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Reactions of thiocyanate in the mixture of nitrite and hydrogen peroxide under acidic conditions: Investigation of the reactions simulating the mixture of saliva and gastric juice

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Abstract

Nitrite and SCN^- in saliva can mixes with H_2O_2 in the stomach. The mixing can result in the formation of ONOOH. It is not yet known how salivary SCN^- reacts with ONOOH. An objective of the present study was to elucidate the reaction between ONOOH and SCN^-. In nitrite/H_2O_2 systems at pH 2, SCN^- inhibited the consumption of nitrite and the formation of NO_3^-. SCN^- enhanced the decomposition of ONOOH and H_2O_2 in HNO_2/H_2O_2 systems. Accompanying the reactions, sulfate was formed, suggesting that ONOOH oxidized SCN^-. SCN^- inhibited the nitration of phenolics induced by HNO_2/H_2O_2. The inhibition is discussed taking SCN^--dependent reduction of ONOOH to HNO_2 into consideration. SCN^- also inhibited H_2O_2-induced consumption of nitrite and nitration of phenolics in acidified saliva. The result obtained in this study suggests that salivary SCN^- can reduce ONOOH to NO_2^-/HNO_2 inhibiting nitrating reactions in the stomach.

Keywords: *Nitrous acid, peroxynitrous acid, saliva, SCN^-, SCN^--dependent reduction of peroxynitrous acid*

Abbreviations: *HPA, 4-hydroxyphenylacetic acid; NO_2HPA, 3-nitro-4-hydroxyphenylacetic acid*

Introduction

Nitrate is normally contained in diet. Dietary nitrate is absorbed from the small intestine into bloodstream [1]. The nitrate in the bloodstream is actively taken up by the salivary glands and secreted into mouth as a component of saliva [2]. The concentration of nitrate in saliva is about ten times higher than that in circulating blood [3]. The nitrate secreted into mouth is reduced to nitrite by buccal bacteria and the normal concentration in human saliva is $0.1 \sim 0.2$ mM [4–6]. The concentration increases to $1 \sim 2$ mM when nitrate rich diet is ingested [7]. In saliva, in addition to nitrite, SCN^- ($pKa = 0.9$), which is formed during detoxification of cyanide in human body, is also contained. Cyanide is incorporated into human body through smoking and ingestion of cyanogenic glycosides as foods [8,9]. SCN^- can also be generated from isothiocyanates that are formed by hydrolysis of glucosinolates contained in plants of Brassicaceae [10–12]. The concentration of SCN^- in saliva is about ten times higher than that in plasma [13] suggesting that SCN^- is also actively taken up by the salivary glands from bloodstream. Its concentration in salvia is around 1 mM [13].

Approximately 1.5 l of saliva (pH 7–8) is produced in a day and the nitrite- and SCN^--rich fluid mixes with acidic gastric juice (pH 2) at the proximal

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stomach. The mixing of saliva with gastric juice results in the protonation of nitrite to nitrous acid (p*K*a = 3.3) that can transform to NO, NO^+, NO_2 and N_2O_3. Thiocyanate is transformed to NOSCN by nitrous acid, and the NOSCN formed can enhance nitrous acid-induced nitrosation of amines [14–16]. Ascorbic acid, which is contained in gastric juice at a concentration of about 0.1 mM [17–19], can reduce nitrosating species NO^+, N_2O_3 and NOSCN to NO [16,20–22] and nitrating species NO_2 to NO_2^- [23]. The reduction of NOSCN by ascorbic acid is discussed to contribute to SCN^--induced enhancement of NO formation by HNO_2/ascorbic acid systems [16,24]. In addition to ascorbic acid, H_2O_2 is also contained in gastric juice at the concentration of 10–600 μM [25]. The formation of H_2O_2 by gastric epidermal cells and macrophages is enhanced when infected by *Helicobactor pylori* [26,27].

Hydrogen peroxide in gastric juice can be transformed to OH radicals by Fenton reaction [25] and the H_2O_2 can react with HNO_2 producing ONOOH (p*K*a = 6.8) that is a potent oxidizing and nitrating agent [23,28–30]. ONOOH can also contribute to nitrosation and hydroxylation [31–33]. It has been reported that SCN^- inhibits not only the nitration of 4-hydroxyphenylacetic acid (HPA) that is induced by HNO_2/H_2O_2 but also Fenton reaction-dependent hydroxylation of salicylic acid [29]. These results indicate that SCN^- can affect the nitration and hydroxylation under acidic conditions in the presence of H_2O_2, but there seems to be no sufficient information how SCN^- inhibits the nitration and hydroxylation in the mixture of saliva and gastric juice. A main objective of the present study is to elucidate the interactions among HNO_2, H_2O_2 and SCN^- under acidic conditions. The result obtained suggests that SCN^- could reduce ONOOH, which was formed by the reaction between H_2O_2 and HNO, to HNO_2 generating sulfate.

Materials and methods

Reagents

HPA, 4-nitrosophenol, 2-nitrophenol, 4-nitrophenol, catechol, and Griess-Romijn reagent for nitrite were obtained from Wako Pure Chemical Industries (Osaka, Japan). 3-Nitro-4-hydroxyphenylacetic acid (NO_2HPA) and 1,2-dihydroxy-4-nitrobenzene were from Aldrich (Milwaukee, USA). Catalase from bovine liver was from Roche Diagnostics GmbH (Mannheim, Germany).

Preparation of saliva

Mixed whole saliva (about 10 ml) was collected, when required, from three volunteers by chewing parafilm at about 10 a.m. after their informed consent had been obtained. The saliva obtained was centrifuged at 20,000*g* for 5 min and the supernatant was used as saliva preparation. Concentrations of nitrite and SCN^- in the saliva preparation were determined using Griess-Romijn reagent and Fe(III), respectively, as reported previously [23].

Determination of concentrations of NO_2^-/HNO_2, NO_3^- *and* SCN^-

Changes in concentrations of NO_2^-/HNO_2 induced by H_2O_2-dependent oxidation were determined using Griess-Romijn reagent. The mixture to determine the concentration of NO_2^-/HNO_2 (1 ml) contained 0.1 ml of 1% (w/v) Griess-Romijn reagent, 0.05 ml of sample and 0.85 ml of 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2.0). After the addition of sample, the mixture was incubated for 10 min at 25°C. The concentration of NO_2^-/HNO_2 was estimated by measuring the absorbance at 540 nm using a standard curve for 0 ~ 0.3 mM $NaNO_2$. Preparation of samples was described in the legend for figures.

We tried to separate and quantify NO_3^-, SCN^- and NO_2^-/HNO_2 by HPLC, and succeeded to separate and quantify the above compounds with a Shim-pack CLC_8 column (6 mm i.d. × 15 cm) (Shimadzu, Kyoto, Japan) under certain conditions. Then, changes in the concentrations of the above compounds were estimated at 210 nm using the above column combined with a spectrophotometric detector with a photodiode array (SPD-M10Avp) (Shimadzu). The mobile phase used was a mixture of methanol and 25 mM KH_2PO_4 (1:4, v/v), pH of which was adjusted to 3.0 by 1 M H_3PO_4, and the flow rate was 1 ml/min.

Measurements of oxidation product of SCN^-

As a final oxidation product of SCN^- was sulfate, we studied whether sulfate was formed or not by $HNO_2/-H_2O_2/SCN^-$ systems using $Ba(NO_3)_2$. It is well known that Ba^{2+} is transformed to insoluble $BaSO_4$ in the presence of sulfate. The reaction mixture (3 ml) contained 1 mM $NaNO_2$, 1 mM H_2O_2, 1 mM NaSCN and 2 mM $Ba(NO_3)_2$ in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2). $BaSO_4$ formed was measured by the increase in turbidity at 600 nm using an UV-240 spectrophotometer (Shimadzu).

