Volatile N-Nitrosamine Formation after Intake of Nitrate at the ADI Level in Combination with an Amine-rich Diet

Ingrid T.M. Vermeer, Danielle M.F.A. Pachen, Jan W. Dallinga, Jos C.S. Kleinjans, and Jan M.S. van Maanen

Department of Health Risk Analysis and Toxicology, Maastricht University, The Netherlands

Formation of nitrite from ingested nitrate can result in several adverse health effects and implies a genotoxic risk as a consequence of endogenous formation of carcinogenic N-nitroso compounds. We studied the formation of volatile N-nitrosamines after intake of nitrate at the acceptable daily intake (ADI) level in combination with a fish meal rich in amines as nitrosatable precursors. Twenty-five volunteers consumed this meal during 7 consecutive days; a diet low in nitrate was consumed during 1 week before and 1 week after the test week. Nitrate intake at the ADI level resulted in a significant rise in mean salivary nitrate and nitrite concentrations. Mean urinary nitrate excretion increased from 76 mg/24 hr in the first control week to 194 and 165 mg/24 hr in the test week, followed by a decline to 77 mg/24 hr in the second control week. The urine samples were analyzed for volatile N-nitrosamines, and both N-nitrosodimethylamine (NDMA) and N-nitrosopiperidine (NPIP) were detected in the samples. Mean urinary NDMA excretion significantly increased from 287 ng/24 hr in the control week to 871 and 640 ng/24 hr in the test week and declined to 383 ng/24 hr in the second control week. Excretion of NPIP was not directly related to the nitrate intake and composition of the diet. Nitrate excretion and NDMA excretion were significantly correlated, as well as salivary nitrate and nitrite concentration and NDMA excretion. We conclude that nitrate intake at the ADI level in combination with a fish meal containing nitrosatable precursors increases NDMA excretion in urine and thus demonstrates increased formation of carcinogenic N-nitrosamines. *Key words:* amines, nitrate, N-nitrosamines, N-nitrosodimethylamine. *Environ Health Perspect* 106:459–463 (1998). [Online 1 July 1998]
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Humans are exposed to nitrate mainly as a consequence of food intake and, to a lesser extent, of drinking water consumption. Vegetables are the principal source of nitrate exposure, providing at least 85% of the average daily nitrate intake when the drinking water nitrate concentration is low (e.g., 1–5 mg/l). The contribution of drinking water intake to the nitrate load is strongly dependent on the type of water supply. The World Health Organization (WHO) guidelines indicate a maximally admissible nitrate concentration in drinking water of 44.3 mg/l (1). Nitrate levels in most European public drinking water supplies seldom exceed 45 mg/l. Private wells, however, may contain as much as 300 mg/l (2). WHO states that nitrate levels in surface waters and groundwaters have markedly increased in the last decades due to increased use of fertilizers, changes in land use, and disposal of waste from intensive animal farming, leading to an increased risk of human exposure (1).

Nitrate per se is relatively nontoxic, but approximately 5% of all ingested nitrate is converted to the more toxic nitrite (3–5). Upon absorption in the bloodstream, nitrite reacts with hemoglobin to form methemoglobin, which is unable to transport oxygen (6). Methemoglobinemia is considered to be the major human health risk associated with nitrate exposure. Infants are especially sensitive to the formation of methemoglobin. Based on the no-adverse-effect level observed in animal studies, WHO has set the acceptable daily intake (ADI) for nitrate and nitrite, respectively, at 3.67 mg/kg body weight and 0.13 mg/kg body weight (expressed as nitrate and nitrite ion) (7).

