The suppression of the N-nitrosating reaction by chlorogenic acid

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N-Nitrosation of a model aromatic amine (2,3-diaminonaphthalene) by the N-nitrosating agent produced by nitrite in acidic solution was inhibited by a polyphenol, chlorogenic acid, which is an ester of caffeic acid and quinic acid. Caffeic acid also inhibited the N-nitrosation, but quinic acid did not. 1,2-Benzenediols and 3,4-dihydroxybenzoic acid had inhibitory activities. Chlorogenic acid, caffeic acid, 1,2-benzenediols and 3,4-dihydroxybenzoic acid were able to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl. Chlorogenic acid was

found to be nitrated by acidic nitrite. The kinetic studies and the nitration observed only by bubbling of nitric oxide plus nitrogen dioxide gases indicated that the nitrating agent was nitrogen sesquioxide. The observations showed that the mechanism by which chlorogenic acid inhibited N-nitrosation of 2,3-diaminonaphthalene is due to its ability to scavenge the nitrosating agent, nitrogen sesquioxide. Chlorogenic acid may be effective not only in protecting against oxidative damage but also in inhibiting potentially mutagenic and carcinogenic reactions *in vivo*.

INTRODUCTION

N-Nitrosamines have been implicated in human carcinogenesis. Although N-nitrosamines are detected in the environment and some foods, endogenous formation has been considered to be most important in the human cancers [1,2]. Endogenous N-nitrosamines are produced both by acid-catalysed nitrosation in the stomach (intragastric N-nitrosamine formation), and by nitrosation with nitric oxide (NO[•])-derived N-nitrosating agents produced by neutrophils and macrophages (extragastric N-nitrosamine formation) [3].

Precursors of N-nitrosamines are secondary amines (or alkylureas) and nitrite, which are taken into the stomach in food with saliva, and the acidity of gastric juice is appropriate for N-nitrosation [2]. Chronic inflammation of the colon and rectum is associated with an increased incidence in colorectal cancer [4]. Reactive intermediates of nitrogen released by leucocytes, which accumulate within the inflamed colonic interstitium, have recently been reported to be important mediators of mutagenesis and carcinogenesis [5–7]. Extravasated neutrophils and macrophages produce NO[•] via the L-arginine-dependent pathway [8–11]. NO[•] rapidly and spontaneously decomposes to yield potent N-

nitrosating agents such as nitrogen dioxide (2NO₂[•] ↔ N₂O₄) and nitrogen sesquioxide (N₂O₃) [12]. These nitrosating agents have been shown to be mutagenic and carcinogenic by virtue of their ability to nitrosate aliphatic and aromatic amines to yield N-nitrosamines.

Chlorogenic acid (3-caffeoyl-D-quinic acid; CGA) is an ester formed between caffeic acid and quinic acid (Figure 1). CGA and other polyphenol compounds are the major phenolic compounds found in many fruits such as apples, pears, peaches, plums, cherries and apricots [13]. CGA is a substrate of polyphenol oxidase which catalyses the oxidation of diphenols to corresponding o-quinones, which upon further reaction lead to brown pigments [14]. CGA has been reported to inhibit lipooxygenase activity in prostaglandin A metabolism [15] and retinoic acid 5,6-epoxidation [16]. CGA, as well as other polyphenolic compounds, are known to be antioxidants. CGA prevents haemolysis and lipid peroxidation of erythrocytes induced by H₂O₂ [17], and caffeic acid, CGA and related catechols suppress the formation of hydroxyl radical (•OH) via a Fenton-type reaction, probably due to chelation with iron ions [18]. Certain antioxidants, such as ascorbic acid, vitamin E and 5-amino-salicylic acid, inhibit acid-catalysed and leucocyte-mediated N-nitrosation reactions [8,19]. We studied the effect of CGA on the N-nitrosating reaction, and found that CGA modulates the potentially mutagenic reaction by scavenging a nitrosating agent, N₂O₃.

