Inhibition of Nitrous Acid-Dependent Tyrosine Nitration and DNA Base Deamination by Flavonoids and Other Phenolic Compounds

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Exposure of tyrosine or DNA bases to acidic nitrite at low pH results in the nitration of tyrosine and the formation of base deamination products, respectively. At pH 1, hypoxanthine and xanthine are formed from the deamination of adenine and guanine, respectively, whereas under the same conditions, uracil is not detected. The yield of 3-nitrotyrosine derived from interaction of equimolar nitrite and tyrosine at pH 1 is approximately 50% of that obtained from equimolar peroxynitrite−tyrosine interactions at pH 7.4. The ability of a range of plant phenolic constituents to prevent damage mediated by acidic nitrite was also examined in comparison with the activity of vitamin C. The epicatechin/gallate family of flavonols, constituents of green tea, red wine, etc., demonstrates the most extensive inhibitory properties against both tyrosine nitration and base deamination. The results also show that ascorbic acid is a poor inhibitor of nitration or deamination under acidic conditions such as those of the stomach. The ability of plant phenolics to scavenge reactive nitrogen species derived from acidic nitrite may contribute to the protective effects of tea polyphenols against gastric cancer.

Introduction

Exposure of humans to excess nitrite from the diet or arising from overproduction of endogenous nitric oxide (e.g., at sites of chronic inflammation) may play a role in the etiology of cancers of the GI tract, such as stomach cancer (*1*, *2*). Overproduction of reactive nitrogen species (RNS)[1] derived from nitrite, e.g., HNO_2 and N_2O_3, can cause damage in several ways. For example, they can lead to N-nitrosation of amines, nitration of aromatic compounds, and deamination of DNA bases (*3*−*10*).

Many studies have shown that the phenolic constituents of the diet are efficient scavengers of reactive oxygen species (*11*, *12*) and reactive chlorine species (*13*), and there is considerable interest in the possibility that they can protect against cancer through such mechanisms. However, they may also act by scavenging RNS. In particular, several phenolic compounds are potent inhibitors of the nitration of tyrosine in vitro by peroxynitrite (*14*, *15*), and they may also scavenge nitric oxide itself (*16*, *17*). It is widely thought that ascorbic acid is also an important agent protecting against RNS in vivo, since it can, under certain circumstances, inhibit nitrosation and nitration reactions (*18*), and inhibit nitration by peroxynitrite (*7*).

The purpose of this study was to model events that may occur in the human stomach after ingestion of nitrite. We examined the ability of plant phenolics (Figure 1) to prevent damage by acidic nitrite in vitro, in comparison with that of vitamin C. Two aspects of damage were examined: nitration of tyrosine and deamination of DNA bases. Both might be involved in gastric damage and cancer development (*18*, *19*)

Experimental Section

Ferulic acid and rutin (HPLC grade) were purchased from Extrasynthese. Epicatechin, catechin (≥98%), 3-nitrotyrosine, caffeic acid, quercetin, gallic acid, 3,4-dihydroxyphenylacetic acid, xanthine, hypoxanthine, and all DNA bases were from Sigma. Epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) were gifts from Unilever Research Laboratories (Colworth, U.K.). Ultrapure water (18.2 MΩ) was used throughout.

Nitrotyrosine Formation from Acidic Nitrite. The time dependency of the reaction between tyrosine and acidic nitrite was followed spectrophotometrically using a Beckman DU7500 spectrophotometer. Stock solutions of 3 mM tyrosine and $NaNO_2$ were prepared in pure water. Tyrosine (2.5 mL) was added to a solution containing $NaNO_2$ in 0.5 M HCl to a total volume of 10 mL (final concentration of 750 μM) and the mixture incubated at 37 °C. Aliquots (1 mL) were removed every hour for 7 h for spectrophotometric analysis. A sample was removed after 4.5 h for analysis by HPLC as described below.

Inhibition of Tyrosine Nitration. Stock solutions of 2.5 mM ascorbic acid and phenolics (ferulic acid, epicatechin, catechin, EGC, ECG, and EGCG) were prepared in pure water. Rutin (75 mM) was initially solubilized in DMSO prior to dilution to 2.5 mM in pure water. The final concentration of DMSO was 0.3%, and appropriate controls and blanks were

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[1] Abbreviations: RNS, reactive nitrogen species; EGC, epigallocatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; DMSO, dimethyl sulfoxide.

