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Quercetin-Dependent Reduction of Salivary Nitrite to Nitric Oxide under Acidic Conditions and Interaction between Quercetin and Ascorbic Acid during the Reduction

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A salivary component, nitrate, is reduced to nitrite in the oral cavity. Polyphenols in foods are mixed with nitrite in the saliva to be swallowed into the stomach. An objective of the present study is to elucidate reactions between a polyphenol quercetin and a nitrite under acidic conditions. Nitric oxide, which is formed by the reactions between nitrous acid and quercetin or ascorbic acid (AA), can be measured using an oxygen electrode in the saliva as well as a buffer solution. The initial oxidation of quercetin was inhibited by AA, and quercetin enhanced the oxidation of AA, suggesting AA-dependent reduction of quercetin radicals, which might be formed during the oxidation of quercetin by nitrous acid. On the basis of the above results, the usefulness of an oxygen electrode for the measurement of nitrite-dependent nitric oxide formation under acidic conditions is proposed and the possible mechanism of reduction of nitrous acid by quercetin and the interaction between quercetin and AA, which is a normal component in the gastric juice, for the reduction of nitrous acid is discussed.

KEYWORDS: Ascorbic acid; detection of nitric oxide; nitrite (nitrous acid); oxygen uptake; quercetin

INTRODUCTION

Fruits and vegetables contain polyphenols including flavonoids. When fruits and vegetables are ingested as foods, flavonoids are dissolved in the saliva during chewing and the dissolved flavonoids may be transformed by some enzymatic reactions in the oral cavity. In the enzymatic reactions, hydrolysis by glycosidases (*1*) and oxidation by salivary peroxidase (*1*, *2*) and leukocytes (*3*) are included. When onion soup, which contains quercetin glucosides as the major flavonols (*1*, *4*, *5*), is ingested, quercetin and the glucosides are found in the saliva (*1*). The amounts of quercetin and the glucosides in the oral cavity decrease slowly, and the flavonols are found even in the mixed whole saliva, which is collected by chewing Parafilm 4 h after the ingestion of onion soup (*1*). This result suggests that polyphenols in foods can stay in the oral cavity for several hours after the ingestion and that polyphenols, which are bound to the epithelial tissues of the oral cavity, are slowly dissolved in saliva and swallowed into the stomach.

On the other hand, saliva normally contains nitrate, and nitrate in the saliva is reduced to nitrite by bacteria in the oral cavity (*6*). Nitrite formed in the oral cavity is also swallowed into the stomach. Because nitrite may be protonated to nitrous acid ($pK_a = 3.3$) in the stomach, reactions between nitrous acid and polyphenols are possible in the stomach. In a previous paper (*7*), we showed that nitrite is reduced to nitric oxide (NO) by quercetin under acidic conditions and that the reaction can also proceed when the pH of saliva is decreased to 2 (*7*). In the investigation, we could not estimate the formation of NO precisely because of the use of a NO-trapping reagent; under the conditions, the NO detected was NO, which had not reacted with molecular oxygen and other components. The reaction of NO with molecular oxygen has been reported (*8*−*11*).

The objectives of the present study are to elucidate the possible mechanism of nitrite-dependent oxidation of quercetin and to study the interaction between quercetin and ascorbic acid (AA), which is a component of gastric juice (*12*, *13*), during nitrite-dependent oxidation of quercetin under acidic conditions. NO formed during the nitrite-dependent oxidation of quercetin was estimated using an oxygen electrode because NO is oxidized consuming molecular oxygen and producing nitrogen oxides (*8*). The reaction of NO with molecular oxygen using an oxygen electrode has been studied around neutral pH values (*9*−*11*). The results obtained in this study indicate that the formation of NO by quercetin/nitrite and AA/nitrite systems could be estimated with an oxygen electrode and suggest that AA inhibited nitrous acid-dependent oxidation of quercetin by reducing the initial oxidation product, quercetin radical. The mechanism of nitrite-dependent oxidation of quercetin under acidic conditions and the significance of the oxidation in the stomach are discussed.

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MATERIALS AND METHODS

Preparation of Saliva. Mixed whole saliva (5−10 mL) was collected from healthy volunteers by chewing Parafilm at about 9 o'clock in the morning. The pH of collected saliva was 7.4−7.8. Cells (detached epithelial cells and leukocytes) contained in the mixed saliva were removed by centrifugation at 20 000*g* for 5 min (4 °C). This saliva preparation was kept on ice and used as centrifuged saliva. When required, centrifuged saliva (10 mL) was dialyzed at 4 °C against 1 L of 10 mM sodium phosphate (pH 7.2) for 24 h. After it was centrifuged at 20 000*g* for 5 min, the supernatant was used as dialyzed saliva. The dialysis tube used cut molecules, the molecular weights of which were larger than about 14 000. Because, during the dialysis, salivary components such as AA, glutathione, uric acid, and SCN^- were expected to be excluded, we could study, using dialyzed saliva, whether salivary proteins could reduce nitrite to NO under acidic conditions.