EXPERIMENTAL

Materials

CGA was the product of Tokyo Kasei (Tokyo, Japan). 2,3-Diaminonaphthalene (DAN) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NO[•] (1810 p.p.m.) and NO₂[•] (2115 p.p.m.) gases were obtained from Seitetsu Kagaku Co., Ltd. All other chemicals were of reagent grade.

Figure 1 Structure of chlorogenic acid

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Abbreviations used: CGA, chlorogenic acid; DAN, 2, 3-diaminonaphthalene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; *k_C*, rate constant for CGA and N₂O₃; *k_N*, rate constant for azide and N₂O₃.

Assay for nitrosamine formation

The nitrosating reaction was started by the addition of 20 mM sodium nitrite into the reaction mixture (final volume of 0.2 ml) containing 50 mM acetate buffer (pH 4.0), 0.2 mM DAN and various concentrations of CGA at room temperature. After a 30 min incubation, 1.8 ml of 50 mM potassium phosphate (pH 7.4) was added to each tube to stop the reaction. The nitrosation of DAN to yield its highly fluorescent N-nitrosated derivative, 2,3-naphthotriazole, was quantified by measuring the fluorescence of each sample using an excitation wavelength of 375 nm and an emission wavelength of 450 nm [20] with a Hitachi 204 fluorescence spectrophotometer.

Assay for scavenging activity against free radicals

The scavenging activity of CGA was assayed by measuring the decrease in absorbance at 517 nm of the stable free radical DPPH [21]. The purple-coloured free radical reacts with scavenger to yield the colourless product 1,1-diphenyl-2-picrylhydrazine.

Assay for nitration of CGA

The nitration was initiated by the addition of sodium nitrite into the reaction mixture which contained the buffered solution at pH 4.0 and polyphenol compounds at the indicated concentrations given in the legends of the Figures and Tables. The nitrated CGA was assayed by measuring the absorbance at 444 nm. Spectrophotometric measurements were performed with a Beckman DU 650 spectrophotometer.

Statistical analysis

Typical results were reported from at least three independent experiments and all data were expressed as an average of three independent determinations with S.E.M.

RESULTS

Inhibition of N-nitrosation of DAN by CGA

Nitrite decomposes in acidic air-saturated solution to generate reactive intermediates of nitrogen [22,23].

NO_2^+ , N_2O_3 and possibly $\text{N}_2\text{O}_4 (\leftrightarrow 2\text{NO}_2^+)$ are potent N-nitrosating agents [24] which are believed to cause the N-nitrosation of DAN to its corresponding fluorescent triazole. Figure 2 illustrates the effects of CGA, caffeic acid and quinic acid on the N-nitrosation of DAN. Caffeic acid and CGA were found to be very effective at inhibiting the nitrosation reaction. The concentrations of caffeic acid and CGA required for half inhibition were 0.25 ± 0.03 and 0.44 ± 0.04 mM respectively. Quinic acid was an ineffective inhibitor of this reaction. The replots of these results show that F/f is proportional to the concentration of inhibitor (Figure 2b), where f and F are the fluorescence intensities in the presence and absence of inhibitor respectively, consistent with the hypothesis that DAN and inhibitor compete for the nitrosating agent. The results also indicate that the reaction between the nitrosating agent and DAN is a simple bimolecular reaction, and that secondary reactions of either DAN or inhibitor are negligible.

Figure 2 Effects of CGA, caffeic acid and quinic acid on the N-nitrosation of DAN to yield 2,3-naphthotriazole

Reaction mixture contained 50 mM acetate buffer (pH 4.0), 20 mM NaNO_2 , 0.2 mM DAN and test compound at the indicated concentrations. The yield of 2,3-naphthotriazole was assayed as described in the Experimental section. A mean value of fluorescence in the absence of test compounds was designated as 100%. Data are transformed to give a plot, F/f versus [test compound], where F and f are the fluorescence intensities in the absence and presence of inhibitor respectively (b). The test compounds were quinic acid (line 1), CGA (line 2) and caffeic acid (line 3).