Other adverse health effects of nitrate exposure have been reported. Exposure to high nitrate levels implies a genotoxic risk for humans due to endogenous formation of carcinogenic N-nitroso compounds. Nitrite derived from nitrate may react *in vivo* with amines and amides to form N-nitroso compounds. About 300 N-nitroso compounds (NOCs) have been tested for carcinogenicity in experimental animals; 85% of the 209 nitrosamines and 92% of the 86 nitrosamides have been shown to be carcinogenic in a variety of species (8). Human exposure to endogenously formed NOCs has been associated with an increased risk of cancer of the stomach, esophagus, and bladder (9–12). Convincing epidemiological evidence for human cancer risk, however, is still lacking. Ward et al. (13) observed that long-term exposure to elevated nitrate levels in drinking water may contribute to the risk of non-Hodgkin's lymphoma. Lin and Ho (14) reported hepatocarcinogenic effects in rats of endogenously formed NOCs after dietary intake of amines in combination with nitrite. Studies with patient groups point to

a role for NOCs in human cancer (15). On the other hand, several studies reported no or even an inverse relationship between nitrate or NOCs and human cancer (16–18).

N-nitrosoproline excretion in urine is frequently used as an indicator of endogenous nitrosation (NPRO test) (19–22). However, limited information is available on excretion of individual (carcinogenic) nitrosamines. In a recent study, we observed an increase in peripheral lymphocyte *hprt* (hypoxanthine-guanine phosphoribosyltransferase) variant frequency in users of well water containing high levels of nitrate, in combination with relatively high urinary excretion levels of the carcinogenic N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosopiperidine (NPIP), and N-nitrosopyrrolidine (NPYR) (23).

The aim of the present study was to investigate nitrosamine formation after nitrate exposure at the ADI level. This paper describes the excretion of volatile nitrosamines in the urine of human volunteers after a nitrate load in water in combination with a fish meal, since fish contains high amounts of amines, which are nitrosatable precursors (24,25). This study can contribute to the discussion of the validity of the current ADI of nitrate and nitrite for preventing human health risks.

Materials and Methods

Study population and protocol. Twenty-five healthy women volunteered to participate and signed an informed consent. They agreed to donate saliva samples, collect 24-hr urine, and to answer a questionnaire on food consumption and lifestyle habits. The participants were nonsmokers and used no medicine or vitamin preparations. The mean weight of the volunteers [± standard deviation (SD)] was 60 ± 6 kg (range 48–75 kg) and the mean age was 23 ± 7 years (range 18–46 years).

The study period consisted of 3 weeks: week 1 was a control week (control week 1), week 2 the experimental week, and week 3

was a control week (control week 2). During the control weeks the subjects refrained from consuming high nitrate-containing food items; on day 7, a 2-ml saliva sample was taken (2 hr after dinner) and 24-hr urine was collected. During the experimental week, the participants received a dinner low in nitrate and containing fish (cod, salmon, shrimp, pollack) at the University of Maastricht, in combination with a KNO_3 solution in water (277 mg KNO_3, corresponding to 170 mg nitrate anion, in 100 ml of distilled water). A nitrate load in water was used instead of nitrate-containing vegetables because this was the most suitable method of giving the volunteers the exact same dose every day. The total nitrate dose was at the ADI level for a person with a body weight of 60 kg (the mean body weight of the participants). Assuming a conservative nitrate intake of 50 mg/day, even with a diet low in nitrate, the volunteers consumed 170 mg of nitrate in water, resulting in a nitrate dose of 220 mg per day. On days 3 and 7, 2-ml saliva samples were taken at 1, 2, 5, and 23 hr after nitrate intake. Twenty-four-hour urine was collected every day, and the volume of the urine was recorded. Urines of days 1, 2, and

3 were pooled, and a 100-ml sample was taken; also urines of days 4–7 were pooled and sampled.

Collection and storage of saliva and urine samples. Two-milliliter saliva samples were produced after stimulating the salivary flow by chewing on parafilm and were collected in plastic vials in which 0.2 ml 1 M NaOH had been evaporated to dryness for stabilization of nitrate and nitrite. The 24-hr urine samples were collected in 2-l containers with 10 g NaOH pellets. Saliva and urine samples were stored at -20°C until analysis.

Analysis of nitrate in fish meals. The meals were homogenized, and 10 g was used for nitrate analysis. Sample preparation and analysis were performed as described in the instructions of the Boehringer Mannheim Nitrate Kit no. 905658 (26).

