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Interaction between Ascorbic Acid and Chlorogenic Acid during the Formation of Nitric Oxide in Acidified Saliva

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When saliva and gastric juice are mixed, salivary nitrite is transformed to nitrous acid to produce nitric oxide (NO). The NO formation in acidified saliva was enhanced by ascorbic acid and chlorogenic acid. Thiocyanate ion (SCN^-) also enhanced the transformation of nitrous acid to NO. During the NO formation in the presence of both ascorbic acid and chlorogenic acid, ascorbic acid was preferentially oxidized. Chlorogenic acid was oxidized after ascorbic acid had been oxidized. Ascorbyl radical was detected during the oxidation of ascorbic acid, and the radical intensity was decreased by chlorogenic acid. The decrease is discussed to be due to the reduction of the oxidation intermediate or product of chlorogenic acid by ascorbyl radical. The result obtained in this study suggests that ascorbic acid was preferentially oxidized and that not only ascorbic acid but also ascorbyl radical could interact with the oxidation intermediate or product of chlorogenic acid when chlorogenic acid was added to the mixture of saliva and gastric juice that contained ascorbic acid.

KEYWORDS: Ascorbic acid; chlorogenic acid; free radicals; nitric oxide; nitrous acid; SCN^-

INTRODUCTION

When saliva is mixed with gastric juice, salivary nitrite (normal concentration, 0.05−0.3 mM) is transformed to nitrous acid. Ascorbic acid, a component of gastric juice, reduces the nitrous acid ($pK_a = 3.3$) to nitric oxide (NO) (*1*−*3*). In addition, phenolics like quercetin, caffeic acid, and chlorogenic acid, which are contained in foods and beverages, can also reduce nitrous acid to NO (*4*−*7*). The function of NO produced in the gastric juice has been discussed in relation to the activity of stomach (*8*−*11*) and the inhibition of the growth of microorganisms (*12, 13*). If NO is produced under aerobic conditions, NO is oxidized by O_2, producing NO_2 that can be transformed to N_2O_3 by reacting with NO. The formation of NO_2 and N_2O_3 from NO has been proposed in the gastric lumen (*14*).

When freeze-dried instant coffee is incubated with nitrite at pH 2, simulating the reactions in the gastric juice, chlorogenic acid in the coffee is transformed to the quinone form and the nitrated compounds producing NO in the absence of thiocyanate ion (SCN^-) (*15*). The quinone form of chlorogenic acid is able to react with a compound that has an SH group (*16, 17*). A salivary component SCN^- ($pK_a = -1.28$) inhibits the formation of the quinone form and the nitrated compounds. During the inhibition, a stable component (*E*)-5′-{3-(7-hydroxy-2-oxobenzo[*d*][1,3]oxathiol-4-yl)acryloyloxy}quinic acid is produced via 2-thiocyanatechlorogenic acid (*15*). The formation of the above stable compound is also observed when coffee and saliva, which contains SCN^-, are mixed under acidic conditions, simulating the mixture of coffee, saliva, and gastric juice. As ascorbic acid, the concentration of which is about 0.05 mM in the gastric juice (*18, 19*), can reduce nitrous acid in the stomach, it is important to understand the interaction between ascorbic acid and *o*-diphenols during the metabolism of nitrous acid in acidified saliva. It is known that ascorbic acid can reduce phenoxyl radicals and quinone forms of phenolics producing the original compounds (*3, 20*) and that ascorbic acid may be able to reduce nitrosyl thiocyanate (ONSCN), which is formed by the reaction between nitrous acid and SCN^-, producing NO (*21, 22*).

Although, at present, the reduction of nitrous acid to NO by chlorogenic acid and ascorbic acid in acidified saliva has been reported (*15, 22*), the interactions between ascorbic acid and chlorogenic acid during the reduction of nitrous acid by the two acids have not been reported. An objective of the present study is the elucidation of the interaction between ascorbic and chlorogenic acids during nitrite-dependent formation of NO in acidified saliva.

MATERIALS AND METHODS

Chemicals. *N*-(Dithiocarboxy)sarcosine sodium salt (DTCS) was obtained from Dojin (Kumamoto, Japan). Chlorogenic acid was from Wako Pure Chem. Co. (Osaka, Japan). This reagent was dissolved in dimethyl sulfoxide at a concentration of 20 mM.

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Preparation of Saliva. Mixed whole saliva (about 10 mL) was collected from volunteers by chewing parafilm at 9−10 o'clock in the morning when required after informed consent had been obtained. The collected saliva was passed through two layers of nylon filter nets [380 mesh (32 μm) net, Sansho, Tokyo, Japan] to remove epithelial cells and other particles. The filtrate was used as saliva. The concentrations of nitrite and SCN^- of saliva were determined using Griess−Romijn reagent and Fe(III), respectively (*22*). The concentration of nitrite was normally 0.05−0.3 mM, and the concentration increased to about 1 mM when nitrate-rich food was ingested (*23*). The concentration of SCN^- was 1.10 ± 0.86 [mean ± standard deviation (SD)] mM (*24*).

Oxygen Uptake. Nitrite-induced O_2 uptake was measured with a Clark type O_2 electrode (Rank Brothers, Cambridge, United Kingdom) at 25 °C. The reaction mixture contained 1 mL of saliva and 0.4−0.5 mL of 50 mM KH_2PO_4−50 mM KCl−HCl (pH 1.36). The pH of the reaction mixture was adjusted to 2.0 by changing the volume of 50 mM KH_2PO_4−50 mM KCl−HCl (pH 1.36). When required, $NaNO_2$, ascorbic acid, and chlorogenic acid were added. Oxygen uptake was also measured using a buffer solution, and the reaction mixture (2 mL) contained 1 mM $NaNO_2$ in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2.0). Ascorbic acid and chlorogenic acid were added within 1 min after the preparation of acidified saliva. When O_2 uptake was measured in the acidic buffer solution, reactions were started by the addition of nitrite.

