Nitrogen Dioxide-Dependent Oxidation of Uric Acid in the Human Oral Cavity under Acidic Conditions: Implications for Its Occurrence in Acidic Dental Plaque

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The pH in dental plaque falls to below 5 after the ingestion of foods, and it may remain low if acid-tolerant bacteria grow in the plaque. Certain nitrate-reducing bacteria in the oral cavity can proliferate in dental plaque at low pH, and nitrite is detected in such plaque. In acidic dental plaque, NO_2 can be produced by self-decomposition of nitrous acid and also by peroxidase-catalyzed oxidation of nitrite, and it may oxidize uric acid, a major antioxidant in the oral cavity. Under experimental conditions that simulate oral cavity, the oxidation of uric acid by nitrite and by nitrite/peroxidase systems was much more rapid at pH 5 than at pH 7, suggesting the more rapid production of NO_2 in dental plaque at lower pH. We propose that if the pH of plaque developed in a dental crevice decreased, NO_2 and other nitrogen oxides produced in the plaque would diffuse into the adjoining gingival tissues. The results of this study seem to contribute to the understanding of the induction of periodontal diseases in the context of nitrite-dependent production of nitrogen oxides in acidic dental plaque.

Introduction

Nitrate contained in food is absorbed in the human body by the intestine, and a part of the absorbed nitrate is secreted into the oral cavity as a component of saliva. Nitrate-reducing bacteria in the human oral cavity reduce salivary nitrate to nitrite (*1*−*4*), which is further reduced to nitric oxide (NO) by nitrite-reducing bacteria (*5, 6*). After the consumption of sugar-containing foods, the pH of dental plaque can fall rapidly to below 5 due to the production of acids (predominantly lactic acid) and then returns slowly to its preingestion value (*7, 8*). The frequency of this pH decrease depends on the frequency of the ingestion of sugar-containing foods (*7, 8*). Frequent or prolonged decreases in plaque pH result in the growth of acid-tolerant bacteria, especially *Lactobacillus* species and mutans streptococci; pH reduction by these bacteria has been discussed in relation to enamel caries (*7, 9*). Nitrite-reducing *Actinomyces* and *Veillonella* species, including the acid-tolerant *Actinomyces naeslundii* (*4*), occur in dental plaque developed in the gingival crevice (*7*), and some of these species (*Actinomyces israelli*, *Actinomyces odontolyticus*, *A. naeslundii*, and *Veillonella parvula*) also occur in dental plaque associated with gingivitis (*7*). The concentration of nitrite in saliva ranges from 0.05 to 1 mM, depending on age and foods consumed (*2, 3, 10*), and its concentration in dental plaque has been reported to be as much as 1.2−2-fold higher (*11, 12*), suggesting nitrate reduction by plaque bacteria.

The growth of acid-tolerant bacteria in dental plaque can maintain a low pH, and at a pH below 5, nitrite in the plaque is protonated to produce nitrous acid with a pK_a of 3.3:

$$NO_2^- + H^+ \leftrightarrow HNO_2 \quad (1)$$

Nitrous acid is transformed to NO, NO_2, and N_2O_3:

$$2HNO_2 \leftrightarrow N_2O_3 + H_2O \leftrightarrow NO + NO_2 + H_2O \quad (2)$$

NO formed by nitrite-reducing bacteria (*5, 6*) can react with O_2:

$$2NO + O_2 \rightarrow 2NO_2 \quad (3)$$

Furthermore, the NO formed can react successively with O_2^-/HO_2 and CO_2 (*13, 14*):

$$NO + O_2^- \text{ or } HO_2 \rightarrow ONOO^- \text{ or } ONOOH \quad (4)$$

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow NO_2 + CO_3^- \quad (5)$$

NO_2 and $ONOO^-$ are strong oxidants and nitrating agents, and N_2O_3 is a weak oxidant and a nitrosating agent (*15, 16*). In addition to reactions 2, 3, and 5, NO_2 can arise through the salivary peroxidase-catalyzed oxidation of nitrite at pH 5 (*10, 17*):

$$2NO_2^- + H_2O_2 + 2H^+ \rightarrow 2NO_2 + 2H_2O \quad (6)$$

Salivary peroxidase has been reported to bind to the cell wall of oral bacteria (*10, 18*), and the peroxidase activity is high in dental plaque (*18*). Furthermore, bacteria in dental plaque possess enzymes that metabolize reactive oxygen species (*7, 18, 19*), implying the formation of O_2^- and H_2O_2 in the plaque.

Uric acid is a major antioxidant in the human oral cavity. Its salivary concentration ranges from 78 to 285 μM with an average concentration of approximately 150 μM (*20*−*22*).

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Therefore, uric acid-dependent scavenging of oral nitrogen oxides is possible in the human oral cavity. If the formation of nitrogen oxides in acidic dental plaque by the above reactions outpaces their scavenging, the nitrogen oxides can diffuse out of the plaque. Thus, nitrogen oxides produced in dental plaque that has developed in a gingival crevice may diffuse into the adjoining gingival tissues. In this report, we have investigated nitrite-dependent oxidation of uric acid under various conditions. On the basis of these results, we proposed that chronically low pH in dental plaque developed in gingival crevices could exacerbate nitrogen oxide-induced damage to the gingival tissues.

Experimental Procedures

Reagents. Uric acid, allantoin, and glucose oxidase (GOX)[1] from *Aspergillus niger* and Griess−Romijn reagent for nitrite were obtained from Wako Pure Chemical Ind. (Osaka, Japan). *N*-(Dithiocarboxy)sarcosine sodium salt (DTCS) and the NO-generating reagent (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR 3) were obtained from Dojin (Kumamoto, Japan). 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was from Sigma Chemical Co. (St. Louis, MO). Uric acid (30 mM) was dissolved in 0.1 M NaOH and then diluted to 10 mM with H_2O.

Preparation of Saliva and Its Fractionation. Mixed whole saliva (5−10 mL) was collected between 9 and 10 a.m. by having volunteers chew parafilm. The collected saliva was passed through two layers of nylon filter net [380-mesh (32 μm), Sansho, Tokyo, Japan] to remove epithelial cells and other particulates. The filtrate was kept on ice and used as whole saliva filtrate. When required, whole saliva filtrate was centrifuged at 20000*g* for 5 min. The sediment was suspended in 50 mM sodium phosphate buffer (pH 5.2 or 7.0), the volume of which was the same as the supernatant, to be used as bacterial fraction. The suspension in the pH 5.2 buffer gave a suspension with pH 5.3. This fraction contains almost all bacterial species found in the oral cavity. The supernatant was dialyzed against 10 mM sodium phosphate (pH 7.0) to be used as salivary peroxidase fraction.

Determination of Uric Acid Concentrations. To quantify uric acid in whole saliva filtrate, 0.2 mL of whole saliva filtrate was mixed with 0.04 mL of methanol. After filtration of the mixture through a cellulose-acetate membrane filter (0.45 μm, Advantec, Tokyo, Japan), an aliquot (10 μL) of the filtrate was applied to a Shim-pack CLC-ODS column (6 mm i.d. × 15 cm) (Shimadzu, Kyoto, Japan). The mobile phase used was the mixture of methanol and 25 mM KH_2PO_4 (1:10, v/v), and the flow rate was 1 mL/min. Uric acid separated by HPLC was detected at 280 nm using a spectrophotometric photodiode array detector (SPD-M10Avp, Shimadzu).

Decreases in uric acid concentration were studied in whole saliva filtrate itself (pH 7.2−7.4) and acidified whole saliva filtrate (pH 4.7−4.8), which was prepared by mixing 0.3 mL of 50 mM KCl-HCl buffer (pH 1.3) with 1 mL of whole saliva filtrate (pH 7.2−7.4). Sodium nitrite (1 mM) was added to these saliva preparations when required. After incubation for defined periods, 0.2 mL of samples was mixed with 0.04 mL of methanol, filtered, and applied to the HPLC column as described above.

Decreases in uric acid concentration were also studied in reaction mixtures (1 mL) containing 0.1 mM uric acid and 1 mM $NaNO_2$ in 50 mM KH_2PO_4. The pH of this mixture was 4.6 after the addition of uric acid and nitrite. After incubation for defined periods, 10 μL of the mixture was applied to the HPLC column. An oxidation product of uric acid allantoin formed in the above reaction mixture was separated by a Shim-pack CLC-ODS column and detected at 210 nm. The mobile phase used was the mixture of methanol and 25 mM KH_2PO_4−HCl (pH 3.0) (1:10, v/v), and the flow rate of the mobile phase was 1 mL/min.