Measurement of H_2O_2

The concentration of H_2O_2 was determined using an oxygen electrode (Rank Brothers, Cambridge, UK). The reaction mixture (1 ml) contained 0.1 or 0.2 mM $NaNO_2$ and 0.2 mM H_2O_2 in 1 ml of 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2.0). After incubation for defined periods, 1 ml of 0.2 M Na_2HPO_4 was added to terminate the reaction between HNO_2 and H_2O_2 (final pH about 7) and then 2600 units of catalase were added. The concentration of H_2O_2 remained in the reaction mixture was calculated from

the amount of oxygen evolved. The combination of oxygen electrode and catalase is commonly used to determine the concentration of H_2O_2.

Nitration

Nitration of HPA was studied in the reaction mixture (1 ml) that contained 1 mM HPA, 0.1 mM $NaNO_2$ and 0.1 mM H_2O_2 in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2.0). After incubation for a defined period, 25 μl of the reaction mixture was applied to an HPLC column. When the nitration was studied using saliva, the reaction mixture (1 ml) contained 1 mM HPA and 0.1 mM H_2O_2 in the mixture of 0.5 ml of saliva and 0.5 ml of 50 mM KH_2PO_4–50 mM KCl–HCl (pH 1.3). The final pH was 1.80 ~ 1.83. After incubation for a defined period, the reaction mixture was filtered using a cellulose acetate filter (0.45 μm; Advantec, Tokyo, Japan), and 25 μl of the filtrate was apply to an HPLC column. HPLC was performed at room temperature using a Shim-pack CLC-ODS column (6 mm i.d. × 15 cm) (Shimadzu) combined with a spectrophotometric detector with a photodiode array (SPD-M10Avp) (Shimadzu). NO_2HPA was separated using a mixture of methanol and 25 mM KH_2PO_4 (pH 4.5) (2:5, v/v) as a mobile phase. Flow rate of the mobile phase was 1 ml/min. NO_2HPA, which was separated by HPLC, was identified by comparing the retention time (10.6 min) and absorption spectrum (peaks; 215, 275 and 357 nm) with standard NO_2HPA.

Nitration, nitrosation and hydroxylation of phenol by $NaNO_2/H_2O_2$ systems were also studied under acidic conditions. The reaction mixture (1 ml) contained 1 mM phenol, 1 mM H_2O_2 and 1 mM $NaNO_2$ in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2.0). Products formed were also separated using a Shim-pack CLC-ODS column. The mobile phase used was a mixture of methanol and 25 mM KH_2PO_4 (pH 4.5) (1:1, v/v) and the flow rate was 1 ml/min. Catechol, 4-nitrosophenol, 2-nitrophenol and 4-nitrophenol were identified by comparing their retention times and absorption spectra with standard reagents. Reaction products in the above reaction mixture were also analyzed by an LC/MS spectrometer combined with a spectrophotometric detector with a photodiode array (1100 LC/MSD SL, Agilent Technologies). The mobile phase used was a mixture of acetonitrile and 5 mM ammonium acetate (1:3, v/v), pH of which was adjusted to 4.5 by acetic acid, and the flow rate was 1 ml/min.

Measurements of decrease in concentration of $ONOO^-$/ONOOH

Peroxynitrite (p*K*a = 6.8) was prepared by adding 0.4 ml of 200 mM $NaNO_2$ to 2.4 ml of 16.7 mM H_2O_2 in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2.0). About 1 s after the addition of $NaNO_2$, 2 ml of 1 M NaOH was added. The final pH was 13.1. The concentration of $ONOO^-$ in the alkaline mixture was estimated by adding 0.1 ml of the $ONOO^-$ preparation to 2 ml of H_2O (final pH 12.3). Molar absorption coefficient used for the estimation was 1.76 mM^{-1} cm^{-1} at 300 nm [34].

Decrease in concentration of $ONOO^-$/ONOOH was measured at 300 nm using a 557 spectrophotometer (Hitachi, Tokyo, Japan) at pH values between 7.6 and 12.3. Reactions were started by adding 0.1 ml of the $ONOO^-$ preparation to 2 ml of H_2O or 50 mM sodium phosphate (pH 7.2 ~ 7.7) in the presence of 0 ~ 5 mM NaSCN. The initial concentration of $ONOO^-$ was about 34 μM.

Data presentation

Since essentially the same result was obtained by two experiments, averages of the experiments were presented when reactions were performed in buffer solutions. Averages with SDs ($n = 3 \sim 7$) were presented when saliva was used.

Results

Effects of SCN^- on the consumption of nitrite

Figure 1 shows time courses of consumption of NO_2^-/HNO_2 in the presence and absence of H_2O_2 in a buffer solution at acidic pH values. The consumption was slow at all pH values examined in the absence of H_2O_2 and no significant effects of SCN^- on the consumption were observed. The consumption of NO_2^-/HNO_2 was significantly enhanced by H_2O_2 at all pH values examined. Rate of the consumption increased as pH was decreased, suggesting that the H_2O_2-dependent consumption of NO_2^-/HNO_2 may be due to oxidation of HNO_2 to ONOOH by H_2O_2. SCN^- (1 mM) inhibited the H_2O_2-induced consumption of NO_2^-/HNO_2. This result suggests that SCN^- reduced ONOOH to NO_2^-/HNO_2. Figure 2 shows effects of concentrations of $NaNO_2$, H_2O_2 and SCN^- on the rate of consumption of NO_2^-/HNO_2. Rate of the consumption increased as functions of $NaNO_2$ (panel A) and H_2O_2 (panel B). SCN^- (1 mM) significantly inhibited the consumption independent of the concentrations of $NaNO_2$ and H_2O_2. The inhibition by SCN^- increased as a function of concentration of SCN^- (panel C).

In the mixture of 0.5 ml of saliva and 0.5 ml of 50 mM KH_2PO_4–50 mM KCl–HCl (pH 1.3) (final concentrations of SCN^- and nitrite, 0.347 ± 0.050 and 0.077 ± 0.008 mM (mean ± SD, $n = 3$), respectively; final pH, about 1.8), no decrease in concentration of NO_2^-/HNO_2 was observed during incubation for 10 min. H_2O_2 (0.1 mM) enhanced the decrease, and time course of the decrease in concentration of NO_2^-/HNO_2 was similar to that at pH