Spectrophotometric Studies. Absorption changes in acidified saliva were measured at about 25 °C using an UV/vis spectrophotometer (557, Hitachi, Tokyo, Japan). The reaction mixture contained 0.8 mL of saliva and 0.36−0.4 mL of 50 mM KH_2PO_4−50 mM KCl−HCl (pH 1.36) (final about pH 2). Oxidation of ascorbic acid was measured by the absorption decrease at 250 nm. This wavelength was determined from the absorption spectra of ascorbic acid in 50 mM KCl−HCl (pH 2.0) (λ_{max} = 240 nm) and acidic reaction mixture without ascorbic acid (λ_{min} = 250 nm). Oxidation of chlorogenic acid was measured by the absorption decrease at 320 nm. Oxidation of ascorbic acid and chlorogenic acid was also measured in 50 mM KCl−HCl (pH 2.0) using an UV/vis spectrophotometer (UV-260, Shimadzu, Kyoto, Japan). The two acids were oxidized by $NaNO_2$ in 50 mM KCl−HCl (pH 2.0). The light path length of the measuring beam was 2 or 4 mm. Ascorbic acid and chlorogenic acid were added within 1 min after the preparation of acidified saliva. When absorption changes were measured in the acidic buffer solution, ascorbic acid and/or chlorogenic acid were added within 30 s after the preparation of reaction mixtures.

The formation of ONSCN was measured using the UV-260 spectrophotometer described above. The reaction mixture (3 mL) contained 10 mM $NaNO_2$ and 10 mM NaSCN in 50 mM KCl−HCl (pH 1.36 and 2.00). Immediately after the preparation of reaction mixture, absorption spectra from 600 to 400 nm were measured. The light path length of the measuring beam was 1 cm, and the scanning speed was 500 nm/min.

Measurements of Electron Spin Resonance (ESR) Spectra. ESR spectra were measured with a JE1XG spectrometer (JEOL, Tokyo, Japan) at about 25 °C using a quartz flat cell (0.05 mL). NO produced was measured with a spin-trapping reagent $Fe(DTCS)_2$ (*15*). The conditions for the measurement were as follows: microwave power, 10 mW; line width, 0.5 mT; amplification, 500-fold; and scanning speed, 5.0 mT/min. The reaction mixture contained 0.2 mL of saliva preparation and 0.08−0.09 mL of 50 mM KH_2PO_4−50 mM KCl−HCl (pH 1.36). After incubation for 30 s under aerobic conditions or by replacing air with argon gas, 0.28−0.29 mL of the solution, which contained 10 mM DTSC and 3 mM $FeCl_3$ in 50 mM sodium phosphate (pH 7.6) [in the following, $Fe(DTCS)_2$], was added to measure a stable radical NO-$Fe(DTCS)_2$ (*25*) in the quartz flat cell. When the formation of NO was measured in a buffer solution, the reaction mixture (0.20 mL) contained 1 mM $NaNO_2$ and 0.2 mM chlorogenic acid, 0.4 mM ascorbic acid, or 1 mM NaSCN in 50 mM KCl−HCl (pH 2.0). After incubation for 30 s under aerobic conditions or by replacing air with argon gas, 0.20 mL of $Fe(DTCS)_2$ was added to measure ESR spectra. The pH after the addition of $Fe(DTCS)_2$ was 7.2−7.4.

Ascorbyl radical was also measured using the ESR spectrometer. The conditions for the measurement were as follows: microwave power, 5 mW; line width, 0.1 mT; amplification, 2000-fold; and scanning speed, 2.5 mT/min. The pH of saliva was decreased to 2 by adding 0.12 mL of 50 mM KCl−50 mM KH_2PO_4−HCl (pH 1.36) to 0.3 mL of saliva. The concentration of ascorbic acid in the mixture was adjusted to 0.47 mM. When acidic buffer solution was used, the reaction mixture (0.5 mL) contained 0.4 mM ascorbic acid and 0.2 mM $NaNO_2$ in 50 mM KCl−HCl (pH 2.0). Immediately after the preparation of the reaction mixtures, an aliquot was withdrawn into the quartz flat cell to measure ESR spectra. It took about 1.5 min to start the measurement of ESR spectra after the preparation of the reaction mixture.

RESULTS

Detection of NO. Typical ESR spectra of NO-$Fe(DTCS)_2$ with three peaks were observed by the addition of $Fe(DTCS)_2$ to acidified saliva (pH 2; nitrite, 0.09 mM) (**Figure 1**, traces in **top** part). Chlorogenic acid (0.1 mM) and ascorbic acid (0.1 mM) increased the signal intensity by about 2- and 5-fold, respectively (traces B and C, respectively). The addition of both ascorbic acid and chlorogenic acid resulted in the further increase in the signal intensity (trace D). This result suggests that ascorbic acid and chlorogenic acid reduced nitrous acid to NO in acidified saliva. No changes in the intensity of the signal of NO-$Fe(DTCS)_2$ were observed during incubation after the addition of $Fe(DTCS)_2$ to acidified saliva. This result indicates that $Fe(DTCS)_2$ did not react with salivary nitrite and SCN^- around neutral pH values.