The hydroxyphenyl compounds having another hydroxyl group at the *ortho*-position, such as CGA, caffeic acid, 3,4-dihydroxybenzoic acid, dopamine and catechol, also inhibited triazole formation, but mono-hydroxyl compounds such as tyrosine, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid and salicylic acid had no ability to inhibit the N-nitrosation (Table 1). Trihydroxyl compounds, such as gallic acid and trihydroxybenzoic acid, had no effect. Neither compounds having a monocarboxyl group, such as histidine, lysine and benzoic acid (except for 3,4-dihydroxybenzoic acid), nor diol compounds, such as ribose and glucose, inhibited N-nitrosation.

Scavenging activity of CGA

Antioxidants have been shown to be potent inhibitors of the N-nitrosation reactions [6,19]. DPPH has been used to assess the scavenging properties of various compounds [21]. It was found

Table 1 Inhibition of N-nitrosation of DAN by CGA and its related compounds

The reaction mixture contained 50 mM acetate (pH 4.0), 0.2 mM DAN, 20 mM sodium nitrite and test compound at the indicated concentrations. After a 30 min incubation reaction, the mixture was diluted 10-fold, and then the yield of 2,3-naphthotriazole was assayed as described in the Experimental section. A mean value of fluorescence in the absence of test compounds was designated as 100%.

Additions	Concentration (mM)	Relative fluorescence (%)
None	—	100.0
CGA	0.2	66.0 ± 5.9
	0.5	43.5 ± 3.7
Caffeic acid	0.2	54.0 ± 3.2
	0.5	36.0 ± 2.5
Quinic acid	0.2	95.5 ± 1.9
	0.5	95.3 ± 2.8
Dopamine	0.2	77.3 ± 3.1
	0.5	56.0 ± 4.6
Catechol	0.2	75.3 ± 3.8
	0.5	56.0 ± 3.0
Serotonin	0.2	76.5 ± 5.4
	0.5	45.5 ± 1.7
2,4-Dihydroxybenzoic acid	0.2	98.5 ± 2.8
	0.5	94.0 ± 1.9
2,5-Dihydroxybenzoic acid	0.2	91.5 ± 2.4
	0.5	84.0 ± 3.1
2,6-Dihydroxybenzoic acid	0.2	95.0 ± 4.7
	0.5	86.0 ± 5.1
3,4-Dihydroxybenzoic acid	0.2	71.8 ± 3.0
	0.5	58.5 ± 2.7
3,5-Dihydroxybenzoic acid	0.2	99.5 ± 1.2
	0.5	97.5 ± 1.5
<i>p</i> -Hydroxyphenylacetic acid	0.5	98.3 ± 1.7
<i>p</i> -Hydroxybenzoic acid	0.5	94.5 ± 5.8
Salicylic acid	0.5	100.0 ± 2.0
Benzoic acid	0.5	97.8 ± 2.7
Gallic acid	0.5	86.5 ± 7.2
Tyrosine	0.5	98.0 ± 1.1
Histidine	0.5	98.0 ± 0.8
Lysine	0.5	98.0 ± 0.9
<i>o</i> -Ribose	0.5	97.0 ± 0.9
Glucose	0.5	92.5 ± 3.7

that only compounds having ability to inhibit N-nitrosation were able to scavenge the stable nitrogen-centred free radical DPPH (Figure 3, Table 2). Ohnishi et al. [17] reported the scavenging activities of CGA, caffeic acid, protocatechuic acid and 3,5-dicaffeoylquinic acid with both DPPH radical-scavenging and superoxide-mediated lipid peroxidation systems. The results indicate that inhibition of N-nitrosation is directly correlated with the scavenging (electron-donating) property for nitrogen-centred free radicals.