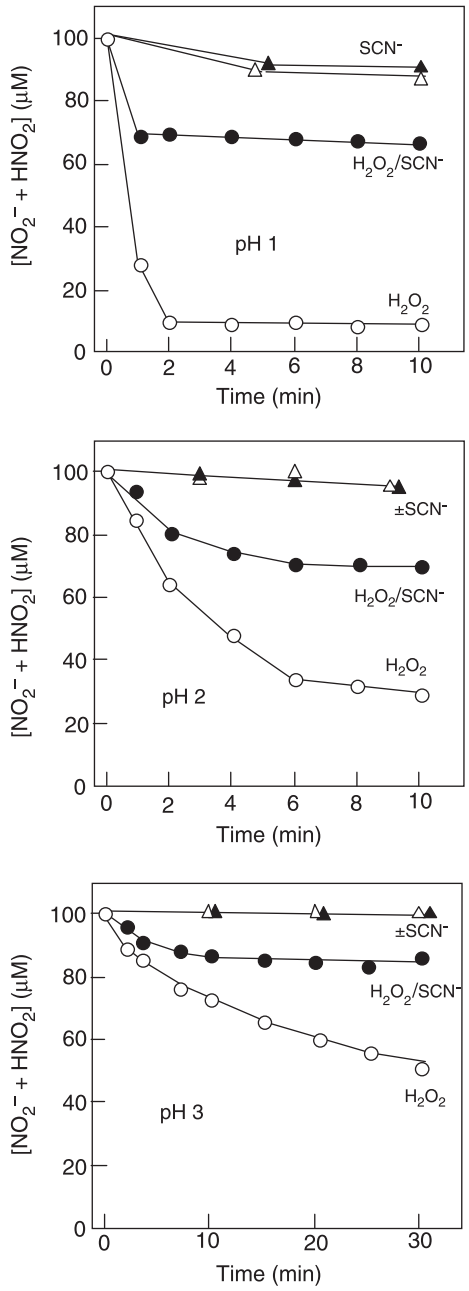

Figure 1. Inhibition of H_2O_2-dependent consumption of NO_2^-/HNO_2 by SCN^-. The reaction mixture (1 ml) contained 0.1 mM $NaNO_2$ in 1 ml of 50 mM KH_2PO_4–50 mM KCl–HCl (pH 1–3). After incubation for defined periods in the presence and absence of 0.1 mM H_2O_2 with or without 1 mM NaSCN, 0.05 ml of the reaction mixture was withdrawn from the mixture to determine the concentration of NO_2^-/HNO_2. △, no addition; ▲, NaSCN; ○, H_2O_2; ●, H_2O_2 + NaSCN. Upper panel, pH 1; middle panel; pH 2 and lower panel, pH 3.

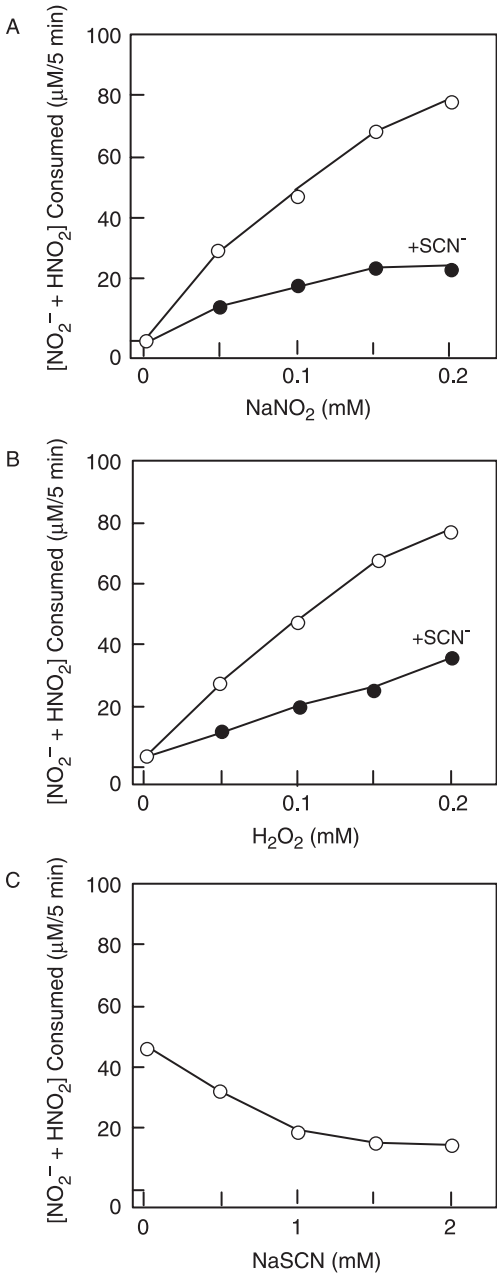

Figure 2. Effects of concentrations of $NaNO_2$, H_2O_2 and NaSCN on the consumption of NO_2^-/HNO_2. Panel A: Effects of concentration of $NaNO_2$. The reaction mixture (1 ml) contained 0.1 mM H_2O_2 and various concentrations of $NaNO_2$ in 1 ml of 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2). ○, no addition; ●, 1 mM NaSCN. Panel B: Effects of concentration of H_2O_2. The reaction mixture (1 ml) contained 0.1 mM $NaNO_2$ and various concentrations of H_2O_2 in 1 ml of the above buffer solution. ○, no addition; ●, 1 mM NaSCN. Panel C: Effects of concentration of NaSCN. The reaction mixture (1 ml) contained 0.1 mM $NaNO_2$, 0.1 mM H_2O_2 and various concentrations of NaSCN in the above buffer solution. After incubation for 5 min, 0.05 ml of the reaction mixture was withdrawn form the mixture to determine the concentration of NO_2^-.

2 in Figure 1. About 55% of the NO_2^-/HNO_2, which was equivalent to 0.042 ± 0.002 mM NO_2^-/HNO_2, was consumed during 10 min of incubation.

Reaction of SCN^- with ONOOH

If ONOOH, which is formed from H_2O_2 and HNO_2, is reduced by SCN^-, SCN^- may enhance the decomposition of ONOOH. Figure 3 shows time courses of the decrease in concentration of $ONOO^-$/ONOOH (p*K*a = 6.8) at various pH values. After the addition of $ONOO^-$ preparation to water (final pH

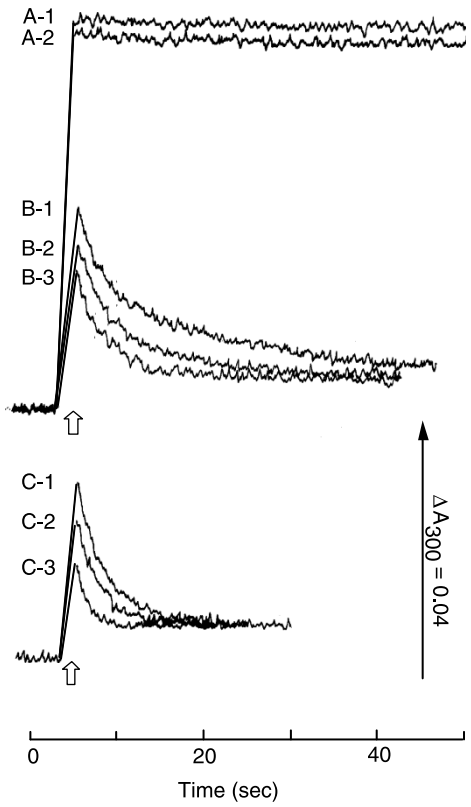

Figure 3. Time courses of decomposition of $ONOO^-$/ONOOH. One hundred μl of the $ONOO^-$ preparation was added to 2 ml of H_2O or 50 mM sodium phosphate (pH 7.6 and 7.4) and then absorbance changes were recorded at 300 nm. Traces A-1 and -2 (in H_2O; final, pH 12.3). A-1, no addition; A-2, 5 mM NaSCN. Traces B-1–B-3 [in 50 mM sodium phosphate (pH 7.6); final pH 8.5]. B-1, no addition; B-2, 1.5 mM NaSCN; B-3, 5 mM NaSCN. Traces C-1–C-3 [in 50 mM sodium phosphate (pH 7.4); final pH 8.0]. C-1, no addition; C-2, 1.5 mM NaSCN; C-3, 5 mM NaSCN. Arrows indicate the addition of the $ONOO^-$ preparation. It took about 3 s to start recording after the addition of $ONOO^-$.