Measurements of Nitrite. Decomposition of nitrite in the saliva was studied in a reaction mixture (1 mL) that contained 0.1 mM nitrite in 1 mL of centrifuged saliva. The pH of the reaction mixture was adjusted to 2 by adding a known amount of 1 M HCl. Because the concentration of nitrite in centrifuged saliva used in this study ranged from 0.08 to 0.16 mM, the concentration was adjusted to 0.1 mM by adding water or $NaNO_2$ solution when the nitrite concentration was higher or lower than 0.1 mM, respectively. Decomposition of nitrite in a buffer solution was also studied. The reaction mixture (1 mL) contained 0.1 mM $NaNO_2$ in 50 mM KCl−HCl (pH 2). AA and quercetin, which were obtained from Wako Pure Chem. Ind. (Osaka, Japan), were added to the above reaction mixtures on requirements. The concentration of nitrite was determined using Griess-Romijn reagent for nitrite detection (Wako Pure Chem. Ind.) as reported previously (*14*). The calibration curve for the determination of nitrite concentration showed a good linearity between absorbance at 540 nm and concentration of $NaNO_2$ in the concentration range from 0.02 to 0.2 mM.

Oxygen Uptake. Oxygen uptake was measured using a Clark type oxygen electrode obtained from Rank Brothers (Cambridge, U.K.). The reaction mixture (1 mL) contained 0.5 mL of centrifuged saliva and 0.5 mL of water at 35 °C. The final concentration of nitrite was 0.08 mM. Reactions were started by adding known amounts of 5 M HCl to the reaction mixture to bring the pH to 1.8. Oxygen uptake was also studied in a reaction mixture (1 mL) that contained 0.1 mM $NaNO_2$ in 50 mM KCl−HCl (pH 1−3). In this reaction mixture, reactions were started by adding $NaNO_2$. AA and quercetin were added to the above reaction mixtures on requirements.

Spectrophotometric Measurements. Oxidation of quercetin and AA was measured by absorbance decreases at 360 and 242 nm, respectively, using a Hitachi 557 spectrophotometer (Tokyo, Japan) at 35 °C. The path length of the measuring beam was 4 mm. The differences in absorption coefficients between the substances and the reaction products were estimated to be 8.8 and 17.5 mM^{-1} cm^{-1} for AA and quercetin, respectively. The reaction mixture (1 mL) contained 0.1 mM $NaNO_2$ in 50 mM KCl−HCl (pH 1−3). When saliva preparations were used, the reaction mixture (1 mL) contained 0.5 mL of saliva and 0.5 mL of 50 mM KCl−HCl (pH 1.3) at 35 °C. The final pH was around 1.8. AA and quercetin were added to the above reaction mixtures on requirements.

High-Performance Liquid Chromatography (HPLC). Oxidation products of quercetin were analyzed by HPLC. After a reaction mixture (1 mL), which contained 0.1 mM $NaNO_2$ and 0.03 mM quercetin in 50 mM KCl−HCl (pH 2.0) at 25 °C, was incubated for defined periods, an aliquot (0.025 mL) was withdrawn from the reaction mixture and applied to a Shim-pack CLC-ODS column (6 mm i.d. × 15 cm, particle size 5 μm) (Shimadzu, Kyoto, Japan). The column was combined with an injector (0.1 mL sample loop; Rheodyne 7125) and an LC-6A pump (Shimadzu). A mixture of methanol and 25 mM KH_2PO_4 (3:2, v/v) was used to quantify quercetin, and a mixture of methanol and 25 mM KH_2PO_4 (1:3, v/v) was used to separate a major oxidation product. The flow rate of the mobile phases was 1 mL min^{-1}. Separated components were detected with a spectrophotometric detector with a photodiode array (SPD-M10A, Shimadzu) at 290 nm. Quercetin and the oxidation products were quantified from the peak areas on chromatograms at the wavelength.

