8-Nitroxanthine, an Adduct Derived from 2′-Deoxyguanosine or DNA Reaction with Nitryl Chloride

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Activated phagocytic cells generate reactive nitrogen species, including nitryl chloride and peroxynitrite, for host defense against invading pathogens. It has been proposed that these reactive nitrogen species may cause DNA damage and thus contribute to the multistage carcinogenesis process associated with chronic infections and inflammation. Previous studies showed that peroxynitrite reacted with guanine, 2′-deoxyguanosine, or DNA forming 8-nitroguanine. We herein report formation of 8-nitroxanthine as the major nitration product in reactions of 2′-deoxyguanosine or calf thymus DNA with nitryl chloride produced by mixing nitrite with hypochlorous acid, and 8-nitroguanine was a minor product in these reactions. 8-Nitroxanthine was characterized by its NMR and laser desorption ionization mass spectra and by deamination of 8-nitroguanine. Formation of 8-nitroxanthine was also detected by xanthine reaction with various reactive nitrogen species, including nitryl chloride, peroxynitrite, nitronium tetrafluoroborate, and heated nitric and nitrous acid. The identity of 8-nitroxanthine in nitryl chloride-treated dG and DNA was confirmed by co-injection with synthetic 8-nitroxanthine and by its reduction to 8-aminoxanthine. Levels of 8-nitroxanthine and 8-nitroguanine in these reactions were quantified by reversed-phase HPLC with photodiode array detection. Once formed, 8-nitroxanthine was spontaneously removed from DNA with a half-life of 2 h at 37 °C and pH 7.4. Therefore, 8-nitroxanthine might be an important DNA lesion derived from reactive nitrogen species in vivo.

Introduction

Chronic infections and inflammation are important risk factors in cancer development (*1*). It has been postulated that peroxynitrite may cause DNA and tissue damage and contribute to the multistage carcinogenesis associated with chronic infections and inflammation (*2*). Peroxynitrite (and peroxynitrous acid, its conjugate acid) was formed from the concomitant release of superoxide anion and nitric oxide (NO)[1] by activated macrophages and neutrophils in inflamed tissues (*3*, *4*). Many of the damaging effect of NO are attributed to the formation of peroxynitrite and/or nitrosoperoxycarbonate ($ONO_2CO_2^-$), the reactive product of peroxynitrite with CO_2, which is abundant in physiological fluids (*5*−*7*). Peroxynitrite is a relatively stable reactive oxygen species with a half-life of ca. one second at physiological pH (*8*, *9*). Therefore, it can diffuse and cross cell membranes before its decomposition (*10*, *11*). It is now well established that peroxynitrite can be formed under oxidative stress in several disease states (*12*−*16*) and in cigarette smoking (*17*, *18*). Peroxynitrite has been reported to induce DNA strand breaks and is mutagenic (*19*). The products of peroxynitrite reaction with DNA bases include 8-nitroguanine (8NG), 8-oxoguanine, base propenals (*20*, *21*), secondary oxidation products of 8-oxoguanine (*22*, *23*), xanthine (*24*), and 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine (*25*). Among them, 8NG induced GC to TA transversion mutations, the same mutations induced by peroxynitrite (*19*).

Another source of 8NG is 2′-deoxyguanosine (dG) reaction with nitryl chloride (NO_2Cl) and/or nitrogen dioxide radical ($NO_2^{\bullet}$), produced in activated phagocytes at the inflammation sites (*26*). Nitryl chloride is capable of nitrating guanine, tyrosine, and lipids as well as chlorinating and oxidizing tyrosine (*26*−*28*). Suzuki and co-workers also reported that 8NG was formed in reaction of dG with NO/O_2 gas mixture under physiological condition (*29*).

In this study, 8-nitroxanthine (8NX) and 8NG were formed in reaction of calf thymus DNA or dG with NO_2-Cl generated in vitro by mixing nitrite with hypochlorous acid in the absence of an enzyme (Scheme 1). Previously found as a side product in reaction of nitrous acid with guanosine and xanthosine (*30*), 8NX was the major nitration product in these two reactions and 8NG was instead a minor product. Formation of 8NX from other

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[1] Abbreviations: 8AG, 8-aminoguanine; 8AX, 8-aminoxanthine; dG, 2′-deoxyguanosine; $^{i}Pr_2EtN$, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; N_2O_5, dinitrogen pentoxide; N_2O_4, dinitrogen tetroxide; H_2O_2, hydrogen peroxide; LDI, laser desorption ionization; NO, nitric oxide; $NO_2^{\bullet}$, nitrogen dioxide radical; 8NG, 8-nitroguanine; $NO_2^+BF_4^-$, nitronium tetrafluoroborate; $NO^+BF_4^-$, nitrosonium tetrafluoroborate; N_2O_3, nitrous anhydride; 8NX, 8-nitroxanthine; $Na_2S_2O_4$, sodium hydrosulfite or sodium dithionite; NaOCl, sodium hypochlorite; $NaNO_2$, sodium nitrite; NO_2Cl, nitryl chloride; ONO_2^-, peroxynitrite; PDA, photodiode array.

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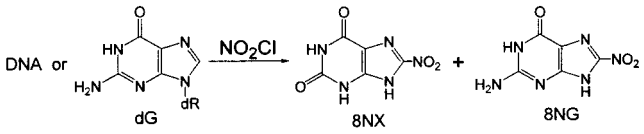
Scheme 1. Reaction of Nitryl Chloride with DNA or dG Forming 8NX and 8NG



reactive nitrogen species was also investigated. Depurination of 8NX from DNA was spontaneous with a half-life of 2 h under physiological conditions. Therefore, 8NX might be an important DNA damage due to the physiological roles of nitrite and hypochlorous acid in inflammation and in cancer development.

Materials and Methods

Materials. Calf thymus DNA and 8-amimoguanosine were from Sigma Chemical Co. (St. Louis, MO). Guanine, diethylenetriamine pentaacetic acid (DTPA), manganese dioxide, nitronium tetrafluoroborate, nitrosonium tetrafluoroborate, sodium hydrosulfite (sodium dithionite, $Na_2S_2O_4$), and sodium hypochloride (NaOCl) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Isoamylnitrite was from T.C.I. Co. (Tokyo, Japan). Hydrogen peroxide was purchased from Acros Organic Chemical Co. (Geel, Belgium) and quantified based on the absorbance at 240 nm ($\epsilon = 43.6\ M^{-1}\ cm^{-1}$) (*31*). Peroxynitrite was synthesized according to the previously described procedures using isoamylnitrite and hydrogen peroxide (*32*) and was stored at −80 °C. The concentration of peroxynitrite was determined by the absorbance at 302 nm in 1N NaOH ($\epsilon = 1670\ M^{-1}\ cm^{-1}$) (*33*). The concentration of hypochlorite was determined by the absorbance at 292 nm (pH 12, $\epsilon = 350\ M^{-1}\ cm^{-1}$) (*34*). Standard 8-aminoguanine was obtained by acid hydrolysis of 8-aminoguanosine (*35*).

Instruments. NMR spectra were recorded on a Bruker DPX 400 MHz (Billerica, MA) instrument. Laser desorption ionization (LDI) mass spectra was performed on a HP G2025A LD-TOF mass spectrometer equipped with a 337 nm nitrogen laser. 8NX and 8AX were dissolved in double distilled water and 1 N HCl, respectively. One microliter of the sample solution was applied on a direct sample probe and dried under reduced pressure. The probe was subsequently sent into the ion source of the mass spectrometer. The accelerating voltage in the ion source was 28 kV. Fast-atom bombardment (FAB) mass spectrum was performed on a VG Trio-2000 spectrometer. HPLC Chromatography was performed by a Hitachi L-7000 pump system with D-7000 interface, a Rheodyne injector, and a L-7450A photodiode array (PDA) detector.

HPLC Conditions: (1) System 1. A Prodigy ODS (3) 250 mm × 10 mm, 5 μm column (Phenomenex, Torrance, CA) was used with the following isocratic conditions: 50 mM ammonium formate (pH = 5.5) buffer at a flow rate of 4.0 mL/min.

(2) System 2. A Prodigy ODS (3) 250 mm × 10 mm, 5 μm column was eluted with H_2O at a flow rate of 4.0 mL/min.

(3) System 3. A Prodigy ODS (3) 250 mm × 4.6 mm, 5 μm column was used with the following isocratic conditions: 50 mM ammonium formate (pH = 5.5) buffer at a flow rate of 1.0 mL/min.

(4) System 4. A Prodigy ODS (3) 250 mm × 4.6 mm, 5 μm column was eluted with 50 mM ammonium formate (pH = 4.0) from 0 to 40 min, followed by a linear gradient of 0−100% MeOH from 40 to 60 min at a flow rate of 1.0 mL/min.