12.3), absorbance increase, which was due to $ONOO^-$, was observed at 300 nm and the increased absorbance did not change during the incubation period (trace A-1). SCN^- (5 mM) did not significantly affect the absorbance change of $ONOO^-$ (trace A-2). When the $ONOO^-$ preparation was added to solutions of sodium phosphate (final pH 7.6 ~ 9.3), absorbance decrease at 300 nm was observed and rate of the decrease increased as pH was decreased. The addition of SCN^- resulted in the enhancement of the decrease in absorbance at all pH values examined. Typical effects of SCN^- are shown in traces B-1 ~ B-3 and traces C-1 ~ C-3 in Figure 3. The result indicates that SCN^- could enhance the decomposition of ONOOH but not $ONOO^-$.

Formation of NO_3^- *and consumption of* SCN^-

Accompanying the formation of ONOOH by H_2O_2/HNO_2 systems, NO_3^- should be formed [35,36]. Then, effects of SCN^- on the formation of NO_3^- were studied (Figure 4). NO_2^-/HNO_2 was detected as a single broad peak at a retention time

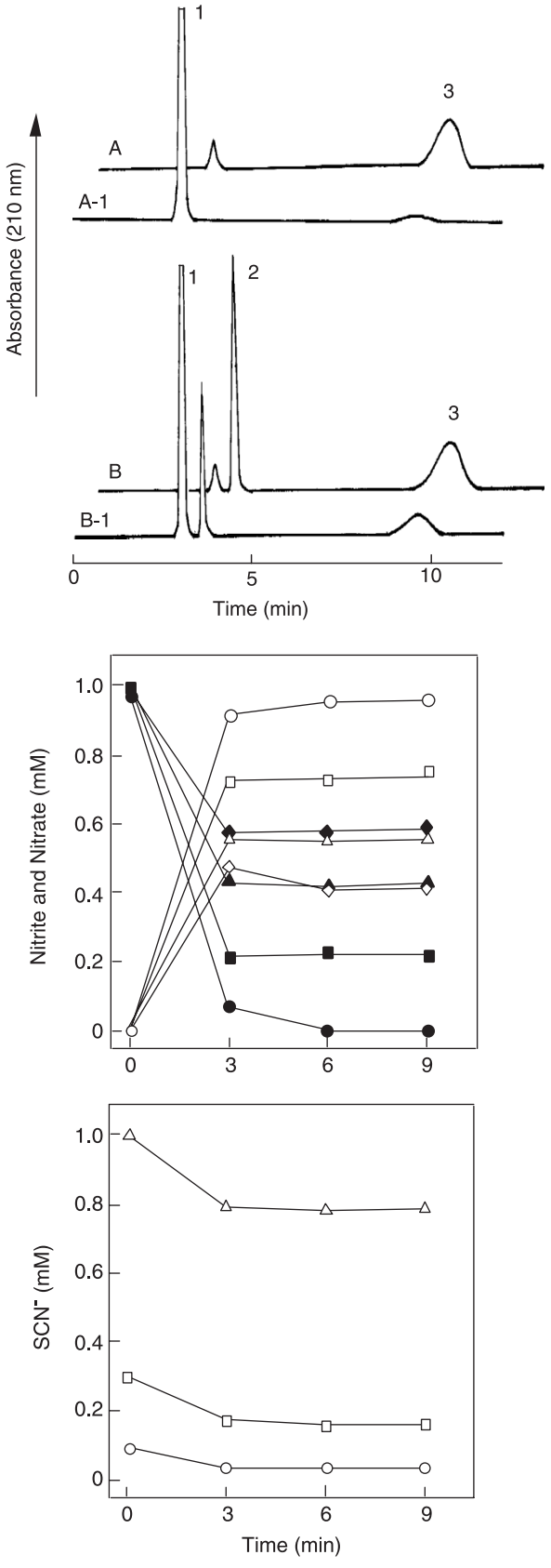

Figure 4. Formation of NO_3^- and consumption of NO_2^-/HNO_2 and SCN^-. Upper panel: Separation of NO_3^-, NO_2^-/HNO_2 and SCN^- by HPLC. Reaction mixtures were prepared using 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2). Trace A, immediately after the preparation of 1 mM $NaNO_2$; trace A-1, 3 min after the preparation

Table I. Effects of concentration of SCN^- on H_2O_2-induced consumption of NO_2^-/HNO_2 and SCN^- in $NO_2^-/HNO_2/SCN^-$ systems.

Concentration of SCN^- ⋆ (mM)	(1) NO_2^-/HNO_2 consumed† (mM)	(2) NO_3^- formed† (mM)	(3) SCN^- consumed† (mM)	(1) + (3) (mM)
0	1.0	0.95	0	1.0
0.1	0.78	0.75	0.07	0.85
0.3	0.58	0.55	0.13	0.71
1.0	0.41	0.40	0.21	0.62

The reaction mixture contained 1 mM H_2O_2, 1 mM $NaNO_2$ and various concentrations of NaSCN in 50 mM KH_2PO_4–50 mM KCl– HCl (pH 2.0) as in Figure 5. The concentrations of NO_2^-/HNO_2 consumed and SCN^- consumed were calculated from the data in Figure 5; ⋆ Initial concentration of NaSCN; † NO_2^-/HNO_2 and SCN^- consumed and NO_2^- formed by 1 mM H_2O_2 after 9 min of incubation.

of 9.5 min when an acidic solution of 1 mM $NaNO_2$ was applied to the HPLC column immediately after the preparation of the solution (Figure 4, upper panel; peak 3 in trace A). Incubation of the reaction mixture for 3 min, which contained 1 mM H_2O_2 and 1 mM $NaNO_2$, resulted in the decrease in the peak area of NO_2^-/HNO_2 and the formation of a new peak (peak 1 in trace A-1). The retention time (3.1 min) and the absorption spectrum of the new peak were identical with those of NO_3^-, indicating the transformation of NO_2^-/HNO_2 to NO_3^-. Time courses of formation of NO_3^- in the presence of 1 mM H_2O_2 and 1 mM $NaNO_2$ in an acidic buffer are shown in Figure 4 (lower panels). As the concentration of NO_2^-/HNO_2 was decreased (closed circles), the concentration of NO_3^- increased (open circles).

When the mixture of 1 mM NaSCN and 1 mM $NaNO_2$ was applied to the HPLC column, SCN^- (retention time, 3.6 min) was separated from NO_2^-/HNO_2 (Figure 4, upper panel; peak 2 in trace B). Incubation of the mixture for 3 min, which contained 1 mM H_2O_2, 1 mM $NaNO_2$ and 1 mM NaSCN in an acidic buffer (pH 2), resulted in the formation of NO_3^- and the decrease in the concentration of SCN^- (trace B-1). The formation of NO_3^- and the consumption of NO_2^-/HNO_2 decreased as the function of concentration of SCN^- (Figure 4, lower panels). The amount of NO_2^-/HNO_2 consumed was nearly the same as that of NO_3^- formed independent of the concentration of SCN^-. During SCN^--dependent inhibition of the formation of NO_3^- and the consumption of NO_2^-/HNO_2, the concentration of SCN^- decreased (Figure 4, lower panels). Although the decrease of the concentration of SCN^- was small, this result suggests that SCN^- was oxidized by HNO_2/H_2O_2 systems. Table I summarizes the data in Figure 4. In the absence of SCN^-, 1 mM NO_2^-/HNO_2 was transformed to about 1 mM NO_3^- consuming almost all H_2O_2 added. As the concentration of SCN^- was increased, sum of the amount of NO_2^-/HNO_2 oxidized to NO_3^- and the amount of SCN^- consumed decreased. For example, in the presence of 1 mM H_2O_2, 1 mM $NaNO_2$ and 1 mM NaSCN, about 0.4 mM HNO_2 was transformed to about 0.4 mM NO_3^- and about 0.2 mM SCN^- was consumed. This result suggests that about 0.6 mM H_2O_2 was used to oxidize HNO_2 and SCN^-. As almost all H_2O_2 added seemed to be consumed during the reaction (this is deduced from the data in the absence of SCN^- in Table I), the data in Table I indicate that not all H_2O_2 added was used to oxidize NO_2^-/HNO_2 and SCN^- in $H_2O_2/HNO_2/SCN^-$ systems.