When 0.2 mL of $Fe(DTCS)_2$ containing 1 mM nitrite (pH 7.6) was mixed with 0.2 mL of 50 mM KCl−HCl (pH 2.0), no formation of NO-$Fe(DTCS)_2$ was observed. This result suggests that $Fe(DTCS)_2$ did not react with nitrous acid that might be produced transiently by the mixing. No formation of NO-$Fe(DTCS)_2$ was observed when 0.2 mL of 50 mM KCl−HCl (pH 2.0), which contained a salivary component SCN^- (1 mM), was mixed with 0.2 mL of $Fe(DTSC)_2$ containing 1 mM nitrite (pH 7.6). This result indicates that ONSCN, which might be formed transiently on the mixing, did not contribute to the formation of NO-$Fe(DTCS)_2$. ESR signals of NO-$Fe(DTCS)_2$ were observed when $Fe(DTCS)_2$ was added to 50 mM KCl−HCl (pH 2.0) with nitrite (**Figure 1**, trace A in the **middle** part). **Figure 1** (**bottom** part, closed circles) shows the time course of the formation of NO-$Fe(DTCS)_2$ when $Fe(DTCS)_2$ was added to the acidic buffer solution of nitrite at defined periods after the preparation of the solution. The result indicates that the concentration of NO increased slowly attaining to a constant value. In addition to chlorogenic acid (trace C) and ascorbic acid (trace D), SCN^- (trace B) also increased the signal intensity of NO-$Fe(DTCS)_2$ (**Figure 1**, **middle** part). Then, the time course of the formation of NO in acidic nitrite solution was also measured in the presence of SCN^- (**Figure 1**, **bottom** part, open circles). The ESR signal intensity due to the formation of NO-$Fe(DTCS)_2$ increased as a function of incubation period, supporting SCN^--dependent reduction of nitrous acid to NO. No changes in the intensity of the signal of NO-$Fe(DTCS)_2$ were observed during incubation after the addition of $Fe(DTCS)_2$ to acidic nitrite solution in the presence of the above chemicals. Replacement of air with argon gas resulted in the enhanced generation of the ESR signal. The enhancement in the presence of 1 mM nitrite was about 1.2-fold, and the enhancements were about 1.2-, 2.7-, and 6.2-fold when 1 mM SCN^-, 0.2 mM chlorogenic acid, and 0.4 mM ascorbic acid were added, respectively, in the presence of 1 mM nitrite. The above results indicate that $Fe(DTCS)_2$ was reacted with NO that had been formed in acidic reaction mixtures and suggests that the NO generated was oxidized by O_2.

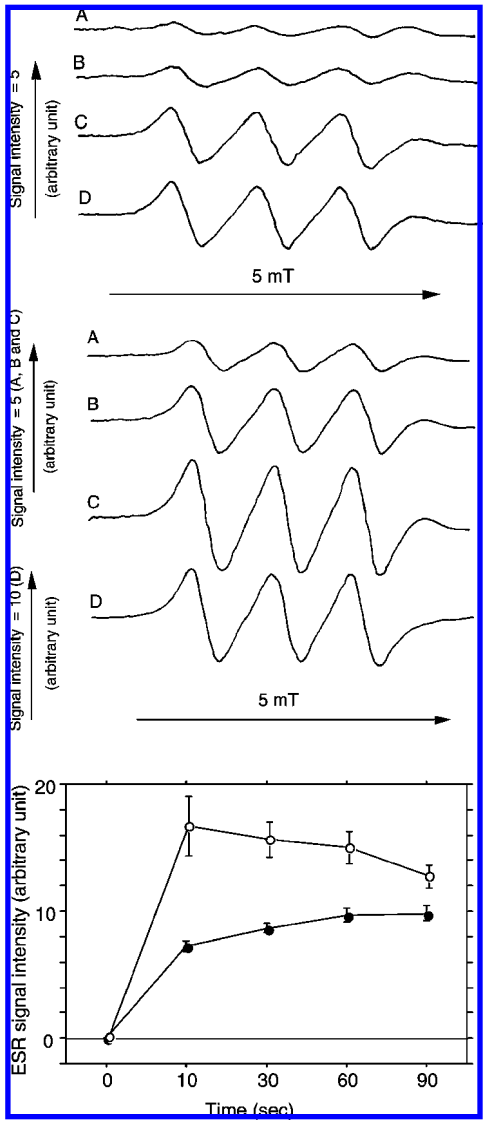

Figure 1. Formation of NO-Fe(DTCS)$_2$ in acidified saliva. (**Top**) ESR spectra in acidified saliva. The reaction mixture (0.56 mL) contained 0.4 mL of saliva and 0.16 mL of 50 mM KH_2PO_4−KCl−HCl (pH 1.36). The pH of the acidified saliva was 2.03, and the concentrations of nitrite and SCN^- were 0.09 and 0.23 mM, respectively. Trace A, no addition; trace B, 0.1 mM chlorogenic acid; trace C, 0.1 mM ascorbic acid; and trace D, 0.1 mM chlorogenic acid + 0.1 mM ascorbic acid. (**Middle**) ESR spectra in 50 mM KCl−HCl (pH 2.0) in the presence of 1 mM $NaNO_2$. Trace A, no addition; trace B, 1 mM NaSCN; trace C, 0.2 mM chlorogenic acid; and trace D, 0.4 mM ascorbic acid. (**Bottom**) Effects of SCN^- on NO-Fe(DTCS)$_2$ formation. Closed circles, no addition; and open circles, 1 mM NaSCN. Each point represents the mean with SD (n = 5).

