STUDIES ON NITROSAMINE FORMATION BY THE INTERACTION BETWEEN DRUGS AND NITRITE. I.
—Measurement of the amount of nitrosamine formed in rat and guinea pig stomachs.—

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Abstract——The amounts of nitrosamine formed by the interaction of several drugs and nitrite in rat and guinea pig stomachs were estimated. The nitrosamine formation from aminopyrine and nitrite was much more in guinea pig stomachs than in rat stomachs. It seemed that this result was due to the difference in gastric contents of these animals. As the nitrosamine formation was also indicated in the interaction of a clinically prescribed dose of aminopyrine and very low doses of nitrite in rat and guinea pig stomachs, there is a possibility that the nitrosation of aminopyrine takes place in human stomachs, too. Minocycline also interacted with nitrite and formed N-nitrosodimethylamine. However, the amount of nitrosamine formed was less in minocycline than in aminopyrine. Oxytetracycline was not found to form nitrosamine in the stomachs of these animals. The effects of several compounds on the nitrosamine formation by the interaction of aminopyrine and nitrite were also investigated. Ascorbic acid, sodium erythorbate, propyl gallate and butylated
hydroxyanisole reduced the nitrosamine formation but the effect of alphatocopherol, sorbic acid and butylated hydroxytoluene was not observed in the stomach.

Key word: drug-interaction, nitrosodimethylamine, nitrosamine-formation, aminopyrine, nitrite, minocycline.

INTRODUCTION

It has been reported that there are a wide variety of drugs which contain tertiary amino groups in their molecules and these drugs interact with sodium nitrite to form potent carcinogenic nitroso compounds in acid conditions in vitro (Lijinsky et al., 1974; Rao and Krishna, 1975). Among them aminopyrine and oxytetracycline were the most reactive substances. Green vegetables contain a high amount of nitrate (Yamada et al., 1963) and nitrite content in saliva was markedly elevated after ingestion of them (Ishiwata et al., 1975; Tannenbaum et al., 1976). Nitrite is also used as a food additive. Therefore it seems important to examine a possibility of formation of nitrosamines by the interaction between nitrite and several tertiary amino compounds in the stomach in vivo.

In the present communication we injected sodium nitrite directly into the lumen of rat and guinea pig stomachs in combination with some drugs which were reported to interact with nitrite in vitro and determined the nitrosamine formed. A preliminary report of these results has appeared (Omori et al., 1979).

METHOD

Rats (Wistar strain, male and female, 8-10 weeks of age, 250-350 g) and guinea-pigs (Hartley strain, male, 300-400 g) were anesthetized with sodium pentobarbital (50 mg/ kg, i. p.) and a small incision was made in the abdominal wall. The stomach was disclosed and the pylorus was ligated. Then the drug solutions were injected directly into the stomach. After 10 to 30 minutes the stomach was excised and the gastric content was washed out with 10 ml of deionized water. The pH value of the content was estimated. 3 minutes after washing out, 5 ml of 20% (w/v) sulfamic acid solution was added to consume nitrite. After centrifugation the precipitate was washed by 5 ml of water and centrifuged again. Both supernatant fractions obtained were mixed and the nitrosamine content in this fraction was colorimetrically determined by the method of Ito et al. (1971). Nitrosamine formed was also analysed by gas chromatography with a Shimazu GC-6AM equipped with a Thermal Energy Analyzer (TEA) detector (Thermo Electron Corporation). The column used was a 1.5 m x 3 mm glass tube packed with 10% PEG 20 M on 60-80 mesh Gaschrom P. The column temperature was 100°C. The injection port temperature was 135°C. The flow of carrier gas (N₂) was 60 ml/min.

The results were expressed by the values calculated from the standard curve established by using N-nitrosodimethylamine (NDMA).