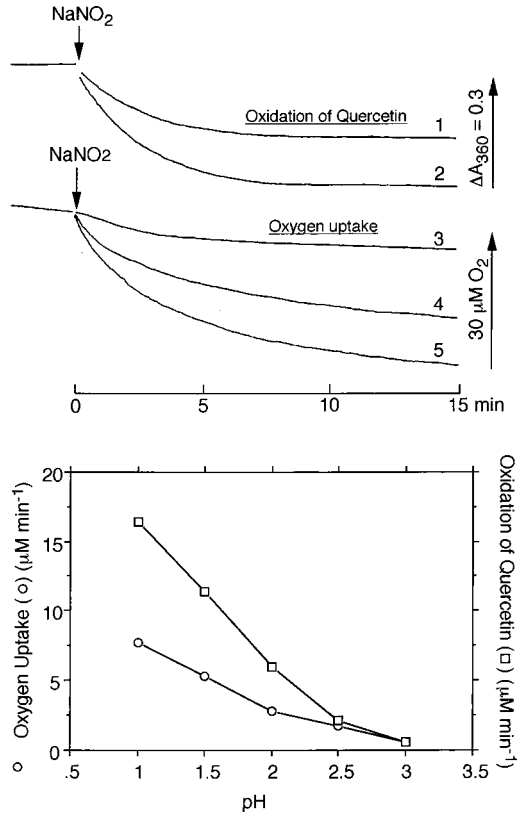

Figure 1. Nitrite-dependent oxidation of quercetin and oxygen uptake. Upper panel: Time courses of oxidation of quercetin (traces 1 and 2) and oxygen uptake (traces 3–5). The reaction mixture (1 mL) contained 0.1 mM $NaNO_2$ in 50 mM KCl–HCl (pH 1.5). Traces 1 and 4, 0.03 mM quercetin; traces 2 and 5, 0.05 mM quercetin; trace 3, without quercetin. Arrows indicate where 0.1 mM $NaNO_2$ was added. Lower panel: Effects of pH on the oxidation of quercetin and oxygen uptake. The reaction mixture (1 mL) contained 0.03 mM quercetin and 0.1 mM $NaNO_2$ in 50 mM KCl–HCl (pH 1–3). Changes in absorbance and oxygen uptake during the initial 1 min were plotted as a function of pH. Circles, oxygen uptake; squares, oxidation of quercetin.

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

RESULTS AND DISCUSSION

Oxidation of Quercetin and Oxygen Uptake. Quercetin is oxidized by nitrite under acidic conditions (*7*). The oxidation is observed as absorbance decreases at 250 and 363 nm and the increase at 293 nm with two isosbestic points at 268 and 325 nm when the concentrations of nitrite were low (0.1−0.3 mM) (*7*). This result suggests the formation of a stable oxidation product(s) of quercetin. Then, we measured the oxidation of quercetin by the absorbance decrease at 360 nm. **Figure 1** (upper panel, traces 1 and 2) shows typical time courses of the oxidation of quercetin by nitrite at pH 1.5. By the addition of nitrite, absorbance decreased to constant values. Initial rates of the oxidation of quercetin were estimated to be 11.4 and 17.1 μM min^{-1} in the presence of 0.03 and 0.05 mM quercetin, respectively. During the absorbance decreases, oxygen uptake (traces 4 and 5) was observed and the initial rates were estimated to be 5.5 and 7.9 μM min^{-1} in the presence of 0.03 and 0.05 mM quercetin, respectively. Slow oxygen uptake was observed in the absence of quercetin (trace 3); this oxygen uptake was due to self-decomposition of nitrite. It is known that nitrite

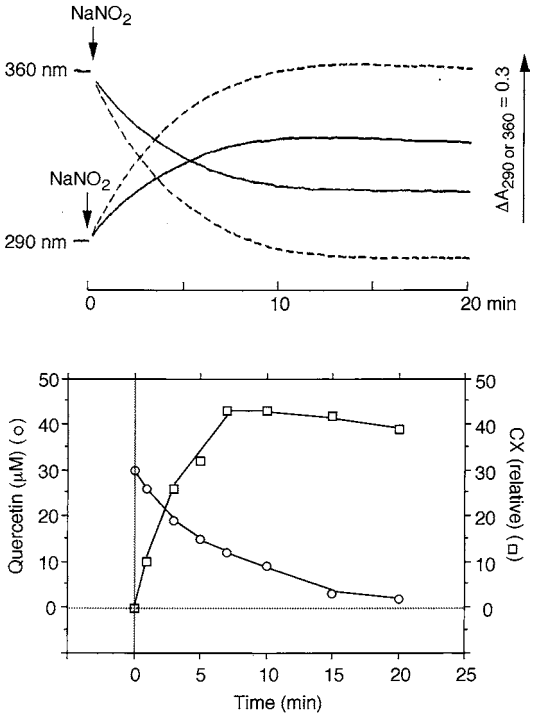

Figure 2. Time courses of oxidation of quercetin and formation of the oxidation product (CX). The reaction mixture (1 mL) contained 0.1 mM $NaNO_2$ in 50 mM KCl–HCl (pH 2.0). Upper panel: Oxidation of quercetin and formation of CX recorded with a spectrophotometer. Solid curves, 0.03 mM quercetin; dashed curves, 0.05 mM quercetin. Arrows indicate where 0.1 mM $NaNO_2$ was added. Lower panel: Oxidation of quercetin and CX formation measured by HPLC. Circles, oxidation of quercetin; squares, formation of CX. The initial concentration of quercetin was 0.03 mM. Because the real concentration of CX could not be determined in this study, the value was expressed as relative value calculated from peak area of CX on HPLC.

