Analysis of Peroxynitrite Reactions with Guanine, Xanthine, and Adenine Nucleosides by High-Pressure Liquid Chromatography with Electrochemical Detection: C8-Nitration and -Oxidation

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Peroxynitrite, the reaction product of nitric oxide and superoxide anion, and a powerful oxidant, was found to nitrate as well as oxidize adenine, guanine, and xanthine nucleosides. A highly sensitive reverse-phase HPLC method with a dual-mode electrochemical detector, which reduces the nitro product at the first electrode and detects the reduced product by oxidation at the second electrode, was applied to detect femtomole levels of 8-nitroguanine and 8-nitroxanthine. This method was used to separate and identify the products of nitration and oxidation from the reactions of nucleosides with peroxynitrite. Peroxynitrite nitrates deoxyguanosine at neutral pH to give the very unstable 8-nitrodeoxyguanosine, in addition to 8-nitroguanine. 8-Nitrodeoxyguanosine, with a half-life of ∼10 min at room temperature and ≤3 min at 37 °C, hydrolyzes at pH 7 to 8-nitroguanine. A decrease in the reaction pH resulted in a decrease in the level of C8-nitration. Peroxynitrite also oxidizes deoxyguanosine in a pH-dependent manner, to give 8-oxodeoxyguanosine with a maximum yield (0.5−0.7%) at pH 5. Guanosine and xanthosine exhibit reactivity similar to that of deoxyguanosine toward peroxynitrite at neutral pH, producing only the corresponding 8-nitronucleosides as well as 8-nitroguanine and 8-nitroxanthine, respectively. 8-Nitroguanosine at pH 7, with a half-life of several weeks at 5 °C and 5 h at 37 °C, was much more stable than 8-nitrodeoxyguanosine. C8-nitration was confirmed by dithionite reduction to the corresponding amino nucleosides, which cochromatographed with synthesized 8-amino nucleoside standards. In contrast to guanine nucleosides, adenine nucleosides undergo peroxynitrite-mediated C8 oxidation even at neutral pH to give the corresponding 8-oxoadenine nucleosides in ∼0.3% yield. Adenine nitration, though minor compared to C8-oxidation, appears to occur at both C2 and C8 positions of the adenine ring. Lowering the reaction pH from 7 to 5 results in 2.4- and 2.2-fold increases in the yields of 8-oxo-dA and 8-oxo-Ado, respectively, but the level of nitration is not altered.

Introduction

The formation of peroxynitrous acid (pernitrous acid) from hydrogen peroxide and nitrous acid, and its nitration and hydroxylation of aromatic compounds such as benzene, had been described by Halfpenny and Robinson many years ago (*1*, *2*). They had also suggested that peroxynitrous acid exhibited this property by undergoing homolytic fission to OH and NO_2 free radicals (*2*). The report that nitric oxide and superoxide can combine to form peroxynitrite, which could be responsible for endothelial injury (*3*), stimulated immense interest during the past decade in the very complex chemistry and biological chemistry of peroxynitrite, which has been reviewed comprehensively (*4*, *5*). The pK_a of peroxynitrite has been reported to be 6.6 or 6.8 (*6*, *7*), which is close to neutral, and the anionic form is relatively stable in alkaline solutions (pH ∼12). Once protonated, the acid form isomerizes spontaneously, mostly to nitrate, with a half-life of ∼1 s at pH 7.0 (*7*). During this isomerization, it also produces a highly potent oxidizing and nitrating species, whose nature has been a great concern to researchers because of its potential to modify biological macromolecules (*3*−*11*). The main targets for modifications by peroxynitrite in biological systems are proteins, lipids, and nucleic acids. Peroxynitrite-mediated tyrosine nitration (*12*−*14*), guanine nitration (*15*), and the further oxidation and nitration of 8-oxodeoxyguanisine (8-oxo-dG)[1] have been extensively investigated (*16*, *17*). However, the reactivity of peroxynitrite[2] toward other nucleic acid components, for instance, guanosine in RNA, or the adenine nucleosides in RNA or DNA, and studies on peroxynitrite-induced oxidative damage to nucleic acids, have been more limited.

Peroxynitrite has been shown to react at neutral pH with DNA in vitro to give 8-oxo-dG (*18*−*21*) and

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[1] Abbreviations: HPLC-ECD, high-pressure liquid chromatography with dual-mode electrochemical detection; EC, electrochemical; 8-NG, 8-nitroguanine; 8-NX, 8-nitroxanthine; DTPA, diethylenetriaminepentaacetate; 8-nitro-dG, 8-nitrodeoxyguanosine; 8-oxo-dG, 8-oxodeoxyguanosine; 8-oxo-Guo, 8-oxoguanosine; 8-nitro-dA, 8-nitrodeoxyadenosine; 8-nitro-Guo, 8-nitroguanosine; 8-nitro-Ado, 8-nitroadenosine; 8-oxo-Ado, 8-oxoadenosine; 8-oxo-dA, 8-oxodeoxyadenosine; 8-NH_2-dA, 8-aminodeoxyadenosine; 8-NH_2-Ado, 8-aminoadenosine.

[2] Throughout this article, the phrases "peroxynitrite reactivity" and "peroxynitrite reactions" refer to the reactivity and reactions of the unstable conjugate acid, peroxynitrous acid, respectively, not those of the peroxynitrite anion itself.

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8-oxodeoxyadenosine (8-oxo-dA) (*22*) in addition to 8-nitroguanine (*15*, *23*). While the nitration of deoxyguanosine in DNA was demonstrated through the formation of 8-nitroguanine (*15*, *23*), the unstable 8-nitrodeoxyguanosine (8-nitro-dG) itself has so far not been observed. In the report on 8-oxo-dA formation, it is not clear at what pH the peroxynitrite reaction was carried out and whether the oxidation may have been due to the contamination of the peroxynitrite used with hydrogen peroxide (*22*). We describe here a systematic study of the reactivity of peroxynitrite with guanine, adenine, and xanthine nucleosides at specific conditions. These studies became possible by the application of the reverse-phase HPLC with dual-mode amperometric detection that had been previously developed for the quantitation of 3-nitrotyrosine (*24*). Two electrodes in series, operated in the reductive and oxidative modes sequentially, are utilized in this procedure. The nitro derivatives of nucleosides and the nucleic acid bases from the reactions of peroxynitrite are reduced at the first (upstream) electrode, and the reduced products are oxidized and detected at the second (downstream) electrode. The second electrode which is always in the oxidative mode can, by itself, detect the 8-oxo derivatives of the purine nucleosides. When the reducing electrode is placed on standby, peaks corresponding to the nitro derivatives disappear, and only the 8-oxo products are detected, providing an additional proof for the identity of both types of compounds. Using this dual-mode electrochemical detection method, femtomole levels of 8-nitroguanine, 8-nitroxanthine, and probably other nitrated bases or nucleosides can be detected in HPLC effluents.

In this study, we find that peroxynitrite reacts with deoxyguanosine or guanosine at neutral pH to give 8-nitro-dG or 8-nitro-Guo, respectively, and that 8-nitroguanine is a product in both reactions; some of the properties of 8-nitro-dG and 8-nitro-Guo are described. Like guanine nucleosides, adenine nucleosides also react with peroxynitrite to undergo nitration at C8, and possibly also at C2. However, unlike guanine nucleosides, adenine nucleosides undergo extensive C8-oxidation, the level of which is several times greater than the level of C8- and C2-nitration, to give 8-oxo-dA or 8-oxo-Ado even at neutral pH. As the pH of the reaction decreases, the level of peroxynitrite-mediated C8-oxidation increases in the cases of both guanine and adenine nucleosides. Our studies at the purine base and nucleoside level suggest that the extent of nitration and oxidative damage caused by peroxynitrite to nucleic acids may have been underestimated and should be reexamined.

Experimental Procedures

Materials and Methods. Guanine was obtained from Aldrich Chemical Co. (Milwaukee, WI); deoxyguanosine was purchased from U.S. Biochemical Corp. (Cleveland, OH). All the other nucleosides, nucleotides, and xanthine were obtained from Sigma Chemical Co. (St. Louis, MO). 8-Oxodeoxyadenosine was a kind gift from S. Emburey of Glen Research (Sterling, VA). The Beckman Ultrasphere ODS columns used for HPLC were obtained from Alltech Co. (Allendale, PA). Peroxynitrite was prepared according to the method of Pryor et al. (*25*). The Fischer model 500 ozone generator, purchased from Fischer Associates (Deerfield, IL), was used to produce ozone for the preparation of peroxynitrite. UV spectra were obtained using a GBC model Centra 40 UV/vis spectrophotometer (GBC Scientific Equipment, Inc., Arlington Heights, IL).

Preparation of 8-Nitroguanine from 8-Aminoguanine. 8-Nitroguanine was prepared by the diazotization of 8-aminoguanine and subsequent reaction of 8-diazoguanine with sodium nitrite in the presence of acid using a published procedure (*26*). The yield of crude 8-nitroguanine was ~40%. It was purified by HPLC. 8-Aminoguanine was obtained by acid hydrolysis of 8-aminoguanosine as previously described (*27*).