The results in Figure 4 confirmed the results in Figures 1 and 2 that SCN^- inhibited H_2O_2-induced consumption of NO_2^-/HNO_2, which was observed using Griess-Romijn reagent, and the results in Figure 4 together the result in Figure 3 suggest that SCN^- could reduce ONOOH. If SCN^- reduces ONOOH to NO_2^-/HNO_2, oxidation products of SCN^- should be formed. Then, we studied whether oxidizing products of SCN^- were formed or not in $H_2O_2/HNO_2/SCN^-$ systems.

Formation of oxidation products of SCN⁻

It is known that an oxidation product of SCN^- is sulfate [38,39]. Then, we studied the formation of sulfate in $H_2O_2/HNO_2/SCN^-$ systems (Figure 5). The production of sulfate was confirmed by the formation of white precipitate when $Ba(NO_3)_2$ was present in the reaction mixture (pH 2) that contained 1 mM $NaNO_2$, 1 mM H_2O_2 and 1 mM NaSCN. If one of the above reagents was removed from the reaction mixture, no apparent absorption increases due to the formation of white precipitate were observed. The result indicates that SCN^- was transformed to sulfate. A lag period was observed for the formation of white precipitate. This may be due to slow aggregation of $BaSO_4$.

of the mixture of 1 mM $NaNO_2$ and 1 mM H_2O_2. Trace B, immediately after the preparation of the mixture of 1 mM $NaNO_2$ and 1 mM NaSCN; trace B-1, 3 min after the preparation of the mixture of 1 mM $NaNO_2$, 1 mM H_2O_2 and 1 mM NaSCN. Peak 1, NO_3^-; peak 2, SCN^-; peak 3, NO_2^-/HNO_2. Lower panels: Time courses of changes in concentrations of NO_3^-, NO_2^-/HNO_2 and SCN^-. Changes in concentration of NO_3^- and NO_2^-/HNO_2. The reaction mixture (1 ml) contained 1 mM $NaNO_2$, 1 mM H_2O_2 and various concentrations of NaSCN in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2). Closed symbols, NO_2^-/HNO_2; open symbols, NO_3^-. ○ and ●, 0 mM; □ and ■, 0.1 mM; △ and ▲, 0.3 mM; ◇ and ◆, 1 mM NaSCN. Changes in concentration of SCN^-. The reaction mixture contained 1 mM $NaNO_2$, 1 mM H_2O_2 and various concentrations of NaSCN in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2). ○, 0.1 mM; □, 0.3 mM; △, 1 mM NaSCN.

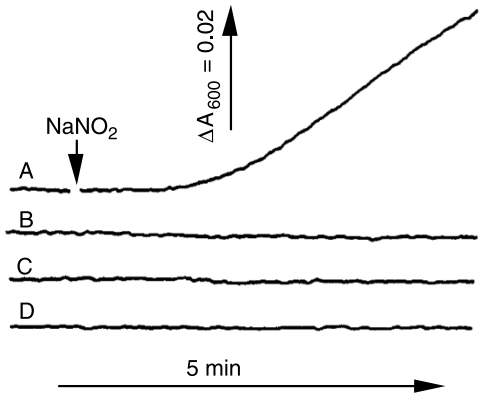

Figure 5. Formation of sulfate from SCN^-. The reaction mixture (2 ml) contained 1 mM $NaNO_2$, 1 mM H_2O_2, 1 mM NaSCN and 2 mM $Ba(NO_3)_2$ in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2) (trace A). An arrow in trace A indicates the addition of $NaNO_2$. Trace B, trace A without the addition of $NaNO_2$; trace C, trace A but the absence of H_2O_2; trace D, trace A but the absence of NaSCN.

Decomposition of H_2O_2

During H_2O_2-dependent consumption of NO_2^-/HNO_2, decomposition of H_2O_2 is possible. Then, changes in concentration of H_2O_2 in the reaction mixtures were studied in the presence and absence of SCN^- (Figure 6). Rate of the decrease in concentration of H_2O_2 was dependent on the concentration of NO_2^-/HNO_2. In contrast to the effect of SCN^- on the consumption of NO_2^-/HNO_2, SCN^- enhanced the decrease in concentration of H_2O_2. When ratio of the initial concentration of H_2O_2 to that of $NaNO_2$ was 1, about 90% of H_2O_2 was decomposed during 5 min of incubation in the presence of 1 mM SCN^-. No significant decrease

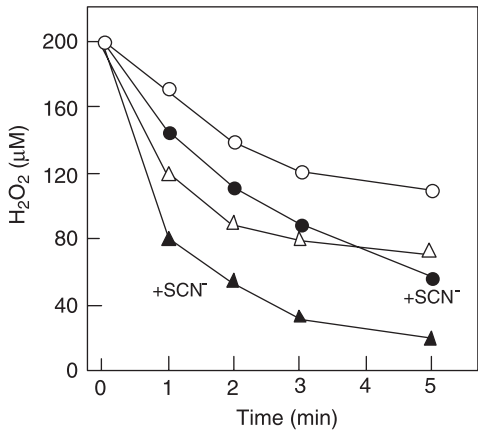

Figure 6. Enhancement of H_2O_2 consumption by SCN^-. The reaction mixture (1 ml) contained 0.2 mM H_2O_2 in 1 ml of 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2). After incubation for defined periods, 1 ml of 0.2 M Na_2HPO_4 and 2600 units of catalase were added successively to estimate H_2O_2 remained. ○, 0.1 mM $NaNO_2$; ●, 0.1 mM $NaNO_2$ + 1 mM NaSCN; △, 0.2 mM $NaNO_2$; ▲, 0.2 mM $NaNO_2$ + 1 mM NaSCN.

of H_2O_2 was observed in the absence of NO_2^-/HNO_2 independent of the presence and absence of SCN^- (data not shown).

Nitration of phenolics

It has been reported that tyrosine and HPA are nitrated in a mixture of nitrite and H_2O_2 under acidic conditions [27,28]. Table II shows that SCN^- effectively inhibited the nitration of HPA induced by 0.1 mM $NaNO_2$ and 0.1 mM H_2O_2 in an acidic buffer solution (pH 2). Ascorbic acid also inhibited the nitration. When 0.1 mM H_2O_2 was added to the mixture of 0.5 ml of saliva and 0.5 ml of the acidic buffer (final pH, about 1.8), slow formation of NO_2HPA was observed.

As ONOOH could participate in hydroxylation and nitrosation in addition to nitration [31–33], effects of SCN^- on nitration, hydroxylation and nitrosation of phenol were studied at pH 2 (Figure 7). In an acidic mixture of phenol and $NaNO_2$, 4-nitrosophenol was detected (peak 2 in trace B). No significant formation of nitrated phenols was observed (peak 6, 4-nitrophenol; peak 7, 2-nitrophenol). Peak 3 in trace B was identified to be NO_2^-/HNO_2 from its retention time and absorption spectrum. SCN^- did not significantly affect the nitrosation (compare trace C with trace B). 2-Nitrophenol and 4-nitrophenol were main products and 4-nitrosophenol and catechol (peak 1) were minor products in the acidic mixture of phenol, $NaNO_2$ and H_2O_2 (trace D). In addition to the above components, peak 5 (absorption maxima; 214, 242, 282 and 399 nm) was detected. Absorption spectrum of peak 3 in trace D (absorption maxima;

Table II. H_2O_2-induced nitration of HPA in a buffer solution and acidified saliva.

