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Oxidation of Quercetin by Salivary Components. Quercetin-Dependent Reduction of Salivary Nitrite under Acidic Conditions Producing Nitric Oxide

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Under acidic conditions, nitrite is protonated to nitrous acid (pK_a = 3.2−3.4) that can be transformed into nitric oxide by self-decomposition and reduction. When sodium nitrite was mixed with quercetin at pH 1−2, quercetin was oxidized producing nitric oxide. In addition to quercetin, kaempferol and quercetin 4′-glucoside were also oxidized by nitrous acid, but oxidation of apigenin, luteolin, and rutin was slow compared to oxidation of the above flavonols. These results suggested that flavonols, which have a free hydroxyl group at carbon position 3, can readily reduce nitrous acid to nitric oxide. When the pH of saliva was decreased to 1−2, formation of nitric oxide was observed. The nitric oxide formation was enhanced by quercetin, and during this process quercetin was oxidized. These results indicate that there is a possibility of reactions between phenolics and nitrous acid derived from salivary nitrite in the stomach.

KEYWORDS: Formation of nitric oxide; nitrite (nitrous acid); oxidation of quercetin; reduction of nitrous acid; saliva

INTRODUCTION

Saliva contains various inorganic components including nitrite and nitrate, and the concentration of nitrite in saliva is between 50 and 200 μM (*1*, *2*). Nitrite in saliva is formed from nitrate (which is secreted as a salivary component from the salivary glands) by bacterial nitrate reductase-dependent reduction in the oral cavity (*2*). Nitrite can react with secondary amines producing carcinogenic nitroso compounds. The formation of nitroso compounds is possible under acidic conditions such as those in the stomach. On the other hand, it has been reported that under acidic conditions, nitrite is protonated to nitrous acid (pK_a = 3.2−3.4) and that nitrous acid decomposes to various nitrogen oxides (*3*, *4*). The decomposition products include nitric oxide, nitric dioxide, and nitrate. The formation of nitric oxide from nitrous acid in the stomach has been reported by Benjamin et al. (*3*) and Lundberg et al. (*5*), and Duncan et al. reported the same reaction in the oral cavity (*1*). The transformation of nitrous acid into nitric oxide in the oral cavity has been discussed in relation to the growth of periodontal disease pathogens (*6*, *7*) and the transformation in the stomach has been discussed in relation to infection by *Helicobactor pylori* (*8*, *9*) and bacteriostasis in the gastrointestinal tract (*10*). In addition, the physiological significance of the formation of nitric oxide in the stomach has been discussed in relation to regulation of mucosal blood flow (*11*, *12*), mucus formation (*13*), and gastric motility (*14*, *15*).

Vegetables and fruit contain flavonoids and other phenolics. When glycosides of the phenolics are ingested, the glycosides can be hydrolyzed to the aglycons during chewing by glycosidases secreted by the oral bacteria. Hirota et al. (*16*) reported that quercetin glucosides, which are contained in onion soup, are hydrolyzed to the aglycon quercetin in the oral cavity. As glycosides and aglycons of phenolics can function as reductants, and the redox potential to reduce nitrous acid to nitric oxide ($HNO_2 + H^+ + e^- \rightarrow NO + H_2O$) is 0.996 V (pH 0), it is possible that the phenolics reduce nitrous acid, producing nitric oxide when these phenolics are mixed with saliva and swallowed into the stomach where the pH is between 1 and 2. This paper deals mainly with quercetin-dependent reduction of nitrous acid to nitric oxide and the effects of quercetin on the formation of nitric oxide in saliva under acidic conditions. In addition, this report also deals with nitrous acid-dependent oxidation of some phenolics other than quercetin.

MATERIALS AND METHODS

Chemicals. Quercetin, rutin, luteolin, benzoic acids, cinnamic acids, and Griess−Romijn reagent for nitrite detection were obtained from Wako Pure Chemical Ind. (Osaka, Japan). Apigenin and kaempferol were from Sigma (St. Louis, MO). *N*-(Dithiocarboxy)sarcosine (DTCS) disodium salt was obtained from Dojin Laboratories (Kumamoto, Japan). Quercetin 4′-glucoside was prepared from onion scales as described previously by Hirota et al. (*17*).

Preparation of Saliva. Whole mixed saliva (5−10 mL) was collected from the authors by chewing Parafilm at about 9:00.

