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Effects of pH on nitrite-induced formation of reactive nitrogen oxide species and their scavenging by phenolic antioxidants in human oral cavity

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Abstract
Nitrite-induced transformation of 3-amino-4-monomethylamino-2′,7′-difluorofluorecein (DAF-FM) to the triazol form (DAF-FMT) was studied using bacterial fraction of mixed whole saliva. The transformation at pH 7 was inhibited by SCN^-, suggesting that nitrosative stresses were small in the oral cavity at the pH value as SCN^- was a normal component of saliva. DAF-FMT formation was much faster at pH 5.2 than 7 and ONSCN generated by the reaction of HNO_2 with SCN^- mainly contributed to its formation at pH 5.2. Coffee and phenolic antioxidants inhibited the DAF-FMT formation less effectively at pH 5.2 than pH 7. The less effectiveness was discussed to be due to slow scavenging of ONSCN, which contributed mainly to the formation of DAF-FMT at pH 5.2, by coffee and phenolics. Since nitrite-induced formation of ONSCN should become faster as the pH decreases, it was suggested that nitrosative stresses to oral tissues became serious under acidic conditions.

Keywords: *Diaminofluorescein, human saliva, ONSCN, pH, phenolic antioxidants, reactive nitrogen oxide species*

Abbreviations: *ABTS, 2,2-azino-bis(3-ethylbenzthianozolin-6-sulphonic acid); DAF-FM, 3-amino-4-monomethylamino-2',7'-difluorofluorecein; DAF-FMT, triazole form of DAF-FM; DTCS, N-(dithiocarboxy)sarcosine; NOR 3, (±)-(E)-4-ethyl-2-[(E)-hydroxyiminol]-5-nitro-3-hexenamide*

Introduction

Nitrate, which is secreted into human oral cavity as a salivary component, is reduced to nitrite and then nitric oxide (NO) by certain bacteria [1–3]. The NO formed is oxidized by O_2 and O_2^-, producing NO_2 and $ONOO^-$ ($pKa = 6.8$), respectively. Salivary peroxidase oxidizes nitrite to NO_2 [4]. Oral bacteria can produce O_2^- and H_2O_2 required for the above reactions [4,5]. The NO_2 formed reacts with NO producing N_2O_3. Local pH of the oral cavity decreases to ~ 5 by the proliferation of acid producing bacteria, which is related to the production of dental plaque [6]. Under the weak acidic conditions, part of the nitrite ion is protonated, producing nitrous acid ($pKa = 3.3$). The nitrous acid formed can be transformed to N_2O_3 and ONSCN by self-decomposition and by the reaction with SCN^- [7,8], respectively. The presence of SCN^- in the saliva is well known. It has been suggested that the formation of some of the above reactive nitrogen oxide species increases as pH is decreased from 7 to 5 in the oral cavity [5]. Although the formation of reactive nitrogen oxide species seems to be dependent on pH, precise mechanisms of their formation under weak acidic and neutral conditions have not been fully elucidated.

On the other hand, chlorogenic acid and quercetin are common components found in foods and beverages and they are strong antioxidants in polyphenols that can react with reactive nitrogen oxide

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species. The polyphenols can inhibit the nitration of 4-hydroxyphenylacetic acid [9] and nitrosation of a diaminofluorescein [10,11]. It has been reported that antioxidative activity of polyphenols is dependent on pH [12,13]. As pKa values of quercetin are 7–7.1, 9.1–9.4 and 11.2 [14,15] and those of chlorogenic acid are 3.3–3.5, 8.2–8.4 and 11–12.5 [15–17], it is supposed that the pH-dependence of the antioxidative activities is due to the ratio of dissociated to non-dissociated forms. Coffee is a common beverage and it contains phenolic antioxidants including chlorogenic acid and its isomers. In spite of this, effects of coffee on the formation of reactive nitrogen oxide species in the human oral cavity have not been fully understood.

This paper, at first, deals with the mechanisms of nitrite-induced transformation of 3-amino-4-monomethylamino-2′,7′-difluorofluorecein (DAF-FM) to its triazol form (DAF-FMT) and then deals with the effects of phenolic antioxidants and coffee on the formation of DAF-FMT using bacterial fraction of saliva at pH values of 5.2 and 7. The reason that DAF-FM was used was that the fluorescence yield of DAF-FMT was nearly constant between pH 5.2 and 12 [18]. The result obtained in this study may provide a scientific background to discuss how to keep the oral cavity in healthy condition from the points of formation and scavenging of reactive nitrogen oxide species.

Materials and methods

Reagents

DAF-FM was obtained from Daiichi Pure Chemicals (Tokyo, Japan). *N*-(Dithiocarboxy)sarcosine disodium salt, dihydrate (DTCS) and (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyiminol]-5-nitro-3-hexenamide (NOR 3) were from Dojin (Kumamoto, Japan). Horseradish peroxidase (type II) and 2,2-azino-bis(3-ethylbenzthianozolin-6-sulphonic acid) (ABTS) were from Sigma (Tokyo, Japan). Phenolics were obtained from Wako Pure Chem. Ind. (Osaka, Japan). Catalase from beef liver was from Roche Diagnostics GmbH (Mannheim, Germany). Freeze-dried instant coffee was obtained from a local market. Uric acid (100 mM) was dissolved in 0.1 M NaOH and then diluted with H_2O to 10 mM to be used for experiments.

Preparation of bacterial fraction

Mixed whole saliva (~10 ml) was collected from volunteers by chewing parafilm at 9–10 o'clock in the morning when required after informed consent had been obtained. The collected saliva was passed through two layers of nylon filter nets [380-mesh (32 μm) net, Sansho, Tokyo, Japan] to remove epithelial cells and other particles. The filtrate was centrifuged at 20 000 × g for 5 min and the sediment was suspended in 50 mM sodium phosphate (pH 5.2 and 7) to be used as bacterial fraction although the fraction contained salivary peroxidase. It has been reported that salivary peroxidase binds to cell walls of oral bacteria [19]. When the sediment was suspended in 50 mM sodium phosphate (pH 5.2), the pH changed slightly and the final pH was between pH 5.2 and 5.3. A significant change in pH was observed only when $Fe(DTCS)_2$ was added (see below).

Preparation of crude salivary peroxidase

After centrifugation of mixed whole saliva at 20 000 × g for 5 min, the supernatant (5 ml) was dialysed against 1 l of 10 mM sodium phosphate (pH 7) for one night. Although the dialyzate contained not only salivary peroxidase but also other proteins, the dialyzate was used as a salivary peroxidase fraction.

Fluorescence measurement

Fluorescence increase due to the transformation of DAF-FM to DAF-FMT was measured using a spectrofluorometer (RF-1500, Shimadzu, Kyoto, Japan). The excitation light (490 nm) was passed through a glass-filter G530 (Hoya, Tokyo, Japan) and a sheet of semi-transparent drug packing paper to weaken the intensity of the excitation light. Fluorescence from DAF-FMT was measured at 515 nm [16]. The reaction mixture (0.5 ml) contained 7 μM DAF-FM and 0.2 mM $NaNO_2$ in 50 mM sodium phosphate buffer or bacterial fraction (pH 5.2 and 7). Reactions were started by the addition of sodium nitrite. When required, NaSCN, phenolics, coffee and other reagents were added to the above reaction mixtures after incubation for 8–10 min with sodium nitrite.

