# Oxygen uptake during the mixing of saliva with ascorbic acid under acidic conditions: possibility of its occurrence in the stomach

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**Abstract Human saliva, which contains nitrite, is normally mixed with gastric juice, which contains ascorbic acid (AA). When saliva was mixed with an acidic buffer in the presence of 0.1 mM AA, rapid nitric oxide formation and oxygen uptake were observed. The oxygen uptake was due to the oxidation of nitric oxide, which was formed by AA-dependent reduction of nitrite under acidic conditions, by molecular oxygen. A salivary component $SCN^-$ enhanced the nitric oxide formation and oxygen uptake by the AA/nitrite system. The oxygen uptake by the AA/nitrite/$SCN^-$ system was also observed in an acidic buffer solution. These results suggest that oxygen is normally taken up in the stomach when saliva and gastric juice are mixed.**


*Key words:* Ascorbic acid; Gastric juice; Nitric oxide; Nitrite; Oxygen uptake; Saliva

## 1. Introduction

Saliva, which contains nitrite (0.05–0.2 mM) ($pK_a = 3.3$) [1,2], is normally swallowed into the stomach and mixed with gastric juice, which contains ascorbic acid (AA) (0.05–0.3 mM) [3,4], and it is well known that nitrite is reduced by AA, producing nitric oxide under acidic conditions [5–9]. According to these reports, it is supposed that nitric oxide is formed when saliva is mixed with gastric juice. In fact, formation of nitric oxide in the stomach has been reported [10–12], and the function has been discussed in relation to regulation of mucosal blood flow [13,14], mucus formation [15] and gastric motility [16,17]. If nitric oxide is formed by the reaction between salivary nitrite and gastric AA, consumption of molecular oxygen is possible in the stomach. This is deduced from the fact that molecular oxygen is consumed producing nitrite when pure nitric oxide is added to aqueous solutions [18–20]. The objective of the present study is to measure oxygen consumption during the reaction between saliva and AA under acidic conditions, which simulates the mixing of saliva with gastric juice. The results obtained suggest that molecular oxygen can be consumed in the stomach when saliva is mixed with gastric juice.

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*Abbreviations:* AA, ascorbic acid; DTCS, *N*-(dithiocarboxy)sarcosine sodium disalt; ESR, electron spin resonance

## 2. Materials and methods

### 2.1. Reagents

Griess–Romijn nitrite reagent was obtained from Wako Pure Chem. (Osaka, Japan) and *N*-(dithiocarboxy)sarcosine sodium disalt (DTCS) was from Dojin Laboratories (Kumamoto, Japan).

### 2.2. Preparation of saliva

Mixed whole saliva (about 10 ml each) was collected from four volunteers at the daytime by chewing parafilm. The collected saliva was centrifuged at $20\,000 \times g$ for 5 min and used as centrifuged saliva. When required, centrifuged saliva (10 ml) was dialyzed against 1 l of 10 mM sodium phosphate (pH 7.4) and used as dialyzed saliva.

### 2.3. Quantification of nitrite and $SCN^-$

Concentrations of nitrite in centrifuged saliva were determined with Griess–Romijn reagent as reported previously [1]. The mixture (1.0 ml) contained 0.05 ml of centrifuged saliva, 0.1 ml of 1% Griess–Romijn reagent and 0.85 ml of 50 mM $KH_2PO_4$–KCl–HCl (pH 1.5). After addition of the saliva preparation, the mixture was incubated for 10 min at 35°C and absorbance at 540 nm was determined. Concentrations of $SCN^-$ in centrifuged saliva were determined from the formation of $Fe(SCN)^{2+}$ [21]. The mixture (1.0 ml) contained 0.1 ml of sample, 0.1 ml of 0.1 M $FeCl_3$ and 0.8 ml of 0.1 M HCl. After addition of the saliva preparation, absorbance at 450 nm was determined.

