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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

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Abstract
Salivary nitrate is reduced to nitric oxide (NO) via nitrite in the human oral cavity. The nitrite and NO formed can be transformed to reactive nitrogen oxide species (RNOS). In this investigation, RNOS formed in mixed whole saliva and its fractions were detected by the oxidation of aminophenyl fluorescein (APF) and the transformation of 3-amino-4-monomethylamino-2′,7′-difluorofluorecein (DAF-FM) to its triazol form (DAF-FMT). Nitrite-induced oxidation of APF and formation of DAF-FMT increased as pH was decreased from 7 to 5 and SCN^- inhibited the oxidation of APF and the formation of DAF-FMT around neutral pH and enhanced at pH about 5. The SCN^--dependent inhibition was due to the suppression of salivary peroxidase and the enhancement was due to the formation of NOSCN from HNO_2 and SCN^-. It is deduced that the increase in the concentrations of nitrite and H^+ in the oral cavity may result in the enhanced formation of RNOS.

Keywords: *Nitrite, pH, reactive nitrogen oxide species, saliva, thiocyanate*

Abbreviations: *APF, aminophenyl fluorescein; DAF-FM, 3-amino-4-monomethylamino-2′,7′-difluorofluorecein; DAF-FMT, triazol form of DAF-FM; DTPA, diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid; HRP, horseradish peroxidase; SOD, superoxide dismutase*

Introduction

Dietary nitrate that is absorbed from small intestine is secreted into the oral cavity as a salivary component [1]. The nitrate secreted is reduced to nitrite and then nitric oxide (NO) by certain bacteria [2–6]. The NO formed has been suggested to have an anti-bacterial function [5–8]. On the other hand, it has been reported that NO formed from nitrite ion by UV-A in human skin can protect against UV-induced cell damages [9]. If NO is formed under aerobic conditions, it is oxidized by O_2 and $O_2^-\cdot$ as follows;

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$NO + O_2^-\cdot \rightarrow ONOO^- \quad (2)$$

NO as well as nitrite may also be oxidized to NO_2 by peroxidase-catalysed reactions [10–13];

$$2NO_2^- + H_2O_2 + 2H^+ \rightarrow 2NO_2 + 2H_2O \quad (3)$$

$$NO + H_2O_2 \rightarrow NO_2 + H_2O \quad (4)$$

H_2O_2 required for the reactions is produced in the oral cavity [12,14] and SCN^-, a physiological substrate of salivary peroxidase [14], is an inhibitor of the above reactions [12]. When SCN^- is inhibiting reactions 3 and 4, an antimicrobial agent $OSCN^-$ is produced. Normal concentrations of nitrite and

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SCN^- in saliva are 0.1 ~ 0.3 mM [15,16] and 0.3 ~ 2 mM [16,17], respectively. The production of NO_2 and $ONOO^-$/ONOOH (pKa = 6.8) may result in the oxidation and nitration of cellular components of the oral cavity, because these reactive nitrogen oxide species are not only strong oxidants but also nitrating reagents [18]. The NO_2 formed reacts with NO as follows;

$$NO + NO_2 \leftarrow \rightarrow N_2O_3 \quad (5)$$

N_2O_3 is known to be a nitrosating agent and can react with amines, thiols or hydroxyl groups to form nitrosated species [19].

The production of NO_2, N_2O_3 and $ONOO^-$/ONOOH in the presence of nitrite has been reported using a bacterial fraction prepared from mixed whole saliva at pH 7 [12,20,21]. It is known that local pH in the oral cavity decreases from neutral or slightly alkaline pH to pH ~ 5 when acid producing bacteria proliferate [22]. One of the objectives of this study is the investigation of the effect of pH on the production of reactive nitrogen oxide species like NO_2, N_2O_3 and $ONOO^-$/ONOOH using mixed whole saliva and bacterial fraction prepared from the saliva. On the other hand, it has been reported that SCN^- can inhibit the formation of NO_2 and N_2O_3 in the human saliva at pH 7 [12]. The second objective of this study is to investigate the effect of SCN^- on the formation of the reactive nitrogen oxide species under acidic conditions using saliva as SCN^- can be transformed to NOSCN in the presence of nitrous acid (pKa = 3.3) [23]. In the present study, the reactive nitrogen oxide species were detected using aminophenyl fluorescein (APF) and 3-amino-4-monomethylamino-2′,7′-difluorofluorecein (DAF-FM). APF is oxidized by highly reactive oxygen and nitrogen oxide species like ·OH, $ONOO^-$/ONOOH and OCl^-/HOCl (pKa = 7.5) and by horseradish peroxidase (HRP) producing fluorescein [24]. DAF-FM can react with N_2O_3 or its equivalents producing a fluorescent trianzol form of DAF-FM (DAF-FMT) and the quantum efficiency is nearly constant at pH values between 5.3–11 [25]. The uses of APF and DAF-FM for the detection of ·OH and $ONOO^-$/ONOOH [26,27] and N_2O_3 derived from NO [28,29], respectively, have been reported.

Experimental procedures

Reagents

APF and DAF-FM were obtained from Daiichi Pure Chemicals (Tokyo, Japan). (±)-(*E*)-4-Ethyl-2-[(*E*)-hydroxyiminol]-5-nitro-3-hexenamide (NOR 3), *N*-(dithiocarboxy)-sarcosine (DTCS) and diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) were from Dojin (Kumamoto, Japan). Fluorescein and superoxide dismutase (SOD) from bovine erythrocytes were from Wako Pure Chemicals (Osaka, Japan). HRP (type II) was from Sigma (Tokyo, Japan).

Preparation of saliva

Mixed whole saliva (~ 10 ml) was collected at 9:00–10:00 am from healthy volunteers when required by chewing parafilm after their informed consent had been obtained as described previously [11]. The collected saliva was passed through two layers of nylon filter nets [380-mesh (32 μm) net, Sansho, Tokyo, Japan] to remove epithelial cells and other particles. The whole saliva filtrate was diluted with buffer solution or was fractionated into bacterial and salivary peroxidase fractions. On the dilution, whole saliva filtrate was mixed with equal volume of 50 mM sodium phosphate (pH 7.0) and the mixture was used as saliva filtrate. The main purpose of the dilution was to decrease the viscosity of saliva and to prevent the increase in pH due to evaporation of CO_2 gas. The concentrations of nitrite and SCN^- in the whole saliva filtrate were 0.05 ~ 0.25 and 0.3 ~ 1.1 mM, respectively, when the concentrations were determined using Griess-Romijn reagent for nitrite and acidic Fe(III).

The whole saliva filtrate (5 ml) was centrifuged at 20 000 × g for 5 min and the sediment was suspended in 5 ml of 50 mM sodium phosphate (pH 7.0). This suspension mainly contained bacteria with some contamination of leukocytes when observed using a microscope and used as a bacterial fraction. The supernatant (5 ml) obtained after the centrifugation was dialysed against 10 mM sodium phosphate (pH 7.0) (1 liter) for one night and then centrifuged at 20 000 × g for 5 min. The supernatant was used as a salivary peroxidase fraction.