Preparation of 8-Nitroxanthine from Xanthine. 8-Nitroxanthine was prepared by nitration of xanthine with a mixture of acetic acid and nitric acid according to the published procedure (*28*). Xanthine (0.22 g) and 1.9 mL glacial acetic acid were heated in an oil bath to 120 °C; concentrated nitric acid (0.3 mL) was added dropwise, and the mixture was heated at 120 °C for 1 h. An additional portion of 0.5 mL of glacial acetic acid was added, followed by 0.2 mL of concentrated nitric acid, and the temperature was increased to reflux the reaction mixture for 1 h. The mixture was then allowed to cool to room temperature, and the precipitate was collected by filtration, washed with cold water, and air-dried. Recrystallization from 0.5 N hydrochloric acid gave 60 mg (20% yield) of the product. HPLC analysis of the product showed 8-nitroxanthine was the only product, but was contaminated with some unreacted xanthine, necessitating HPLC purification. 8-Nitroxanthine obtained by this method exhibited UV spectral and chromatographic properties that were the same as those of 8-nitroxanthine prepared from the reaction of peroxynitrite with xanthine, and also the same chemical properties, such as reduction by sodium dithionite to give 8-aminoxanthine.

HPLC and ECD Instrumentation. Shimadzu model LC-600 HPLC pumps controlled by the Shimadzu model SCL-6B solvent programmer were used for solvent delivery. The HPLC column effluent was analyzed using the Waters model 996 photodiode array detector (Waters Corp., Millford, MA) downstream from the Bioanalytical Systems (West Lafayette, IN) BAS LC-4B EC detector; chromatographic data were processed using the Waters Millennium Chromatography Manager software. All electrodes were obtained from BAS.

The dual-mode EC detector, consisting of two LC-4B controllers and one CC4 electrochemical cell compartment, was configured to operate in accordance with the manufacturer's instructions. All applied potentials were referenced against the Ag/AgCl/3 M NaCl electrode. The flow cell was equipped with one of the three working dual-electrode combinations. These consisted of either (a) glassy carbon and gold/mercury, (b) glassy carbon and gold, or (c) two glassy carbon electrodes in series, with the glassy carbon electrode always downstream. The preparation and maintenance of the Au/Hg electrode was described in detail in an earlier publication (*24*). To maintain a satisfactory sensitivity, all working electrodes must be periodically refurbished according to the manufacturer's instructions.

HPLC Solvent Systems. The solvent systems used for elution consisted of 50 mM sodium acetate (pH adjusted to 4.7 by addition of ~6 mL of glacial acetic acid) with either 1% (solvent system 1), 2% (solvent system 2), 5% (solvent system 3) or 8% methanol (solvent system 4) or of 100 mM sodium acetate (pH adjusted to 4.5 with ~20 mL of glacial acetic acid) containing 5% methanol (solvent system 5). The flow rate was 1 mL/min.

Reaction of Peroxynitrite with Guanine and Xanthine. Peroxynitrite, prepared by the ozonation of sodium azide according to the published procedure (*25*), was stored at −20 or −80 °C. After dilution with 0.1 N NaOH, and just before the reaction had started, the peroxynitrite concentration was determined by its absorbance at 301 nm ($\epsilon = 1670\ M^{-1}\ cm^{-1}$).

The final concentration of peroxynitrite in the reaction mixture was 10−12 mM; the purine base concentration was 6−10 mM. The reactions were conducted at 37 °C or room temperature. The purine base was dissolved or suspended in 0.5 M sodium phosphate buffer in 0.1 mM diethylenetriaminepentaacetate (DTPA) at pH 7.0 and equilibrated at either 37 °C or room temperature prior to the addition of peroxynitrite. Peroxynitrite was added all at once while the mixture was

vortexed, and the reaction mixture was returned immediately to a bath maintained at either 37 °C or room temperature. Incubation with shaking was continued for an additional 5 min. For control runs, peroxynitrite was decomposed with 1 N HCl prior to the addition to the reaction mixture.

To compare the reactivity of peroxynitrite with xanthine and guanine, both reactions were performed under the same conditions, at the same time and with the same batch of peroxynitrite. The purine bases, 1 mg (~6 μmol), were dissolved or suspended in 1 mL of sodium phosphate buffer at pH 7 and 37 °C, and an equal amount of peroxynitrite (12 μmol) was added to each of the mixtures. The rest of the procedure was followed as described above.

8-Nitroxanthine and 8-nitroguanine were reduced to the corresponding 8-amino derivatives by addition of an excess amount of solid sodium dithionite (3−10 mg) directly to the appropriate fractions of the HPLC effluent. The mixture was vortexed for a few seconds and used for further studies. 8-Aminoguanine and 8-aminoxanthine are quite stable and can be stored in a refrigerator for several months.

HPLC Analysis and Hydrodynamic Voltammetry of 8-Nitroguanine and 8-Nitroxanthine. Characterization of 8-nitroguanine and 8-nitroxanthine by hydrodynamic voltammetry was performed by HPLC with EC detection, using two Ultrasphere ODS 3 μm columns (0.46 cm × 7.5 cm) connected in series and protected by a 0.46 cm × 4.6 cm Ultrasphere ODS 5 μm guard column. The columns were eluted isocratically at a flow rate of 1 mL/min with a solution of 1 or 2% methanol in 50 mM sodium acetate (pH 4.7), which was continually sparged with helium. To determine the optimum potential for the electrochemical reduction of 8-nitroguanine or 8-nitroxanthine, a fixed amount of the standard nitro derivative was injected into the HPLC system and analyzed repeatedly at various reducing potentials while the oxidation potential was kept constant. To determine the optimum oxidation potential, the procedure was repeated with a constant reducing potential and varying oxidation potentials. In all cases, only the current due to oxidation at the glassy carbon electrode was recorded.

The HPLC system used for the analysis of the products of reactions of purine bases was the same as that described above for the hydrodynamic voltammetry. The level of UV absorption of the HPLC effluent was measured at 253 and 375 nm, and the dual-electrode EC detector was set up in the sequential reduction and oxidation modes. After optimization of the EC detector conditions by hydrodynamic voltammetry, the upstream Au electrode was used for reduction at −0.7 or −0.9 V and the downstream glassy carbon electrode was used for oxidation at 0.6 V unless otherwise specified.

Preparation of 8-Oxo- and 8-Aminopurines and the Corresponding Nucleoside Standards. 8-Aminoguanosine was obtained from the Sigma Chemical Co. 8-Aminoguanine and 8-oxoguanine were prepared by the acid hydrolysis of the corresponding ribonucleosides following the procedure described previously (*27*). 8-Aminoadenine and 8-oxoadenine were prepared by acid hydrolysis of 8-aminodeoxyadenosine and 8-oxodeoxyadenosine, respectively, in 1 N HCl at 90 °C for 20 min. 8-Aminodeoxyguanosine, 8-aminodeoxyadenosine, and 8-aminoxanthosine were prepared from the corresponding bromo derivatives as described in the literature (*29*). 8-Oxoxanthosine, 8-aminoxanthosine, and 8-oxoguanosine were also prepared according to published procedures (*30*, *31*). 8-Aminoadenosine and 8-oxoadenosine standards were prepared by the sequential enzymatic hydrolysis of 8-aminoadenosine 3′,5′-cyclic monophosphate or 8-hydroxyadenosine 3′,5′-cyclic monophosphate (Sigma Chemical Co.), respectively, with 3′,5′-cyclic nucleotide phosphodiesterase and alkaline phosphatase (Sigma Chemical Co.).

Reaction of Purine Nucleosides with Peroxynitrite at pH 7.0 and 5.0. For the reactions of adenine and guanine nucleosides, the concentration of peroxynitrite was 10−12 mM; for the reactions of 9-methylguanine and xanthosine, the peroxynitrite concentration was 7−8 mM. The concentrations of the nucleosides and the bases used in the various reactions were 3 mM deoxyguanosine, 3.5 mM guanosine, 5 mM deoxyadenosine or adenosine, 10 mM xanthosine, or 8.5 mM 9-methylguanine. All reaction mixtures contained 0.1 mM DTPA and 0.5 M phosphate buffer at pH 7.0 or 0.2 M sodium acetate buffer at pH 5.0. The buffer solutions were prepared with MilliQ-filtered water and used as such, and no effort was made to exclude oxygen or carbon dioxide from either the buffer solutions or the reaction mixtures. Enough peroxynitrite to achieve the required concentration was added all at one time, using a glass micropipet or an Eppendorf pipet, while the reaction mixture was vortexed. The reaction mixtures of adenine and guanine nucleosides were analyzed by HPLC immediately after the addition of peroxynitrite and stored on ice, or used immediately for dithionite reduction or other reactions. In the case of deoxyguanosine, an aliquot of the reaction mixture should be injected within at least 20 s of the addition of peroxynitrite to observe the elution of the nitrated deoxynucleoside without excessive decomposition. The yields of the nitropurines and -nucleosides were estimated as the corresponding amino derivatives after reduction with sodium dithionite, using standard curves obtained with the 8-aminonucleoside and 8-aminopurine standards (*29*−*32*). Reduction was carried out at room temperature or 5 °C by direct addition of excess solid sodium dithionite to the reaction mixture or to the HPLC-purified product in solution at pH 7. All yields are expressed in terms of the percentage of the parent nucleoside concentration.

For the reactions of deoxyguanosine at various pH values, a mixed buffer of 0.25 M sodium phosphate and 0.2 M sodium acetate was used for pH 7 and 6. For reactions carried out at pH 5−3, 0.2 M sodium acetate buffer was used after adjusting the pH to the desired value by addition of glacial acetic acid. All reaction mixtures contained 0.1 mM DTPA.

Control runs were conducted under the same conditions, with the same concentrations of the starting materials for each experiment, except that peroxynitrite that had been allowed to decompose in the same buffer was substituted for peroxynitrite.

HPLC-EC Analysis of the Nucleoside Reaction Mixtures. The HPLC system and the UV and ECD instrumentation were similar to those described above for the hydrodynamic voltammetry of 8-nitroguanine and 8-nitroxanthine. HPLC columns were eluted with 50 mM sodium acetate (pH 4.7) with 5 or 8% MeOH or 100 mM sodium acetate (pH 4.5) with 5% MeOH, and the flow rate was 1 mL/min for the analyses of all of the nucleoside reactions.

