak of the yield at around pH 7.7 was observed (Fig. 4B). In the presence of Cl^- , the yields of 8-Cl-Guo were significantly increased under acidic conditions (pH < 4.5) with or without nitrite (Fig. 4, A and B). On the other hand, more 8-oxo-Guo was formed at acidic pH in the presence of nitrite with or without Cl^- or in the absence of nitrite with Cl^- (Fig. 4, C and D). The yield of 8-oxo-Guo was maximum at pH ~ 6.5 in the absence of both nitrite and Cl^- (Fig. 4D). The optimal pH for formation of 8-nitro-Guo from guanosine with HOCl and nitrite was 8.5 (Fig. 4E) with or without Cl^- . The yield of 8-Cl-Guo at pH 7.4 from the reaction of 0.5 mM each of guanosine, HOCl, and nitrite was 20–30 times higher than those of 8-oxo-Guo and 8-nitro-Guo.

The presence of 100 mM NaCl before the addition of 0.5 mM HOCl with or without 0.5 mM nitrite significantly increased the yield of 8-Cl-Guo only under acidic conditions (pH < 4.5) but not at pH > 5 (Figs. 4 and 5). As reported previously for 5-Cl-dCyd formation (4), the order of addition of Cl^- and HOCl was important for the chlorination of guanosine at acidic pH. Addition of Cl^- after HOCl to the reaction mixture (pH 4.5) did not increase the yield of 8-Cl-Guo. The presence of Cl^- did not affect the formation of 8-oxo-Guo by HOCl in the presence of nitrite (Fig. 4C) but inhibited it in the absence of nitrite (Fig. 4D). Cl^- did not affect formation of 8-nitro-Guo by HOCl and nitrite (Fig. 4E).

Effects of Tertiary Amines and Related Compounds on the Formation of Modified Guanosine Derivatives by HOCl—Prütz (26) recently reported that tertiary amines such as TMA and quinine accelerate HOCl-mediated oxidation reactions. We examined the effects of various tertiary amines and related compounds on the formation of 8-Cl-, 8-oxo-, and 8-nitro-Guo under physiological conditions (pH 7.4). As shown in Fig. 6A, the presence of tertiary amines such as TMA, nicotine, and quinine at 50 μM led to a 3–4-fold enhancement in the yield of 8-Cl-Guo formed from 0.5 mM each of guanosine and HOCl. More notably, when 0.5 mM nitrite was present, these tertiary amines increased yields of 8-Cl-Guo 25–30-fold, compared with that

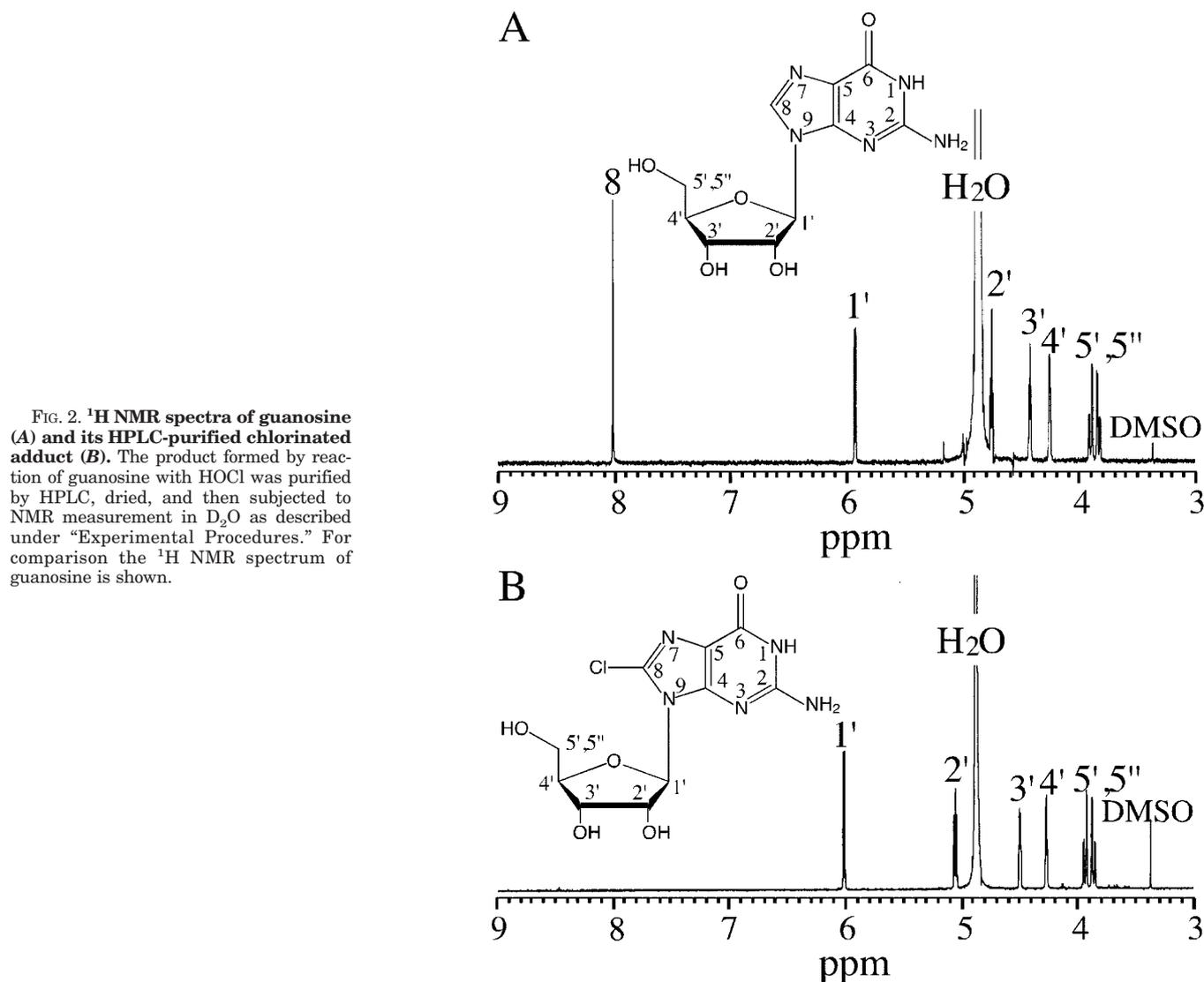


FIG. 2. ^1H NMR spectra of guanosine (A) and its HPLC-purified chlorinated adduct (B). The product formed by reaction of guanosine with HOCl was purified by HPLC, dried, and then subjected to NMR measurement in D_2O as described under "Experimental Procedures." For comparison the ^1H NMR spectrum of guanosine is shown.

without tertiary amines. A nicotine-related compound with a tertiary amine structure (*N*-methylpyrrolidine) catalyzed chlorination, but those without such a structure (nornicotine and pyridine) did not. Other tertiary and quaternary amines such as cotinine, caffeine, creatinine, betaine, and trimethylamine *N*-oxide did not exert enhancing effects on the formation of 8-Cl-Guo by HOCl. Nicotine enhanced formation of 8-oxo-Guo by HOCl 30- and 250-fold in the absence and the presence of nitrite, respectively (Fig. 6B). TMA and quinine also strongly enhanced the formation of 8-oxo-Guo by HOCl with or without nitrite (Fig. 6B). Formation of 8-nitro-Guo by HOCl and nitrite was also enhanced 2-fold by nicotine and TMA (Fig. 6C). As shown in Fig. 7A, the yield of 8-Cl-Guo formed from 0.5 mM each of guanosine and HOCl with or without 0.5 mM nitrite increased dose-dependently with increasing concentrations of nicotine and TMA up to 50–100 μM , but higher concentrations (> 150 μM) decreased the yield. Nicotine and TMA also enhanced the yields of 8-oxo-Guo and 8-nitro-Guo dose-dependently (Fig. 7, B and C).