Determination of Nitrite and SCN^- Concentrations. Nitrite in saliva was determined in reaction mixtures (1 mL) that contained 0.05 mL of whole saliva filtrate, 0.85 mL of 50 mM KCl-HCl buffer (pH 1.76), and 0.1 mL of 1% (w/v) Griess−Romijn reagent (*10*). After incubation for 15 min at room temperature (25 °C), absorbance at 540 nm was measured. Nitrite concentrations were determined using a standard curve. To quantify SCN^- in bacterial fraction, a bacterial fraction was prepared using water. The reaction mixture (1 mL) contained 0.1 mL of bacterial fraction, 0.1 mL of 0.1 M $FeCl_3$, and 0.8 mL of 0.1 M HCl (*10*). The concentration of SCN^- was calculated from a standard curve.

Detection of NO Using $Fe(DTCS)_2$. Dithiocarbamate derivatives have been reported to be able to trap NO (*23, 24*). Thus, we studied nitrite-induced formation of NO using $Fe(DTCS)_2$. DTCS (5 mM) was prepared in 50 mM KH_2PO_4, and then, $FeCl_3$ solution (100 mM in H_2O) was added to give 1.5 mM $FeCl_3$. This mixture, hereafter referred to as $Fe(DTCS)_2$ solution, had a pH of 5.2. It was kept on ice in the dark. NO formation was initiated by the addition of nitrite to $Fe(DTCS)_2$ solution. After the addition of nitrite, an aliquot of the solution was immediately withdrawn into a quartz flat cell (0.05 mL). Recording of electron spin resonance (ESR) spectra at about 25 °C began 1.5 min after nitrite addition using an ESR spectrometer (type JE1XG; JEOL, Tokyo, Japan) with the following settings: microwave power, 10 mW; scanning speed, 5 mT/min; line width, 0.5 mT; and amplification, 500-fold (*6*).

GOX-Induced Oxidation of Uric Acid. GOX-induced oxidation of uric acid was studied in reaction mixtures that contained 0.1 mM uric acid, 2 mM glucose, and 0.2 μg of GOX in 1 mL of bacterial fraction (pH 7.0 or 5.3). The mixtures of 2 mM glucose and 0.2 μg of GOX/mL in 50 mM sodium phosphate buffer (pH values 7.0 or 5.3) consumed O_2 at a rate of about 2 μM/min when measured using a Clark type oxygen electrode (*10*). This result indicated that GOX-catalyzed formation of H_2O_2 was 2 μM/min in the above reaction mixtures.

The oxidation of uric acid was determined by measuring $\Delta A_{284-320}$ at pH 5.3 and $\Delta A_{288-320}$ at pH 7.0 using a 557 dual-wavelength spectrophotometer (Hitachi, Tokyo, Japan). Uric acid exhibited an absorption peak at 284 (Figure 1, trace b) and 288 nm (not shown) at pH 5.3 and 7.0, respectively, with no absorbance in the wavelength range from 320 to 400 nm. The absorbance of the above reaction mixtures decreased at 284 and 288 nm at pH 5.3 and 7.0, respectively, but this was not accompanied by an absorption increase at 300−316 nm that would have indicated the formation of nitrated uric acid (*25*). This result suggested that the observed absorbance decrease was mainly due to the decomposition of the initial uric acid oxidation product, uric acid radical (*26*), without nitration and nitrosation. Allantoin, an oxidation product of uric acid, did not absorb light in the wavelength range from 260 to 400 nm (not shown).

Measurements of Salivary Peroxidase Activity. Salivary peroxidase activity was studied using ABTS as a substrate (*18*). When salivary peroxidase fraction was used, reaction mixtures (1 mL) contained 0.1 mM ABTS, 2 mM glucose, 0.2 μg of GOX, and 0.05 mL of salivary peroxidase fraction in 0.1 M sodium phosphate buffer (pH 4.6). When a bacterial fraction (pH 5.3) was used, the reaction mixture contained 0.1 mM ABTS, 2 mM glucose, and 0.2 μg of GOX or contained 0.1 mM ABTS and 0.1 mM H_2O_2 in 1 mL of bacterial fraction. The oxidation of ABTS was determined by measuring $\Delta A_{414-460}$.

Results

Nitrite-Induced Oxidation of Uric Acid in Acidic Solution. Nitrite induced the decrease in the concentration of uric acid in 50 mM KH_2PO_4 (pH 4.6) when uric acid was quantified by HPLC as described in the Experimental Procedures (Table 1). Sodium thiocyanate (1 mM) did not significantly

[1] Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); GOX, glucose oxidase; DTCS, *N*-(dithiocarboxy)sarcosine sodium salt.

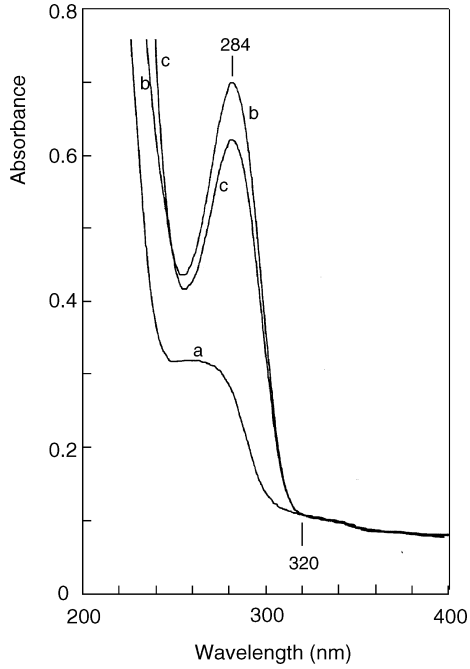

Figure 1. Change in the absorption spectrum of uric acid during the oxidation. The reaction mixture (1 mL) contained 2 mM glucose in bacterial fraction (pH 5.3). Trace a, no addition; trace b, 0.1 mM uric acid; and trace c, 15 min after the addition of 0.2 mM sodium nitrite and 0.2 μg of GOX.

Table 1. Nitrite-Induced Decrease in the Concentration of Uric Acid in an Acidic Buffer Solution[a]

	rate of decrease (μM/h)	
	0.01 mM uric acid	0.1 mM uric acid
no addition	3.9 ± 0.3	19.0 ± 1.9
+1 mM NaSCN		18.3 ± 1.4
+1 mM NaN_3		1.7 ± 1.2

[a] Reaction mixture contained 1 mM nitrite in 50 mM KH_2PO_4 (pH 4.6). Each value represents the mean with the SD (n = 3).

affect the decrease in uric acid concentration, suggesting that nitrosyl thiocyanate (ONSCN), generated by reaction of SCN^- with HNO_2 (*27*), did not contribute to the decrease in uric acid concentration. This result was consistent with the reported ineffectiveness of SCN^- on the nitrite-induced decrease in concentration of uric acid at pH 2 (*28*). The decrease in uric acid concentration was inhibited by approximately 90% by 1 mM sodium azide, a scavenger of N_2O_3 (*29*−*32*). This suggested the participation of NO_2 formed by reaction 2 in the decrease in uric acid concentration in acidic solution. The decrease in uric acid concentration led to a decrease in the absorption peak of uric acid at 284 nm, without detectable increases in the wavelength range from 300 to 400 nm (cf. Figure 1). The absence of absorbance increases in the wavelength from 300 to 400 nm suggested that the decrease in uric acid concentration was due mainly to oxidative degradation. This idea was supported by the result that allantoin was detected by HPLC in the mixture of 0.1 mM uric acid and 1 mM nitrite in 50 mM KH_2PO_4 (pH 4.6).