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Immediately after collection, the saliva was centrifuged at 20 000*g* for 5 min at 4 °C. The supernatant, pH of which was 7.5−7.8, was kept on ice and used for experiments as soon as possible. Nitrite in centrifuged saliva was measured with Griess−Romijn reagent. The mixture (1.0 mL) contained 0.1 mL of sample, 0.8 mL of 50 mM KCl−HCl buffer (pH 2.0), and 0.1 mL of Griess-Romijn reagent, which was dissolved in water at a concentration of 1% (w/v). After addition of sample, the mixture was incubated for 15 min at 35 °C and absorbance at 540 nm was measured.

Nitrite-Dependent Oxidation of Quercetin. Nitrite-dependent oxidation of quercetin was measured with a 557 double-beam spectrophotometer (Hitachi, Tokyo, Japan). The reaction mixture (1 mL) contained 50 μM quercetin and 100−500 μM $NaNO_2$ in 50 mM KCl−HCl buffer (pH 1−3.2). Reactions were started by adding $NaNO_2$. The oxidation of quercetin was measured by monitering the decrease in absoption at 360 nm. Nitrite-dependent oxidation of other phenolics was also measured spectrophotometrically in reaction mixtures (1 mL) that contained 50 μM phenolic and 0.2 mM $NaNO_2$ in 50 mM KCl−HCl (pH 2.0). The oxidation rates were estimated from the decreases in absorption and the absorption coefficients at the peak wavelengths.

When acid-dependent oxidation of quercetin was measured using centrifuged saliva, quercetin was added to the saliva (1.0 mL) to a final concentration of 50 μM. The pH of the salivary solution (initial pH 7.5−7.8) was deceased to 1−3 by adding 2.5 M HCl, and the solution was incubated for 1 min at 25 °C. During incubation, pH was determined with a glass electrode. Immediately after the incubation period, the acidic solution was extracted twice with 3 mL of ethyl acetate and the ethyl acetate extracts were combined. After evaporating ethyl acetate with a rotary evaporator, the residue was dissolved in methanol to quantify quercetin by HPLC (see below). Aliquots of 10 μL of the methanol solution were applied to an HPLC column. The time courses of acid-dependent oxidation of quercetin by centrifuged saliva were measured by adding 80 μL of 2.5 M HCl to 1.0 mL of centrifuged saliva containing 50 μM quercetin. The final pH was 1. After defined incubation periods, the mixtures were extracted twice with 3 mL of ethyl acetate. The methanol solution to quantify quercetin by HPLC was prepared as described above.

HPLC. HPLC was performed using an LC-10AS (Shimadzu, Kyoto, Japan) combined with a Shim-pack CLC-ODS column (6 mm i.d. × 15 cm; particle size 5 μm) (Shimadzu) and an injector (Rheodyne 7125) with a 100-μL sample loop. The volume of sample applied to the column was 10 μL. Quercetin separated by HPLC was detected with a spectrophotometric detector with a photodiode array (SPD-M10A, Shimadzu) at 360 nm. A mixture of methanol and 25 mM KH_2PO_4 (2:3, v/v) was used as the mobile phase and the flow rate was 1 mL min^{-1}.

Electron Spin Resonance (ESR) Spectra. ESR spectra were measured using a JEOL JES-FE1XG spectrometer at about 25 °C with a quartz flat cell (0.05 mL) under the following conditions: microwave power, 10 mW; line width, 0.2 mT; amplification, 2000-fold; scanning speed, 0.625 mT min^{-1}. Nitric oxide formed from $NaNO_2$ was trapped by Fe-$(DTCS)_2$ (*18*) as follows. The reaction mixture (0.5 mL) contained 50 μM quercetin and 0.2 mM $NaNO_2$ in 50 mM KCl−HCl buffer (pH 2). Reactions were started by adding $NaNO_2$. After defined incubation periods, 0.5 mL of a mixture containing 5 mM DTCS and 1.5 mM $FeCl_3$ in 0.1 M sodium phosphate buffer (pH 7.6) was added. The final pH was about 7. Nitric oxide formation was stopped and stable NO-Fe-$(DTCS)_2$ complex was formed at neutral pH.

When nitric oxide in saliva was measured, the reaction mixture contained 0.5 mL of centrifuged saliva with or without quercetin. Nitric oxide formation was started by adding 10 μL of 2.5 M HCl, which resulted in a decrease in salivary pH to 1.9. Immediately after addition of HCl, the mixture was mixed with a test tube shaker (1000 rpm, 5 s) and incubated for defined periods. After incubation, 0.5 mL of a mixture containing 5 mM DTCS and 1.5 mM $FeCl_3$ in 0.1 M sodium phosphate (pH 7.6) was added to the salivary solutions and stirred for 5 s as described above. An aliquot of 50 μL was withdrawn into the flat cell, and the ESR spectrum of NO−Fe−$(DTCS)_2$ complex was measured. The concentration of NO−Fe−$(DTCS)_2$ complex was estimated using 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy radical as a standard.