Effects of Scavengers and Ferric or Ferrous Ion on the Formation of 8-Cl-, 8-Oxo-, and 8-Nitro-Guo by HOCl and Nicotine—As shown in Fig. 8, *N*-acetylcysteine, ascorbic acid, sodium azide, glutathione, taurine, and seleno-DL-methionine at 5 mM (10 times higher concentrations than HOCl) completely inhibited the formation of 8-Cl-Guo and 8-oxo-Guo by the reaction of 0.5 mM each of guanosine and HOCl in the presence of

0.05 mM nicotine with or without 0.5 mM nitrite. Even at 0.5 mM, these compounds inhibited the formation of 8-Cl-Guo strongly. In contrast, mannitol and dimethyl sulfoxide inhibited the formation of 8-Cl-Guo and 8-oxo-Guo by HOCl and nicotine only slightly. *N*-Acetylcysteine, ascorbic acid, glutathione, and seleno-DL-methionine also strongly inhibited the formation of 8-nitro-Guo by HOCl and nitrite in the presence of nicotine.

Ferric (Fe^{3+}) and ferrous (Fe^{2+}) ions did not affect the formation of 8-Cl- and 8-nitro-Guo from guanosine and HOCl, with or without nitrite. However, the yields of 8-oxo-Guo formed by HOCl without nitrite were about 20 times greater in the presence of Fe^{3+} and Fe^{2+} than without these metal ions, suggesting that HOCl in the presence of Fe^{3+} or Fe^{2+} generates HO^\cdot . The presence of nitrite reduced the formation of 8-oxo-Guo by HOCl and Fe^{3+} or Fe^{2+} by ~30–40% (Table I).

Formation of Chlorinated Nucleosides by HOCl in the Presence and Absence of Nitrite and/or Nicotine—We examined the reactions of various nucleosides with HOCl in the presence and the absence of nitrite and tertiary amines. As shown in Fig. 9A, 2'-deoxyguanosine, 2'-deoxycytidine, and 2'-deoxyadenosine reacted with HOCl to form 8-Cl-dGuo, 5-Cl-dCyd, and 8-Cl-dAdo, respectively, when the reactions were terminated by adding *N*-acetylcysteine or methionine. When the reaction of 2'-deoxycytidine with HOCl was not terminated by the addition of excess *N*-acetylcysteine or methionine, *N*-chloro-2'-deoxy-

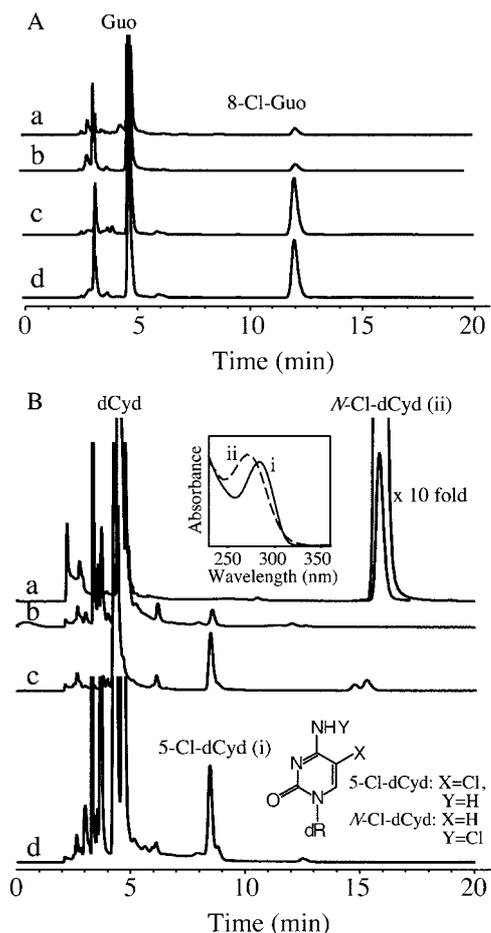


FIG. 3. Typical reverse-phase HPLC chromatograms from analyses of reaction products of guanosine (A) and 2'-deoxycytidine (B) incubated with HOCl in the absence or presence of nicotine and the effects of termination of the reaction by excess *N*-acetylcysteine. The reaction mixture containing 0.5 mM nucleoside and 0.5 mM HOCl in 50 mM sodium phosphate buffer (pH 7.4) was incubated at room temperature for 15 min in the absence (a and b) or presence (c and d) of 0.05 mM nicotine. The effects of termination of the reactions by adding excess *N*-acetylcysteine were also examined. The mixture was analyzed without (a and c) or with (b and d) addition of excess (5 mM) *N*-acetylcysteine. Inset, UV spectra and structures of 5-Cl-dCyd (i) and *N*-chloramine of 2'-deoxycytidine (*N*-Cl-dCyd) (ii).

tidine was a major product (Fig. 3B). Similarly, *N*-chloro-2'-deoxyadenosine was exclusively formed if the reaction was not terminated by *N*-acetylcysteine or methionine (data not shown). In contrast, there were no significant differences in chromatograms analyzed for (2'-deoxy)guanosine-HOCl reaction products with or without *N*-acetylcysteine or methionine (Fig. 3A). Under the present conditions, thymidine did not react with HOCl to form modified compounds. No significant loss of thymidine or formation of new products were detected by our HPLC-UV method. In the absence of nitrite and tertiary amine, the yields of 5-Cl-dCyd and 8-Cl-dGuo from the reaction of 0.5 mM each of nucleosides and HOCl were $\sim 10 \mu\text{M}$, but the yield of 8-Cl-dAdo was several times lower than the others (Fig. 9A). Nicotine enhanced the chlorination of 2'-deoxycytidine and of guanosine in the presence or absence of nitrite at neutral pH. Interestingly, nicotine inhibited the formation of *N*-chloro-2'-deoxycytidine by HOCl (Fig. 3B, line c). Nitrite and nicotine inhibited formation of 8-Cl-dAdo from 2'-deoxyadenosine and HOCl (Fig. 9A).

To compare the reaction rates for chlorination of various 2'-deoxynucleosides, we carried out the reaction with HOCl in the presence of two different nucleosides both at 0.5 mM. For-

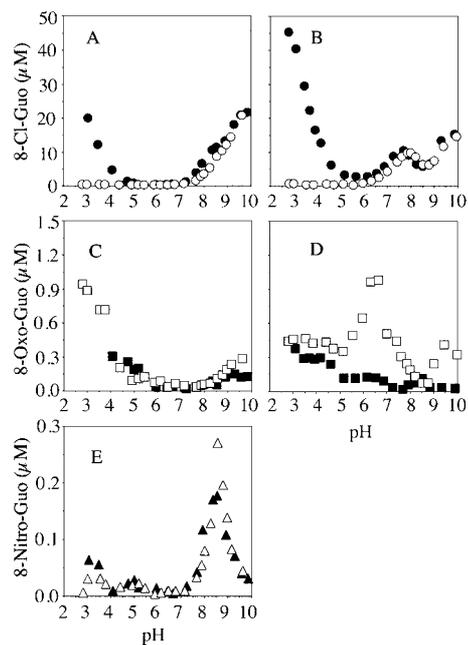


FIG. 4. Effects of pH and chloride ion on the formation of 8-Cl-, 8-oxo-, and 8-nitro-Guo. The reaction mixture containing 0.5 mM guanosine and 0.5 mM HOCl in 50 mM sodium phosphate buffer was incubated at room temperature for 15 min in the absence (open symbols) or presence (closed symbols) of 100 mM NaCl. The reaction mixtures also contained 0.5 mM nitrite (A, C, and E).