Fluorescence measurements

Fluorescence increases due to the oxidative transformation of APF to fluorescein [24] and the transformation of DAF-FM to DAF-FMT [25] were measured with a spectrofluorometer RF-550 (Shimadzu, Kyoto, Japan). The excitation and emission wavelengths were 490 and 515 nm, respectively. The excitation light was passed through glass filters ND-13 and B-440 from Hoya (Tokyo, Japan). The reaction mixture (0.5 ml) contained 10 μM APF or 7 μM DAF-FM in 50 mM sodium phosphate, bacterial fraction or saliva filtrate (pH 7). Reactions were started by the addition of nitrite or NOR 3 and then SCN^- or SOD was added. Oxidation of APF by salivary peroxidase fraction was also measured fluorometrically. The reaction mixture (0.5 ml) contained 10 μM APF, 10 μl of salivary peroxidase fraction and various concentrations of $NaNO_2$ in 50 mM sodium phosphate (pH 5.3 and 7.0). Reactions were started by the addition of H_2O_2.

Effects of pH on the fluorescence increase were measured in buffer solution (50 mM sodium phosphate), bacterial fraction and saliva filtrate. Bacterial fraction with various pH values was prepared by suspending the sediment, which was obtained after centrifugation of saliva filtrate, in 50 mM sodium phosphate (pH 5 ~ 7). Saliva filtrate was mixed with equal volume of 50 mM sodium phosphate (pH 5.3 ~ 7.0) or 50 mM NaH_2PO_4/50 mM CH_3COOH (pH 4.3 ~ 4.7) to obtain saliva filtrate with various pH values. Anaerobic experiments were performed under a stream of argon gas.

Spectrophotometric studies

Quercetin is oxidized in bacterial fraction and the main part of the oxidation is catalysed by salivary peroxidase [12]. The oxidation was studied in the reaction mixture (1 ml) that contained 20 μM quercetin in bacterial fraction (pH 5.2 and 7) using a 557 spectrophotometer (Hitachi, Tokyo, Japan) with dual-wavelength mode. Wavelengths used were $\Delta A_{360-295}$ and $\Delta A_{380-330}$ at pH values of 5.2 and 7, respectively. The wavelengths were determined by measuring the change in absorption spectra by HRP/H_2O_2/quercetin systems. It has been reported that absorption spectrum of a major oxidation product of quercetin [2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(*2H*)-benzofuranone] is significantly affected by pH [30].

Measurement of NO formation

ESR (electron spin resonance) spectra were measured using a JE1XG spectrometer (JEOL, Tokyo, Japan) at ~25°C using a quartz flat cell (0.05 mL). NO trapped by a spin-trapping reagent Fe-$(DTSC)_2$ complex [20] was detected under the following conditions: microwave power, 10 mW; line width, 0.5 mT; amplification, 2000-fold; scanning speed, 2.5 mT/min. Fe-$(DTCS)_2$ solution was prepared by adding 0.03 ml of 100 mM $FeCl_3$ to 1 ml of 10 mM DTCS. This solution (0.25 ml) was mixed with 0.25 ml of 50 mM sodium phosphate or bacterial fraction. Immediately after the mixing, 0.2 mM $NaNO_2$ was added and an aliquot of the mixture was withdrawn into the quartz cell to measure ESR spectra. Final pH values of the reaction mixtures are given in the legend for Figure 7.

HPLC

Fluorescein and DAF-FMT formed from APF and DAF-FM, respectively, were separated by HPLC using a Shim-pack CLC-ODS column (15 cm × 6 mm i.d.) (Shimadzu) combined with a spectrofluorometric detector (RF-550) (Shimadzu). The mobile phase used was a mixture of methanol and 10 mM sodium phosphate (pH 7.0) (1:2, v/v), and the flow rate was 1 ml/min. Excitation and emission wavelengths were 490 and 515 nm, respectively. The retention times of fluorescein and DAF-FMT were ~17 and 6 min, respectively. When nitrite-induced formation of fluorescein and DAF-FMT was measured using buffer solution, the reaction mixture was directly applied to the HPLC column after incubation for defined periods. When bacterial fraction and saliva filtrate were used, 0.2 ml of methanol was added to 0.2 ml of the reaction mixture after incubation to precipitate proteins and then the mixture was filtered with a cellulose acetate filter (0.45 μm; Advantec, Tokyo, Japan) to apply to the HPLC column.

The amount of DAF-FMT formed could not be quantified because standard DAF-FMT was not obtained. Instead, the decrease in concentration of DAF-FM was measured by HPLC as a function of fluorescence increase due to the formation of DAF-FMT. A linear relation was obtained between the fluorescence increase and the decrease in concentrations of DAF-FM. DAF-FM was quantified from the peak area at 490 nm using a Shim-pack CLC-ODS column and a spectrophotometric detector with a photodiode array (SPD-M10Avp, Shimadzu). The mobile phase used was a mixture of methanol and 10 mM sodium phosphate (pH 7.0) (1:1, v/v) and the flow rate was 1 ml/min. The retention time of DAF-FM was 4.9 min.

After incubation of quercetin (20 μM) in bacterial fraction (pH 5.2 and 7) for 30 min, the fraction was extracted with 50% methanol and the oxidation product described above was separated by the above HPLC system. The mobile phase used was a mixture of methanol and 25 mM KH_2PO_4 (3:2, v/v) and the flow rate was 1 ml/min. The product was detected at 290 nm and its retention time was 3.4 min.

Statistical analysis

When required, data were shown as means ± SDs and were statistically evaluated by Student's *t*-test. $p < 0.05$ was regarded as statistically significant.

Results

Oxidation of APF by NOR 3 and nitrite

Figure 1 shows typical time courses of fluorescence increase due to the oxidation of APF at pH 7.0. NOR 3-induced fluorescence increase was dependent on the concentration of NOR 3 (traces in A). NOR 3 is known to produce NO by self-decomposition [31–33] and the fluorescence increase may be due to the formation of fluorescein [24]. The formation of fluorescein was confirmed by HPLC by comparing the retention time and the excitation and emission spectra with standard fluorescein. NaSCN (2 mM) slightly inhibited the fluorescence increase