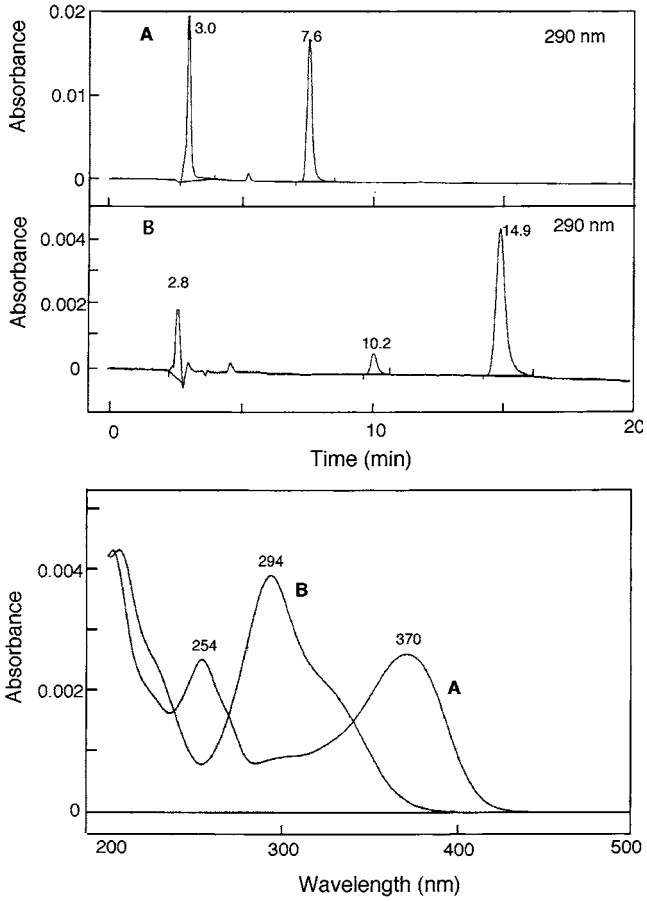

Figure 3. HPLC of quercetin and the oxidation product (CX). Upper panel: HPLC. Quercetin (0.03 mM) was oxidized in a reaction mixture (1 mL) that contained 0.1 mM $NaNO_2$ in 50 mM KCl–HCl (pH 2.0). After they were incubated for defined periods, aliquots (0.025 mL) of the reaction mixture were withdrawn and applied to an HPLC column. Trace A, methanol and 25 mM KH_2PO_4 (3:2, v/v). A component with a retention time of 7.6 min was quercetin. Trace B, methanol and 25 mM KH_2PO_4 (1:3, v/v). A component with a retention time of 14.9 min was CX. Lower panel: Absorption spectra. Curve A, quercetin; curve B, CX.

decomposes to nitrate under acidic conditions producing NO, NO_2, and N_2O_3 as intermediates (*8*). The oxidation of quercetin and the oxygen uptake were dependent on pH (**Figure 1**, lower panel); as the pH was decreased from 3 to 1, the rate increased suggesting the participation of nitrous acid in the oxidation of quercetin. When the pH was lower than 2, the stoichiometry of oxygen uptake to quercetin oxidation in rate was around 0.45. The result in **Figure 1** suggests that the oxygen uptake can be a measure of the formation of NO, since nitrous acid is reduced to NO by quercetin (*7*) and NO is oxidized by molecular oxygen (*9*−*11*).

Oxidation Product. As, during oxidation of quercetin, the absorbance around 293 nm increases (*7*), we compared time courses of absorbance increase at 290 nm with those of absorbance decrease at 360 nm in the presence of 0.03 and 0.05 mM quercetin (**Figure 2**, upper panel). Half-rise times at 290 nm and half-decay times at 360 nm were about 2.6 min independent of the concentrations of quercetin, suggesting that the oxidation of quercetin and the formation of the oxidation product follow a first-order reaction kinetic. This is confirmed by the data that the increase and the decrease in absorbance were parallel when the absorbance changes were plotted semilogarithmically as a function of incubation time. The oxidation products of quercetin were analyzed by HPLC (**Figure 3**, upper panel). When a mixture of methanol and 25 mM KH_2PO_4 (3:2, v/v) was used as a mobile phase, two main peaks were detected (trace A). One peak with a retention time of 7.6 min was quercetin, and the other peak with a retention time of 3.0 min was the oxidation products. Because the peak with a retention time of 3.0 min might be composed of plural components, we tried to separate them using a mixture of methanol and 25 mM KH_2PO_4 (1:3, v/v) as a mobile phase (trace B): one major peak was detected at a retention time of 14.9 min (in the following CX). The absorption spectrum of CX had a peak at 294 nm in the mobile phase (lower panel, curve B).