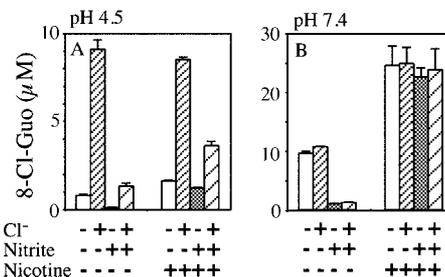


FIG. 5. Formation of 8-Cl-Guo by HOCl with and without chloride ion, nitrite, and/or nicotine at pH 4.5 (A) and at pH 7.4 (B). The reaction mixture containing 0.5 mM guanosine and 0.5 mM HOCl in 50 mM sodium phosphate buffer (pH 4.5 or 7.4) was incubated at room temperature for 15 min in the absence or presence of the compound as indicated. The reactions were terminated by adding 5 mM *N*-acetylcysteine. The means \pm S.D. ($n = 3$) are presented.

mation of 8-Cl-dGuo by HOCl in the absence of tertiary amine was enhanced by $\sim 20\%$ in the presence of 2'-deoxyadenosine but was inhibited by 5 and 40% in the presence of thymidine and 2'-deoxycytidine, respectively. Formation of 5-Cl-dCyd was not affected by 2'-deoxyadenosine but was inhibited by 80% by the presence of 2'-deoxyguanosine. 8-Cl-dAdo formation was completely inhibited by the presence of 2'-deoxyguanosine and 2'-deoxycytidine. On the basis of these experiments, the order of ease of chlorination of different nucleosides by HOCl was determined to be as follows: 2'-deoxyguanosine \geq 2'-deoxycytidine \gg 2'-deoxyadenosine (and no reaction with thymidine).

Modification of Guanosine and Other Nucleosides by the Myeloperoxidase- $\text{H}_2\text{O}_2\text{-Cl}^-$ System—We have examined chlorination, oxidation, and nitration reactions mediated by the human myeloperoxidase- $\text{H}_2\text{O}_2\text{-Cl}^-$ system, using guanosine as a probe. 8-Cl-Guo was formed in a time- and myeloperoxidase dose-dependent manner during 60 min of incubation in the presence of H_2O_2 and Cl^- . Similar to the enhancement of HOCl-mediated chlorination of guanosine by nicotine, the myeloperoxidase-mediated formation of 8-Cl-Guo was also dramatically (50–80 times) enhanced by nicotine, compared with

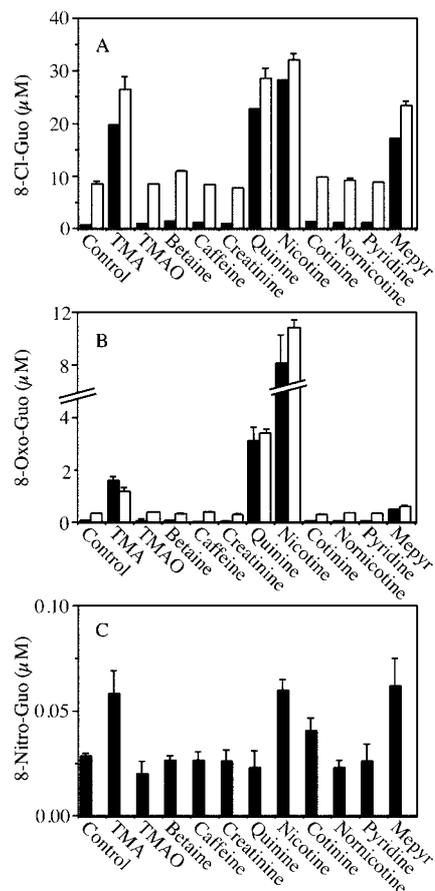


FIG. 6. Effects of tertiary amines and related compounds on the formation of 8-Cl-, 8-oxo-, and 8-nitro-Guo by HOCl. The reaction mixture containing 0.5 mM guanosine and 0.5 mM HOCl with (closed bars) or without (open bars) 0.5 mM nitrite in 50 mM sodium phosphate buffer (pH 7.4) was incubated at room temperature for 15 min in the absence (control) or presence of 0.05 mM tertiary amines. The reactions were terminated by adding 5 mM *N*-acetylcysteine. TMAO, trimethylamine *N*-oxide; Mepyr, *N*-methylpyrrolidine. The means \pm S.D. ($n = 3$) are presented.

the reaction without nicotine (Fig. 10A). The presence of nitrite did not affect chlorination. In contrast, the level of 8-oxo-Guo was decreased rapidly (< 5 min incubation) in the complete system, its level being half of the background level of 8-oxo-Guo that was originally present in the guanosine solution (Fig. 10B). This decrease was dependent on the myeloperoxidase dose. Omission of Cl^- further accelerated the decrease in 8-oxo-Guo levels. The decrease in 8-oxo-Guo was faster in the presence of nitrite than in its absence (data not shown). Small amounts of 8-nitro-Guo were formed by myeloperoxidase in the presence of H_2O_2 and nitrite (Fig. 10C), and this formation was time- and myeloperoxidase dose-dependent (data not shown).

The yields of 8-Cl-Guo and 8-oxo-Guo formed by the reaction of guanosine with the human myeloperoxidase- H_2O_2 - Cl^- system were increased significantly in the presence of nicotine, especially at $> 5 \mu\text{M}$ (Fig. 11, A and B). Similarly to chemical reactions with HOCl, various chlorinated nucleosides were also formed by this system, and nicotine enhanced chlorination of nucleosides (Fig. 9B). 8-Cl-dAdo was formed by the human myeloperoxidase system only in the presence of nicotine and without nitrite, but the yield was 20 times lower than those of 8-Cl-dGuo and 5-Cl-dCyd.

Activated Human Neutrophils Can Chlorinate, Oxidize, and Nitrate Guanosine—As shown in Fig. 12, formation of 8-Cl-Guo by human neutrophils required neutrophil activation by β -phorbol myristate acetate. Nonactivated neutrophils also

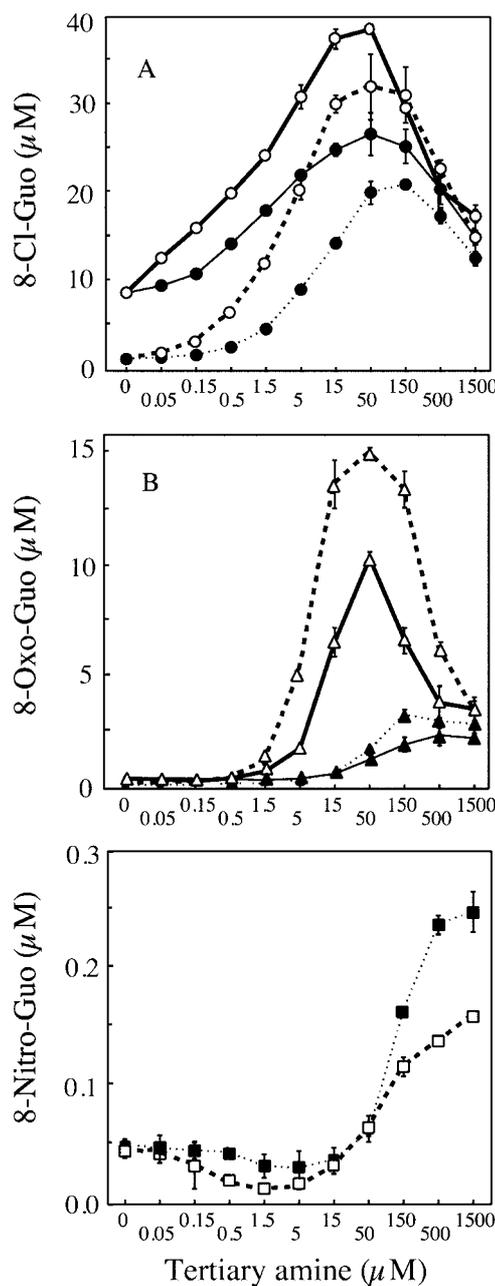


FIG. 7. Effect of nicotine and TMA concentration on the formation of 8-Cl-, 8-oxo-, and 8-nitro-Guo by HOCl. The reaction mixture contained 0.5 mM guanosine and 0.5 mM HOCl with (dotted lines) or without (solid lines) 0.5 mM nitrite in 50 mM sodium phosphate buffer (pH 7.4). It was incubated at room temperature for 15 min in the presence of various concentrations of nicotine (open symbols) or TMA (closed symbols). The reactions were terminated by adding 5 mM *N*-acetylcysteine. The means \pm S.D. ($n = 3$) are presented.