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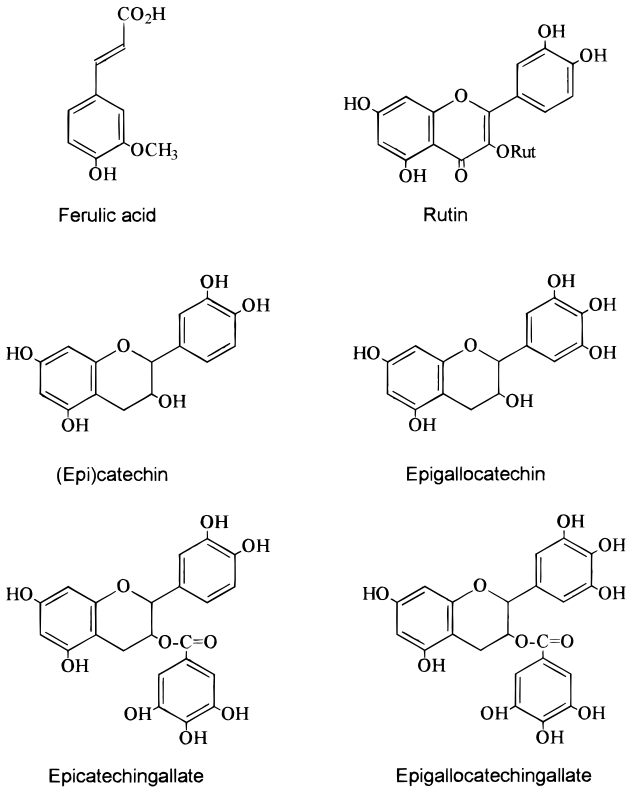

Figure 1. Structures of the phenolic compounds.

included. Each compound was added (final concentration of 0−400 μM) to a solution containing sodium nitrite and tyrosine (400 μM final concentration for each) in 0.5 M HCl (final concentration) to a total volume of 1 mL (final pH of <1). The reaction was allowed to continue at 37 °C for 4.5 h and then stopped by placing the mixture on ice. Each sample was diluted 2-fold with the mobile phase prior to HPLC analysis.

HPLC Analysis of Inhibition of Nitrotyrosine Formation. An isocratic reverse phase HPLC method was developed on a Waters HPLC system for the analysis of tyrosine and nitrotyrosine (calibration ranges of 25−200 and 2.5−15 μM, respectively) and the phenolics, ferulic acid, epicatechin, catechin, ECG, EGC, EGCG, and rutin (10−200 μM calibration ranges). It consisted of an autosampler with a peltier temperature controller, a model 626 pump with a model 600S controller, a model 996 photodiode array detector, and the Millennium software system which controlled all the equipment and carried out data processing. A Nova-Pak C^{18} column (4.6 mm × 250 mm) with a 4 μm particle size was used and the temperature maintained by the column oven set at 30 °C. Injections (10 μL) were made by an autosampler with a 100 μL fixed loop.

The solvent system (flow rate of 0.5 mL min^{-1}) consisted of 98% (v/v) solvent A [50 mM KCl and 7.8 mM HCl (pH 2.2) in 20% methanol] and 2% (v/v) solvent B (acetonitrile) for the phenolics studied, with the exception of rutin. Rutin was analyzed by a linear gradient system where solvent A was held at 98% for 10 min and then decreased linearly to 80% solvent A at 15 min and held at these conditions for a further 20 min before returning to 98% solvent A at 45 min, being held at these conditions for a further 5 min. Detection was by diode array at 357 and 275 nm. Intra- and interassay variations were 0.4 and 3.2% for tyrosine and 0.6 and 9.9% for nitrotyrosine, respectively. None of the phenolics interfered with the HPLC analysis of nitrotyrosine. Sinapic acid (2 μg/mL) was used as an external standard.