Oxygen Uptake. If NO is formed under aerobic conditions, O_2 uptake by the autoxidation of NO can be observed (*22, 26*−*28*). **Figure 2** shows time courses of O_2 uptake in acidified saliva. No detectable O_2 uptake was observed when the pH of saliva was decreased to 2 (trace **A**-1). By the addition of 0.1 mM chlorogenic acid to the acidified saliva, about 8 μM O_2 was taken up during 4 min of incubation (trace **A**-2). The O_2 taken up by the addition of 0.1 mM ascorbic acid was about 43 μM O_2 during 4 min of incubation (trace **A**-3). Chlorogenic acid seemed to enhance the O_2 uptake only slightly in the presence of ascorbic acid (trace **A**-4). The addition of 1 mM $NaNO_2$ to the acidified saliva resulted in the slow O_2 uptake (trace **B**-1), and the O_2 uptake was enhanced by chlorogenic acid (trace **B**-2) and ascorbic acid (trace **B**-3). The amount of O_2 taken up during

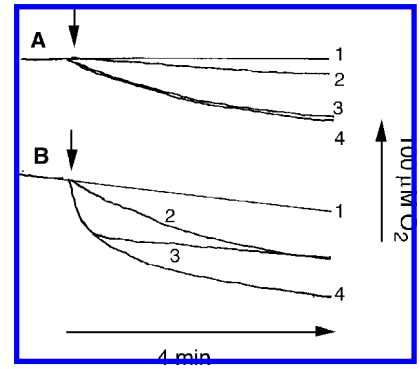

Figure 2. Effects of chlorogenic acid and ascorbic acid on O_2 uptake in acidified saliva. The reaction mixture (1.4 mL) contained 1 mL of saliva and 0.4 mL of 50 mM KH_2PO_4−KCl−HCl (pH 1.36). The pH of the acidified saliva was 2.03, and the concentrations of nitrite and SCN^- were 0.09 and 0.23 mM, respectively. (**A**) Acidified saliva itself. Trace 1, no addition; trace 2, 0.1 mM chlorogenic acid; trace 3, 0.1 mM ascorbic acid; and trace 4, 0.1 mM chlorogenic acid + 0.1 mM ascorbic acid. Arrows indicate the addition of reagents. (**B**) Acidified saliva plus 1 mM nitrite. Trace 1, no addition; trace 2, 0.1 mM chlorogenic acid; trace 3, 0.1 mM ascorbic acid; and trace 4, 0.1 mM chlorogenic acid + 0.1 mM ascorbic acid.

4 min of incubation in the presence of 0.1 mM ascorbic acid (43 μM) was similar to that in the presence of chlorogenic acid. The time course of the O_2 uptake observed by the addition of both chlorogenic acid and ascorbic acid seemed to be the additive of traces **B**-2 and **B**-3, and the amount of O_2 taken up during 4 min of incubation was about 70 μM (trace **B**-4). The result suggests that NO, which was produced by ascorbic acid- and chlorogenic acid-dependent reduction of nitrous acid, contributed to the O_2 uptake.

Nitrite-induced O_2 uptake was also studied in 50 mM KCl−HCl (pH 2.0) (data not shown). By the addition of 1 mM $NaNO_2$, slow O_2 uptake was observed, and the rate of the O_2 uptake was enhanced by 0.1 mM chlorogenic acid and 0.1 mM ascorbic acid. The enhancement by ascorbic acid was larger than that by chlorogenic acid as shown in **Figure 2**. The amount of O_2 taken up during 5 min of incubation in the presence of ascorbic acid (48 μM) was similar to that in the presence of chlorogenic acid. In the mixture of chlorogenic acid and ascorbic acid, the time course of the O_2 uptake was the additive of the time course in the presence of chlorogenic acid and that in the presence of ascorbic acid, and the amount of O_2 taken up during 5 min of incubation was about 98 μM. SCN^- (1 mM) did not clearly affect the O_2 uptake induced by the addition of chlorogenic acid as reported for caffeic acid (*3*) but enhanced the rate induced by the addition of ascorbic acid more than 3-fold without affecting the amount of O_2 taken up. In the presence of both ascorbic acid and SCN^-, the effect of chlorogenic acid on the initial rate of the O_2 uptake was not clear as in **Figure 2B**. The result obtained in buffer solution supports that the NO produced by the reaction between nitrous acid and ascorbic acid or chlorogenic acid contributed to the O_2 uptake and suggests that ONSCN preferentially reacted with ascorbic acid.

In **Figure 1**, nitrite-induced formation of NO-Fe(DTCS)$_2$ was enhanced by SCN^-. If SCN^- enhanced the NO formation, O_2 uptake by autoxidation of NO should be enhanced by the reagent. Rates of O_2 uptake in the presence of 1 mM nitrite in 50 mM KCl−HCl (pH 2.0) were 0.72 ± 0.08 and 1.11 ± 0.21 μM/min [means ± SDs (n = 5), p value = 0.0049] in the absence and presence of 1 mM NaSCN, respectively, supporting SCN^--dependent reduction of nitrous acid to NO.

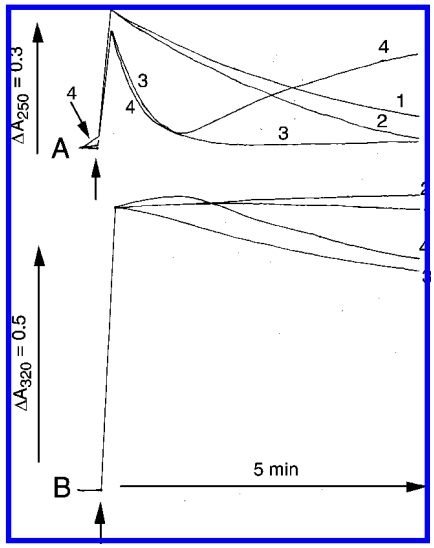

Figure 3. Changes in absorption of ascorbic acid and chlorogenic acid in acidified saliva. The reaction mixture (1.4 mL) contained 0.8 mL of saliva and 0.32 mL of 50 mM KH_2PO_4−KCl−HCl (pH 1.36). The pH and the concentrations of nitrite and SCN^- of acidified saliva were the same as in **Figure 1**. (**A**) Oxidation of ascorbic acid (0.1 mM). Trace 1, no addition; trace 2, 0.1 mM chlorogenic acid; trace 3, 0.2 mM $NaNO_2$; and trace 4, 0.1 mM chlorogenic acid + 0.2 mM $NaNO_2$. (**B**) Oxidation of chlorogenic acid (0.1 mM). Trace 1, no addition, trace 2, 0.1 mM ascorbic acid; trace 3, 0.2 mM $NaNO_2$; and trace 4, 0.1 mM ascorbic acid + 0.2 mM $NaNO_2$. Upward arrows in **A** and **B** indicate the addition of ascorbic acid and chlorogenic acid, respectively.