Experiments under anaerobic conditions were performed as follows; the reaction mixture (0.5 ml) in a cuvette (2.5 cm high, 0.2 cm wide, 1 cm long) was bubbled with argon gas for a few minutes and then fluorescence increase was measured under the stream of argon gas. Reagents were added to the reaction mixture, agitating the mixture with argon gas.

Electron spin resonance (ESR) spectra

ESR spectra were measured with a JE1XG spectrometer (JEOL, Tokyo, Japan) at ~25°C using a quartz flat cell (0.05 ml). NO produced was detected with an NO-trapping reagent $Fe(DTCS)_2$ as reported previously [5]. The conditions for the measurement were as followings: microwave power, 10 mW; line width, 0.5 mT; amplification, 1000-fold; scanning speed, 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, which were dissolved in H_2O. This mixture (0.15 ml) was mixed with 0.2 ml of 50 mM sodium phosphate buffer (pH 5.2 and 7) or 0.2 ml of

bacterial fraction (pH 5.2 and 7) and the final pH values were ~5.6 and 7.1, respectively. Immediately after the mixing, 0.2 mM $NaNO_2$ and other reagents were added to withdraw into the quartz cell.

HPLC

The decrease in the concentration of DAF-FM was measured by HPLC using Shim-pack CLC-ODS column (15 cm × 6 mm i.d.) combined with a spectrophotometric detector with a photodiode array (SPD-10MAvp, Shimadzu). After incubation of the reaction mixture (0.5 ml) for 10 min, which contained 7 μM DAF-FM and 0.2 mM $NaNO_2$ in bacterial fraction, the mixture was passed through a cellulose acetate filter (0.45 μm, Advantec, Tokyo, Japan) and 10 μl of the filtrate was applied to the HPLC column. When incubated in buffer solution, the reaction mixture was directly applied to the column. The mobile phase was a mixture of methanol and 5 mM sodium phosphate (pH 7.0) (1:1, v/v) and the flow rate was 1 ml/min. DAF-FM was detected at 490 nm and the retention time was ~4 min.

DAF-FMT was separated by the HPLC column described above and detected with a spectrofluorometric detector (RF-550, Shimadzu). The reaction mixture (0.5 ml), which contained 7 μM DAF-FM and 0.2 mM $NaNO_2$ in bacterial fraction, was incubated for 10 min, and then 1 μl of the mixture was directly applied the HPLC column. The excitation and emission wavelengths were 490 and 515 nm, respectively, and the mobile phase was a mixture of methanol and 5 mM sodium phosphate (pH 7.0) (1:2, v/v). The flow rate was 1 ml/min.

Decreases in the concentration of chlorogenic acid and its isomers in coffee were also measured using the HPLC column described above. The reaction mixture (1 ml) contained 2 μg of coffee and 1 mM $NaNO_2$ in bacterial fraction. After incubation for 30 min, the reaction mixture was passed through a cellulose acetate filter (0.45 μm, Advantec, Tokyo, Japan) to apply to the HPLC column. The mobile phase was a mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v), pH of which was adjusted to 3.0 by 1 M H_3PO_4 and the flow rate was 1 ml/min. Chlorogenic acid was detected at 320 nm and the retention time was 5.6 min. In addition to chlorogenic acid, two isomers of chlorogenic acid with retention times of 4.1 and 6.0 min were also detected at 320 nm.

HPLC was also performed to determine the concentration of quercetin in the reaction mixture using a Shim-pack CLC-ODS column. After incubation of bacterial fraction (0.5 ml), which contained 10 μM quercetin, for defined periods (5 and 15 min in the presence and absence of horseradish peroxidase (10 μg/ml), respectively), the bacterial fraction was passed through a cellulose acetate filter (0.45 μm) to apply to the HPLC column. The mobile phase was a mixture of methanol and 25 mM KH_2PO_4 (3:2, v/v, pH 4.5) and the flow rate was 1 ml/min. Quercetin was detected at 360 nm and the retention time was 7.3 min.

Spectrophotometric measurements

Oxidation of ABTS by salivary peroxidase was measured by the absorption increase at 734 nm [20] using a spectrophotometer (UV-260, Shimadzu). The reaction mixture (1 ml) contained 0.05 mM ABTS, 0.01 mM H_2O_2 and 0.05 ml of salivary peroxidase fraction in 50 mM sodium phosphate (pH 5.2 and 7).

Results

Formation of DAF-FMT

Figure 1 shows HPLC of fluorescent components produced by nitrite in bacterial fraction. At pH 7, a major fluorescent component (retention time, 5.2 min) was detected (Figure 1A). The component was also detected when DAF-FM was incubated with an NO generating reagent (NOR 3) as reported previously [5], suggesting that the fluorescent component was DAF-FMT. SCN^- inhibited the formation of DAF-FMT at pH 7 (Figure 1B). At pH 5.2, DAF-FMT was also the major fluorescent component independent of the presence and absence of SCN^-

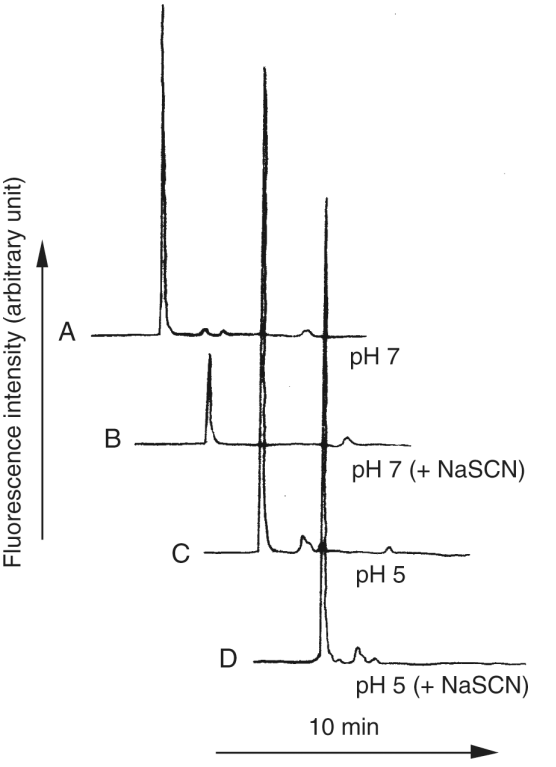

Figure 1. Separation of fluorescent products by HPLC. The reaction mixture (0.5 ml), which contained 7 μM DAF-FM and 0.2 mM $NaNO_2$ in 0.5 ml of bacterial fraction (pH 7 and 5.2), was incubated for 10 min. Traces A and B, pH 7; traces C and D, pH 5.2. Traces A and C, no addition; traces B and D, 1 mM NaSCN.