Synthesis of 8NX (1) from Peroxynitrite. Xanthine (2.0 mg, 13.2 μmol) dissolved in 0.5 mL of 0.2 N HCl was added peroxynitrite (200 mM in 56 mM NaOH and 50 mM ammonium formate) and 962 μL of 56 mM NaOH containing 50 mM ammonium formate with vigorous stirring at room temperature for 2 min. **(2) from Nitryl Chloride.** A solution containing xanthine (0.2 mg, 1.31 μmol) dissolved in 0.2 N HCl (0.2 mL) was added 298 μL of a solution of $NaNO_2$ (4.4 mM in 0.4 M potassium phosphate buffer, pH 12.8) and 4.5 μL of a solution of NaOCl (0.29 M in 0.4 M potassium phosphate buffer, pH 12.8) with stirring. Final pH of the reaction mixtures was between 7 and 7.4. The reaction mixture was filtered through a 0.22 μm nylon syringe filter (Advantec, MFS, Inc.) and collected from 17 to 20 min by HPLC using system 1. The collected fractions were combined and evaporated to dryness. The mixture was reconstituted in water and injected to HPLC using system 2 collecting from 14 to 16 min to obtain salt-free 8NX. UV_{max} (pH 5.5) λ 205, 376 nm; $\epsilon_{382} = 7673\ M^{-1}\ cm^{-1}$ (pH 7.0); MS (LDI-) 196 ($[M - H]^-$); ^1H NMR (DMSO-d_6) δ 7.0 (broad s, 1H, N9−H), 10.2 (s, 1H, N3−H), 10.9 (s, 1H, N1−H).

Synthesis of 8-Nitroguanine (8NG). 8-Nitroguanine was synthesized by a modified method (*36*). Briefly, a solution containing guanine (0.3 mg, 0.53 μmol) in 0.95 mL of 0.2 N HCl and 50 mM ammonium formate was added 38 μL of peroxynitrite (200 mM in 0.67 N NaOH and 50 mM ammonium formate) and 962 μL of 0.67 N NaOH containing 50 mM ammonium formate with vigorous stirring at room temperature for 2 min. Final pH of the reaction was between 6.8 and 7.0. The reaction mixture was concentrated to ca. 1 mL, filtered through a 0.22 μm Nylon syringe filter, and collected repeatedly from 15 to 16 min, by HPLC using system 1. The collected fractions were combined and evaporated to dryness. The mixture was reconstituted in water, injected to HPLC using system 2, and collected from 5 to 7 min to obtain salt-free 8NG. MS (LDI-) *m*/*z* 195 ($[M - H]^-$), 179 ($[M-17]^-$); UV_{max} (pH 5.5) λ 210, 231, 258, 393 nm. $\epsilon_{400} = 9144\ M^{-1}\ cm^{-1}$ (pH 7.0).

Synthesis of 8AX. 8NX (1.4 mg, 8.4 μmol) in 1.0 mL of water was added sodium hydrosulfite (5.85 mg, 33.6 μmol) at room temperature with stirring for 10 min. The reaction mixture was concentrated to ca. 1 mL, filtered through a 0.22 μm Nylon syringe filter, adjusted to pH 5.5, and collected repeatedly from 13.5 to 14.5 min, by HPLC using system 1. The collected fractions were combined and evaporated to dryness. The mixture was reconstituted in water and injected to HPLC using system 2 collecting from 5 to 7 min to obtain salt-free 8AX. UV_{max} (H_2O) λ 207, 288 nm. MS (MALDI+) 168 ($[M + H]^+$); MS (LDI-) 166 ($[M - H]^-$).

Reduction of 8NG to 8AG. 8NG (2.0 mg, 10.2 μmol) in 1.0 mL of water was added sodium hydrosulfite (3.55 mg, 20.4 μmol) at room temperature with stirring for 10 min as described (*37*). The reaction mixture was adjusted to pH 5.5 and analyzed by HPLC using system 3. The retention time and UV spectrum were compared with standard 8-aminoguanine obtained from acid hydrolysis of 8-aminoguanosine (*35*). UV_{max} (pH 5.5) λ 226, 250, 291 nm. MS (FAB+) 167 ($[M + H]^+$).

8NG Reaction with *tert*-Butylnitrite. A solution of 8NG (2.5 μmol) in 4.0 mL of 3 N HCl was added ^{t}BuONO (30 μL, 250 μmol) with stirring at room temperature for 1 h. The reaction mixture was adjusted to pH 5.5 and analyzed by HPLC system 3. A portion of the reaction mixture (80 μL) was co-injected with synthetic 8NX (35 nmol) described above and analyzed by HPLC system 3. Another portion of the reaction mixture (3.0 mL) was collected by HPLC system 3 and reduced by sodium hydrosulfite (500 μg). One thirtieth of the reduced mixture with added synthetic 8AX (6.3 nmol) described above and analyzed by HPLC system 3.

Effect of pH on Nitration of Xanthine or Guanine. Xanthine or guanine (0.1 mg, 0.66 μmol in 0.1 mM HCl) was added 0.4 M potassium phosphate buffer (pH 3.0−10.0), $NaNO_2$ (0.66 μmol), and NaOCl (0.66 μmol) at room temperature with vortexing for 2 min and the final volume was 0.4 mL. The reaction mixture was adjusted to pH 5.5 and analyzed by HPLC system 3. Quantification of 8NX and 8NG was monitored at 376 and 393 nm, respectively.

Xanthine or Guanine Reaction with Nitric or Nitrous Acid. Xanthine or guanine (7.4 mg, 49 μmol) was added 5.0 mL of 1.0 M nitric acid or nitrous acid and heated at 90 °C for 3 h. An aliquot (100 μL) of the reaction mixture was adjusted to pH 5.5 and analyzed by HPLC system 3.

Xanthine or Guanine Reaction with Nitronium Tetrafluoroborate or Nitrosonium Tetrafluoroborate. Xanthine or guanine (1.2 mg, 7.9 μmol) dissolved in 3.0 mL of anhydrous DMF was added $NO_2^+BF_4^-$ (250 mg, 1.88 mmol) or $NO^+BF_4^-$ (92 mg, 790 μmol) with stirring at room temperature for 5 min. An aliquot (50 μL) of the reaction mixture was adjusted to pH 5.5 and analyzed by HPLC system 3.

dG Reaction with Nitryl Chloride. A solution containing 2′-dG (0.6 mg, 2.25 μmol) and $NaNO_2$ (7.8 mg, 113 μmol) dissolved in 0.5 mL of 0.2 M potassium phosphate buffer (pH 7.0) was added a solution containing 470 μL of NaOCl (0.24 M, 113 μmol) with stirring at room temperature for 2 min. The reaction mixture was adjusted to pH 4.0 and an aliquot (50 μL) was analyzed by HPLC using system 4.

Reaction of DNA with Nitryl Chloride. Calf thymus DNA (0.4 mg in 0.4 mL 0.1 M potassium phosphate, pH 7.0) was added 520 μL of a solution containing $NaNO_2$ (0.52 M) and 340 μL of a solution of NaOCl (66 mM) with vortexing for 2 min. The final pH of the reaction mixture was 7.0. The reaction mixture was added 3 mL cold ethanol and centrifuged at 15 000 rpm at 4 °C for 5 min. The precipitated DNA was washed with 70% ethanol (3 mL × 2) and evaporated to dryness.

Hydrolysis of Nitryl Chloride-Treated Calf Thymus DNA. After calf thymus DNA reacted with nitryl chloride, the reaction mixture was divided into two equal portions. Both portions were precipitated, centrifuged, washed, and evaporated. One portion of DNA was added 0.3 mL of 0.1 N HCl and heated at 100 °C (acid hydrolysis). The other portion of DNA was hydrolyzed in 0.3 mL of H_2O heating at 100 °C (neutral hydrolysis). After 30, 60, 90, 120, and 180 min, an aliquot (50 μL) of each of the hydrolysate was removed, adjusted to pH 4.0, and analyzed by HPLC system 4 at 376 nm.

Identity of 8NX in Nitryl Chloride-Treated dG and Calf Thymus DNA. (1) dG. One milliliter solution containing 2′-dG (final concentration 2.32 mM), $NaNO_2$ (final concentration 116 mM), and 0.1 M potassium phosphate buffer (pH 7.0) was added a solution of NaOCl (final concentration 116 mM) with stirring at room temperature for 2 min. **2. DNA.** Calf thymus DNA (2.0 mg in 2.0 mL 0.2 M potassium phosphate buffer, pH 7.0) was added 770 μL of a solution containing $NaNO_2$ (0.58 M) and 372 μL of a solution of NaOCl (0.30 M). After vortexing for 2 min, DNA was precipitated, washed, and hydrolyzed with 0.1 N HCl (400 μL) at 100 °C for 30 min. Both of the reaction mixtures of dG and DNA were adjusted to pH 5.5 and a 100 μL aliquot from each reaction mixture was analyzed by HPLC using system 3. The remaining reaction mixtures were collected from 20 to 22 min by HPLC using system 3. The collected 8NX fraction was concentrated and reconstituted in 400 μL of water. A portion of the aliquot (100 μL) was analyzed by HPLC system 3 at 376 nm and another portion (100 μL) was added synthetic 8NX (1.0 μg) and analyzed under the same condition. The remaining aliquot (200 μL) was reduced by sodium hydrosulfite and 100 μL of the aliquot was analyzed by HPLC system 3 at 287 nm. The rest of the reduction mixture (100 μL) was added 8AX (48 ng) and analyzed by HPLC system 3 at 287 nm.

