Dosing Time with Ascorbic Acid and Nitrater, Gum and Tobacco Chewing, Fasting, and Other Factors Affecting N-Nitrosoproline Formation in Healthy Subjects Taking Proline with a Standard Meal

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Abstract

The N-nitrosoproline (NPRO) test measures the potential for intragastric formation of carcinogenic nitrosamines in humans. Nitrate and L-proline are administered to volunteers. Noncancerigenic NPRO is produced by an acid-catalyzed reaction of proline (a model for ingested amines) with nitrate-derived nitrite in the stomach. It is then absorbed and excreted in the urine, which is for intragastric formation of carcinogenic nitrosamines. Therefore, the NPRO test is considered safe because NPRO has been shown to be noncancerigenic and is quantitatively excreted in urine. Ohshima and Bantsch (4), who developed the test in 1981, found that a fasting man who was fed 325 mg nitrate and 500 mg L-proline excreted 23 pg (160 nmol) of NPRO. One g of ASC taken with the proline inhibited NPRO formation by 81%. The test is considered safe because NPRO has been shown to be noncancerigenic and is quantitatively excreted in urine. In previous tests, proline was given 1 h after or between meals. Urines were collected for 24 h, and samples were analyzed for NPRO by published methods. This standard test yielded 26 ± 2 (mean ± SE) nmol NPRO compared with 5 ± 1 nmol NPRO when proline alone was taken.

In variations of the standard test, NPRO yield was not significantly affected by the subjects’ gender, the time at which the standard meal was eaten, the size of the meal, or the drinking of extra water after the meal. Doses of 100 and 200 mg nitrate had lesser effects on NPRO yield than did the dose of 400 mg nitrate. Nitrate (400 mg) produced the most NPRO when it was given 1 h before the meal. Fasting increased NPRO yield by 3–4 times compared to giving proline with a meal. One g of ASC given 5 or 2 h before, with, or 1 or 2 h after the meal with proline inhibited NPRO formation by mean values of 0, 71, 71, 67, and 19%, respectively. Chewing gum or tobacco for 2–3 h after the test meal did not increase NPRO formation or salivary nitrate levels, but salivary nitrate levels were reduced, especially when gum was chewed. When nitrate was not taken, chewing tobacco appeared to increase salivary nitrate and nitrate levels. The weak carcinogen N-nitrososarcosine (NSAR) was also detected in some tests, and the standard group showed 21 ± 3 nmol NSAR. A high NSAR result (44 ± 7 nmol) for women undergoing the standard test should be reexamined. We discuss applying these results to the conduct of future NPRO tests, as well as their implications for reducing the potential production of carcinogenic nitrosamines in the stomach.

Introduction

The NPRO test in humans involves feeding nitrate and L-proline to volunteers and determining NPRO levels in urine collected over the next 24 h. There is evidence that NPRO is formed by the reaction of proline with nitrite in the stomach, absorbed into the blood, and quantitatively excreted in the urine. Ohshima and Bartsch (4), who developed the test in 1981, found that a fasting man who was fed 325 mg nitrate and 500 mg L-proline excreted 23 μg (160 nmol) of NPRO. One g of ASC taken with the proline inhibited NPRO formation by 81%.

Rate of NPRO formation = k (proline) (nitrite)² (A)

Apart from exposure to nitrosamines in tobacco smoke (7), most NOC exposure may occur because of acid-catalyzed ni-

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3 The abbreviations used are: NPRO, N-nitrosoproline; ASC, ascorbic acid; vitamin C; CHO, carbohydrate; NOC, N-nitroso compound; NSAR, N-nitrososarcosine; oz., ounce (1 oz. = 28.3 g); UNMC, University of Nebraska Medical Center.
trosation in the stomach. After ingested nitrate is absorbed, 25% is actively secreted by the salivary glands, and 20% of salivary nitrate is reduced to nitrite by oral bacteria and swallowed (8). Of the gastric nitrite (its normal level in fasting gastric juice is 120 μg/liter; Ref. 9), 80% arises from salivary nitrite and 20% from nitrite ingested as such (8). It is important to assess the potential for gastric nitrosation because this process may be involved in the etiology of cancer of the stomach (9), esophagus (10), nasopharynx, and other organs. After giving proline but not nitrate, NPRO excretion was raised in areas with high incidences relative to areas with low incidences of stomach cancer in Japan and Colombia (11, 12), cancer of the esophagus in northern China (11), and cancer of the nasopharynx in southern China (13). Wu et al. (14) examined pooled urine samples from 30 men from each of 2 villages/county in 69 counties in China with different cancer incidences. Significant associations were found between (a) the yields of NPRO and NSAR, a nitrosamino acid that is a weak esophageal carcinogen in rats (15), and the decrease in NPRO after giving ASC; and (b) the incidences of leukemia and cancer of the esophagus, nasopharynx and breast, but not of gastric cancer. NOCs can induce tumors at all of these sites in rodents (16).