(Figure 1C and D). DAF-FMT was also detected when DAF-FM was incubated with 0.2 mM nitrite in 50 mM sodium phosphate buffer (pH 5.2) in the presence and absence of SCN^- (data not shown).

Effects of catalase, H_2O_2 and SCN^- on DAF-FMT formation

Figure 2 shows typical time courses of nitrite-induced fluorescence increase due to the formation of DAF-FMT at pH values of 7 and 5.2 under various conditions. At pH 7, any fluorescence increase could not be detected by the successive addition of nitrite and SCN^- to buffer solution with DAF-FM (Figure 2A). In bacterial fraction, nitrite induced fluorescence increase and the fluorescence increase was decreased to $43 \pm 6\%$ (mean $\pm$ SD, $n = 4$) by catalase (Figure 2B). Rate of the nitrite-induced fluorescence increase under anaerobic conditions was $53 \pm 23\%$ ($n = 6$) of that under aerobic conditions and H_2O_2 enhanced the fluorescence increase $\sim$2-fold (Figure 2C). SCN^- inhibited the nitrite-induced fluorescence increase and the rate was $18 \pm 4\%$ ($n = 3$) of that in the absence of SCN^- (Figure 2D). In the presence of SCN^-, no significant effects of catalase and H_2O_2 were observed (Figure 2D and E). The result suggests that a salivary peroxidase/H_2O_2/nitrite system contributed to the fluorescence increase observed at pH 7. It is known than SCN^- is a substrate of salivary peroxidase in the oral cavity [17].

At pH 5.2, nitrite induced slow fluorescence increase and SCN^- enhanced the increase greatly in buffer solution (Figure 2A). Since SCN^- is known to react with HNO_2 producing ONSCN [7,8] and ONSCN is a nitrosating agent [8,21], the result indicates that ONSCN produced by the reaction between HNO_2 and SCN^- contributed to the formation of DAF-FMT at pH 5.2. The addition of DAF-FM to bacterial fraction resulted in the fluorescence increase and the fluorescence increase was enhanced by nitrite (Figure 2B). The nitrite-induced fluorescence increase was decreased to $38 \pm 10\%$ ($n = 3$) by catalase and SCN^- enhanced the decreased fluorescence increase (Figure 2B). Rate of the fluorescence increase under anaerobic conditions was $27 \pm 11\%$ ($n = 3$) of that under aerobic conditions and the fluorescence increase was enhanced $\sim$5-fold by H_2O_2 (Figure 2C). Slow fluorescence increase was also observed by the addition of DAF-FM to bacterial fraction in the presence of SCN^- and the rate was $27 \pm 1\%$ ($n = 3$) of that in the absence of SCN^- (Figure 2D). Nitrite enhanced the fluorescence increase significantly in the presence of SCN^- (Figure 2D) and the enhancement was comparable to that in the absence of SCN^- (Figure 2B). Catalase only slightly decreased the fluorescence increase in the presence of SCN^- and the rate was $89 \pm 6\%$ ($n = 3$) of that in the absence of the enzyme (Figure 2D).

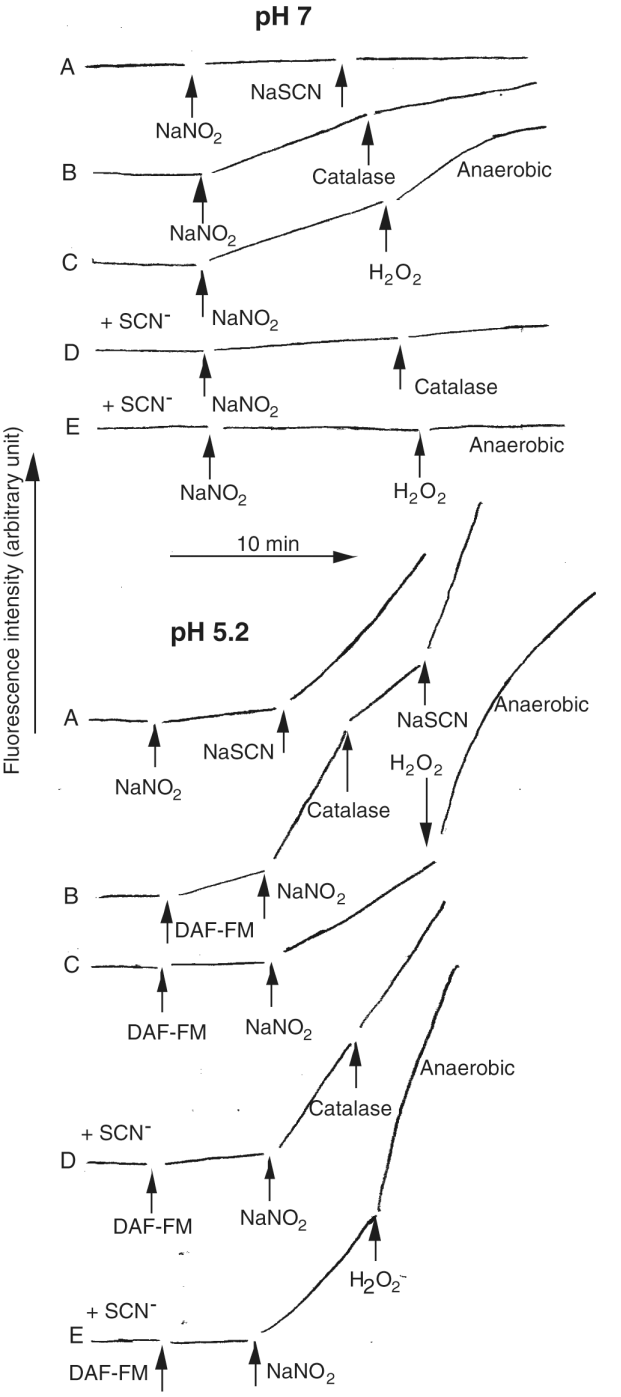

Figure 2. Typical time courses of fluorescence increase due to formation of DAF-FMT. Upper: pH 7. Lower: pH 5.2. The reaction mixture (0.5 ml) contained 7 μM DAF-FM in 50 mM sodium phosphate buffer (pH 7 and 5.2) or bacterial fraction (pH 7 and 5.2). Traces A, buffer solution. DAF-FM had been added before the addition of $NaNO_2$. Traces B–E, bacterial fraction. Traces B and D, aerobic conditions; traces C and E, anaerobic conditions. Traces D and E, with 1 mM NaSCN. Where indicated by arrows, DAF-FM (7 μM), $NaNO_2$ (0.2 mM), catalase (1400 units/ml), H_2O_2 (10 μM) or NaSCN (1 mM) was added.

Nitrite-induced fluorescence increase under anaerobic conditions was $52 \pm 27\%$ ($n = 3$) of that under aerobic conditions in the presence of SCN^- and H_2O_2 enhanced the fluorescence increase significantly

(Figure 2E). The effects of catalase, H_2O_2 and SCN^- suggest that salivary peroxidase-dependent and -independent reactions contributed to the fluorescence increase at pH 5.2.