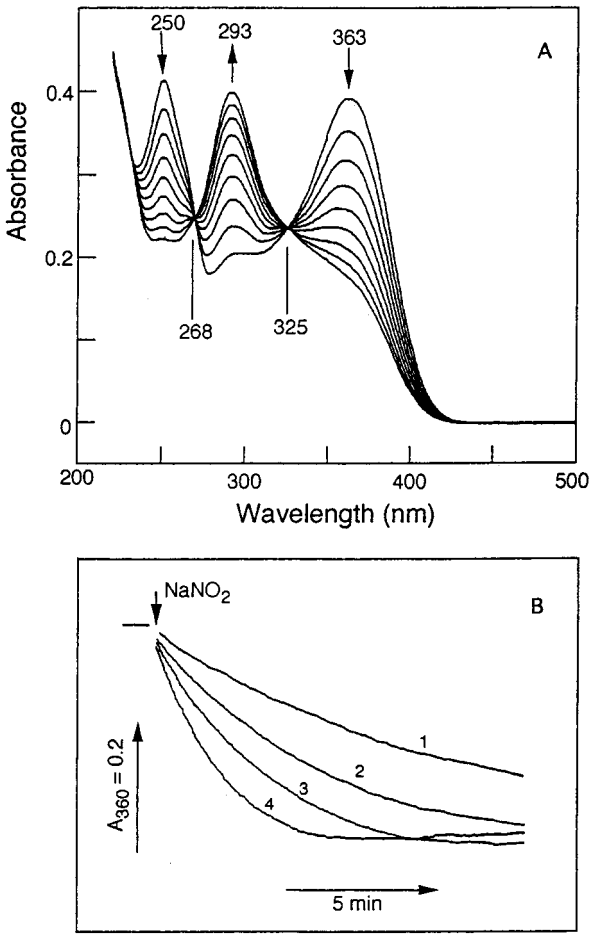

Figure 1. Oxidation of quercetin by nitrite. (A) Changes in absorption spectrum. Scanning was repeated every 1 min from 500 to 220 nm at 300 nm min^{-1}. The reaction mixture (1 mL) contained 50 μM quercetin and 0.1 mM $NaNO_2$ in 50 mM KCl–HCl (pH 2.1). Upward arrow, absorbance increase; downward arrows, absorbance decrease. (B) Time courses of oxidation of quercetin. The reaction mixture (1 mL) contained 50 μM quercetin and various concentrations of $NaNO_2$ in 50 mM KCl–HCl (pH 2.1). Trace 1, 0.1 mM; trace 2, 0.2 mM; trace 3, 0.3 mM; and trace 4, 0.5 mM $NaNO_2$. $NaNO_2$ was added where indicated. The length of the light path was 4 mm.

Data Presentation. Measurements were repeated at least three times and essentially the same results were obtained. Typical data or data of three measurements are presented.

RESULTS AND DISCUSSION

Oxidation of Quercetin by Nitrous Acid. Figure 1A shows changes in the absorption spectrum of 50 μM quercetin in 50 mM KCl−HCl buffer (pH 2.1) when 0.1 mM $NaNO_2$ was added. On addition, quercetin was oxidized, which was observed as decreases in absorbance at 250 and 363 nm and an increase in absorbance at 293 nm. Isosbestic points were observed at 268 and 325 nm, indicating that the oxidation products of quercetin are not readily transformed further under the conditions shown in **Figure 1A**. When 50 μM quercetin was oxidized by 0.3 mM $NaNO_2$, the isosbestic point at 325 nm was obscure, suggesting that the oxidation products were transformed further during incubation in the presence of high concentrations of $NaNO_2$. The transformation of the oxidation products of quercetin was supported by the results shown in **Figure 1B**: in the presence of 0.5 mM $NaNO_2$, absorbance at 360 nm increased after reaching the minimal absorbance (trace 4). The initial rate