ASC inhibits nitrosation in chemical systems and in vivo (6, 17). This may explain why the intake of ASC-rich fruits and vegetables is negatively correlated with many types of cancer, although ASC may also react with free radicals in tissues, thereby blocking promotion (18, 19). ASC was shown to inhibit NPRO formation in most of the quoted NPRO studies. In vivo inhibition of nitrosation by vegetable and fruit juices was shown to be due to both ASC and polyphenols (20). The ASC fraction was the predominant inhibitor in tomato juice (21).

The NPRO test has been used to compare populations with different cancer incidences (see above), to study patients with elevated gastric pH (who show reduced NPRO formation), and to demonstrate that cigarette smoking increases in vivo nitrosation (11). We found that the presence of nitrate in the drinking water consumed by rural Nebraskans was correlated with urinary NPRO (22). However, the effects of variation in dietary practices on the NPRO test has not been sufficiently studied. Such changes could be important in determining the extent of gastric nitrosation and in standardizing the NPRO test. In the present project, we studied some dietary and other factors that could affect the NPRO test in humans under relatively controlled conditions. In previous NPRO studies, proline was given 1 h after a meal in most of the tests in high cancer incidence areas (11, 13, 14), between meals (20, 23, 24), or while fasting (4). In the present study, proline was administered together with a meal because most nitrosatable amines (for which proline was a model) are components of the diet and because most food is consumed as meals. The proline was administered with a standard light meal (typical for the United States) to help obtain reproducible results that would apply in this country.

Materials and Methods

General Procedures. Table 1 lists the groups undergoing each treatment. We obtained L-proline from Ajinomoto Chemical Company (Raleigh, NC), which sells the product to health food stores, and nitrate and ascorbic acid (both United States pharmaceutical grade) from Mallinckrodt, Inc. (St. Louis, MO). The subjects were medical students or laboratory technologists (19–50 years old) at UNMC, nonsmokers, and all men except for those in group 5. They completed a brief medical questionnaire (they were excluded if they had conditions affecting the stomach or chronic diseases), signed informed consent forms, and received $60/experiment. Each “experiment” (a test carried out together by, usually, 10 men) lasted 5 days (6 days for groups 27–29). During the entire experiment, the volunteers avoided foods that contained NPRO, including nitrate-preserved or smoked meat and fish products, beer, wine, and yellow cheese (25, 26); gelatin because of its high proline content; and celery, beets, radishes, and lettuce because of their high nitrate content (8). On days 4 and 5 of the experiment, the volunteers also excluded foods rich in ASC, including citrus fruit, melons, strawberries, pineapples, tomato products, cabbage family, spinach, potatoes, vitamin-fortified foods, and vitamin supplements. On these days they did not chew gum or tobacco except where this was specified. Food and beverage intakes were recorded on days 4 and 5, including approximate amounts and times of consumption.

The tests were conducted on days 4 and 5 (or days 4–6 in groups 27–29). Tests on each of these days were usually unrelated. Each group in Tables 1 and 2 was subject to a different procedure. Each subgroup refers to a set of subjects in a single experiment. In the standard test (group 4), sodium nitrate (548 mg = 400 mg nitrate) was given in 4 oz. unfortified commercial apple juice 1 h before the test meal. Most groups received the standard test meal at noon. For groups 1–24, this meal (“meal A”) consisted of 2 slices of whole wheat bread, 2.5 oz. roast beef, 0.5 oz. Monterey Jack cheese, mustard and mayonnaise (optional), 1 oz. potato chips, 4 oz. orange sherbet, and 8 oz. (1 cup) of unfortified apple juice containing 500 mg added L-proline. (The volume of this meal, measured by displacement of water, was 400 ml) The meal was consumed in about 15 min. It contained 700 calories, of which 53% arose from CHO, 31% from fat, and 17% from protein. The chemical and test meals were consumed under supervision at the Center for Human Nutrition. Urine was collected for 24 h after each test meal in bottles containing H2SO4 (ammonium sulfate reagent)(22). No food or liquids were allowed for 2 h before and after the test meal.