### 2.4. Oxygen uptake

Oxygen uptake was measured at 35°C using an oxygen electrode obtained from Rank Brothers (Cambridge, UK). When saliva preparations were used, the reaction mixture (1.0 ml) contained 0.5 ml of a saliva preparation and 0.5 ml of 50 mM $KH_2PO_4$–KCl–HCl (pH 1.0–2.5). The final pHs, which were determined with a glass electrode, were between 1.3 and 5.2. Oxygen uptake was also measured in 50 mM $KH_2PO_4$–KCl–HCl (pH 1.0–4.5).

### 2.5. Spectrophotometric measurements

Oxidation of AA by nitrite under acidic conditions was measured by the decrease in absorbance at 242 nm using a double beam spectrophotometer (UV-260, Shimadzu, Kyoto, Japan). The reaction mixture (1 ml) contained 0.1 mM AA and 0.05 mM $NaNO_2$ in 50 mM $KH_2PO_4$–KCl–HCl (pH 2.0). Reactions were started by adding AA. The light path of the measuring beam was 4 mm.

### 2.6. Electron spin resonance (ESR) measurements

ESR spectra were measured using a JEOL JES-FE1GX spectrometer at about 25°C with a quartz flat cell (0.05 ml) under the following conditions: microwave power, 10 mW; line width, 0.2 mT; amplification, 1000-fold; scanning speed, 0.625 mT $min^{-1}$. Nitric oxide formed was trapped by $Fe–(DTCS)_2$ [22] as follows. The reaction mixture (0.4 ml) contained 0.1 mM $NaNO_2$ in 50 mM $KH_2PO_4$–KCl–HCl (pH 2.0). Reactions were started by adding $NaNO_2$. After 30 s of incubation at 35°C, 0.5 ml of a mixture containing 10 mM DTCS and 1.5 mM $FeCl_3$ in 0.1 M sodium phosphate (pH 7.6) was added and an aliquot of 0.05 ml was withdrawn into the flat cell. The final pH was about 7. Nitric oxide formation was stopped and stable NO–Fe–$(DTCS)_2$ complex was formed around neutral pH.

When nitric oxide formed in the saliva was measured, the reaction

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mixture (0.4 ml) contained 0.2 ml of centrifuged saliva and 0.2 ml of 50 mM $KH_2PO_4$–KCl–HCl (pH 1.5). The final pH of this reaction mixture was 1.97. After incubation for 30 s, 0.5 ml of a mixture containing 10 mM DTCS and 1.5 mM $FeCl_3$ in 0.1 M sodium phosphate (pH 7.6) was added and an aliquot of 0.05 ml was withdrawn into the flat cell to measure NO–Fe–$(DTCS)_2$ complex.

*2.7. Data presentation*

Measurements were repeated for at least three saliva preparations and essentially the same results were obtained. Typical data or data of two to three measurements are presented.

## 3. Results and discussion

Rapid oxygen uptake was observed when 0.1 mM AA was added to a mixture of 0.5 ml of centrifuged saliva and 0.5 ml of 50 mM $KH_2PO_4$–KCl–HCl (pH 1.5) (final pH about 1.9) (Table 1). Since saliva preparations used in this study contained 0.08–0.16 mM nitrite which could be reduced to nitric oxide by AA, the oxygen uptake was deduced to be due to the formation of nitric oxide. In fact, centrifuged saliva produced nitric oxide under acidic conditions as reported previously [22] and the production was enhanced by AA (Fig. 1, compare traces S-1 and S-2). No oxygen evolution was observed on the addition of catalase (1300 unit $ml^{-1}$) to the reaction mixture, the pH of which had been increased to 7, after completion of the oxygen uptake by AA/nitrite systems under acidic conditions. This result suggests that even if the formation of hydrogen peroxide contributed the oxygen uptake, the contribution was not so much under the conditions of this study. If $H_2O_2$ is formed under acidic conditions, part of the $H_2O_2$ may be scavenged by peroxidase in the saliva because peroxidase in the saliva was still active at pH 2; peroxidase activity in the saliva at pH 2 (3–6 μM guaiacol oxidation $min^{-1}$) was about 2% of the activity at pH 7.6 when the activity was measured in the presence of 0.1 mM $H_2O_2$ and 1 mM guaiacol. Further studies are required for the production of hydrogen peroxide when saliva is mixed with gastric juice.