Analysis of nitrate and nitrite in saliva. Saliva samples were deproteinized by adding 50 µl 1 M $ZnSO_4$ to 0.5 ml of saliva. The mixture was vortexed, placed on ice for 15 min, and centrifuged for 10 min at 12,000g. The supernatant was heated at

100°C for 10 min for further deproteinization. After centrifugation for 10 min at 12,000g, the nitrate and nitrite concentrations in the supernatant were determined by HPLC using anion-exchange chromatography as previously described (27).

Analysis of nitrate and nitrite in urine. One milliliter of urine was centrifuged for 10 min at 12,000g, and 50 µl of the supernatant was analyzed for nitrate content with the spectrophotometric method from Boehringer (Boehringer Mannheim Kit no. 905658) using nitrate reductase (26). We calculated the amount of nitrate excreted in urine by multiplying the urinary nitrate concentration with the volume of the 24-hr urine. Urine samples were screened for nitrite content with nitrite test strips (Merckoquant nitrit-test; Merck, Germany).

Analysis of volatile nitrosamines in urine. Volatile nitrosamines, as listed in Table 1, were determined by gas chromatography mass spectrometry (GC-MS) as previously described (23) with some modifications. A 20-ml urine aliquot was extracted with 1 ml dichloromethane; 0.5 µl of the dichloromethane solution was splitlessly injected into the GC-MS system, consisting of an HP5890 Series II gas chromatograph (Hewlett-Packard, Avondale, Pennsylvania) and a Jeol SX102A mass spectrometer (Jeol Ltd., Tokyo). The column used was a WCOT fused silica column (Chrompack, New Jersey), length 50 m, inside diameter 0.25 µm, stationary phase CP-Sil-8 CB-MS, and film thickness 0.25 µm. Helium was used as the carrier gas (1 ml/min), and the injector temperature was 250°C. The column temperature was 50°C for 0.8 min, then rising

Table 1. The five N-nitrosamines analysed in urine

N-Nitrosamine	M^{+} ion (m/z)	Retention time (min)
N-Nitrosodimethylamine	74.05	3.6
N-Nitrosomethylethylamine	88.06	4.7
N-Nitrosodiethylamine	102.08	5.85
N-Nitrosopiperidine	114.08	8.93
N-Nitrosomorpholine	116.06	8.50

Table 2. Nitrate excretion in urine (mg/24 hr) during the study protocol

Time	Nitrate excretion in urine (mg/24 hr), mean ± SD	Nitrate excretion as percent of total dose
Control week 1	76 ± 42	
Test week, days 1–3	194 ± 45*	88
Test week, days 4–7	165 ± 40*	75
Control week 2	77 ± 41	

SD, standard deviation.
*Significant increase compared with control week 1 and control week 2 (Wilcoxon, $p = 0.0001$).
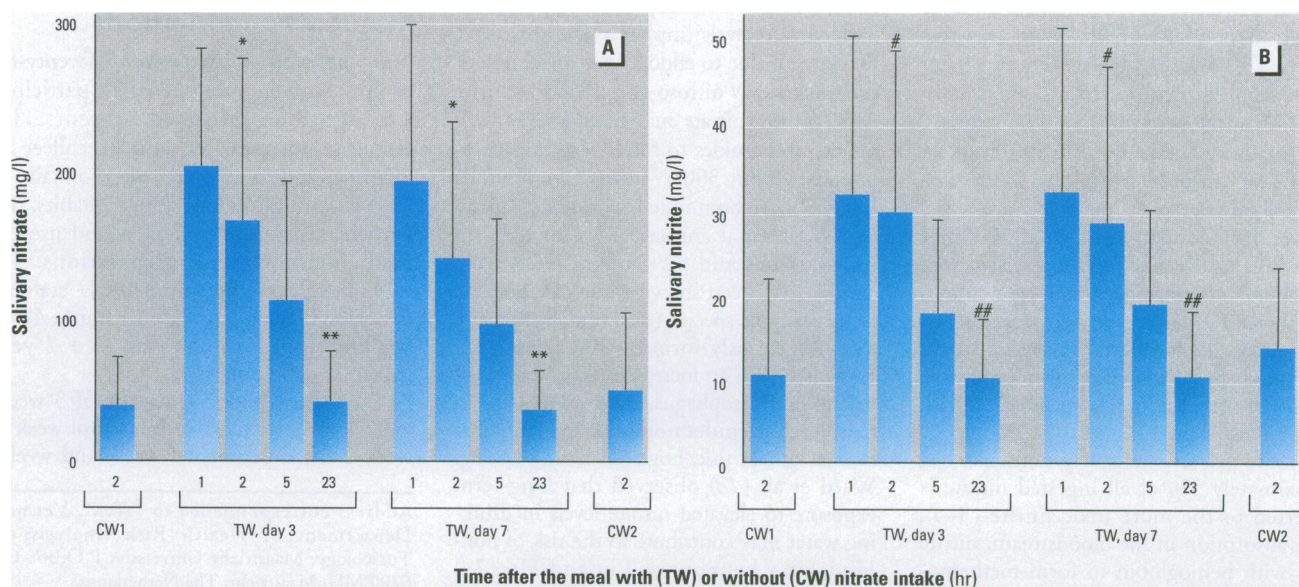