Specific Procedures for Each Group. Table 1 lists the amount and timing of the nitrate dose in each group. In groups 1–3, proline and/or nitrate were omitted as indicated. Apple juice was given with the meal when proline was omitted, but was not given 1 h before the meal in group 3, in which nitrate was omitted. The 17 subgroups of control group 1 and the 9 subgroups of standard group 4 were interspersed among the other groups. In group 5, the standard test was taken as in group 4, but the subjects were adult women. In groups 6 and 7, nitrate was given 15 min or 2 h before instead of 1 h before the standard meal. In groups 8 and 9, 100 or 200 (instead of 400) mg nitrate were given 1 h before the test meal.

In group 10, the subjects fasted overnight for 12 h, took 400 mg nitrate in 4 oz. apple juice at 8:00 a.m., took 500 mg proline in 8 oz. tap water at 8:30 a.m., and started the 24-h urine collection. Fasting was continued until 1 p.m. In groups 11 and 12, the standard meal was given as breakfast at 8 a.m. or as supper at 5:30 p.m., with nitrate given 1 h before the test meal. Urine collections began after the standard meal. In group 13, the amounts of all test meal items were reduced to one-half of the usual levels, 400 mg nitrate was given in 2 oz. apple juice at 11:00 a.m., and 500 mg proline was given in 4 oz. apple juice with the meal. In group 14, all meal items (except for the nitrate and proline doses) and the volumes of apple juice were increased to twice the normal amounts. In group 15, the standard test was conducted, but 12 oz. (1.5 cups) of tap water were consumed 30 min after the end of the standard meal. In group 16, a high CHO meal (2 cups of spaghetti, 1 cup of canned
Table I  Effect of various treatments on NPRO and NSAR excretion

<table>
<thead>
<tr>
<th>Group</th>
<th>Conditions</th>
<th>Proline Time, nitrate to meal (min)</th>
<th>NPRO (nmol/24 h)</th>
<th>NSAR (nmol/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of subgroups</td>
<td>No. of samples</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>1</td>
<td>No nitrate, no proline</td>
<td>–</td>
<td>–</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>Nitrate, no proline</td>
<td>–</td>
<td>60</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Proline, no nitrate</td>
<td>+</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Standard</td>
<td>+</td>
<td>60</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>Standard, women</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Nitrate 15 min before meal</td>
<td>+</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>Nitrate 2 h before meal</td>
<td>+</td>
<td>120</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>100 mg nitrate</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>200 mg nitrate</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>Fasting</td>
<td>+</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>Breakfast as test meal</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>Supper as test meal</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>Half meal</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>Double meal</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>Extra water</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>High CHO meal</td>
<td>+</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>17</td>
<td>170 g beets with meal</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>18</td>
<td>105 g cabbage with meal</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>19</td>
<td>1 g ASC 5 h before meal</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>1 g ASC 2 h before meal</td>
<td>+</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>21</td>
<td>1 g ASC with meal</td>
<td>+</td>
<td>60</td>
<td>3</td>
</tr>
<tr>
<td>22</td>
<td>1 g ASC 1 h after meal</td>
<td>+</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>23</td>
<td>1 g ASC 2 h after meal</td>
<td>+</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>1 g ASC with meal, nitrate 15 min before meal</td>
<td>+</td>
<td>15</td>
<td>1</td>
</tr>
</tbody>
</table>

* Nitrate was given in all groups except for groups 1, 3, and 29. The nitrate dose was 400 mg except for groups 8 and 9.
* Seven NPRO results in this group were in the range 33–188 nmol. An additional result of 7 nmol was excluded.
* The standard meal was slightly modified in these groups (see "Materials and Methods").

cooked tomatoes, 2 slices of French bread, 1 pat of butter, 12 oz. of apple juice, 1/3 of a cup of sherbet, and 2 carrot sticks) was given instead of the standard meal. This meal contained 1040 calories, of which 71% was due to CHO, 20% to fat, and 8% to protein.

In groups 17 and 18, 1 cup (170 g) of cooked beets or 1 cup (105 g) of raw cabbage containing 500 mg of added proline was served with the standard meal, and 400 mg of nitrate was given 1 h before the meal. In groups 19–24, 1 g ASC was given in 4 oz. apple juice at the indicated times before, with, or after the meal, except that in group 21 the ASC was added to the apple juice containing proline and was given with the meal. In group 24, 1 g ASC was taken with the meal, and 400 mg nitrate were taken 15 min before the meal.