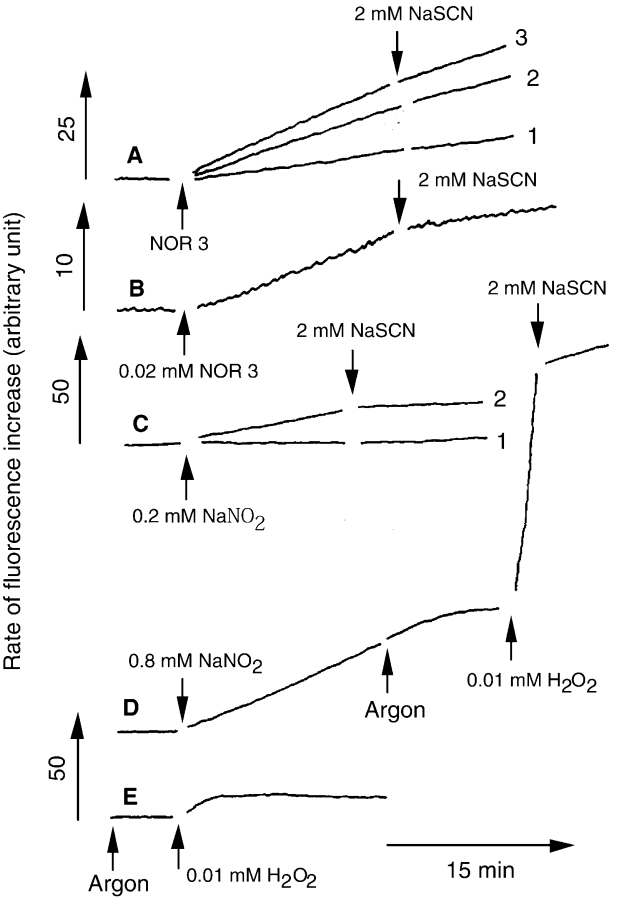

Figure 1. NOR 3- and nitrite-induced fluorescence increase in the presence of APF. (A) NOR 3-induced fluorescence increase in buffer solution. The reaction mixture (0.5 ml) contained 10 μM APF in 50 mM sodium phosphate (pH 7.0). NOR 3 and NaSCN were added as indicated by arrows. Trace 1, 5 μM; trace 2, 10 μM; trace 3, 20 μM NOR 3. (B) NOR 3-induced fluorescence increase in bacterial fraction. The reaction mixture (0.5 ml) contained 10 μM APF in bacterial fraction (pH 7). Where indicated, NOR 3 and NaSCN were added. (C) Nitrite-induced fluorescence increase. The reaction mixture (0.5 ml) contained 10 μM APF in buffer solution (50 mM sodium phosphate, pH 7) or in bacterial fraction (pH 7). Trace 1, buffer solution; trace 2, bacterial fraction. Where indicated, $NaNO_2$ and NaSCN were added. (D) Effects of argon and H_2O_2 on nitrite-induced fluorescence increase. The reaction mixture (0.5 ml) contained 10 μM APF in bacterial fraction (pH 7). After the addition of nitrite (0.8 mM), air was replaced with argon and then H_2O_2 (10 μM) and NaSCN (2 mM) were added. (E) H_2O_2-induced fluorescence increase under anaerobic conditions. The reaction mixture (0.5 ml) contained 10 μM APF in bacterial fraction (pH 7). Where indicated, H_2O_2 was added.

and degree of the inhibition in the presence of 20 μM NOR 3 was $13.5 \pm 13\%$ (mean $\pm$ SD, $n = 3$). The NOR 3-induced fluorescence increase was nearly completely inhibited by the replacement of air with argon. NOR 3 also oxidized APF to fluorescein in bacterial fraction although the fluorescence increase was slow (trace B). The increase was inhibited by 2 mM SCN^- and degree of the inhibition in the presence of 20 μM NOR 3 was $62 \pm 4\%$ (mean $\pm$ SD, $n = 3$).

When nitrite was added to buffer solution, fluorescence increased very slowly, whereas when added to bacterial fraction, rapid increase due to the formation of fluorescein was observed (traces in C). NaSCN (2 mM) did not affect the nitrite-induced fluorescence increase in buffer solution, but clearly inhibited the increase in bacterial fraction. The fluorescence increase induced by nitrite was nearly completely inhibited by replacing air with argon in bacterial fraction (trace D). The slow fluorescence increase under anaerobic conditions was greatly enhanced by H_2O_2 and SCN^- inhibited the H_2O_2-induced increase. In the absence of nitrite, H_2O_2 induced small fluorescence increase not only under anaerobic (trace E) but also aerobic (not shown) conditions.

Figure 2 (upper panel) shows effects of SCN^- and SOD on fluorescence increase due to the oxidation of APF in bacterial fraction as a function of concentration of nitrite at pH 7. Rate of the oxidation increased and the inhibitory effects of SCN^- and SOD became smaller and larger, respectively, as the concentration of nitrite was increased.

Fluorescence increase was observed by the addition of APF to saliva filtrate although the rate was much slower than that in bacterial fraction. This may be due to the presence of SCN^- and reductants like uric acid in saliva filtrate. The addition of nitrite enhanced the fluorescence increase (Figure 2, lower panel A). Neither SCN^- nor SOD affected the increase in saliva filtrate itself (B-1), but the reagents inhibited the fluorescence increase that had been enhanced by the addition of nitrite (B-2 and B-3). Here again, fluorescein was the only fluorescent product.

Effects of nitrite on salivary peroxidase-catalysed oxidation of APF

Fluorescence increase induced by salivary peroxidase fraction/H_2O_2 at pH 7 was slow in the absence of nitrite (Figure 3A). Nitrite enhanced the fluorescence increase as a function of its concentration (Figure 3A and C). The maximal rate was calculated to be $\sim$50-times the rate in the absence of nitrite by double reciprocal plots and Km for nitrite was $\sim$0.3 mM in the presence of 10 μM H_2O_2. The amount of fluorescein formed also increased as a function of concentration of nitrite (Figure 3A). Rate of the fluorescence increase in the presence of 0.2 mM nitrite increased nearly linearly as a function of concentration of H_2O_2 in the concentration range from 0–10 μM (data not shown). Nitrite also enhanced the fluorescence increase at pH 5.3 and the enhancement was somewhat smaller than that at pH 7 (Figure 3B).

Effects of pH on nitrite-induced oxidation of APF

In 50 mM sodium phosphate (pH $5.3 \sim 7$), fluorescence increase due to the oxidation of APF became

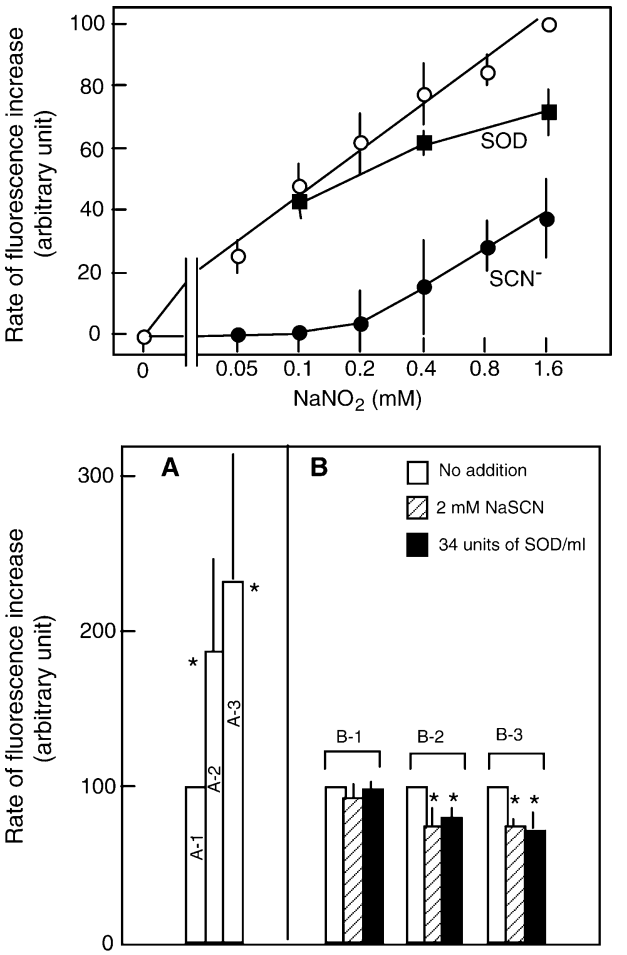

Figure 2. Effects of nitrite, SCN^- and SOD on fluorescence increase in the presence of APF. Upper panel: Bacterial fraction. The reaction mixture (0.5 ml) contained 10 μM APF in bacterial fraction (pH 7). Reactions were started by the addition of $NaNO_2$. After incubation for 10–12 min, NaSCN (2 mM) or SOD (34 units/ml) was added. Rate of fluorescence increase at each concentration of $NaNO_2$ was normalized by the rate of fluorescence increase at 1.6 mM $NaNO_2$ in the absence of NaSCN and SOD. ○, no addition; ●, 2 mM NaSCN; ■, 34 units of SOD/ml. Each data point represents average with SD ($n = 3 \sim 4$). Lower panel: Saliva filtrate. The reaction mixture (0.5 ml) contained 10 μM APF in saliva filtrate (pH 7). (A) Effects of concentration of nitrite. A-1, no addition; A-2, 1 mM $NaNO_2$; A-3, 2 mM $NaNO_2$. (B) Effects of SCN^- and SOD. B-1, no addition; B-2, 1 mM $NaNO_2$; B-3, 2 mM $NaNO_2$. Open columns, neither addition of NaSCN nor SOD (represented as 100); dashed columns, 2 mM NaSCN; filled columns, 34 units of SOD/ml. Each column represents average with SD ($n = 3 \sim 4$). $^\star p < 0.05$ (compared with no addition).