Nitration of CGA by nitrite in acidic solution

In order to clarify the reaction mechanism of CGA with N-nitrosating agents, the changes in the spectrum of CGA were recorded after the addition of nitrite in oxygen-saturated acidic solution. As shown in Figure 4(a), the spectrum of CGA was altered in a time-dependent manner upon the addition of nitrite. The observed peak in the visible region was at 444 nm. This spectrum is similar to those of 4-hydroxy-3-nitrophenylacetic acid, nitrotyrosine and peroxynitrite-modified superoxide dismutase which contains 3-nitrotyrosine [25–27]. We could not confirm the augmentation of absorbance at 444 nm in alkaline

Figure 3 The scavenging activities of CGA, caffeic acid or quinic acid towards DPPH radical

The reaction mixture contained 50 mM acetate buffer (pH 4.0), 0.1 mM DPPH and test compound at the indicated concentrations. The spectra were recorded just after the addition of test compound. The test compounds were caffeic acid (line 1), CGA (line 2) and quinic acid (line 3).

Table 2 Scavenging activity of CGA and its related compounds

Assay conditions were the same as in the legend for Figure 3.

Test compounds (50 μM)	ΔA_{517}
CGA	0.56 ± 0.04
Catechol	0.80 ± 0.07
<i>p</i> -Hydroxybenzoic acid	0
Salicylic acid	0
Benzoic acid	0
Protocatechuic acid	0.39 ± 0.02

solution, because CGA was rapidly autoxidized in alkaline solution and changed its colour to orange. The peak of product in the visible region disappeared on the addition of dithionite, which reduces the nitro-group of nitrotyrosine to a colourless amine [28]. The absorbance at 444 nm increased linearly with time up to a 15 min reaction time, and then the rate gradually decreased. After a 30 min reaction time, the increase of absorbance at 444 nm stopped, after which absorbance at 444 nm slowly decreased. The final product(s) after 24 h of reaction cannot be identified. Caffeic acid also reacted with N-nitrosating agents and resulted in a similar spectrum to that of CGA (Figure 4b). The absorbance peak was 472 nm. On the other hand, nitrate had no action on CGA and caffeic acid.

Nitration of CGA as a function of the concentration of nitrite

The rate of nitration was assayed by measuring the absorbance increase at 444 nm. The initial rate of CGA nitration increased with an increase in nitrite concentration (Figure 5). The data were transformed to give a plot of nitration rate versus [total nitrite]² (inset in Figure 5). The plot was linear, suggesting that the reacting species with CGA is N₂O₃, as discussed below.

Figure 4 Spectral changes of CGA (a) and caffeic acid (b) caused by the addition of sodium nitrite to the acidic solution

The reaction mixture contained 50 mM acetate buffer (pH 4.0), 20 mM sodium nitrite and 68 μ M CGA (a) or caffeic acid (b). The spectrum was recorded after 0 (line 1), 2 (line 2), 6 (line 3), 10 (line 4), 15 (line 5) and 30 min reaction (line 6) in the case of (a), and 0 (line 1), 0.5 (line 2), 2 (line 3), 5 (line 4) and 16 min reaction (line 5) in the case of (b).

CGA was nitrated by N_2O_3

From results that the nitration rate increased linearly with $[\text{nitrite}]^2$ (Figure 5), we examined the reaction of CGA with pure NO^* , NO_2^* , or NO^* plus NO_2^* gases. CGA in buffered solution at pH 4.5 was bubbled with each gas or their combination under anaerobic conditions, because NO^* reacts with molecular oxygen to form NO_2^* (eqn. 3). CGA was nitrated only when both NO^* and NO_2^* gases were bubbled through the reaction mixture (Figure 6). The spectrum of CGA was changed in a time-dependent manner. The spectrum of product was same as that obtained by the reaction of CGA with acidic nitrite (Figure 4a).