Fig. 2 (upper panel, open circles) shows the effects of AA concentration on the oxygen uptake by centrifuged saliva under acidic conditions. When AA was not added, the rate of oxygen uptake was low; the rate increased nearly linearly as the concentration of AA was increased. The nitrite concentration of the reaction mixture containing centrifuged saliva was 0.06 mM, then AA-dependent oxygen consumption was studied in a mixture that contained 0.05 mM $NaNO_2$ in 50 mM $KH_2PO_4$–KCl–HCl (pH 2.0); the rates of oxygen uptake were significantly lower in the buffer solution than in centrifuged saliva at any concentration of AA examined. The lower

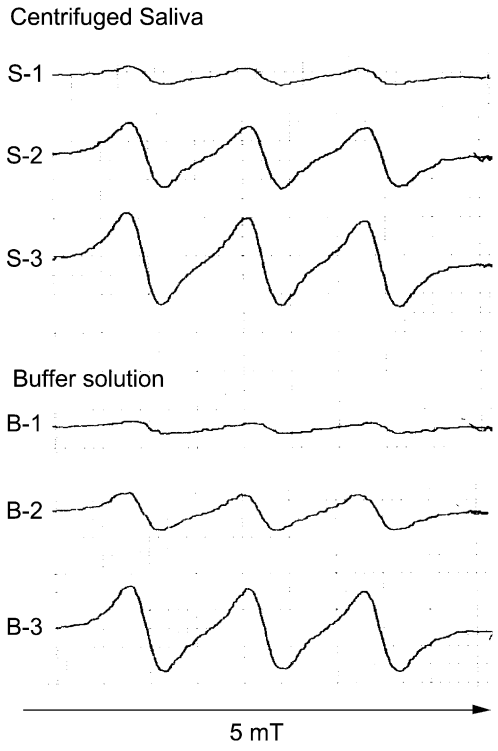


Fig. 1. Nitric oxide formation in saliva (upper panel) and buffer solution (lower panel). Upper panel: The reaction mixture (0.4 ml) contained 0.2 ml of centrifuged saliva, which contained 0.13 mM $NO_2^-$ and 0.33 mM $SCN^-$, and 0.2 ml of 50 mM $KH_2PO_4$–KCl–HCl (pH 1.5). After incubation for 30 s, 0.5 ml of Fe–$(DTCS)_2$ complex was added. S-1, no addition; S-2, 0.1 mM AA; S-3, 0.1 mM AA+1 mM NaSCN. Lower panel: The reaction mixture (0.4 ml) contained 0.1 mM $NaNO_2$ in 50 mM $KH_2PO_4$–KCl–HCl (pH 2.0). After incubation for 30 s, 0.5 ml of Fe–$(DTCS)_2$ complex was added. B-1, no addition; B-2, 0.1 mM AA; B-3, 0.1 mM AA+ 1 mM NaSCN.

panel of Fig. 2 shows effects of concentration of $NaNO_2$ on AA-dependent oxygen uptake. The rates were much higher in centrifuged saliva than in the buffer solution at any concentration of $NaNO_2$ when the concentration of AA in centrifuged saliva and the buffer solution was adjusted to 0.1 mM. The results in Fig. 2 show that there were components in centrifuged saliva which could enhance AA-dependent oxygen uptake.

As $SCN^-$ enhanced the reaction between AA and nitrite under acidic conditions [23], effects of $SCN^-$ on the oxygen uptake were studied (Table 1). AA/nitrite-dependent oxygen

Table 1
Oxygen uptake during mixing saliva with AA under acidic conditions

| | Saliva ($n=12$) | Dialyzed saliva ($n=3$) | | Buffer ($n=3$) | |
|---|---|---|---|---|---|
| $SCN^-$ (μM) | 0.14 ± 0.01[a] | 0 | 0.2 | 0 | 0.2 |
| $NO_2^-$ (μM) | 57 ± 20[a] | 50 | 50 | 50 | 50 |
| pH | 1.92 ± 0.06[a] | 1.91 | 1.91 | 2.0 | 2.0 |
| AA (mM) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| $O_2$ uptake (μM/min) | 19.2 ± 9.3[a] | 8.5 ± 0.6[a] | 24.3 ± 4.1[a] | 3.8 ± 0.7[a] | 14.5 ± 2.4[a] |
| NO formation[b] (μM/min) | 76.8 | 34 | 97.2 | 15.2 | 58.0 |