In groups 25–29, meal A was modified in that apple juice was replaced by a soft drink (Sprite), potato chips by corn chips, and orange sherbet by fudge sticks. In this meal (totaling 700 calories as with meal A), 42% of the calories were due to CHO, 40% to fat, and 18% to protein. These modifications were made to minimize the consumption of ASC and polyphenols that could react with nitrite. In group 26, a common brand of sugarless chewing gum was chewed for 2 h starting immediately after the test meal. The gum was replaced every 20 min. In groups 27 and 28, 0.8 g plugs of a common brand of chewing tobacco were kept in the mouth for 3 h starting immediately after the meal. The plugs were replaced every 45 min. Each plug or piece of gum was actively chewed for at least 10 min. All subjects had chewed tobacco previously, at least occasionally. We observed the initial chewing of the gum and tobacco, and the volunteers continued chewing on their own. Nitrate and proline were given in groups 26 and 27 but not in group 28. The tests in groups 25 and 26 were conducted on days 4 and 5.
NPRO Formation in Healthy Subjects taking Proline and Mitrate

Significantly different from value in group 28. with | This group was tested at a different time from the other groups. The subjects consumed the usual low-NPRO, low-nitrate diet.

Proline was taken with and nitrate without stimulation 2 h after a free-choice lunch (not the standard meal).

respectively, of a 5-day experiment. The tests in groups 27–29 were conducted on days 4–6, respectively, of a 6-day experiment.

In groups 25–29, urine was collected as usual and 4 ml of saliva samples were collected in screw-capped test tubes (containing 1 ml of 0.5 N Na2CO3) 1 and 2 h after the end of the test meal. The saliva was collected without stimulation in control groups 25 and 27 and while chewing the gum or tobacco (the current sample was chewed for at least 10 min before saliva collection began) in groups 26, 28, and 29. The time needed to collect each saliva sample was recorded.

Chemical Analyses: Analysis of Urine Collections for NPRO and NSAR. As before (22), the 24-h urine collections were mixed, their volumes were recorded, and samples were analyzed for specific gravity and creatinine at a clinical laboratory to check completeness of the collections (all urines contained >1 g creatinine). Other samples were analyzed for NPRO by the method of Stillwell et al. (12) as modified by us (22). In brief, 20 ml of urine containing 400 ng N-nitrosopiper- colic acid (added as an internal standard) were absorbed on Celic columns and eluted with CH2Cl2:methanol 92:8. The eluates were treated with BF3 in methanol to produce the nitrosamino acid methyl esters, which were analyzed by gas chromatography-thermal energy analysis.

Typically, NPRO and nitrosopipercolic methyl esters were eluted at 9.1 and 10.9 min, respectively. A frequent peak eluting at about 6.0 min was identified as NSAR methyl ester by comparing its retention time to that of a sample prepared from authentic NSAR. During the study, it appeared that some of the NPRO decomposed on lengthy storage of the urine samples at −15°C in the presence of acid, presumably due to denitrosation. Subsequent samples were collected under acidic conditions as before but were then made alkaline with 5 N NaOH (as in Ref. 11), stored at −15°C, and analyzed within 3 months. All urine samples were analyzed in full duplicate. If individual results differed by >15% from the mean values (>30% for results with <10 nmol NPRO/urine), additional analyses were performed.

Analysis of Saliva. Salivary nitrate and nitrite were measured as before (22). Nitrate was determined after conversion to nitrobenzene (27). In brief, saliva samples (1.0 ml) were mixed with 0.5 ml water and then with Ag2SO4 and ZnSO4, incubated at 70°C, and filtered by using a Millipore vacuum filter unit with 5-μm filters. Filtrate samples (0.5 ml) were heated with 1.0 ml concentrated H2SO4 and 0.1 ml benzene for 15 min at 70°C, cooled in ice, and made basic. Nitrobenzene was extracted with ethyl acetate, which was analyzed by gas chromatography. Nitrate levels were based on nitrobenzene standards because recoveries of NaN3 calculated from nitrobenzene standards were 80–90%.