For detection of the products from the reactions of nucleoside with peroxynitrite by HPLC-ECD with dual-mode EC detection, the Au and glassy carbon dual electrode was used, with the upstream Au electrode reducing at −0.7 V and the downstream glassy carbon electrode oxidizing at 0.6 V. For the analysis of adenine nucleoside reaction mixtures only, a potential of 0.8 V was applied to the downstream oxidizing electrode and −0.7 V was applied to the upstream reducing electrode.

Results

HPLC Analysis. The reverse-phase HPLC method with dual-mode electrochemical detection as described here was originally used in our laboratory for the determination of 3-nitrotyrosine (*24*). With a minor modification of the eluting solvent, it was possible to apply it to the detection of femtomole amounts of 8-nitroguanine and 8-nitroxanthine. When the methanol concentration of the eluting solvent was increased, the separation and detection of products from the reactions of various nucleosides with peroxynitrite also became feasible. Figure 1 was obtained with 8-nitroguanine, 8-nitroxanthine, and several other standard 8-amino- and 8-oxoguanine nucleosides that had been prepared according to published procedures (*26*−*31*). This figure illustrates the separations and the resolution typically

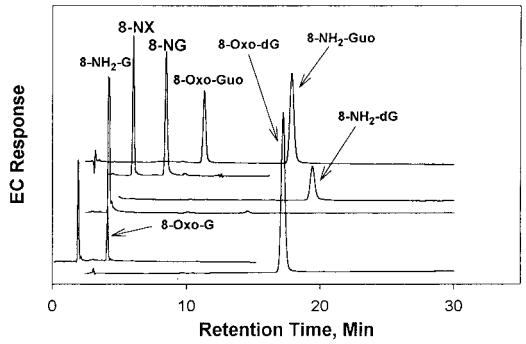

Figure 1. HPLC-ECD profiles of standard nucleoside derivatives. Oxidizing electrode response, with the reducing electrode W2 (Au electrode) at −0.9 or −0.7 V and the oxidizing electrode W1 at 0.6 V. 8-NG, 8-nitroguanine; 8-NX, 8-nitroxanthine; 8-NH_2-G, 8-aminoguanine; 8-Oxo-G, 8-oxo-guanine. HPLC solvent system 3 was used at a rate of 1.0 mL/min.

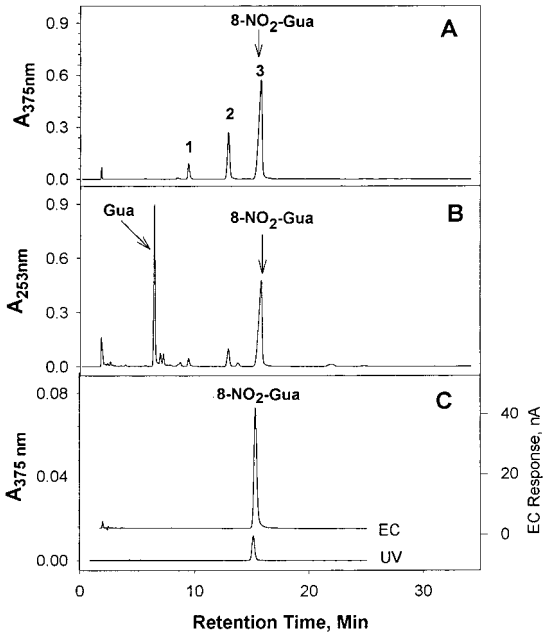

Figure 2. HPLC profiles of the 8-nitroguanine obtained by synthesis from 8-aminoguanine as described in Experimental Procedures: (A) chromatogram monitored for UV absorption at 375 nm, (B) chromatogram monitored at 253 nm, showing the side product guanine, and (C) rechromatography of 8-nitroguanine, peak 3, collected from HPLC. UV, photodiode array detector response at 375 nm; EC, dual-mode electrochemical detector response with the reducing electrode W2 (Au/Hg electrode) at −0.9 V and the oxidizing electrode at 0.6 V. HPLC solvent system 1 was used at a rate of 1 mL/min.

achieved using the method. Unlike the nitropurines, the 8-amino- and 8-oxonucleosides do not require reduction prior to detection at the downstream oxidation electrode; hence, these species can be detected in femtomole levels using either the dual- or single-detection mode.

Preparation and Detection of 8-Nitroguanine. The 8-nitroguanine standard was prepared by the diazotization of 8-aminoguanine to 8-diazoguanine and the subsequent reaction of 8-diazoguanine with sodium nitrite to give the product (*26*). Since analysis showed guanine as a major side product, 8-nitroguanine, designated as product **3** in Figure 2A, was purified by reverse-phase HPLC (Figure 2C). Its identity was evident from its characteristic UV absorption maximum at 385 nm (*33*), and by its reduction to 8-aminoguanine (Figure 3, trace 3), which is a known compound (*27*). The other

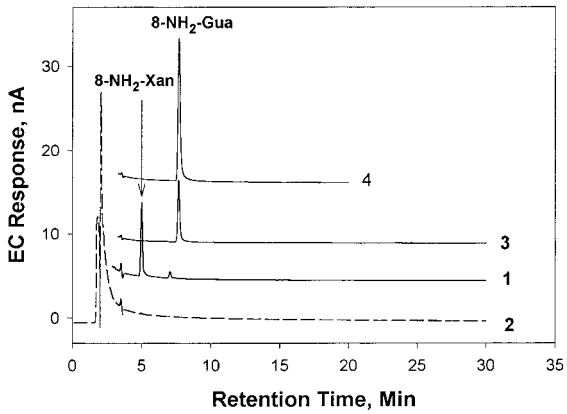

Figure 3. HPLC-ECD profiles of products 1 (trace 1), 2 (trace 2), and 3 (trace 3) in Figure 2A, purified by HPLC, after dithionite reduction. Trace 4 represents cochromatography of the reduced product 3 with the 8-aminoguanine standard that was prepared by acid hydrolysis of 8-aminoguanosine. The traces represent the response of the oxidizing electrode (W1) with the reducing electrode (W2) on standby. HPLC solvent system 1 was used at a rate of 1 mL/min.

products, **1** and **2** in Figure 2A, each with a UV absorption maximum at approximately 375 nm, were also purified by HPLC and reduced with sodium dithionite. Only product **1**, which we identified in later experiments as 8-nitroxanthine, underwent reduction with dithionite to give a product (8-aminoxanthine) that produced a detector response using the oxidative mode alone (Figure 3).

With HPLC conditions similar to those used to quantitate 3-nitrotyrosine (*24*), i.e., 50 mM sodium acetate (pH 4.7) containing 1% methanol as the eluant, 8-nitroguanine elutes with a retention time of 15 min (or an elution volume of 15 mL). Using dual-mode amperometric detection, this species could be detected at very low concentrations. When the reducing electrode is turned off or put on standby, the peak corresponding to the nitro compound disappears from the chromatogram, providing additional evidence for the presence of the nitro function. In the work described here, the upstream electrode consisted of either an Au/Hg or Au electrode programmed to operate in the reduction mode at −0.9 or −0.7 V, and the downstream electrode in all cases was a glassy carbon electrode operating in the oxidation mode at 0.6 V unless otherwise specified. Under these conditions, a detection limit of 190 fmol per injection could be achieved with the 8-nitroguanine standard. Because of the characteristic long-wavelength UV absorption spectrum of nitro compounds, low detection levels with good selectivity can also be achieved for 8-nitroguanine using only the photodiode array detector; however, the sensitivity is about 100 times lower than that of dual-mode ECD, or ∼15 pmol per injection.

Analysis by hydrodynamic voltammetry at various oxidation potentials and a fixed reduction potential of −0.9 V shows that 8-nitroguanine can be detected at oxidative potentials as low as 0.2 V when the gold amalgam electrode is used (Figure 4). The use of such low potentials has the advantage of providing increased selectivity and enhanced detection limits during the analysis of biological samples, since fewer interfering compounds will be oxidized under those conditions. Figure 4 also shows that 8-nitroguanine can be easily reduced on a gold or carbon electrode, although at somewhat higher negative potentials.

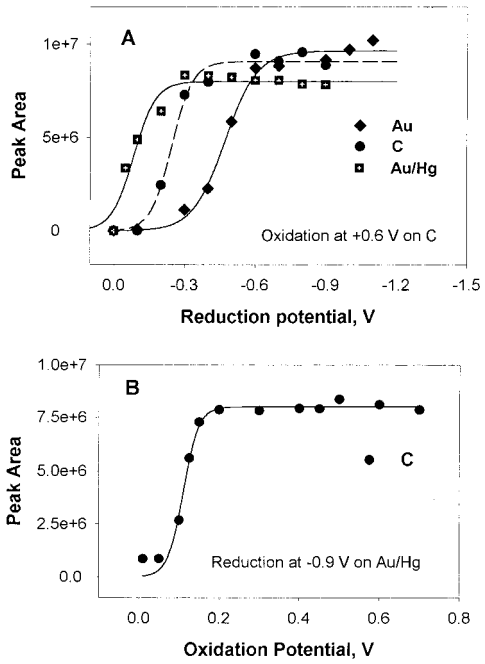

Figure 4. Hydrodynamic voltammetry of 8-nitroguanine. In panel A, different electrode materials at various negative potentials are used for the reducing upstream electrode, while the oxidation potential is fixed at 0.6 V at the downstream glassy carbon electrode. In panel B, various positive potentials are applied to the downstream glassy carbon electrode while the reduction potential is kept constant at −0.9 V for the Au/Hg upstream electrode.