Chemicals: Aminopyrine, minocycline hydrochloride and diphenhydramine hydro-
chloride were obtained from Daiichi Seiyaku Co. LTD, (Nihonbashi, Chuo-ku, Tokyo), Lederle (Japan).LTD, (Kyobashi, Chuo-ku, Tokyo), and Tanabe Seiyaku Co. LTD, (Dosshomachi, Higashi-ku, Osaka), respectively. Oxytetracycline hydrochloride and Oleandomycin phosphate were obtained from Phizer Taito Co. LTD, (Nishishinbashi, Minato-ku, Tokyo). l-Ascorbic acid, sodium erythorbate and sorbic acid were purchased from Tokyo Kasei Co. LTD, (Toshima, Kita-ku, Tokyo). Butylated hydroxyanisole, Butylated hydroxytoluene and n-propyl gallate were purchased from Ueno Seiyaku Co. LTD, (Koraibashi Higashi-ku, Osaka), nikki Universal Co. LTD, (Ohtemachi, Chiyoda -ku, Tokyo), Nakarai Chemicals LTD, (Chuo-ku, Kyoto), respectively. Alpha-tocopherol and HCD 60 were obtained from Eizai Co. LTD, (koishikawa, Bunkyo-ku, Tokyo).

RESULTS

Scheunig et al. (1976) reported that the nitroso compounds besides N-nitrosodimethylamine (NDMA) were extracted from a reaction mixture of aminopyrine and sodium nitrite by dichromomethane and colorimetrically proved positive. So we estimated NDMA by gas chromatography with a TEA detector and compared it with the value estimated by the colorimetric method. There was a tendency that the former value was greater than the latter but there was a good correlation between the two values

Table 1. Comparison of the amounts of N-nitrosodimethylamine estimated by gas chromatography with the ones of nitrosamine by colorimetric method.

Samples were extracts from the reaction mixture of aminopyrine and sodium nitrite in rat stomachs by dichromomethane.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>gas chromatography (A) (μmole/tube)</th>
<th>colorimetry (B) (μmole/tube)</th>
<th>ratio (A/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.17</td>
<td>0.86</td>
<td>1.36</td>
</tr>
<tr>
<td>2</td>
<td>1.47</td>
<td>1.09</td>
<td>1.35</td>
</tr>
<tr>
<td>3</td>
<td>1.13</td>
<td>0.88</td>
<td>1.28</td>
</tr>
<tr>
<td>4</td>
<td>1.17</td>
<td>0.90</td>
<td>1.30</td>
</tr>
</tbody>
</table>

(Table 1). So it was suggested that most of the nitroso compound determined by the colorimetric method was NDMA. Therefore in this report nitrosamine formed was estimated by the colorimetric method.

The reaction of aminopyrine (0.4 mmole/kg) with sodium nitrite (1.0 mmole/kg) proceeded rapidly in the stomach and the level of nitrosamine reached a submaximal level in less than 10 minutes after injection of them (Fig. 1). 11.5% of injected aminopyrine changed to nitrosamine in rats and 33.5% in guinea pigs. The gastric contents of rats were mostly solid and the pH values of the mixture washed out were 4 to 5. On the other hand, those of guinea pigs were rather fluid and the pH values were 2 to 3. In many cases the lower the pH value was, the higher the nitrosamine formation was.
Fig. 1. Formation of nitrosamine by the interaction of aminopyrine (0.4 mmole/kg) with sodium nitrite (1.0 mmole/kg) in the stomach. Each point represents the mean ± S. E. of 4-5 animals.

Fig. 2. Formation of nitrosamine by the interaction of aminopyrine (0.4 mmole/kg) with various doses of sodium nitrite in the male rat stomach for 10 min. Each point represents the mean ± S. E. of 4-5 rats.

The formation of nitrosamine showed a dose dependent increase with the dose of nitrite as in Fig. 2. As the dose of nitrite was increased from 0.125 mmole/kg to 1.0 mmole/kg with 0.4 mmole/kg aminopyrine, the yield of nitrosamine increased from 1.48 ± 0.13 μmole NDMA/kg to 71.5 ± 13.5 μmole NDMA/kg. These yields were 0.4% and 16.2% of injected aminopyrine, respectively. On the other hand as the dose of aminopyrine was increased from 0.05 mmole/kg to 0.4 mmole/kg in combination with 1.0 mmole/kg of sodium nitrite, the yield of nitrosamine increased 20.7 ± 1.1 μmole NDMA/kg to 45.9 ± 6.7 μmole NDMA/kg (Fig. 3). These yields were 41.4% and 11.4% of injected aminopyrine, respectively. A clinical dose of aminopyrine (10 μmole/kg) was also investigated (Fig. 4). At the dose of sodium nitrite 10 μmole/kg, nitrosamine was not detected in all rats, but was detected in 3 out of 4 guinea pigs. Nitrosamine was detected in 5 out of 7 rats at 20 μmole/kg of sodium nitrite and detected in all rats and guinea pigs at 30 μmole/kg. In these cases the yield of nitrosamine was also more in guinea pig stomachs than in rats.