Results and Discussion

The subjects consumed their normal diets except that they excluded food items with large amounts of NPRO, proline, and nitrate. On days 3 and 4 of the 5-day experiment, they also avoided high ASC foods, ate standard lunches with 500 mg added l-proline, took nitrate (usually 400 mg) and/or 1 g ASC at defined times, and collected 24-h urine samples. Table 1 shows the results expressed as nmol/24 h of NPRO and (in some tests) NSAR. Kruskal-Wallis analysis of the overall results for NPRO showed significant (P = 0.0001) differences between individual groups. The only subgroups that showed significant differences within groups were in groups 1 and 3. In group 1, 1 of 18 subgroups showed unusually high NPRO results and was excluded. In group 3, subgroup 1 showed 3.00 ± 0.54 nmol NPRO and 4.74 ± 1.06 nmol NSAR (mean ± SE), and subgroup 2 showed 7.33 ± 1.44 nmol NPRO and 15.88 ± 2.05 nmol NSAR (significantly different, P < 0.01, for both compounds). However, the difference between 3.0 and 7.3 nmol NPRO is not important because both values are very low.

The same applies (although less so) to NSAR.

Statistics. For the NPRO and NSAR results, Student’s t test was used to compare two groups, and ANOVA was used to compare more than 2 groups. If the ANOVA showed significant differences, pair-wise comparisons were made by using Benferrion’s adjustments. Results for saliva nitrate and nitrite were compared by Student’s t test.

Table 2. Effect of chewing gum and chewing tobacco on salivary nitrite and nitrate

<table>
<thead>
<tr>
<th>Group</th>
<th>Experiment</th>
<th>Nitrate and proline</th>
<th>No. of samples</th>
<th>Salivation rate (ml/min, mean ± SE)</th>
<th>Level in saliva (mg/l, mean ± SE)</th>
<th>Mean % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nitrite</td>
<td>Nitrate</td>
</tr>
<tr>
<td>25</td>
<td>Control for group 26</td>
<td>+</td>
<td>20</td>
<td>0.8 ± 0.1</td>
<td>64 ± 8</td>
<td>211 ± 19</td>
</tr>
<tr>
<td>26</td>
<td>Chewing gum</td>
<td>+</td>
<td>20</td>
<td>1.5 ± 0.1</td>
<td>37 ± 5</td>
<td>223 ± 16</td>
</tr>
<tr>
<td>27</td>
<td>Control for group 28</td>
<td>+</td>
<td>20</td>
<td>0.9 ± 0.3</td>
<td>77 ± 14</td>
<td>148 ± 34</td>
</tr>
<tr>
<td>28</td>
<td>Chewing tobacco</td>
<td>+</td>
<td>19</td>
<td>2.6 ± 0.7</td>
<td>41 ± 11</td>
<td>196 ± 43</td>
</tr>
<tr>
<td>29</td>
<td>Control for group 29</td>
<td>−</td>
<td>11</td>
<td>0.3 ± 0.1</td>
<td>6 ± 2</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>27</td>
<td>Chewing tobacco</td>
<td>−</td>
<td>20</td>
<td>2.4 ± 0.7</td>
<td>28 ± 9</td>
<td>97 ± 29</td>
</tr>
</tbody>
</table>

*Proline was taken with and nitrate was taken 1 h before the test meal except in the last two groups, where neither compound was administered.

1 The same numbers are used as in Table 1.

2 Significantly different from values in the appropriate control group, with P < 0.01.

3 Significantly different from values in the appropriate control group, with P < 0.05.

4 This group was tested at a different time from the other groups. The subjects consumed the usual low-NPRO, low-nitrate diet for 2 days. On day 2 they collected saliva without stimulation 2 h after a free-choice lunch (not the standard meal).

5 Significantly different from value in group 28, with P < 0.05.
except that the subjects were women. The NPRO results were similar for groups 4 and 5, i.e., there was no gender difference for NPRO.

**Timing and Dose of Nitrate.** When 400 mg nitrate was taken 15 min, 1 h, or 2 h before the meal with proline (groups 6, 4, and 7, respectively), mean NPRO yields were 17, 26, and 19 nmol nitrate, respectively. Although ANOVA for these three groups did not show significant differences, nitrate given 1 h before the meal (group 4) gave a 37–55% higher mean NPRO value than nitrate given at the other times. The apparent 1-h delay between the intake of nitrate and maximum NPRO formation is attributed to the time required for nitrate to be absorbed, secreted into the saliva, reduced to nitrite by oral bacteria, and swallowed. This 1-h delay is consistent with the findings that 1–2 h were required for saliva nitrite to peak after taking a dose of nitrate (28, 29) and that total gastric NOCs peaked 2 h after 200 mg nitrate was taken (30). Nitrate was given 1 h before the test meal in all subsequent experiments.