At pH 7, 1 mM SCN^- inhibited nitrite-induced fluorescence increase ~80%, but the effect of SCN^- on the fluorescence increase was small at pH 5.2 (Figures 1 and 2). Then we studied effects of the concentration of SCN^- on nitrite-induced fluorescence increase at pH 5.2 under anaerobic conditions excluding the effects of O_2 and its reduction products on the fluorescence increase (Figure 3). In buffer solution, SCN^- enhanced the fluorescence increase as a function of the concentration of SCN^- (○). In bacterial fraction, low concentrations of SCN^- (0.04 and 0.1 mM) inhibited the fluorescence increase. The effect of SCN^- turned to enhancement when the concentration was increased beyond 0.3 mM (●). The increase as a function of the concentration of SCN^- was similar to that in buffer solution, suggesting that ONSCN formed by the reaction of HNO_2 with SCN^- [7,8] also contributed to the formation of DAF-FMT in bacterial fraction at pH 5.2.

Consumption of DAF-FM

A diaminofluorescein can be transformed to its triazol form by nitrosation and oxidative nitrosylation [22,23]. To understand the mechanism of DAF-FMT formation at pH values of 7 and 5.2, we measured the consumption of DAF-FM (Table I). No detectable consumption of DAF-FM was observed when nitrite or nitrite plus SCN^- were added to buffer solution at pH 7. In an acidic buffer solution (pH 5.2), consumption of DAF-FM was observed in the presence of both nitrite and SCN^-. In bacterial fraction, nitrite-induced consumption of DAF-FM was faster at pH 7 than pH 5.2. SCN^- inhibited the consumption by ~80% at pH 7 as SCN^- did the formation of DAF-FMT at pH 7 (Figures 1 and 2), suggesting that oxidation intermediates formed during the consumption of DAF-FM were used to produce DAF-FMT at pH 7. At pH 5.2, the consumption was inhibited by ~50% by SCN^-, although SCN^- only slightly inhibited the formation of DAF-FMT (Figures 1 and 2), suggesting that SCN^- itself contributed to the DAF-FMT formation at pH 5.2.

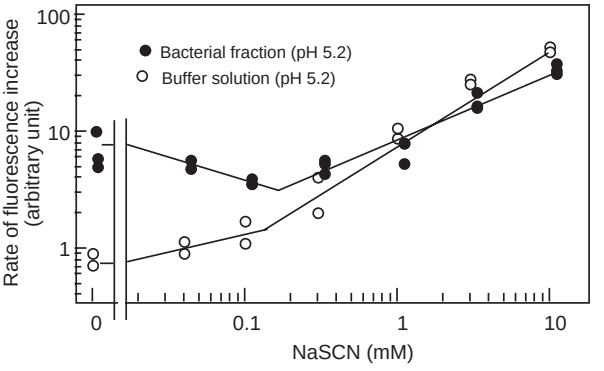

Figure 3. Effects of concentration of SCN^- on fluorescence increase in buffer solution and bacterial fraction under anaerobic conditions. The reaction mixture (0.5 ml) contained 7 μM DAF-FM, 0.2 mM $NaNO_2$ in buffer solution or bacterial fraction (pH 5.2). After the addition of $NaNO_2$, various amounts of NaSCN were added successively. Rate of the fluorescence increase was calculated from the fluorescence increase during 8–10 min of incubation after the addition of NaSCN.

Detection of NO

Figure 4 shows nitrite-induced formation of NO-Fe(DTCS)$_2$ in buffer solution and bacterial fraction. In buffer solution, the formation of NO-Fe(DTCS)$_2$ was faster at pH 5.6 than pH 7.1 (Δ), as reported previously [5]. SCN^- did not significantly affect the nitrite-induced NO-Fe(DTCS)$_2$ formation at pH 5.6 (▽) as well as pH 7.1 in buffer solution (not shown). In bacterial fraction, slow formation of NO-Fe(DTCS)$_2$ was observed at pH 5.6 but not at pH 7.1 when nitrite was not added (○), indicating the NO formation using an endogenous substrate at pH 5.6. Nitrite enhanced the formation of NO-Fe(DTCS)$_2$ at the two pH values in bacterial fraction (●). SCN^- enhanced its formation at pH 5.6 but did not at pH 7, independent of the presence (□) and absence (■) of added nitrite and the SCN^--dependent enhancement in the presence of nitrite (● → □) was similar to that in the absence of nitrite (○ → ■), suggesting that SCN^- enhanced NO formation that was dependent on the endogenous substrate but not the added nitrite. Under anaerobic conditions, nitrite-induced formation of NO-Fe(DTCS)$_2$ was also observed at pH values of 7.1 and 5.6 (data not shown).

Effects of azide on formation of DAF-FMT

Azide (pKa = 4.6–4.7) can inhibit nitrosation by scavenging NO^+ donor such as N_2O_3 [22,24–26] and can inhibit peroxidase activity [27]. Then, we

Table I. Nitrite-induced consumption of DAF-FM.

	− SCN^- (nM/min)	+ 1mM SCN^- (nM/min)
Buffer pH 7.0	BD	BD
Buffer pH 5.2	BD	48 ± 36
Bacterial fraction pH 7.0 (without $NaNO_2$)	8 ± 14	BD
Bacterial fraction pH 7.0 (with 0.2mM $NaNO_2$)	198 ± 48	41 ± 28
Bacterial fraction pH 5.2 (without $NaNO_2$)	BD	BD
Bacterial fraction pH 5.2 (with $NaNO_2$)	63 ± 21	30 ± 11

The consumption of DAF-FM was measured as described in Materials and methods. Data represent means ± SDs (n = 3–6). BD, below the level of detection.

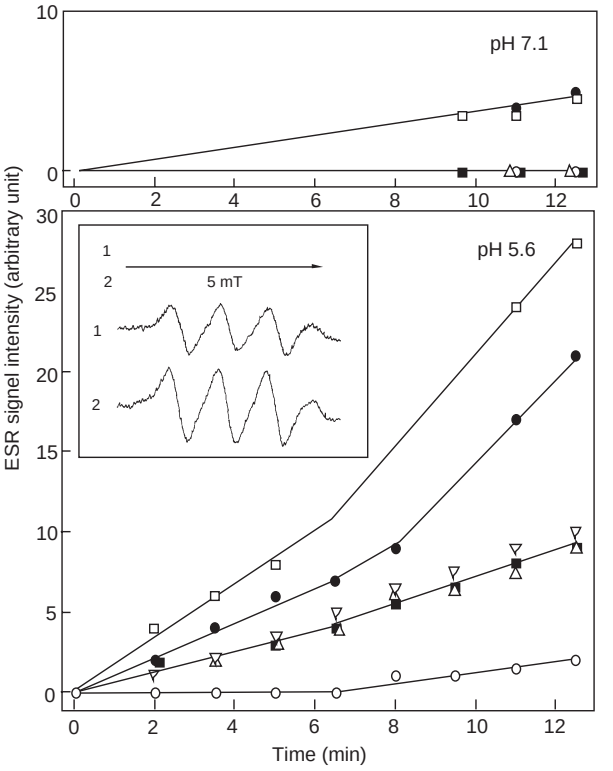

Figure 4. NO formation in bacterial fraction. The reaction mixture contained 0.2 mM $NaNO_2$, 3.8 mM DTCS and 1.3 mM $FeCl_3$ in 50 mM sodium phosphate buffer or bacterial fraction. Upper panel, pH 7.1; lower panel, pH 5.6. (○), bacterial fraction; (●), 0.2 mM $NaNO_2$; (■), 2 mM NaSCN; (□), 0.2 mM $NaNO_2$ + 2 mM NaSCN. (△), 0.2 mM $NaNO_2$ in buffer; (▽), 0.2 mM $NaNO_2$ + 2 mM NaSCN in buffer. Inset: typical ESR spectra of NO-Fe(DTCS)$_2$ complex. 1, buffer (pH 5.6); 2. bacterial fraction (pH 5.6). The reaction mixture was incubated for ~12 min and then ESR spectra were measured.