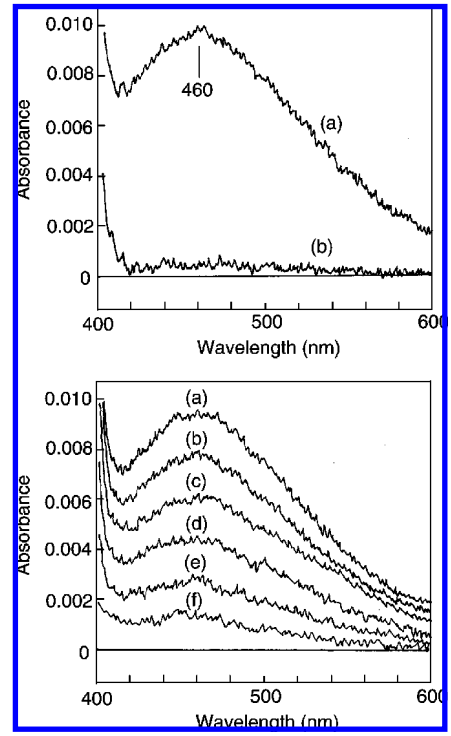

Figure 4. Formation of ONSCN in acidic solutions of nitrite and SCN^-. (**Top**) Effects of pH. (a) pH 1.36 and (b) pH 2.00. (**Bottom**) Effects of ascorbic acid. (a−f) 0, 1, 2, 3, 4, and 5 mM ascorbic acid, respectively.

Spectrophotometric Measurements. If ascorbic acid and chlorogenic acid reduced nitrous acid to NO, oxidation of the two acids should be observed. **Figure 3** shows time courses of the oxidation of ascorbic acid (**A**) and chlorogenic acid (**B**) in acidified saliva that contained 0.09 mM nitrite and 0.23 mM SCN^-. By the addition of 0.1 mM ascorbic acid, absorption increased at 250 nm due to the absorbance of ascorbic acid, and the increased absorbance decreased slowly (trace **A**-1). The absorption decrease was enhanced by 0.1 mM chlorogenic acid (trace **A**-2). In trace **A**-2, the absorption increase observed by the addition of chlorogenic acid had been subtracted. When 0.2 mM $NaNO_2$ was added to acidified saliva and then ascorbic acid was added, rapid oxidation of the ascorbic acid added was observed (trace **A**-3). The addition of both 0.2 mM $NaNO_2$ and 0.1 mM chlorogenic acid to acidified saliva resulted in the observation of slow absorption increase at 250 nm (a sloped arrow in trace **A**-4). By the addition of ascorbic acid, the absorption due to ascorbic acid decreased rapidly and then increased (trace **A**-4). The absorption increase was due to the oxidation of chlorogenic acid as reported previously (*15*). The result suggests that ascorbic acid reduced the oxidation intermediate or product of chlorogenic acid and that the oxidation of chlorogenic acid became to be observed after almost all ascorbic acid had been extinguished. No changes in absorption were observed when ascorbic acid (0.1 mM) was added to 50 mM KCl−HCl (pH 2.0).

Chlorogenic acid has absorption peaks at about 320 nm, and the absorption decreases during the oxidation (*15*). By the addition of 0.1 mM chlorogenic acid to acidified saliva, slow absorption decrease following slow absorption increase was observed (**Figure 3**, trace **B**-1). The addition of chlorogenic acid in the presence of 0.1 mM ascorbic acid resulted in the continuous slow absorption increase (trace **B**-2). At present, it is not clear how the absorption increased. When 0.2 mM $NaNO_2$ was added to acidified saliva and then chlorogenic acid was added, a clear absorption decrease due to the oxidation of chlorogenic acid was observed (trace **B**-3). The absorption decrease was inhibited by ascorbic acid, suggesting that ascorbic acid reduced the oxidation intermediate or product of chlorogenic acid to the original compound (trace **B**-4). The inhibition disappeared during incubation. The disappearance was related to the extinguishment of ascorbic acid from the reaction mixture as described above. The relation became clear by comparing time courses of absorption changes in **Figure 3A**,**B**.

In 50 mM KCl−HCl (pH 2) that contains 0.01 mM ascorbic acid and 0.01 mM chlorogenic acid, preferential oxidation of ascorbic acid (about 4 μM/min) was observed at the initial period of incubation when 0.1 mM $NaNO_2$ was added (data not shown). The oxidation of chlorogenic acid appeared when almost all ascorbic acid had been oxidized, and chlorogenic acid (0.02 and 0.4 mM) enhanced the oxidation of ascorbic acid, which was induced by 0.1 mM $NaNO_2$, about 1.5- and 2-fold, respectively, supporting the oxidation of ascorbic acid by the oxidation intermediate or product of chlorogenic acid. The enhancement by chlorogenic acid became obscure in the presence of 0.1 and 1 mM NaSCN that enhanced oxidation of ascorbic acid 3- and 8-fold, respectively. This was due to much larger enhancement of the oxidation of ascorbic acid by SCN^- than that by chlorogenic acid.

