Endogenous Nitrosation in Relation to Nitrate Exposure from Drinking Water and Diet in a Danish Rural Population

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ABSTRACT

Increasing levels of nitrate in drinking water is of concern due to the possibility of an associated increase in long-term exposure to endogenously formed carcinogenic N-nitroso compounds. Excretion of N-nitroso-L-proline in 12-h overnight urine after intake of 500-mg L-proline was used to quantify the rate of endogenous nitrosation in 285 individuals in an area in northern Denmark with large variation in nitrate concentration of the drinking water. Nitrate intake was measured in a 24-h duplicate diet sample. The crude association between nitrate concentration in drinking water and rate of endogenous nitrosation in individuals is only weakly positive and not quite statistically significant (P = 0.08). The risk of having detectable nitrosation increases significantly with total nitrate intake and tobacco smoking. In nonsmokers, nitrosation is determined by nitrate intake. Smokers have increased nitrosation which does not depend on nitrate intake. Effect modification through dietary factors was evaluated and indicated a protective effect of tea consumption, while the effect of eating vegetables was not clear-cut. The experimental design (12-h urine sample; proline dose taken in the evening) is likely to underestimate the effect of nitrate in drinking water relatively to nitrate in the diet.

INTRODUCTION

The average concentration of nitrate (NO₃⁻) in ground water used as drinking water in Denmark increased from 3.0 mg NO₃⁻/liter to 13.3 mg/liter over the period 1940 to 1983 (1). The maximum nitrate concentration in Denmark should not exceed 50 mg/liter and it is recommended that water with more than 25 mg/liter is not used as drinking water. However, by 1983 some 6–7% of the Danish population had water supplies with more than 50 mg/liter, and 13–14% used water with more than 25 mg/liter (1). Health hazards related to a high intake of nitrate include the rare, acute toxic effect methaemoglobinemia in bottle-fed infants (2), and the possibility of increased exposure to endogenously formed carcinogenic N-nitroso compounds (3–8). It has been suggested that gastric cancer is associated with nitrate intake through this mechanism (9–15), although the more recent evidence, especially from Britain, does not show this association (16–19).

In a previous paper we demonstrated that drinking water may be a major source of total nitrate intake (20). With 50 mg nitrate per liter drinking water, the average Danish consumer is exposed to 90 mg nitrate per day, more than half of which is derived from the drinking water. Consumption of vegetables is the other important source of nitrate and provides on average about 40 mg per day. The purpose of the present study was to examine whether a high-nitrate source of drinking water is associated with increased exposure to endogenously formed N-nitroso compounds, and to examine the association between total nitrate intake and endogenous nitrosation.

MATERIALS AND METHODS

Endogenous nitrosation was investigated in a Danish rural population through measurement of urinary excretion of the nontoxic and noncarcinogenic compound NPRO in 12-h urine samples following oral intake of 500 mg L-proline. This method is a modification of the assay developed by Ohshima and Bartsch (4) which has been used intensively to quantify the potential for endogenous nitrosation in humans (21–22).

The Population. In the rural area of west Jutland in northern Denmark, age- and sex-stratified samples of 18 persons were selected from the population being served by each of 21 waterworks, i.e., altogether 378 persons. The 21 water supplies had deliberately been selected to provide high variation in nitrate concentration in the drinking water of the participants in the study. Eight supplies had 0–5 mg NO₃⁻/liter, five had 35–59 mg/liter, and eight had 60 mg/liter or more. Thirty-nine persons were permanently or temporarily living away from the "permanent" address obtained from the Danish Central Population Register, and were considered not eligible for the study. Of 339 eligible persons, 294 (87%) participated in the study. Of these, 281 provided sufficient information to be included in all statistical analyses. The participation rate was uniform over the three groups of water supplies.