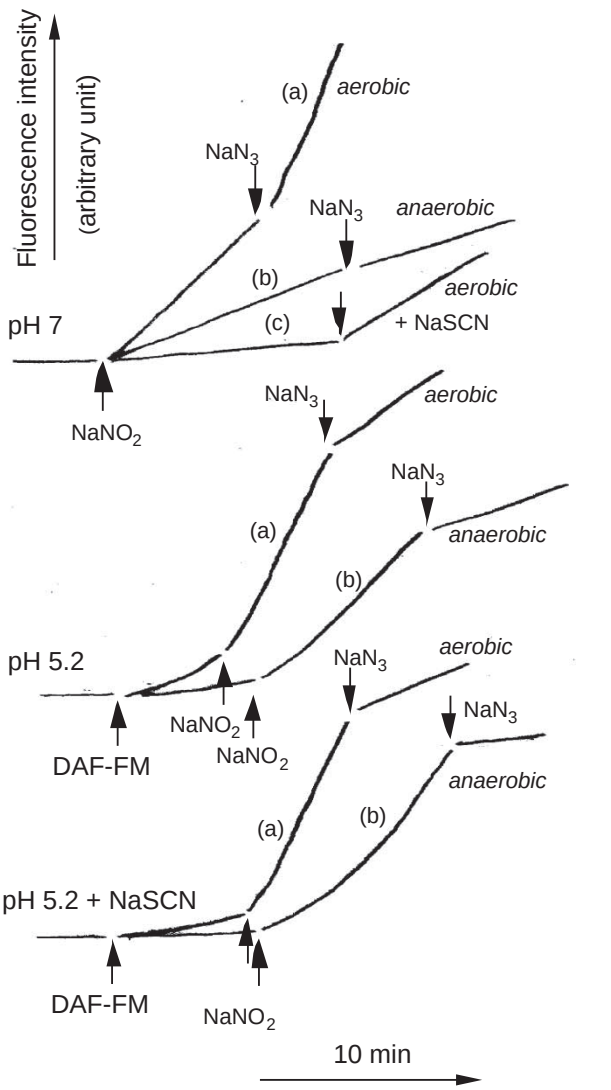

Figure 5. Effects of azide on nitrite-induced fluorescence increase. The reaction mixture (0.5 ml) contained 7 μM DAF-FM in bacterial fraction (pH 7). At pH 5.2, DAF-FM (7 μM) was added as shown in the figure. Other reagents were added as shown in each trace. The concentrations of $NaNO_2$, NaSCN and NaN_3 were 0.2, 2 and 1 mM, respectively. Traces A, aerobic conditions; traces B, anaerobic conditions; trace C, in the presence of SCN^-.

studied the effects of azide on nitrite-induced fluorescence increase using bacterial fraction in the presence of DAF-FM (Figure 5). Azide (1 mM) enhanced the fluorescence increase, although degree of the enhancement depended on the preparation of bacterial fraction at pH 7 (Figure 5A in the top). Azide also enhanced the nitrite-induced fluorescence increase in the presence of SCN^- (Figure 5C). Under anaerobic conditions, azide did not enhance the fluorescence increase (Figure 5B). At pH 5.2, azide inhibited the nitrite-induced fluorescence increase 60–70% under both aerobic (Figure 5A) and anaerobic (Figure 5B) conditions (traces in the middle). Degrees of the inhibition by azide in the presence of SCN^- were 85 and 95% under aerobic (Figure 5A) and anaerobic (Figure 5B) conditions, respectively (traces in the bottom). A major fluorescent component formed in the presence of azide was separated by HPLC and the component was identical with DAF-FMT (not shown). Azide (1 mM) also inhibited nitrite-induced fluorescence increase more than 90% in 50 mM sodium phosphate buffer (pH 5.2) in the presence and absence of 1 mM SCN^- under aerobic as well as anaerobic conditions (data not shown).

Inhibition of salivary peroxidase by azide

Effects of azide on the oxidation of ABTS by a salivary peroxidase were studied in the presence of 10 μM H_2O_2. The oxidation of ABTS was ~10-fold faster at pH 5.2 than pH 7. Azide (1 mM) completely inhibited the reduction at pH values of 7 and 5.2, independent of the presence and absence of 0.1 mM nitrite. SCN^- (2 mM) also nearly completely inhibited the oxidation of ABTS at both pH values of 7 and 5.2. This result can be explained by the

preferential oxidation of SCN^- to $OSCN^-$/HOSCN (pKa = 5.3), even when ABTS was present [28].

Inhibition of DAF-FMT formation by salivary antioxidants and phenolics

Table II shows effects of various antioxidants on the formation of DAF-FMT in bacterial fraction at pH values of 7 and 5.2 under aerobic conditions. Uric acid (0.1 mM) (pKa = 5.4) [20], a main antioxidant in saliva [29], inhibited the formation of DAF-FMT at pH 7 but did not at pH 5.2. Ascorbic acid inhibited the DAF-FMT formation at pH values of 7 and 5.2. Although 1 μM ascorbic acid strongly inhibited the fluorescence increase, the inhibition became smaller during incubation, attaining to the rate before the addition of ascorbate. The periods required for the recovery were 7 ± 1 and 32 ± 10 min ($n = 4$) at pH values of 7 and 5.2, respectively. From the values, average rates of the oxidation of 1 μM ascorbic acid were calculated to be ~140 and 30 nM/min at pH values of 7 and 5.2, respectively. SCN^- (2 mM) prolonged the recovery period at pH 7 ~3-fold, but no detectable effects of SCN^- were observed at pH 5.2. Glutathione (10 μM) also inhibited the DAF-FMT formation at pH values of 7 and 5.2. Phenolic antioxidants inhibited the formation of DAF-FMT more effectively at pH 7 than pH 5.2. Fifty per cent inhibition of the DAF-FMT formation by quercetin was observed at ~0.3 and 20 μM at pH values of 7 and 5.2, respectively.

Effects of ascorbic acid (10 μM), glutathione (0.1 mM) and quercetin (10 μM) on the formation of DAF-FMT were studied in the presence of SCN^- at pH 5.2 (Table II). Ascorbic acid and glutathione inhibited the formation of DAF-FMT more than 80%, but quercetin ~40%, indicating that SCN^- did not significantly affect the inhibition by the above reagents.