Figure 4 (**top**) shows the absorption spectra of acidic solutions of 10 mM nitrite and 10 mM SCN^-. At pH 1.36 (trace a), a yellow component with an absorption peak at 460 nm was formed. This component has been reported to be ONSCN (*29*). In the mixture of nitrite and SCN^- at pH 2.0 (trace b), ONSCN seemed to be detected. If the signal was due to ONSCN, the concentration at pH 2 was calculated to be about 4 μM using an extinction coefficient of ONSCN (ϵ_{460} = 100 M^{-1} cm^{-1}) (*29*). When ascorbic acid was added to trace a in the **top** panel,

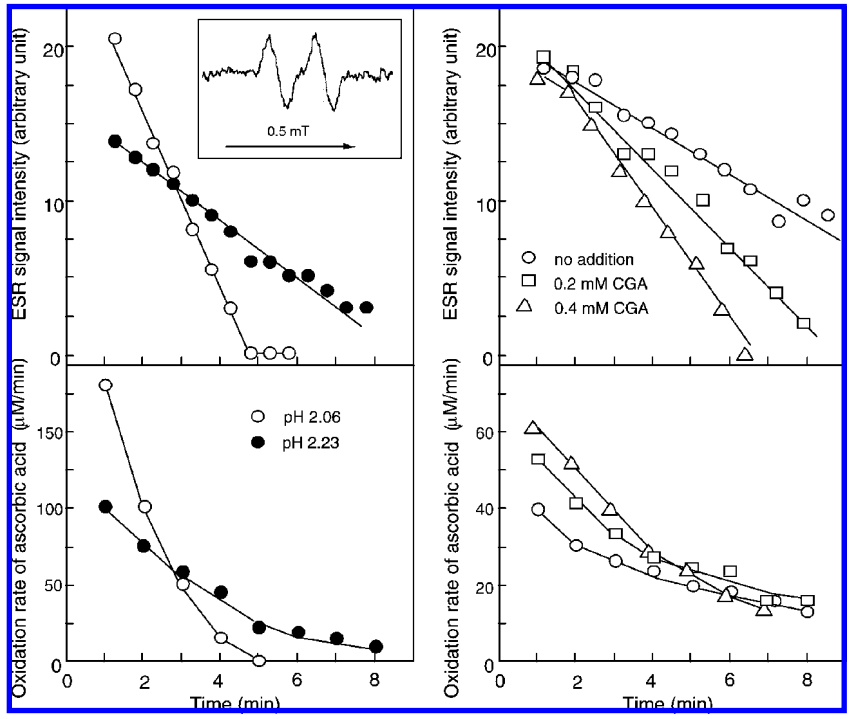

Figure 5. Changes in the concentration of ascorbyl radical (**top** panels) and the oxidation of ascorbic acid (**bottom** panels) as a function of incubation time. (**Left**) Acidified saliva. The reaction mixture (0.42 mL) contained 0.47 mM ascorbic acid in 0.3 mL of saliva and 0.12 mL of 50 mM KCl−50 mM KH_2PO_4−HCl (pH 1.36). Final pH, 2.06 (○) and 2.23 (●). (Inset) A typical ESR spectrum of ascorbyl radical. (**Right**) Acidic buffer solution. The reaction mixture (0.5 mL) contained 0.4 mM ascorbic acid and 0.2 mM $NaNO_2$ in 50 mM KCl−HCl (pH 2.0). Symbols: ○, no addition; □, 0.2 mM chlorogenic acid; and Δ, 0.4 mM chlorogenic acid.

the absorption of ONSCN decreased rapidly, and about 90% of the absorption of ONSCN disappeared by the addition of 5 mM ascorbic acid (**Figure 4**, **bottom**).

Detection of Ascorbyl Radical. If ascorbic acid reduced nitrous acid producing NO, a radical of ascorbic acid should be generated. As the rate constant of the dismutation of ascorbyl radical is small (*30*), the radical generated can be detected by an ESR technique. **Figure 5** (inset) shows an ESR spectrum observed when 0.47 mM ascorbic acid was added to acidified saliva. The spectra ($a^H = 0.18$ mT) were the same as those of ascorbyl radical (*31*), although the hyperfine structure could not be observed due to low signal intensity. The ESR signal intensity of ascorbyl radical and the oxidation rate of ascorbic acid, which was determined spectrophotometrically, were plotted as a function of incubation time using acidified saliva at pH values of 2.06 and 2.23 (**Figure 5**, **left**). The result indicates that the concentration of ascorbyl radical reflected the oxidation rate of ascorbic acid.

Figure 5 (**right**) shows changes in the intensity of ESR signal of ascorbyl radical and the oxidation rate of ascorbic acid in the reaction mixture that contained 0.4 mM ascorbic acid and 0.2 mM nitrite in 50 mM KCl−HCl (pH 2.0). The oxidation of ascorbic acid in the buffer solution was much slower than that in acidified saliva (pH 2.06). One of the reasons for the slow oxidation might be due to the absence of SCN^- in the buffer solution, as SCN^- largely enhanced the oxidation of ascorbic acid (*22*). Both the signal intensity and the oxidation rate in the buffer solution decreased as a function of incubation time as in the **left** panels in **Figure 5**. Chlorogenic acid did not significantly affect the intensity of ESR signal at the initial period of incubation, although the compound enhanced the oxidation rate of ascorbic acid. Furthermore, during an incubation period between 4 and 8 min, chlorogenic acid decreased the intensity of ascrobyl radical, although the oxidation rate of ascorbic acid in the presence of chlorogenic acid was roughly the same as that in the absence of chlorogenic acid. The result in **Figure 5** suggests that there were interactions between the ascorbyl radical and the oxidation intermediate or product of chlorogenic acid. No detectable formation of ascorbyl radical was observed when 0.4 mM ascorbic acid was added to 50 mM KCl−HCl buffer (pH 2.0).