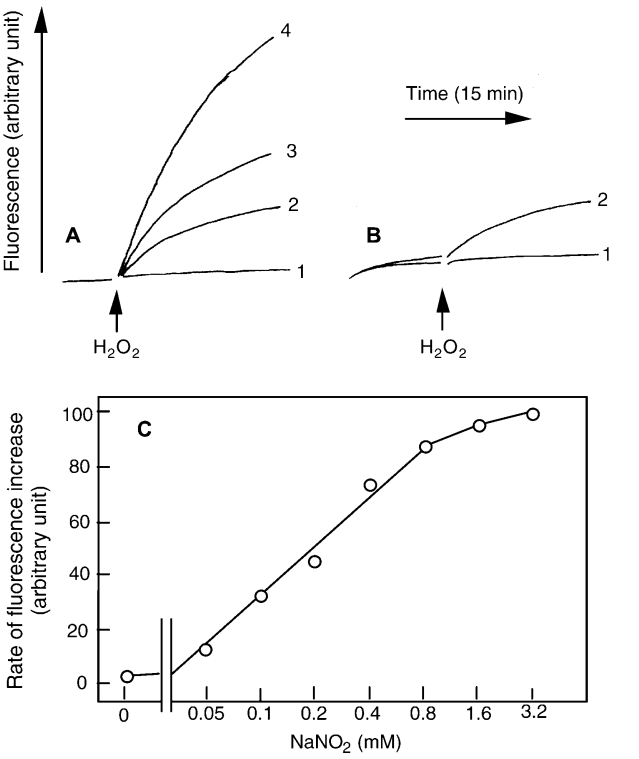

Figure 3. Salivary peroxidase-catalysed oxidation of APF. (A) and (B) Time courses of fluorescence increase at pH 7.0 and 5.3, respectively. The reaction mixture (0.5 ml) contained 10 μM APF and 10 μl of salivary peroxidase fraction in 50 mM sodium phosphate (pH 7.0 and 5.3). A-1, 0 mM $NaNO_2$; A-2, 0.2 mM $NaNO_2$; A-3, 0.8 mM $NaNO_2$; A-4, 3.2 mM $NaNO_2$. B-1, 0 mM $NaNO_2$; B-2, 0.2 mM $NaNO_2$. Where indicated, 10 μM H_2O_2 was added. (C) Effect of concentration of nitrite on rate of the fluorescence increase. The reaction mixture (0.5 ml) contained 10 μM APF, 10 μM H_2O_2, 10 μl of salivary peroxidase preparation and various concentrations of $NaNO_2$ in 50 mM sodium phosphate (pH 7.0). Each data point represents average of two experiments.

faster as pH was decreased (Figure 4A, open circles). SCN^- enhanced the increase at pH 5.3 and 6.1, but no clear effect of SCN^- was observed at pH 7 (closed circles). The formation of fluorescein also increased as pH was decreased (triangles) and its formation was enhanced by SCN^- (data not shown).

In the bacterial fraction, fluorescence increase in the presence of APF had an optimum pH at about 6 (Figure 4B, open circles). The formation of fluorescein increased as pH was decreased from 7.1 to 5.3 (triangles) and the rates at pH values of 5.3, 6.1 and 7.1 were 8.5 ± 3.1, 6.4 ± 3.5 and 2.7 ± 1.9 nM/min (means $\pm$ SDs, $n = 3$), respectively. SCN^- inhibited the fluorescence increase at pH values between 6.1–7.1, but slightly enhanced it at pH 5.3 (closed circles). SOD (34 units/ml) inhibited the increase at all pH values examined in the presence of 1 mM nitrite. The rates in the presence of SOD were 88 ± 11, 84 ± 18 and $70 \pm 9^\star$% (means $\pm$ SDs, $n = 4$; $^\star p < 0.05$) of those in the absence of SOD at pH values of 5.3, 6.1 and 7.1, respectively.

In saliva filtrate, the rate of the fluorescence increase observed by the addition of APF was nearly constant at pH values between 6–7.1 and much higher at pH 5.3 (Figure 4C, open circles). The rate of the formation of fluorescein determined by HPLC was less than 0.1 nM/min in the pH range from 6–7.1 and the rate was ~ 0.3 nM/min at pH 5.3 in a saliva filtrate. The addition of 2 mM NaSCN to the saliva filtrate did not significantly affect the