Figure 2 (lower panel) shows time courses of oxidation of quercetin and formation of CX, which were measured using HPLC. During the oxidation of quercetin, CX was formed. The time courses of absorbance changes in the upper panel were essentially the same as those of the decrease and increase in the concentrations of quercetin and CX, respectively, in the lower panel. The result in **Figure 2** supports that nitrous acid-dependent oxidation of quercetin and formation of CX could be estimated spectrophotometrically. Although some oxidation products of quercetin, which have been obtained by oxidase- and peroxidase-catalyzed reactions, have been identified (*15*−*17*), further studies are required to characterize CX found in this study.

Effects of AA on Oxidation of Quercetin in a Buffer Solution. When quercetin in the saliva is mixed with the gastric juice, nitrite-dependent oxidation of quercetin may be affected by AA in the juice. **Figure 4** shows time courses of nitrite-

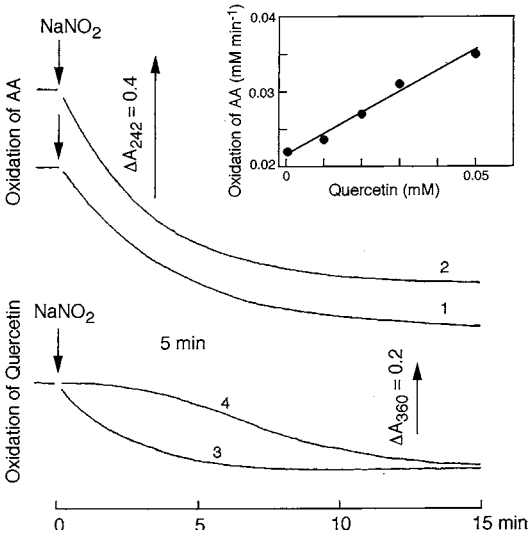

Figure 4. Time courses of oxidation of AA and quercetin. The reaction mixture (1 mL) contained 0.1 mM $NaNO_2$ in 50 mM KCl–HCl (pH 1.7). Traces 1 and 2, oxidation of AA; traces 3 and 4, oxidation of quercetin. Trace 1, 0.1 mM AA; traces 2 and 4, 0.1 mM AA plus 0.03 mM quercetin; trance 3, 0.03 mM quercetin. Downward arrows indicate the addition of $NaNO_2$. The inset shows effects of quercetin concentration on the oxidation of 0.1 mM AA by 0.1 mM $NaNO_2$ dissolved in 50 mM KCl–HCl (pH 1.7).

Table 1. Rates of Nitrous Acid-Dependent Oxidation of AA and Quercetin and Oxygen Uptake in a Buffer Solution and the Saliva

Oxidation

	rate[c] (μM min^{-1})	
	in a buffer[a]	in saliva[b]
AA		
0.1 mM AA	22.5	53.9
0.1 mM AA + 0.03 mM quercetin	31.2	63.9
quercetin		
0.03 mM quercetin	9.3	6.4
0.1 mM AA + 0.03 mM quercetin	0.0	0.0

Oxygen Uptake

	rate[c] (μM min^{-1})	
	in a buffer[a]	in saliva[b]
no addition	1.1	4.4
0.1 mM AA	10.7	36.0
0.03 mM quercetin	4.6	8.9
0.1 mM AA + 0.03 mM quercetin	15.7	46.9

[a] The reaction mixture contained 0.1 mM $NaNO_2$ in 50 mM KCl–HCl (pH 1.7) as in **Figure 4**. [b] Oxidation reactions were measured in a reaction mixture (1 mL) that contained 0.5 mL of saliva with 0.16 mM nitrite and 0.5 mL of 50 mM KCl–HCl (pH 1.3). The final pH was 1.8. Oxygen uptake was measured in a reaction mixture (1 mL) that contained 0.5 mL of saliva with 0.16 mM nitrite and 0.5 mL of water. The pH of the reaction mixture was adjusted to 1.8 by adding a known amount of 5 M HCl. [c] Initial rates of oxidation and oxygen uptake.