formed 8-Cl-Guo but only in low yields. The presence of 0.05 mM nicotine enhanced 4–7-fold the formation of 8-Cl-Guo by activated neutrophils both in the presence and the absence of nitrite (Fig. 12, A and B). The presence of 6 mM methionine (a scavenger of HOCl), 10 mM sodium azide (a heme enzyme inhibitor), and 10 $\mu\text{g}/\text{ml}$ catalase (a scavenger of H_2O_2) blocked the formation of 8-Cl-Guo by activated neutrophils in the presence of 0.05 mM nicotine. Heat-inactivated catalase did not inhibit chlorination. Addition of 10 $\mu\text{g}/\text{ml}$ superoxide dismutase increased the yield of 8-Cl-Guo 3–4-fold, probably because it increased the supply of H_2O_2 or protected myeloperoxidase from inactivation (13). 8-Oxo-Guo levels decreased during incubation of guanosine with activated neutrophils to a level

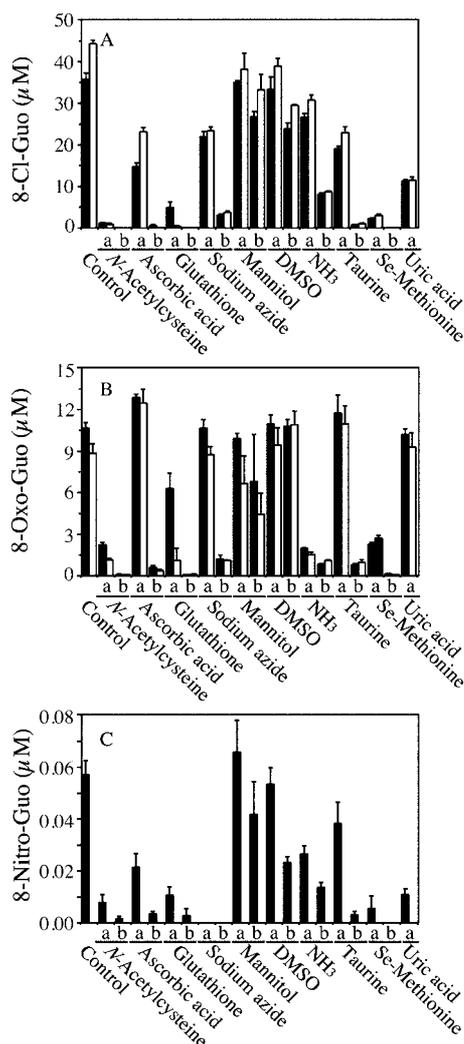


FIG. 8. Effect of scavengers and anti-oxidants on the formation of 8-Cl- (A), 8-oxo- (B), and 8-nitro-Guo (C) by HOCl. The reaction mixture contained 0.5 mM guanosine, 0.5 mM HOCl and 0.05 mM nicotine with (closed bars) or without (open bars) 0.5 mM nitrite in 50 mM sodium phosphate buffer (pH 7.4). It was incubated at room temperature for 15 min in the absence (control) or presence of 0.5 mM (a) or 5 mM (b) of test compounds. The reactions were terminated by adding 5 mM *N*-acetylcysteine. DMSO, dimethyl sulfoxide. The means \pm S.D. ($n = 3$) are presented.

lower than the background present in the guanosine solution, indicating that 8-oxo-Guo was decomposed during the reaction (Fig. 10E). 8-Nitro-Guo was also formed by human neutrophils, but the yields were very low ($< 0.005 \mu\text{M}$) even in the presence of 0.05 mM nicotine (Fig. 10F). We examined the effect of nicotine concentration on the formation of 8-Cl-Guo by activated human neutrophils (Fig. 11C). The yield of 8-Cl-Guo significantly increased with increasing nicotine concentrations ($> 5 \mu\text{M}$) with or without nitrite. The yield of 8-Cl-Guo formed by activated neutrophils in the presence of 150 μM nicotine was about 10 times higher than without nicotine (Fig. 11C). Nicotine did not increase the formation of 8-oxo-Guo mediated by activated neutrophils (Fig. 11D). Activated human neutrophils also generated 8-Cl-dAdo, 8-Cl-dGuo, and 5-Cl-dCyd from the corresponding nucleosides (Fig. 9C). The yield of 8-Cl-dAdo was, however, 10 and 20 times lower than those of 8-Cl-dGuo and 5-Cl-dCyd, respectively.

Formation of Chlorinated Nucleosides in DNA and RNA—To study whether chlorinated nucleosides are formed in DNA and RNA, we have developed a tandem mass spectrometry-based method for their detection. The positive electrospray ionization

TABLE I
Effect of ferrous and ferric ions on HOCl-mediated formation of 8-Cl-, 8-oxo-, and 8-nitro-Guo

The reaction mixture contained 0.5 mM guanosine, 0.5 mM HOCl, with or without 0.5 mM nitrite and/or 0.05 mM FeCl_2 or FeCl_3 and 0.05 mM EDTA in 0.5 ml of 50 mM sodium phosphate buffer, pH 7.4. The reaction was carried out at room temperature for 15 min and terminated by adding excess (5 mM) *N*-acetylcysteine. The means \pm S.D. ($n = 3$) are presented.

	8-Oxo-Guo			
	-HOCl		+HOCl	
	- Nitrite	+ Nitrite	- Nitrite	+ Nitrite
	μM			
Control	0.06 \pm 0.00	0.06 \pm 0.00	0.17 \pm 0.01	0.06 \pm 0.00
FeCl_3	0.54 \pm 0.05	0.57 \pm 0.07	3.43 \pm 0.34	2.37 \pm 0.15
FeCl_2	0.79 \pm 0.12	0.52 \pm 0.04	3.18 \pm 0.55	2.01 \pm 0.14
	+HOCl			
	8-Cl-Guo		8-Nitro-Guo	
	- Nitrite	+ Nitrite	- Nitrite	+ Nitrite
	μM			
Control	9.81 \pm 0.11	1.25 \pm 0.03	0	0.07 \pm 0.001
FeCl_3	9.47 \pm 0.18	1.19 \pm 0.14	0	0.06 \pm 0.003
FeCl_2	9.64 \pm 0.25	1.20 \pm 0.11	0	0.07 \pm 0.001

mass spectra of the different chlorinated nucleosides exhibit major intense ions corresponding to the protonated molecular ion $[\text{M} + \text{H}]^+$. The sodium adduct ion was also detectable. As expected, the $[\text{M} - \text{H}]^-$ ion was the main intense ion obtained in the negative mode for all studied nucleosides. However, the detected ions were more intense in the positive mode; thus the latter mode was chosen to improve the sensitivity of the detection. In the positive mode, the main fragmentation of the protonated molecular ion of the chlorinated 2'-deoxynucleosides corresponds to the cleavage of the *N*-glycosidic bond. This leads to the loss of the 2-deoxyribose moiety and the release of the protonated base $[\text{M} - (2\text{-deoxyribose}) + 2\text{H}]^+$, as already observed for other 2'-deoxynucleosides (23). For example, the collision-induced dissociation of the protonated molecular ion of 8-Cl-dGuo at $m/z = 302$ gave rise to a major fragment at $m/z = 186$. Collision-induced dissociation of the protonated molecular ion of the corresponding ribonucleoside, 8-Cl-Guo at $m/z = 318$, generated an intense ion at $m/z = 186$. This corresponds to the loss of the sugar moiety (Fig. 13). Similar patterns of fragmentation were observed for the 8-chloroadenine and 5-chlorocytosine nucleosides (data not shown).