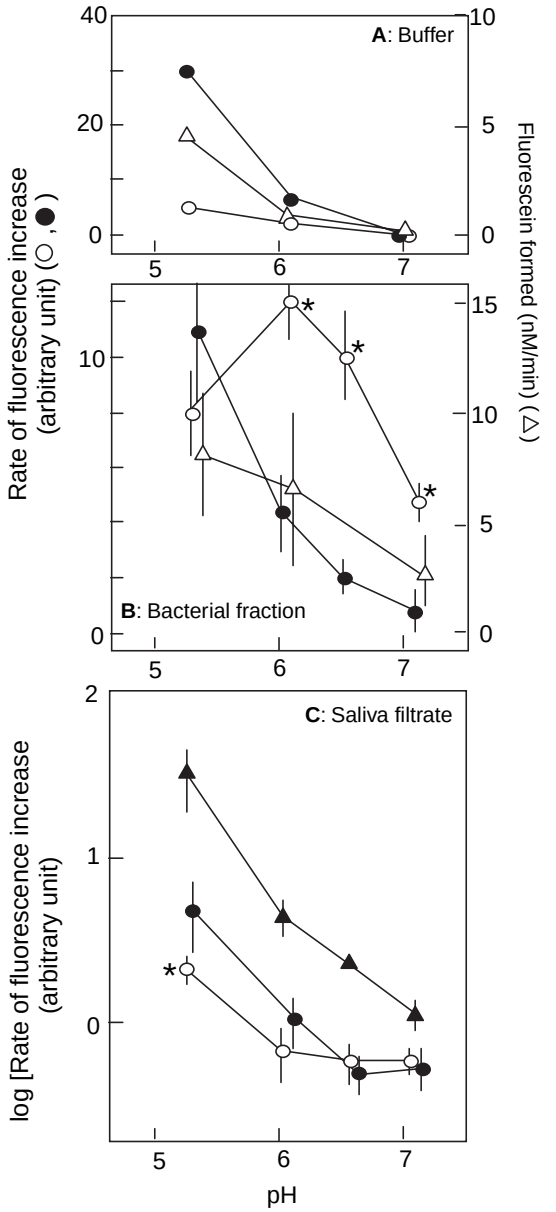

Figure 4. Effects of pH on the fluorescence increase in the presence of APF. (A) Buffer solution. The reaction mixture (0.5 ml) contained 10 μM APF and 0.2 mM $NaNO_2$ in 50 mM sodium phosphate (pH 5.3 ~ 7.0). ○, no addition; ●, 2 mM NaSCN; △, fluorescein formation in the absence of NaSCN. Each data point represents average of two experiments. (B) Bacterial fraction. The reaction mixture (0.5 ml) contained 10 μM APF and 0.2 mM $NaNO_2$ in bacterial fraction (pH 5.3–7.1). ○, no addition; ●, 2 mM NaSCN; △, fluorescein formation in the absence of NaSCN. Each data point represents average with SD ($n = 3 \sim 4$). $^{\star}p < 0.05$ (compared with ●). (C) Saliva filtrate. The reaction mixture (0.5 ml) contained 10 μM APF in the mixture of 0.25 ml of saliva filtrate and 0.25 ml of buffer solutions with various pH values. ○, no addition; ●, 2 mM NaSCN; ▲, 1 mM $NaNO_2$. Each data point represents average with SD ($n = 3 \sim 4$). $^{\star}p < 0.05$ (compared with ○).

fluorescence increase in a pH range from 6–7.1 but enhanced the increase at pH 5.3 (closed circles). The addition of 1 mM nitrite enhanced the fluorescence increase at all pH values examined (triangles).

Effects of pH on nitrite-induced transformation of DAF-FM

Nitrite (0.2 mM) induced fluorescence increase in the presence of DAF-FM and SCN^- enhanced the fluorescence increase ~10-fold in buffer solution at pH values of 5 and 6 (Figure 5A). In the bacterial fraction, slow fluorescence increase was observed in the presence of DAF-FM (Figure 5B, open circles) and the increase was enhanced largely by 0.2 mM nitrite (closed circles) at all pH values examined. The similarity of the effect of pH under the two conditions (with and without 0.2 mM nitrite) suggests that the fluorescence increase without the addition of nitrite might be due to nitrite that was contained in the bacterial fraction. SCN^- inhibited the fluorescence increase at pH values of 6 and 7, but slightly enhanced at pH 5.3 in the presence of 0.2 mM nitrite (triangles). SOD (34 units/ml) enhanced the fluorescence increase in the presence of 0.2 mM nitrite and degrees of the enhancement were calculated to be 1.17 ± 0.12, 1.37 ± 0.24 and $1.42 \pm 0.23^{\star}$ (means ± SDs, $n = 4$; $^{\star}p < 0.05$) at pH 5.3, 6 and 7, respectively.

By the addition of DAF-FM to saliva filtrate, slow fluorescence increase was observed and the rate increased as pH was decreased (Figure 5C, open circles). Nitrite (1 mM) enhanced the fluorescence increase at all pH values examined (closed circles). SCN^- (2 mM) enhanced the fluorescence increase at pH 5.3 but did not at pH values of 6 and 7.1 (triangles). SOD (34 units/ml) enhanced the nitrite-induced fluorescence increase in the presence of 1 mM nitrite and degrees of the enhancement were calculated to be 1.07 ± 0.07, $1.36 \pm 0.26^{\star}$ and $1.88 \pm 0.49^{\star}$ (means ± SDs, $n = 4$; $^{\star}p < 0.05$) at pH values of 5.3, 6.0 and 7.1, respectively.

A major fluorescent product with a retention time of ~6 min was separated by HPLC after incubation of DAF-FM with 20 μM NOR 3 in 50 mM sodium phosphate (pH 7.0) suggesting that this component was DAF-FMT. In addition to DAF-FMT, three minor components (retention times, 9.4, 11.2 and 19.2 min) were also separated and fluorescence intensities of the three components were less than 2% of DAF-FMT. DAF-FMT was also formed as a major component when DAF-FM was incubated in buffer solution and bacterial fraction in the presence of 0.2 mM nitrite at pH values of 5 and 7 and the formation of DAF-FMT was enhanced by 2 mM SCN^- at pH 5 as in Figure 5. When DAF-FM was incubated in saliva filtrate at pH values of 5 and 7, DAF-FMT was detected. The formation of DAF-FMT was enhanced by 2 mM SCN^- and 1 mM nitrite as in Figure 5. The result indicates that DAF-FMT was formed as a major fluorescent component under the conditions of this study. In the present

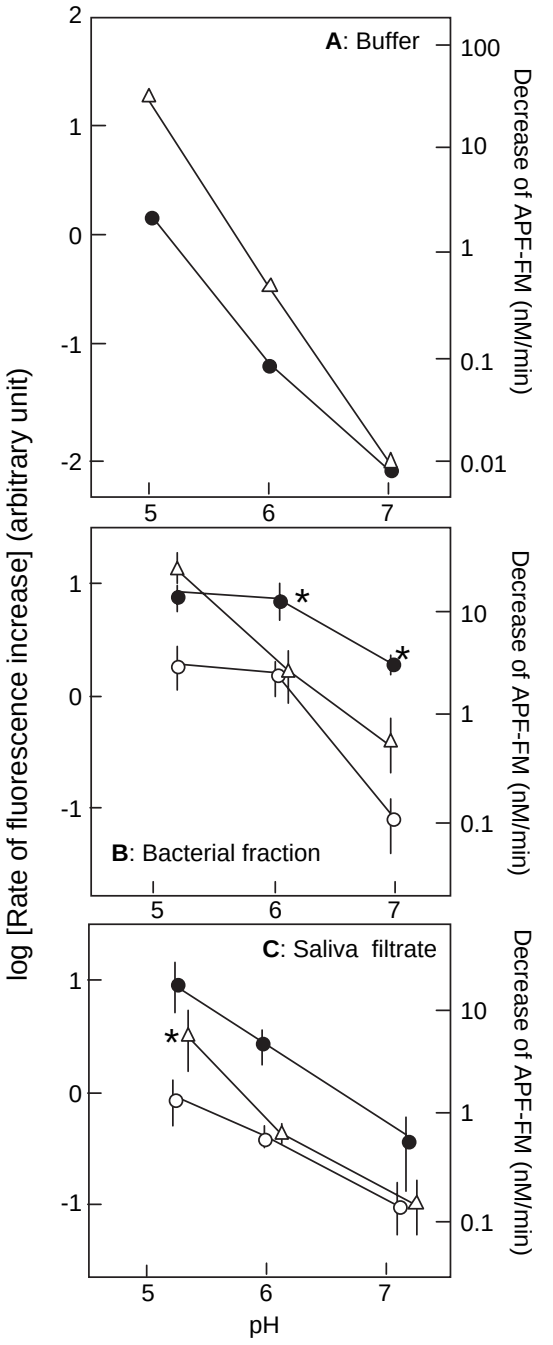

Figure 5. Effects of pH on the fluorescence increase in the presence of DAF-FM. (A) Buffer solution. The reaction mixture (0.5 ml) contained 7 μM DAF-FM and 0.2 mM $NaNO_2$ in 50 mM sodium phosphate (pH 5.3–7.0). ●, no addition; △, 2 mM NaSCN. Each data point represents average of two experiments. (B) Bacterial fraction. The reaction mixture (0.5 ml) contained 7 μM DAF-FM in bacterial fraction (pH 5.3 ~7.1). ○, no addition; ●, 0.2 mM $NaNO_2$; △, 0.2 mM $NaNO_2$ plus 2 mM NaSCN. Each data point represents average with SD ($n=4$). $^{\star}p<0.05$ (compared with △). (C) Saliva filtrate. The reaction mixture (0.5 ml) contained 7 μM DAF-FM in the mixture of 0.25 ml of saliva filtrate and 0.25 ml of buffer solutions with various pH values. ○, no addition; ●, 1 mM $NaNO_2$; △, 2 mM NaSCN. Each data point represents average with SD ($n=5$). $^{\star}p<0.05$ (compared with ○). The relation between fluorescence increase and decrease in the concentration of DAF-FM was determined as described in the Experimental procedures section.

study, components other than DAF-FMT remained to be identified.