The nitrate dose was also varied. ANOVA for groups 3, 8, 9, and 4 with 0, 100, 200, and 400 mg nitrate, respectively, given 1 h before the meal with proline, showed $P = 0.0001$ for overall differences between the groups, with $P < 0.05$ for group 3 versus group 4 (0 versus 400 mg nitrate). The results for 100 and 200 mg nitrate (groups 8 and 9) were less than one-half of those for 400 mg nitrate (group 4) but were not significantly different from those in group 3 or group 4. However, NPRO for combined groups 8 and 9 differed significantly from that for standard group 4 ($P < 0.05$). The standard meal was estimated (from Table 3-5 of Ref. 8) to contain 2 mg nitrate and 0.3 mg nitrite, which are negligible compared to the nitrate doses used. The sharp rise in NPRO from groups 8 and 9 to group 4 is similar to the finding when nitrate and proline were taken by a fasting man (4).

**Fasting.** In group 10, the men fasted overnight and during and after taking nitrate and proline. Because proline was taken 30 min after nitrate in this group, the results were compared with those for groups 4 and 6, in which proline was taken with a meal 1 h and 15 min, respectively, after the nitrate. According to ANOVA, NPRO in group 10 was significantly greater than it was in groups 4 and 6 ($P < 0.05$). Fasting increased the NPRO yield 4.5-fold (for group 10 versus group 4) or 3.0-fold (for group 10 versus group 6). This enhancement is logical because the meal eaten by volunteers in groups 4 and 6 would have diluted the proline and nitrite, and the nitrosation rate should be proportional to the cube of reactant concentrations (Eq. A). Similarly, the 26 nmol NPRO that formed when proline was given with a meal in group 4 was less than the 160 nmol NPRO reported for proline given while fasting (4) and the 42 nmol NPRO reported for proline given between meals (20). Therefore, nitrosatable sugars such as sorbitol (31) should probably be taken with and not between meals to reduce their in vivo nitrosation.

**Time and Size of Test Meal and Extra Water.** Eating the test meal as breakfast or supper (groups 11 and 12) instead of lunch (group 4) did not affect NPRO yield. Eating lunches one-half or double the size (groups 13 and 14, respectively) lowered NPRO yield to 19–21 nmol from the 26 nmol found for group 4 (not significant). In group 15, drinking 12 oz. (340 ml) of water 30 min after the test meal lowered NPRO yield by 55% from the group 4 value to 16 nmol (not significant). A reduction by extra water had been expected because it would lower the concentrations of gastric nitrate and proline (Eq. A).

**High CHO Meal.** Eating a high CHO meal (group 16) lowered mean NPRO to 8 nmol, 31% of that in group 4 ($P < 0.001$). We had expected gastric pH with the high CHO meal to be lower than with the standard meal because we thought that protein in the standard meal would buffer the stomach contents more effectively than the smaller amount of protein in the high CHO meal. This lower pH should have increased the yield of NPRO. In rat experiments, gastric methylnitrosourea formation from methyurea and nitrite was raised 70% when a high CHO meal was fed (32). The low NPRO yield in group 16 could have been due to the cooked tomato sauce, which may still have contained ASC and polyphenols despite the cooking (21).

**Vegetables.** Vegetable and fruit consumption is negatively correlated with the incidence of many cancers (18), probably in part because these foods contain ASC and polyphenols that inhibit gastric nitrosation. This effect may be counterbalanced by the high nitrate content of some vegetables. Average values are 2400 mg nitrate and 100 mg ASC/kg for beets and 520 mg nitrate and 470 mg ASC/kg for raw cabbage (8, 33). Hence, we expected beets to enhance and cabbage to inhibit NPRO formation. In fact, cooked beets (group 17) reduced NPRO from 26 nmol in group 4 to 15 nmol (not significant), whereas raw cabbage (group 18) had no effect. Tests on other vegetables were not performed because Helser et al. (20) published an extensive study of this question.

**Timing of ASC.** In groups 19–23, 1 g ASC was taken at times varying from 5 h before to 2 h after the meal with proline, and nitrate was given 1 h before the meal. ANOVA showed significant differences between groups 4 and 19–23 ($P < 0.001$). There were significant differences between groups 21 and 4, between groups 22 and 4, between groups 19 and 20, and between groups 21 and 23 ($P = 0.05$). Percentage inhibition of NPRO formation by ASC was calculated after subtracting the 5 nmol “background” NPRO in group 3 (with proline alone) from the results for each group, as done by Leaf et al. (23). For ASC given 5 and 2 h before, with, and 1 and 2 h after the meal with proline (groups 19–23), the mean inhibitions of NPRO formation were 0, 71, 71, 67, and 19%, respectively (Fig. 1). (These values are corrected from those in our abstracts; Refs. 2, 3.) ASC also inhibited NPRO formation by 41% when we gave it with the meal and gave nitrate 15 min before the meal (group 24) and compared the results with those in group 6 (not significant). These results extend previous findings that were made when ASC and proline were given between meals or while
fasting (4, 23) in that ASC inhibition was now observed when proline was taken with a meal.