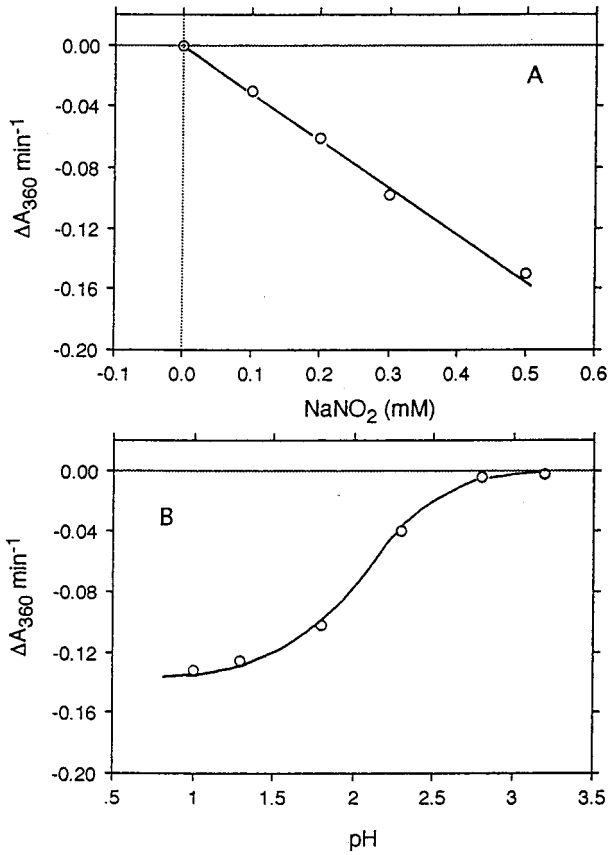

Figure 2. Effects of $NaNO_2$ concentration and pH on oxidation of quercetin. (A) Effect of $NaNO_2$ concentration. The reaction mixture (1 mL) contained 50 μM quercetin and various concentrations of $NaNO_2$ in 50 mM KCl–HCl (pH 2.0). (B) Effect of pH. The reaction mixture (1 mL) contained 50 μM quercetin and 0.2 mM $NaNO_2$ in 50 mM KCl–HCl at various pH values.

of oxidation of quercetin as a function of $NaNO_2$ concentration was nearly linear (**Figure 2A**). The oxidation of quercetin by 0.1 mM $NaNO_2$ was slow at pH 3.2, and the initial rate increased as pH was decreased (**Figure 2B**). The half-maximal rate of quercetin oxidation was observed at a pH value of about 2.

Nitric Oxide Formation by Nitrous Acid/Quercetin Systems. Figure 3A (inset) shows the ESR spectrum when nitric oxide, which was generated by the reaction between 50 μM quercetin and 0.2 mM nitrite at pH 2, was trapped by Fe−(DTCS)$_2$ complex. The signal was not observed in the absence of 0.2 mM $NaNO_2$. A small signal was observed in the presence of 0.2 mM $NaNO_2$ but the absence of quercetin. No nitric oxide was detected in the reaction mixture of 50 μM quercetin and 0.2 mM $NaNO_2$ at pH 7. These results indicated that in the absence of quercetin, nitric oxide is produced from nitrous acid but not from nitrite by the reactions suggested by Benjamin et al. (*3*) and McKnight et al. (*4*).

$$3NHO_2 \rightarrow H_2O + 2NO + NO_3^- \quad (1)$$

$$2HNO_2 \rightarrow H_2O + N_2O_3 \quad (2)$$

$$N_2O_3 \rightarrow NO + NO_2 \quad (3)$$

The observation that nitrite oxidized quercetin under acidic conditions (**Figures 1** and **2**) accompanying the generation of nitric oxide (**Figure 3A**) indicated that the following reaction for the formation of nitric oxide is also possible:

$$QH_2 + HNO_2 \rightarrow QH\bullet + NO + H_2O \quad (4)$$

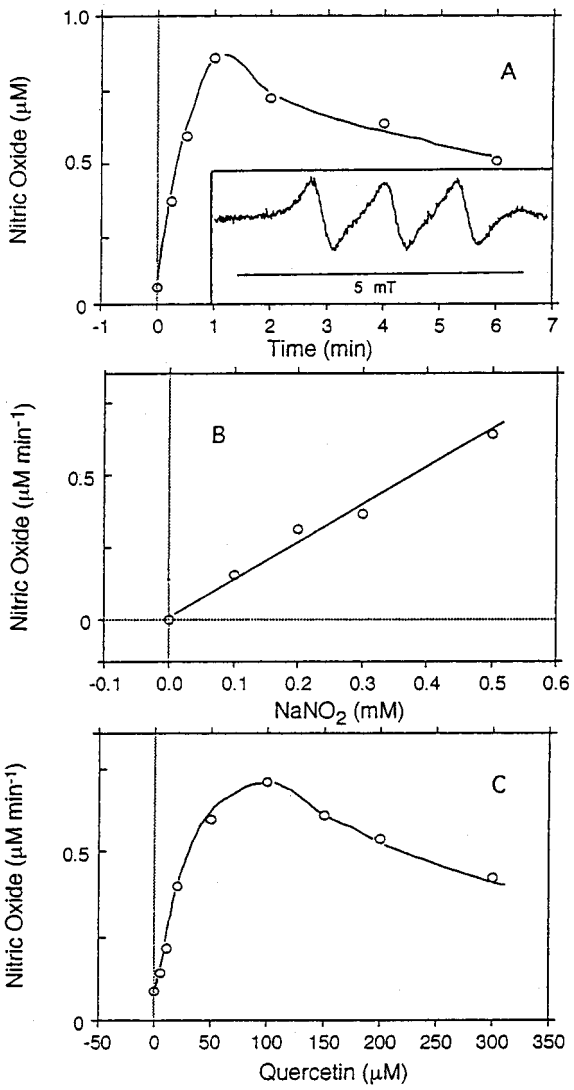

Figure 3. Nitric oxide formation by nitrous acid/quercetin system. (A) Time course of changes in the concentration of nitric acid. The reaction mixture (0.5 mL) contained 50 μM quercetin and 0.2 mM $NaNO_2$ in 50 mM KCl–HCl buffer (pH 2.0). After defined incubation periods, 0.5 mL of Fe–(DTCS)$_2$ complex was added. The inset shows a typical ESR spectrum of NO–Fe–(DTCS)$_2$. (B) Effects of $NaNO_2$ concentration on the formation of nitric oxide. The reaction mixture (0.5 mL) contained 50 μM quercetin and various concentrations of $NaNO_2$ in 50 mM KCl–HCl buffer (pH 2.0). After incubation for 15 s, 0.5 mL of Fe–(DTCS)$_2$ complex was added. (C) Effects of quercetin concentration on the formation of nitric oxide. The reaction mixture (0.5 mL) contained various concentrations of quercetin and 0.2 mM $NaNO_2$ in 50 mM KCl–HCl buffer (pH 2.0). After incubation for 30 s, 0.5 mL of Fe–(DTCS)$_2$ complex was added. In all experiments, reactions were started by the addition of $NaNO_2$.

where QH_2 is quercetin and QH• is quercetin radical. QH• may be transformed further to the oxidation products such as 3,4-dihydroxybenzoic acid and 2,4,6-trihydroxyohenylglyoxylic acid (*19*, *20*). The reduction of nitrous acid by redox reactions as shown in eq 4 was supported by the previous reports that ascorbic acid reduces nitrous acid producing nitric oxide (*1*, *21*). According to the above reactions, reduction of nitrous acid by quercetin competes with self-decomposition of nitrous acid; therefore, quercetin can inhibit the reaction in eq 2 suppressing the formation of N_2O_3, which is a nitroso reagent (*22*).

The concentration of nitric oxide was increased during incubation of sodium nitrite with quercetin at pH 2.0 when the