Figure 1. (A) Mean (± standard deviation) salivary nitrate concentrations (mg/l); (B): mean salivary nitrite concentrations (mg/l) 2 hr after a meal low in nitrate during the control weeks (CW) and 1, 2, 5, and 23 hr after a meal in combination with nitrate intake at 2 different days during the test week (TW).
*Significantly increased compared with CW 1 and CW 2, Wilcoxon, $p<0.0004$; **significantly decreased compared with 1, 2 and 5 hours after nitrate intake, Wilcoxon, $p<0.0004$; #significantly increased compared with CW 1 and CW 2, Wilcoxon, $p<0.0016$; ##significantly decreased compared with 1, 2 and 5 hr after nitrate intake, Wilcoxon, $p<0.0011$.

to 100°C at 9°C/min. After 0.5 min at 100°C, the temperature rose to 290°C at 35°C/min and was maintained at 290°C for 5 min. The mass spectrometer generated ions by electron ionization at 70 eV. The molecular ions ($M^{+\cdot}$) were detected using high-resolution (3000), single-ion monitoring (HR-SIM). Both retention time and elemental composition of the ions were used as proof of the identity of the measured components. Quantification was performed by a calibration curve for each of the nitrosamines, using a nitrosamine mixture for EPA method 8270 (Aldrich, Zwijndrecht, the Netherlands). The calibration curves (in urine) were analyzed analogously to the samples, adjusting for a possible loss of nitrosamines during workup. The detection limit for each nitrosamine was 1 pg/µl of dichloromethane solution, corresponding to 50 pg/µl of urine.

In our previous study, we mainly detected *N*-nitrosopyrrolidine in urine samples from subjects exposed to drinking water with medium or high nitrate levels (*23*). However, in a recent study with volunteers consuming meals with fish and vegetables rich in nitrate and using mouthwashes, we predominantly detected NDMA and no NPYR (*28*). Therefore, in this study with comparable nitrosating precursors, the GC-MS run detecting five volatile nitrosamines after one injection including NDMA and excluding NPYR was performed.

Statistical methods. Results are expressed as means ± SD. Statistical comparisons of the data with respect to nitrate and nitrite concentrations in saliva, nitrate reduction, and nitrate and nitrosamine excretion in urine were performed by the nonparametric Wilcoxon signed-ranks test. A *p*-value <0.05 was considered significant, except for NDMA excretion in urine, where the *p*-value

was compared with an adjusted α of 0.01 because five comparisons were made within one set of data (*29*). We used linear regression analysis to examine the relationship between nitrate and nitrosamine excretion in urine and between the log transformed nitrate and nitrite concentration in saliva, and nitrate and nitrosamine excretion in urine and between nitrate reduction and nitrosamine excretion in urine. A *p*-value <0.05 was considered significant.

Results

The mean nitrate intake via the fish meals consumed during the test week was 17 ± 15 mg. The nitrate content of the meals consumed during days 1–3 was slightly higher than the nitrate content of the meals consumed during days 4–7 (mean nitrate content 21 and 13 mg, respectively).