Depurination of 8NX in Nitryl Chloride-Treated Calf Thymus DNA. After calf thymus DNA (4.8 mg in 4.8 mL of 0.1 M potassium phosphate, pH 7.0) was added 2.4 mL of $NaNO_2$ (2.24 M) and 7.9 mL of NaOCl (0.17 M) at room temperature, the reaction mixture was incubated at 37 °C. An aliquot (1.26 mL) of the reaction mixture was removed at various time points. DNA was precipitated with cold ethanol (2.5 mL), centrifuged, washed with 2.5 mL of 70% cold ethanol twice, and evaporated. DNA was reconstituted in 400 μL of 0.1 M phosphate buffer and hydrolyzed at 100 °C for 3 h and pH of the mixture was adjusted to 4.0, filtered through a 0.22 μm Nylon syringe filter, and analyzed by HPLC system 4 at 376 nm. The ethanol solutions were combined, evaporated, adjusted pH to 4.0, filtered through a 0.22 μm Nylon syringe filter, and analyzed by HPLC system 4 at 376 nm for 8NX released in the medium.

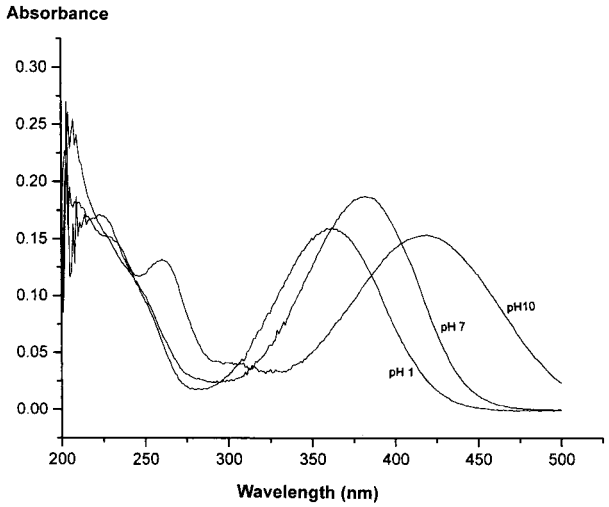
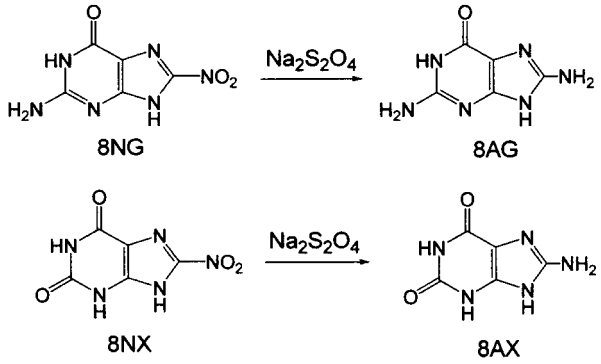


Figure 1. UV spectrum of 8NX at pH 1, 7, and 10.

Scheme 2. Reduction of 8NG and 8NX



Results

Characterization of 8NX. Like 8NG, 8NX has a characteristic UV−vis absorption above 350 nm. At neutral pH, 8NX and 8NG exhibited an absorbance maximum at 383 and 400 nm, respectively. A hypsochromic shift was observed for 8NX with decreasing pH (Figure 1) as was reported for 8NG (*36*). As reduction with sodium hydrosulfite converted 8NG to 8-aminoguanine (8AG), same reagent reduced 8NX to 8-aminoxanthine (8AX), which was evident by mass spectroscopic analysis (Scheme 2).

The negative laser desorption ionization (LDI) mass spectra of 8NX and 8AX showed the *m*/*z* 196 and 166 as the $[M - H]^-$ ions, respectively (Figure 2). The odd molecular weight of 8NX and 8AX determined by LDI mass spectrometry was supported by the nitrogen rule of odd number of molecular weight for compounds containing odd nitrogen atoms. Reduction of the nitro groups turned the yellow 8NX and 8NG to the colorless 8AX and 8AG, respectively. The 8AG produced from 8NG reduction was identical to that from acid hydrolysis of commercially available 8-aminoguanosine as evidenced by their mass and UV spectra, their retention times and coelution on reversed-phase HPLC. The UV spectrum of 8AX has the absorption maximum at 206 and 287 nm as compared to that for 8AG at 250 and 291 nm at pH 5.5.

To further confirm that the nitro group of 8NX was at the C-8 position as that in 8NG, 8NG was treated with acidic *tert*-butylnitrite, which was known to deaminate guanine to xanthine (*38*). In the presence of the deamination agent, 8NG was converted to 8NX (Scheme 3 and

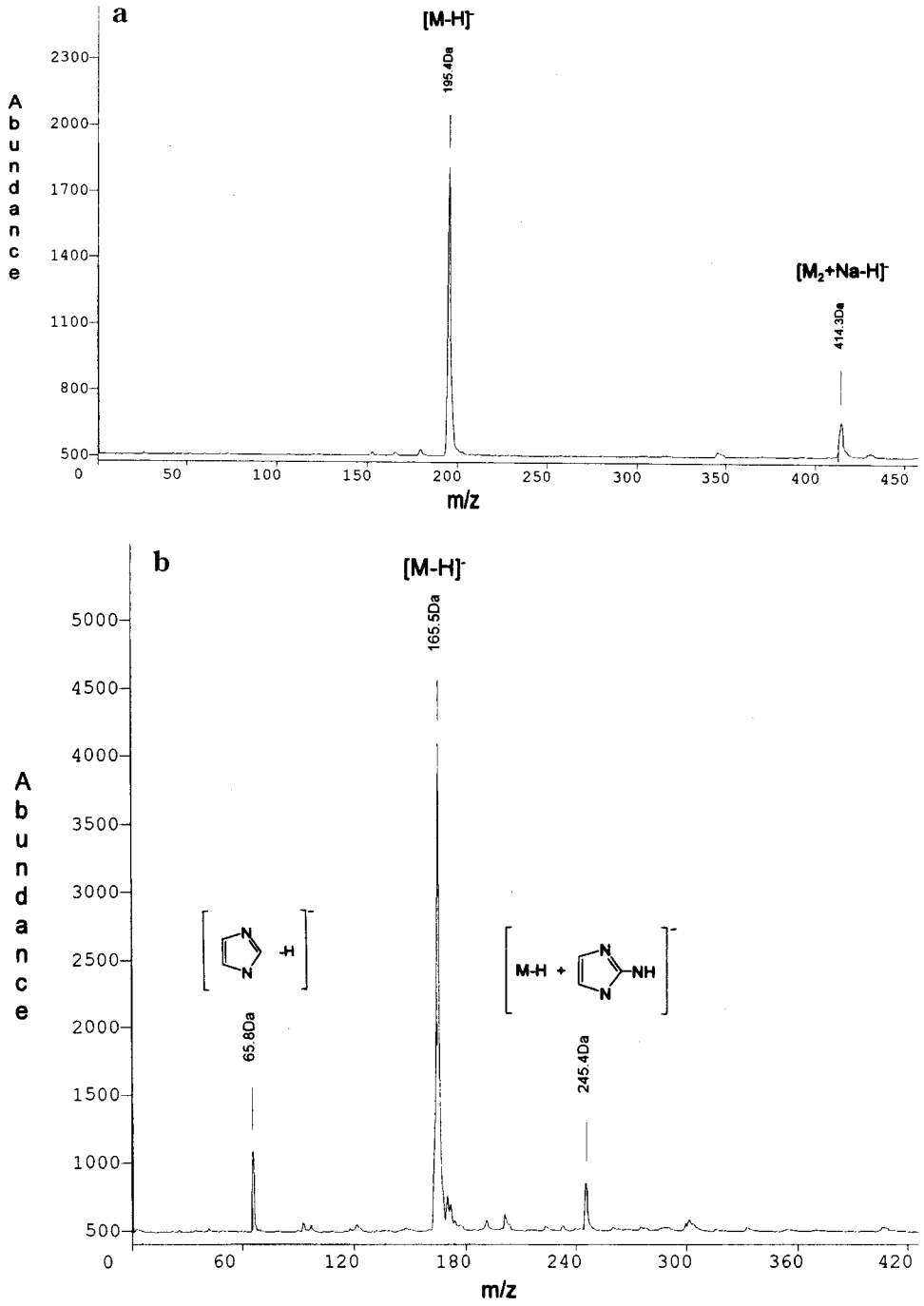
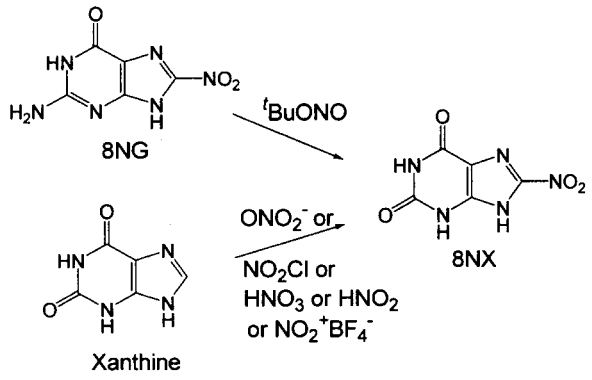


Figure 2. Laser desorption ionization mass spectrum of (a) 8NX and (b) 8AX.