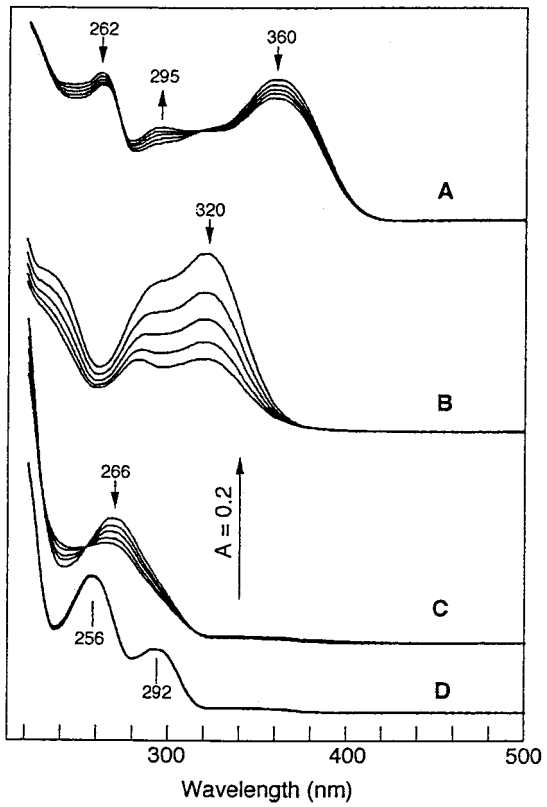

Figure 4. Nitrous acid-dependent oxidation of phenolics. The reaction mixture (1 mL) contained 50 μM phenolic and 0.2 mM $NaNO_2$ in 50 mM KCl–HCl (pH 2.0). Scanning was repeated every 2.6 min from 500 to 220 nm at 120 nm min^{-1}. A, kaempferol; B, caffeic acid; C, 3,4,5-trihydroxybenzoic acid (gallic acid); D, 3,4-dihydroxybenzoic acid. Upward arrow, absorbance increase; downward arrows, absorbance decrease. The length of the light path was 4 mm.

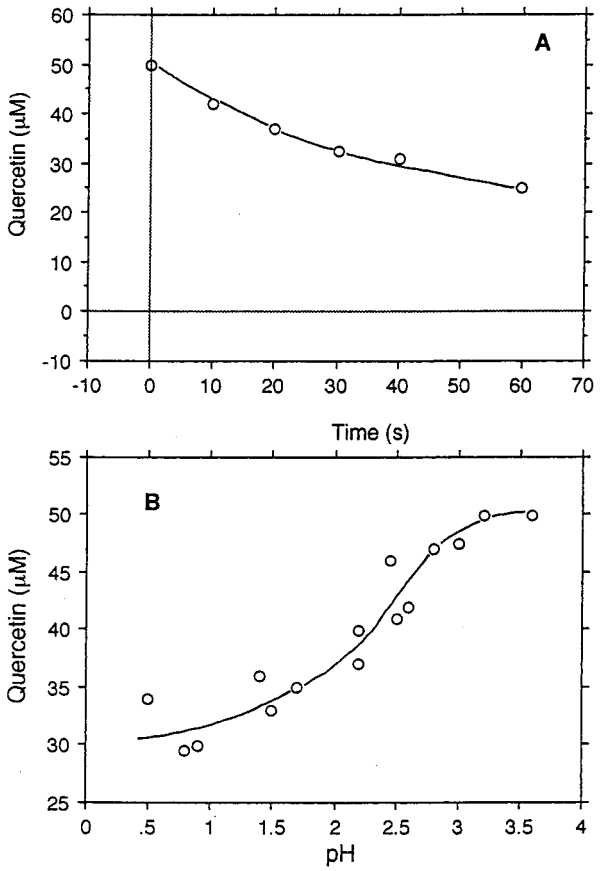

Figure 5. Oxidation of quercetin by saliva under acidic conditions. The reaction mixture (1 mL) contained 1 mL of centrifuged saliva and 50 μM quercetin. (A) Typical time course of oxidation of quercetin. The mixture was incubated for defined periods at pH 1 and extracted with ethyl acetate to quantify quercetin. (B) Effects of pH on quercetin oxidation. After incubation of the mixture for 1 min at various pH values, quercetin was extracted by ethyl acetate to allow quantification. Data from three different saliva preparations are presented.

incubation period was shorter than 1 min, and then decreased slowly (**Figure 3A**). The decrease in concentration of nitric oxide may be due to the decrease in rate of nitric oxide formation by the decreases in concentrations of quercetin and nitrous acid. The decrease in concentration of quercetin during incubation is shown in **Figures 1** and **2**. The decrease in concentration of nitrous acid is also possible by the reactions in eqs 1, 2, and 4, and this was confirmed by measuring the changes in concentration of nitrite during incubation (data not shown). The initial rate of nitric oxide formation was roughly proportional to the concentration of $NaNO_2$ (**Figure 3B**) similarly to the oxidation rate of quercetin as a function of $NaNO_2$ concentration (**Figure 2A**). When the rate of formation of nitric oxide was plotted against the concentration of quercetin (**Figure 3C**), the rate reached a maximum at 100 μM quercetin and then decreased. The decrease in the level of nitric oxide at higher concentrations of quercetin may be due to the reaction of quercetin with nitric oxide. Reactions between phenolics and nitric oxide have been reported by Paquay et al. (*23*) and van Acker et al. (*24*). The possibility of reaction between quercetin radicals and nitric oxide cannot be excluded because nitric oxide, in general, reacts readily with free radicals (*25*).

