Studies on the Formation of Nitrosamines (V)

The Effects of Citrate, Tartrate and Thiocyanate on the Rates of Nitrosation

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The effects of citrate, tartrate and thiocyanate on the initial rates of nitrosation of hydantoic acid (HA), ethyl N-ethylcarbamate (EEC), dimethylamine (DMA) and sarcosine (Sar) were studied under the simulated condition occurred in the human stomach after ingestion of foods. Citrate and tartrate are present in foods widely, and thiocyanate is present in human saliva. The rates of reaction were calculated by measuring the absorbance spectra of reaction mixture periodically.

Thiocyanate accelerated the rates of nitrosation of DMA and Sar (secondary amines), but did not influence those of HA and EEC (amide compounds). On the other hand, citrate and tartrate accelerated the rates of nitrosation of HA and EEC, but did not influence those of DMA and Sar.

1. Introduction

In the reaction of amines or amides with nitrite, several compounds such as ascorbic acid are shown to inhibit the nitrosation, whereas thiocyanate and halide ions are shown to accelerate the nitrosation. Thiocyanate is contained in human saliva, especially in smoker's saliva, and was reported to accelerate the nitrosation of secondary or tertiary amines. There is a possibility that when the substances which accelerate nitrosation reaction are ingested simultaneously with nitrosatable compounds and nitrite, they might affect the rates of nitroso compounds formation in digestive tracts.

In the previous papers, we reported that citrate, tartrate and thiocyanate accelerated the carboxymethylnitrosourea (CMNU) formation from nitrite and glycocyamine (GC), one of the guanido compounds. Citrate and tartrate are present widely in foods, and are used as food additives. However, there has been no report regarding the effects of these compounds on the rates of nitrosation. Therefore, the effects of citrate, tartrate and thiocyanate on the nitrosation were studied. The experiments were carried out at pH 2.5 or 3.4, since the pH in the human stomach would be higher during digestion of meal though the pH of the human gastric juice is 1~2. Hydantoic acid (HA) and ethyl N-ethylcarbamate (EEC) which are amide compounds, and dimethylamine (DMA) and sarcosine (Sar) which are secondary amines were studied as nitrosatable compounds.

2. Methods

2.1 Measurements of the rates of nitrosation

Determination of the rates of nitrosation were made in the same manner as described in the previous paper. To aqueous solution of HA, EEC, DMA or Sar, aqueous solution of sodium nitrite was added. The pH of the mixed solution was adjusted with hydrochloric acid immediately to 2.5 or 3.4 and incubated at 37°C. The initial rates of nitrosation were measured spectrophotometrically as described in the previous paper. The final concentrations of the nitrosatable compounds and nitrite shown in Table 1 were chosen to get the suitable rates of nitrosation for measurements. The pH values of reaction mixtures and the wavelength for measurements were also listed in Table 1.

To determine the effects of sodium citrate, citric acid, sodium tartrate, sodium thiocyanate and sodium chloride, aqueous solutions of these
Table 1. The Reaction Conditions for Determination of the Rates of Nitrosation

<table>
<thead>
<tr>
<th>Nitrosatable compounds</th>
<th>[NaNO₂] (mM)</th>
<th>pH</th>
<th>Wavelength for measurement (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMA</td>
<td>50</td>
<td>10</td>
<td>3.4</td>
</tr>
<tr>
<td>Sar</td>
<td>5</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20</td>
<td>3.4</td>
</tr>
<tr>
<td>HA</td>
<td>20</td>
<td>50</td>
<td>2.5</td>
</tr>
<tr>
<td>EEC</td>
<td>20</td>
<td>100</td>
<td>2.5</td>
</tr>
</tbody>
</table>

anions were mixed with aqueous solutions of HA, EEC, DMA and Sar. Other procedures were the same as described above.

In each experiment, the reaction mixture omitting HA, EEC, DMA or Sar was incubated, and the absorbance of light the solution was measured as a blank value. Each blank value was subtracted from the corresponding experimental value.