Incubation of Free Bases with Sodium Nitrite. Solutions of DNA bases were prepared by dissolving adenine, guanine, and cytosine in 0.1 M HCl to a concentration of 1.4 mM. For the pH dependence study, the bases at the same concentration were dissolved in potassium phosphate buffer (50 mM) for pH 3 and 7.4 or in sodium carbonate buffer (50 mM) for pH 9. The bases were preincubated for 10 min at 37 °C, and aqueous solutions of sodium nitrite (50 μL) were added, making the final concentration range 0.05−3.2 mM. For studies of the time course and pH dependence, a final nitrite concentration of 0.45 mM was used. After incubation for 1 h at 37 °C, the mixture (0.5 mL) was neutralized with 1 M KOH (10 μL) and then mixed with 0.5 mL of phosphate buffer (50 mM, pH 3.0). This preparation was subjected to HPLC analysis.

HPLC Analysis of Deaminated Products of the Free Bases. A Gynkotek HPLC system consisting of a model 480 solvent delivery pump, a GINA 50 autosampler, a UVD 340S photodiode array UV detector, and an INTRO electrochemical detector was used. The separation of DNA bases and their deaminated products was achieved on a reverse phase Hypersil C^{18} column (250 mm × 4.6 mm, 5 μm) which was eluted at a flow rate of 1 mL/min with an isocratic mobile phase, a potassium phosphate buffer (50 mM, pH 3) containing 2 mM triethylamine and 0.05 M EDTA. The deaminated products hypoxanthine, uracil, and xanthine were detected by UV spectroscopy at 250 and 265 nm, respectively. Xanthine was also detected by the electrochemical detector at a potential of 0.9 V.

Inhibition of the Deamination of the Free Bases. The stock solutions of the phenolic compounds were prepared by dissolving in water at a concentration of 20 mM, and then dilution was carried out to obtain 10, 5, 2.5, and 1.25 mM solutions. An aqueous solution of sodium nitrite (9 mM) was used. To DNA base solutions (0.9 mL) were added the phenolics (50 μL) to final concentrations of 1, 0.5, 0.25, 0.125, and 0.065 mM. The mixture was preincubated at 37 °C for 10 min, and then sodium nitrite (50 μL) was added to the mixture to a final concentration of 0.45 mM. After incubation for 1 h at 37 °C, 0.5 mL of the incubation mixture was neutralized with 1 M KOH (10 μL), mixed with 0.5 mL of phosphate buffer (50 mM, pH 3), and subjected to HPLC analysis.

Results

Nitration of Tyrosine by Acidic Nitrite. Lowering the pH of a sodium nitrite solution to <1 changes its spectrum to give multiple peaks in the region of 357 nm, consistent with the formation of nitrosating/nitrating species arising from HNO_2. The interaction between tyrosine at equimolar (400 μM) concentrations and acidified nitrite at 37 °C results in the formation of 3-nitro-L-tyrosine, which has an enhanced absorbance at 357 nm. The time course of this reaction demonstrates that completion is reached after 4.5 h with a maximum yield of 15 μM. No product is formed in the absence of acid. When analyzed by HPLC, tyrosine and nitrotyrosine have sharp reproducible peaks with retention times of 6 ± 0.2 and 8 ± 0.5 min, respectively.

Direct Reactions of Phenolics with Acidic Nitrite. Figure 2 shows the UV/visible spectra of epicatechin, epigallocatechin (EGC), ferulic acid, and rutin in HCl and the resulting changes in their absorbance spectra after reaction with acidic nitrite. In the case of epicatechin, after interaction for 10 min with acidic nitrite, a pronounced shoulder appears at 350 nm on the 280 nm band. On prolonged interaction, the shoulder disappears with a concomitant further increase in absorbance at 280 nm. Epigallocatechin responds in a similar way with a dramatic transformation of the major shoulder at 280 nm to a peak at 280 nm with a 4-fold increase in absorbance, accompanied by a pronounced shoulder at 350 nm as for epicatechin. The spectrum of pure rutin exhibits two bands: band I with a peak at 350 nm due to the phenolic