Oxidation of Phenolics by Nitrous Acid. As vegetables and fruits contain various phenolics, we studied the oxidation of some flavonoids, cinnamic acids, and phenolic acids (50 μM) by 0.2 mM $NaNO_2$ in 50 mM KCl−HCl (pH 2.0) spectrophotometrically. The oxidation rates of kaempferol (**Figure 4A**) and quercetin 4′-glucoside were estimated to be 0.96 and 1.0 μM min^{-1}, respectively, whereas the rates of rutin (quercetin 3-rutinoside), apigenin, and luteolin were below 0.2 μM min^{-1}. The oxidation rate of quercetin was estimated to be 6.2 μM min^{-1}. These results suggested that the hydroxyl group at carbon position 3 of flavonols can be readily attacked by nitrous acid. Such flavonols also have potent antioxidative activities (*26*). $NaNO_2$-dependent changes in absorbance of caffeic acid (**Figure 4B**) were much faster than those of *p*-coumaric acid, suggesting that caffeic acid is an effective reductant for nitrous acid. The oxidation rate of caffeic acid was estimated to be 4.1 μM min^{-1}. Oxidation of 4-hydroxybenzoic and 3,4-dihydroxybenzoic (**Figure 4D**) acids was not clearly detected, but the oxidation of 3,4,5-trihydroxybenzoic acid (gallic acid) (**Figure 4C**) was observed by an increase and decrease in absorbance at 240 and 266 nm, respectively, with an isosbestic point at 254 nm. The oxidation rate was estimated to be 1.1 μM min^{-1}. These results indicated that not all phenolics can readily reduce nitrous acid to nitric oxide.

Oxidation of Quercetin by Acidified Saliva. When the pH of centrifuged saliva was decreased to 1−3 in the presence of quercetin, quercetin was oxidized. **Figure 5A** shows the typical time course of quercetin (50 μM) oxidation by centrifuged saliva at pH 1. The oxidation rates increased as pH was decreased, and the half-maximal oxidation rate was observed at a pH of about 2 (**Figure 5B**). The results shown in **Figure 5** indicate that salivary components participate in the oxidation of quercetin under acidic conditions. One such component may be nitrite

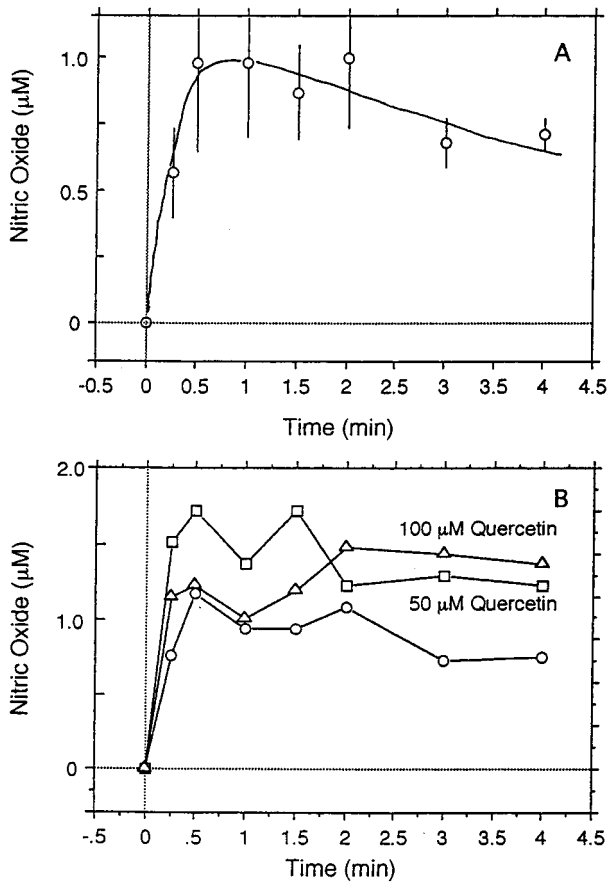

Figure 6. Nitric oxide formation by saliva under acidic conditions. The reaction mixture (0.5 mL) contained centrifuged saliva. Formation of nitric oxide was started by decreasing the pH to 1.9, and after defined incubation periods 0.5 mL of Fe–$(DTCS)_2$ complex was added to measure the ESR spectra of NO–Fe–$(DTCS)_2$. (A) Time course of nitric oxide formation in the absence of quercetin. Values are means ± SD ($n = 3$ or 4). (B) Effects of quercetin on nitric oxide formation. Centrifuged saliva containing quercetin was incubated for defined periods at pH 1.9 and ESR spectra were measured. Circles (○), no addition; squares (□), 50 μM quercetin; triangles (△), 100 μM quercetin.

because the pH value for the half-maximal rate of oxidation of quercetin by centrifuged saliva (**Figure 5B**) was similar to that for $NaNO_2$-dependent oxidation of quercetin (**Figure 2**). The presence of nitrite in saliva preparations used in this study (0.25 ± 0.05 mM, $n = 4$) also supported the participation of nitrite in the oxidation of quercetin by centrifuged saliva at low pH. In addition, the concentration of nitrite decreased when saliva was incubated in the absence of quercetin and the decrease was enhanced by quercetin (data not shown). This result further supported the reaction between nitrous acid and quercetin in acidified saliva. The half-maximal rate of nitrite-dependent nitric oxide formation has been reported to be observed around pH 2 (*1*, *4*).