Effects of coffee on the formation of DAF-FMT

Figure 6 shows coffee-dependent inhibition of nitrite-induced fluorescence increase in bacterial fraction. At pH 7, 50% inhibition was observed at ~2 μg of coffee/ml (△). Although it is not shown in the Figure, 1 mM SCN^- did not significantly affect the coffee-dependent inhibition. Coffee was less effective for the inhibition of the fluorescence increase at pH 5.2 and 50% inhibition was observed at ~20 μg of coffee/ml (○). No significant effects of SCN^- on the inhibitory effect of coffee were observed (●). Coffee also inhibited the fluorescence increase in buffer solution (7 μM DAF-FM and 0.2 mM $NaNO_2$ in 50 mM sodium phosphate at pH 5.2) and the concentrations required for 50% inhibition were ~20 and 4 μg of coffee/ml in the presence and absence of 1 mM NaSCN, respectively. Amount of chlorogenic acid and its isomers in coffee was 64 ± 3.3 nmol per mg of coffee, as reported previously [9,30].

Consumption of chlorogenic acid and quercetin

Table III shows rates of consumption of chlorogenic acid plus its isomers in coffee in bacterial fraction in the presence of nitrite, since rate of the oxidation of chlorogenic acid in the absence of nitrite is quite slow [9]. The consumption was faster at pH 7 than pH 5.2 and SCN^- inhibited the consumption at the pH values, suggesting the faster production of H_2O_2 at pH 7 than pH 5.2. The oxidation of quercetin by bacterial fraction was also faster at pH 7 than pH 5.2 and the enhancement by nitrite was small, as reported previously [5,31] (Table IV). The enhancement of the oxidation of quercetin by horseradish peroxidase was

Table II. Inhibition of the formation of DAF-FMT by phenolics, ascorbic acid and glutathione in bacterial fraction.

	Rate of DAF-FMT formation (relative)		
	pH 7.0	pH 5.2	pH 5.2 + 2 mM SCN^-
No addition	100	100	100
Uric acid (100 μM)	43 ± 4 ($n = 3$)	105 ± 5 ($n = 3$)	
Ascorbic acid (1 μM)	25 ± 7 ($n = 3$)	15 ± 2 ($n = 3$)	
(10 μM)	4 ± 2 ($n = 3$)	5 ± 2 ($n = 3$)	5 ± 3 ($n = 3$)
Glutathione (10 μM)	84 ± 5 ($n = 5$)	57 ± 4 ($n = 5$)	
(100 μM)	12 ± 8 ($n = 5$)	14 ± 10 ($n = 5$)	17 ± 5 ($n = 3$)
Quercetin (10 μM)	5	63	58 ± 6 ($n = 3$)
Catechin (10 μM)	26	90	
Chlorogenic acid (10 μM)	26	100	
Caffeic acid (10 μM)	13	88	
Ferulic acid (10 μM)	45	89	
Gallic acid (10 μM)	2	50	

The reaction mixture (0.5 ml) contained 7 μM DAF-FM and 0.2 mM $NaNO_2$ in bacterial fraction (pH 5.2 and 7.0). After incubation for ~10 min, each reagent was added and the rate of formation of DAF-FMT relative to that before the addition was calculated. The rate of fluorescence increase before the addition was represented as 100. Each data is average of two or more experiments (mean ± SD).

Table III. Consumption of chlorogenic acid plus its isomers in coffee by bacterial fraction.

	Oxidation of chlorogenic acid (nM/min)	
	No addition	+ 1 mM NaSCN
pH 7.0	3.23±0.90	0.60±0.63
pH 5.2	1.13±0.53	0.40±0.17

The reaction mixture contained 2 μg of coffee in 1 ml of bacterial fraction that contained 1 mM $NaNO_2$. The initial concentration of chlorogenic acid plus its isomers in the reaction mixture was equivalent to ~0.14 μM chlorogenic acid. Data represent means±SDs ($n=5$).

larger at pH 7 than pH 5.2 (Table IV), supporting the faster production of H_2O_2 at pH 7 than pH 5.2.

Discussion

In a previous paper [5], we have discussed that N_2O_3 generated by the reaction between NO and NO_2 participates in the nitrite-induced transformation of DAF-FM to DAF-FMT in bacterial fraction prepared from mixed whole saliva at pH 7. The discussion is based on the results that NO can be produced by nitrite-reducing bacteria and that NO_2 can be generated by the auto-oxidation of NO and the salivary peroxidase-catalysed oxidation of nitrite. In the present investigation, catalase and H_2O_2 inhibited and enhanced, respectively, the transformation of DAF-FM to DAF-FMT at pH 7 (Figure 2), supporting the participation of a salivary peroxidase/H_2O_2 system in the formation of DAF-FMT, but azide, a scavenger of N_2O_3, did not inhibit the formation of DAF-FMT (Figure 5). The failure of the inhibition suggests that the contribution of N_2O_3 in the formation of DAF-FMT was not significant in the formation of DAF-FMT. If N_2O_3 did not contribute to the formation of DAF-FMT, the fluorescent compound might be formed by oxidative nitrosylation [22,23]. Two reactions are possible for the oxidation of DAF-FM. One is the oxidation of DAF-FM by a salivary peroxiadse/H_2O_2 system itself and the other is its oxidation by NO_2 formed by a salivary peroxidase/H_2O_2/nitrite system (Figure 7, Scheme 1). The result that nitrite significantly enhanced the consumption of DAF-FM (Table I) supports the latter. The formation of NO_2 by a salivary peroxidase/H_2O_2/nitrite system has been reported [31–33]. The contribution of NO_2 in the formation of DAF-FMT is supported further by the result that uric acid, which can react with not only $ONOO^-$ [34,35] but also NO_2 [36,37], inhibited the fluorescence increase (Table II). It has been reported that uric acid neither affects the formation of NO in bacterial fraction [38] nor inhibits the salivary peroxidase-catalysed oxidation reaction [32]. The contribution of $ONOO^-$ formed from NO and O_2^- to the oxidation of DAF-FM [22,23] is excluded as superoxide dismutase enhances the formation of DAF-FMT [5].