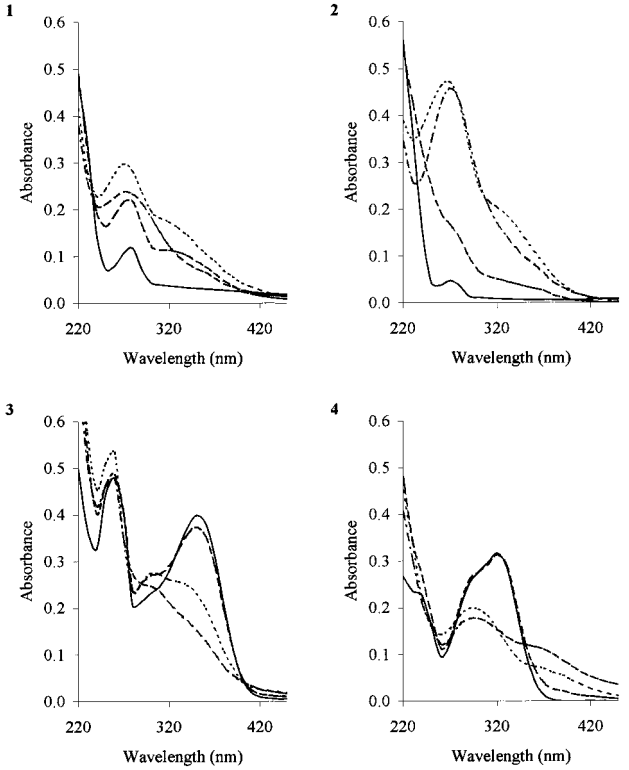

Figure 2. Spectral changes on the interaction between a 25 μM phenolic and 250 μM acidic nitrite at 37 °C: (1) epicatechin, (2) epigallocatechin, (3) rutin, and (4) ferulic acid at (—) 0, (– –) 0.5, (---) 10, and (-–-) 270 min.

B ring and band II with a maximum at 270 nm representing the A ring (Figure 1). When band I is exposed to acidic nitrite for 10 min, its intensity declines and its profile changes, and on prolonged incubation for up to 270 min, the intensity of band I decreases further. For band II, there is a transient small increase in the absorbance at 10 min, which has returned to the original position at 270 min. For ferulic acid, reaction with acidic nitrite leads to the replacement of the main peak at 320 nm (with its characteristic shoulder at 290 nm) by a single peak with a lower intensity at 295 nm, accompanied by the formation of a broad shoulder at 350 nm. Ascorbic acid also reacted directly with acidic nitrite, resulting in the rapid (<1 min) loss of the peak at 245 nm.

Inhibition of Tyrosine Nitration. To investigate the ability of the phenolics to inhibit nitrotyrosine formation mediated by acidic nitrite, ferulic acid (4-hydroxy-3-methoxycinnamic acid), rutin (quercetin-3-rutinoside), epicatechin, catechin, EGC, ECG, and EGCG (final concentrations of 0−400 μM) or ascorbic acid (400 μM) were added to nitrite/tyrosine solutions (final concentrations of 400 μM). HPLC analysis gave good baseline separation (Figure 3) and reproducible retention times for all the compounds tested; there was no interference with the HPLC detection of 3-nitrotyrosine. All inhibited the formation of nitrotyrosine, the concentrations of compound giving 50% inhibition (Table 1) being in the following order of effectiveness: EGCG ≈ ECG ≈ catechin > epicatechin > EGC ≈ ferulic acid ≈ rutin. Ascorbic acid had no inhibitory effect on tyrosine nitration by acidic nitrite up to concentrations of 400 μM. At 50 μM, EGCG and ECG exerted a 2-fold greater inhibitory effect than ferulic acid and rutin. At 100 μM (25% of that of the tyrosine substrate), EGCG, ECG, and catechin completely suppressed tyrosine nitration, whereas 100 μM epicatechin or EGC decreased it by about 90%. At this concentration, rutin and ferulic acid produced 55−60% inhibition.