The $\varepsilon$ values used for calculation of the initial rates of nitrosation were $\varepsilon_{394\text{nm}}$ = 88 for CMNU (from HA), $\varepsilon_{403\text{nm}}$ = 80 for ethyl N-ethylnitrosocarbamate (EENC), $\varepsilon_{330\text{nm}}$ = 95.5 for dimethylnitrosamine (DMNA), $\varepsilon_{260\text{nm}}$ = 1100 and $\varepsilon_{343\text{nm}}$ = 86 for nitrososarcosine (NS).

2.2 Thin-layer chromatography of reaction products

Forty or fifty ml of the reaction mixtures were incubated at 37°C for 30 min under the conditions described in Table 1. After decomposing nitrite with sulfamic acid, the reaction mixtures were extracted three times with dichloromethane to obtain nitrosation of DMA and with ethyl acetate for others. The organic solvent layer was dried over anhydrous sodium sulfate, and was concentrated to about 1 ml with a rotary evaporator. Then, thin-layer chromatography was performed. The adsorbent was silica gel HF₂₅₄+₃₆₆ (Merck) and the developing solvents were as follows.

CMNU: chloroform–t-butanol–acetic acid (6:3:1)
EENC: petroleum ether–ethyl ether–acetic acid (90:10:1)
DMNA: hexane–ethyl ether–dichloromethane (4:3:2)
NS: methyl acetate–iso-propanol–concentrated ammonium hydroxide (9:7:4)

After development, spots were detected with ultra violet lamps (253.6 nm and 365.0 nm) or Griess reagent as described in the previous paper, comparing with authentic nitroso compounds except EENC which detection was described in § 3.1. The Rf value of each compound was 0.59 for CMNU, 0.35 for EENC, 0.24 for DMNA, 0.40 for syn conformer of NS and 0.47 for anti conformer of NS.

3. Results

3.1 Identification of the reaction products

The absorption spectra at 310~420 nm of the reaction mixture of nitrite and HA, EEC, DMA or Sar were the same as those of the corresponding nitroso compounds, respectively, in the presence or absence of accelerating substances. Each $\lambda_{\text{max}}$ of the spectrum was coincident with reference. (CMNU: 394 nm, EENC: 403 nm, DMNA: 330 nm, NS: 343 nm) After extracting the reaction mixture with the organic solvent described in § 2.2, the aqueous layer exhibited no absorption maximum except 300 nm of nitric acid which was formed from sodium nitrite by incubation in acidic medium. The reaction products were identified by thin-layer chromatography. EENC which authentic sample was not available was identified as follows. On thin-layer chromatogram of the extract from reaction mixture of EEC and nitrite in the presence and absence of sodium citrate, only one spot was detected with ultraviolet lamps and Griess reagent. The spot was eluted with dichloromethane and the absorption spectrum was measured. The $\lambda_{\text{max}}$ was coincident with that of EENC reported by Mirvish ($\lambda_{\text{max}}$: 403 nm, 422 nm). In the reaction of HA and nitrite in the presence and absence of sodium citrate or sodium tartrate, only CMNU which was also formed from GC and nitrite was detected as a product. In the reaction of DMA or Sar with nitrite at pH 3.4 in the presence and absence of sodium thiocyanate, DMNA or NS was the only spot which had ultraviolet absorption on thin-layer chromatogram, respectively. In the reaction of Sar and nitrite at pH 2.5 in the presence and absence of sodium thiocyanate, another spot (Rf 0.63) besides NS was detected with ultraviolet lamps on thin-layer chromatogram, but the spot was also observed on thin-layer chromatogram of the blank solution Sar being omitted. Consequently, it was confirmed that the nitrosation of HA,
EEC, DMA or Sar could be followed by measuring the light absorbance at each $\lambda_{\text{max}}$ of the corresponding nitroso compound except the nitrosation of Sar at pH 2.5, where the absorbance was measured at 260 nm because of co-existence of a by-product that had light absorbance at 343 nm.