The chlorinated nucleosides were detected in the multiple reaction monitoring mode using specific transitions (see "Experimental Procedures"). Under our HPLC conditions, the different chlorinated nucleoside derivatives were totally separated as illustrated in Fig. 14. The results, expressed as the number of modified nucleosides/ 10^5 dGuo or Guo for DNA or RNA, respectively, are reported in Table II. Chlorinated nucleosides were detected in both DNA and RNA samples treated with HOCl. It is interesting to note that 5-Cl-dCyd was a major chlorinated nucleoside found in the DNA samples, whereas 8-Cl-Guo was detected at the highest level in the RNA samples. The presence of nicotine increased the level of 8-Cl-Guo about 10-fold in RNA samples compared with those treated with HOCl alone.

DISCUSSION

We have demonstrated that HOCl, human myeloperoxidase in the presence of H_2O_2 and Cl^- , and activated human neutrophils in the presence of Cl^- can react with various (2'-deoxy)nucleosides to form chlorinated (2'-deoxy)nucleosides. In addition to previously reported 5-Cl-(d)Cyd (4, 11, 12) and 8-Cl-(d)Ado (9, 10), we have identified 8-Cl-(d)Guo as a major

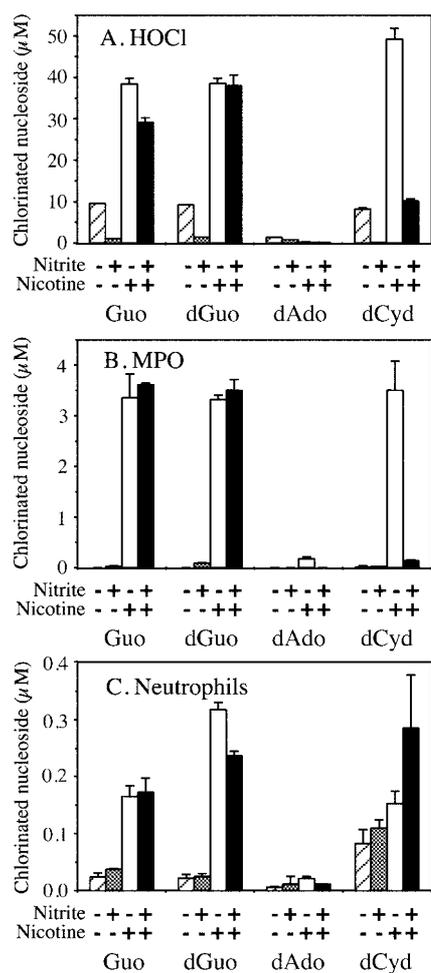


FIG. 9. Formation of chlorinated nucleosides by HOCl (A), myeloperoxidase (B), and activated human neutrophils (C) with and/or without nitrite and nicotine. A, the reaction mixture contained 0.5 mM nucleoside (guanosine, 2'-deoxyguanosine, 2'-deoxyadenosine, or 2'-deoxycytidine) and 0.5 mM HOCl with and/or without 0.5 mM nitrite and 0.05 mM nicotine in 50 mM sodium phosphate buffer (pH 7.4). It was incubated at room temperature for 15 min. B, the reaction mixture contained 0.5 mM nucleoside with and/or without 0.5 mM nitrite and 0.05 mM nicotine in 50 mM sodium phosphate buffer (pH 7.4). It was incubated at 37 °C for 30 min in the presence of 80 nM human myeloperoxidase, 0.1 mM H₂O₂ and 100 mM NaCl. C, the reaction mixture contained 0.5 mM nucleoside with and/or without 0.1 mM nitrite and 0.05 mM nicotine in 0.5 ml of Hank's buffered salt solution (pH 7.4). It was incubated with human neutrophils (5×10^5 cells/ml) activated by 200 nM β -phorbol myristate acetate at 37 °C for 60 min. All of the reactions were terminated by adding 5 mM methionine. The means \pm S.D. ($n = 3$) are presented.

new product formed by the reaction of (2'-deoxy)guanosine with HOCl as well as with the human myeloperoxidase system and activated neutrophils. Furthermore, using electrospray ionization tandem mass spectrometry, we have shown that several chlorinated nucleosides including 8-Cl-dGuo and 8-Cl-Guo are formed following exposure of calf thymus DNA or calf liver RNA to HOCl. Although the reaction of HOCl and nucleosides such as guanosine was reported in the 1970s to form unknown products (27), this study is the first to isolate the product and identify it as 8-Cl-(d)Guo. The yield of 8-Cl-Guo under our standard conditions (0.5 mM each of guanosine and HOCl, pH 7.4) was 1.6%, whereas about 30% of the guanosine disappeared during the reaction with HOCl. This suggests that the majority of the compounds were not detected by our HPLC-UV method. We are currently identifying other products formed by the reaction between (2'-deoxy)guanosine and HOCl.

Recent studies show that nitrating and oxidizing agents such

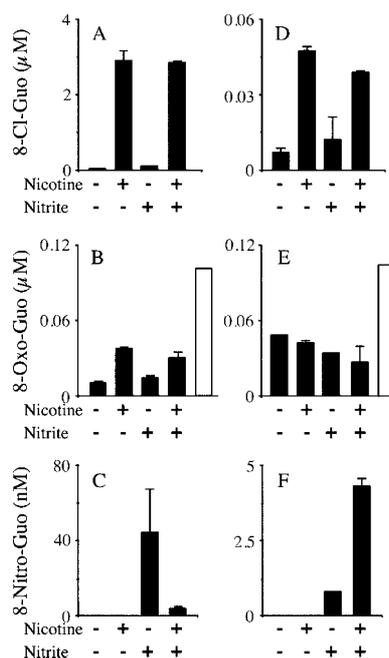


FIG. 10. Effects of nicotine and nitrite on the formation of 8-Cl-Guo (A and D), 8-oxo-Guo (B and E), and 8-nitro-Guo (C and F) by human myeloperoxidase (A-C) and activated human neutrophils (D-F). A-C, the reaction mixture contained 0.5 mM guanosine with and/or without 0.5 mM nitrite and 0.05 mM nicotine in 50 mM sodium phosphate buffer (pH 7.4). It was incubated at 37 °C for 30 min in the presence of 80 nM human myeloperoxidase, 0.1 mM H₂O₂, 100 mM NaCl with and/or without 0.1 mM nitrite and 0.05 mM nicotine. D-F, the reaction mixture contained 0.5 mM nucleoside with and/or without 0.1 mM nitrite and 0.05 mM nicotine in 0.5 ml of Hank's buffered salt solution (pH 7.4). It was incubated with human neutrophils (5×10^5 cells/ml) activated by 200 nM β -phorbol myristate acetate at 37 °C for 60 min. All of the reactions were terminated by adding 5 mM *N*-acetylcysteine. Open bars (B and E) indicate the concentrations of 8-oxo-Guo, which was originally present in the guanosine solution used for the reaction. The means \pm S.D. ($n = 3$) are presented.

as NO₂Cl, HO[•], and singlet oxygen are formed from HOCl in the presence of nitrite, O₂⁻, and metallic ions (Equations 1-6) (6-8, 13, 15). For these reasons, we compared the relative chlorination, oxidation, and nitration activities of HOCl, myeloperoxidase, and activated neutrophils in the presence and the absence of nitrite by analyzing 8-Cl-, 8-oxo-, and 8-nitro-Guo, respectively, in the reactions with guanosine. We found that chlorination occurs more easily than oxidation or nitration in each reaction.