The effectiveness of ASC given 2 h before the meal with proline suggests that sufficient ASC persisted in the stomach for 2 h for it to inhibit NPRO formation. The relative effectiveness of ASC given 1 h after the meal and the ineffectiveness of ASC given 2 h after the meal suggests that most proline nitrosation occurred between 1 and 2 h after the meal. This is reasonable because it takes 1–2 h for gastric contents to become reacidified after a meal (34) and 3 h for 80% of a normal meal to empty from the stomach (35).

A dose of ASC given 5 h before the test meal should have emptied long before the meal was taken but could still have inhibited gastric nitrosation because it probably raised the level of plasma ASC, and hence, could have increased the active secretion of ASC into the stomach. Gastric secretion of ASC was indicated because the normal level of ASC plus dehydro-ASC is 50 mg/liter gastric juice and 7 mg/liter plasma (36) and is estimated to proceed at a rate of 0.4 mg/h (37). The lack of effect of the 1 g dose of ASC given 5 h before the meal suggests that gastric secretion of ASC was not important when proline was taken with a meal, which would have diluted the secreted ASC. In contrast, when proline was taken between meals, NPRO formation was inhibited by 44% for 470 mg ASC given 5 h before the proline (24). In that experiment, ASC may have inhibited NPRO formation because this formation occurred in a nearly empty stomach (35). Under this condition, an increased secretion of ASC due to its ingestion may have significantly reduced the gastric level of nitrite.

In a 5.25-year intervention study in China (38), volunteers took 120 mg ASC once daily. No effect was observed on esophageal and gastric cancer deaths. This negative result could have occurred even if NOCs were involved in the etiology because: (a) NOCs had initiated carcinogenesis many years before the study began; (b) ASC was not always taken with meals; or (c) the ASC dose was too low (we used doses of 1 g). Our results show the importance of taking ASC with meals. Because our study indicates that taking ASC inhibits nitrosation for a maximum of about 3 h, it supports the recommendation by the National Cancer Institute and the American Cancer Society to eat fresh vegetables or fruits 5 times/day with each meal or as a snack. With regard to the ASC dose, our test used 1 g ASC, whereas in the field studies of the Bartsch group (see “Introduction”), 100–200 mg ASC inhibited NPRO formation. In those tests, ASC and proline were taken 1 h after meals when ASC may be more effective than when it and proline are taken with a meal. NPRO formation was significantly inhibited by 45–90 mg ASC when this was given with the proline between meals (23).

Chewing Gum and Tobacco. We examined the effect of chewing gum or tobacco for 2–3 h after the test meal, with 400 mg nitrate given 1 h before the meal (groups 25–29). It seemed likely that chewing tobacco would stimulate saliva flow and, thereby, increase nitrate secretion into the mouth and its reduction to nitrite. Therefore, we expected an increase in NPRO yield when tobacco was chewed. Gum was included as a control for the tobacco. The standard meal was slightly modified in these tests to further reduce its level of ASC and polyphenols. Although most of the chewing was not directly supervised, follow-up questions and the results for group 29 (see below) make us confident that the materials were chewed as instructed. Both the gum and tobacco groups (groups 26 and 28) showed identical mean NPRO yields to those of their control groups 25 and 27.

Group 29, in which tobacco was chewed without taking nitrate and proline, was included to indicate the contribution of preformed NPRO in the tobacco. (It is unlikely that chewing gum contains NPRO.) If we assume the mean values are accurate, it appears that (a) the 20 nmol NPRO in group 29 (tobacco without nitrate and proline) is composed of 7 nmol as a baseline (from results for group 1 without nitrate and proline) and 13 nmol derived from NO PRO in the tobacco; (b) the 26 nmol NPRO in group 4 (nitrate plus proline) is composed of 7 nmol as a baseline and 19 nmol due to in vivo nitrosation; and (c) the 26 nmol NPRO in group 28 (tobacco plus nitrate plus proline) is composed of 7 nmol baseline NPRO, 13 nmol derived from NPRO in the tobacco and only 6 nmol due to in vivo nitrosation.