	NO_2HPA formed (μM/4 min)
In buffer solution*	
No addition	0.0
0.1 mM H_2O_2	5.6
0.1 mM H_2O_2 + 1 mM SCN^-	0.4
0.1 mM H_2O_2 + 0.1 mM ascorbic acid	0.0
0.1 mM H_2O_2 + 1 mM SCN^- + 0.1 mM ascorbic acid	0.1
Acidified saliva†	
No addition	0.0
0.1 mM H_2O_2	0.17 ± 0.06

* The reaction mixture (1 ml) contained 1 mM HPA and 0.1 mM $NaNO_2$ in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2.0). After incubation for 4 min, the concentration of NO_2HPA formed was determined by HPLC. Values are average of two experiments; † The reaction mixture (1 ml) contained 0.5 ml of saliva and 0.5 ml of 50 mM KH_2PO_4–50 mM KCl–HCl (pH 1.3). The pH of the reaction mixture was about 1.8, and the concentrations of nitrite and SCN^- in the reaction mixture were 0.11 ± 0.07 and 0.34 ± 0.08 mM (mean ± SD), respectively ($n = 7$). After incubation for 4 min, the concentration of NO_2HPA was determined by HPLC. The value is mean ± SD ($n = 7$).

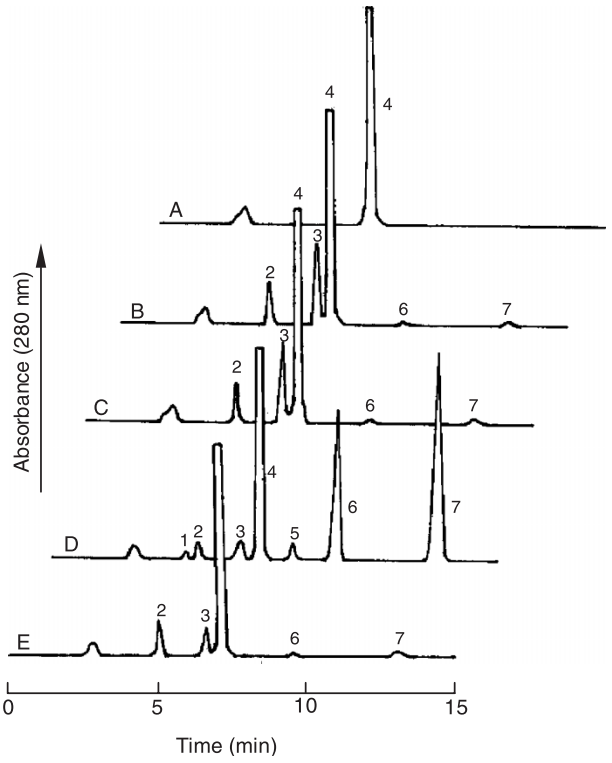

Figure 7. Effects of SCN^- on nitration, nitrosation and hydroxylation of phenol. The reaction mixture (1 ml) contained 1 mM phenol in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2). After incubation for 4 min, 0.1 ml of the reaction mixture was directly applied to HPLC column. A, no addition; B, 1 mM $NaNO_2$; C, 1 mM $NaNO_2$ + 1 mM NaSCN; D, 1 mM $NaNO_2$ + 1 mM H_2O_2; E, 1 mM $NaNO_2$ + 1 mM H_2O_2 + 1 mM NaSCN. Peak 1, catechol; peak 2, 4-nitrosophenol; peak 3, nitrite (traces B, C and E) or 3,4-dihydroxynitrobenzene (trace D); peak 4, phenol; peak 5, isomer of 3,4-dihydroxynitrobenzene; peak 6, 4-nitrophenol; peak 7, 2-nitrophenol.

210, 242 and 348 nm) was different from that of NO_2^-/HNO_2. SCN^- significantly inhibited the formation of nitrated products catechol and peak 5, and slightly enhanced the formation of 4-nitrosophenol (trace E). Peak 3 in trace E was NO_2^-/HNO_2. This result indicates that the component with absorption maxima at 210, 242 and 348 nm trance D was also inhibited by SCN^-.

When the reaction mixture for trace D in Figure 7 was analyzed in the mobile phase for LC/MS, peaks 3 and 5 were detected at retention times of 8.4 and 10.7 min, respectively. Molecular ion of the two components was *m/z* 154 [$(M\text{-}H)^-$]. The component of peak 3 was identified to be 3,4-dihydroxynitrobenzene (molecular weight, 155) by comparing retention time and absorption spectrum with standard reagent. The component of peak 5 was estimated to be an isomer of 3,4-dihydroxynitrobenzene from its molecular ion. Inhibition of the formation of the nitrated catechols by SCN^- was confirmed in the mobile phase for LC/MS.

Discussion

It is known that HNO_2 reacts with H_2O_2 producing ONOOH under acidic conditions [28–30]. The ONOOH formed is transformed to $NO_3^- + H^+$ by intermolecular rearrangement or decomposed to $\cdot NO_2$ and $\cdot OH$ or other products including molecular oxygen [30,35,36]. In acidic solutions (pH < 3), the major product formed from ONOOH is $NO_3^- + H^+$[35,36]. SCN^- inhibited not only H_2O_2-dependent decreases in the concentration of NO_2^-/HNO_2 but also H_2O_2-dependent formation of NO_3^- (Figures 1, 2 and 4). On the other hand, SCN^- was oxidized by HNO_2/H_2O_2 systems (Figure 4) and SCN^- enhanced the decomposition of ONOOH but not $ONOO^-$ (Figure 3). The above results indicate that SCN^- could reduce ONOOH to NO_2^-/HNO_2, which might be formed by HNO_2/H_2O_2 systems at pH 2. Hydrogen peroxide-dependent enhancement of the decomposition of NO_2^-/HNO_2 was also observed in acidified saliva. This result suggests that the formation of ONOOH is possible in the mixture of saliva and gastric juice if H_2O_2 is present. The decrease in the concentration of salivary nitrite (about 55% after 10 min of incubation) was smaller than that expected from Figure 1 in a buffer solution (more than 70% after 10 min of incubation) at pH 1.8. This might be due to the reduction of ONOOH to NO_2^-/HNO_2 by a salivary reductant like SCN^-.