Nitric Oxide Formation by Acidified Saliva. Nitric oxide was formed in centrifuged saliva itself under acidic conditions (**Figure 6A**). The amount of nitric oxide formed was dependent on saliva preparations especially during the initial period of incubation. As nitric oxide can be formed by self-decomposition and reduction of nitrous acid as described above, the variation can be attributed to the differences in concentrations of nitrite and reductants in saliva preparations. Sodium thiocyanate, which is found in saliva at 0.5−2 mM (*2*), may also contribute to the formation of nitric oxide, as the mixture of 1 mM NaSCN and 0.2 mM $NaNO_2$ in 50 mM KCl−HCl (pH 2.0) produced nitric oxide. The time course of the change in concentration of NO−Fe−$(DTCS)_2$ (**Figure 6A**) showed that nitric oxide was formed rapidly with the decrease in pH and that the concentration of nitric oxide decreased slowly after attaining a maximal level (around 1 min after the decrease in pH). When Fe−$(DTCS)_2$ complex was added to centrifuged saliva, pH of which was 7.5, no ESR signal was detected. Precise experiments are required to detect nitric oxide in saliva under physiological conditions using Fe−$(DTCS)_2$ complex because there have been reports on salivary nitric oxide measuring concentrations of nitrite in saliva (*27*−*29*).

The effects of quercetin on nitric oxide formation are shown in **Figure 6B**. As the variation of nitric oxide formation in the control saliva (without quercetin) was large as shown above, typical data are presented. Although the effects of 50 and 100 μM quercetin on the time course of nitric oxide formation were different, the flavonol enhanced the formation of nitric oxide. The stimulation of nitric oxide formation by quercetin was independent of saliva preparations used in this study, and the stimulation by quercetin can be explained by quercetin-dependent reduction of nitrous acid as quercetin was oxidized by nitrous acid (**Figures 1** and **2**) and by centrifuged saliva under acidic conditions (**Figure 5**).

CONCLUDING REMARKS

The results of the present study indicate that some phenolics, including quercetin, can reduce nitrous acid, producing nitric oxide. Lundberg et al. (*5*) reported the formation of nitric oxide in chewed lettuce when the pH was decreased to 1, and Sobala et al. (*21*) reported the enhancement of nitric oxide formation by ascorbic acid in the stomach. As lettuce contains ascorbate and various phenolic compounds, it is a possible that phenolics as well as ascorbic acid stimulate the formation of nitric oxide in the stomach when lettuce is ingested. The increase in concentration of nitric oxide by some phenolics indicates that nitric oxide formed by reactions between phenolics and nitrous acid is not effectively scavenged by the phenolics, although there have been reports on the reaction between nitric oxide and phenolics (*23*, *24*). In addition to nitric oxide, NO_2 and N_2O_3 can be formed from nitrous acid and nitric oxide (*30*). Peroxynitric acid may also be formed by the reaction between nitric oxide and HO_2 when HO_2 is formed in the stomach. Phenolics may be able to scavenge these reactive nitrogen species directly or indirectly, as some phenolics can inhibit nitrous acid (*30*, *31*)- and ONOOH (*32*)-dependent nitration of tyrosine. A salivary phenolic component, 4-hydroxyphenylacetic acid, may also contribute to the scavenging of reactive nitrogen species in the stomach (*33*). Therefore, we deduced that the concentrations of reactive nitrogen species such as NO_2, N_2O_3, and ONOOH might not be elevated even if phenolics in foods stimulated the formation of nitric oxide from nitrous acid in the stomach. As nitric oxide can regulate gastric mucosal blood flow, resulting in preservation of mucosal integrity, mucus production, and gastric motility as described in the Introduction, if adequate amounts of phenolics that have quercetin-like activity are ingested it is possible that the phenolics may participate in the improvement of gastric motility scavenging reactive nitrogen species.

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