This discussion indicates that chewing tobacco did not increase (and probably inhibited) in vivo nitrosation. Our finding about in vivo nitrosation should not detract from the fact that chewing tobacco contains large amounts of carcinogenic nitrosamines (7) and nicotine.

The reason why chewing tobacco and gum may not have affected NPRO yield may be that the rate of saliva secretion increased while these materials were chewed (Table 2). This additional saliva may have diluted the gastric nitrate and proline and, hence, reduced NPRO formation (Eq. A). Saliva samples from groups 25–29 were collected 1 and 2 h after chewing commenced and were analyzed for nitrite and nitrate (Table 2). Results for the two saliva collections in each group were similar and are combined in Table 2. The 2–8-fold increase in the rate of saliva secretion caused by chewing in groups 26, 28, and 29 (Table 2) was expected because saliva stimulated by chewing was secreted at 2.0 ml/min, compared to 0.4 ml/min for unstimulated saliva (39). These rates are similar to those in Table 2. When nitrate was taken, the concentration of salivary nitrite, but not of salivary nitrate, was reduced during the chewing (groups 26 and 28), and this difference was significant for the test on gum. The lower nitrite level is attributed to reduced contact time with nitrate-reducing oral bacteria caused by the increased flow of saliva. In group 29, where nitrate was not taken, chewing tobacco increased the salivary nitrite and nitrate levels relative to a control group, who only collected saliva, but this conclusion is tentative because the control and experimental tests were conducted under slightly different conditions.

People who chewed betel nuts, tobacco, and betel nut–tobacco mixtures showed increased NPRO in saliva and in 6-h urine collections (40), but doses of nitrate and proline were not taken, each group contained only 3–6 subjects, and the effects were not significant. United States moist snuff contained an average of 12 ppm NPRO in 1985 (41). If this was the NPRO content of the tobacco used here, this would correspond to 38.4 µg (266 nmol) of NPRO in the 4 plugs (3.2 g) used in each test. This is 20 times the 13 nmol urinary NPRO that was estimated to be derived from the tobacco. The apparent low extraction of NPRO from the tobacco may have occurred because NPRO may have been present mostly as NH2-terminal NPRO in polypeptides, as in nitrite-cured meat (25, 26). The brand of tobacco used here contains mean values of 1080 µg/g of nitrate, 64 µg of nitrite and 600–1200 µg/g of free proline, estimated by its ability to form NPRO.4 Ingestion of these NPRO precursors may have contributed to the urinary NPRO.

Yield of NSAR. For some groups, we also calculated the yield of the nitrosamino acid NSAR. Standard group 4 showed a

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4. D. Hoffmann, personal communication.
mean of 21 nmol NSAR/24 h, nearly as much as the 26 nmol NPRO found in the same group. NSAR yield was only 10 nmol/day in group 3, where proline but no nitrate was given (P < 0.05 for difference from group 4) and was 13 nmol/day in group 19, where ASC was given with the meal. These results suggested that NSAR was derived from ingested nitrate by gastric nitrosation of sarcosine. However, group 1 (with no nitrate and no proline) showed a relatively high mean NSAR of 24 nmol/day due to a high value of 48 ± 8 nmol/day for 1 of 4 subgroups. NSAR showed little variation between most of the other groups except for group 5, which showed the highest mean NSAR (44 nmol/day), with P = 0.001 for the difference from group 4. This increased NSAR excretion in women may have been due to experimental variations and should be confirmed because NSAR is a weak carcinogen (15).

Conclusions. These results should be useful for delineating possible confounding factors when future NPRO tests are being planned. Our most important conclusions are: (a) NPRO formation after taking standard doses of nitrate and proline was surprisingly constant because it was not affected by a number of factors, including gender and the time and size of the meal; (b) intake of 400 mg nitrate produced significant amounts of NPRO when proline was given with a meal, but lower amounts of nitrate had a lesser effect. This confirms previous studies in which proline was given while fasting; (c) fasting increased NPRO yield 3–4 times compared to giving proline with a meal; this suggests that nitrosatable drugs should be taken with and not between meals to minimize their in vivo nitrosation; (d) according to our tests with 1 g ASC, this vitamin must be taken between 2 h before and 1 h after a meal to efficiently inhibit the nitrosation of meal components; (e) the same ASC tests suggest that most gastric nitrosation occurs between 1 and 2 h after meals; and (f) chewing gum or tobacco does not increase in vivo nitrosation.

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References


Dosing time with ascorbic acid and nitrate, gum and tobacco chewing, fasting, and other factors affecting N-nitrosoproline formation in healthy subjects taking proline with a standard meal.


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