Effects of CO_2 and a chelator DTPA in bacterial fraction

Sodium bicarbonate (20 mM) did not significantly affect the fluorescence increase in the presence 0.2 mM nitrite and APF or DAF-FM. The oxidation of APF by 0.2 mM nitrite was not affected by 1 mM DTPA and inhibited 10–20% by DTPA plus SOD (34 units/ml) at pH 7. This result suggests that even if ·OH, which was formed by Fenton reaction, participated in the oxidation of APF, its participation was small compared to the participation of other oxidants. DTPA and DTPA plus SOD enhanced the formation of DAF-FMT by $31\pm8^{\star}$% and $138\pm40^{\star}$% (mean ± SD, $n=3$; $^{\star}p<0.05$), respectively, in the bacterial fraction in the presence of 0.2 mM nitrite at pH 7. No detectable effects of DTPA were observed at pH 5.3 when DAF-FMT formation and oxidation of APF were measured in the presence of 0.2 mM nitrite.

Oxidation of quercetin by bacterial fraction

Figure 6 shows time courses of oxidation of quercetin at pH values of 5.2 and 7. By the addition of quercetin, increases in absorbance were observed. During the incubation, absorption decrease was observed and the decrease was faster at pH 7 (trace 2) than pH 5.2 (trace 1). SCN^- inhibited the absorbance decrease and HRP, activity of which was not inhibited by SCN^- [12], enhanced the absorbance

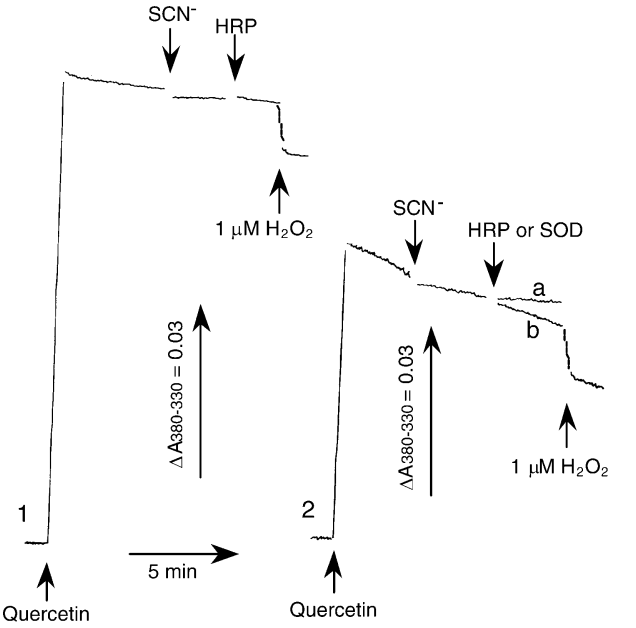

Figure 6. Time courses of oxidation of quercetin. Trace 1: pH 5.2. Upward and downward arrows indicate the addition of reagents. Quercetin, 20 μM; NaSCN, 1 mM; HRP, 10 μg/ml; H_2O_2, 1 μM. Trace 2: pH 7. (a) addition of SOD (34 units/ml); (b) addition of HRP (10 μg/ml).

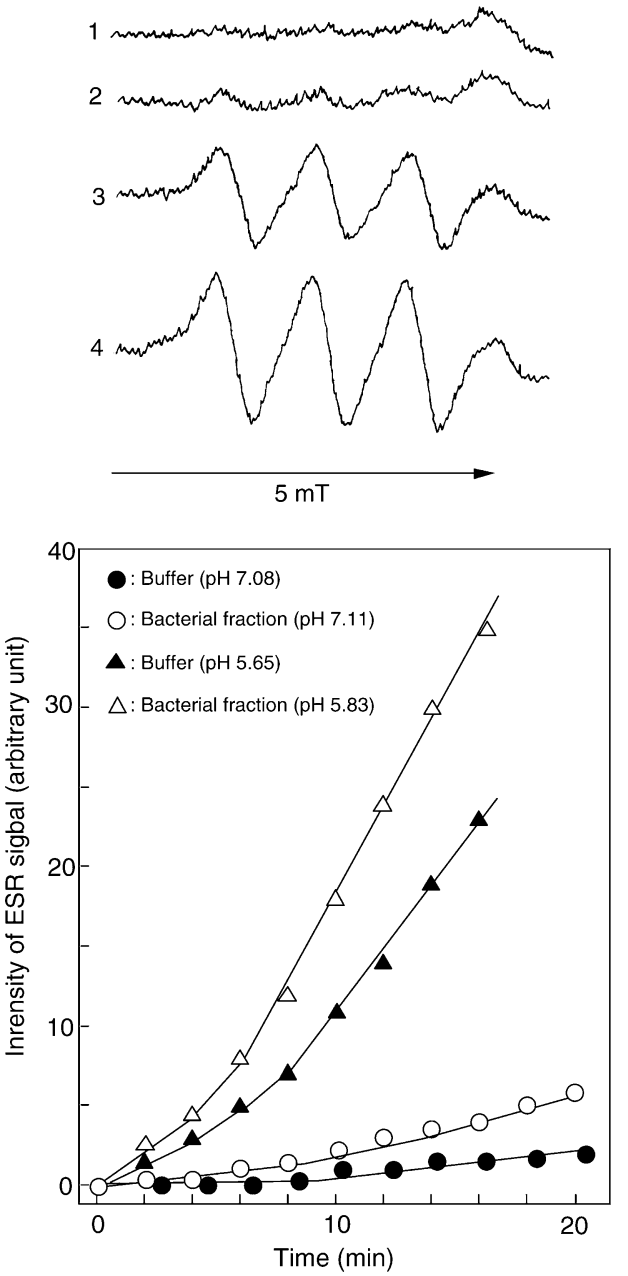

Figure 7. NO formation in buffer solution and bacterial fraction. Upper panel: ESR spectra 16 min after the preparation of reaction mixture. 1, buffer (pH 5.65); 2, bacterial fraction (pH 7.11); 3, buffer (pH 5.65); 4, bacterial fraction (pH 5.80). Lower panel: Time courses of increase in ESR signal. ESR spectrum was recorded every 2 min after the preparation of the reaction mixture and the signal intensity was plotted as a function of incubation time. ●, buffer (pH 7.08); ○, bacterial fraction (pH 7.11); ▲, buffer (pH 5.65); △, bacterial fraction (pH 5.80).

decrease in the presence of SCN^- (b in trace 2). When SOD (a in trace 2) or DTPA (not shown) was added in the presence of SCN^-, further inhibition of the absorbance decrease was observed at pH 7, suggesting the participation of $O_2^-\cdot$ and $\cdot OH$ formed by Fenton-type reactions in the oxidation of quercetin. At pH 5.2, the effect of SOD and DTPA was not studied as SCN^- nearly completely inhibited the absorbance decrease. The above result indicates that rate of production of $O_2^-\cdot$ as well as H_2O_2 was slower at pH 5.2 than pH 7. Rates of the oxidation of quercetin were estimated referring absorbance changes induced by 1 μM H_2O_2 and the values were 28 ± 7 and 225 ± 47 nM/min ($n = 3$) at pH values of 5.2 and 7, respectively. The rate of formation of the oxidation product of quercetin at pH 5.2 was ~14% of that at pH 7. No significant effects of 0.2 mM nitrite were observed for the oxidation of quercetin at pH values of 5.2 and 7.