The concentration of SCN^- decreased when SCN^- was inhibiting H_2O_2-induced formation of NO_3^- (Figure 4) and sulfate was formed in the $HNO_2/H_2O_2/SCN^-$ system (Figure 5). The result indicates that SCN^- was oxidized to sulfate by ONOOH. The data in Table I indicate that not all H_2O_2 added was used to decrease the concentration of NO_2^-/HNO_2 and SCN^-, and the data in Figure 6 shows that SCN^- enhanced the decomposition of H_2O_2. Taking the above results and oxidation of SCN^- to sulfate into consideration, we can deduce that H_2O_2 was not only used to oxidize HNO_2 to ONOOH but also used to oxidize the oxidation intermediates of SCN^-. Possible oxidation intermediates are HOSCN (p*K*a = 5.5) and $SCN\cdot$ formed by the following reactions:

$$ONOOH + SCN^- + H^+ \rightarrow HNO_2 + HOSCN \quad (1)$$

$$ONOOH + SCN^- + H^+ \rightarrow \cdot NO_2 + SCN\cdot + H_2O \quad (2)$$

The $SCN\cdot$ formed may dimerize to $(SCN)_2$ that can be hydrolyzed [37,38];

$$(SCN)_2 + H_2O \rightarrow HOSCN + SCN^- + H^+. \quad (3)$$

If HOSCN is produced under acidic conditions, HOSCN can react with H_2O_2 as following [39,40]:

$$HOSCN + H_2O_2 \rightarrow HOOSCN + H_2O \quad (4)$$

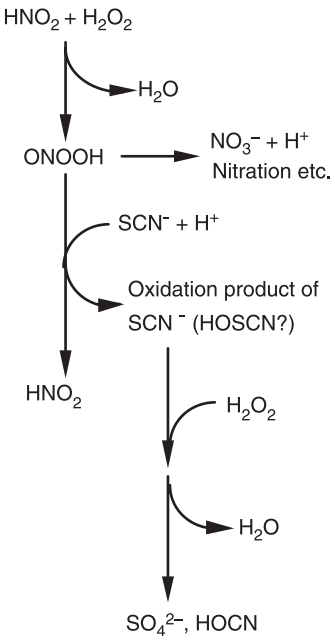

Figure 8. Possible reactions in the mixture of HNO_2, H_2O_2 and SCN^-. For details, see text.

$$HOOSCN + H_2O_2 \rightarrow HSO_3^+ + H^+ + HOCN \quad (5)$$

$$HSO_3^- + H_2O_2 + H^+ \rightarrow SO_4^{2-} + 2H^+ + H_2O \quad (6)$$

If the above reactions proceed, consumption of small amounts of SCN^- during the inhibition of H_2O_2-dependent oxidation of NO_2^-/HNO_2 and SCN^--dependent enhancement of H_2O_2 consumption can be explained. HOCN formed by reactions (5) can be hydrolyzed to carbon dioxide and ammonia. The latter is transformed to ammonium ion under acidic conditions. In addition to H_2O_2-dependent oxidation of HOSCN, ONOOH-dependent oxidation of HOSCN cannot be excluded although we did not have any evidence for the reaction.

Nitration of HPA and phenol, which was induced by H_2O_2/HNO_2 systems, was effectively inhibited by SCN^- in an acidic buffer solution (Table II and Figure 7). If ONOOH oxidized phenolics, radicals of the phenolics and $\cdot NO_2$ are formed [30]. The two radical species react each other generating nitrated phenolics. SCN^- can inhibit the ONOOH-dependent nitration by scavenging the oxidant by reactions (1) and/or (2). It is possible that $\cdot NO_2$ formed by reaction (2) oxidizes phenolics to the radicals that can react with another molecule of $\cdot NO_2$ [41]. As SCN^- has been reported not to be effective enough to scavenge $\cdot NO_2$ [42], effective inhibition of nitration reactions by SCN^- (Table II and Figure 8) suggests that reaction (2) seems not to be a major reaction to scavenge ONOOH. If ONOOH decomposes to $\cdot NO_2$ and $\cdot OH$ [30,36], $\cdot OH$ can oxidize phenolics to the radicals. The radicals of phenolics may react with $\cdot NO_2$ producing nitrated phenolics. SCN^- can inhibit the nitration of phenolics induced by $\cdot OH$ and $\cdot NO_2$ by scavenging $\cdot OH$ [30]. The result that SCN^- inhibited the hydroxylation of phenol to catechol (Figure 7) suggests that SCN^- can scavenge $\cdot OH$ if the radical is generated. When SCN^- scavenges $\cdot OH$, $(SCN)_2$, which can be hydrolyzed by reaction (3), may be formed. As the mechanism for formation of nitrated catechols, nitration of catechol and/or hydroxylation of nitrated phenols are possible. Kinetical studies are required to distinguish between the two reactions.

Nitrosation of phenol was induced by NO_2^-/HNO_2 (Figure 7). H_2O_2 significantly inhibited the nitrosation, suggesting that H_2O_2 suppressed the formation of nitrosating species by reacting with HNO_2. SCN^- enhanced the nitrosation of phenol in the presence of both H_2O_2 and HNO_2. The amount of 4-nitrosophenol formed in the $SCN^-/H_2O_2/HNO_2$ system (trace E) was similar to that of 4-nitrosophenol formed in the SCN^-/HNO_2 system (trace C). This result indicates that nitrosation of phenolics was not significantly affected by H_2O_2 in the presence of SCN^-.

Figure 8 summarizes reactions that may proceed in the mixture of HNO_2, H_2O_2 and SCN^-. ONOOH formed from HNO_2 and H_2O_2 is reduced by SCN^- producing HNO_2 and oxidation product of SCN^-. H_2O_2 seems to oxidize the oxidation intermediates of SCN^- to sulfate. The H_2O_2-dependent oxidation of the oxidation intermediates of SCN^- to sulfate may contribute to the enhanced consumption of H_2O_2. The SCN^--dependent enhancement of the decomposition of H_2O_2 can result in the inhibition of $\cdot OH$ formation by Fenton reaction. It has been reported that SCN^- inhibits hydroxylation of 2-hydroxybenzoic acid by H_2O_2/Fe(II) in the presence of NO_2^-/HNO_2 [29]. SCN^- strongly inhibited ONOOH-induced nitration of phenolics. Rate of ONOOH-induced nitration of HPA was quite slow in acidified saliva. The slow nitration in acidified saliva suggests that salivary SCN^- can also participate in the reduction of ONOOH that may be formed in the mixture of saliva and gastric juice. The inhibitory effects of SCN^- were comparable to those of ascorbic acid, supporting that SCN^- was also a potent inhibitor of the nitration in the stomach as well as in the oral cavity [43]. In this way, SCN^- in the mixture of saliva and gastric juice seems to have important roles to protect stomach from oxidative damages by reducing ONOOH and H_2O_2 when H_2O_2 formation in the stomach is enhanced by infection etc.

References

[1] McColl KEL. When saliva meets acid: Chemical warfare at the oesophagogastric junction. Gut 2005;54:1–3.

[2] Bartholomew B, Hill MJ. The pharmacology of dietary nitrite and the origin of urinary nitrate. Food Chem Toxicol 1984; 22:789–795.