Preparation, Hydrodynamic Voltammetry, and Detection of 8-Nitroxanthine. In general, the HPLC method described above could be useful for the detection and estimation of any nitro derivatives that easily undergo reversible electrochemical reduction. While several nitro derivatives of purine bases may be generated by reaction with peroxynitrite, so far only the production of 8-nitroguanine has been adequately described (*33*). Since guanine can be deaminated in vivo to xanthine, which can then be oxidized to uric acid, it seems possible that 8-nitroguanine might also be metabolized in vivo to 8-nitroxanthine or even to 8-aminoxanthine. If indeed this is the case, a method for the quantitation of 8-nitroxanthine in body fluids could be extremely useful as an indicator of overall nucleic acid damage specifically produced by peroxynitrite.

We prepared 8-nitroxanthine from the reaction of xanthine with peroxynitrite and separated the products by reverse-phase HPLC using the same conditions that were used for the analysis of 8-nitroguanine described above, except that the methanol composition in the eluent was increased to 2% (Figure 5A, traces 1 and 3). Interestingly, though 8-nitroxanthine is more nonpolar than 8-nitroguanine, it eluted earlier than 8-nitroguanine at pH 4.7, perhaps because of specific interactions of the latter with the column packing. The peak corresponding to 8-nitroxanthine was collected and used for further characterization. The UV absorption maximum of 8-nitroxanthine was observed to shift to a longer wavelength at basic pH (Figure 6B) as described in a previous report (*28*). Reduction of 8-nitroxanthine with sodium dithionite gives 8-aminoxanthine, as evidenced by the disappearance of the longer-wavelength absorption ($\lambda_{max} = 380$ nm) due to the nitro functional group and the appearance of a new absorption maximum at 287 nm (spectrum not

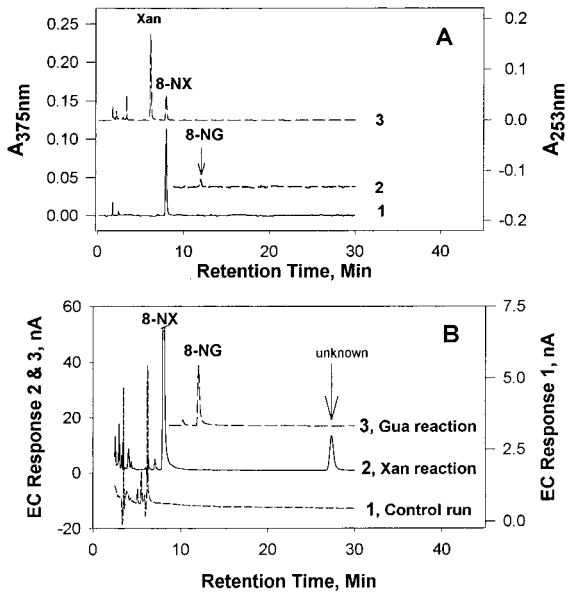

Figure 5. Reactivity of peroxynitrite with xanthine and with guanine. (A) Chromatograms of the reaction products of peroxynitrite and xanthine monitored for UV light absorption at 375 (trace 1) and 253 nm (trace 3) and of the reaction mixture of peroxynitrite and guanine, monitored at 375 nm (trace 2). (B) HPLC-ECD profiles of the reaction products of peroxynitrite with xanthine (traces 1 and 2) and with guanine (trace 3). For the control run (trace 1), the peroxynitrite was allowed to decompose prior to being used. The ECD was in the dual mode, using the conditions described in the legend of Figure 2. HPLC system 2 was used at a flow rate of 1 mL/min.

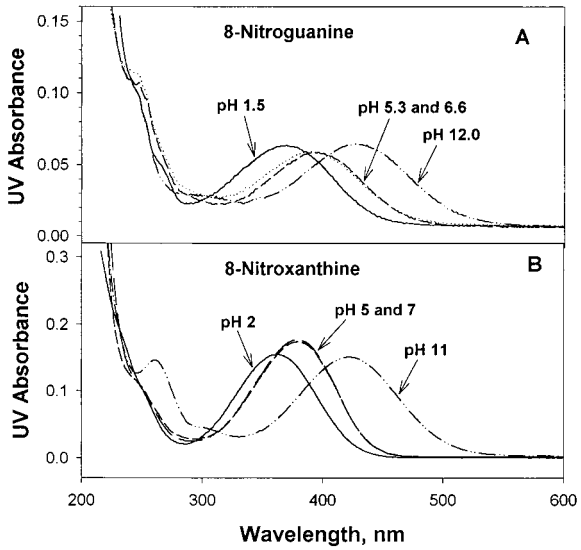

Figure 6. UV spectra at various pH values of (A) 8-nitroguanine and (B) 8-nitroxanthine, both obtained from the reaction of peroxynitrite with guanine or xanthine, respectively, and purified by HPLC.

shown). The 8-aminoxanthine standard, which was prepared by acid hydrolysis of 8-aminoxanthosine, exhibits a UV spectrum similar to that of the dithionite reduction product of 8-nitroxanthine. 8-Nitroxanthine prepared by an alternate route from xanthine (*28*) exhibited UV spectral, HPLC elution, and electrochemical properties similar to those of the 8-nitroxanthine obtained from the peroxynitrite reaction with xanthine. It also underwent dithionite reduction to give 8-aminoxanthine. Though the reducing power of sodium dithionite is at its maximum in slightly alkaline solutions, the nitropurines described

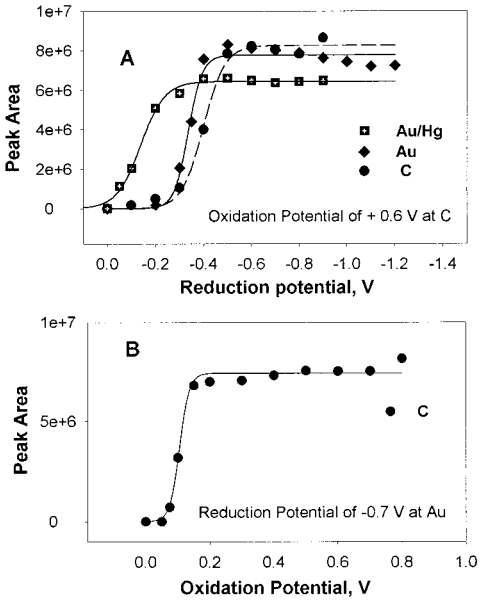

Figure 7. Hydrodynamic voltammetry of 8-nitroxanthine. (A) Different electrode materials were used for the upstream electrode at various reduction potentials, while the oxidation potential was fixed at 0.6 V for the downstream glassy carbon electrode. (B) The downstream glassy carbon electrode was set to various oxidation potentials, while the reduction potential was kept constant at −0.7 V at the gold upstream electrode.

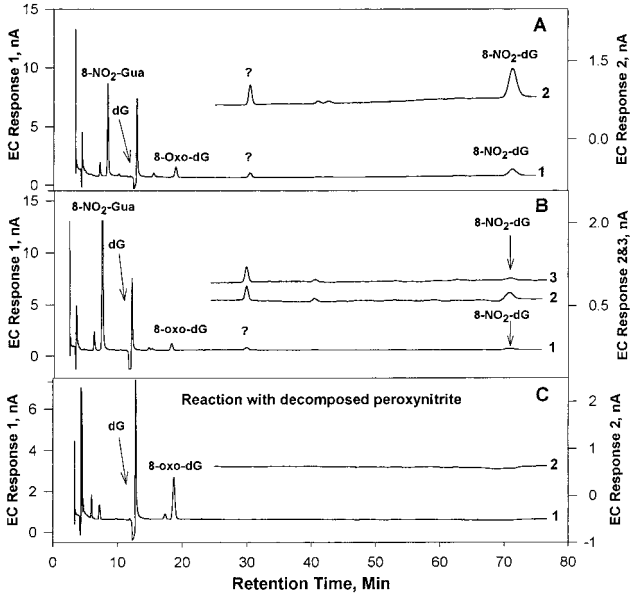

Figure 8. HPLC-ECD profiles of the reaction products of peroxynitrite with deoxyguanosine. In panel A, the reaction was carried out at pH 7 and 0−5 °C, with immediate analysis of products. In panel B, traces 1 and 2, the reaction was carried out at pH 7 and 37 °C; the mixture was then allowed to shake gently at 37 °C for 3 min before analysis. Trace 3 shows the products of the reaction at 0−5 °C after they were allowed to be shaken at 37 °C for 5 min prior to analysis. In panel C is shown the control incubation with decomposed peroxynitrite, carried out under the same conditions described for panel A. For clarity, only the dual-mode ECD profiles, obtained with the reducing electrode W2 at −0.7 V and the oxidizing electrode W1 at 0.6 V, are shown. The retention time of deoxyguanosine is 13 min (does not give ECD response). Trace 2 in all figures is a portion of trace 1 at a higher sensitivity, with the scale as shown on the right *Y*-axis. HPLC solvent system 3 was used at a flow rate of 1 mL/min.

here could be reduced even in sodium acetate buffer at pH 5, or in just water or water/methanol solutions.

8-Nitroxanthine, prepared by the reaction of peroxynitrite with xanthine or by the nitration of xanthine with nitric acid (*28*), exhibits a UV spectrum similar to that of product **1** in Figure 2, one of the side products in the 8-nitroguanine preparation, and also cochromatographs with peak **1**. Peak **1** in Figure 2 undergoes reduction with sodium dithionite to give a product similar in UV and HPLC elution properties to 8-aminoxanthine obtained by the reduction of 8-nitroxanthine. 8-Nitroxanthine, moreover, is an expected side product formed by hydrolytic deamination during the diazotization of 8-aminoguanine. Parenthetically, we found xanthine as a major product from the reaction of guanine with nitronium tetrafluoroborate, which also gave 8-nitroguanine, albeit in a very poor yield (results not shown).