3.2 Determination of the rates of nitrosation

a) CMNU formation

The initial rates of CMNU formation from 20 mM of HA and 50 mM of sodium nitrite at pH 2.5 and 37°C, in the presence and absence of 100 mM of sodium citrate, citric acid, sodium tartrate or sodium thiocyanate were shown in Table 2. The formation of CMNU was accelerated by sodium citrate, citric acid and sodium tartrate, but not influenced by sodium thiocyanate. The extents of accelerating effects of citric acid and sodium citrate on CMNU formation were almost similar. Since there are some reports that chloride ion affects nitrosation, the pH of reaction mixture was adjusted with perchloric acid instead of hydrochloric acid. However, the rates of CMNU formation and accelerating effects of sodium citrate, citric acid and sodium tartrate were not affected by use of perchloric acid instead of hydrochloric acid. When 50~150 mM of sodium citrate was added to reaction mixture, the rates of CMNU formation from HA and nitrite were accelerated with increasing sodium citrate concentration, as shown in Fig. 1. When 50~350 mM of sodium thiocyanate was added to reaction mixture, no effects were observed in the rates of CMNU formation. (Fig. 1). In the reaction of HA and nitrite at pH 2 as well, sodium thiocyanate did not influence the rate of CMNU formation.

b) EENC formation

The initial rates of EENC formation from 20 mM of EEC and 100 mM of sodium nitrite at pH 2.5 and 37°C in the presence and absence of 200 mM of sodium citrate, citric acid, sodium thiocyanate were added to reaction mixture. The reactions were carried out at pH 2.5 and 37°C. The final concentrations of the components were 20 mM of EEC, 100 mM of sodium nitrite and 200 mM of each anion.

### Table 2. Effects of Some Anions on Initial Rate of CMNU Formation from HA and Nitrite

<table>
<thead>
<tr>
<th>Anion</th>
<th>Hydrochloric* acid (millimoles)</th>
<th>Rate** × 10^6 (M/min)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.8</td>
<td>9.46</td>
<td>1</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>12</td>
<td>22.10</td>
<td>2.34</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.6</td>
<td>20.45</td>
<td>2.16</td>
</tr>
<tr>
<td>Sodium tartrate</td>
<td>12</td>
<td>20.29</td>
<td>2.14</td>
</tr>
<tr>
<td>Sodium thiocyanate</td>
<td>1.4</td>
<td>10.91</td>
<td>1.15</td>
</tr>
</tbody>
</table>

*: The amount of hydrochloric acid for pH adjustment in 40 ml of reaction mixture

**: The average of two experiments

### Table 3. Effects of Some Anions on Initial Rate of EENC Formation from EEC and Nitrite

<table>
<thead>
<tr>
<th>Anion</th>
<th>Rate* × 10^6 (M/min)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.57</td>
<td>1</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>22.77</td>
<td>1.81</td>
</tr>
<tr>
<td>Citric acid</td>
<td>20.51</td>
<td>1.63</td>
</tr>
<tr>
<td>Sodium tartrate</td>
<td>18.48</td>
<td>1.47</td>
</tr>
<tr>
<td>Sodium thiocyanate</td>
<td>15.23</td>
<td>1.20</td>
</tr>
</tbody>
</table>

*: The average of two experiments
tartrate or sodium thiocyanate were shown in Table 3. The rates of EENC formation were accelerated by addition of sodium citrate, citric acid and sodium tartrate. However, particularly high effect was not observed with sodium thiocyanate.

c) DMNA formation

The initial rates of DMNA formation from 50 mM of DMA and 100 mM of sodium nitrite at pH 3.4 and 37°C in the presence and absence of 100 mM of sodium citrate, citric acid, sodium tartrate or sodium thiocyanate were shown in Table 3. The rates of EENC formation were accelerated by addition of sodium citrate, citric acid and sodium tartrate. However, particularly high effect was not observed with sodium thiocyanate.