Concentrations of $SCN^-$ and $NO_2^-$ are values in reaction mixtures. When saliva was used, the reaction mixture (1.0 ml) contained 0.5 ml of saliva and 0.5 mM of 50 mM $KH_2PO_4$–KCl–HCl (pH 1.5). $SCN^-$ and $NO_2^-$ were derived from saliva and final pHs were determined after the measurements of oxygen uptake. Dialyzed saliva (0.5 ml) was mixed with 0.5 ml of 50 mM $KH_2PO_4$–KCl–HCl (pH 1.0) and the final pH is given in the table. The buffer solution (1.0 ml) used was 50 mM $KH_2PO_4$–KCl–HCl (pH 2.0). Reactions were started by adding AA.

[a] Means ± S.D.

[b] Values calculated from oxygen uptake (see text).

uptake in dialyzed saliva was enhanced about three-fold by 0.2 mM NaSCN. Such enhancement was also observed in a buffer solution (Table 1). These results suggest that the difference in rate of oxygen uptake between centrifuged saliva and a buffer solution (Fig. 2) might be due to the presence and absence of $SCN^-$. In fact, centrifuged saliva used in this study contained about 0.3 mM $SCN^-$. It is known that the concentration of $SCN^-$ in the saliva is around 1 mM [2]. To confirm whether $SCN^-$ enhances oxygen uptake by AA/nitrite systems, effects of $SCN^-$ concentration on the oxygen uptake were studied (Fig. 3, upper panel); as the concentration of $SCN^-$ was increased, the rate of oxygen uptake increased in both centrifuged saliva and a buffer solution. No significant enhancement of oxygen uptake by 1 mM $SCN^-$ was observed in the absence of AA and no decrease in concentration of $SCN^-$ was observed during the oxygen uptake (data not shown). In addition, $SCN^-$ stimulated nitric oxide formation