Nitrite-Induced Formation of NO-Fe(DTCS)$_2$. Because N_2O_3 can decompose by reaction 2 to produce NO, we studied nitrite-induced formation of NO at pH 5.2 using $Fe(DTCS)_2$ (Figure 2). No ESR signal was observed when uric acid or azide was added to the $Fe(DTCS)_2$ solution as a control (trace A). The addition of nitrite resulted in the formation of NO-$Fe(DTCS)_2$ (trace B). The greater signal intensity of the right peak in trace B than that of the left peak indicated that the

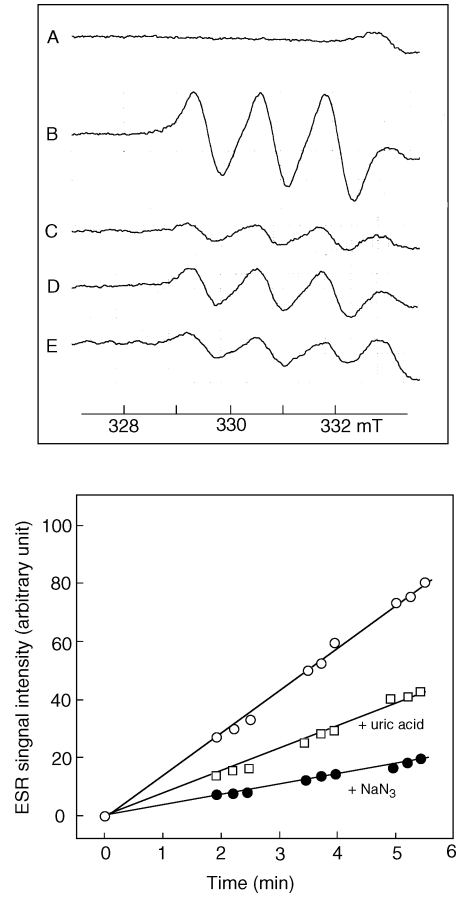

Figure 2. Nitrite-induced formation of NO under acidic conditions. Upper panel: Formation of NO-$Fe(DTCS)_2$. (A) No addition, 1 mM NaN_3, or 0.1 mM uric acid; (B) 1 mM $NaNO_2$; (C) 1 mM $NaNO_2$ + 1 mM NaN_3; (D) 1 mM $NaNO_2$ + 0.1 mM uric acid; and (E) 0.1 mM NOR 3. The recording of each spectrum was started about 1.5 min after the addition of reagents. Lower panel: Time courses of formation of NO-$Fe(DTCS)_2$. Key: ○, 1 mM $NaNO_2$; ●, 1 mM $NaNO_2$ + 1 mM NaN_3; and □, 1 mM $NaNO_2$ + 0.1 mM uric acid. One representative experiment of three is shown.

concentration of NO-$Fe(DTCS)_2$ increased during incubation. The formation of NO-$Fe(DTCS)_2$ was inhibited by azide (trace C) or uric acid (trace D). Trace E represents the ESR signal recorded after the addition of the NO generating reagent NOR 3 (*33*) to the $Fe(DTCS)_2$ solution, supporting the interpretation that ESR signals in traces B−D were derived from NO-$Fe(DTCS)_2$.

The intensity of the NO-$Fe(DTCS)_2$ signal increased linearly with incubation time. Azide and uric acid inhibited the increase by 75 and 45%, respectively (Figure 2, lower panel). The inhibition by azide suggests that NO produced by reaction 2 contributed to the formation of NO-$Fe(DTCS)_2$, even though dithiocarbamate−Fe complexes cannot always be used as direct NO-trapping reagents (*34, 35*). The contribution of NO to the formation of NO-$Fe(DTCS)_2$ supports the contribution of NO_2 formed by reaction 2 to the nitrite-induced oxidative degradation of uric acid at pH 4.6. The inhibition of NO-$Fe(DTCS)_2$ formation by uric acid indicates that uric acid could not reduce N_2O_3 and HNO_2 to NO as ascorbate does (*15, 16*).

Nitrite-Induced Oxidation of Uric Acid in Whole Saliva Filtrate. No decrease in uric acid concentration was observed when whole saliva filtrate (pH 7.2−7.4) was incubated for 1 h with or without 1 mM nitrite (Table 2). A decrease in uric acid concentration occurred only when acidified whole saliva filtrate (pH 4.7−4.8) was incubated in the presence of 1 mM nitrite (Table 2).

Table 2. Nitrite-Induced Decrease in the Concentration of Uric Acid in Whole Saliva Filtrate[a]

		uric acid (μM)	
		no addition	1 mM $NaNO_2$
pH 7.2−7.4	initial concentration	166 ± 5	166 ± 5
	after incubation for 1 h	174 ± 4	172 ± 1
pH 4.7−4.8	initial concentration	164 ± 4	164 ± 4
	after incubation for 1 h	167 ± 5	136 ± 4

[a] Reaction mixtures of pH values of 7.2−7.4 and 4.7−4.8 were prepared as described in the Experimental Procedures. After incubation for 1 h, the concentration of uric acid was determined. Each value represents the mean with the SD (n = 3−4).

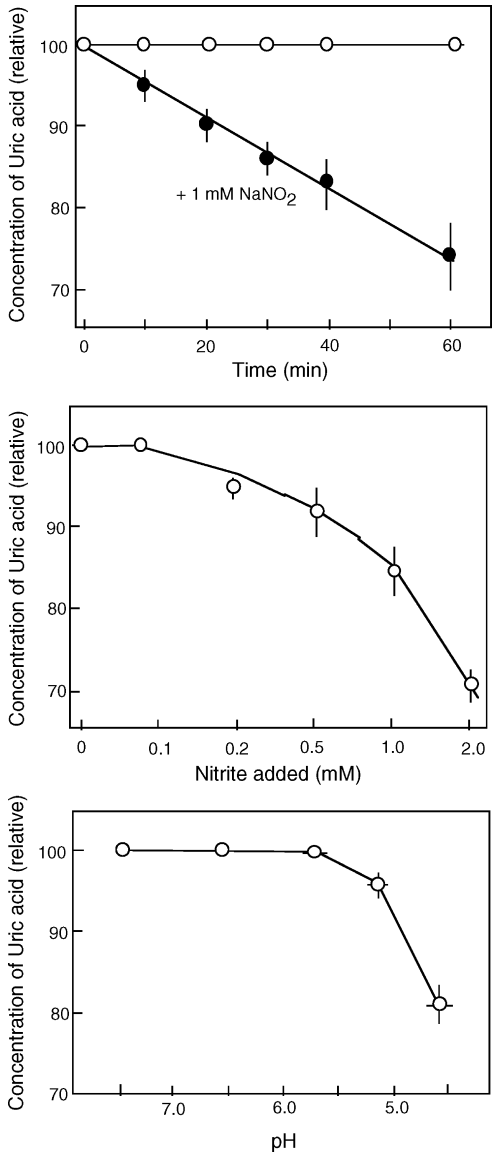

Figure 3. Nitrite-induced oxidation of uric acid in acidified whole saliva filtrate. Upper panel: Time courses. Key: ○, acidified whole saliva filtrate (pH 4.7−4.8); and ●, +1 mM $NaNO_2$. Middle panel: Effect of concentration of nitrite. Various concentrations of $NaNO_2$ were added to acidified whole saliva filtrate and then incubated for 1 h. Lower panel: Effects of pH. Whole saliva filtrate was incubated for 1 h at various pH values in the presence of 1 mM $NaNO_2$. Each data point represents the mean with the SD (n = 3−4).

The initial concentration of uric acid in whole saliva filtrate, which was used for the experiments in Figure 3, was 144 ± 23 μM (mean ± SD) (n = 5). The decrease in uric acid concentration was observed at the rate of 30.4 ± 7.0 μM/h (n = 5) in the presence of 1 mM nitrite, while no decrease in

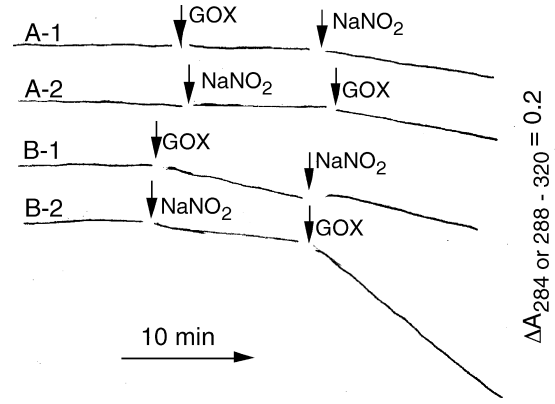

Figure 4. Oxidation of uric acid by nitrite and GOX. The reaction mixture (1 mL) contained 2 mM glucose and 0.1 mM uric acid in bacterial fraction (pH 7.0 or 5.3). A-1 and A-2, pH 7.0; B-1 and B-2, pH 5.3. Where indicated, 0.3 mM $NaNO_2$ or 0.2 μg of GOX was added. One representative experiment of three is shown.

the concentration was evident in the absence of added nitrite in acidified whole saliva filtrate (Figure 3, upper panel), consistent with the results presented in Table 2. The nitrite-induced decrease in uric acid concentration increased with the concentration of nitrite (Figure 3, middle panel) and was observed only at pH values below 6 in the presence of 1 mM nitrite (Figure 3, lower panel). The nitrite concentration in whole saliva filtrate used for the experiments shown in Figure 3 was 0.14−0.28 mM.