The amount of nitrosamine formed in the stomachs of female rats was more than in male rats (Table 2).

The yields of nitroso compound by the reaction between several other drugs and nitrite in the stomachs of rats and guinea pigs are given in Table 2. Oxytetracycline (0.2 and 0.4 mmole/kg), oleandomycin (0.4 mmole/kg), erythromycin (0.4 mmole/kg) and diphenhydramine (0.4 mmole/kg) did not form detectable nitrosamine in both species.
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Fig. 3. Formation of nitrosamine by the interaction of various doses of aminopyrine with sodium nitrite (1.0 mmole/kg) in the male rat stomach for 10 min.

Each point represents the mean ± S. E. of 4-5 rats.

Fig. 4. Formation of nitrosamine by the interaction of a clinical dosage of aminopyrine (10 μmole/kg) with sodium nitrite for 10 min.

Each point represents the mean ± S. E. of 4-5 animals.

Table 2. Formation of nitrosamine by the interaction of several drugs with sodium nitrite in rat and guinea pig stomachs.

<table>
<thead>
<tr>
<th>Drug and Dose</th>
<th>SN</th>
<th>Time (min.)</th>
<th>Rat (μmole/kg)</th>
<th>Yield of Nitrosaminea</th>
<th>Guinea pig (μmole/kg)</th>
<th>(%)f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopyrine</td>
<td>0.01</td>
<td>0.01</td>
<td>10</td>
<td>N. D. d</td>
<td>M(4)e</td>
<td>0.81±0.39 (4)f</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.02</td>
<td>10</td>
<td>0.50±0.21 M(7)</td>
<td>5.0</td>
<td>1.03±0.52 (4)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.0</td>
<td>10</td>
<td>33.1±3.3 M(4)</td>
<td>33.1</td>
<td>0.81±0.39 (4)</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>1.0</td>
<td>10</td>
<td>45.9±6.7 M(6)</td>
<td>11.5</td>
<td>134.6±10.5 (5)</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>1.0</td>
<td>10</td>
<td>60.3±5.6 F(5)</td>
<td>15.1</td>
<td>33.5</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>1.0</td>
<td>20</td>
<td>55.4±4.0 F(5)</td>
<td>13.9</td>
<td>33.5</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>0.2</td>
<td>1.0</td>
<td>10</td>
<td>N. D. M(4)</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>1.0</td>
<td>20</td>
<td>N. D. M(4)</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>Minocycline</td>
<td>0.02</td>
<td>0.1</td>
<td>10</td>
<td>N. D. M(5)</td>
<td>0.85±0.18 (5)</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.2</td>
<td>10</td>
<td>0.67±0.33 M(5)</td>
<td>3.35</td>
<td>3.11±0.60 (3)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td>10</td>
<td>0.74±0.22 M(5)</td>
<td>0.74</td>
<td>15.34±2.17 (5)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.0</td>
<td>10</td>
<td>6.59±2.59 M(5)</td>
<td>6.60</td>
<td>30.31±4.29 (4)</td>
</tr>
<tr>
<td>Oleandomycin</td>
<td>0.4</td>
<td>1.0</td>
<td>20</td>
<td>N. D. M(4)</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.4</td>
<td>1.0</td>
<td>20</td>
<td>N. D. M(4)</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>0.4</td>
<td>1.0</td>
<td>20</td>
<td>N. D. M(4)</td>
<td>N. D.</td>
<td>N. D.</td>
</tr>
</tbody>
</table>

a: The yields of nitrosamine were determined by the colorimetric method using NDMA as a standard and given as mean ± S. E.

b: Figures in parentheses indicate the number of animals.

c: Calculated from the amount of drugs.

d: N. D. not detected.

e: M: male rats.

f: Female rats.
On the other hand, minocycline reacted with sodium nitrite to form nitrosamine, which was found to be NDMA by gas chromatography with a TEA detector. In rats the yield of nitrosamine from minocycline was about one-fifth as much as the one from aminopyrine. However, the yield of nitrosamine from minocycline (0.1 mmole/kg) and sodium nitrite (1.0 mmole/kg) in guinea pigs was almost the same as that from aminopyrine (0.1 mmole/kg) and sodium nitrite (1.0 mmole/kg) in rats. So it was thought that under suitable conditions a relatively high amount of nitrosamine could be formed from minocycline even in human stomachs.