Nitrite-induced fluorescence increase was also observed under anaerobic conditions at pH 7 in the absence of SCN^- (Figure 2). Under anaerobic conditions, contribution of N_2O_3, H_2O_2, NO_2 and $ONOO^-$ for the formation of DAF-FMT was impossible. If DAF-FM was oxidized during the reduction of nitrite to NO by nitrite-reducing bacteria, the formation of DAF-FMT was possible. The production of NO under anaerobic conditions was confirmed in this study using $Fe(DTCS)_2$. SCN^--dependent

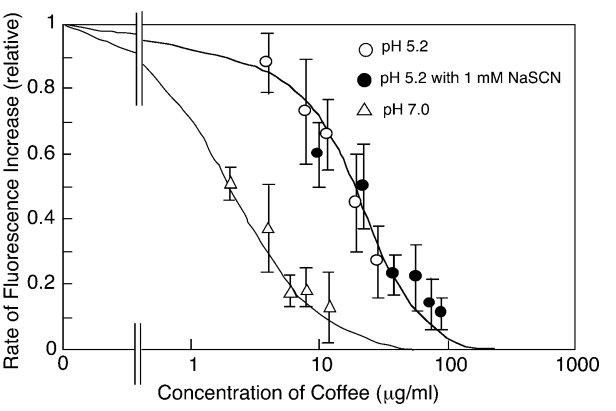

Figure 6. Effects of coffee on the nitrite-induced DAF-FMT formation in bacterial fraction. The reaction mixture (0.5 ml) contained 7 μM DAF-FM and 0.2 mM $NaNO_2$ in bacterial fraction (pH 5.2 and 7). The reaction mixture was incubated for ~10 min after the addition of nitrite and then coffee was added to calculate the rate of fluorescence increase relative to that before the addition of coffee.

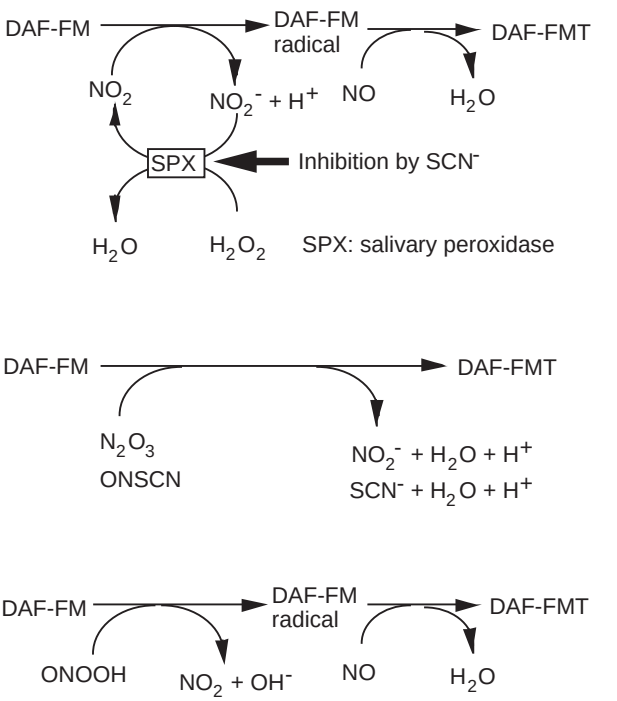

Figure 7. Possible reactions to generate DAF-FMT in human oral cavity. The postulation that DAF-FM radical may react with NO is included in the figure.

Table IV. Oxidation of quercetin in bacterial fraction.

	Oxidation of quercetin (nM/min)		
	No addition	+ $NaNO_2$	+ Horseradish peroxidase
pH 7.0	165±70 (n=9)	207±43 (n=7)	715±104 (n=4)
pH 5.2	54±38 (n=9)	87±48 (n=7)	408±100 (n=4)

Concentrations of quercetin, horseradish peroxidase and $NaNO_2$ were 10 μM, 10 μg/mL and 0.2 mM, respectively. Data represent means ± SDs.

inhibition of the nitrite-induced fluorescence increase under anaerobic conditions (Figure 2) could be explained if SCN^- inhibited the oxidation of DAF-FM since SCN^- did not inhibit the formation of NO (Figure 4). The transformation of 4,5-diaminofluorescein (DAF-2) to its triazol form is nearly completely inhibited by the replacement of air with argon gas [4]. The difference may be due to the difference in the functional group between DAF-2 and DAF-FM. The more hydrophobicity of DAF-FM than DAF-2 may make DAF-FM easier to access to the site where diaminofluoresceins can be oxidized.

In a previous paper [5], we have discussed that the N_2O_3, which is generated by (i) auto-oxidation of NO, (ii) reaction of NO_2 produced by salivary peroxidase-catalysed oxidation of nitrite with NO and (iii) self-decomposition of nitrous acid, contributes to the nitrite-induced formation of DAF-FMT at pH 5.3 under aerobic conditions in the absence of SCN^- (Figure 7, Scheme 2). In this study, we observed azide-dependent inhibition of the nitrite-induced formation of DAF-FMT at pH 5.2 (Figure 5), supporting the contribution of N_2O_3 in the formation of DAF-FMT, but if NO_2 formed by the peroxidase-catalysed reaction contributed for the formation of N_2O_3, azide could also inhibit the formation of N_2O_3 by suppressing salivary peroxidase. The contribution of a salivary peroxidase/H_2O_2 system can be deduced from the catalase-dependent inhibition of the formation of DAF-FMT (Figure 2). The contribution of the oxidative nitrosylation of DAF-FM should be smaller at pH 5.2 than pH 7, because the oxidation of DAF-FM was slower at pH 5.2 than pH 7 (Table I). The contribution of $ONOO^-$ formed from NO and O_2^- can be excluded as superoxide dismutase enhances the transformation of DAF-FM to its triazol form [5]. Alternatively, the contribution of ONOOH produced by the reaction of HNO_2 with H_2O_2 was possible for the oxidation of DAF-FM to react with NO (see below). Azide also inhibited the nitrite-induced formation of DAF-FMT under anaerobic conditions at pH 5.2 in the absence of SCN^- (Figure 5). The inhibition could be explained by the scavenging of N_2O_3 generated by self-decomposition of nitrous acid. The production of NO by chemical reaction in the mouth has been proposed [39].

In a previous paper [5], we have also discussed that ONSCN produced by the reaction between SCN^- and HNO_2 [7,8] can contribute to the nitrite-induced formation of DAF-FMT under acidic and aerobic conditions in the presence of SCN^- (Figure 7, Scheme 2). Catalase and the removal of O_2 did not largely inhibit the nitrite-induced formation of DAF-FMT (Figure 2), suggesting that the contribution of a peroxidase/H_2O_2/nitrite system to the formation of DAF-FMT was small. A scavenger of ONSCN, azide [40], greatly suppressed the formation of DAF-FMT (Figure 5), supporting the significant contribution of ONSCN to the formation of DAF-FMT. The enhancement of the formation of DAF-FMT by SCN^- in the presence of catalase (Figure 2) also supports the participation of ONSCN in the formation of DAF-FMT. The small effects of SCN^- on the nitrite-induced formation of DAF-FMT at pH 5.2 in contrast to its effects at pH 7 (Figure 2) may be explained if SCN^--dependent formation of ONSCN exceeded SCN^--dependent inhibition of salivary peroxidase-catalysed oxidation of DAF-FM.

Azide largely inhibited the nitrite-induced formation of DAF-FMT in bacterial fraction as well as in buffer solution (pH 5.2) in the presence of 1 mM SCN^- under anaerobic conditions (Figure 5), indicating that ONSCN also contributed to the formation of DAF-FMT in bacterial fraction under anaerobic conditions. The contribution of ONSCN in the bacterial fraction seemed to increase as the concentration of SCN^- was increased beyond 0.3 mM (Figure 3). The inhibition of DAF-FMT formation by low concentrations of SCN^- (< 0.1 mM) may be explained by the suppression of the oxidation of DAF-FM.