Table 1. IC_{50} Values of Dietary Phenolics with Respect to Their Action on Acidic Nitrite-Mediated Tyrosine Nitration[a]

compound	IC_{50}[b]
rutin	75.8 ± 23.6
ferulic acid	73.0 ± 15.1
epigallocatechin	64.8 ± 12.8
epicatechin	50.2 ± 8.2
catechin	41.6 ± 3.7
epicatechin gallate	38.4 ± 7.3
epigallocatechin gallate	35.8 ± 9.0

[a] Results are means ± SD. The concentration of tyrosine and nitrite was 400 μM (for details, see the text). [b] IC_{50} is the concentration (micromolar) of the compound giving 50% inhibition of nitrotyrosine formation.

In all cases, HPLC analysis showed complete loss of the native polyphenol in the mixture by incubation for 4.5 h accompanied by the appearance of new peaks with different absorbance spectra. In the case of ferulic acid, a peak appears with a retention time of 16.7 min, the absorbance of which increases as the initial ferulic acid concentration increases. This was spectrally identical to nitroferulic acid (*15*). With rutin a large number of new bands appeared (1−12), with the absorbance increasing as the initial rutin concentration increased. An unidentified peak appears in the solvent front with all the catechins.

The loss of the phenolics with the catechol structure is consistent with inhibition of tyrosine nitration through a mechanism of oxidation (*15*, *20*).

Deamination of DNA Bases and Their Inhibition. Sodium nitrite in acid (pH 1) caused deamination of adenine and guanine, forming hypoxanthine and xanthine as measured by HPLC. Figure 4 shows a representative separation. The extent of formation of xanthine and hypoxanthine increased linearly in the range of 0.056−0.9 mM sodium nitrite (Figure 5). At equimolar concentrations of free bases and sodium nitrite, about 3% of the guanine and 0.4% of the adenine were converted to their deaminated products, suggesting that the reaction between acidified nitrite and guanine is favored over that with adenine. No uracil, the deamination product of cytosine, was detected until a very high concentration of acidified nitrite (7 mM) was used, at which only about 0.01% of the cytosine was converted to uracil, suggesting that cytosine is a poor substrate for deamination by acidified nitrite. A time course study showed that the deamination of the free bases was complete at about 2 h for hypoxanthine and at about 4 h for xanthine (data not shown).

The deamination of the free bases was pH-dependent. About 30- and 2-fold more xanthine and hypoxanthine, respectively, were formed at pH 1 than at pH 3, while no deaminated products were detectable at pH >7.4. Examination of the ability of phenolic compounds to inhibit deamination showed that all were effective. As indicated by the IC_{50} values (Table 2), catechin compounds and caffeic acid exhibited a similar efficacy in inhibiting the deamination of free bases by the acidified nitrite with quercetin being significantly less effective. However, the inhibitory effects of catechol, gallic acid, and 3,4-dihydroxyphenylacetic acid (DHPAA) were found