[3] Mcnight GM, Smith LM, Drummond RS, Duncan CW, Golden M, Benjamin N. Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. Gut 1997;40: 211–214.
[4] Granli T, Dahl R, Brodin P, Granli T, Dahl R, Brodin P, Bockman OC. Nitrate and nitrite concentrations in human saliva; variations with salivary flow-rate. Food Chem Toxicol 1989;27:675–680.
[5] van Maanen JM, van Geel AA, Kleinjans JC. Modulation of nitrate–nitrite conversion in the oral cavity. Cancer Detect Prev 1996;20:590–596.
[6] Ruddell WSJ, Blendis LM, Walters CL. Nitrite and thiocyanate in the fasting and secreting stomach and in saliva. Gut 1977;18:73–77.
[7] Pannal AS, Mani AR, Spencer JPE, Skinner V, Bruckdorfer KR, Moore KP, Rice-Evans CA. The effect of dietary nitrate on salivary, plasma and urinary nitrate metabolism in humans. Free Radic Biol Med 2003;34:576–584.
[8] Heaney RK, Fenwick GR. Natural toxins and protective factors in Brassica species, including rapeseed. Nat Toxins 1995;3:233–237.
[9] Seawright AA. Directly toxic effects of plants chemicals which may occur in human and animals foods. Nat Toxins 1995;3: 227–232.
[10] Srivastava VK, Hill DC. Thiocyanate ion formation in rapeseed meals. Can J Biochem 1975;53:630–633.
[11] Borek V, Morra MJ. Ionic thiocyanate (SCN^-) production from 4-hydroxybenzyl glucosinolate contained in *Sinapis alba* seed meal. J Agric Food Chem 2005;53:8650–8654.
[12] Heaney RK, Fenwick GR. Methods for glucosinolate analysis. In: Waterman PG, editor. Method in plant biochemistry., Vol. 8 London: Academic Press; 1993. p 531–550.
[13] Tsuge K, Kataoka M, Seto Y. Cyanide and thiocyanate levels on blood and saliva of healthy adult volunteers. J Health Sci 2000;46:343–350.
[14] Boyland E, Walker SA. Effect of thiocyanate on nitrosation of amines. Nature 1974;248:601–602.
[15] Fan T-Y, Tannenbaum SR. Factors influencing the rate of formation of nitrosomorpholine for morpholine and nitrite: Acceleration by thiocyanate and other anions. J Agric Food Chem 1973;21:237–240.
[16] Licht WR, Tannenbaum SR, Deen WM. Use of ascorbic acid to inhibit nitrosation: Kinetic and mass transfer consideration for an *in vitro* system. Carcinogenesis 1988;9:365–372.
[17] Rathbone BJ, Johnson AW, Wyatt JI, Kelleher J, Heatley R, Losowsky MS. Ascorbic acid: A factor concentrated in human gastric juice. Clin Sci 1989;76:237–241.
[18] Waring AJ, Drake IM, Schorah CJ, White KLM, Lynch DAF, Axon ATR, Dixon MF. Ascorbic acid and total vitamin C concentrations in plasma, gastric juice, and gastrointestinal mucosa: Effects of gastritis and oral supplementation. Gut 1996;38:171–176.
[19] Dabrowska-Ufniarz E, Dzieniszewski J, Jarosz M, Wartanowicz M. Vitamin C concentration in gastric juice in patients with precancerous lesions of stomach and gastric cancer. Med Sci Monit 2002;8:CR96–CR103.
[20] Mirvish SS, Wallcave L, Eagan M, Shubik P. Ascorbate-nitrite reaction: Possible means of blocking the formation of carcinogenic *N*-nitroso compounds. Science 1972;177:65–67.
[21] Archer MC, Tannenbaum SR, Fan T-Y, Weisman M. Reaction of nitrite with ascorbate and its relation to nitrosamine formation. J Natl Cancer Inst 1975;54:1203–1205.
[22] Mirvish SS. Experimental evidence for inhibition of *N*-nitroso compound formation as a factor in the negative correlation between vitamin C consumption and the incidence of certain cancers. Cancer Res 1994;54:1948s–1951s.
[23] Takahama U, Hirota S, Yamamoto A, Oniki T. Oxygen uptake during the mixing of saliva with ascorbic acid under acidic conditions: Possibility of its occurrence in the stomach. FEBS Lett 2003;550:64–68.
[24] Huie RE. The reaction kinetics of NO_2. Toxicology 1994;89: 193–216.
[25] Nalini S, Ramakrishna BS, Mohanty A, Balasubramanian KA. Hydroxyl radical formation in human gastric juice. J Gastroenterol Hepatol 1992;7:497–501.
[26] Chaturvedi R, Cheng Y, Asim M, Bussiere FI, Xu H, Gobert AP, Hacker A, Casero RA, Wilson K. Induction of polyamine oxidase 1 by *Hericobactor pylori* causes macrophage apoptosis by hydrogen peroxide release and mitochondrial membrane deporalization. J Biol Chem 2004;279: 40161–40173.
[27] Xu H, Chaurvedi R, Cheng Y, Bussiere FI, Asim M, Yao MD, Potosky D, Meltzer SJ, Rhee JG, Kim SS, Moss SF, Hacker A, Wang Y, Casero RB, Wilson KT. Spermine oxidation induced by *Helicobactor pylori* results in apoptosis and DNA damage: Implication for gastric carcinogenesis. Cancer Res 2004;64: 8521–8525.
[28] Oury TD, Tatro L, Ghio AJ, Piantadosi CA. Nitration of tyrosine by hydrogen peroxide and nitrite. Free Radic Res 1995;23:537–547.
[29] Takahama U, Oniki T. Salivary thiocyanate/nitrite inhibits hydroxylation of 2-hydroxybenzoic acid induced by hydrogen peroxide/Fe(II) systems under acidic conditions: Possibility of thiocyanate/nitrite-dependent scavenging of hydroxyl radicals in the stomach. Biochim Biophys Acta 2004;1675: 130–138.
[30] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford, UK: Oxford University Press; 1999.
[31] Carroll RT, Galatsis P, Borosky S, Kopec KK, Kumar V, Althaus JS, Hall ED. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) inhibits peroxynitrite-mediated phenol nitration. Chem Res Toxicol 2000;13:294–300.
[32] Uppu RM, Lemercier JN, Squadrito GL, Zhang H, Bolzan RM, Pryor WA. Nitrosation by peroxynitrite: Use of phenol as a probe. Arch Biochem Biophys 1998;358: 1–16.
[33] Santos CX, Bonini MG, Augusto O. Role of the carbonate radical anion in tyrosine nitration and hydroxylation by peroxynitrite. Arch Biochem Biophys 2000;377:146–152.
[34] Schmidt K, Klatt P, Mayer B. Reaction of peroxynitrite with oxyhaemoglobin: Interference with photometrical determination of nitric oxide. Biochem J 1994;301: 645–647.
[35] Pfeiffer S, Gorren ACF, Schmidt K, Werner ER, Hansert B, Bohle DS, Mayer B. Metabolic fate of peroxynitrite in aqueous solution. Reaction with nitric oxide and pH-dependent decomposition to nitrite and oxygen in a 2:1 stoichiometry. J Biol Chem 1997;272:3465–3470.
[36] Kirsch M, Korth H-G, Wensing A, Sustmann R, de Groot H. Product formation and kinetic simulation in the pH range 1–14 account for a free-radical mechanism of peroxynitrite decomposition. Arch Biochem Biophys 2003; 418:133–150.
[37] Thomas EL, Aune TM. Lactoperoxidase, peroxide, thiocyanate antimicrobial system: Correlation of sulfhydryl oxidation with antimicrobial action. Infect Immun 1978;20: 456–463.
[38] Barnet JJ, Stanbury DM. Formation of trithiocyanate in the oxidation of aqueous thiocyanate. Inorg Chem 2002;41: 164–166.
[39] Pruitt KM, Tenovuo J. Kinetics of hypothiocyanate production during peroxidase-catalyzed oxidation of thiocyanite. Biochim Biophys Acta 1982;704:204–214.
[40] Wilson IR, Harris GM. The oxidation of thiocyanate ion by hydrogen peroxide II. The acid-catalysed reaction. J Am Chem Soc 1961;83:286–289.

[41] van der Vliet A, Eiserich JP, Halliwell B, Cross CE. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. J Biol Chem 1997;272: 7617–7625.

[42] Takahama U, Hirota S, Oniki T. Thiocyanate cannot inhibit the formation of reactive nitrogen species in the human oral cavity in the presence of high concentrations of nitrite: Detection of reactive nitrogen species by 4,5-diaminofluorescein. Chem Res Toxicol 2006;19:1066–1073.

[43] Takahama U, Hirota S, Nishioka T, Oniki T. Human salivary peroxidase-catalyzed oxidation of nitrite and nitration of salivary components 4-hydroxyphenylacetic acid and proteins. Arch Oral Biol 2003;48:679–690.