Figure 3a). The identity of the product in this reaction was confirmed by their UV spectra and by coelution in reversed-phase HPLC with the authentic 8NX synthesized from xanthine reaction with peroxynitrite (Figure 3b). Furthermore, the product was collected from reversed-phase HPLC, reduced by sodium hydrosulfite, and co-eluted with 8AX synthesized from reduction of 8NX obtained from xanthine reaction with peroxynitrite (Figure 3c). The reduced product also has identical UV spectrum with synthetic 8AX. The sharp singlets at 10.9 and 10.2 ppm in ^{1}H NMR of 8NX in DMSO-d_6 are the N1- and N3-protons and the N9-proton is the broad peak around 7.0 ppm. The absence of the C8−H around 8.0 ppm, as that of xanthine, indicated that the C8−H is substituted with the nitro group.

Scheme 3. Synthesis of 8NX



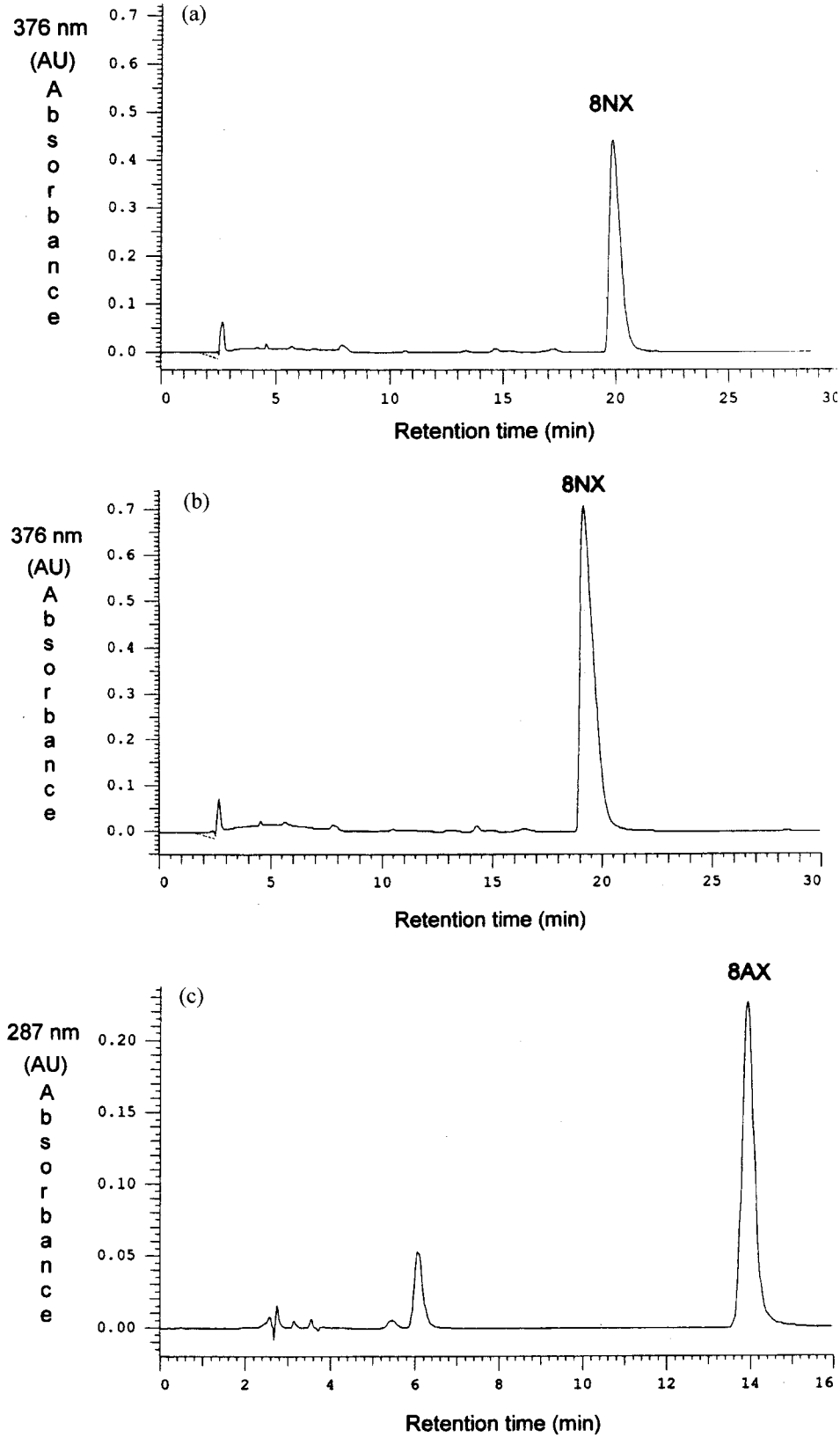


Figure 3. HPLC Chromatogram of reaction of 8NG with ^{t}BuONO to form 8NX. (a) A solution of 8NG (2.5 μmol) in 4.0 mL of 3 N HCl was added ^{t}BuONO (250 μmol) with stirring at room temperature for 1 h; (b) a portion of the reaction mixture (80 μL) was co-injected with synthetic 8NX (35 nmol); (c) a portion of the reaction mixture (3.0 mL) was collected and reduced by sodium hydrosulfite (500 μg). One thirtieth of the reduced mixture was added synthetic 8AX (6.3 nmol) and analyzed by HPLC as described in the Materials and Methods.

Formation of 8NX and 8NG. The pH-dependent nitration of xanthine by nitryl chloride was examined. In reaction of nitryl chloride with equal amount of xanthine, maximum yield of 8NX was achieved at slightly alkaline pH (Figure 4), which is physiologically significant. The pH profile was consistent with that in tyrosine nitration by mixing $NaNO_2$ and NaOCl (*27*). This result suggests that hypochlorous acid (HOCl) and nitrite ion

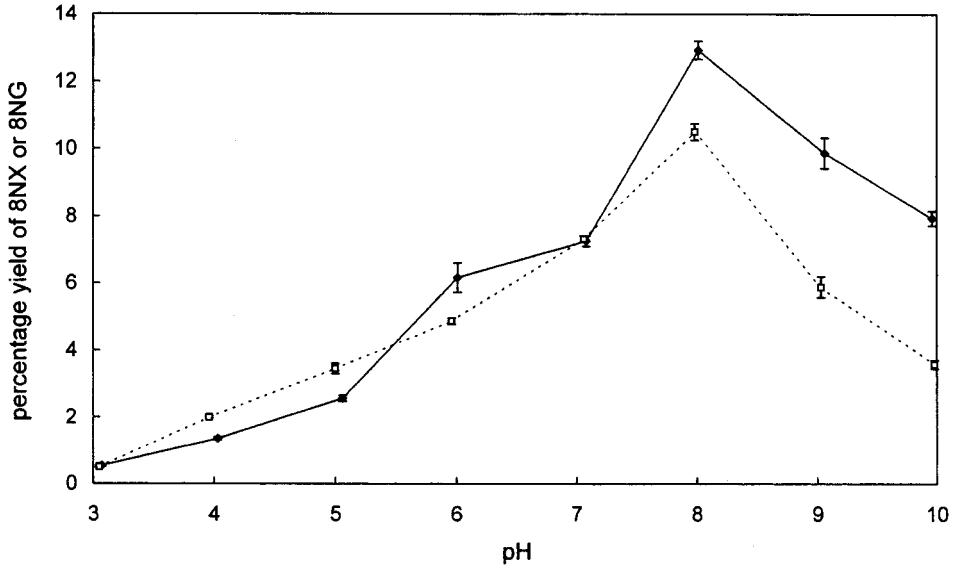


Figure 4. Effect of pH on nitration of xanthine or guanine with equal amount of nitryl chloride forming the respective 8NX (◆) or 8NG (□). Xanthine or guanine (0.66 μmol in 0.1 mM HCl) was added 0.4 M potassium phosphate buffer (pH 3.0−10.0), $NaNO_2$ (0.66 μmol), and NaOCl (0.66 μmol) at room temperature with a final volume of 0.4 mL. Results are expressed as mean ± SD of triplicate experiments.