H_2O_2-induced fluorescence increase was much faster at pH 5.2 than pH 7 in the absence of SCN^- (Figure 2). This result may be explained by the faster oxidation of nitrite to NO_2 at pH 5.2 than pH 7. It has been reported that salivary peroxidase can oxidize nitrite at pH 5.2 [31] and that catalytic activities of salivary peroxidase are much faster at pH 5 than 7 (in this study and Pruitt et al. [28]). In spite of the higher catalytic activity of salivary peroxidase at pH 5.2 than pH 7, the consumption of DAF-FM, chlorogenic acids, quercetin and ascorbic acid in bacterial fraction was faster at pH 7 than pH 5.2 and SCN^- inhibited their consumption (Tables I, III and IV). The result suggests that H_2O_2 production would be faster at pH 7 than pH 5.2. The faster production of H_2O_2 is

supported by the result that the oxidation of quercetin in the presence of horseradish peroxidase was faster at pH 7 than pH 5.2 (Table IV) and the faster production may result in the increase in concentration of $OSCN^-$/HOSCN, which is an antibacterial agent, in the oral cavity [17].

H_2O_2 enhanced the fluorescence increase in the presence of SCN^- at pH 5.2 but not at pH 7 (Figure 2). The contribution of a salivary peroxidase/H_2O_2/nitrite system to the formation of DAF-FMT may be small because salivary peroxidase preferentially oxidizes SCN^- to $OSCN^-$ when SCN^- is present [19,28,41]. Alternatively, the enhancement may be attributed to an ONOOH/NO system (Figure 7, Scheme 3) since ONOOH is produced by the reaction of H_2O_2 with HNO_2 [20,42–44] and can oxidize DAF-FM [22,23]. NO_2 produced during the ONOOH-dependent oxidation of DAF-FM may also contribute to the oxidation of DAF-FM. The contribution of the ONOOH is supported by the result that H_2O_2 did not enhance the formation of DAF-FMT at pH 7 in the presence of SCN^-, where the concentration of HNO_2 was calculated to be $\sim$1/60th of that at pH 5.2 (Figure 2). The above discussion suggests the formation of ONOOH is possible in the oral cavity when the pH was decreased to $\sim$5.

SCN^- inhibited the fluorescence increase observed by the addition of DAF-FM to bacterial fraction at pH 5.2 in the absence of nitrite (Figure 2). On the other hand, SCN^- enhanced the formation of NO at pH 5.6 (Figure 4). The results suggest that the SCN^--dependent enhancement of the formation of NO using an endogenous substrate was not fast enough to overcome the SCN^--dependent suppression of the oxidation of DAF-FM. The replacement of air with argon gas also inhibited the fluorescence increase observed by the addition of DAF-FM to bacterial fraction, suggesting the contribution of O_2 to the oxidation of DAF-FM in the absence of nitrite.

According to the above discussion, it seemed that $NO_2 + NO$ had a major role in the formation of DAF-FMT in the absence of SCN^- and that SCN^- inhibited the formation of NO_2 by salivary peroxidase-catalysed oxidation of nitrite, resulting in the inhibition of the formation of DAF-FMT at pH 7. When the pH was decreased to 5.2, in addition to $NO_2 + NO$, N_2O_3 seemed to contribute to the formation of DAF-FMT in the absence of SCN^-, but the contribution of ONSCN seemed to be increased as the concentration of SCN^- was increased. The effective inhibition of DAF-FMT formation by ascorbic acid and glutathione at pH values of 7 and 5.2 (Table II) suggests effective scavenging of NO_2 and N_2O_3 by the reductants at the pH values. The ineffectiveness of uric acid at pH 5.2 (Table II) may be explained by the small contribution of NO_2 in the formation of DAF-FMT and the ineffective scavenging of N_2O_3.

Phenolics inhibited the formation of DAF-FMT and the inhibition was greater at pH 7 than pH 5.2. According to the mechanisms of DAF-FMT formation, the difference can be explained by faster reaction of phenolics with NO_2 than N_2O_3. Nitrite-induced enhancement of the oxidation of chlorogenic acid [9] and quercetin (Table IV) supports the reaction of NO_2 with phenolics. The inhibition of DAF-FMT formation by ascorbic acid, glutathione and quercetin was not affected by SCN^- (Table II), suggesting that the reagents could also scavenge ONSCN. It has been reported that ascorbic acid and quercetin can react with ONSCN [45,46]. In addition, H^+-dissociation of phenolics may also concern the difference in the inhibitory effect between pH 7 and 5.2. For example, part of the phenolic OH groups of chlorogenic acid and quercetin is dissociated at pH 7 but not pH 5.2 according to their pKa values (see Introduction). The important function of H^+-dissociated phenolics on the scavenging of N_2O_3 may be supported by the effect of quercetin on the NOR 3-induced formation of DAF-FMT; quercetin inhibited the formation of DAF-FMT $\sim$20 and 97% at pH values of 5.2 and 7, respectively. The formation of DAF-FMT was faster at pH 7 than pH 5.2. If N_2O_3 contributed to the NOR 3-induced formation of DAF-FMT, we can deduce that H^+-dissociated quercetin reacted more rapidly with N_2O_3 than non-dissociated one.

It has been reported that chlorogenic acid and its isomers in coffee can react with NO_2 at pH 7 [11]. The concentration of coffee required for 50% inhibition of the formation DAF-FMT at pH 5.2 was much higher than that at pH 7. According to the above discussion, the different effect can be explained by the faster reaction of antioxidants in coffee with NO_2 than N_2O_3 and ONSCN. In addition to chlorogenic acid and its isomers, coffee melanoidins can also react with NO_2, N_2O_3 and ONSCN [47]. The inhibitory effects of coffee on nitrite-induced fluorescence increase were decreased by SCN^- in buffer solution (pH 5.2). The decrease can be explained by the much faster formation of nitrosating reagents, which can transform DAF-FM to DAF-FMT, in the presence than the absence of SCN^- (Figure 2).

The result obtained in this study indicates (i) that when pH in the oral cavity is $\sim$7 and the concentrations of nitrite and SCN^- are $\sim$0.2 and 1 mM, respectively, the formation of reactive nitrogen oxide species to generate DAF-FMT is quite slow and (ii) that when the pH is decreased to $\sim$5 under the above conditions, the formation of DAF-FMT is much faster than that at pH 7 and ONSCN may mainly contribute for the formation of DAF-FMT. The different effects of phenolics at pH values of 7 and 5.2 were discussed to be due to the difference in reactivity of phenolics with NO_2, N_2O_3 and ONSCN. The increase in the formation of ONSCN may result

in the rapid consumption of ascorbic acid and SH compounds and the increased nitrosation stresses in the oral cavity. It has been reported that the decrease in pH is related to the enhanced formation of dental plaque that has been discussed in relation to periodontal diseases [6] and the increased formation of DAF-FMT in whole saliva [5]. Therefore, the prevention of the decrease in pH in the oral cavity may be important to protect the cavity from nitrosative stresses induced by ONSCN.

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