Data Collection. A detailed description of the data collection procedure has been given elsewhere (20). In short, the following information and samples were collected: (a) a questionnaire on smoking habits, history of gastric disease, and dietary habits; (b) a 24-h duplicate portion of the total diet on the day of participation from early morning to late night. Separate containers were provided for beverages (including tap water) and more solid dietary items; (c) a record of all dietary items consumed during the 24-h period of collection of the duplicate portion; (d) a 12-h overnight urine sample collected from 1 h after the evening meal to (and including) the first void in the following morning. Capsules with 500-mg L-proline were taken by the participants at the start of the period of urine collection. An aliquot of the urine sample was conserved with NaOH and stored at −20°C; (e) two samples of tap water from the residence. The data and biological samples were collected from September 1986 to March 1987.

Chemical Analyses. Nitrate in drinking water samples, duplicate diet portions, and urine samples were measured following homogenization and extraction (20, 23). Urinary creatinine was determined by the alkaline picrate method (24).

The method used for measurement of NPRO was based on the work of Ohshima and Bartsch (4, 25). NPIP and NPRO was kindly supplied by Dr. Bartsch, IARC, Lyon, and by the Danish Institute of Protein Chemicals, Harsholm. Other chemicals were analytical grade.

To 15 ml of thawed, centrifuged urine in a separating funnel were added 2 ml 20% ammonium sulfamate solution in 1.8 M sulfuric acid, 4 g sodium chloride, and 247 µg of NPIC as internal standard. The urine was extracted three times with 25 ml dichloromethane containing 10% methanol, and the organic phase was filtered though anhydrous sodium sulfate into a 100-ml pear-shaped evaporation flask. The sodium sulfate was washed with extraction solvent, the combined extracts were evaporated in rotary evaporator at 35°C and dissolved in 1 ml ethylacetate.

A diazomethane generator was mounted in the flask and cooled in an ice bath. Diazomethane was generated from approx. 40 mg Nmethylnitrosourea by addition of 300 µl 6 N NaOH. After methylation

3117

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: NPRO, N-nitroso-L-proline; NPIC, N-nitroso-L-pipecolic acid.
for 1 h the yellow extracts were transferred into 2-ml autosampler vials closed with teflon-lined septa. Ten µl extract was injected on a 4-mm i.d. stainless steel column packed with 10% Superox 4 on Chromosorb W with the following program: 140°C isotherm for 5 min, 4°C/min to 220°C, which was kept for 2 min. The separated nitrosocompounds were detected with a Thermal Energy Analyser model 502 using a pyrolyzer temperature at 475°C. The retention times of NPIC and NPRO were 22.0 min and 23.7 min.

Contents of NPRO was calculated from peak heights corrected for recovery of NPIC using a standard curve with NPIC and NPRO methylated in the same way as the urine extracts. The standard curve was linear from 0 to at least 300 ng NPRO/ml extract. The detection limit was better than 1.0 ng NPRO/12 h.

Statistical Analysis. First, the three groups of persons with water supplies with low, intermediate, and high nitrate concentration in the drinking water were compared. The Wilcoxon rank sum test was used to evaluate differences between medians in NPRO and other factors. Secondly, the crude association of NPRO excretion with nitrate concentration in drinking water and with total nitrate intake were evaluated, and χ² test for trend was used. The relative risk of having NPRO exceeding 1 µg/day was used as the measure of association. Thirdly, by means of multivariate maximum likelihood logistic regression analysis (26) using the CATMOD program in the SAS-package (27), the determinants of endogenous nitrosation measured as urinary excretion of NPRO was further evaluated. Due to the extremely skewed distribution of urinary NPRO, this variable was analyzed as a discrete outcome phenomenon. One µg NPRO or more in the 12-h urinary sample was considered a positive outcome, while urinary excretion of less than 1 µg was considered a negative outcome. Similarly, separate regression models were evaluated to identify factors influencing the risk of exceeding 5 µg and 10 µg NPRO in the 12-h urine sample. The effect modification of dietary factors on the association between nitrate intake and endogenous nitrosation, was further evaluated among nonsmokers. Tobacco smoking was quantified as "cigarette equivalents per day": one cigar = one pipe-full = 2.5 cigarillos = five cigarettes.