Table 1. Reactions of Xanthine or Guanine with Various Reactive Nitrogen Species

reaction	yield of 8NX[a]	reaction	yield of 8NX	yield of 8NG[a]
X + NO_2Cl (1:1)[b]	7.3%	G + NO_2Cl (1:1)[b]	ND[e]	7.3%
X + ONO_2^- (1:1)[b]	9.2%	G + ONO_2^- (1:1)[b]	ND	8.9%
X + HNO_3 (1:100)[c]	4.0%	G + HNO_3 (1:100)[c]	0.1%	3.7%
X + HNO_2 (1:100)[c]	2.5%	G + HNO_2 (1:100)[c]	13%	ND
X + $NO_2^+BF_4^-$ (1:240)[d]	0.4%	G + $NO_2^+BF_4^-$ (1:9)[d]	ND	1.5%

[a] Average of duplicate experiments. [b] Room temperature, pH 7.0, 5 min. [c] 90 °C, 3 h. [d] Room temperature, 5 min. Prolonged reaction time led to decrease of the product. [e] Not detectable.

(NO_2^-), rather than hypochlorite (OCl^-) and nitrous acid (HNO_2), are involved in formation of this nitration species since pK_a of HOCl and HNO_2 is 7.5 and 3.4, respectively.

Synthesis of 8NX was achieved by reactions of xanthine with excessive amount of NO_2Cl or peroxynitrite (Scheme 3). To isolate pure 8NX, it was collected from the reaction mixture by reversed-phase HPLC eluting with a buffer at pH 5.5 to separate it from unreacted xanthine, followed by removal of the salt with a second HPLC eluting with water. In reaction of xanthine with peroxynitrite, 8NX was the only product, whereas other side products were present in the reaction mixture of xanthine with nitryl chloride. Therefore, collection of 8NX from the reaction mixture of xanthine with peroxynitrite gave large quantity of the pure product more efficiently than from the nitryl chloride reaction.

8-Nitroxanthine was also formed as the major product in reaction of xanthine with nitric or nitrous acid heated at 90 °C for 3 h, a condition that reactive nitrogen oxides, such as nitrogen dioxide ($NO_2^•$), dinitrogen tetroxide (N_2O_4), and dinitrogen pentoxide (N_2O_5) were generated. Nitric acid gave 8NX in a yield (4.0%) slightly higher than nitrous acid (2.5%) (Table 1). Upon heating, nitric acid generates NO_2 and its dimer N_2O_4, which are in equilibrium with each other. The anhydride of nitric acid, N_2O_5, is also generated upon heating HNO_3. Among these reactive nitrogen species, NO_2, N_2O_4, and N_2O_5 are nitrating agents. When heated nitric acid reacted with guanine, yield of 8NG (3.7%) was slightly less than its reaction with xanthine. Only trace amount of 8NX (0.1%) was obtained as a result of nitration of xanthine, the deamination product of guanine, also present in the reaction mixture. Heating nitrous acid generates nitrous anhydride (N_2O_3), $NO_2^•$, and $NO^•$. Neither $NO^•$ nor N_2O_3 is a nitrating agent, but in the presence of O_2, $NO^•$ is oxidized to $NO_2^•$ ($k = 2 \times 10^6$ M^{-2} s^{-1}), which is capable of nitrating aromatic compounds (*39*, *40*). On the other hand, nitration of xanthine by the nitrosating agent N_2O_3 is presumably via oxidation of the intermediate 8-nitrosoxanthine (*41*). In reaction of guanine with nitrous acid, all the guanine was deaminated to xanthine and high yield (13%) of 8NX was obtained without detectable amount of 8NG. This result suggested that formation of the diazonium salt at the N2-amino enhanced yield of nitration at the C8 position and that 8NG was not an intermediate in formation of 8NX.

Formation of 8NX was observed when xanthine was treated with nitronium tetrafluoroborate ($NO_2^+BF_4^-$) (Scheme 3), presumably via electrophilic attack of the nitronium ion. Nitration of xanthine or guanine by $NO_2^+BF_4^-$ was less effective than other nitrating agents described (Table 1), possibly due to the rapid hydrolysis of nitronium ion. On the other hand, reaction of xanthine with nitrosonium tetrafluoroborate ($NO^+BF_4^-$) gave a product with UV spectrum and retention time in HPLC distinct from those of 8NX. These findings further confirmed that 8NX is a result of nitration, not nitrosylation. The identity of 8NX in reactions of xanthine with heated nitric or nitrous acid or $NO_2^+BF_4^-$ were confirmed by comparing their UV spectrum and by coelution with 8NX synthesized from xanthine with peroxynitrite. Furthermore, 8NX in each reaction mixture was isolated

Table 2. 8NX and 8NG Formation in Reactions of dG or DNA with Nitryl Chloride or Peroxynitrite

reaction	yield of 8NX	yield of 8NG[a]
dG + NO_2Cl (1: 50)[b]	2.5%	0.50%
DNA + NO_2Cl (1: 250)[b]	0.71%	0.30%

[a] Average of duplicate experiments. [b] Room temperature, pH 7.0, 5 min.

from reversed phase HPLC, reduced by sodium hydrosulfite, and the reduced adduct coeluted with authentic 8AX obtained from reduction of synthetic 8NX.

Reaction of dG or DNA with Nitryl Chloride. Reactivity of nitryl chloride toward dG or calf thymus DNA was much lower as compared to its reaction with xanthine or guanine, and the product yield was dependent on the amount of nitryl chloride. Both 8NX and 8NG were formed in reaction of dG with nitryl chloride. On the other hand, only 8NG, but not 8NX was detected in reaction of dG or DNA with peroxynitrite. Molar yields of 8NX and 8NG in reaction of dG with nitryl chloride 50 times in molarity were much higher than in DNA reaction with 250 times of nitryl chloride (Table 2). (The adducts were merely detectable in reaction of DNA with 50 times of nitryl chloride.) In both reactions, 8NX was the major product, and the ratio of 8NX versus 8NG was 5.0 and 2.3 in reaction of dG and DNA, respectively. The ratio of 8NX versus 8NG indicated that hydrogen bondings of guanine in DNA protected it from deamination. Depurination of 8-nitro-2′-deoxyxanthosine in reaction of dG with nitryl chloride, appeared to be very fast since that the 8-nitro nucleoside was not detected even when HPLC analysis was performed immediately after the reaction.

Formation of xanthine was not detected in reaction of dG with nitryl chloride nor in the hydrolysate of nitryl chloride-treated DNA, indicating that formation of 8NX in dG and in DNA was not through the intermediacy of xanthine. When isolated 8NG was added excess nitryl chloride, only very small amount (1.8%) of 8NX was detected (data not shown), suggesting that nitryl chloride is not a good agent for deamination. With the preferred formation of 8NX over 8NG in reactions of nitryl chloride with dG or DNA, the possibility that 8NX detected in these reactions being the end product of 8NG deamination is ruled out.

Yield of 8NX in nitryl chloride-treated DNA was not affected by time of acid (0.1 N HCl, 100 °C, 30−180 min) or neutral thermal hydrolysis (0.1 M phosphate buffer, pH 7.0, 100 °C, 1−24 h) (data not shown). These results indicate that nitryl chloride is short-lived and that 8NX is stable under these hydrolysis conditions. There were more peaks in HPLC chromatogram of the DNA hydrolysate obtained after acid hydrolysis than neutral hydrolysis. The identity of 8NX in DNA hydrolysate and in dG reaction was confirmed by coelution of fractions collected from the reaction mixtures with 8NX synthesized from xanthine reaction with peroxynitrite and analyzed by reversed-phase HPLC with photodiode array detection. In addition, the 8NX elution of these two reactions were collected, reduced, and co-injected with standard 8AX obtained from reduction of synthetic 8NX (Figure 5).

Depurination of 8NX in DNA. The stability and half-life of 8NX formed in nitryl chloride-treated calf thymus DNA was investigated under physiological conditions. When nitryl chloride-treated calf thymus DNA was incubated at 37 °C, pH 7.0, it underwent spontaneous depurination. The half-life of 8NX in nitryl chloride-treated calf thymus DNA was 2 h as measured by the time-dependent release of 8NX in the medium and 8NX remained in DNA at various intervals. The latter was analyzed after DNA precipitation followed by hydrolysis. The sum of 8NX in the medium and 8NX remained in DNA was nearly constant, indicating that the adduct was completely released after 24 h (Figure 6).

Discussion

Since chronic infections and inflammation is implicated in cancer development, it is of interest to study the chemistry of these reactive species with DNA. Among the reactive species, peroxynitrite and nitryl chloride are capable of nitrating biomolecules. The nitration product of guanine, 8NG, can be derived from peroxynitrite reaction with guanine or DNA (*21*, *36*). It is also formed in the myeloperoxidase-hydrogen peroxide-nitrite system of activated human neutrophils with dG (*26*), presumably by NO_2Cl or $NO_2^{\bullet}$ formed via the one-electron oxidation of the nitrite ion by compound I or compound II of peroxidases (*42*).