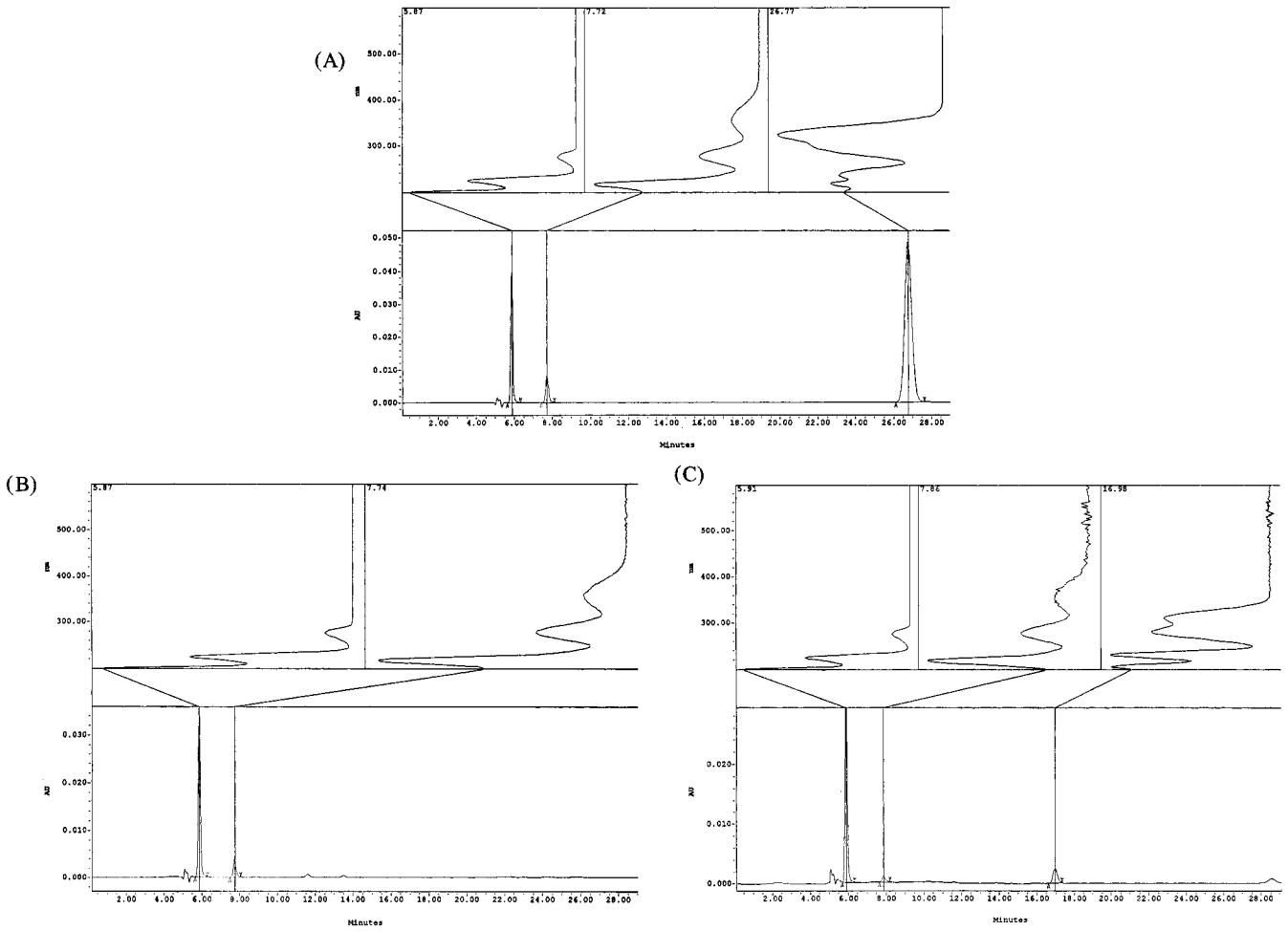

Figure 3. Chromatograms and the corresponding spectra of (A) standard solutions of tyrosine (400 μM), nitrotyrosine (30 μM), and ferulic acid (400 μM) and (B and C) tyrosine (400 μM) and the resulting nitrotyrosine in the absence and presence of ferulic acid (150 μM), respectively, after exposure to acidic nitrite (400 μM) at 37 °C for 4.5 h.

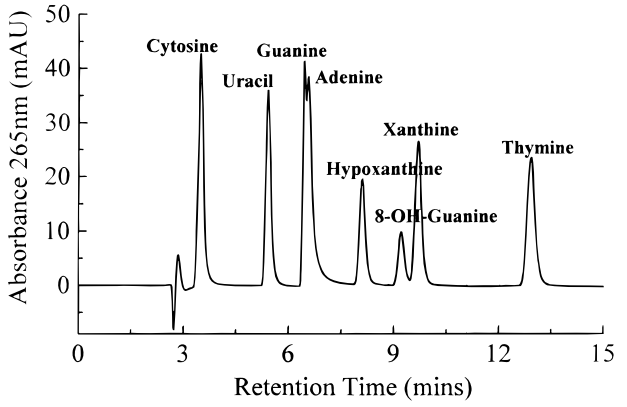

Figure 4. Chromatogram of HPLC separation of DNA bases (for conditions, see the text).