Formation of NO in bacterial fraction

Figure 7 (upper panel) shows nitrite-induced formation of NO under weak alkaline and acidic conditions. When nitrite was incubated for 16 min in buffer solution (pH 5.65), an ESR spectrum due to NO-Fe-$(DTSC)_2$ was detected (trace 3). The signal intensity was larger in the bacterial fraction (pH 5.80) than in the buffer solution. No clear ESR spectra due to NO-Fe-$(DTSC)_2$ could be detected in the buffer solution at pH 7.08 (trace 1). Bacteria enhanced the formation of NO (trace 2). Time courses of NO-Fe-$(DTSC)_2$ formation (lower panel) indicate that NO formation by chemical reactions was faster under weak acidic conditions and that reduction of NO_2^- to NO by bacteria was also faster under weak acidic conditions. SCN^- (1 mM) did not affect the formation of NO under the conditions of Figure 7.

Discussion

NOR 3 induced the oxidation of APF in buffer solution and the oxidation was not observed under anaerobic conditions (Figure 1A). The result suggests that an oxidation product of NO oxidized APF. NO_2 is the candidate because $\cdot OH$, $ONOO^-$/ONOOH and OCl^-/HOCl might not be formed in the buffer solution used in this study. Oxidation of APF by NO_2 is possible as $E^{\circ\prime}$ of NO_2/NO_2^- (0.99 V) is similar to that of $HOCl/(H_2O + Cl^-)$ (1.1 V) [18]. If NO_2 participates in the oxidation of APF, the result in Figure 1A supports that SCN^- is not an effective scavenger of NO_2 [12]. SCN^- inhibited the NOR 3-induced fluorescence increase in bacterial fraction (Figure 1B). This result suggests that NO_2 or its equivalent was formed from NO not only by auto-oxidation but also by salivary peroxidase-catalysed oxidation of NO [12] (Figure 8, scheme 1). H_2O_2 required for the peroxidase-catalysed reactions is produced in the bacterial fraction and the bacterial fraction contains salivary peroxidase [12,14].

Nitrite-induced fluorescence increase due to the oxidation of APF was much faster in the bacterial fraction than in the buffer solution and the fluorescence increase in the bacterial fraction was inhibited by SCN^- at pH 7 (Figures 1 and 2). APF itself was not a good substrate of salivary peroxidase/H_2O_2 and

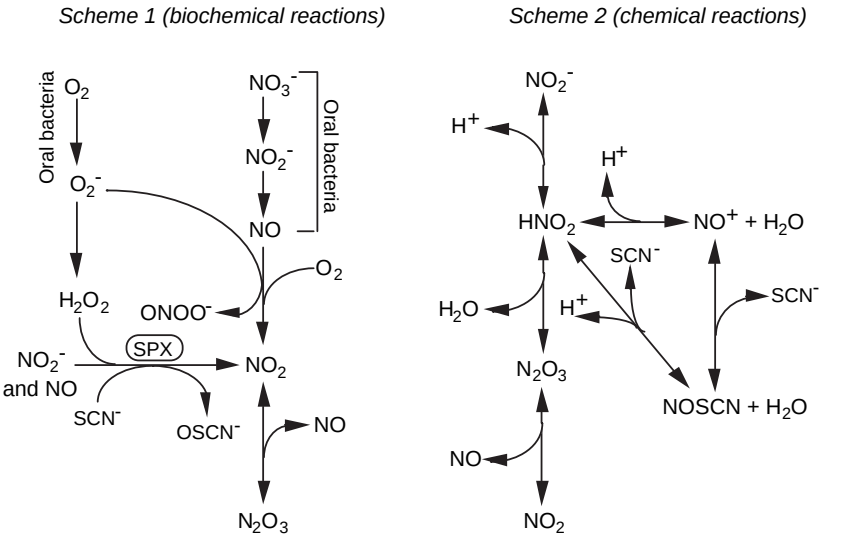

Figure 8. Possible biochemical (scheme 1) and chemical (scheme 2) reactions during the metabolism of nitrite and nitrous acid. SPX, salivary peroxidase. For details, see text.

both rate and amount of the oxidation of APF by salivary peroxidase/H_2O_2 increased as a function of concentration of nitrite (Figure 3). The result suggests that APF was oxidized by NO_2 or its equivalent formed by salivary peroxidase/H_2O_2/nitrite (Figure 8, scheme 1) [10–13,34]. As a compound equivalent to NO_2, peroxidase combined with ONOOH, which may be formed by the reaction between H_2O_2 and NO_2^- bound to peroxidase, is possible [13]. Oxidation of nitrite by salivary peroxidase/H_2O_2 has also been suggested by measuring nitration of phenolic compounds [35]. In addition, formation of NO_2 or its equivalent is also possible by the oxidation of NO by salivary peroxidase/H_2O_2 as NO is formed from nitrite in bacterial fraction.

Inhibitory effects of SCN^- and SOD decreased and increased, respectively, as the concentration of nitrite was increased at pH 7 in bacterial fraction (Figure 2). The decrease in the effect of SCN^- suggests that APF was oxidized by peroxidase-independent reactions at higher concentrations of nitrite. As a peroxidase-independent reaction, production of NO_2 by auto-oxidation of NO is possible. It has been reported that the reduction of nitrite to NO by oral bacteria increases as a function of concentration of nitrite [2]. The other oxidant is $ONOO^-$/ONOOH generated by reaction 2. The inhibition by SOD supports the occurrence of reaction between NO and $O_2^-\cdot$ in bacterial fraction as reported previously [12]. The production of $O_2^-\cdot$ in the bacterial fraction has been reported [14,36,37] and is suggested in Figure 6. The participation of $\cdot$OH to the nitrite-induced oxidation of APF in the bacterial fraction seemed to be small.

Oxidation of APF in saliva filtrate was not affected by externally added SCN^- and SOD at pH 7 (Figure 2). The oxidation was enhanced by the addition of nitrite and SCN^- and SOD partly inhibited the enhanced oxidation (Figure 2). The result suggests that endogenous SCN^- could not fully inhibit the peroxidase-catalysed formation of NO_2 or its equivalent and that the reaction between $O_2^-\cdot$ and NO proceeded, producing $ONOO^-$/ONOOH in the presence of high concentration of nitrite. The increase in concentration of salivary nitrite has been observed after the ingestion of nitrate-rich food [38,39].