Sodium azide (1 mM) inhibited the decrease in uric acid concentration in acidified whole saliva filtrate (pH 4.7−4.8) in the presence of 1−2 mM sodium nitrite by approximately 90%. Because azide serves not only as a scavenger of N_2O_3 (*29*−*32*) but also as an inhibitor (*36*) of salivary peroxidases (*10, 18*), a decrease in the uric acid concentration in the acidified whole saliva filtrate could be induced by NO_2 generated by reaction 6 as well as by reaction 2. Therefore, we examined the effect of H_2O_2 on the decrease in uric acid concentration in acidified whole saliva filtrate. The generation of H_2O_2 using a glucose/GOX system in acidified whole saliva filtrate did not enhance the decrease in uric acid concentration in the presence or absence of nitrite, indicating that NO_2 produced by reaction 2 but not by reaction 6 decreased the concentration of uric acid by oxidation. The lack of enhancement by H_2O_2 might be attributable to the presence of SCN^-, a substrate and an inhibitor of salivary peroxidase (*36, 37*), in whole saliva filtrate.

We then examined whether or not the concentration of nitrite decreased during the nitrite-induced oxidation of uric acid. When acidified whole saliva containing 0.14−0.28 mM nitrite was incubated for 1 h, no decrease in the concentration of nitrite was detected, nor did it decrease upon 1 h of incubation after the addition of 1 mM nitrite. These results suggested that the transformation of nitrite to nitrate was very slow under the conditions used. Similarly, the concentration of nitrite has been reported to decrease slowly at pH 1.9 (*28*).

GOX-Induced Oxidation of Uric Acid in Bacterial Fraction. Because H_2O_2 is produced in dental plaque, the effects of H_2O_2 on nitrite-dependent uric acid oxidation were studied in bacterial fraction (Figure 4). At pH 7.0, no uric acid oxidation was detected in the bacterial fraction in the presence of GOX until nitrite was added (trace A-1). When nitrite alone was added, no detectable oxidation of uric acid was observed (trace A-2). This result indicates that the reduction of nitrite to NO by nitrite-reducing bacteria was too slow to produce NO_2 by the autoxidation of NO (reaction 3). GOX induced uric acid oxidation in the presence of nitrite (trace A-2). The rate of uric acid oxidation induced by GOX was 29.4 ± 8.4 μM/h (n = 4),

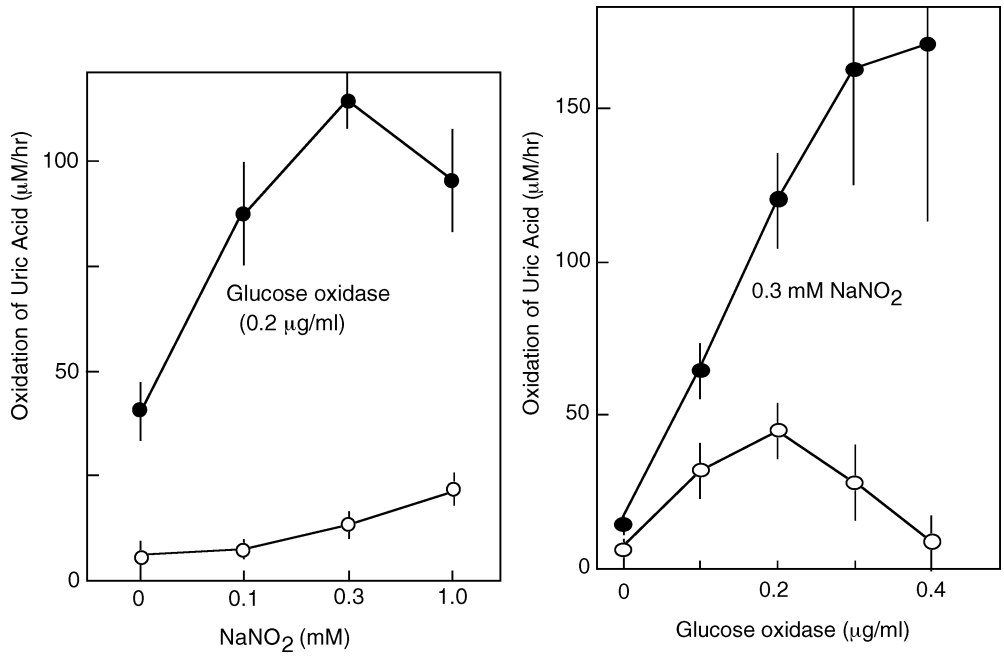

Figure 5. Effects of concentrations of nitrite and GOX on uric acid oxidation. Left panel: Nitrite. The reaction mixture contained 0.1 mM uric acid and 2 mM glucose in 1 mL of bacterial fraction (pH 5.3). Key: ○, without GOX; ●, +0.2 μg of GOX/mL. Right panel: GOX. The reaction mixture was the same as that in left panel. Key: ○, without nitrite; and ●, +0.3 mM $NaNO_2$. Each data point represents the mean with the SD ($n = 3-4$).

and 1 mM SCN^- inhibited the oxidation by 65 ± 18% ($n = 4$). The K_m value for the oxidation of SCN^- by salivary peroxidase at pH 8 was estimated to be about 1 mM in the presence of 10 μM H_2O_2 using the data in ref *36*. Thus, the inhibition by SCN^- indicated the participation of salivary peroxidase in bacterial fraction (*10, 18*) in the GOX-dependent oxidation of uric acid in the presence of nitrite. This interpretation is supported by the observation that the oxidation of uric acid (1 mM) by the glucose/GOX system in 50 mM sodium phosphate buffer (pH 7.0) did not occur in the absence of peroxidase.

In an acidified bacterial fraction (pH 5.3) with the glucose/GOX system, uric acid was oxidized at a rate of 46.2 ± 5.4 μM/h ($n = 3$) in the absence of nitrite (trace B-1). This oxidation was inhibited by approximately 90% by 1 mM SCN^-, indicating the participation of peroxidases present in the bacterial fraction (not shown), whereas it was enhanced by approximately 15% by addition of 0.3 mM nitrite (trace B-1). The addition of 0.3 mM nitrite alone to acidic bacterial fraction resulted in slow oxidation of uric acid, which was enhanced by GOX to a rate of 121.1 ± 16.3 μM/h ($n = 3$) (trace B-2). Subsequent addition of 1 mM SCN^- inhibited this rate by 75 ± 7% ($n = 7$), and 50% inhibition was observed at about 0.2 mM SCN^-. This partial inhibition can be understood if SCN^- inhibited NO_2 formation by reaction 6 but not by reaction 2.

The rate of uric acid oxidation in bacterial fraction (pH 5.3) increased with an increase in the concentration of nitrite in the absence of GOX (Figure 5, left panel). The rate was unaffected by 1 mM SCN^- but nearly completely inhibited 1 mM azide (not shown), indicating that N_2O_3 mainly contributed to uric acid oxidation under these conditions. GOX enhanced the rate of oxidation, and the effect was maximal at 0.3 mM nitrite. This was due to inhibition of GOX by nitrite when the concentration increased above 0.3 mM (*10*).

The right panel of Figure 5 shows GOX concentration dependence on uric acid oxidation in bacterial fraction (pH 5.3). In the absence of nitrite, the oxidation rate was maximal at 0.2 μg of GOX/mL. Endogenous peroxidase, in addition to the H_2O_2-generating system, may contribute to the oxidation of uric acid. Thus, the reduction of oxidation rate above 0.2 μg of GOX/mL may be explained by H_2O_2-induced inhibition of peroxidase present in the bacterial fraction. Nitrite (0.3 mM) enhanced the GOX-induced uric acid oxidation over the entire range of concentrations of GOX, indicating that nitrite relieved the inhibition of peroxidase.