dependent oxidation of AA and quercetin in a buffer solution at pH 1.7. Half-times of the oxidation of AA and quercetin were about 3 (trace 1) and 1.8 min (trace 3), respectively. Initial rates of the oxidation reactions are shown in **Table 1**. AA inhibited the oxidation of quercetin (trace 4), and quercetin enhanced the oxidation of AA (trace 2). The initial rate of oxidation of AA in the presence of quercetin could be estimated because AA nearly completely inhibited the initial oxidation of quercetin. The value was 31.2 μM min^{-1}. This value was similar to the sum of oxidation rates of AA (22.5 μM min^{-1}) and quercetin (9.3 μM min^{-1}). The oxidation rate of AA increased as the concentration of quercetin was increased (**Figure 4**, inset). These results suggest that AA and quercetin were oxidized independently and that an oxidation intermediate of quercetin, which may be quercetin radical, is reduced by AA. It has been reported that AA can readily reduce phenoxy radicals (*18*). During the oxidation of 0.1 mM AA and 0.03 mM quercetin, oxygen uptake was observed as shown in **Table 1**. The stoichiometry of oxygen uptake to oxidation of AA (0.48) in the presence of 0.1 mM AA was similar to that of oxygen uptake to quercetin oxidation (0.49) in the presence of 0.03 mM quercetin. The rate of oxygen uptake in the presence of 0.1 mM AA and 0.03 mM quercetin was 15.7 μM min^{-1}. The stoichiometry of this value to oxidation of AA in the presence of both AA and quercetin (31.2 μM min^{-1}) was about 0.5. Because AA reduces nitrite to NO under acidic conditions (*19*), the observation of the AA-dependent oxygen uptake supports the above conclusion that the oxygen uptake by quercetin/nitrite systems under acidic conditions can be a measure of the formation of NO. As the oxidation product of AA is dehydroascorbic acid (*20*, *21*), which is formed by two electron oxidation of AA via monodehydroascorbic acid radical, and the stoichiometry of oxygen uptake/AA oxidation was similar to that of oxygen uptake/quercetin oxidation, we deduced that quercetin might also be transformed to CX by two electron oxidation of quercetin via quercetin radical. We confirmed the formation of monodehydroascorbic acid radical during nitrite-dependent oxidation of AA under acidic conditions using an electron spin resonance spectrometer (data not shown).

Effects of AA on Oxidation of Quercetin in Saliva. When the pH of saliva, which contained 0.08 mM nitrite, was decreased to 1.8 in the presence of AA or quercetin, these reagents were oxidized showing time courses similar to those in **Figure 4**. Oxidation of 0.1 mM AA was complete within 5 min with a half-time of 1.2 min. Quercetin was oxidized slowly, and it took more than 10 min for the complete oxidation; the half-time was 3 min. Initial rates of oxidation of AA and quercetin are summarized in **Table 1**. The oxidation rate of AA was faster than that in a buffer solution, but the rate of quercetin was slower than that in a buffer solution (see **Table 1)**. As a reason for the slower oxidation of quercetin in saliva, the difference in concentration of nitrite between the saliva preparation used for this experiment (0.08 mM) and a buffer solution (0.1 mM) is possible, although other reasons such as reduction of quercetin radical by salivary components, etc. cannot be excluded. The faster oxidation of AA in the saliva than in a buffer solution may be explained by the presence and absence of SCN^-; it is reported that saliva contains 1.2 ± 0.7 mM SCN^- (*6*). This is supported by the fact that oxidation of 0.1 mM AA by nitrite in 50 mM KCl−HCl (pH 2) was enhanced by 0.1−1 mM NaSCN (about 3- and 9-fold at 0.1 and 1 mM NaSCN, respectively) as reported previously (*20*, *21*), but the oxidation of quercetin was only slightly enhanced by the concentrations of NaSCN; maximal stimulation was about 20% at pH 2. Nitrite-dependent oxidation of caffeic acid was not enhanced by NaSCN, either. These results suggest that oxidation of phenolics by nitrite is not significantly enhanced by the nitrosyl compound of SCN^- (NOSCN), which may participate in the enhancement of nitrous acid-dependent oxidation of AA (*20*, *21*). The difference in the effects of SCN^- on the oxidation of AA and quercetin may be explained by the difference in redox potential between the two reductants.

The initial rate of oxidation of 0.1 mM AA in the presence of 0.03 mM quercetin could be estimated in saliva because AA completely inhibited the initial oxidation of quercetin as shown in **Figure 4**, and the rate was 63.9 μM min^{-1}. This value was