Table 4. Effects of Some Anions on Initial Rate of DMNA Formation from DMA and Nitrite

<table>
<thead>
<tr>
<th>Anion</th>
<th>Rate* × 10^4 (M/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.36</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>8.54</td>
</tr>
<tr>
<td>Citric acid</td>
<td>12.89</td>
</tr>
<tr>
<td>Sodium tartrate</td>
<td>9.57</td>
</tr>
<tr>
<td>Sodium thiocyanate</td>
<td>31.62</td>
</tr>
</tbody>
</table>

*: The average of two experiments

The reactions were carried out at pH 3.4 and 37°C. The final concentrations of the components were 50 mM of DMA, 100 mM of sodium nitrite and 100 mM of each anion.

Fig. 2. Effects of various concentrations of sodium citrate and sodium chloride on the initial rates of DMNA formation from DMA and sodium nitrite.

-○-, sodium citrate; -△-, sodium chloride.

The reactions were carried out at pH 3.4 and 37°C. The concentration of DMA was 50 mM and that of sodium nitrite was 100 mM.

tartrate or sodium thiocyanate were shown in Table 4. The rate of DMNA formation was accelerated 2.6 times by 100 mM of sodium thiocyanate, slightly inhibited by 100 mM of sodium citrate or sodium tartrate, and scarcely influenced by 100 mM of citric acid. When sodium citrate was added to reaction mixture in concentrations of 50, 100 and 200 mM, the rates of DMNA formation were decreased with increasing sodium citrate concentration as shown in Fig. 2. When 100 mM or 200 mM of sodium chloride was added to reaction mixture, the rates of DMNA formation were also decreased with increasing sodium chloride concentration. (Fig. 2). In the reaction of DMA and nitrite at pH 2 as well, the rate of DMNA formation was not influence by citric acid.

d) NS formation

The initial rates of NS formation from 5 mM of Sar and 10 mM of sodium nitrite at pH 2.5 and 37°C in the presence and absence of 20 mM of sodium citrate, citric acid, sodium tartrate or sodium thiocyanate were shown in Table 5.

Table 5. Effects of Some Anions on Initial Rates of NS Formation from Sar and Nitrite

<table>
<thead>
<tr>
<th>Anion</th>
<th>Final concentration of anion (mM)</th>
<th>Rate* × 10^4 (M/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20</td>
<td>1.61</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>20</td>
<td>1.59</td>
</tr>
<tr>
<td>Citric acid</td>
<td>20</td>
<td>1.66</td>
</tr>
<tr>
<td>Sodium tartrate</td>
<td>20</td>
<td>1.51</td>
</tr>
<tr>
<td>Sodium thiocyanate</td>
<td>5</td>
<td>8.22</td>
</tr>
</tbody>
</table>

*: The average of two experiments

The rates of NS formation were scarcely influenced by 20 mM of sodium citrate, citric acid or sodium tartrate, but strongly accelerated by 5 mM of sodium thiocyanate. This acceleration was dependent on the concentration of sodium thiocyanate as shown in Fig. 3. High concentration of sodium citrate inhibited NS formation, but marked inhibition was not observed when sodium chloride was added to reaction mixture (Fig. 4(a)). In the reaction of Sar and nitrite at pH 3.4, NS formation was inhibited more strongly by sodium citrate and sodium chloride.
Fig. 3. Effect of sodium thiocyanate concentration on the initial rate of NS formation from Sar and sodium nitrite.

The reactions were carried out at pH 2.5 and 37°C. The concentration of Sar was 5 mM and that of sodium nitrite was 10 mM.

with increasing the concentration of those (Fig. 4(b)).