Figure 5 Nitration of CGA as a function of the concentration of sodium nitrite

The reaction mixture contained 50 mM acetate buffer (pH 4.0), 68 μ M CGA and sodium nitrite at the indicated concentrations. Inset: data are transformed to give a plot of nitration rate versus $[\text{sodium nitrite}]^2$.

Figure 6 The spectra of CGA after reactions with NO^* , NO_2^* and NO^* plus NO_2^* gases

N_2 (line 1), NO^* (line 2), NO_2^* (line 4), or NO^* plus NO_2^* (line 3) was bubbled at the rate of 100 ml/min through the reaction mixture containing 50 mM potassium phosphate (pH 4.5) and 73 μ M CGA. After reaction for 50 min the spectrum was recorded.

The absorbance at 444 nm increased with time. The absorbance increase did not stop during the observed period (90 min). NO_2^* was less reactive towards CGA.

Effect of pH on the nitration of CGA

The maximal rate of nitration of CGA (Figure 7) was observed at pH 3.4 which is the $\text{p}K_a$ value of nitrite. The results suggest

Figure 7 Effect of pH on the nitration of CGA

CGA at $68 \mu\text{M}$ was incubated with 20 mM sodium nitrite for 30 min in buffered solution at the indicated pH values. Glycine/HCl (pH 2–3.5), acetate (pH 4–5.5), potassium phosphate (pH 6–7.5) and glycine/NaOH (pH 8) buffers were used at 50 mM. The nitration in the presence (line 1) or absence (line 2) of sodium nitrite was measured.

that nitration seems to be dependent on the balance between the increase of non-ionized HNO_2 and the decrease of ionized CGA, as the pH drops. The $\text{p}K$ value of CGA is calculated to be 2.66 using $K = 2.2 \times 10^{-3}$ at 27°C which appears in the Merck Index.

Reaction rate constant between CGA and the nitrating agent N_2O_3

A competition assay is widely used to determine the reaction rate constant based on the known reaction rate of the reference substance. Since the results described above indicated that CGA and caffeic acid inhibited N-nitrosation in a simple linear manner (Figure 2b) and the nitrosating/nitrating agent was N_2O_3 (Figure 5), the rate constant (k_c) for the reaction between CGA and N_2O_3 can be calculated with the published rate constant (k_n) for the reaction of azide with N_2O_3 ($2 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$) [29]. In the present system, a nitrating agent, N_2O_3 , generated by acidic nitrite may react further in three ways: (1) the nitration of CGA

$$v = d[\text{CGA}]/dt = k_c[\text{CGA}][\text{N}_2\text{O}_3] \quad (5)$$

(2) the reaction of azide with N_2O_3

$$d[\text{N}_2\text{O}_3]/dt = k_n[\text{NaN}_3][\text{N}_2\text{O}_3] \quad (6)$$

and (3) the hydrolysis of N_2O_3 to form nitrite

$$d[\text{N}_2\text{O}_3]/dt = k_h[\text{N}_2\text{O}_3] \quad (7)$$

The hydrolysis of N_2O_3 is negligible, because this reaction is too slow to be relevant ($k_h = 1000 \text{ s}^{-1}$) [30]. Therefore the generation rate of N_2O_3 is given as follows:

$$V = -d[\text{N}_2\text{O}_3]/dt = k_c[\text{CGA}][\text{N}_2\text{O}_3] + k_n[\text{NaN}_3][\text{N}_2\text{O}_3] \quad (8)$$

In the absence of azide, the generation rate of N_2O_3 is proportional to the initial rate of CGA nitration. By dividing eqn. (8) by v , eqn. (9) is obtained.

$$V/v = 1 + k_n[\text{NaN}_3]/k_c[\text{CGA}] \quad (9)$$

Figure 8 Rate constant determination for the reaction between CGA and N_2O_3

CGA at 0.2 mM was incubated in 50 mM acetate buffer at pH 4.0 with different concentrations of sodium azide. The reactions were started by the addition of 5 mM sodium nitrite and the rates of nitration were measured immediately after. V and v indicate the initial nitration rates of CGA in the absence and the presence of sodium azide respectively.