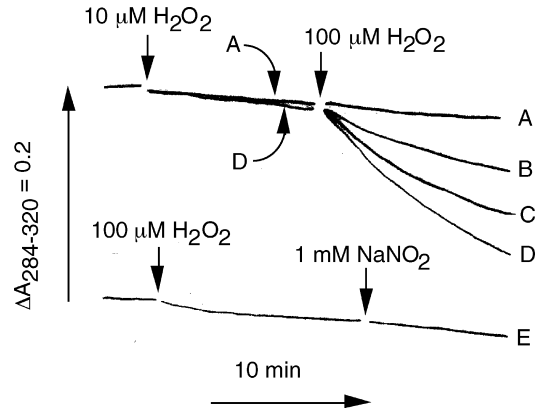

Figure 6. H_2O_2-dependent oxidation of uric acid. The reaction mixture (1 mL) contained 2 mM glucose, 0.1 mM uric acid, and various concentrations of nitrite in bacterial fraction (pH 5.3). (A) Without nitrite, (B) 0.1 mM $NaNO_2$, (C) 0.3 mM $NaNO_2$, and (D) 1 mM $NaNO_2$. H_2O_2 (10 or 100 μM) was added as indicated. (E) The bacterial fraction (pH 5.3) was incubated with 100 μM H_2O_2 plus 0.1 mM uric acid, and then, 1 mM $NaNO_2$ was added as indicated. One representative experiment of three is shown.

If H_2O_2 generated by the glucose/GOX system inhibited salivary peroxidase, the reagent H_2O_2 should also inhibit the activity, and the inhibition should be protected by nitrite. Figure 6 shows the effects of nitrite on H_2O_2-induced oxidation of uric acid in acidic bacterial fraction. In the absence of nitrite, slow oxidation of uric acid was observed following the successive addition of 10 and 100 μM H_2O_2 (trace A). However, 100 μM H_2O_2-induced uric acid oxidation became faster with an increasing nitrite concentration (traces A−D). In trace E, the bacterial fraction was incubated with 100 μM H_2O_2 for approximately

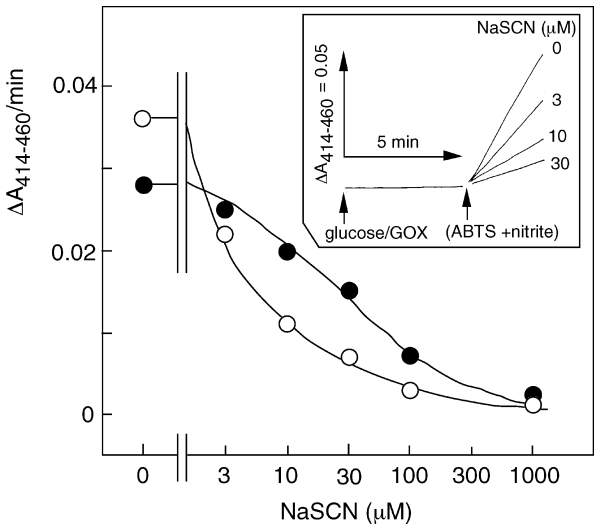

Figure 7. Inhibition of salivary peroxidase activity by H_2O_2/SCN^- systems. Inset: Typical time courses of ABTS oxidation. The reaction mixtures, which contained 0.05 mL of salivary peroxidase fraction, 2 mM glucose, 0.2 μg of GOX, and various concentrations of NaSCN in 1 mL of 50 mM KH_2PO_4 (pH 4.6), were incubated for 5 min, and then, 0.1 mM ABTS + 0.3 mM nitrite were added. Main panel: Effects of SCN^- concentrations on oxidation of ABTS. Key: ○, addition of 0.1 mM ABTS + 0.3 mM nitrite to bacterial fraction incubated with a glucose/GOX system as inset; and ●, addition of 0.1 mM ABTS to a bacterial fraction incubated with a glucose/GOX system in the presence of 0.3 mM nitrite.

10 min in the presence of uric acid before the addition of nitrite. Under these conditions, nitrite addition enhanced the oxidation of uric acid only slightly, as was also seen in Figure 4, trace B-1. This result suggests that H_2O_2 inhibited endogenous peroxidase activity in the bacterial fraction under acidic conditions in the absence of nitrite.

Participation of SCN^- in H_2O_2-Induced Inhibition of Salivary Peroxidase. The salivary peroxidase fraction was incubated with a glucose/GOX system in the absence of nitrite, and then, ABTS + nitrite was added as shown in the inset of Figure 7. The oxidation of ABTS was observed after the addition of ABTS + nitrite, and the oxidation was inhibited by approximately 50% by 3 μM SCN^- (○). The salivary peroxidase fraction was incubated with the glucose/GOX system in the presence of nitrite, and then, ABTS was added. The oxidation of ABTS observed after the addition of ABTS was inhibited by approximately 50% by 30 μM SCN^- (●). The results indicate (i) that H_2O_2/SCN^- inhibited salivary peroxidase activity and (ii) that nitrite protected the peroxidase from the inhibition, although nitrite slightly suppressed ABTS oxidation in the absence of SCN^-.

Hydrogen peroxide-induced oxidation of ABTS was studied using the bacterial fraction (pH 5.3), which contained 34 ± 7 μM SCN^- (n = 3). The oxidation of ABTS was not affected by nitrite (compare traces A and B in Figure 8), although nitrite partly inhibited its oxidation by salivary peroxidase fraction in the absence of SCN^- (see Figure 7). The different effects of nitrite might be due to the presence of SCN^- in the bacterial fraction. The incubation of the bacterial fraction with H_2O_2 resulted in the inhibition of peroxidase activity by approximately 60% (compare traces A and C). The inhibition was due to the presence of SCN^- or due to the decrease of H_2O_2 concentration during the incubation. The latter could be excluded by the result that addition of H_2O_2 to trace C did not significantly enhance the oxidation of ABTS. The bacterial fraction was incubated with H_2O_2 as trace C, and then, ABTS + nitrite was added (trace D). The oxidation of ABTS observed after the addition of ABTS + nitrite was slow, and the oxidation was enhanced by H_2O_2. The rate of the H_2O_2-enhanced ABTS oxidation in trace D was comparable to that of the oxidation of ABTS observed after the addition of H_2O_2 to trace C. The oxidation of ABTS in the bacterial fraction, which had been incubated in the presence of both H_2O_2 and nitrite (trace E), was faster than the oxidation of ABTS in trace D. The oxidation was also enhanced by H_2O_2, and the rate after the addition of H_2O_2 (trace E) was comparable to that of the H_2O_2-induced oxidation in trace A. Essentially the same results as Figure 8 were obtained when bacterial fraction (pH 5.3) was incubated with glucose/GOX systems. The above results also indicate that H_2O_2/SCN^- systems could inhibit salivary peroxidase activity and that nitrite could protect the peroxidase from the inhibition in acidic bacterial fraction.

Discussion

Nitrite induced the oxidation of uric acid in an acidic solution. The oxidation appeared to be due to NO_2 generated by reaction 2 because uric acid can be oxidized by oxidants including NO_2 (*38, 39*). Azide inhibited both the nitrite-induced oxidation of uric acid and the formation of NO. If azide scavenges N_2O_3 as shown below (*40, 41*), azide can inhibit the formation of both NO_2 and NO by reaction 2.

$$N_2O_3 + N_3^- + H^+ \rightarrow N_3NO + NO_2^- + H^+ \quad (7)$$

$$N_3NO \rightarrow N_2 + N_2O \quad (8)$$

Uric acid inhibited the nitrite-induced formation of NO-$Fe(DTCS)_2$, that is, the formation of NO (Figure 2), although Pietraforte et al. (*28*) reported that uric acid enhanced nitrite-induced formation of NO at pH 1.9. The absence of such an enhancement in the present study suggests that uric acid-dependent reduction of nitrite might be much slower at pH 5.2 than at pH 1.9. This follows from the fact that the concentration of nitrous acid (pK_a = 3.3) is much lower at pH 5.2 than at pH 1.9.

If NO and NO_2 are produced by reaction 2 in the presence of nitrite in acidic solution, uric acid radical is initially produced by reaction 9:

$$\text{uric acid} + NO_2 \rightarrow \text{uric acid radical} + NO_2^- \quad (9)$$

The uric acid radical can react rapidly with NO, producing nitroso derivatives of uric acid (*28, 39*). This occurrence would explain uric acid-dependent inhibition of the formation of NO-$Fe(DTCS)_2$. However, nitration/nitrosation products of uric acid were not detected, suggesting that the reaction of NO with uric acid radical (or other radicals derived from uric acid radical) was slow as compared to oxidative degradation of the radical. Further studies are required to elucidate how uric acid decreased the concentration of NO.