It was known that various antioxidants inhibited the formation of NDMA by the reaction of aminopyrine and sodium nitrite in vitro. So we investigated the effect of some antioxidants on the formation of nitrosamine in rat stomachs (Fig. 5 and Table 3). The abilities of ascorbic acid and sodium erythorbate to inhibit nitrosamine formation were high. They prevented the formation of nitrosamine by the reaction of aminopyrine (40 μmole/kg) and sodium nitrite (200 μmole/kg) at the dose of 25 μmole/kg. The inhibitions of the formation by them were dose-dependent and the dose of 100 μmole/kg prevented the nitrosamine formation more than 50% (Fig. 5). Sorbic acid, which was reported to inhibit the formation of nitrosamine from the reaction of some secondary amines and sodium nitrite (Tanaka et al., 1978), did not inhibit at 100 μmole/kg and α-tocopherol, which was reported to inhibit the formation of NDMA from the reaction of aminopyrine and sodium nitrite (Mergen et al., 1978), showed a slight inhibition but it was not significant. Butylated hydroxyanisole (BHA) significantly inhibited the nitrosamine formation at 100 μmole/kg but the rate of inhibition was low.

<table>
<thead>
<tr>
<th>Table 3. The effect of several drugs on the formation of nitrosamine.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug (100 μmole/kg)</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Ex. 1</td>
</tr>
<tr>
<td>Controla</td>
</tr>
<tr>
<td>Sorbic acidb</td>
</tr>
<tr>
<td>Ex. 2</td>
</tr>
<tr>
<td>Controlb</td>
</tr>
<tr>
<td>α-tocopherolc</td>
</tr>
<tr>
<td>Ex. 3</td>
</tr>
<tr>
<td>Controla</td>
</tr>
<tr>
<td>Butylated hydroxyanisoled</td>
</tr>
<tr>
<td>Butylated hydroxytoluenee</td>
</tr>
<tr>
<td>Propyl gallate*</td>
</tr>
</tbody>
</table>

a: Control rats were given aminopyrine (40 μmole/kg) and sodium nitrite (200 μmole/kg).
b: Sorbic acid was suspended in 1% CMC solution.
c: Butylated hydroxyanisole, butylated hydroxytoluene and propyl gallate were suspended in 2% CMC solution.
d: α-tocopherol was suspended in 10% HCD-66 solution.
e: All drugs were injected directly into the stomach at the dose of 100 μmole/kg.
f: The yields of nitrosamine were determined by the colorimetric method using NDMA as a standard and given as means±S.E.
g: Figures in parentheses indicate the number of animals.
h: Calculated from the amount of drugs.
* : Significant difference from control at p<0.05.
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Fig. 5. The effect of ascorbic acid and sodium erythorbate on the formation of nitrosamine in the male rat stomach.

Control rats were given aminopyrine (40 μmole/kg) and sodium nitrite (200 μmole/kg). Treated rats were simultaneously injected with ascorbic acid or sodium erythorbate (100 μmole/kg) into the stomach.

Each value is the average obtained from 4 to 5 rats and results are expressed as a percentage of control rats.

whereas butylated hydroxytoluene (BHT) did not inhibit at all. Propyl gallate inhibited the nitrosamine formation by 55% at the dose of 100 μmole/kg and was thought to have a relatively strong inhibitory effect.

DISCUSSION

The formation of NDMA by the interaction of aminopyrine and nitrite was observed in rat and guinea pig stomachs, and the yield of NDMA was about 2 to 3 times greater in guinea pigs than in rats. (Fig. 1, Table 2). Gastric absorption of NDMA was rather low in rats (Phillips et al., 1975) and in guinea pigs (Ishiwata et al., 1977). Rao et al. (1975) reported that the yield of NDMA by the reaction of aminopyrine and nitrite in vitro was about twice greater in pH 2 or 3 than in pH 4, and in pH 5 the yield was very low. The amount of nitrosamine produced in the stomach had a tendency to be much more when the gastric contents were fluid and the pH values were low. Therefore these species differences observed between guinea pigs and rats seemed to be caused by the differences in the nature of the stomach contents.