The competitive inhibition of CGA nitration by azide is shown in Figure 8. From the competition of CGA with azide for N_2O_3 , the rate constant of reaction between CGA and N_2O_3 (k_c) is calculated to be $4.1 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$. Using this value and data in Figure 2, the rate constants of the reactions between DAN and N_2O_3 and between caffeic acid and N_2O_3 were calculated to be 8.6×10^9 and $6.9 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively.

DISCUSSION

Nitrite can react with secondary amines and N-substituted amides under the acidic conditions of the stomach to form N-nitrosamines and N-nitrosamides, collectively called N-nitroso compounds. Most N-nitroso compounds are carcinogenic. In this study, we showed that CGA and related polyphenols inhibited competitively the nitrosation reaction of DAN by a nitrosating agent produced by nitrite under acidic conditions. The intake of ascorbic acid or vitamin E is negatively correlated with cancer of several organs [31,32]. Although action of these compounds remains controversial, Block reported that free-radical scavengers, ascorbic acid and vitamin E, protected lipid membranes from peroxidation, and the homeostasis of the immune system could therefore be maintained [31,32]. 5-Aminosalicylic acid and other anti-inflammatory and immunosuppressive drugs are potent antioxidants [33] and are inhibitors of neutrophil-mediated N-nitrosation of DAN [8]. Grisham and Miles showed that the antioxidant (electron-donating) property correlated positively with the ability to inhibit N-nitrosation [34]. There are many reports on the antioxidant activity of the polyphenol compound CGA, although the detailed mechanism is still unknown. CGA prevents lipid peroxidation mediated by superoxide, peroxy radical, carbon tetrachloride and ^{60}Co -irradiation [17,35,36], on which the protective effect was comparable with that by Trolox, the water-soluble vitamin E [35]. CGA also prevents γ -radiation-induced chromosomal damage [37]. CGA and caffeic acid are more effective radical scavengers

than α -tocopherol and ascorbic acid are [17]. We confirmed that CGA inhibited the formation of the spin adduct of 5,5-dimethyl-1-pyrroline-*N*-oxide with $\cdot\text{OH}$ generated via a Fenton-type reaction, and can react with $\cdot\text{OH}$ generated by pulse radiolysis with a rate constant of 10^9 – $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ to form a different product from that formed by the reaction of CGA with N_2O_3 (Y. Kono and H. Shibata, unpublished work). Caffeic acid [18] and CGA are well known to form complexes with metal ions. Thus, CGA and related compounds could act as excellent antioxidants by not only scavenging free radicals but also by chelating heavy metal ions, such as iron and copper ions [18]. Polyphenol compounds, which have scavenging activity towards DPPH radicals (Figure 3 and Table 2), also showed an inhibitory effect on the N-nitrosating reaction (Figure 2 and Table 1), indicating the good relationship between the ability to scavenge free radicals and to inhibit N-nitrosation.

In this study the formation of nitrated CGA by the nitrosating agent was shown. The data obtained here indicate that the mechanism by which CGA inhibits N-nitrosation is due to its ability to scavenge or decompose the nitrosating agent. Using pure $\text{NO}^\cdot$ or $\text{NO}_2^\cdot$ gas, the nitrating (and/or nitrosating) agent was found to be N_2O_3 , which is easily formed by mixing of $\text{NO}^\cdot$ and $\text{NO}_2^\cdot$ (eqn. 4). $\text{NO}^\cdot$ had no ability to nitrate CGA, consistent with the view that $\text{NO}^\cdot$ itself is not a nitrosating agent. $\text{NO}_2^\cdot$ was able to nitrate CGA, but the yield of nitrated CGA was 19% of that obtained when both gases were combined. Nitration of aromatic molecules can be mediated by a free-radical mechanism involving addition of $\text{NO}_2^\cdot$ to phenoxyl radicals of aromatic molecules [38]. $\text{NO}_2^\cdot$ itself hardly reacts with aromatic molecules to form phenoxyl radicals, due to an extremely slow reaction. N_2O_3 could nitrate CGA by the following sequences of reactions.