Uric acid (0.1 mM) was oxidized at a rate close to 20 μM/h in 50 mM KH_2PO_4 (pH 4.6) in the presence of 1.0 mM nitrite and 30 μM/h in acidified whole saliva filtrate (pH 4.7−4.8) containing approximately 0.14 mM uric acid and 1.2 mM nitrite. Azide inhibited uric acid oxidation by about 90% in both acidic solution and acidified whole saliva filtrate. The data indicate that oxidation of uric acid, which was induced by NO_2 formed by reaction 2, was as rapid in the acidified whole saliva filtrate as in acidic solution. Taking these ideas into consideration with the above result, we can propose that the production of NO_2 by reaction 2 can occur in dental plaque when the pH falls below

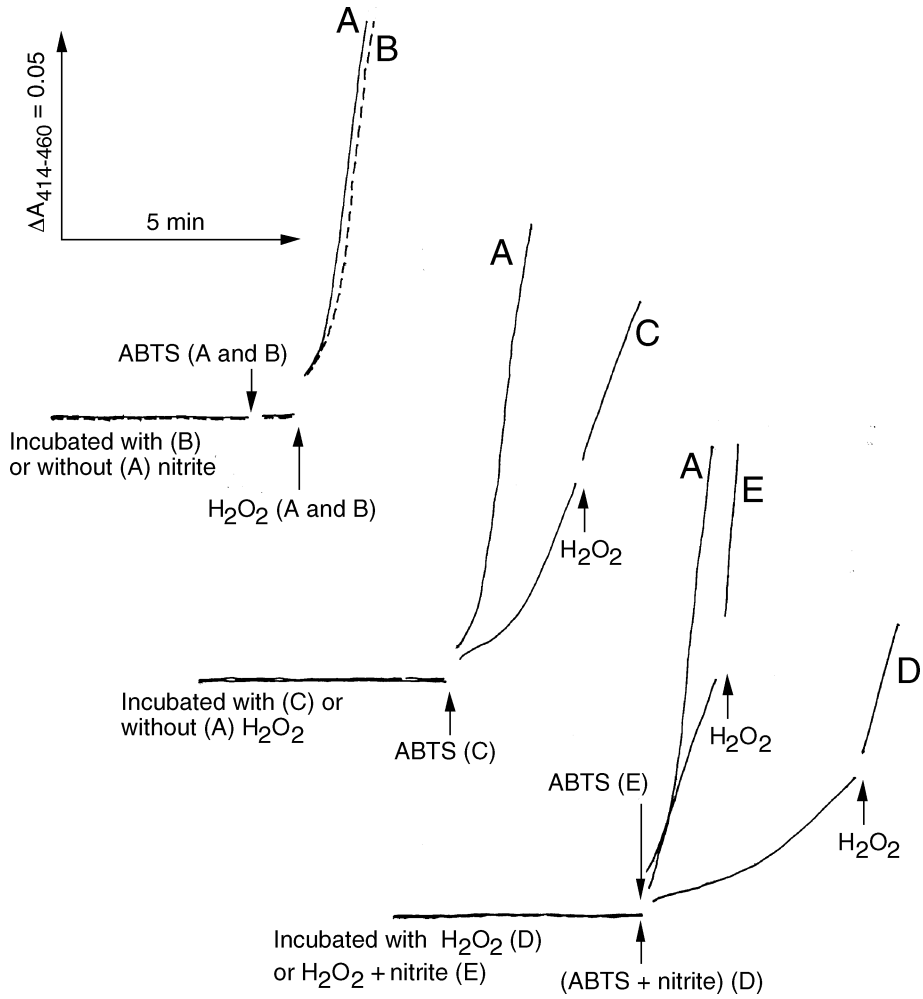

Figure 8. H_2O_2-induced inhibition of peroxidase activity. The bacterial fraction itself (pH 5.3) with various reagents was incubated for 4−5 min. Trace A, no addition; trace B, 0.3 mM nitrite; traces C and D, 0.1 mM H_2O_2; and trace E, 0.3 mM nitrite + 0.1 mM H_2O_2. Upward and downward arrows. Addition of 0.1 mM H_2O_2, 0.1 mM ABTS, or 0.1 mM ABTS + 0.3 mM nitrite.

5, as depicted in Figure 9. The existence of nitrate-reducing bacteria in dental plaque (*4*) and the higher concentration of nitrite in dental plaque than in saliva (*11, 12*) support this proposal. The more rapid formation of N_2O_3 or its equivalent at pH values near 5 than at pH 7 in bacterial fraction in the presence of nitrite (*42, 43*) also lends further support to the proposal. Saliva contains ascorbic acid and glutathione (*20*−*22*), but these may have been oxidized during the preparation of whole saliva filtrate, because the oxidation rate of uric acid in acidified whole saliva filtrate was similar to that in acidic solution.

In addition to reaction 2, the decomposition of $ONOOCO_2^-$ (reaction 5) could also contribute to the formation of NO_2. However, because CO_2 can react with $ONOO^-$ but not with ONOOH ($pK_a = 6.6$) (*44*), the formation of $ONOOCO_2^-$ would be expected to be slow at pH 5. Therefore, the formation of NO_2 by reaction 5 likely does not proceed under the acidic conditions of the present study.

At pH 7, GOX induced the oxidation of uric acid in the presence of nitrite but not in its absence (Figure 4). This result indicates that uric acid is not a good substrate for the peroxidase occurring in bacterial fraction and that uric acid was instead oxidized by NO_2 generated via peroxidase-catalyzed oxidation of nitrite (reaction 6). The peroxidase-catalyzed oxidation of nitrite to NO_2 near neutral pH has been reported for peroxidases from various sources (*17, 45*−*48*).

At pH 5, NO_2 generated by reaction 6 also contributed to uric acid oxidation in bacterial fractions in the presence of both nitrite and GOX. This is supported by the result that nitrite-induced uric acid oxidation was enhanced by GOX and that GOX-induced oxidation of uric acid was slower in the absence than in the presence of nitrite (Figures 4 and 5). Enhancement of GOX-induced uric acid oxidation by nitrite was greater at pH 5 than at pH 7 (Figure 4), suggesting more rapid generation of NO_2 by reaction 6 at pH 5 than at pH 7, as illustrated in Figure 9. It has been reported (i) that GOX induced nitration of 4-hydroxyphenylacetic acid in the presence of nitrite in bacterial fraction, (ii) that this nitration occurred much more rapidly at pH 5 than pH 7, and (iii) that the nitration was inhibited by uric acid (*10*). Because the nitration is related to the production of NO_2, those results support that generation of NO_2 by reaction 6 is faster at pH 5 than pH 7. The finding that salivary peroxidase-catalyzed oxidation of nitrite is faster at pH 5 than at pH 7 (*17*) lends even further support.

In the absence of nitrite, GOX-induced oxidation of uric acid was inhibited by high concentrations of GOX at pH 5 (Figure 5, right panel). The inhibition by GOX was prevented by the prior addition of nitrite to the reaction mixture (Figure 5, right panel). These results suggest that in the absence of nitrite, H_2O_2 generated by the GOX-catalyzed reaction participated not only in the peroxidase-catalyzed oxidation of uric acid but also in the inhibition of peroxidase activity. Because it has been reported

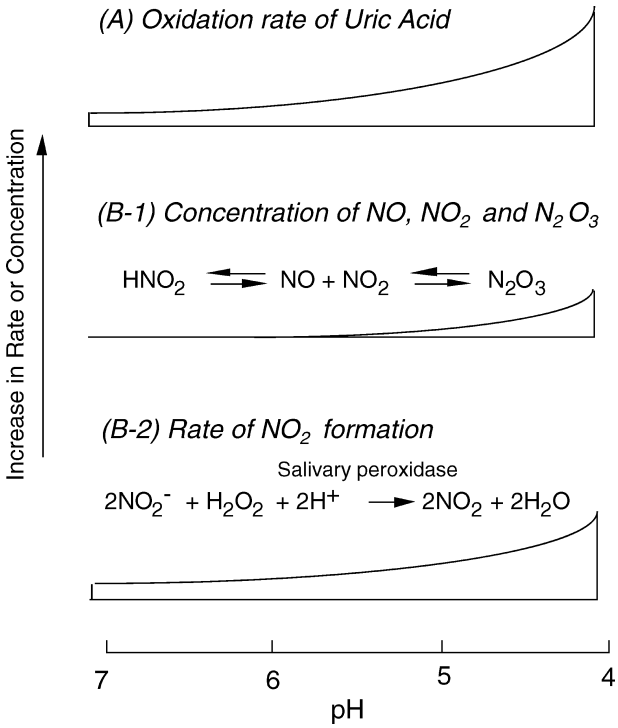

Figure 9. Oxidation rate of uric acid, concentrations of NO, NO_2, and N_2O_3, and rate of NO_2 formation with decrease in pH. (A) Oxidation rate of uric acid. (B-1) Concentrations of NO, NO_2, and N_2O_3. The concentrations of NO, NO_2, and N_2O_3 were estimated from reactions 1 and 2. (B-2) Rate of NO_2 formation. The rate of NO_2 formation was estimated from the rate of salivary peroxidase-catalyzed oxidation of uric acid. References *12*, *14*, *16*, *42*, and *43* were taken into consideration for the preparation of the figure.