Effects of 0.05 mM SCN^- on changes in the concentration of ascrobyl radical and the oxidation rate of ascorbic acid were studied in the presence of 0.4 mM ascorbic acid and 0.2 mM nitrite in 50 mM KCl−HCl (pH 2.0). The intensity of ESR signal of ascrobyl radical and the oxidation rate of ascorbic acid at 1.5 min after the start of reactions were increased by about 1.5- and 2.4-fold, respectively, by SCN^-. The concentration of ascrobyl radical decreased as the rate of oxidation of ascorbic acid decreased. The oxidation rate of ascorbic acid (about 100 μM/min) in the mixture of 0.4 mM ascorbic acid, 0.2 mM nitrite, and 0.05 mM SCN^- in 50 mM KCl−HCl could be obtained in the mixture of 0.45 mM nitrite and 0.4 mM ascorbic acid in the acidic buffer solution. Then, the concentration of ascrobyl radical and the oxidation rate of ascorbic acid were measured as a function of incubation time in the latter mixture. There were no significant differences in not only time courses of the decrease in the oxidation rate of ascorbic acid but also time courses of the decrease in the ESR signal intensity between the two reaction conditions. The result suggests that ascorbyl radical did not react with ONSCN.

DISCUSSION

The formation of NO-$Fe(DTCS)_2$ was observed in this study. Its formation was mainly due to NO derived from nitrous acid in acidified saliva and acidic buffer solution. Direct reactions of $Fe(DTCS)_2$ with nitrous acid and ONSCN, when $Fe(DTCS)_2$ was added to acidic nitrite solution (*32*), were excluded in this study (see the Results). In addition, the contribution of

NO−ascorbic acid, which might be formed in acidified saliva via *S*-nitrosothioles and *S*-nitrosothiones (*33, 34*), in the formation of NO-Fe(DTCS)$_2$ in the presence of ascorbic acid might also be excluded. This is deduced from the result that ESR signal intensity of NO-Fe(DTCS)$_2$ after the addition of Fe(DTCS)$_2$ (final pH, 7.2−7.4) did not change. If NO−ascorbic acid significantly contributed to the formation of NO-Fe(DTCS)$_2$, the signal intensity of NO-Fe(DTCS)$_2$ should be increased after the addition of Fe(DTCS)$_2$ as NO−ascorbic acid could donate NO. It has been reported that when Fe(DTCS)$_2$ is added to a solution of ethyl nitrite around neutral pH values, the ESR signal intensity of NO-Fe(DTCS)$_2$ increases during incubation (*35*).

The formation of NO-Fe(DTCS)$_2$ and the O_2 uptake in acidified saliva were enhanced by ascorbic acid. The enhancements have been reported to be due to the enhanced formation of NO by the following reactions (*3, 22*):

$$\text{ascorbic acid} + 2HNO_2 \rightarrow 2NO + \text{dehydroascorbic acid} + 2H_2O \quad (1)$$

If ONSCN is generated by the following reaction using salivary SCN^-:

$$HNO_2 + SCN^- + H^+ \rightleftharpoons ONSCN + H_2O \quad (2)$$

ascorbic acid can reduce ONSCN as following:

$$\text{ascorbic acid} + 2ONSCN \rightarrow 2NO + \text{dehydroascrobic acid} + 2SCN^- + 2H^+ \quad (3)$$

In addition to the above reactions, the oxidation of ascorbic acid by N_2O_3 generated by the self-decomposition of HNO_2 was also possible

$$\text{ascorbic acid} + N_2O_3 \rightarrow 2NO + \text{dehydroascorbic acid} + H_2O \quad (4)$$

The NO formed by the above reactions can react with Fe(DTCS)$_2$, producing NO-Fe(DTCS)$_2$, and with O_2 as follows:

$$2NO + O_2 \rightarrow 2NO_2 \quad (5)$$

The amount of O_2 taken up in the presence of 0.1 mM ascorbic acid was about 43 μM in acidified saliva, independent of the presence and absence of 1 mM nitrite (**Figure 2**). The value in the acidic buffer solution was 48 μM. If ascorbic acid was preferentially oxidized by reactions 1, 3, and 4, producing NO, it is expected that the amount of O_2 consumed in the presence of 0.1 mM ascorbic acid was 0.1 mM as NO formed was transformed to NO_2 by reaction 5. The reaction between ascorbic acid and NO_2 has been reported to be fast ($k = \sim 10^7\ M^{-1}\ s^{-1}$) (*36*). Then, the following reaction should be taken into account for the oxidation of ascorbic acid in addition to reactions 1, 3, and 4:

$$\text{ascorbic acid} + 2NO_2 \rightarrow 2HNO_2 + \text{dehydroascorbic acid} \quad (6)$$

The formation of NO_2 from nitrous acid via N_2O_3 seemed not to be significant in the presence of ascorbic acid, as N_2O_3 formed from nitrous acid could be reduced by ascorbic acid by reaction 4. If NO_2 did not react with ascorbic acid, N_2O_3 might be generated by the following reaction:

$$NO + NO_2 \rightarrow N_2O_3 \quad (k = \sim 10^9\ M^{-1}\ s^{-1}) \quad (7)$$

The N_2O_3 formed could also react with ascorbic acid as reaction 4. However the formation of N_2O_3 by reaction 7 might be slow when the concentration of ascorbic acid was high relative to NO. According to the above discussion, the consumption of 43 μM O_2 in the presence of 0.1 mM ascorbic acid in acidified saliva may suggest that 43% of the ascorbic acid added was consumed by reactions 1, 3, and 4, and the remaining was by NO_2 and/or N_2O_3 generated by reactions 5 and 7.