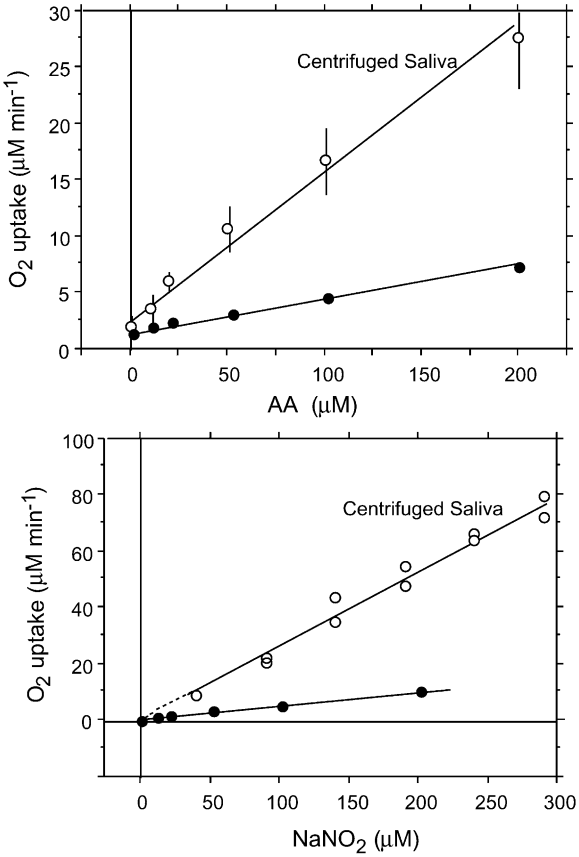


Fig. 2. Effects of concentrations of AA and $NaNO_2$ on oxygen uptake in centrifuged saliva and buffer solution. Upper panel: Effects of AA concentration. When saliva was used, the reaction mixture (1.0 ml) contained 0.5 ml of saliva, which contained 0.24 mM $SCN^-$ and 0.12 mM nitrite, and 0.5 ml of 50 mM $KH_2PO_4$–KCl–HCl (pH 1.5). The final pH was 1.97. The reaction mixture for buffer solution (1.0 ml) contained 0.05 mM $NaNO_2$ in 50 mM $KH_2PO_4$–KCl–HCl (pH 2.0). Open circles, saliva (means ± S.D., $n = 3$); closed circles, buffer solution (means of two measurements). Lower panel: Effects of concentration of $NaNO_2$. When saliva was used, the reaction mixture (1.0 ml) contained 0.5 ml of saliva, which contained 0.24 mM $SCN^-$ and 0.08 mM nitrite, and 0.5 ml of 50 mM $KH_2PO_4$–KCl–HCl (pH 1.5). The final pH was 1.92. The reaction mixture for buffer solution (1.0 ml) contained 0.1 mM AA in 50 mM $KH_2PO_4$–KCl–HCl (pH 2.0). Open circles, saliva (data from two measurements); closed circles, buffer solution (means of two measurements). Initial rate of oxygen uptake was plotted.

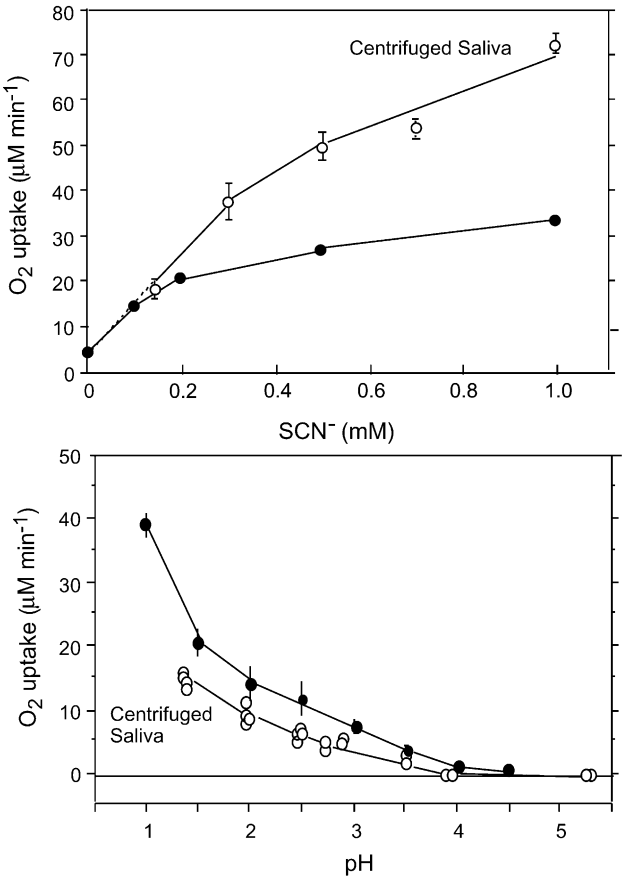


Fig. 3. Effects of concentration of $SCN^-$ and pH on oxygen uptake in centrifuged saliva and buffer solution. Upper panel: Effects of $SCN^-$ concentration. When saliva was used, the reaction mixture (1.0 ml) contained 0.5 ml of saliva and 0.5 ml of 50 mM $KH_2PO_4$–KCl–HCl (pH 1.5). The final concentrations of AA and nitrite were 0.1 mM and 0.08 mM, respectively, and final pH was 1.98. The reaction mixture for buffer solution (1.0 ml) contained 0.05 mM $NaNO_2$ and 0.1 mM AA in 50 mM $KH_2PO_4$–KCl–HCl (pH 2.0). Open circles, saliva (means ± S.D., $n = 3$); closed circles, buffer solution (means of two measurements). Lower panel: Effects of pH. When saliva was used, the reaction mixture (1.0 ml) contained 0.5 ml of saliva and 0.5 ml of 50 mM $KH_2PO_4$–KCl–HCl at various pHs. The final concentrations of AA, nitrite and $SCN^-$ were 0.1, 0.04–0.05 and 0.12–0.14 mM, respectively. The reaction mixture for buffer solution (1.0 ml) contained 0.1 mM AA, 0.05 mM $NaNO_2$ and 0.2 mM NaSCN in 50 mM $KH_2PO_4$–KCl–HCl (pH 1–4.5). Open circles, saliva; closed circles, buffer solution (means ± S.D., $n = 3$). Initial rate of oxygen uptake was plotted.