that H_2O_2/SCN^- systems inhibit the activity of salivary peroxidase under acidic conditions (*36, 48*), we can postulate that SCN^- present at low concentrations in the bacterial fraction may contribute to the H_2O_2-dependent inhibition of peroxidase activity. In this study, the presence of SCN^- in bacterial fraction and the inhibition of salivary peroxidase by H_2O_2/SCN^- systems were shown (Figure 7). Nitrite protected salivary peroxidase from H_2O_2-dependent inhibition not only when uric acid was used as the substrate (Figures 4 and 5) but also when ABTS was used as the substrate (Figures 7 and 8). The protection may be due to competition between nitrite and SCN^- as the peroxidase substrate (*49*).

The above discussion suggests that nitrite may protect peroxidase present in acidic bacterial fraction from inhibition by H_2O_2/SCN^- systems. The concentration of SCN^- in dental plaque is estimated to be 0.030 ± 0.02 mM (*50*). We found approximately 50% inhibition of nitrite-dependent uric acid oxidation in bacterial fraction (pH 5.3) by 0.2 mM SCN^-, and 0.03 mM SCN^- inhibited peroxidase-catalyzed oxidation of ABTS by approximately 50% in the presence of nitrite (Figure 7). These results indicate that NO_2 can be produced in acidic dental plaque by reaction 6. Although SCN^- inhibits reactions catalyzed by salivary peroxidase, it can enhance nitrosation by peroxidase-independent reactions under acidic conditions (*42, 43*). However, the SCN^--dependent nitrosation by nitrite/SCN^- systems in acidic dental plaque would appear to be insignificant due to the low concentration of SCN^- in plaque (*50*).

If NO, NO_2, and N_2O_3 are produced in acidic dental plaque by reactions 2 and 6, a portion of the NO_2 and N_2O_3 might be scavenged via reduction and hydrolysis, while the remainder may diffuse away from the plaque. Thus, in the plaque developed in gingival crevices, diffusion of NO_2 and N_2O_3 produced in the plaque into adjacent gingival tissue is possible (Figure 10). NO remaining in dental plaque after NO_2 scavenging reactions may also diffuse into adjoining tissues, where it can be transformed to NO_2, N_2O_3, and $ONOO^-$ (Figure 10).

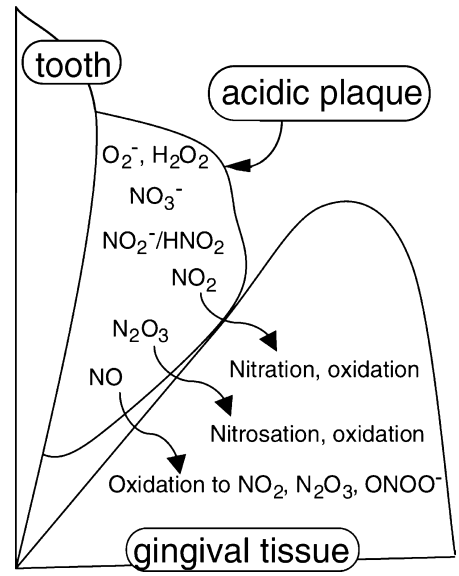

Figure 10. Diffusion of nitrogen oxides from acidic dental plaque into gingival tissue. Wavy lines, diffusion of each nitrogen oxide.

Accordingly, if plaque developed in gingival crevices is maintained at chronically low pH, this would increase the concentration of nitrite and enhance the production of nitrogen oxides in the plaque, allowing these nitrogen oxides to diffuse into the adjoining tissues, whereas if the plaque pH remains close to neutrality and the nitrite concentration is low, the generation of nitrogen oxides remains slow. Thus, the result of this study suggests that if concentrations of nitrite and H_2O_2 are elevated in acidic plaque developed in gingival crevices, gingival tissues in the crevices can be injured by nitrogen oxides formed in the acidic plaque.

References

(1) Zetterquist, W., Pedroletti, C., Lundberg, J. O., and Alving, K. (1999) Salivary contribution to exhaled nitric oxide. *Eur. Respir. J. 13*, 327–333.
(2) Pannala, A. S., Mani, A. R., Spencer, J. P. E., Skinner, Y., Bruckdorfer, K. R., Moore, K. P., and Rice-Evans, C. A. (2003) The effect of dietary nitrate on salivary, plasma and urinary nitrate metabolism in humans. *Free Radical Biol. Med. 34*, 576–584.
(3) Doel, J. J., Hector, M. P., Amirtham, C. V., Al-Anzan, L. A., Benjamin, N., and Allaker, R. P. (2004) Protective effect of salivary nitrate and microbial nitrate reductase activity against caries. *Eur. J. Oral Sci. 112*, 424–428.
(4) Doel, J. J., Benjamin, N., Hector, M. P., Rogers, M., and Allaker, R. P. (2005) Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur. J. Oral Sci. 113*, 14–19.
(5) Palmerini, C. A., Palombari, R., Perito, S., and Arienti, G. (2003) NO synthesis in human saliva. *Free Radical Res. 37*, 29–31.
(6) Takahama, U., Hirota, S., and Oniki, T. (2005) Production of nitric oxide-derived reactive nitrogen species in human oral cavity and their scavenging by salivary redox components. *Free Radical Res. 39*, 737–745.
(7) Marsh, P., and Martin, M. V. (1999) *Oral Microbiology*, 4th ed., Wright, Oxford, United Kingdom.
(8) Edger, M., and Higham, S. (2004) Saliva and the control of plaque pH. In *Saliva and Oral Health*, 3rd ed (Edger, M., Dawes, C., and O'Mullane, D., Eds.) pp72−86, British Dental Association, London.
(9) McNeill, K., and Hamilton, I. R. (2003) Acid tolerance response of biofilm cells *Streptococcus mutans*. *FEMS Microbiol. Lett. 221*, 25–30.
(10) Takahama, U., Imamura, H., and Hirota, S. (2009) Nitration of the salivary component 4-hydroxyphenylacetic acid in the human oral cavity: Enhancement of nitration under acidic conditions. *Eur. J. Oral Sci. 117*, 555–562.