RESULTS

Table 1 shows the marked variation in the nitrate concentration of drinking water reflecting the selection of the three groups of persons. The nitrate content of the 24-h duplicate diet portion (including beverages) and urinary nitrate varies significantly with the measured nitrate concentration in drinking water. Urinary creatinine is identical in the three groups. The median of urinary excretion of NPRO is highest in the group with the highest nitrate concentration in the drinking water, but none of the pairwise differences between median NPRO excretion in the three groups are statistically significant.

Fig. 1 shows the distribution of NPRO in individual urine samples. About half of the samples contained less than 0.5 µg NPRO while the other half contained from 0.5 to 119 µg. Consumption of cured meat products during the 24-h period before urine collection is associated with a high urinary level of NPRO. The odds-ratio for exceeding more than 1 µg/12 h associated with consumption of cured meat is 3.68 (1.27–10.67) for having more than 1 µg NPRO/12 h, which increases to 4.63 (1.54–13.87) and 9.30 (2.04–42.40) for excreting more than 5 µg/12 h and more than 10 µg/12 h, respectively. This means that consumers of cured meat contribute markedly to the upper tail of the distribution of urinary NPRO measurements (Fig. 1). It is well known that cured meat may contain up to several gram NPRO per kilo meat product (28). Like NPRO formed in the stomach, ingested NPRO is not metabolized but is rapidly excreted in the urine. It is thus likely that the increased urinary NPRO associated with consumption of cured meat represent ingested NPRO rather than intragastric formation.

Tables 2 and 3 show a positive association between nitrate concentration in drinking water and nitrate intake respectively on the prevalence of NPRO excretion exceeding 1 µg in persons who do not smoke tobacco. NPRO excretion increases with nitrate concentration in drinking water in nonsmokers, although not statistically significant (χ² test for trend; P = 0.08) (Table 2) or in smokers and nonsmokers combined (P = 0.08). NPRO excretion increases with total nitrate intake in nonsmokers (χ² test for trend; P < 0.01) (Table 3) and in smokers and nonsmokers combined (χ² test for trend; P < 0.01).

Results of the logistic regression analysis of these individual data are shown in Table 4. From the model including sex, age, history of gastric disease, batch of NPRO analysis, urinary creatinine concentration, and consumption of cured meat it can be estimated that the odds-ratio for excretion of 1 µg NPRO or more is 1.9 for an increase in nitrate intake of 50 mg/day among nonsmokers; this is highly significant (95% confidence interval = 1.39–2.58). Nitrate intake was in this model evaluated as a continuous variable with a unit = 50 mg/day. Among smokers of 1–20 cigarette-equivalents per day, nitrosation is significantly increased compared with nonsmokers and nitrate intake is not a strong determinant within this group. In the group of heavy smokers (21+ cigarette-equivalents) nitrosation is also increased compared with nonsmokers, but the effect of

Table 1 Quantitative information from samples of drinking water, 24-h duplicate dietary samples, and overnight urinary samples in three drinking water categories

<table>
<thead>
<tr>
<th>Drinking water category</th>
<th>Low nitrate (L) eight water supplies (0-5 mg/liter)</th>
<th>Intermediate (I) five water supplies (35-59 mg/liter)</th>
<th>High nitrate (H) eight water supplies (60+ mg/liter)</th>
<th>P value of pairwise comparison of medians</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrato concentration in drinking water (mg/liter)</td>
<td>Mean ± standard deviation (n)</td>
<td>Mean ± standard deviation (n)</td>
<td>Mean ± standard deviation (n)</td>
<td>L &amp; I</td>
</tr>
<tr>
<td>Mean; quartile range</td>
<td>0.3 ± 0.7 (115)</td>
<td>46.5 ± 6.9 (76)</td>
<td>84.4 ± 19.2 (103)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total nitrate in 24-h duplicate portion (mg)</td>
<td>Mean; quartile range</td>
<td>46.5 ± 43.6 (114)</td>
<td>102.4 ± 46.3 (76)</td>
<td>143.6 ± 79.8 (103)</td>
</tr>
<tr>
<td>Nitrato in urinary sample (mg)</td>
<td>Mean; quartile range</td>
<td>37.2 ± 17.5-58.3</td>
<td>89.3; 72.6-123.0</td>
<td>122.8; 87.5-187.5</td>
</tr>
<tr>
<td>Urinary creatinine (mM)</td>
<td>Mean; quartile range</td>
<td>36.3 ± 23.8 (112)</td>
<td>54.9 ± 26.9 (71)</td>
<td>72.6 ± 38.0 (100)</td>
</tr>
<tr>
<td>Urinary NPRO (µg/12 h)</td>
<td>Mean; quartile range</td>
<td>9.6 ± 4.7 (115)</td>
<td>9.9 ± 5.0 (73)</td>
<td>9.7 ± 4.8 (103)</td>
</tr>
<tr>
<td>Median; quartile range</td>
<td>2.90 ± 9.15 (112)</td>
<td>1.86 ± 2.85 (74)</td>
<td>3.75 ± 12.57 (99)</td>
<td>0.00; 0.00-3.05</td>
</tr>
</tbody>
</table>