Nitrite (1 mM) enhanced the oxygen uptake (**Figure 2**). This result might be explained by the production of NO by self-decomposition of nitrous acid and the formation of nitrosyl compounds, which could decompose, producing NO, by the reaction of nitrous acid with salivary components. The amount of O_2 taken up in ascorbic acid/nitrous acid systems in 50 mM KCl−HCl (pH 2) was not affected by SCN^-. This result suggests effective reduction of ONSCN to NO by ascorbic acid without producing NO−ascorbic acid and supports the proceeding of reaction 3 in the mixture of saliva and gastric juice. Ascorbic acid-dependent oxygen uptake in acidified saliva was enhanced by 1 mM nitrite. According to the above discussion, the enhanced oxygen uptake could be attributed to the increase in rates of reactions 1, 3, and 4 to produce NO, which resulted in the increase in the rate of reaction 5.

The enhancement of O_2 uptake by chlorogenic acid (**Figure 2**) was due to the reduction of nitrous acid to NO by the acid, and the enhancement of oxidation of ascorbic acid by chlorogenic acid (**Figure 3**) was due to the reduction of the oxidation intermediate or product of chlorogenic acid by ascorbic acid. As the oxidation intermediate and product, chlorogenic acid radical (*4*) and the quinone form (*7*) were possible. When ascorbic acid had been extinguished, the quinone form might be accumulated. The quinone form can react with SCN^-, producing a stable component as described in the Introduction, although SCN^- did not enhance the reduction of nitrous acid to NO by chlorogenic acid as reported previously (*4, 6*).

The result that the intensity of ascrobyl radical decreased as the decrease in the oxidation rate of ascorbic acid (**Figure 5**) indicates that the radical intensity reflected the rate of formation of ascorbyl radical. The result that chlorogenic acid accelerated the decrease in ESR signal intensity of ascorbyl radical (**Figure 5**) seems to suggest that ascorbyl radical also reacted with the oxidation intermediate or product of chlorogenic acid such as chlorogenic acid radical (*4*) and the quinone form (*15*). The decrease in concentration of ascorbyl radical was faster at a higher concentration of chlorogenic acid, supporting the reaction of ascorbyl radical with the oxidation intermediate or product of chlorogenic acid. The absence of reactions of ascorbyl radical with ONSCN suggests that in the mixture of saliva and gastric juice, ascorbyl radical is scavenged by dismutation, reaction with nitrous ac, etc. if the phenolics are not present.

SCN^- enhanced nitrite-induced formation of NO-Fe(DTCS)$_2$ (**Figure 1**) and oxygen uptake (**Figure 2**). Furthermore, the formation of ONSCN was detected at pH 2 (**Figure 4**). It has been reported that ONSCN can decompose, producing NO (*29*). Therefore, the enhanced formation of NO-Fe(DTCS)$_2$ and oxygen uptake was due to NO produced by the reaction between nitrous acid and SCN^-, suggesting that the formation of NO by the reaction between nitrous acid and SCN^- was possible in the mixture of saliva and gastric juice. When the concentration of nitrite and SCN^- in the stomach was 0.2 and 1 mM, respectively (*23, 24*), the concentration of ONSCN at pH 2 could be calculated from the concentration of ONSCN in **Figure 4** using reaction 2. The value was about 8 nM. Almost all absorption due to ONSCN disappeared by the addition of 5 mM ascorbic acid in the presence of 10 mM SCN^- and 10 mM nitrite (**Figure 4**). This result supports that ascorbic acid reduced

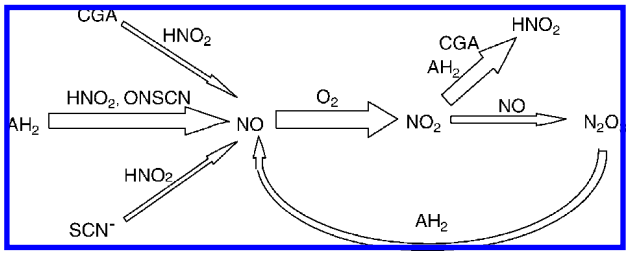

Figure 6. Possible reactions in the mixture of saliva and gastric juice. The width of the arrow indicates the postulated relative rate of each reaction. AH_2, ascorbic acid; and CGA, chlorogenic acid.

ONSCN by reaction 3. Furthermore, the stoichiometry supports that almost all ONSCN formed was reduced to NO by ascorbic acid without generating NO−ascorbic acid as discussed above.

The result obtained in this study indicates (i) that nitrous acid could be reduced to NO by SCN^- as well as chlorogenic and ascorbic acids and (ii) that ascorbyl radical seemed to reduce the oxidation intermediate or product of chlorogenic acid as ascorbic acid did. Under a condition of this study (0.2 mM nitrite, 0.1 mM ascorbic acid, 0.1 mM chlorogenic acid, and 0.23 mM SCN^- and pH 2), the relative rate of NO formation and the consumption of reactive nitrogen oxide species could be summarized as shown in **Figure 6**. The interaction between ascorbic acid and chlorogenic acid prevented the formation of toxic quinones of *o*-diphenols (not shown in **Figure 6**). When ascorbic acid was extinguished, quinone forms of *o*-diphenols might be scavenged by SCN^- (*15*). Because the protection of gastric injuries by dietary nitrate (*37*−*39*) may relate to the formation of nitrite and NO, ascorbic acid/*o*-diphenols/nitrous acid systems in the stomach increase the activity of stomach by producing bioactive component NO (*14, 40*−*42*) scavenging quinones and radicals derived from *o*-diphenols that are formed by the reduction of nitrous acid by the phenols.

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