Surprisingly, we find that at neutral pH and 37 °C peroxynitrite reacts more extensively with xanthine than with guanine to give a 10−15% yield of 8-nitroxanthine, as estimated from the initial concentration of xanthine (Figure 5). One reason for this may be the better solubility of xanthine compared to guanine. However, we also observe that xanthosine undergoes nitration by peroxynitrite to a greater extent than guanosine does.

Under conditions similar to those described above for 8-nitroguanine, a detection limit of 150 fmol per injection could be reached for 8-nitroxanthine. In comparison, the photodiode array detector, at 375 nm, achieved a detection limit of 7 pmol per injection for this base. The 8-nitroguanine and 8-nitroxanthine we prepared were found to be stable and can be stored in solution at 4−5 °C and pH 5 for several months.

As shown by hydrodynamic voltammetry in Figure 7, 8-nitroxanthine could also be detected with high-level sensitivity using the gold or carbon electrode for reduction (the glassy carbon electrode was used for oxidation in all cases). The gold electrode appears to be the best for reduction of 8-nitroxanthine and the glassy carbon electrode for the reduction of 8-nitroguanine. As in the case of 8-nitroguanine, an oxidative potential as low as 0.2 V can be used to detect 8-nitroxanthine without a loss in the level of sensitivity. For routine analyses, the gold electrode may be preferred for reduction because of the lower background noise level that was obtained.

Reaction of Deoxyguanosine and Guanosine with Peroxynitrite at pH 7. During vortexing, peroxynitrite was added to the solution of deoxyguanosine at pH 7 that had been pre-equilibrated to the desired temperature. The reaction mixture was analyzed by HPLC with dual-mode ECD as soon as possible, i.e., within 30 s of addition of peroxynitrite, to minimize the decomposition of the nitrodeoxynucleoside product (Figure 8). The late-eluting peak at 72 min and 8-nitroguanine eluting at ∼8 min are formed only upon reaction with peroxynitrite; neither of these products is formed when the reaction is carried out with decomposed peroxynitrite (Figure 8C). These species can be detected by the dual-mode electrochemical detector only when a potential is applied to the reducing electrode. 8-Oxo-dG formation was not observed in the reactions of deoxyguanosine with peroxynitrite at pH 7. When the reaction mixture was allowed to stand at room temperature, or when the reaction was carried out at 37 °C, the 72 min peak disappeared and the level of 8-nitroguanine increased (Figure 8B), suggesting a precursor−product relationship, with the 72 min peak likely representing 8-nitrodeoxyguanosine (8-nitro-dG). Because the substance responsible for the 72 min peak was

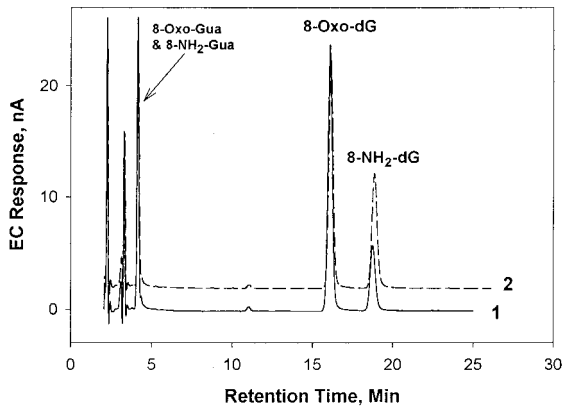

Figure 9. HPLC-ECD profiles of the reaction products of peroxynitrite and deoxyguanosine after reduction with sodium dithionite (trace 1) and cochromatography with the 8-aminodeoxyguanosine (8-NH_2-dG) standard (trace 2). Chromatographic traces that are shown were obtained using ECD in the single oxidative mode, with the oxidizing electrode W1 at 0.6V and the reducing electrode W2 on standby. The HPLC solvent system is the same as that described in the legend of Figure 8.

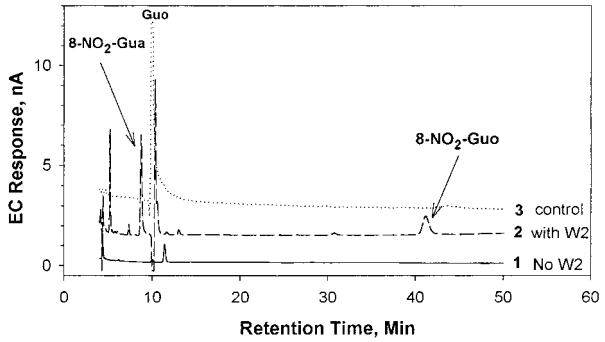

Figure 10. HPLC-ECD analysis of the reaction products of guanosine and peroxynitrite at pH 7 and 0−5 °C. Trace 1 was obtained with the ECD in the single oxidative mode, with the oxidizing electrode W1 at 0.6 V and the reducing electrode W2 on standby. Trace 2 was obtained with dual-mode ECD with the reducing electrode W2 at −0.7 V and the oxidizing electrode W1 at 0.6 V. Trace 3 is the control, run with decomposed peroxynitrite, using the same dual-mode ECD conditions described for trace 2. The retention time of guanosine is ∼10 min (does not give an ECD response). The HPLC solvent system is the same as that described in the legend of Figure 8.

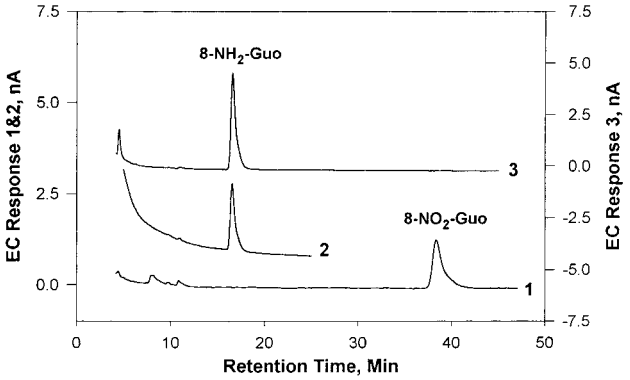

Figure 11. Trace 1, rechromatography of 8-nitroguanosine (8-NO_2-Guo) that had been purified by HPLC; trace 2, the same after reduction with sodium dithionite; and trace 3, cochromatography of the reduced product in trace 2 with the 8-aminoguanosine (8NH_2-Guo) standard. ECD was used only in the single oxidative mode, with the oxidizing electrode W1 at 0.6 V and the reducing electrode W2 on standby. The HPLC solvent system is the same as that described in the legend of Figure 8.

so unstable and disappeared even when stored at 5 °C overnight, it was impractical to collect it from HPLC for further characterization. Therefore, without attempting its isolation, we studied its properties in reaction mixtures that had been placed on ice immediately after the addition of peroxynitrite. When sodium dithionite was added to the reaction mixture at 5 °C within 5 min of the peroxynitrite addition, both the 72 min peak and the 8-nitroguanine at 8 min disappeared, along with the appearance of peaks corresponding to 8-aminodeoxyguanosine (8-amino-dG), 8-oxo-dG, 8-aminoguanine, and 8-oxoguanine (Figure 9**)**. Since 8-nitroguanine yields 8-aminoguanine upon reduction with dithionite (*15*, *33*), the 8-amino-dG must be the reduction product of 8-nitro-dG, the latter being responsible for the 72 min peak.

The addition of sodium dithionite to the control mixture gave neither 8-amino-dG nor 8-oxo-dG. Therefore, we assume that the 8-oxo-dG, which is formed upon treatment of the reaction mixture with dithionite, results from the reduction of an unknown product of the reaction of peroxynitrite with deoxyguanosine that could not be detected with our analytical method. This unknown either gives no response with the electrochemical detector under the conditions that were used or may have a very low elution time and thus cannot be distinguished from peaks due to salts, which also elute early. The unknown could be the 4,8-endoperoxide of the guanine nucleoside, or might be structurally related to the products reported in the reaction of peroxynitrite with deoxyguanosine, such as the 4-hydroxy- or 4-nitrosooxy-dG adduct (*34*, *35*). This highly interesting phenomenon is currently under further investigation.

Using HPLC with dual-mode ECD, we were able to investigate the stability of 8-nitro-dG in the reaction mixtures. From the time-dependent decreases in the areas of the 72 min peak, we estimate the half-life of 8-nitro-dG to be 44 h at −20 °C, 7 h at 0 °C, ∼10 min at room temperature, and <3 min at 37 °C (Figure 8B). After reduction to the corresponding 8-amino derivatives, the levels of formation of 8-nitro-dG and 8-nitroguanine in the reaction of deoxyguanosine with peroxynitrite at pH 7 and 5 °C are estimated to be 1.7 and 1.5 μM, or 0.06 and 0.05% yields, from 3 mM deoxyguanosine, respectively. With an increase in the reaction temperature, the level of 8-nitroguanine increases and that of 8-nitro-dG decreases.