3118

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nitrate appears to be reversed although the confidence intervals are very wide.

Table 5 shows the results of further analyses of dietary determinants of nitrosation in nonsmokers. When information on consumption of selected foods is added individually to the baseline model given in Table 4, increased nitrosation is seen for green vegetables, cabbage, and other vegetables, although only the effect of green vegetables is statistically significant. Consumption of tea and vegetables on sandwiches provides significant protection against nitrosation.

The 24-h duplicate portions were collected with use of separate containers for beverages and solid dietary items. Nitrate in these subsamples was measured separately. It is therefore possible to estimate the effect of nitrate in the liquids (mainly from drinking water but also from fruit juices etc.) and in the of more solid dietary items [mainly from vegetables but also from drinking water used in cooking (20)]. An odds ratio of 1.88 (1.37–2.57) among nonsmokers for 50 mg total nitrate breaks down to 1.48 (0.95–2.32) for 50 mg nitrate in the liquids subsample and 2.41 (1.46–3.96) for 50-mg nitrate in the subsample of solid dietary items, when these are considered simultaneously in the regression model.

DISCUSSION

It was the purpose of the present study to examine the association between nitrate intake and endogenous nitrosation in view of the hypothesized relationship between nitrate, nitrosation, and gastric cancer. Examination of the general population exposed to various levels of nitrate is of particular importance in view of the often high and increasing exposure to nitrate from drinking water which result from intensive agricultural practices.

While no overall association is found between nitrosation in three groups of individuals with household water-supplies with 0.3–4.6, 5–8.4, and >8.4 mg nitrate/liter, respectively, nitrosation of proline is strongly associated with nitrate intake in individuals who do not smoke tobacco. The nitrosation rate should theoretically depend on the square of the concentration of nitrate (29). Inclusion of the square of nitrate intake in the regression analysis did not improve the goodness-of-fit. Ohshima & Bartsch (4) found, in a nonsmoking individual, very low nitrosation rates at nitrate intakes below 200 mg/day and strongly increasing nitrosation with higher nitrate intake. Few individuals in the present study had a nitrate intake of 200 mg/day or more, and it appears that nitrate intake increases endogenous nitrosation in nonsmokers, also at the level of 20–150 mg/day typically found in the Danish population.

The odds-ratio of excreting more than 5 μg NPRO/12 h and 10 μg NPRO/12 h for a change of 50 mg nitrate per day are not substantially different from the 1.90 obtained with a threshold at 1 μg NPRO/12 h. The effect of nitrate intake in nonsmokers is thus generally to increase the rate of endogenous nitrosation.

Endogenous nitrosation is associated with tobacco smoking and apparently independent of nitrate intake in smokers. Thiocyanate is a strong catalyst of nitrosation of amines, and smokers have three to four times higher levels of thiocyanate in saliva than nonsmokers (30). Also, thiocyanate and nitrate compete at the level of active transportation and salivary recirculation (31). Increased salivary thiocyanate levels may therefore increase the rate of endogenous nitrosation in the stomach and at the same time, for any level of nitrate intake, reduce the flow of recirculated nitrate in saliva. Our findings of an increased excretion of NPRO in smokers is in line with previous observations (32).