In the absence of an enzyme, our results demonstrate the formation of 8NX, in addition to 8NG, as the major nitration product in reaction of 2′-deoxyguanosine or calf thymus DNA with nitryl chloride, generated in vitro from sodium nitrite and sodium hypochlorite under neutral pH. 8-Nitroxanthine was also detected in xanthine reaction with various reactive nitrogen species, including nitryl chloride, peroxynitrite, nitronium tetrafluoroborate, and heated nitric or nitrous acid. Multiple lines of experiments suggested that the reaction pathway of forming 8NX was independent of 8NG formation. First, addition of excess nitryl chloride to purified 8NG produced negligible amount of 8NX. Second, a much higher yield of 8NX was obtained in reaction of nitrous acid with guanine as compared to the same reaction with xanthine, indicating that formation of the diazonium ion at the N2-amino group of guanine may play a key role to enhance the nitration yield at the C8 position. Another supplementary support can be rendered by the ratio of 8NX versus 8NG in nitryl chloride-treated DNA, which was lower than that in nitryl chloride reaction with dG, suggesting that the Watson−Crick type hydrogen bondings of guanine in DNA hindered the diazonium ion formation to yield 8NX. Furthermore, yield of 8NX in nitryl chloride-treated dG or DNA exceeded that of 8NG, while xanthine was not detectable in the reaction mixtures. Collectively, these results indicate that formation of 8NX in nitryl chloride-treated dG or DNA is not through the intermediacy of 8NG, but rather the formation of 8NX and 8NG is ascribed to two competing processes.

A tentative mechanism proposed for the formation of 8NX and 8NG is depicted in Scheme 4. Upon reaction of nitryl chloride with dG or the dG moiety in DNA, a neutral radical, dG(−H)$^{\bullet}$, is obtained through the one-electron oxidation and simultaneous deprotonation at the C-8 position. Additional nitryl chloride can attack this radical at C-8, resulting in 8-nitro-dG which subsequently depurinates to give 8NG. Alternatively, nitryl chloride can react with the N-2 amino group forming diazonium ion radical intermediate **I**. This intermediate **I** reacts rapidly with another nitryl chloride at C-8 to form the diazonium intermediate **II**, which is subsequently hydrolyzed to yield 8-nitro-2′-deoxyxanthosine and 8NX

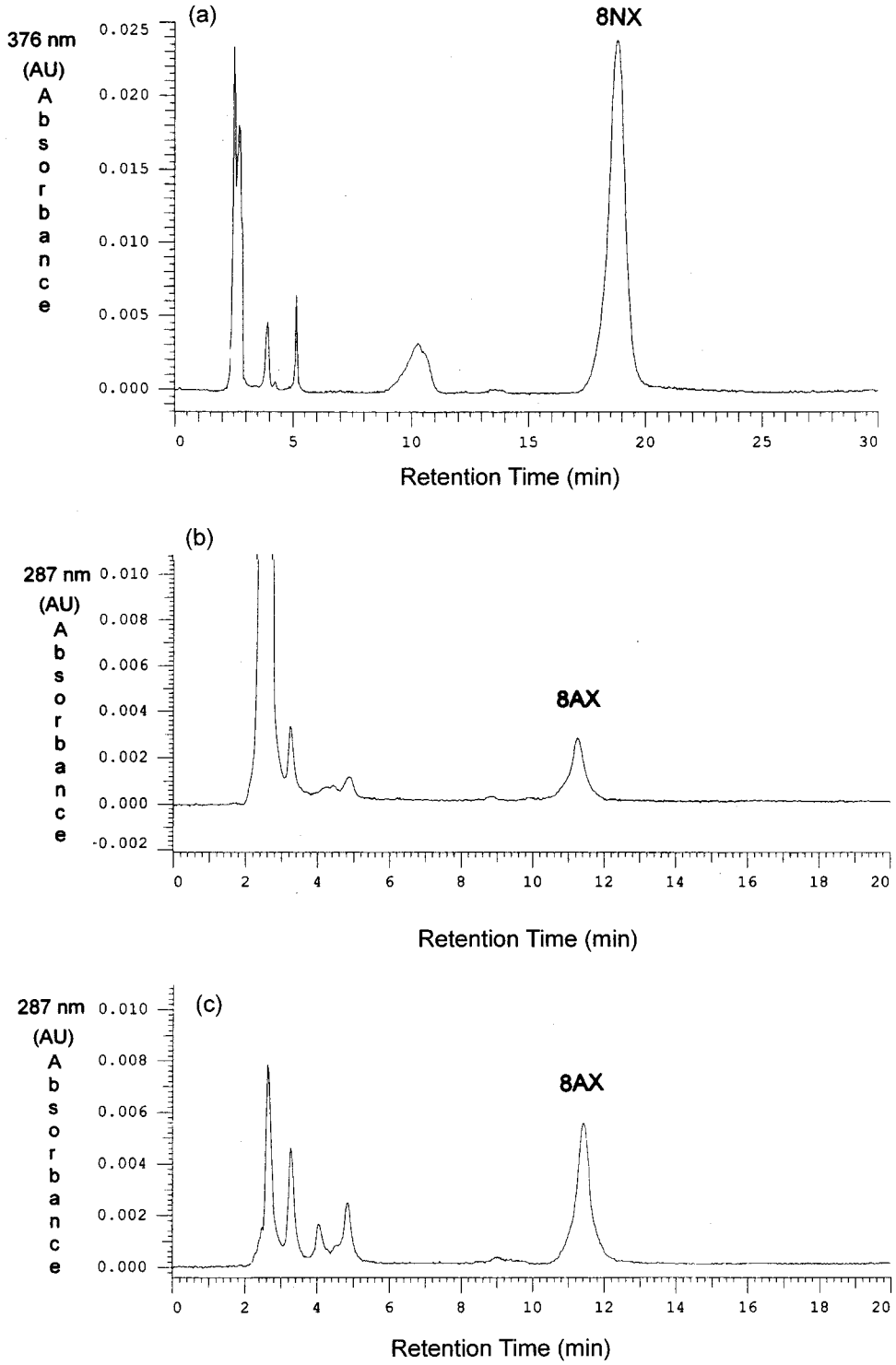


Figure 5. 8NX in reaction of calf thymus DNA with nitryl chloride. (A) Hydrolysate of calf thymus DNA reaction with nitryl chloride. Calf thymus DNA (2.0 mg in 2.0 mL 0.2 M potassium phosphate buffer, pH 7.0) was added 770 μL of a solution containing $NaNO_2$ (0.58 M) and 372 μL of a solution of NaOCl (0.30 M). After vortexing for 2 min., DNA was precipitated, washed, and hydrolyzed with acid as described under the Materials and Methods. (B) Reduction mixture of 8NX collected from hydrolysate of nitryl chloride-treated DNA. (C) Co-injection of 8AX (48 ng) with the reduction mixture of 8NX collected from hydrolysate of nitryl chloride-treated calf thymus DNA.

after depurination. Nitration of intermediate **I** to form intermediate **II** is expected to be a highly exergonic, low energy barrier process due to the possible resonance induction via the electron-withdrawing group (i.e., the diazonium ion). Accordingly, the bimolecular rate constant k_4 for the decomposition of intermediate **I** forming intermediate **II** should be much greater than k_2 and k_3, resulting in a steady-state approximation for the intermediate **I**, i.e., $d[\mathbf{I}]/dt \approx 0$, while the k_2 and k_3 are rate determining steps for the decomposition of dG(−H)•. In reaction of dG, the yield of 8NX is much greater than 8NG, indicating $k_3 > k_2$. Whereas in reaction of DNA, the rate of formation of intermediate **I**, k_3, is relatively slow due to the hydrogen bonding formation at the N-2 amino position, resulting in an increase of the relative yield of 8NG. Since intermediate **I** is likely to reach a