Nitrate and nitrite concentrations in saliva increased significantly after intake of nitrate at the ADI level (Fig. 1). Peak levels of both nitrate and nitrite were observed 1 hr after nitrate ingestion. Nitrate concentrations reached a maximum value of 202 ± 80 mg/l (mean ± SD) at day 3 and 192 ± 107 mg/l at day 7. Maximum nitrite levels were 32 ± 18 mg/l at day 3 and 32 ± 19 mg/l at day 7. Nitrate and nitrite levels 2 hr after nitrate intake were significantly increased compared with levels 2 hr after a meal low in nitrate during the control weeks (Wilcoxon, *p*<0.0016). Twenty-three hours after nitrate intake, salivary nitrate and nitrite concentrations had decreased to baseline levels.

A significant difference was observed between nitrate reduction levels (at 2 hr after the meal) during the control weeks and during the test week (Wilcoxon, *p* = 0.0011). Nitrate reduction during the

control weeks was 25 ± 15% and during the test week was 18 ± 11%.

Table 2 presents data on nitrate excretion in urine. Mean 24-hr nitrate excretion increased significantly during the test week compared with the control weeks (*p* = 0.0001). Nitrate excretion during the test week, during days 1–3 and days 4–7, expressed as percentage of the total nitrate dose (220 mg/day), was 88 and 75%, respectively. Nitrite was not detected in the urine samples.

Two *N*-nitrosamines were detected in urine samples: NDMA and NPIP. The results of NDMA excretion in urine are shown in Figure 2. Mean NDMA excretions during control week 1, test week days 1–3, test week days 4–7, and control week 2 were 287 ± 223, 871 ± 430, 640 ± 277, and 383 ± 168 ng/24 hr, respectively. One observation (during the test week, days 1–3) was identified as an outlier (more than five times the SD) and was excluded from all analyses. Comparisons were made between NDMA excretions in control week 1 and test week days 1–3, control week 1 and test week days 4–7, control week 1 and control week 2, test week days 1–3 and control week 2, and test week days 4–7 and control week 2. We compared *p*-values with an adjusted α of 0.010. A significant increase in NDMA excretion was observed during test week days 1–3 and test week days 4–7 compared with control week 1 and control week 2 (*p*<0.0002). There was no significant difference in NDMA excretion between the two control weeks (*p* = 0.03).

Mean urinary NPIP excretion significantly increased during the study relative to control week 1. During control week 1, NPIP excretion was 69 ± 36 ng/24 hr, followed by an excretion of 86 ± 49 ng/24 hr during test week days 1–3 (compared with
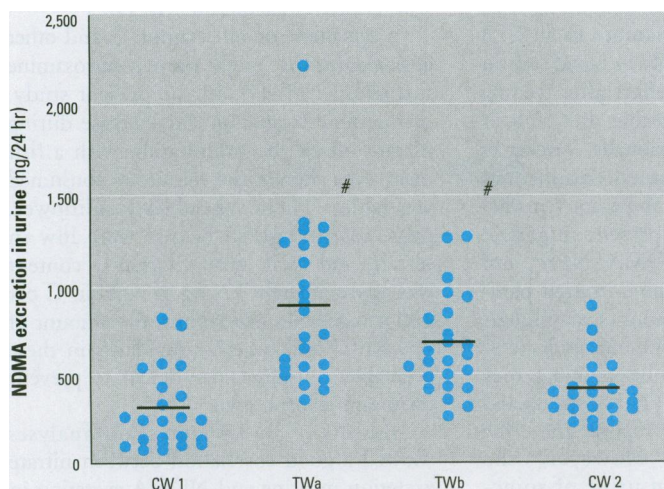