Unlike 8-nitro-dG, which undergoes depurination very quickly under mild conditions, the corresponding ribonucleoside, 8-nitro-Guo, should be more stable and allow for better characterization. Guanosine also reacted with peroxynitrite to give 8-nitroguanosine, eluting with a retention time of ∼42 min (8-nitro-Guo), and also 8-nitroguanine, eluting at ∼8 min (Figure 10). The yield of 8-nitro-Guo, estimated by reduction to 8-aminoguanosine, was ∼0.5 μM, or 0.02%, from 3 mM guanosine. 8-Nitro-Guo was found to be much more stable than 8-nitro-dG; the reaction mixture could be stored in the refrigerator for several days with minimal changes in composition. Because of its stability, the product could be collected from the HPLC column and used for studying its properties, and for reduction with sodium dithionite to 8-aminoguanosine to confirm its identity (Figures 10 and 11). The formation of 8-amino- and 8-oxoguanosine was observed only when the reaction mixture, but not the control mixture, was reduced with sodium dithionite, as was the case with deoxyguanosine. Dithionite reduction

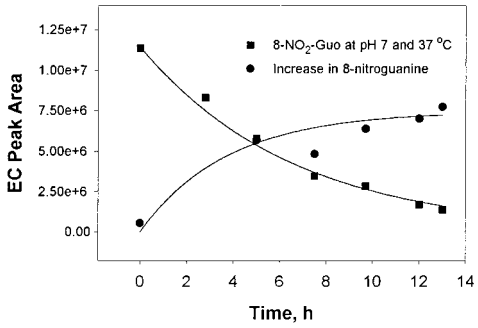

Figure 12. Stability of 8-nitro-Guo at pH 7 and 37 °C. HPLC-purified 8-nitro-Guo, as in Figure 11, was incubated at 37 °C, and aliquots were taken and analyzed at various times by HPLC-ECD.

of HPLC-purified 8-nitro-Guo gave only 8-amino-Guo, again suggesting that the source of the 8-oxoguanosine was not 8-nitro-Guo but some other unidentified product. While 8-nitro-dG exhibited a half-life of only a few minutes, 8-nitro-Guo exhibited a half-life of ∼5 h at pH 7 and 37 °C (Figure 12). At higher temperatures, for instance, 80−90 °C, it hydrolyzed completely to 8-nitroguanine within 5−10 min.

Interestingly, the formation of 8-nitroguanine was observed in the guanosine reaction at 0−5 °C and neutral pH, at the level of ∼0.85 μM, even when analyzed almost immediately after the addition of peroxynitrite (Figure 10). Thus, it is unlikely that the source of the 8-nitroguanine is the decomposition of 8-nitro-Guo, which has a relatively long half-life. Since the guanosine used in the reactions with peroxynitrite was analyzed by HPLC and found to contain no trace contamination with guanine, the 8-nitroguanine also could not be the product of a direct reaction of peroxynitrite with guanine. We suggest, as a possible explanation, that the 8-nitroguanine that was formed must have been the product of the direct reaction of peroxynitrite with the ribosyl or deoxyribosyl nucleoside. We hypothesize that $ONOO^-$, perhaps acting as a one-electron oxidant (*36*, *37*), oxidizes deoxyguanosine or guanosine to a radical cation, which then undergoes glycosidic bond hydrolysis and, at the same time, reacts with another peroxynitrite molecule to give 8-nitroguanine.

We find similarly that the reaction of xanthosine with peroxynitrite gives 8-nitroxanthine in addition to 8-nitroxanthosine (Figure 13A). The extent of nitration by peroxynitrite at neutral pH is higher in the xanthosine reaction than in the guanosine reaction. 8-Nitroxanthosine was stable when the reaction mixture was stored at 5 °C; thus, it was possible to collect the product from HPLC and study its properties. At 37 °C, 8-nitroxanthosine exhibited a half-life of more than 12 h at pH 7 and 37 °C (figure not shown). The product of 8-nitroxanthosine decomposition was 8-nitroxanthine. As in the case of 8-nitroguanosine, 8-nitroxanthosine hydrolyzed to 8-nitroxanthine in 5 min at 90 °C. Upon reduction with dithionite, it converted to the amino product, which cochromatographed with the synthesized 8-aminoxanthosine standard (not shown in the figure).

We also examined the reaction of peroxynitrite with 9-methylguanine, which does not have a glycosidic bond. 8-Nitro-9-methylguanine, the late-eluting peak at 43 min in Figure 13B, required electrochemical reduction and subsequent oxidation to be detected, and was found to be stable even at 90 °C, as expected. The HPLC-purified

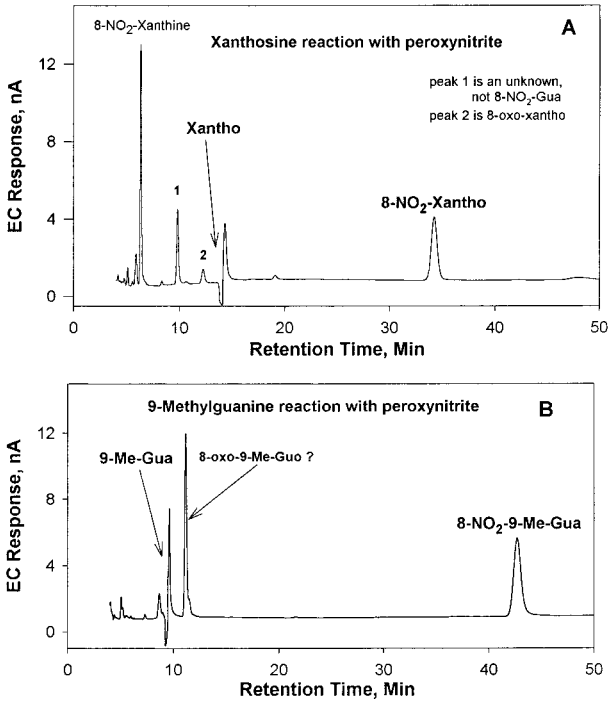

Figure 13. HPLC-ECD of the reaction of peroxynitrite with (A) xanthosine and (B) 9-methylguanine at pH 7 and 0−5 °C. HPLC-ECD conditions are as described in the legend of Figure 8.

8-nitro-9-methylguanine exhibits a UV spectrum similar to that of 8-nitroguanine, with the absorption maximum of 385 nm at pH 5 and 420 nm at pH 11. Thus, C8-nitration of purines may be a general reaction of peroxynitrite.

In contrast to Burney et al. (*38*), who reported that 8-nitroguanine reacts more rapidly with peroxynitrite than does 8-oxo-dG, we find that 8-nitroguanine does not react with peroxynitrite to the same extent as 8-oxo-dG. In our hands, at pH 7 and an initial concentration of the 8-oxodeoxynucleoside of 100 μM, 1 mM peroxynitrite reacted completely, with the loss of all of the initial 8-oxo-dG. In contrast, 8-nitroguanine exhibited much less reactivity toward peroxynitrite. Peroxynitrite, 0.5 mM, exhibited no detectable reaction with 100 μM 8-nitroguanine at pH 7, and 10 mM peroxynitrite reacted with only 14 and 18% of the 8-nitroguanine present in the reaction mixtures at initial concentrations of 100 and 20 μM, respectively. We have observed that the storage of reaction mixtures of peroxynitrite with nucleosides after the reaction is over, even at −20 °C, can result in the loss of the original products and the formation of additional side products, since the nitrite, which is formed in addition to nitrate during the decomposition of peroxynitrite (*39*, *40*), can react further with the nucleoside products. This might be one possible reason for differences in interlaboratory results. 8-Nitroguanine, purified by HPLC, and thus devoid of peroxynitrite breakdown products, can be stored at 4−5 °C in a sodium acetate solution at pH 5 for several months without degradation.

pH-Dependent Formation of 8-Oxodeoxyguanosine from the Reaction of Deoxyguanosine with Peroxynitrite. The reaction of deoxyguanosine with peroxynitrite at various pH values was conducted at 0−5 °C as described in Experimental Procedures. An aliquot of the reaction mixture was submitted to HPLC immediately after the addition of peroxynitrite, before side

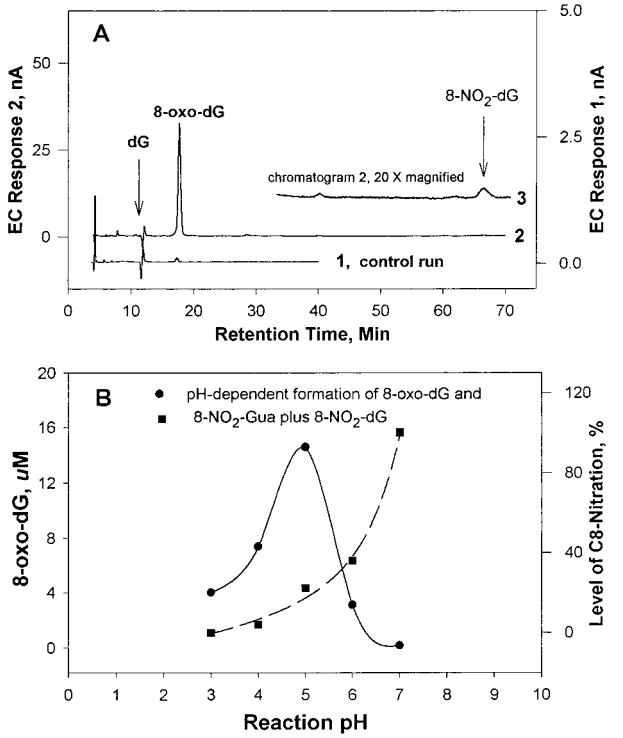

Figure 14. (A) HPLC-ECD profiles of the reaction products of peroxynitrite with deoxyguanosine at pH 5 and 0−5 °C. (B) pH-dependent formation of 8-oxo-dG (●), and level of C8-nitration (■) estimated from the combined amounts of 8-NG and 8-NO_2-dG. The level of 8-NG and 8-NO_2-dG from the reaction run at pH 7 is considered 100%. HPLC-ECD conditions for panels A and B are as described in the legend of Figure 8.