Generally, vegetables are the major source of nitrate intake (15) but in Danish communities with nitrate concentration in drinking water exceeding about 50 mg/liter (20) this was not found to be the case. Vegetables contain nitrate which may increase endogenous nitrosation, but vegetables also contain ascorbate, a strong nitrosation inhibitor (33). Our results (Table 5) show that consumption of vegetables increases the endogenous formation of NPRO. The effect is strongest for green vegetables (OR = 2.37) but the effects of cabbage (OR = 1.82), and other vegetables (OR = 1.43) is in the same direction, although these effects are not statistically significant. On the other hand, consumption of other sources of ascorbate: vege-

Table 2  Prevalence of excretion of 1 μg NPRO or more in overnight urine, in relation to nitrate concentration in drinking water

<table>
<thead>
<tr>
<th>Nitrate concentration in drinking water (mg/liter)</th>
<th>Amount NPRO in overnight urinary sample</th>
<th>Nonsmokers*</th>
<th>Crude rate-ratio</th>
<th>Smokers*</th>
<th>Crude rate-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥1 μg</td>
<td>&lt;1 μg</td>
<td>% ≥1 μg</td>
<td>≥1 μg</td>
<td>&lt;1 μg</td>
</tr>
<tr>
<td>0–24</td>
<td>22</td>
<td>36</td>
<td>37.9</td>
<td>1.00</td>
<td>20</td>
</tr>
<tr>
<td>25–49</td>
<td>9</td>
<td>14</td>
<td>9.1</td>
<td>1.03</td>
<td>11</td>
</tr>
<tr>
<td>50–74</td>
<td>18</td>
<td>23</td>
<td>43.9</td>
<td>1.16</td>
<td>11</td>
</tr>
<tr>
<td>75–99</td>
<td>10</td>
<td>9</td>
<td>52.6</td>
<td>1.39</td>
<td>11</td>
</tr>
<tr>
<td>100+</td>
<td>7</td>
<td>4</td>
<td>63.6</td>
<td>1.68</td>
<td>3</td>
</tr>
</tbody>
</table>

* x2 = 2.9; P = 0.08 for trend.

* x2 = 0.60; P = 0.44 for trend.
Table 3 Prevalence of excretion of 1 \mu g NPRO or more in overnight urine, in relation to total nitrate intake

<table>
<thead>
<tr>
<th>Total nitrate intake (mg/24 h)</th>
<th>Amount NPRO in overnight urinary sample</th>
<th>Crude rate-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\geq 1 \mu g</td>
<td>&lt;1 \mu g</td>
</tr>
<tr>
<td>0–49</td>
<td>12</td>
<td>29</td>
</tr>
<tr>
<td>50–99</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>100–149</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>150–199</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>200+</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>

* Adjusted for sex, age, history of gastric disease, batch of NPRO analysis, and consumption of cured meat.

Table 4 Logistic regression analysis of overnight NPRO excretion in relation to total nitrate intake and tobacco smoking

<p>| Odds-ratios with 95% confidence intervals for having 1 \mu g NPRO or more in the urine sample. |
|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Smoking, cigarette equivalents per day</th>
<th>Nitrate intake (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.00 (1.39–2.58)</td>
</tr>
<tr>
<td>1–20</td>
<td>2.20 (1.11–5.15)</td>
</tr>
<tr>
<td>21+</td>
<td>1.31 (0.17–6.92)</td>
</tr>
</tbody>
</table>

* Adjusted for sex, age, history of gastric disease, batch of NPRO analysis, urinary creatinine concentration, and consumption of cured meat.

Table 5 Further logistic regression analysis of urinary NPRO excretion in nonsmokers