We indicated that the formation of nitrosamine occurred by the interaction of a clinically prescribed dose of aminopyrine and very low doses of nitrite (Fig. 4). Nitrate or Nitrite is contained in green vegetables, cured meats, cheese and so on and it was reported that 500 mg of nitrite/l was detected in the saliva 1-2 hours after ingesting vegetable juice (Tannenbaum et al., 1976). So there is a probability that the nitrosamine formation from aminopyrine takes place in humans who are eating foods containing nitrite or nitrate. According to the fact that the pH value of the gastric content of human beings is normally from 1.6 to 2.0, it is probable that the amount of nitrosamine formed in the human stomach is relatively high.

We also estimated the amount of nitrosamine formed by several other drugs and
nitrite in rat stomachs (Table 2) and found that minocycline reacted with nitrite and formed NDMA. Oxytetracycline, which was reported to interact with nitrite in vitro (Lijinsky et al., 1972a, b), did not form nitrosamine in this study. According to Roper et al. (1978), among several different tetracycline antibiotics, namely achromycin, aureomycin, anhydroaureomycin, ledermycin, minocycline, oxytetracycline and doxycycline, minocycline was the best reactant to nitrite and formed the highest amount of NDMA. So even in rat stomachs where the pH value was 4 to 5, we could find the formation of nitrosamine by the interaction of minocycline and nitrite. On the contrary, oxytetracycline is a poor reactant as compared with minocycline, so it was thought that the amount of nitrosamine formed was too small to be detected in rat and guinea pig stomachs.

There are many reports concerning the inhibitory effect of antioxidants on the formation of nitrosamine by the interaction between amines and nitrite. Ascorbic acid was known to prevent hepatotoxicity in rats caused by the simultaneous feeding of sodium nitrite and aminopyrine (Kamm et al., 1973) and ascorbate or erythorbate was able to inhibit formation of NDMA in frankfurters (Fiddler et al., 1973). Sorbic acid, a food preservative, was found to react with nitrite in acidic solutions and inhibited the in vitro formation of NDMA from dimethylamine and nitrite (Tanaka et al., 1978). Propyl gallate was reported to reduce the amount of nitrosamine in fried bacon (Sen et al., 1976) and phenolic antioxidants inhibited hepatotoxicity caused by dimethylamine and sodium nitrite (Astill et al., 1977). The mechanism of the protective effect of these compounds is believed to be due to the ability to compete with amines for the available nitrite ion. Therefore the rate of inhibition seems to depend on the difference between the rates of reaction of amines and these compounds to nitrite. And these reaction rates depend on the pH value of the reaction mixture. The nitrosamine formation from aminopyrine is relatively rapid (Mirvish, 1975), so it is thought that sorbic acid failed to inhibit the nitrosamine formation in our experiment (Table 3). This result agreed with the fact that sorbic acid had no inhibitory effect on the nitrosation of N-methylanilin which proceeds rapidly (Tanaka et al., 1978). According to Mergens et al. (1978), α-tocopherol as well as ascorbate reacted to nitrite and reduced the elevation of serum GPT activity in rats administered sodium nitrite and aminopyrine. In our experiment the inhibition of α-tocopherol on the nitrosamine formation in rat stomachs was weak (Table 3). At pH 5, α-tocopherol scarcely reacted with nitrite (Mergens et al., 1978) and the pH value in rat stomach contents was 4 to 5, so it was probable that the inhibition rate was low.

Though there are many reports about nitrosation of drugs, almost all reports were based on the experiment in vitro. In the present experiment we estimated nitrosation of these drugs in rat and guinea pig stomachs and found some differences between in vitro and in vivo and species differences. Therefore, it is thought that when we assess the risks of nitrosatable drugs, we should consider these differences.
ACKNOWLEDGEMENT

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REFERENCES


Nitrosamine formation by drug interaction. I.