Henderson *et al.* (4) recently reported that formation of 5-Cl-dCyd is enhanced by the presence of Cl⁻ under acidic conditions but not under neutral conditions. On the basis of these findings, they proposed that a Cl₂-like species, formed by the reaction of HOCl with Cl⁻ and H⁺ (Equation 1), is the chlorinating intermediate in 5-Cl-dCyd formation. In our experiments, 8-Cl-Guo formation by HOCl was similarly enhanced by the presence of Cl⁻ under acidic conditions, suggesting that a Cl₂-like species is involved in the reaction. On the other hand, the yield of 5-Cl-dCyd even in the presence of Cl⁻ is low at neutral pH, compared with that under acidic conditions (4), whereas the yield of 8-Cl-Guo at pH > 7.4 is high even in the absence of Cl⁻. Higher yields of 8-Cl-Guo are formed under more alkaline conditions (pH > 8) (Fig. 4A). These results suggest that 8-Cl-Guo formation at neutral pH is mediated by a chlorinating intermediate that may be different from a Cl₂-like species. On the basis of these findings and other published data, we propose a mechanism for the formation of 8-Cl- and 8-oxo-Guo by the reaction of guanosine with HOCl under neutral conditions (Fig. 15). In the reaction of HOCl, the Cl⁺

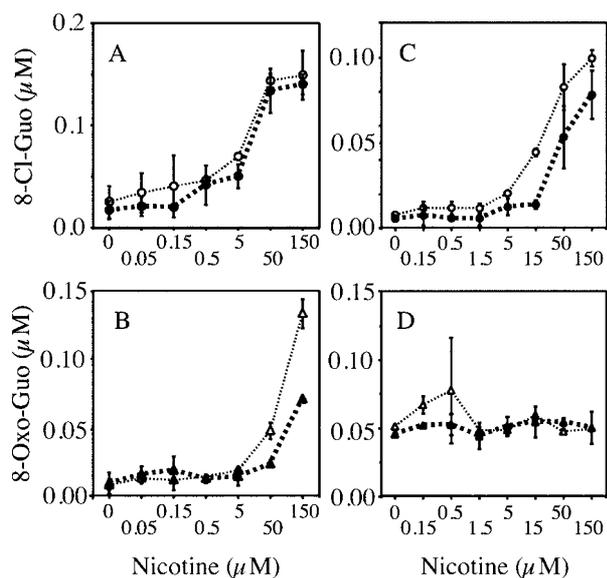


FIG. 11. Effect of nicotine concentration on the formation of 8-Cl- (A and C) and 8-oxo-Guo (B and D) mediated by myeloperoxidase (A and B) and by activated human neutrophils (C and D). A and B, the reaction mixture contained 0.5 mM guanosine, 20 nM myeloperoxidase, 0.1 mM H_2O_2 , 100 mM NaCl with (closed symbols) or without (open symbols) 0.1 mM nitrite. It was incubated in the presence of various concentrations of nicotine (0–150 μM) at 37 °C for 30 min. C and D, the reaction mixture contained 0.5 mM guanosine and human neutrophils (5×10^5 cells/ml) activated by 200 nM β -phorbol myristate acetate with (closed symbols) or without (open symbols) 0.1 mM nitrite in 0.5 ml of Hank's buffered salt solution. It was incubated in the presence of various concentrations of nicotine (0–150 μM) at 37 °C for 60 min. All of the reactions were terminated by adding 5 mM *N*-acetylcysteine. The means \pm S.D. ($n = 3$) are presented.

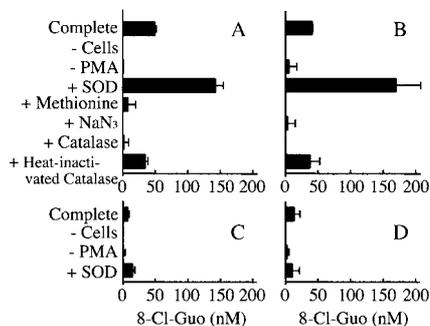


FIG. 12. Requirements for the formation of 8-Cl-Guo by human neutrophils with and without nitrite and/or nicotine. The complete system consisted of 0.5 mM guanosine, human neutrophils (5×10^5 cells/ml), and 200 nM β -phorbol myristate acetate (PMA) in 0.5 ml of Hank's buffered salt solution (pH 7.4). In addition, 0.1 mM nitrite was present (B and D) or absent (A and C) and 0.05 mM nicotine was present (A and B) or absent (C and D). Where indicated, 10 $\mu\text{g}/\text{ml}$ superoxide dismutase (SOD), 6 mM methionine, 10 mM sodium azide, and 10 $\mu\text{g}/\text{ml}$ catalase or heat-inactivated catalase were present, or human neutrophils and β -phorbol myristate acetate were omitted. The reaction mixture was incubated at 37 °C for 60 min. All of the reactions were terminated by adding 5 mM *N*-acetylcysteine. The means \pm S.D. ($n = 3$) are presented.

moiety ($\text{HO}^{\delta-}\text{Cl}^{\delta+}$) has been proposed to serve as an electrophilic species (28, 29). Like various electrophilic carcinogens that react at N-7 of guanine, the most nucleophilic position, to form N-7 adducts, Cl^+ could react first with N-7 of guanine to form an N-7 chlorine adduct cation as an intermediate, which might then be converted to 8-Cl-Guo by a chlorine migration (Fig. 15). Another strong electrophile, a nitrenium ion ($\text{R}-\text{NH}^+$) arising from hydroxylamine, has been reported to react with 2'-deoxyguanosine to form an adduct at C-8 along with several other products including 8-oxo-dGuo (30, 31). These

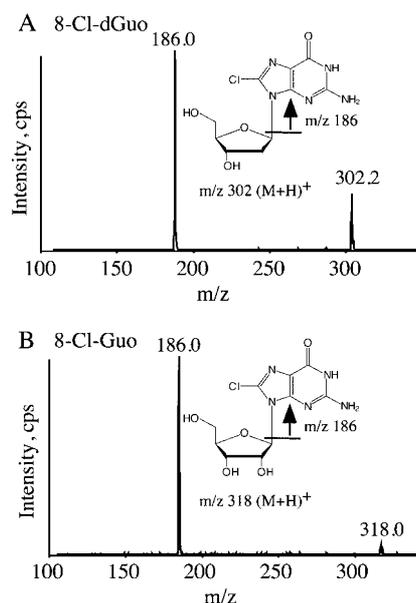


FIG. 13. Collision-induced dissociation mass spectra of ion m/z 302 for 8-Cl-dGuo and ion m/z 318 for 8-Cl-Guo obtained in the positive ionization mode.

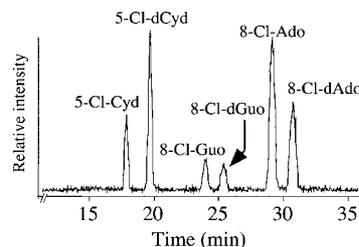


FIG. 14. HPLC-MS/MS chromatogram of the six measured chlorinated ribo- and 2'-deoxy-nucleosides. A mixture containing six chlorinated nucleosides (1 pmol each) was analyzed as described under "Experimental Procedures."

TABLE II

Levels of chlorinated nucleosides formed in DNA and RNA following exposure to HOCl *in vitro*

Calf thymus DNA or calf liver RNA (1 mg/ml) were incubated at 37 °C for 15 min in the presence or absence of 200 μM HOCl with or without 20 μM nicotine in 1 ml of 50 mM sodium phosphate buffer, pH 7.4. The means \pm S.D. ($n = 3$) are presented. ND, not detected.