<table>
<thead>
<tr>
<th>Factor</th>
<th>Odds-ratio*</th>
<th>Adjusted odds-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetables on sandwiches</td>
<td>0.37</td>
<td>0.41 (0.16–0.87)</td>
</tr>
<tr>
<td>Tea</td>
<td>0.44</td>
<td>0.40 (0.16–1.01)</td>
</tr>
<tr>
<td>Regular use of vitamins</td>
<td>0.83</td>
<td>0.85 (0.36–2.18)</td>
</tr>
<tr>
<td>Fresh fruits</td>
<td>0.09</td>
<td>0.09 (0.04–0.28)</td>
</tr>
<tr>
<td>Potatoes</td>
<td>1.08</td>
<td>1.08 (0.54–2.17)</td>
</tr>
<tr>
<td>Coffee</td>
<td>1.31</td>
<td>1.31 (0.63–2.71)</td>
</tr>
<tr>
<td>Other vegetables</td>
<td>1.43</td>
<td>1.43 (0.64–3.19)</td>
</tr>
<tr>
<td>Cabbage</td>
<td>1.86</td>
<td>1.86 (0.66–5.03)</td>
</tr>
<tr>
<td>Green vegetables</td>
<td>2.37</td>
<td>2.37 (1.02–5.49)</td>
</tr>
</tbody>
</table>

* Adjusted for sex, age, history of gastric disease, batch of NPRO analysis, and urinary creatinine concentration, and consumption of cured meat.

ENDOGENOUS NITROSATION IN RELATION TO NITRATE EXPOSURE

The finding that endogenous nitrosation depends strongly on nitrate intake seems to support the model for nitrate-induced gastric cancer proposed by Correa (11–13). It is clear however, that endogenous formation of N-nitroso compounds in nonsmokers in this Danish population. Given that many N-nitroso compounds are strong carcinogens in animal systems (39) our results indicate that it may be advisable to limit the intake of nitrate. A general decrease in vegetable consumption is not advisable because vegetables are an important source of calories, fibers, trace minerals, and vitamins, but it would seem well advised to reduce the intake of nitrate from drinking water. It may be considered also to concentrate on cultivars with lower nitrate content, or to agricultural practices which produce low nitrate vegetables.

The data offer a more direct means of evaluation of the effect of dietary nitrate (predominantly from vegetables) and water-derived nitrate. The 24-h duplicate dietary portions were collected in two containers which were analyzed separately. The odds-ratio of 1.88 for an increase in total nitrate intake of 50 mg breaks down to 1.48 for 50 mg in the subsample of beverages and 2.41 for 50 mg in the subsample of solid dietary items. Although the two subsamples of the duplicate portions do not perfectly separate nitrate from vegetables and nitrate from drinking water, this result is in line with the finding of increased nitrosation being associated with consumption of vegetables.

Drinking water contains no nitrosation inhibitors while the diet contains at least some ascorbate and other inhibitors. Nitrate in drinking water is therefore expected to convey a comparable or even stronger risk than nitrate from the diet. However, persons who use a nitrate-free source of drinking water obtain all their nitrate from vegetables, most of which are taken with the evening meal, i.e., at about the same time as the proline-dose. By contrast, persons who use a high-nitrate source of drinking water are exposed to nitrate during most of the day. The closer to the proline intake and to the start of the collection period a nitrate ion is ingested, the higher is the chance for this particular ion to become part of a formed NPRO molecule. The 12-h method of urine collection and the timing of the proline-dose very close to the evening mean may therefore not provide a complete reflection of the nitrosation potential of water-derived nitrate. The effect of tobacco smoking may also, for this reason, be somewhat magnified in the present study since many smokers like to smoke after their evening meal. These limitations of the present protocol should be taken into account in future studies.

In conclusion, nitrate intake is an important determinant of endogenous formation of N-nitroso compounds in nonsmokers in this Danish population. Given that many N-nitroso compounds are strong carcinogens in animal systems (39) our results indicate that it may be advisable to limit the intake of nitrate. A general decrease in vegetable consumption is not advisable because vegetables are an important source of calories, fibers, trace minerals, and vitamins, but it would seem well advised to reduce the intake of nitrate from drinking water. It may be considered also to concentrate on cultivars with lower nitrate content, or to agricultural practices which produce low nitrate vegetables.

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that nitrate in drinking water can only be one of several deter-
minants of gastric cancer incidence in Denmark where the inci-
dence rate of this disease has decreased steadily for several
decades. A full understanding of the epidemiology of gastric
cancer in Denmark requires, within the framework of Correa's
model, that changes in the intake of nitrate from other sources
disturb drinking water and changes in the intake of nitrosation
inhibitors in this century are given consideration.

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