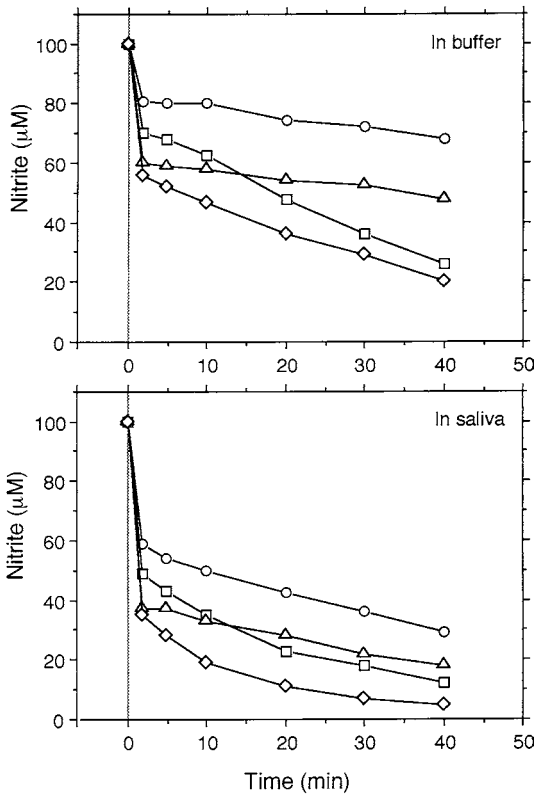

Figure 5. Effects of quercetin and AA on decomposition of nitrite. Upper panel: In a buffer solution. The reaction mixture (1 mL) contained 0.1 mM $NaNO_2$ in 50 mM KCl–HCl (pH 2). Lower panel: In saliva. The reaction mixture (1 mL) was 1 mL of centrifuged saliva, which contained 0.1 mM $NaNO_2$. The pH of the reaction mixture was adjusted to 2 by adding a known amount of 1 M HCl. Circles, no addition; squares, 0.03 mM quercetin; triangles, 0.1 mM AA; diamonds, 0.03 mM quercetin plus 0.1 mM AA.

larger than that of AA oxidation in the absence of quercetin (53.9 μM min^{-1}) and suggests that AA and quercetin were oxidized independently and that quercetin radical might be reduced by AA as discussed above. Oxidation of quercetin was observed after almost all AA had been oxidized. Oxygen was taken up when the pH of centrifuged saliva was decreased to 1.8 (**Table 1**); saliva itself consumed oxygen on acidification, and the rate was increased by AA and quercetin. The stoichiometries of oxygen uptake to oxidation of AA and quercetin in rate were larger than those in a buffer solution, 0.67 and 0.73 in the presence of 0.1 mM AA and 0.1 mM AA plus 0.03 mM quercetin, respectively. Quercetin (0.03 mM) dissolved in acidified saliva (pH 1.8) was also transformed to CX when the oxidation product was analyzed by HPLC (methanol: 25 mM KH_2PO_4 = 3:1, v/v). Here again, CX was the main oxidation product of quercetin.

Effects of AA and Quercetin on Decomposition of Nitrite. **Figure 5** (upper panel) shows time courses of decomposition of nitrite in a buffer solution at pH 2. In the absence of AA and quercetin, nitrite decomposed slowly after the initial rapid decomposition (about 10 μM min^{-1}). AA (0.1 mM) increased the initial rate about 2-fold but did not significantly affect the decomposition observed between 5 and 40 min of incubation. Quercetin (0.03 mM) increased the initial rate of decomposition of nitrite about 50% and also increased the decomposition observed between 5 and 40 min of incubation. The initial rate of decomposition of nitrite in the presence of both AA (0.1 mM) and quercetin (0.03 mM) was somewhat higher than that in the presence of AA only, and the rate of the decomposition observed between 5 and 40 min of incubation in the presence of both AA and quercetin was similar to that in the presence of quercetin only. The quercetin-dependent enhancement of decomposition of nitrite, which was observed between 5 and 40 min of incubation, suggests that an oxidation product of quercetin, CX, might also contribute to the decomposition of nitrite although the rate by CX was not as fast as that by quercetin. The reaction between CX and nitrite is deduced from the data that the concentration of CX decreased slowly after attaining a maximal concentration (**Figure 2**, lower panel).

Figure 5 (lower panel) shows time courses of decomposition of nitrite in centrifuged saliva at pH 2. Not only the initial rate but also the rate between 5 and 40 min were faster in the saliva than in a buffer solution (**Figure 5**, upper panel) in the absence of AA and quercetin. One of the reasons for the faster decomposition may be due to the presence of AA (<0.02 mM) (*22*), glutathione (about 2.2 μM), and uric acid (about 0.2 mM) (*23*) in the saliva. In addition, phenolics such as tyrosine residues and SH and NH_2 groups in salivary proteins may also contribute to not only the increase in the initial but also the later decomposition of nitrite. The reduction of nitrite to NO by salivary components is deduced from the result that centrifuged saliva produces NO on acidification (*7*), and the enhancement of acid-dependent decomposition of nitrite and oxygen uptake by dialyzed saliva (not shown) supports that participation of salivary proteins in the reduction of nitrous acid. Effects of AA and quercetin on the decomposition of nitrite in centrifuged saliva were essentially the same as those of the reagents on the decomposition of nitrite in a buffer solution.