Treatment	Lesions/ 10^5 (d)Guo			
	8-Cl-(d)Guo	5-Cl-(d)Cyd	8-Cl-(d)Ado	
DNA	None	ND	ND	
	HOCl	2.8 ± 0.8	218 ± 58	6.9 ± 1.6
	HOCl + nicotine	8.6 ± 6.2	278 ± 37	11.5 ± 1.7
RNA	None	ND	ND	
	HOCl	5.5 ± 1.1	1.0 ± 0.2	ND
	HOCl + nicotine	51.5 ± 2.6	4.6 ± 2.7	ND

studies also suggested that an N-7 adduct cation is first formed as an intermediate by the reaction of dGuo with $\text{R}-\text{NH}^+$, followed by arylamine migration from N-7 to C-8 to form the C-8 arylamine adduct (30, 31). Higher yields of 8-Cl-Guo under alkaline conditions (Fig. 4A) could be attributable to deprotonation of H-8 from the N-7 chlorine adduct cation by OH^- that would facilitate the migration of the chlorine atom from N-7 to C-8 (Fig. 15).

In the present study, 8-oxo-Guo was also formed easily in the reaction of guanosine with HOCl under neutral conditions. The lack of effects of addition of dimethyl sulfoxide, a scavenger of HO^\bullet (Fig. 8), and of D_2O , an enhancer of $^1\text{O}_2^*$, upon the yield of 8-oxo-Guo (data not shown) rules out the possibility of involve-

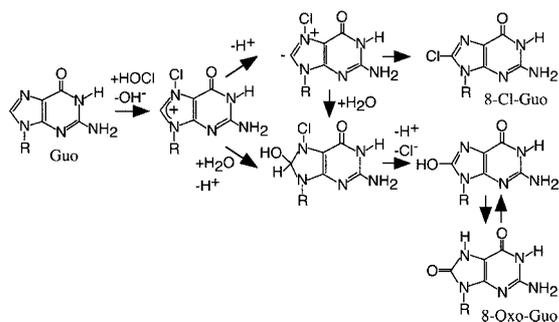
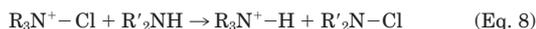
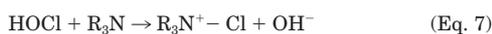


FIG. 15. Proposed mechanism for formation of 8-Cl- and 8-oxo-Guo by the reaction of guanosine with HOCl under neutral conditions.

ment of HO^\cdot and $^1\text{O}_2^*$. Thermodynamic calculations indicate that HOCl can act as either a one- or a two-electron oxidant (32). However, it is a relatively poor one-electron oxidant, having an estimated one-electron reduction potential (E°') in the range of +0.17 to +0.26 V at pH 7 (32) and therefore would not oxidize guanosine in neutral solution, because the one-electron oxidation potential of guanosine is +1.29 V at pH 7 (33). On the other hand, HOCl is a strong two-electron oxidant, having an E°' of +1.08 V, and might oxidize guanosine, resulting in guanosine $^+$ via a two-electron oxidation. However, it has been reported that 8-oxo-dGuo is not formed from 2'-deoxyguanosine by another two-electron oxidant, an artificial nuclease Mn-TMPyP/KHSO₅ (34). On the basis of these biophysical data, we propose that 8-oxo-(d)Guo formation by HOCl is not mediated by a two-electron oxidation reaction but can be mediated through formation of an N-7 chlorine adduct cation from guanosine and Cl^+ and a further reaction with H_2O (Fig. 15). Interpretation of analyses of 8-oxo-Guo formed by the human myeloperoxidase system and activated neutrophils is complicated because 8-oxo-Guo, once formed, can be also easily converted to further oxidation products by oxidants (35). We have recently identified diastereomers of spiroiminodihydroantoin nucleoside as an oxidized product formed by reaction of 8-oxo-dGuo with HOCl or with a myeloperoxidase- H_2O_2 - Cl^- system (36).

The presence of physiologically relevant concentrations (μM) of tertiary amines such as nicotine and TMA dramatically enhanced chlorination of free (2'-deoxy)nucleosides and those in RNA but not in DNA by HOCl. Oxidation and nitration of guanosine by HOCl were also enhanced by tertiary amines. Interestingly, the presence of nicotine completely inhibited the formation of N-chlorinated 2'-deoxycytidine by HOCl, suggesting that nicotine reacted more rapidly with HOCl than the primary amino group of 2'-deoxycytidine. Prütz (26) recently reported that chlorination of salicylate is enhanced by several orders of magnitude by catalytic amounts of tertiary amines such as TMA and quinuclidine. It has been reported that tertiary amines react with HOCl to form reactive quaternary chlorammonium ions ($\text{R}_3\text{N}^+-\text{Cl}$) (Equation 7) that readily chlorinate secondary amines to form N-chloro secondary amines (Equation 8) (37, 38).



It is therefore possible that the quaternary chlorammonium ions may chlorinate (2'-deoxy)guanosine at the N-7 position to form first its N-7 chlorine adduct via a Cl^+ -like species or direct transfer of chlorine atom, followed by migration of chlorine to generate 8-Cl-Guo (Fig. 15).

An alternative mechanism for chlorination of nucleosides involves free radicals. The quaternary chlorammonium ion may generate a chlorine radical (Cl^\cdot) together with a tertiary amine cation radical (Equation 9), as suggested for the myeloperoxidase-catalyzed oxidation of aminopyrine (39). Cl^\cdot could then react with guanosine to form a C-8 guanosine radical that might react further with Cl^\cdot , HO^\cdot , and NO_2 to form 8-Cl-, 8-oxo-, and 8-nitro-Guo, respectively. However, the fact that metallic ions enhanced oxidation but not chlorination (Table I) suggests that involvement of free radicals in chlorination is less likely.

The presence of physiologically relevant concentrations (μM) of nicotine and TMA dramatically enhanced the chlorination damage of (2'-deoxy)nucleosides not only by HOCl but also by human myeloperoxidase and activated neutrophils. Because the nicotine content of mainstream smoke from commercial cigarettes ranges from <0.3 to 2.5 mg/cigarette (40), its concentration in the respiratory tract could easily reach the μM range. Cigarette smoking is a major risk factor for a number of diseases, including lung cancer, chronic obstructive pulmonary disease, arteriosclerosis, cardiovascular disease, cerebrovascular disease, and Crohn's disease. Inflammation is common to the pathogenesis of these diseases. It has been reported that nicotine is chemotactic for neutrophils (41) and cigarette smoke primes neutrophils (42). The present study suggests that nicotine present in tobacco smoke may further enhance tissue and DNA damage through HOCl-mediated chlorination by myeloperoxidase secreted from neutrophils, contributing to lung carcinogenesis and other smoking-related diseases. It is interesting to note that the G-463A polymorphism of the *MPO* gene, which strongly reduces myeloperoxidase mRNA expression (43), is associated with a reduced risk of lung cancer (44–46). Similarly, TMA is present at high concentrations in salted and dried fish products whose consumption has been associated with an increased risk of stomach cancer (47, 48). Consumption of 100 g of fish such as haddock and cod may increase the concentration of TMA to > 20 μM in the stomach, if the volume of gastric content is assumed to be 1 liter (47). One can postulate that tissue or DNA damage induced by neutrophils in the stomach with *Helicobacter pylori* infection could be accelerated by dietary TMA, contributing to gastric carcinogenesis. In fact, feeding of fish meal markedly enhanced *H. pylori*-induced gastritis in Mongolian gerbils (49). Although 8-Cl-(d)Guo has not been studied for mutagenicity, it has been shown that incorporation of both dAMP and dCMP by human DNA polymerases occurred opposite 8-bromo-2'-deoxyguanosine present in DNA templates, possibly inducing G:C to T:A transversion mutation (50). However, in contrast to the templates containing 8-oxo-dGuo, for which incorporation of dAMP is favored over dCMP, dCMP is favored for incorporation opposite 8-bromo-2'-deoxyguanosine (50). Further studies are needed to elucidate the genotoxicity and types of mutation induced by incorporation of 8-Cl-dGuo into DNA or those caused by formation of 8-Cl-dGuo in DNA.