Rate of nitrite-induced fluorescence increase by the oxidation of APF increased as pH was decreased in buffer solution (Figure 4A) although the fluorescence yield of fluorescein was low at lower pH values [40]. The formation of fluorescein also increased as pH was decreased (Figure 4A). The result suggests that the oxidation of APF was faster at lower pH values. Here again, chemical species, which oxidized APF to fluorescein, might be NO_2 because $\cdot$OH, $ONOO^-$/ONOOH and OCl^-/HOCl were not formed in the buffer solution. NO_2 can be formed by the reverse reaction of reaction 5 (equilibrium constant, 4.8×10^{-3} m) [23] if N_2O_3 is generated from nitrite (pKa = 3.3) by the following reaction (Figure 8, scheme 2);

$$2HNO_2 \leftarrow\rightarrow N_2O_3 + 2H_2O \qquad (6)$$

In addition, NO^+ formed by the reaction between HNO_2 and H^+ (Figure 7, scheme 2) may also contribute to the oxidation of APF because $E^{\circ\prime}$ of NO^+/NO (1.21 V) is comparable to that of HOCl/($H_2O + Cl^-$) (1.1 V) [18]. SCN^--dependent enhancement of the fluorescence increase under acidic conditions (Figure 4A) may be attributed to NOSCN formed by the following reaction [41,42];

$$SCN^- + HNO_2 + H^+ \rightarrow NOSCN + H_2O \qquad (7)$$

If NOSCN dissociates to NO^+ and SCN^- [23], oxidation of APF by the NO^+ is possible.

Nitrite-induced fluorescence increase due to the oxidation of APF had an optimum pH at ~6 in the bacterial fraction, whereas the formation of fluorescein increased as the decrease in pH from 7 to 5.3 in the absence of SCN^- (Figure 4B). The discrepancy could be explained by the lower quantum efficiency of fluorescein at pH 5.3 than pH 6~7 [40]. The increase in fluorescein formation as the decrease in pH suggests that the production of NO^+, NO_2 and $ONOO^-$/ONOOH was enhanced at low pH values. In the reactive nitrogen oxide species, the enhanced formation of $ONOO^-$/ONOOH seemed not to be probable because the inhibitory effect of SOD was not significant at low pH values. The enhanced formation of NO^+ is possible as discussed above. Possible reactions for the enhanced formation of NO_2 were, in addition to the dissociation of N_2O_3 formed by reaction 6, (i) salivary peroxidase-catalysed oxidation of nitrite and NO and (ii) auto-oxidation of NO produced by salivary bacteria. Possibility (ii) is supported by the result in Figure 7 that the decrease in pH was associated with the increased production of NO not only by chemical but also by biochemical reactions. It has been reported that the decrease in pH is associated with the increase in the reduction of nitrite to NO by nitrite-reducing bacteria in the human oral cavity [43]. If the production of NO is increased, possibility (i) may also be included in the enhanced formation of NO_2 by reaction 4, although rate of H_2O_2 formation estimated from the oxidation of quercetin was slower at pH 5.2 than 7 (Figure 6).

SCN^- inhibited the nitrite-induced fluorescence increase at pH values between 6 and 7 in the presence of APF in bacterial fraction (Figure 4B). The mechanism of the inhibition is discussed above. At pH 5.3, SCN^- slightly enhanced the fluorescence increase. The result suggests that NOSCN participated in the oxidation of APF in addition to NO^+ and NO_2 produced by the auto-oxidation of NO, which was generated by nitrite-reducing bacteria and that SCN^--dependent inhibition of NO_2 formation by salivary peroxidase/H_2O_2/(nitrite and NO) was recovered by the formation of NOSCN from HNO_2 and SCN^-. In saliva filtrate, rate of the fluorescence increase due to the oxidation of APF was fast at lower pH values and the addition of SCN^- enhanced the oxidation at pH 5.3 (Figure 4C). The former result may be explained by the increased participation of NO_2 and NOSCN at lower pH values. NO_2 can be formed from salivary HNO_2 via N_2O_3 and by auto-oxidation of NO formed by salivary bacteria and NOSCN can be formed by the reaction between endogenous HNO_2 and SCN^-. The latter result supports the participation of NOSCN in the oxidation of APF in saliva.

Nitrite-induced formation of DAF-FMT increased as pH was decreased in buffer solution (Figure 5A). The result suggests that N_2O_3 formed by reaction 6 participated in the formation of DAF-FMT. The enhancement of DAF-FMT formation by SCN^- may be due to the formation of NOSCN as NOSCN is a nitrosating agent [19,23].

In the bacterial fraction, nitrite also induced the formation of DAF-FMT (Figure 5B). At pH values of 6 and 7, N_2O_3, which could react with DAF-FM, might be produced by the reaction between NO and NO_2. NO is generated by nitrite-reducing bacteria and NO_2 is formed by auto-oxidation of NO and by salivary peroxidase/H_2O_2/(nitrite and NO) (Figure 8, scheme 1). SCN^--dependent inhibition of the formation of DAF-FMT at pH values of 6 and 7 supports the participation of salivary peroxidase/H_2O_2/(nitrite and NO) in the formation of N_2O_3. It has been discussed that NO_2 formed by salivary peroxidase-catalysed reactions can contribute to the formation of N_2O_3 using NO generated by nitrite-reducing oral bacteria [12]. Under acidic conditions, the following reactions might contribute to the formation of N_2O_3 in the absence of SCN^-; (i) auto-oxidation of NO produced by salivary bacteria, (ii) reaction 6 and (iii) reaction of NO_2, which was produced by salivary peroxidase-catalysed reactions, with NO. Enhancement of the formation of DAF-FMT by SCN^- at pH 5.3 (Figure 5B) can be attributed to the formation of NOSCN. In saliva filtrate, SCN^- also enhanced the formation of DAF-FMT at pH 5.3 (Figure 5C). The result may support the idea that NOSCN can be formed in saliva under acidic conditions. Degree of the enhancement of DAF-FMT formation by SOD was small at low pH values in bacterial fraction and saliva filtrate, supporting the slow formation of $O_2^-\cdot$ at lower pH values. DTPA enhanced DAF-FMT formation at pH 7. The enhancement may be explained by the increase in concentration of H_2O_2, which can be used for the oxidation of nitrite by salivary peroxidase/H_2O_2, due to DTPA-dependent inhibition of Fenton reaction.

From the result obtained in this study, it is concluded (i) that SCN^- effectively inhibits the formation of NO_2 and N_2O_3, which can oxidize APF and can transform DAF-FM to DAF-FMT, respectively, around neutral pH values when the concentration of nitrite is low but not when the concentration was high, and (ii) that at pH~5, in addition to NO^+, NO_2 and N_2O_3, NOSCN seems to participate in the oxidation of APF and the formation of DAF-FMT in the presence of SCN^-. Conclusion (i) suggests that SCN^- can prevent the formation of reactive nitrogen oxide species, which can give damages to cells and tissues of oral cavity, in the presence of the normal concentration of nitrite in saliva as discussed previously [12] and conclusion (ii) suggests that SCN^- can enhance nitrite-dependent damages under acidic conditions. Taking the above discussion and nitrite-induced inactivation of bacteria under acidic conditions [44,45] into consideration,

we can suggest that nitrite is able to injure the cells and tissues of the oral cavity when the concentrations of nitrite and H^+ were increased at the same time.

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