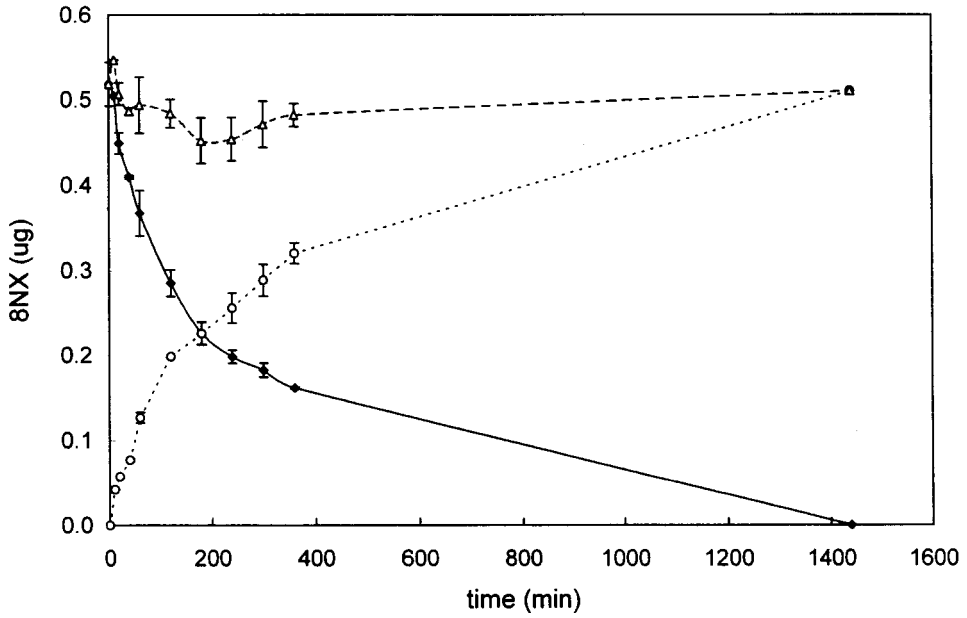
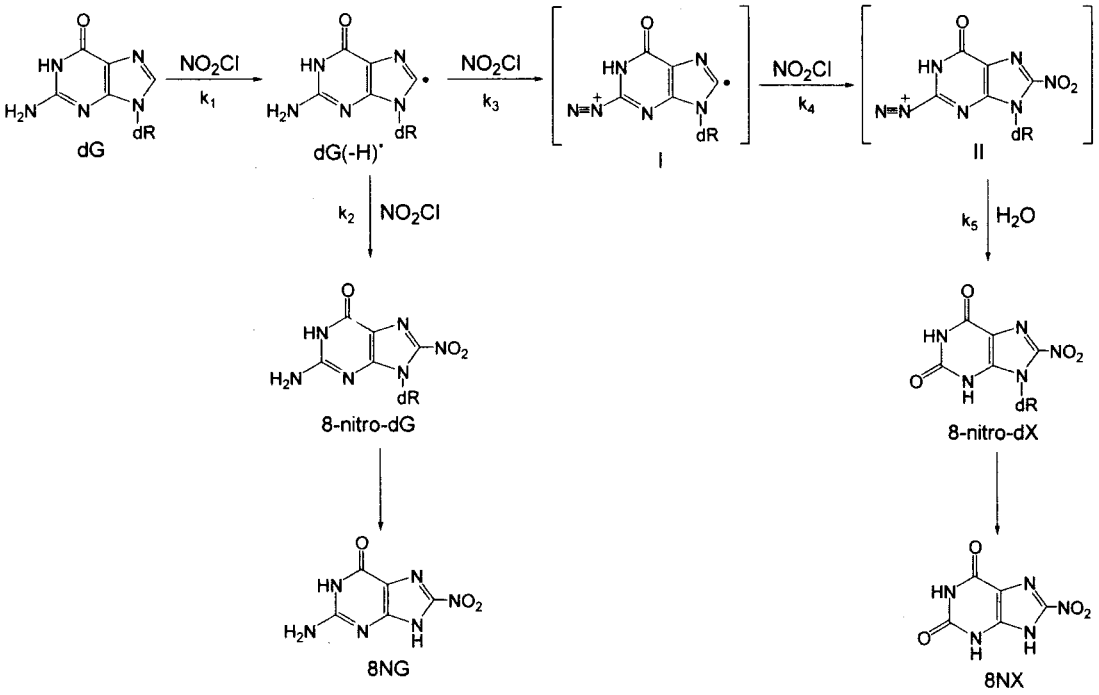


Figure 6. Release of 8NX from DNA under physiological conditions. Calf thymus DNA (1.0 mg/mL) was treated with nitryl chloride at room temperature, followed by incubation at 37 °C, pH 7.0. At various time intervals, an aliquot (0.4 mL) of the reaction mixture was removed, precipitated with cold ethanol, and washed twice with 70% cold ethanol. The ethanol solutions were combined and evaporated for analysis of 8NX released in the medium. DNA was dissolved in phosphate buffer (pH 7.0) and hydrolyzed at 100 °C for 3 h. (◆) 8NX in DNA; (○) 8NX in the medium; (△) total 8NX. Results are expressed as mean ± SD of duplicate experiments.

Scheme 4. A Proposed Mechanism for the Formation of 8NX and 8NG



steady state due to the large bimolecular rate constant k_4, it qualitatively explains the fact that xanthine, its hydrolysis product, was not detected in DNA hydrolysate.

The glycosidic linkage of 8NX in DNA is more labile than that of 8NG. Once formed in DNA, 8NX underwent spontaneous depurination with a half-life of 2 h when incubated under physiological conditions. Therefore, 8NX might be a more mutagenic lesion than 8NG in forming apurinic sites since the half-life of 8NG in peroxynitrite-treated calf thymus DNA was 4 h (*21*). Although the role of 8NX in carcinogenesis remains to be determined, it might be an important DNA damage due to nitration by reactive nitrogen species generated in vivo by phagocytes at sites of inflammation.

Although nitryl chloride has weaker nitrating activity toward phenolics, such as tyrosine and acetaminophen, than peroxynitrite (*43*, *44*), it might be significant in nitration, chlorination, and oxidation of many biomolecules, including nucleic acids, lipids, and sterols. Nitrite ion, being a substrate and inhibitor of myeloperoxidase (*45*), plays an important role in the antimicrobial activity of reactive nitrogen species (*46*). Concentrations of nitrite in extracellular fluids vary between sub-micromolar in plasma to ca. 200 μM in saliva, which can be increased

to millimolar range after intake of nitrate from diet (*47*) or due to inflammation (*48*, *49*). The elevated level of nitrite during inflammation appears to be a result of overproduction of NO by inducible nitric oxide synthase (*50*). Since at least 28% of the oxygen consumed by human neutrophils is converted to HOCl (*51*, *52*), production of nitryl chloride in vivo is highly plausible when HOCl meets nitrite in the biological fluids (*53*). Therefore, increased levels of both HOCl and nitrite during inflammation might contribute significantly to endogenous nitration of biomolecules. Detection of nitrated and chlorinated tyrosine residues of protein in phagocyte activation of human neutrophils (*54*, *55*) indicated that elevated production of NO and HOCl at inflammatory sites and that nitryl chloride is at least one of the reactive species (*56*). Thus, 8NX and 8NG may be useful biomarkers for the exposure of DNA to endogenous nitrating species, including nitryl chloride and peroxynitrite.

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References

(1) Ohshima, H., and Bartsch, H. (1994) Chronic infections and inflammatory processed as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat. Res.* **305**, 253−264.

(2) Gordon, L. I., and Weitzman, S. A. (1993) Inflammation and Cancer. *Cancer J.* **6**, 257−261.

(3) Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* **5**, 834−842.

(4) Ischiropoulos, H., Zhu, L., and Beckman, J. S. (1992) Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.* **298**, 446−451.

(5) Lymar, S. V., and Hurst, J. K. (1995) Rapid reaction between peroxynitrite ion and carbon dioxide: Implications for biological activity. *J. Am. Chem. Soc.* **117**, 8867−8868.

(6) Lymar, S. V., and Hurst, J. K. (1996) Carbon Dioxide: Physiological Catalyst for Peroxynitrite-Mediated Cellular Damage or Cellular Protectant? *Chem. Res. Toxicol.* **9**, 845−850.

(7) Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996) Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. *Biochemistry* **35**, 7855−7861.

(8) Lemercier, J. N., Squadrito, G. L., and Pryor, W. A. (1995) Spin trap studies on the decomposition of peroxynitrite. *Arch. Biochem. Biophys.* **321**, 31−39.

(9) Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. (1990) Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1620−1624.

(10) Marla, S. S., Lee, J., and Groves, J. T. (1997) Peroxynitrite rapidly permeates phospholipid membranes. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 14243−14248.

(11) Denicola, A., Souza, J. M., and Radi, R. (1998) Diffusion of peroxynitrite across erythrocyte membranes. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3566−3571.

(12) Spencer, J. P. E., Wong, J., Jenner, A., Aruoma, O. I., Cross, C. E., and Halliwell, B. (1996) Base modification and strand breakage in isolated calf thymus DNA and in DNA from human skin epidermal keratinocytes exposed to peroxynitrite or 3-morpholinosydnonimine. *Chem. Res. Toxicol.* **9**, 1152−1158.

(13) Douki, T., Cadet, J., and Ames, B. N. (1996) An adduct between peroxynitrite and 2′-deoxyguanosine: 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine. *Chem. Res. Toxicol.* **9**, 3−7.

(14) Arteel, G. E., Kadiiska, M. B., Rusyn, I., Bradford, B. U., Mason, R. P., Raleigh, J. A., and Thurman, R. G. (1999) Oxidative stress occurs in perfused rat liver at low oxygen tension by mechanisms involving peroxynitrite. *Mol. Pharmacol.* **55**, 708−715.

(15) Wu, G. S., Zhang, J., and Rao, N. A. (1997) Peroxynitrite and oxidative damage in experimental autoimmune uveitis. *Invest. Ophthalmol. Visual Sci.* **38**, 1333−1339.

(16) Koppal, T., Drake, J., Yatin, S., Jordan, B., Varadarajan, S., Bettenhausen, L., and Butterfield, D. A. (1999) Peroxynitrite-induced alterations in synaptosomal membrane proteins: insight into oxidative stress in Alzheimer's disease. *J. Neurochem.* **72**, 310−317.

(17) Pennathur, S., Jackson-Lewis, V., Przedborski, S., and Heinecke, J. W. (1999) Mass spectrometric quantification of 3-nitrotyrosine, ortho-tyrosine, and o,o′-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice, a model of oxidative stress in Parkinson's disease. *J. Biol. Chem.* **274**, 34621−34628.