Acknowledgments—NMR measurements were performed by B. Fenet at the Center de Résonance Magnétique Nucléaire (Université Claude Bernard, Lyon I, France). We thank Dr. J. Cheney for editing the manuscript and P. Collard for secretarial assistance.

REFERENCES

- Ohshima, H., and Bartsch, H. (1994) *Mutat. Res.* **305**, 253–264
- Ames, B. N., Gold, L. S., and Willett, W. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5258–5265
- Ohshima, H., Chazotte-Aubert, L., Li, C. Q., Masuda, M., and Pignatelli, B. (2000) in *Free Radicals in Chemistry, Biology and Medicine* (Yoshikawa, T., Toyokuni, S., Yamamoto, Y., and Naito, Y., eds) pp. 92–101, OICA International, London
- Henderson, J. P., Byun, J., and Heinecke, J. W. (1999) *J. Biol. Chem.* **274**, 33440–33448
- Saran, M., Beck-Speier, I., Fellerhoff, B., and Bauer, G. (1999) *Free Radic.*

- Biol. Med.* **26**, 482–490
6. Eiserich, J. P., Cross, C. E., Jones, A. D., Halliwell, B., and van der Vliet, A. (1996) *J. Biol. Chem.* **271**, 19199–19208
 7. Khan, A. U., and Kasha, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 12362–12364
 8. Candeias, L. P., Stratford, M. R., and Wardman, P. (1994) *Free Radic. Res.* **20**, 241–249
 9. Whiteman, M., Jenner, A., and Halliwell, B. (1997) *Chem. Res. Toxicol.* **10**, 1240–1246
 10. Whiteman, M., Jenner, A., and Halliwell, B. (1999) *Biomarkers* **4**, 303–310
 11. Patton, W., Bacon, V., Duffield, A. M., Halpern, B., Hoyano, Y., Pereira, W., and Lederberg, J. (1972) *Biochem. Biophys. Res. Commun.* **48**, 880–884
 12. Gould, J. P., Richards, J. T., and Miles, M. G. (1984) *Water Res.* **18**, 991–999
 13. Byun, J., Mueller, D. M., and Heinecke, J. W. (1999) *Biochemistry* **38**, 2590–2600
 14. Hazen, S. L., and Heinecke, J. W. (1997) *J. Clin. Invest.* **99**, 2075–2081
 15. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and van der Vliet, A. (1998) *Nature* **391**, 393–397
 16. Ohshima, H., Celan, I., Chazotte, L., Pignatelli, B., and Mower, H. F. (1999) *Nitric Oxide* **3**, 132–141
 17. Jiang, Q., and Hurst, J. K. (1997) *J. Biol. Chem.* **272**, 32767–32772
 18. Hazen, S. L., Hsu, F. F., Gaut, J. P., Crowley, J. R., and Heinecke, J. W. (1999) *Methods Enzymol.* **300**, 88–105
 19. Morita, Y., Iwamoto, H., Aibara, S., Kobayashi, T., and Hasegawa, E. (1986) *J. Biochem. (Tokyo)* **99**, 761–770
 20. Heinecke, J. W., Li, W., Daehnke, H. L., and Goldstein, J. A. (1993) *J. Biol. Chem.* **268**, 4069–4077
 21. Yermilov, V., Rubio, J., and Ohshima, H. (1995) *FEBS Lett.* **376**, 207–210
 22. Ravanat, J. L., Remaud, G., and Cadet, J. (2000) *Arch. Biochem. Biophys.* **374**, 118–127
 23. Frelon, S., Douki, T., Ravanat, J. L., Pouget, J. P., Tornabene, C., and Cadet, J. (2000) *Chem. Res. Toxicol.* **13**, 1002–1010
 24. Ryu, E. K., and MacCoss, M. (1981) *J. Org. Chem.* **46**, 2819–2823
 25. Kasai, H., Crain, P. F., Kuchino, Y., Nishimura, S., Ootsuyama, A., and Tanooka, H. (1986) *Carcinogenesis* **7**, 1849–1851
 26. Prutz, W. A. (1998) *Arch. Biochem. Biophys.* **357**, 265–273
 27. Hayatsu, H., Pan, S., and Ukita, T. (1971) *Chem. Pharm. Bull. (Tokyo)* **19**, 2189–2192
 28. Arnhold, J., Panasenko, O. M., Schiller, J., Vladimirov, Y., and Arnold, K. (1995) *Chem. Phys. Lipids* **78**, 55–64
 29. Brezonik, P. L. (1994) *Chemical Kinetics and Process Dynamics in Aquatic Systems*, Lewis Publishers, London
 30. Humphreys, W. G., Kadlubar, F. F., and Guengerich, F. P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 8278–8282
 31. Guengerich, F. P., Mundkowsky, R. G., Voehler, M., and Kadlubar, F. F. (1999) *Chem. Res. Toxicol.* **12**, 906–916
 32. Koppenol, W. H. (1994) *FEBS Lett.* **347**, 5–8
 33. Steenken, S., and Jovanovic, S. V. (1997) *J. Am. Chem. Soc.* **119**, 617–618
 34. Viala, C., Praviel, G., Claparols, C., and Meunier, B. (1998) *J. Am. Chem. Soc.* **120**, 11548–11553
 35. Niles, J. C., Burney, S., Singh, S. P., Wishnok, J. S., and Tannenbaum, S. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 11729–11734
 36. Suzuki, T., Masuda, M., Friesen, M. D., and Ohshima, H. (2001) *Chem. Res. Toxicol.* **14**, 1163–1169
 37. Ellis, A. J., and Soper, F. G. (1954) *J. Chem. Soc.* 1750–1755
 38. Higuchi, T., Hussain, A., and Pltman, I. H. (1969) *J. Chem. Soc.* 626–631
 39. Sayo, H., and Saito, M. (1990) *Xenobiotica* **20**, 957–965
 40. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (1986) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Tobacco Smoking*, Vol. 38, International Agency for Research on Cancer, Lyon, France
 41. Totti, N., McCusker, K. T., Campbell, E. J., Griffin, G. L., and Senior, R. M. (1984) *Science* **223**, 169–171
 42. Koethe, S. M., Kuhnmuench, J. R., and Becker, C. G. (2000) *Am. J. Pathol.* **157**, 1735–1743
 43. Piedrafito F. J., Molander, R. B., Vansant, G., Orlova, E. A., Pfahl, M., and Reynolds, W. F. (1996) *J. Biol. Chem.* **271**, 14412–14420
 44. London, S. J., Lehman, T. A., and Taylor, J. A. (1997) *Cancer Res.* **57**, 5001–5003
 45. Cascorbi, I., Henning, S., Brockmoller, J., Gephart, J., Meisel, C., Muller, J. M., Loddenkemper, R., and Roots, I. (2000) *Cancer Res.* **60**, 644–649
 46. Le Marchand, L., Seifried, A., Lum, A., and Wilkens, L. R. (2000) *Cancer Epidemiol. Biomarkers Prev.* **9**, 181–184
 47. Zeisel, S. H., and DaCosta, K. A. (1986) *Cancer Res.* **46**, 6136–6138
 48. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (1993) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins*, Vol. 56, International Agency for Research on Cancer, Lyon, France
 49. Tanigawa, T., Kawamori, T., Iimuro, M., Ohta, T., Higuchi, K., Arakawa, T., Sugimura, T., and Wakabayashi, K. (2000) *Jpn. J. Cancer Res.* **91**, 769–773
 50. Efrati, E., Tocco, G., Eritja, R., Wilson, S. H., and Goodman, M. F. (1999) *J. Biol. Chem.* **274**, 15920–15926