Mechanism and Significance of Quercetin Oxidation. In this study, we suggested that nitrite-dependent oxygen uptake under acidic conditions could be a measure for the formation of NO. Because both AA and quercetin might be oxidized by two electron oxidation processes as discussed above, we could postulate that the mechanism of oxidation of quercetin was similar to that of AA. The participation of nitrous acid on the oxidation is supported by the data that rates of oxidation of both AA and quercetin were increased as pH was decreased (**Figure 1**). As nitrous acid is transformed to oxidation equivalents such as $H_2NO_2^+$ (or NO^+) and N_2O_3 (*8*, *21*), these oxidants may directly contribute to the oxidation. If NOSCN participated in the SCN^--dependent stimulation of AA oxidation, NOSCN might be formed by the reaction between SCN^- and $H_2NO_2^+$ (or NO^+) (*20*, *21*). If the oxidation of AA and quercetin proceeds in the stomach, the concentration of NO is increased. The increase in concentration of NO in the stomach has been discussed in relation to the regulation of mucosal blood flow (*24*, *25*), mucus formation (*26*), gastric motility (*27*, *28*), and gastric acid secretion (*29*). The inhibition of bacterial growth may also be a function of NO (*30*−*32*).

The data that stoichiometries of oxygen uptake to oxidation of AA and quercetin (in the following, XH_2) were 0.4−0.5 in a buffer solution. This may be explained if one postulates that the following reactions proceed, mainly

$$XH_2 + 2HNO_2 \rightarrow X + 2NO + 2H_2O$$

$$4NO + O_2 + 2H_2O \rightarrow 4HNO_2$$

The sum of the above reactions is as follows

$$2XH_2 + O_2 \rightarrow 2X + 2H_2O$$

According to the equations, rates of NO formation could be

roughly estimated from the rates of oxidation of XH_2 and oxygen uptake; the values calculated from the data in **Table 1** were about 18 μM min^{-1} for 0.03 mM quercetin. This value was much larger than NO formation estimated using an NO-trapping reagent (between 0.6 and 0.8 μM min^{-1} in the presence of 0.1 mM $NaNO_2$ and 0.05 mM quercetin at pH 2.0) (*7*). Because the NO detected in ref *7* was NO that had not reacted with molecular oxygen and other components before the addition of the NO-tapping reagent, the value obtained using an NO-trapping reagent should be lower than the true value.

In saliva, the stoichiometries were larger than those in a buffer solution. This may be explained by reduction of nitrous acid to NO by not only AA and quercetin but also salivary components. In addition, oxygen-dependent oxidation of salivary components may also contribute to the larger stoichiometries. These are deduced from the data that saliva itself consumed oxygen (**Table 1**). If NO is formed, the compound is transformed to various nitrogen oxides. These nitrogen oxides may be scavenged by AA and quercetin. The oxidation of AA (*8*, *20*, *33*) and polyphenols (*8*, *34*−*37*) by nitrogen oxides has been reported. Scavenging of reactive nitrogen species by quercetin as well as AA may prevent nitrosation of amines and nitration of tyrosine residues in proteins (*8*, *21*, *38*, *39*).

The oxidation of AA by centrifuged saliva, which contained 0.08 mM nitrite, was about 2.5-fold faster than that by 0.1 mM $NaNO_2$. We discussed above that this result could be explained by SCN^--dependent enhancement of AA oxidation. This is confirmed by the experiments that the oxidation of 0.1 mM AA by dialyzed saliva, to which 0.1 mM $NaNO_2$ was added, was stimulated by NaSCN and that degree of the stimulation was increased depending on the concentration of NaSCN (0.1−0.4 mM) (about 3.5- and 6-fold at 0.1 and 0.4 mM NaSCN, respectively). Because AA is contained in the gastric juice, it is probable that when saliva, which contained both nitrite and SCN^-, is mixed with gastric juice, NO is rapidly formed consuming AA. Nitrous acid-dependent oxidation of quercetin (**Figure 4**) and slow decomposition of nitrous acid by quercetin (**Figure 5**) were observed after almost all AA had been oxidized. This result suggests that quercetin-dependent formation of NO and a decrease in concentration of nitrous acid continue in the stomach even after AA-dependent reduction of nitrous acid has been completed. When the pH of saliva was decreased to 1.8, oxygen uptake was observed and the oxygen uptake was enhanced by AA and quercetin (**Table 1**). These results suggest that if saliva is swallowed into the stomach, the concentration of molecular oxygen in the gastric juice is decreased and the decrease is enhanced by AA in the gastric juice, and that if quercetin is contained in saliva, enhancement of the oxygen consumption may continue in the stomach even after almost all AA has been oxidized. Because lipid peroxidation and formation of hydroxyl radical (*40*−*42*) are possible in the gastric juice, the decrease in concentration of molecular oxygen may slow the formation of reactive oxygen species and reactive oxygen-dependent reactions in the stomach.

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