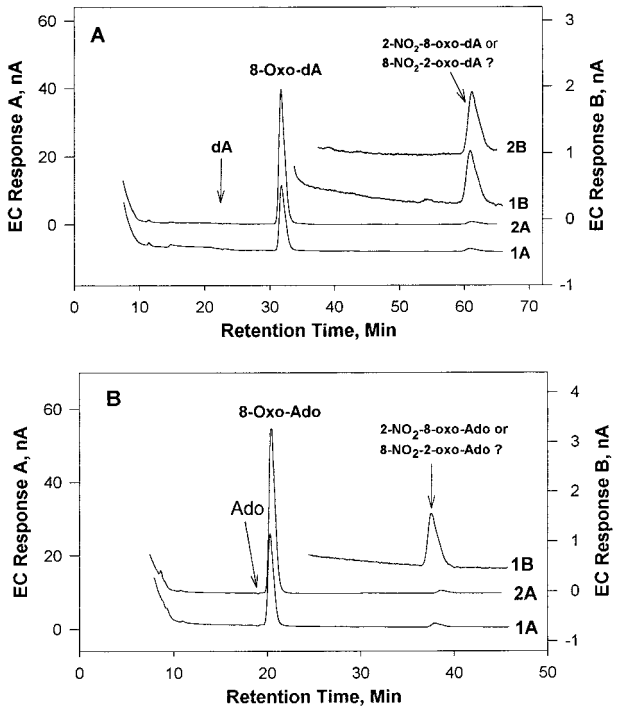

Figure 15. (A) HPLC-ECD profiles of the reaction products of peroxynitrite with deoxyadenosine at pH 7 (trace 1A) and 5 (trace 2A), both at 0−5 °C. To show the nitrated deoxyadenosine product more clearly, traces 1A and 2A are redrawn with higher magnification as traces 1B and 2B, respectively. The reducing electrode W2 was set at −0.7 V and the oxidizing electrode W1 at 0.8 V. Deoxyadenosine (no ECD response) elutes at a retention time of ∼22 min and can be observed by its UV trace at 253 nm (not shown). (B) HPLC-ECD profiles of the reaction products of peroxynitrite with adenosine at pH 7 (trace 1A) and 5 (trace 2A) and 0−5 °C. Trace 1B represents increased vertical magnification of trace 1A. The same ECD conditions were used that were used for panel A. Adenosine (no ECD response) elutes at a retention time of ∼19 min and can be observed by its UV absorption at 253 nm (not shown). HPLC solvent system 5 was used at a flow rate of 1 mL/min for both panels A and B.

reactions could take place, to determine accurately the yield of 8-oxo-dG. The formation of 8-oxo-dG appeared to reach a maximum level of 15 μM (∼0.5% yield) at pH ∼5, as estimated from the initial deoxyguanosine concentration (Figure 14). This pH-dependent C8-oxidation of deoxyguanosine is mediated by peroxynitrite and is not due to reaction with side products, since control reactions using decomposed peroxynitrite and run at the same pH values showed no increase in the level of 8-oxo-dG. The inability to observe the formation of 8-oxo-dG at pH 7 is probably due to its faster reaction with peroxynitrite at pH 7 than at pH 5. While all of the 100 μM 8-oxo-dG was lost in the reaction with 1 mM peroxynitrite at pH 7, 10% of the 100 μM 8-oxo-dG remained unchanged when the reaction pH was lowered to 5. At pH 7, since 8-oxo-dG reacts faster with peroxynitrite than does deoxyguanosine (*11*), even the amount of 8-oxo-dG normally present as a contaminant decreases by 40−50% when commercial deoxyguanosine is reacted with peroxynitrite. We found that 96% of 100 μM and 100% of 10 μM 8-oxo-dG were destroyed at pH 5 by 10 mM peroxynitrite; the latter is the average concentration of peroxynitrite used in our reactions. Therefore, the amount of 8-oxo-dG observed is probably less than that actually formed. In the reaction at pH 5, the initial concentration of deoxyguanosine decreased by 2% compared to the control run, while the yield of 8-oxo-dG was ∼0.5% of the starting nucleoside. When the reaction mixtures of deoxyguanosine and peroxynitrite were stored frozen at −20 °C, the amount of 8-oxo-dG from the reaction carried out at pH 5 decreased by half, and in the case of reactions carried out at an even lower pH, the 8-oxo-dG disappeared completely. 8-Oxo-dG might be lost by reacting with nitrous acid, which is formed from the nitrite at acidic pH, to give unknown products; the expected product, 8-oxodeoxyxanthosine, was not observed.

While the peroxynitrite-mediated C8-oxidation of deoxyguanosine reached a maximum at pH 5, the level of C8-nitration decreased as the pH under which the reaction was carried out decreased. The reaction at pH 7 produced the maximum level of C8-nitration of deoxyguanosine, measured as the amount of 8-nitroguanine and 8-nitro-dG. When compared to that of the pH 7 reaction, the yield of 8-nitroguanine decreased by 75% at pH 6 and by 90% at pH 5, while the yield of 8-nitro-dG decreased by only 35% at both pH 6 and 5 (Figure 14B). 8-Nitroguanine formation in the peroxynitrite reaction with deoxyguanosine does not appear to be catalyzed by acidic pH. As the pH of the reaction was decreased further to 3, neither 8-nitroguanine nor 8-nitro-dG was formed. At this pH, some formation of 8-oxo-dG (∼0.1% yield) was observed.

Reaction of Deoxyadenosine and Adenosine with Peroxynitrite at pH 7 and 5. Unlike guanine nucleosides, 5 mM deoxyadenosine and adenosine react with peroxynitrite at pH 7 and 5 °C, or room temperature, to give 16 μM 8-oxo-dA and 17 μM 8-oxo-Ado (∼0.3% yield), respectively, in addition to thus far unidentified nitration products (Figure 15). Probably because of their lower reactivities with peroxynitrite, it is possible to observe the formation of 8-oxo-dA and 8-oxo-Ado even in reactions

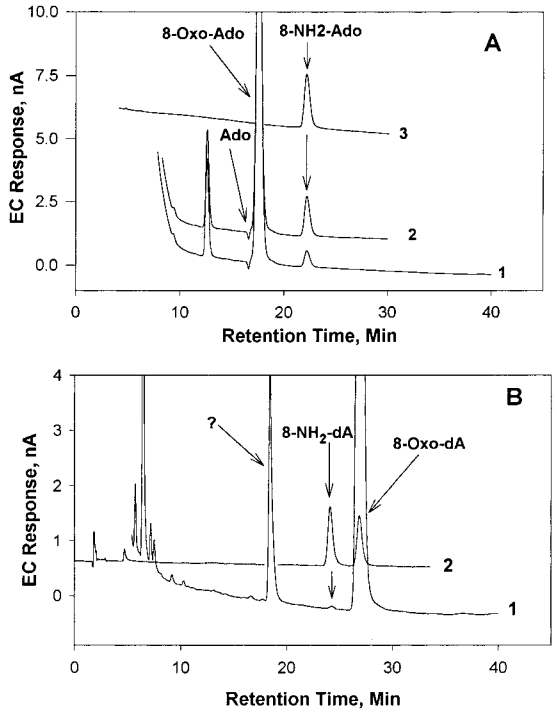

Figure 16. (A) HPLC-ECD analysis of the products from the adenosine−peroxynitrite reaction at pH 7 after reduction with dithionite (trace 1), the products from the adenosine−peroxynitrite reaction at pH 7 after reduction with dithionite cochromatographed with the 8-NH_2-Ado standard (trace 2), and the HPLC-ECD profile of the 8-NH_2-Ado standard alone (trace 3). HPLC conditions were like those described in the legend of Figure 15. 8-Aminoadenine and 8-oxoadenine standards (not shown) eluted at ∼7 and ∼8 min, respectively. (B) HPLC-ECD analysis of the products from the reaction of deoxyadenosine and peroxynitrite at pH 7 after dithionite reduction (trace 1) and of 8-NH_2-dA and 8-oxo-dA standards (trace 2). HPLC system 4 was used. Deoxyadenosine (not shown), which does not give an ECD response, elutes at ∼19 min.

carried out at neutral pH. Indeed, we found that while 100 μM 8-oxo-dG was destroyed completely by reacting with 1 mM peroxynitrite at pH 7 and 5 °C, only 20% of the 100 μM 8-oxo-dA was lost under these conditions. As evident from the HPLC profiles in Figure 15, the level of oxidation of adenine nucleosides at C8 was several-fold higher than that of nitration, even at neutral pH. Lowering the reaction pH to 5 resulted in 2.4- and 2.2-fold increases in the yields of 8-oxo-dA and 8-oxo-Ado, respectively, but did not alter the level of nitration.

The late-eluting peaks, at ∼60 min in the case of the dAdo reaction with peroxynitrite (Figure 15A) and at ∼38 min in case of the Ado reaction (Figure 15B), were observed only when a suitable potential was applied to the reducing electrode, suggesting that they represent nitrated nucleosides. For the detection of these species, a reducing potential of −0.7 V and an oxidizing potential of 0.6 V were sufficient. However, increasing the oxidation potential to 0.8 V at the downstream electrode enabled the detection of 8-oxoadenine nucleosides together with the nitration products (Figure 15). Dithionite reduction of the reaction mixtures led to formation of 8-aminoadenine nucleosides, which could be detected at an oxidation potential of 0.8 V (Figure 16A,B) even when the reducing electrode was turned off. However, compared to the area of the peaks at 60 and 38 min in Figure 15, the areas of the peaks corresponding to the amino derivatives appear to be much smaller, suggesting, at first, an incomplete reduction or insufficient oxidation potential. The former possibility is unlikely since both of the peaks corresponding to nitrated products disappear completely after dithionite reduction of the reaction mixture. The latter possibility is also unlikely since analysis by hydrodynamic voltammetry showed that the ECD response to 8-NH_2-dA reaches a plateau at oxidizing potentials of ≥0.8 V; at <0.7 V, there is no response (data not shown). The amount of 8-NH_2-dA obtained from dithionite reduction of the deoxyadenosine reaction mixture was ∼20 times smaller than the amount of 0.3−0.4 μM 8-NH_2-Ado obtained after reduction of the adenosine reaction mixture, suggesting that the level of C8 nitration was correspondingly lower in the case of the deoxynucleoside.