in acidified saliva and in a buffer solution (Fig. 1). These results suggest that $SCN^-$ is a catalyst for the reaction between AA and nitrite, as suggested previously [23]. Other dialyzable salivary components, glutathione and uric acid, did not significantly affect the oxygen uptake by 0.1 mM nitrite in 50 mM $KH_2PO_4$–KCl–HCl (2.0). As $SCN^-$ enhanced oxygen uptake by AA/nitrite systems under acidic conditions, the effects of pH on the oxygen uptake were studied in a buffer solution in the presence of 0.2 mM NaSCN (Fig. 3, lower panel); rates of oxygen uptake increased as pH was decreased. In centrifuged saliva which contained 0.12–0.14 mM $SCN^-$, rates of oxygen uptake also increased as pH was decreased. Effects of pH similar to Fig. 3 were observed for the oxygen uptake by an AA/nitrite system in a buffer solution that did not contain $SCN^-$ (data not shown).

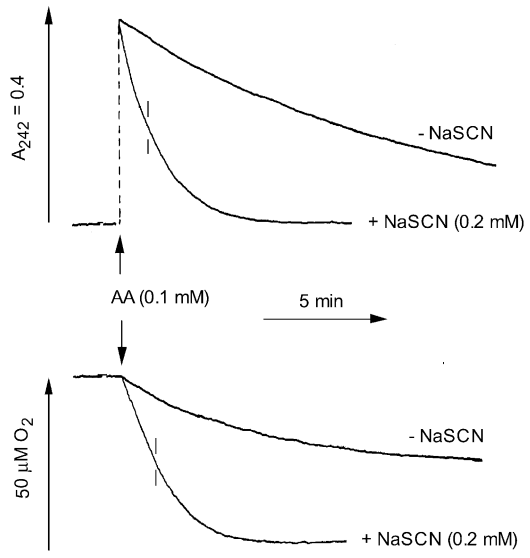


Fig. 4. Time courses of AA oxidation and oxygen uptake. The reaction mixture (1 ml) contained 0.1 mM AA and 0.05 mM $NaNO_2$ in 50 mM $KH_2PO_4$–KCl–HCl (pH 2.0) with or without 0.2 mM NaSCN. Upper traces, AA oxidation; lower traces, oxygen uptake. Arrows indicate the addition of 0.1 mM AA.

Time courses of oxygen uptake were compared with those of AA oxidation in a buffer solution (Fig. 4). In the absence of $SCN^-$, slow oxygen uptake and slow oxidation of AA were observed. The oxygen uptake and AA oxidation were significantly enhanced by 0.2 mM $SCN^-$ and the half-time for the completion of oxygen uptake (1.3 min) was similar to that of AA oxidation (1.2 min). This result indicates that oxidation of AA participates in the oxygen uptake. During oxidation of 0.1 mM AA, about 0.05 mM $O_2$ was consumed in the presence of 0.2 mM NaSCN. The amounts of oxygen taken up during oxidation of 0.1 and 0.2 mM AA were 0.046 and 0.091 mM, respectively, in acidified centrifuged saliva.