(18) Aoyama, K., Matsubara, K., Fujikawa, Y., Nagahiro, Y., Shimizu, K., Umegae, N., Hayase, N., Shiono, H., and Kobayashi, S. (2000) Nitration of manganese superoxide dismutase in cerebrospinal fluids is a marker for peroxynitrite-mediated oxidative stress in neurodegenerative diseases. *Ann. Neurol.* **47**, 524−527.

(19) Juedes, M. J., and Wogan, G. N. (1996) Peroxynitrite-induced mutation spectra of pSP189 following replication in bacteria and in human cells. *Mutat. Res.* **349**, 51−61.

(20) Yermilov, V., Yoshie, Y., Rubio, J., and Ohshima, H. (1996) Effects of carbon dioxide/bicarbonate on induction of DNA single-strand breaks and formation of 8-nitroguanine, 8-oxoguanine and base-propenal mediated by peroxynitrite. *FEBS Lett.* **399**, 67−70.

(21) Yermilov, V., Rubio, J., and Ohshima, H. (1995) Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination. *FEBS Lett.* **376**, 207−210.

(22) Burney, S., Niles, J. C., Dedon, P. C., and Tannenbaum, S. R. (1999) DNA damage in deoxynucleosides and oligonucleotides treated with peroxynitrite. *Chem. Res. Toxicol.* **12**, 513−520.

(23) Tretyakova, N. Y., Niles, J. C., Burney, S., Wishnok, J. S., and Tannenbaum, S. R. (1999) Peroxynitrite-induced reactions of synthetic oligonucleotides containing 8-oxoguanine. *Chem. Res. Toxicol.* **12**, 459−466.

(24) Muller, T., Haussmann, H. J., and Schepers, G. (1997) Evidence for peroxynitrite as an oxidative stress-inducing compound of aqueous cigarette smoke fractions. *Carcinogenesis* **18**, 295−301.

(25) Pryor, W. A., and Stone, K. (1993) Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Ann. N. Y. Acad. Sci.* **686**, 12−27.

(26) Byun, J., Henderson, J. P. Mueller, D. M., and Heinecke, J. W. (1999) 8-Nitro-2′-deoxyguanosine, a specific marker of oxidation by reactive nitrogen species, is generated by the myeloperoxidase-hydrogen peroxide-nitrite system of activated human phagocytes. *Biochemistry* **38**, 2590−2600.

(27) Eiserich, J. P., Cross, C. E., Jones, A. D., Halliwell, B., and van der Vliet, A. (1996) Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid. A novel mechanism for nitric oxide-mediated protein modification. *J. Biol. Chem.* **271**, 19199−19208.

(28) Schmitt, D., Shen, Z., Zhang, R., Colles, S. M., Wu, W., Salomon, R. G., Chen, Y., Chisolm, G. M., Hazen, S. L. (1999) Leukocytes utilize myeloperoxidase-generated nitrating intermediates as physiological catalysts for the generation of biologically active oxidized lipids and sterols in serum. *Biochemistry* **38**, 16904−16915.

(29) Suzuki, T., Yamada, M., Kanaori, K., Tajima, K., Morii, T., and Makino, K. (1999) Formation of 8-nitroguanine from 2′-deoxyguanosine by NO/O_2 system. *Nucleic Acids Symp. Ser.* **42**, 155−156.

(30) Shapiro, R., and Pohl, S. H. (1968) The reaction of ribonucleosides with nitrous acid. Side products and kinetics. *Biochemistry* **7**, 448−455.

(31) Nelson, D. P., and Kiesow, L. A. (1972) Enthalpy of decomposition of hydrogen peroxide by catalase at 25 degrees C. *Anal. Biochem.* **49**, 474−478.

(32) Uppu, R. M., and Pryor, W. A. (1996) Biphasic synthesis of high concentrations of peroxynitrite using water-insoluble alkyl nitrite and hydrogen peroxide. *Method Enzymol.* **269**, 322−329.

(33) Beckman, J. S., Chen, J., Ischiropoulos, H., and Crow, J. P. (1994) Oxidative chemistry of peroxynitrite. *Methods Enzymol.* **233**, 229−240.

(34) Morris, J. C. (1966) The acid ionization constant of HOC1 from 5 to 35 degrees. *J. Phys. Chem.* **70**, 3798−3805.

(35) Sodum, R. S., Nie, G., and Fiala, E. S. (1993) 8-Aminoguanine: a base modification produced in rat liver nucleic acids by the hepatocarcinogen 2-nitropropane. *Chem. Res. Toxicol.* **6**, 269−276.

(36) Yermilov, V., Rubio, Becchi, M., Friesen, M. D., Pignatelli, B., and Ohshima, H. (1995) Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite in vitro. *Carcinogenesis* **16**, 2045−2050.

(37) Redemann, C. T., and Redemann, C. E. (1955) 5-Amino-2,3-dihydro-1,4-phthalazinedione. *Org. Synth. Coll.* **3**, 69−70.

(38) Wu, K. Y., Scheller, N., Ranasinghe, A., Yen, T. Y., Sangaiah, R., Giese, R, and Swenberg, J. A. (1999) A gas chromatography/electron capture/negative chemical ionization high-resolution

mass spectrometry method for analysis of endogenous and exogenous N7-(2-hydroxyethyl)guanine in rodents and its potential for human biological monitoring. *Chem. Res. Toxicol.* **12**, 722−729.

(39) Olah, G. A., Malhotra, and Narang, S. C. (1989) *Nitration. Methods and Mechanisms*, p 92−132, VCH Publishers, New York.

(40) Lewis, R. S., and Deen, W. M. (1994) Kinetics of the reaction of nitric oxide with oxygen in aqueous solutions. *Chem. Res. Toxicol.* **7**, 568−574.

(41) Lewis, R. S., Tannenbaum, S. R., and Deen, W. M. (1995) Kinetics of N-nitrosation in oxygenated nitric oxide solutions at physiological pH: Role of nitrous anhydride and effects of phosphate and chloride. *J. Am. Chem. Soc.* **117**, 3933−3939.

(42) van der Vliet, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1997) Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem.* **272**, 7617−7625.

(43) Ohshima, H., Celan, I., Chazotte, L., Pignatelli, B., and Mower, H. F. (1999) Analysis of 3-nitrotyrosine in biological fluids and protein hydrolyzates by high-performance liquid chromatography using a postseparation, on-line reduction column and electrochemical detection: Results with various nitrating agents. *Nitric Oxide* **3**, 132−141.

(44) Lakshmi, V. M., Hsu, F. F., Davis, B. B., and Zenser, T. V. (2000) Nitrating reactive nitric oxygen species transform acetaminophen to 3-nitroacetaminophen. *Chem. Res. Toxicol.* **13**, 891−899.

(45) van Dalen, C. J., Winterbourn, C. C., Senthilmohan, R., and Kettle, A. J. (2000) Nitrite as a substrate and inhibitor of myeloperoxidase. Implications for nitration and hypochlorous acid production at sites of inflammation. *J. Biol. Chem.* **275**, 11638−11644.

(46) Klebanoff, S. J. (1993) Reactive nitrogen intermediates and antimicrobial activity: Role of nitrite. *Free Radical Biol. Med.* **14**, 351−360.

(47) Tannenbaum, S. R., Weisman, M., and Fett, D. (1976) The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet. Toxicol.* **14**, 549−552.

(48) Leone, A. M., Francis, P. L., Rhodes, P., and Moncada, S. (1994) A rapid and simple method for the measurement of nitrite and nitrate in plasma by high performance capillary electrophoresis. *Biochem. Biophys. Res. Commun.* **200**, 951−957.

(49) Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982) Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.* **126**, 131−138.

(50) Kharitonov, S. A., Chung, K. F., Evans, D., O'Connor, B. J., and Barnes, P. J. (1996) Increased exhaled nitric oxide in asthma is mainly derived from the lower respiratory tract. *Am. J. Respir. Crit. Care Med.* **153**, 1773−1780.

(51) Weiss, S. J., Klein, R., Slivka, A., and Wei, M. (1982) Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. *J. Clin. Invest.* **70**, 598−607.

(52) Foote, C. S., Goyne, T. E., and Lehrer, R. I. (1983) Assessment of chlorination by human neutrophils. *Nature* **301**, 715−716.

(53) Whiteman, M., Spencer, J. P., Jenner, A., and Halliwell, B. (1999) Hypochlorous acid-induced DNA base modification: potentiation by nitrite: Biomarkers of DNA damage by reactive oxygen species. *Biochem. Biophys. Res. Commun.* **257**, 572−576.

(54) Hazen, S. L., Hsu, F. F., Mueller, D. M., Crowley, J. R., and Heinecke, J. W. (1996) Human neutrophils employ chlorine gas as an oxidant during phagocytosis. *J. Clin. Invest.* **98**, 1283−1289.

(55) Evans, T. J., Buttery, L. D., Carpenter, A., Springall, D. R., Polak, J. M., and Cohen, J. (1996) Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 9553−9558.

(56) Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and van der Vliet, A. (1999) Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* **391**, 393−397.

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