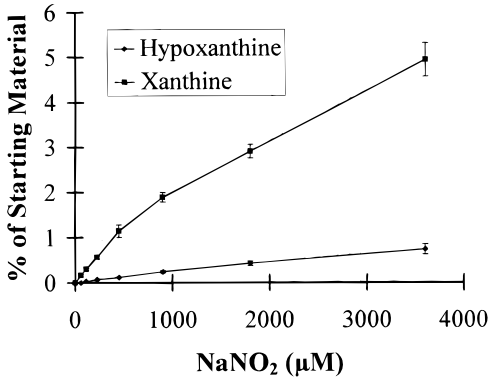

Figure 5. Nitrite-induced conversion of adenine and guanine to hypoxanthine and xanthine (for conditions, see the text).

to be much weaker, equally so for the inhibition of xanthine and hypoxanthine formation.

The effect of ascorbic acid on the deamination was also investigated. Ascorbic acid did not inhibit the formation of xanthine and hypoxanthine significantly. A weak inhibitory effect on the deamination of free bases was observed only when the concentration of ascorbic acid used was raised to 1 mM, when about 40% inhibition was achieved.

Discussion

The nitrosating species arising from aqueous acidic nitrite are depicted in the following equations (*10*):

$$NO_2^- + H^+ \rightleftarrows HNO_2$$

$$HNO_2 + H^+ \rightleftarrows H_2NO_2^+ \rightleftarrows H_2O + NO^+$$

$$2HNO_2 \rightleftarrows N_2O_3 + H_2O$$

$$N_2O_3 \rightleftarrows NO^+ - NO_2^-$$

Table 2. IC_{50} Values of the Phenolics for the Inhibition of Formation of Hypoxanthine and Xanthine by the Nitrite-Dependent Deamination of Adenine and Guanine, Respectively[a]

compound	IC_{50} (μM)	
	hypoxanthine	xanthine
epigallocatechin gallate	44.67 ± 10.56	31.19 ± 7.67
caffeic acid	53.01 ± 2.15	62.91 ± 14.58
catechin	67.22 ± 7.64	67.89 ± 2.82
epicatechin	69.91 ± 6.20	64.98 ± 9.19
epigallocatechin	73.97 ± 1.24	63.99 ± 2.48
quercetin	85.30 ± 2.49	75.67 ± 5.69
catechol	144.14 ± 12.94	274.53 ± 3.40
gallic acid	150.52 ± 19.55	226.18 ± 4.25
3,4-dihydroxyphenylacetic acid	171.90 ± 33.58	247.29 ± 11.46

[a] Results are means ± SD.

Several phenolic antioxidants have been shown to decrease the extent of nitration of tyrosine by peroxynitrite in vitro (*14*, *15*) and that of nitrosation and nitration of several amino acids in vivo (*3*). The results reported in this study demonstrate that tyrosine is nitrated by nitrite at acidic pH (<1) to form 3-nitrotyrosine. The yield of 3-nitrotyrosine derived from equimolar interactions of nitrite and tyrosine is about 8%, increasing to 14% with a nitrite:tyrosine molar ratio of 2:1. Equivalent yields of 3-nitrotyrosine from peroxynitrite−tyrosine interactions at equimolar concentrations at pH 7.4 are reported to be in the region of 14% (*7*). While the concentration of nitrite (400 μM) is relatively high, studies suggest that these levels can be achieved in gastric juice derived from humans consuming 100 g of spinach (which can contain up to 500 mg of nitrate) (*21*, *22*)

All the phenolic compounds tested were effective in inhibiting acidic nitrite-induced tyrosine nitration, epigallocatechin gallate, epicatechin gallate, epicatechin, epigallocatechin, and catechin being almost maximally effective at 25% of the tyrosine concentration and ferulic acid and rutin at 50% of the tyrosine concentration. For ferulic acid, the results suggest a competitive nitration, the identification of nitroferulic acid being consistent with the findings of Pannala et al. (*15*) in studies on peroxynitrite-induced nitration of phenolics. Spectral studies on the reactions of the phenolics with acidic nitrite suggest that the mechanism of action with the catechin family and rutin may proceed via oxidation of flavonols and flavone in scavenging reactive nitrogen species, whereas for the hydroxycinnamate, ferulic acid, nitration is the mode of action (*14*, *15*).