The results obtained in this study suggest that oxygen uptake by AA/nitrite systems was due to $O_2$-dependent oxidation of nitric oxide. This is supported by the data that nitrite-dependent nitric oxide formation was enhanced by AA and that nitric oxide formation by AA/nitrite systems was enhanced by $SCN^-$ (Fig. 1). It has been reported that nitric oxide formation can be measured using an oxygen electrode [18–20]. As a mechanism of nitric oxide formation by AA/nitrite systems under acidic conditions, reduction of nitrous acid by AA is possible [5–9]:

$$AA + 2HNO_2 \rightarrow \text{Dehydroascorbic acid} + 2NO + 2H_2O \quad (1)$$

The formed nitric oxide may react with molecular oxygen-producing nitrous acid under acidic conditions by the following reaction as reported previously [18–20]:

$$4NO + O_2 + 2H_2O \rightarrow 4HNO_2 \quad (2)$$

As a sum of the above reactions, the following reaction can be postulated:

$$2AA + O_2 \rightarrow 2\text{Dehydroascorbic acid} + 2H_2O \quad (3)$$

This chemical equation indicates that the stoichiometry between AA oxidation and oxygen uptake is 0.5. The stoichiometry measured (about 0.5 in Fig. 4) suggests that the oxidation of AA and oxygen uptake proceeded by the above reactions. Values slightly lower than 0.5 were obtained for the stoichiometry in centrifuged saliva. This result suggests that, in acidified centrifuged saliva, oxygen was also taken up by reactions similar to those in a buffer solution. The lower values might be explained as follows: oxidation of AA by salivary components which were oxidized by $NO_2$ and $N_2O_3$. These nitrogen oxides may be formed as the intermediates of reaction 2 [18]. If the above reactions are accepted, rates of nitric oxide formation can be estimated from the rates of oxygen uptake. The results are given in Table 1.

According to the above discussion, the effects of AA on the decomposition of nitrite should be small under the conditions of this study. This was confirmed when the concentration of AA was 0.1 mM; about 20 and 30% of 0.1 mM nitrite were decomposed in the absence and presence of 0.1 mM AA, respectively, after incubation for 2 min. When the concentration of AA was high (1 mM), the amount of nitrite decomposed was about 70%. The significant decomposition by 1 mM AA can be explained by reactions between AA and nitrogen oxides formed as the intermediates of reaction 2, producing products other than nitrite/nitrous acid.

As oxidation equivalents formed from nitrite under acidic conditions, $H_2NO_2^+$ (or $NO^+ + H_2O$) and $N_2O_3$ are possible. The former may be formed by protonation of $HNO_2$ (reaction 4) and the latter may be formed by an acid–base reaction (reaction 5) [23,24]:

$$HNO_2 + H^+ \leftrightarrow H_2NO_2^+ \leftrightarrow NO^+ + H_2O \quad (4)$$

$$HNO_2 + NO_2^- + H_3O^+ \leftrightarrow N_2O_3 + 2H_2O \quad (5)$$

If $N_2O_3$ participates in the oxidation of AA, the optimum pH should be around the $pK_a$ value ($pK_a = 3.3$) because the formation of $N_2O_3$ must be highest around the $pK_a$ value [23], but the oxygen uptake increased as pH was decreased (Fig. 3). This result suggests that $H_2NO_2^+$ or $NO^+$ may mainly participate in the oxidation of AA under the conditions of this study [25]. $SCN^-$ can also react with $H_2NO_2^+$ or $NO^+$ producing $NOSCN + H_2O$ or NOSCN [23,24–26]. The stimulation of oxygen uptake by $SCN^-$ may be explained by faster reaction of NOSCN than $H_2NO_2^+$ or $NO^+$ with AA producing NO and $SCN^-$. The rapid reaction between AA and NOSCN may keep the concentrations of $H_2NO_2^+$, $NO^+$ and NOSCN low in a mixture of saliva and gastric juice.

According to the result obtained in this study and the above discussion, oxygen in the gastric juice is consumed when saliva is swallowed into the stomach and the maximal amounts of oxygen uptake may mainly depend on the concentration of AA in the gastric juice (0.05–0.3 mM) [3,4]. As lipid peroxidation and formation of a hydroxyl radical are possible in the gastric juice [27–29], the decrease in concentration of molecular oxygen may slow down the formation of reactive oxygen species and oxygen-dependent reactions such as lipid peroxidation in the gastric juice. Further studies are required to elucidate the physiological significance of the oxygen uptake induced by the AA/nitrite/$SCN^-$ system in the gastric juice.

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