4. Discussion

In the reaction mixture containing sodium citrate or sodium tartrate, an appreciable amount of hydrochloric acid was needed for pH adjustment, while only a small amount of hydrochloric acid was needed in the reaction mixture containing sodium thiocyanate or citric acid, as shown in Table 2. Therefore, appreciable amounts of sodium ion and chloride ion were present in the reaction mixture containing sodium citrate or sodium tartrate, but these ions were scarcely present in the reaction mixture containing citric acid.

In the nitrosation of HA or EEC, the extents of accelerating effects of sodium citrate and citric acid were almost similar. Consequently, the rate of nitrosation of HA or EEC was not considered to be influenced by sodium chloride or chloride ion. On the other hand, DMNA formation from DMA and nitrite was inhibited by sodium citrate, but not influenced by citric acid. Since DMNA formation was inhibited by sodium chloride with increasing the concentra-

Fig. 4. Effects of various concentrations of sodium citrate and sodium chloride on the initial rates of NS formation from Sar and sodium nitrite at pH 2.5 and pH 3.4.

---, sodium citrate; ---, sodium chloride.

(a) The reactions were carried out at pH 2.5 and 37°C. The concentration of Sar was 5 mM and that of sodium nitrite was 10 mM.

(b) The reactions were carried out at pH 3.4 and 37°C. The concentration of Sar was 20 mM and that of sodium nitrite was 20 mM.
tion (Fig. 2), the inhibitory effect of sodium citrate on nitrosation of DMA was probably due to the salt effect.

In the reaction of Sar and nitrite at pH 2.5, NS formation was inhibited by high concentration of sodium citrate, but a slightly inhibitory effect was observed in the presence of sodium chloride (Fig. 4 (a)). According to Mirvish et al.\textsuperscript{10} and Hildrum et al.,\textsuperscript{17} the inhibitory effect of sodium chloride on the rate of nitrosation was more intense at pH 3.4 than pH 2.5. Therefore, Sar was reacted with nitrite at pH 3.4 in the presence of sodium citrate or sodium chloride. NS formation was inhibited by sodium citrate or sodium chloride more strongly at pH 3.4 than pH 2.5 (Fig. 4). When the rates of nitrosation in the presence of anions were compared with those in the absence of anions, the effect of sodium chloride which was formed from sodium nitrite and hydrochloric acid for pH adjustment is negligible, because equal amount of sodium chloride was formed from them, irrespective of presence and absence of anions.

According to Mirvish,\textsuperscript{10} nitrite is first converted to nitrous acid (HNO\textsubscript{2}) in acidic medium, and then nitrous acid is converted to active nitrosating species, e.g.; nitrous anhydride (N\textsubscript{2}O\textsubscript{3}), nitrosyl thiocyanate (NOSCN), nitrosyl halide (NOX) or nitrous acidium ion (H\textsubscript{2}NO\textsubscript{2}+) which is formed proportionally to [H\textsuperscript{+}]. The main nitrosating agent in the reaction condition might be N\textsubscript{2}O\textsubscript{3} for nitrosation of many secondary amines, H\textsubscript{2}NO\textsubscript{2}+ for nitrosation or ureas, guanidines or carbamates, and NOSCN or NOX for nitrosation in the presence of thiocyanate or halide ion. It has been reported that nitrosation reaction in the presence of thiocyanate was accelerated more strongly at pH 2 than pH>2.\textsuperscript{13-15,17} Therefore, DMA or HA was reacted with nitrite at pH 2 in the presence of citric acid or sodium thiocyanate respectively, but no effect was observed in the rate of DMNA or CMNU formation.

In the present study, thiocyanate accelerated nitrosation of DMA and Sar, but did not influence nitrosation of HA and EEC. On the other hand, citrate and tartrate accelerated nitrosation of HA and EEC, but did not influence nitrosation of DMA and Sar. Consequently, it is considered that activating mechanisms of citrate and tartrate in nitrosation are different from that of thiocyanate. It is under investigation to elucidate why citrate or tartrate accelerate nitrosation of ureas or urethanes.

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References