(11) Carossa, S., Pera, P., Doglio, P., Lombardo, S., Colagrande, P., Brussino, L., Rolle, G., and Bucca, C. (2001) Oral nitric oxide during plaque deposition. *Eur. J. Clin. Invest. 31*, 876–879.
(12) Bayindir, Y. Z., Polat, M. F., and Seven, N. (2005) Nitirc oxide concentrations in saliva and dental plaque in relation to caries experience and oral hygiene. *Caries Res. 39*, 130–133.
(13) Zhang, H., Squadrito, G. L., and Pryor, W. A. (1997) The mechanism of the proxynitrite-carbon dioxide reaction probed using tyrosine. *Nitric Oxide 1*, 301–307.
(14) Lancaster, J. R., Jr. (2006) Nitroxidative, nitrosative, and nitrative stress: Kinetic predictions of reactive nitrogen species chemistry under biological conditions. *Chem. Res. Toxicol. 19*, 1160–1174.
(15) Halliwell, B., and Gutteridge, J. M. C. (1999) *Free Radicals in Biology and Medicine*, Oxford University Press, New York.
(16) Espey, M. G., Miranda, K. M., Pluta, R. M., and Wink, D. A. (2000) Nitrosative capacity of macrophages is dependent on nitric-oxide synthase induction signals. *J. Biol. Chem. 275*, 11341–11347.
(17) Hitora, S., Takahama, U., Ly, T. N., and Yamauchi, R. (2005) Quercetin-dependent inhibition of nitration induced by peroxidase/H_2O_2/nitrite systems in human saliva and characterization of an oxidation product of quercetin formed during the inhibition. *J. Agric. Food Chem. 53*, 3265–3272.
(18) Tenovuo, J. O. (1989) Nonimmunoglobulin defense factors in human saliva. In *Human Saliva: Clinical Chemistry and Microbiology* (Tenovuo, J. O., Ed.) Vol. 2, pp 55−91, CRC Press, Boca Raton.
(19) Marquis, R. E. (1995) Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. *J. Ind. Microbiol. 15*, 198–207.
(20) Zappacosta, B., Persichilli, S., De Sole, P., Mordente, A., and Giardina, B. (1999) Effect of smoking one cigarette on antioxidant metabolites in the saliva of healthy smoker. *Arch. Oral Biol. 44*, 485–488.
(21) Nagler, R. M., Klein, I., Zarzhevsky, N., Drigues, N., and Renznick, A. (2002) Characterization of the differentiated antioxidant profile of human saliva. *Free Radical Biol. Med. 32*, 268–277.
(22) Diab-Ladki, R., Pellat, B., and Chahine, R. (2003) Decrease in the total antioxidant activity of saliva in patients with periodontal diseases. *Clin. Oral Invest. 7*, 103–107.
(23) Fujii, S., Yoshimura, T., and Kamada, H. (1996) Nitric oxide trapping efficiencies of water-soluble iron (III) complexes with dithiocarbamate derivatives. *Chem. Lett.* 785–786.
(24) Kalyanaraman, B. (1996) Detection of nitric oxide by electron spin resonance in chemical, photochemical, cellular, physiological, and pathophysiological systems. *Methods Enzymol. 268*, 168–187.
(25) Skinner, K. A., White, C. R., Patel, R., Tan, S., Barnes, S., Kirk, M., Darley-Usmar, V., and Parks, D. A. (1996) Nirtrosation of uric acid by peroxynitrite. Formation of a vasoactive nitric oxide donor. *J. Biol. Chem. 272*, 24491–24497.
(26) Maples, K. R., and Mason, R. P. (1988) Free Radical metabolite of uric acid. *J. Biol. Chem. 263*, 1709–1712.
(27) Doherty, A. M. M., Garley, M. S., Haine, N., and Stedman, G. (1997) Formation of an adduct between thiocyanate ion and nitrosyl thiocyanate. *J. Chem. Soc., Dalton Trans.* 2163–2166.
(28) Pietraforte, D., Castelli, M., Metere, A., Scorza, G., Samoggia, P., Menditto, A., and Minetti, M. (2006) Salivary uric acid at the acidic pH of the stomach is the principal defense against nitrite-derived reactive species: Sparing effects of chlorogenic acid and serum albumin. *Free Radical Biol. Med. 41*, 175301763.
(29) Caulfield, J. L., Wishnok, J. S., and Tannenbaum, S. R. (1998) Nitric oxide-induced deamination of cytosine and guanine in deoxynucleosides and oligonucleosides. *J. Biol. Chem. 237*, 12689–12695.
(30) Espey, M. G., Thomas, D. D., Miranda, K., and M., Wink, D. A. (2002) Focusing of nitric oxide mediated nitrosation and oxidative nitrosylation as a consequence of reaction with superoxide. *Proc. Natl. Acad. Sci. U.S.A. 99*, 11127–11132.
(31) Lakshmi, V. M., Nauseef, W. M., and Zenser, T. V. (2005) Myeloperoxidase potentiates nitric oxide-mediated nitrosation. *J. Biol. Chem. 280*, 1746–1753.
(32) Wink, D. A., Nims, R. W., Darbyshire, J. F., Christodoulou, D., Hanbauer, L., Cox, G. W., Laval, F., Laval, J., Cook, J. A., Krishna, M. C., DeGraff, W. G., and Mitchell, J. B. (1994) Reaction kinetics for nitrosation of cysteine and glutathione in aerobic nitric oxide solution at neutral pH. Insights into the fate and physiological effects of intermediates generated in the NO/O_2 reaction. *Chem. Res. Toxicol. 7*, 519–525.
(33) *Dojindo Catalogue*, 25th ed., (2007) Dojindo Laboratories: Kumamoto, Japan, pp 154−156.
(34) Hiramoto, K., Tomiyama, S., and Kikugawa, K. (1997) Appearance of electron spin resonance signals in the interaction of dithoicarbamate-Fe(II) with nitrogen dioxide and nitrite. *Free Radical Res. 27*, 505–509.
(35) Vanin, A. F., Liu, X., Samouilov, A., Stukan, R. A., and Zwier, J. L. (2000) Redox properties of iron-dithiocarbamates and their nitrosyl derivatives: Implications for their use as traps of nitric oxide in biological systems. *Biochim. Biophys. Acta 1474*, 365–377.
(36) Mansson-Rahemtulla, B., Rahemtulla, F., Baldone, D. C., Pruitt, K. M., and Hjerpe, A. (1988) Purification and characterization of salivary peroxidase. *Biochemistry 27*, 233–239.
(37) Pruitt, K. M., Mansson-Rahemtulla, B., Baldone, D. C., and Rahemtulla, F. (1988) Steady-state kinetics of thiocyanate oxidation catalyzed by human salivary peroxidase. *Biochemistry 27*, 240–245.
(38) Ford, E., Hughes, M. N., and Wardman, P. (2002) Kinetics of the reaction of nitrogen dioxide with glutathione, cystein, and uric acid at physiological pH. *Free Radical Biol. Med. 32*, 1314–1323.
(39) Vasquez-Vivar, J., Santos, A. M., Junqueira, V. B. C., and Augusto, O. (1996) Peroxynitrite-mediated formation of free radicals in human plasma: EPR detection of ascrobyl, albumin-thiyl and uric acid-derived free radicals. *Biochem. J. 314*, 869–876.
(40) Galbraith, J. M., and Schaefer, H. F., III (1996) The nitrosyl azide potential energy hypersurface: A high-energy-density boom or burst? *J. Am. Chem. Soc. 118*, 4860–4870.
(41) Doherty, A. M. M., Dadcliffe, M. D., and Stedman, G. (1999) Kinetics of oxidation of nitrogen compounds by cerium (IV). *J. Chem. Soc., Dalton Trans.* 3311–3316.
(42) Takahama, U., Ryu, K., Oniki, T., and Hirota, S. (2007) Dual-function of thiocyanate on nitrite-induced formation of reactive nitrogen oxide species in human oral cavity: Inhibition under neutral and enhancement under acidic conditions. *Free Radical Res. 41*, 1289–1300.
(43) Takahama, U., Hirota, S., and Kawagishi, S. (2009) Effects of pH on nitrite-induced formation of reactive nitrogen oxide species and their scavenging by phenolic antioxidants in human oral cavity. *Free Radical Res. 43*, 250–261.
(44) Goldstein, S., Lind, J., and Merenyi, G. (2005) Chemistry of peroxynitrites as compared to peroxinitrates. *Chem. Rev. 105*, 2457–2470.
(45) van der Vliet, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1997) Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem. 272*, 7617–7625.
(46) Burner, U., Furtmuller, P. G., Kettle, A. J., Koppenol, W. H., and Obinger, C. (2000) Mechanism of reaction myeloperoxidase with nitrite. *J. Biol. Chem. 275*, 20597–20601.
(47) Bian, K., Gao, Z., Weisbrodt, N., and Murad, F. (2003) The nature of heme/iron-induced protein tyrosin nitration. *Proc. Natl. Acad. Sci. U.S.A. 100*, 5712–5717.
(48) Nishioka, T., Kimura, M., and Takahama, U. (1996) Effects of pH and thiocyanate on hydrogen peroxide-induced evolution of molecular oxygen in human saliva. *Arch. Oral Biol. 41*, 911–917.
(49) Takahama, U., Hirota, S., and Oniki, T. (2006) Thiocyanate cannot inhibit the formation of reactive nitrogen species in the human oral cavity in the presence of high concentration of nitrite: Detection of reactive nitrogen species with 4,5-diaminofluorescein. *Chem. Res. Toxicol. 19*, 1066–1073.
(50) Tenovuo, J., and Anttonen, T. (1980) Peroxidase-catalyzed hypothiocyanite production in human salivary sediment in relation to oral health. *Caries Res. 14*, 269–275.

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