Since eqn. (12) could be more rapid than eqn. (11), N_2O_3 formation becomes rate-limiting. When CGA is nitrated by N_2O_3 , the kinetics of nitration could follow eqn. (13).

$$\text{Rate} = k[\text{total CGA}][\text{total nitrite}]^2 \quad (13)$$

where [total CGA] and [total nitrite] indicate the molar concentrations of ionized CGA plus free CGA, and those of $\text{NO}_2^\cdot$ plus HNO_2 , respectively, and k , the stoichiometric rate constant, depends on pH. The linear relationship between the rate of CGA nitration and [total nitrite]² (Figure 5) indicates that the active nitration agent is N_2O_3 . Nitrosation of simple aliphatic secondary amines and certain amino acids by acidic nitrite have been shown to follow the same kinetics [2,39], showing that the nitrosating agent is also N_2O_3 . The kinetic data exclude the participation of the nitrosonium ion (H_2NO_2^+), another possible nitrosating agent, in the present nitration and/or nitrosation reaction.

Wink et al. suggested that the nitrating agents generated in the $\text{NO}^\cdot/\text{O}_2$ system are not one of the commonly proposed molecules, such as $\text{NO}_2^\cdot$, N_2O_3 , $\text{NO}^\cdot$ or $\text{OONO}^\cdot$ [40]. They used ferrocyanide and azide as the detector molecule of the nitrating agent and as the competitor respectively. In spite of their prediction that at 10 mM azide only 40% of the observed $\text{Fe}(\text{CN})_6^{3-}$ formation by the eqns. (3) and (4) should be quenched, complete quenching was observed. The complete inhibition of CGA nitration by azide was observed in our system. It has been reported, however, that $\text{NO}_2^\cdot$ is neither formed nor accumulated in quantities from $\text{NO}^\cdot$ in an oxygen-containing solution [41]. The extremely low concentration of $\text{NO}_2^\cdot$ formed in the $\text{NO}^\cdot/\text{O}_2$ system and the published second-order rate constants for the reactions of $\text{NO}_2^\cdot$

with $\text{NO}^\cdot$ ($1.1 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$) and $\text{Fe}(\text{CN})_6^{4-}$ ($3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) [30,42] lead us to conclude that the competitive inhibition of $\text{Fe}(\text{CN})_6^{3-}$ formation by azide observed by Wink et al. is due to its competition for N_2O_3 .

The nitration rate showed a maximum value at pH 3.4 (pK_a of HNO_2) (Figure 7). The balance between $[\text{CGA}^-]$ and $[\text{HNO}_2]$ may reflect this pH dependence. The effect of [nitrite] is predominant, because it is squared as in eqn. (13).

Serotonin was able to inhibit N-nitrosation (Table 1), in spite of the inability of mono-hydroxyl compounds to inhibit. Serotonin, and possibly dopamine, has a free amino group that can compete with DAN for N-nitrosation, as suggested by Wilcox et al. [43].

The present study shows that CGA and related polyphenol compounds are potent scavengers for nitrogen-centred free radicals and are inhibitors of the N-nitrosation reaction, suggesting that polyphenols may be effective not only in protection against oxidative damage but also in inhibiting the formation of potent mutagenic and carcinogenic N-nitroso compounds *in vivo*. CGA and related compounds are widely present in various agricultural products in substantial quantities, e.g. 3.4–14 mg/100 g fresh weight in several varieties of potatoes, 12–31 mg in 100 ml of apple juice, 89 mg/100 g of dry tea shoots and approx. 250 mg in a cup of coffee [13,44–47]. Thus, these polyphenol compounds are important in human health and may be useful as antioxidants and anti-cancer agents in drugs, food, toiletries and so on.

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