When the product responsible for the 38 min peak from the adenosine reaction with peroxynitrite was purified by HPLC, reduced with dithionite, and then analyzed, we observed the appearance of two new, thus far unidentified, electrochemically active products, which eluted earlier than adenosine. However, little 8-NH_2-Ado formation was observed, even though the reduction was complete. This suggests that the 8-NH_2-Ado, which was observed after dithionite reduction of the reaction mixture, did not arise from the material in the 38 min peak. Therefore, neither the 38 min peak from the adenosine reaction nor the 60 min peak from the deoxyadenosine reaction is likely to represent 8-nitroadenosine or 8-nitrodeoxyadenosine. It is possible that adenine nucleosides can be nitrated at the C2 position. Such 2-nitroadenine derivatives would yield the corresponding 2,6-diaminopurines upon dithionite reduction. However, while 2-nitroadenine nucleosides would be expected to produce a signal at the downstream electrode of the dual-mode ECD, we find that 2,6-diaminopurine nucleosides are not electrochemically active under any of the ECD conditions used in this report. This possibility is therefore not likely.

As perhaps more likely candidates for the unknowns, 2-nitro-8-oxoadenine and 8-nitro-2-oxoadenine or the corresponding nucleosides may be expected to produce an ECD signal in the dual mode prior to dithionite reduction, and in the single oxidative mode after dithionite reduction. This, in fact, is observed for the 38 min peak obtained from the adenosine reaction. Therefore, we tentatively suggest that the late-eluting peaks at 60 and 38 min in the reactions of peroxynitrite with deoxyadenosine or adenosine may represent nucleosides of 2-nitro-8-oxoadenine or 2-oxo-8-nitroadenine, although the possibility of 2,8-dinitroadenine nucleoside is also not excluded. Overall, nitration appears to be a minor route in the reactions of peroxynitrite with adenine nucleosides; as estimated from the size of the peaks corresponding to 8-oxoadenine nucleoside and the late-eluting peaks corresponding to nitroadenine nucleosides, nitration is at least 50-fold less extensive than C8-oxidation. We also find that while adenine nucleosides react easily with peroxynitrite, the adenine base itself exhibited little reactivity and did not yield any significant nitration products; this, however, may also be due, in part, to its poor solubility.

Discussion

Several studies have been published on the reactivity of peroxynitrite with deoxyguanosine and 8-oxo-dG, and on the peroxynitrite-induced formation of 8-nitroguanine in DNA (*11*, *15*−*17*, *23*, *33*, *35*, *38*, *41*). To our knowledge,

however, comparative studies on the extents of peroxynitrite-induced oxidative damage and nitration have not been reported, nor, apparently, has the nitration of ribonucleosides and deoxyribonucleosides been examined.

Central to the present work is the use of a highly selective and sensitive method for the detection of nitrated purines and nucleosides, based on HPLC with a dual-mode amperometric detector. Highly sensitive dual-mode electrochemical detection involving sequential reduction and oxidation of biologically important molecules such as 3-nitrotyrosine, glutathione, glutathione disulfide, and 8-nitroguanine has been used in the past (*24*, *41*, *42*). Other workers (*41*) have used a coulometric detector equipped with several electrodes, which also employed reduction and subsequent oxidation, for the detection of 8-nitroguanine formation in the reactions of calf thymus DNA with peroxynitrite in the presence of various antioxidants; however, the parent deoxynucleoside, 8-nitro-dG, from which the 8-nitroguanine was derived, was not detected.

The work presented here shows that amperometric detection can be used to achieve high selectivity and sensitivity for compounds, such as 8-nitroguanine and 8-nitroxanthine, that can undergo reversible electrochemical reduction and oxidation at an electrode surface. While the current due to the reduction of the nitro compounds at the upstream electrode is ignored because of its very low signal-to-noise ratio, high sensitivity is obtained in our system by measuring only the current produced at the downstream oxidizing electrode, and by optimizing the ECD conditions for each compound of interest through hydrodynamic voltammetry. The very close proximity of the reducing and oxidizing electrodes in the BAS ECD decreases the possible loss of unstable products produced at the upstream electrode. In the case of nitroaromatic and nitroheterocyclic compounds, turning the upstream reducing electrode off or placing it on standby results in a loss of the signal at the downstream oxidizing electrode. Since signals due to the oxidation of compounds such as 8-oxo-dG and 8-oxo-dA are still observable under this condition, this feature provides enhanced selectivity in addition to that furnished by the high-performance liquid chromatographic separation. The latter has been optimized, in this work, to give high resolution of several purine bases and nucleosides with nonbulky modifications.

Our work shows that peroxynitrite reacts very quickly with all of the purine nucleosides examined at neutral pH, resulting in C8-oxidation as well as in nitration. Although the C8-oxidation of adenine and guanine nucleosides by peroxynitrite had been reported in an earlier work by others (*22*), it is not clear at what pH the reaction was carried out or whether the oxidation may have been partially due to contamination with hydrogen peroxide. In the work presented here, the peroxynitrite was prepared by the ozonation of sodium azide (*25*), excluding the possibility of hydrogen peroxide contamination. Also, peroxynitrite, which had been allowed to decompose under the same conditions as the actual runs, was used in all control incubations. Thus, the formation of 8-oxo-dA and 8-oxo-dG at various pH values that we describe in the reactions of deoxyadenosine and deoxyguanosine is indeed mediated by peroxynitrite. At neutral pH, the inability to observe the formation of 8-oxo-dG in the reactions of deoxyguanosine with peroxynitrite is due to its further reactivity toward peroxynitrite; however, when the rapidity of the 8-oxo-dG−peroxynitrite reaction is considered, it becomes evident that the oxidation at C8 is much more extensive than nitration in the reactions of both guanine and adenine nucleosides at either pH 7 or 5. In contrast to 8-oxo-dG, 8-oxo-dA is less susceptible to further reactions with peroxynitrite or its decomposition products. We propose, therefore, that 8-oxo-dA may prove to be a more accurate marker of the extent of peroxynitrite-mediated oxidative damage to DNA than 8-oxo-dG.

To account for the ability of peroxynitrite to oxidize a wide variety of biological molecules, the formation of a new intermediate species, the "H-bonded caged radical pair", during the isomerization of peroxynitrite to nitrate, has been proposed on the basis of theoretical calculations (*43*). This intermediate species, like hydroxyl radicals, would be highly reactive and capable of causing oxidative damage (*43*). Alternatively, since peroxynitrite is known to act as both a one-electron and two-electron oxidant (*36*, *37*, *44*), the 8-oxo-dG and 8-oxo-dA observed in the reactions of deoxyguanosine and deoxyadenosine may have been produced from the reaction of a one-electron-oxidized purine ring with a water molecule (*45*). The same one-electron-oxidized purine intermediate, after loss of the ribosyl or deoxyribosyl group, could also have given rise to the 8-nitropurine base we observe immediately after the addition of peroxynitrite in the reactions of guanine and xanthine nucleosides.

In contrast to the findings of Byun et al., who reported a retention time of 9 min for 8-nitro-dG and 11 min for 8-nitroguanine in a reverse-phase HPLC system (*46*), we find that the C8-nitro products from the reaction of peroxynitrite with purine nucleosides elute with significantly different retention times, and in a different order, in our system. For instance, with HPLC system 3, deoxyguanosine elutes at 13 min (Figure 8), 8-nitroguanine at ∼9 min (Figure 1), and 8-nitro-dG elutes at 72 min (Figure 8). Generally, the introduction of a nitro group increases the nonpolarity of a compound and thus increases the elution time of the compound on reverse-phase columns; the reason for the large discrepancy between the retention times we obtained and those reported by Byun et al. (*46*) is unknown. Unlike the 8-oxonucleosides, the nitronucleosides were not produced in a sufficiently high yield in our reactions to produce UV signals or spectra, but could be observed by the dual-mode ECD when the reducing electrode W2 was on, as illustrated in Figure 10 for 8-nitro-Guo. At neutral pH and 37 °C, 8-nitro-Guo exhibited a half-life of 5 h, but 8-nitro-dG, which was reported to exhibit a half-life of 4 h in DNA and of 1 h in an oligonucleotide (*15*, *23*), exhibited an apparent half-life of only ≤3 min as the free nucleoside.

Peroxynitrite-mediated C8-nitration of guanine and xanthine nucleosides was confirmed directly by purification of the nitro nucleosides by HPLC, followed by dithionite reduction to give the corresponding 8-amino-nucleosides. In the peroxynitrite reactions with adenine nucleosides, C8-nitration, which occurred to a much lower extent than in the case of guanine and xanthine nucleosides, could only be shown indirectly by the formation of 8-aminoadenine nucleoside after dithionite reduction of the whole reaction mixture. However, the reaction of peroxynitrite with adenine nucleosides appears to be more complex, since the adenine ring is capable of undergoing C8- and C2-nitration, although nitration was

minor when compared to C8-oxidation. Further work to fully characterize the nitration products of adenine nucleosides is in progress in our laboratory. The failure to detect these modifications up to now may simply be the lack of the proper HPLC system and mode of detection. We consider that the results of the studies described here are important for understanding the reactive nature of peroxynitrite and also as prerequisites to the search for analogous products, possibly mutagenic, from the reaction of peroxynitrite with DNA and RNA in vitro and in vivo.

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