The results presented here also demonstrate that acidified nitrite can cause damage to DNA bases, forming xanthine and hypoxanthine from guanine and adenine, respectively, and that this can be inhibited by plant phenolics, epigallocatechin gallate again being the most effective. These deamination products could conceivably play an important role in mutagenic events in cells (*18*, *19*, *23*). The deamination of cytosine and its mutagenic consequence have been extensively studied (*19*). However, in this study, cytosine was shown to be a poor substrate for the acidified nitrite-mediated deamination. This finding suggests that the genotoxcity caused by nitrite or its derived reactive species may not involve deamination of cytosine.

Ascorbic acid and α-tocopherol have been shown to inhibit nitrosation in some human studies and animal models (*24*−*27*), a suggested mechanism being by preferential reaction with nitrite or nitrite-derived species such as nitrosyl cation (NO^+). Inhibition of nitrosation by the hydroxycinnamates, caffeic acid, ferulic acid, and coffee (rich in caffeic acid esters) and tea (rich in catechin/gallates) in vivo has also been demonstrated (*28*, *29*). Furthermore, tannins such as gallic acid (*30*) appear to exert a protective effect against nitrosation. For example, ferulic acid, a disubstituted monohydroxycinnamate, decreases urinary *N*-nitrosoproline levels by 14−45% in vivo in humans, suggesting inhibition of nitrosation (*28*, *29*). In addition, the related dihydroxycinnamate chlorogenic acid (an ester of caffeic acid and quinic acid) suppresses N-nitrosation (by nitrite in acidic solution) of a model amine in vitro (*31*). Other dihydroxyphenols such as dopamine, 3,4-dihydroxybenzoic acid, and catechol also inhibit nitrosation, whereas monosubstituted hydroxy compounds such as tyrosine, *p*-hydroxybenzoic acid, salicylic acid, and *p*-hydroxyphenylacetic acid do not (*31*).

The results described here show that ascorbic acid may be a poor inhibitor of nitration or deamination by nitrite under acidic conditions such as those of the stomach. In neutral aqueous media, ascorbic acid reacts very slowly with nitrite, forming dehydroascorbic acid via a nitrosation reaction (*32*). The rate-limiting step is the formation of the nitrosating agent which is proposed to be either N_2O_3 or $H_2NO_2^+$ (*32*). The reaction is highly pH-dependent, suggesting that nitrite accelerates vitamin C decay in fruit and vegetables under acidic storage conditions (*32*). Dahn et al. (*33*) have observed that the rate of reaction between nitrite and ascorbic acid is slow at physiological concentrations of nitrite in acidic media due to the formation of N_2O_3. It is also known that under the acidic conditions of the stomach, and possibly in the saliva, ascorbic acid can reduce nitrite to nitric oxide, thus regenerating the nitrosating agent (*33*−*35*). The full cycle involves reduction of nitrite to nitric oxide which is then oxidized to NO_2 and N_2O_3 which regenerates nitrite on hydrolysis (*27*).

Many studies have shown that nitric oxide reacts with molecular oxygen to yield a variety of reactive nitrogen species such as NO_2 and N_2O_3 (*36*). These species are potent nitrosating agents and may react with phenolics to form nitroso or nitrophenolics and with primary and secondary aromatic amines to form potentially mutagenic and carcinogenic nitrosamines or to cause deamination of DNA bases via diazotization of the amino group followed by hydrolysis (*8*, *9*). It is known that acidified nitrite can produce NO^+ and N_2O_3. The deamination of DNA bases by acidified nitrite may therefore share the same reaction mechanism as proposed previously for NO and its derived reactive nitrogen species, deamination proceeding through the following potential mechanism:

$$ArNH_2 + NO^+ \rightarrow ArN_2^+ + H_2O \rightarrow ArOH + N_2 + H^+$$

$$ArNH_2 + N_2O_3 \rightarrow ArN_2^+ + NO_2^- + H_2O \rightarrow ArOH + HNO_2 + N_2$$

The ability of plant phenolics to scavenge reactive nitrogen species may account for the protective effects of tea polyphenols against gastric cancer (*37*, *38*), and indeed, the studies of Kuenzig et al. (*39*) suggest that dietary hydroxycinnamates (constituents of fruit and grains) may play a role in the body's defense against

carcinogenesis by inhibiting the formation of *N*-nitroso compounds.

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