Figure 2. *N*-Nitrosodimethylamine (NDMA) excretion in urine (ng/24 hr) of 24 subjects during control weeks (CW 1 and CW 2) and test week days 1–3 and 4–7 (TWa and TWb).
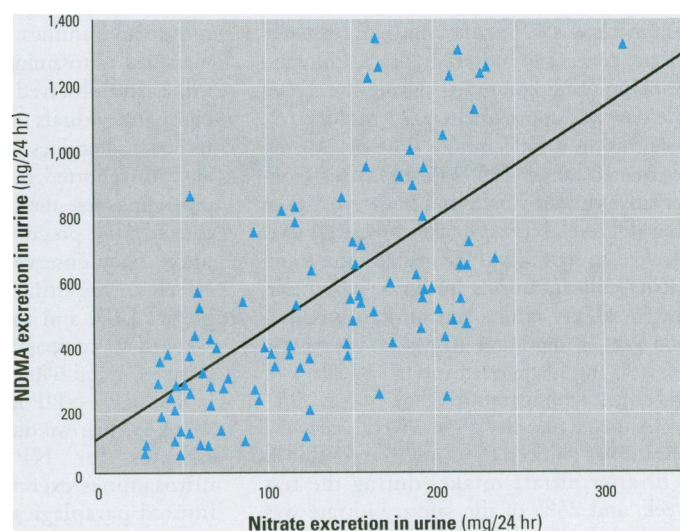#Significantly increased compared with CW 1 and CW 2; Wilcoxon, *p*<0.0002.

Figure 3. Correlation between nitrate excretion in urine and *N*-nitrosodimethylamine (NDMA) excretion in urine (*r* = 0.68; *p* = 0.0001).

control week 1; Wilcoxon, $p = 0.008$), 94 ± 57 ng/24 hr during test week days 4–7 (Wilcoxon, $p = 0.006$), and 104 ± 55 ng/24 hr in control week 2 (Wilcoxon, $p = 0.002$). NPIP levels in urine were approximately 4–10 times lower than NDMA levels.

Linear regression analyses of the data of all urine samples of test and control weeks showed a significant correlation between urinary nitrate excretion and urinary NDMA excretion ($r = 0.68$ and $p = 0.0001$; see Fig. 3). No relationship was observed between nitrate excretion in urine and NPIP excretion in urine; however, a relationship was observed between cumulative nitrate excretion in urine and cumulative NPIP excretion in urine ($r = 0.8$ and $p = 0.0001$). Furthermore, a correlation was found between the log of the nitrate concentration in saliva and nitrate excretion in urine ($r = 0.70$ and $p = 0.0001$). Finally, correlations were found between log salivary nitrate concentration and NDMA excretion in urine ($r = 0.48$; $p = 0.0001$) and between log nitrite concentration in saliva and NDMA excretion in urine ($r = 0.40$; $p = 0.0001$). No correlation was observed between nitrate reduction levels and NDMA excretion in urine.

Discussion

WHO has set guidelines for nitrate and nitrite intake to prevent the occurrence of methemoglobinemia. Nitrate exposure, however, implies other adverse health effects, including the endogenous formation of carcinogenic N-nitroso compounds. The present study was performed because no information is available about N-nitrosamine formation after nitrate intake at the ADI level. The volunteers in this study received a nitrate dose of 170 mg in drinking water in combination with a fish meal low in nitrate during 1 week. Mean nitrate content of the fish meals was 17 mg/day, and the estimated nitrate content of breakfast, lunch, fruit, and drinking water consumed during the rest of the day was approximately 23 mg/day (8), resulting in a total nitrate dose of 210 mg (estimated range: 185–235 mg). This (conservative) nitrate intake will be referred to as the ADI level. Nitrate intake at the ADI level resulted in significantly increased nitrate and nitrite concentrations in saliva. The mean rise in salivary nitrate and nitrite concentration was 73 mg/l and 10 mg/l, respectively, per 100 mg of ingested nitrate, which is in agreement with literature values (4,5,27,30).

Approximately 18% of the salivary nitrate was reduced to nitrite (mean value at 2 hr after nitrate intake) during the test week, and 25% of the salivary nitrate was reduced during the control weeks. Other studies have shown comparable results for nitrate reduction after a nitrate load (4,27).

On the other hand, no results are reported on nitrate reduction after a meal low in nitrate. Nitrate may be reduced more effectively when the amount of nitrate in saliva is low (31), leading to the relatively high level of reduction of nitrate observed during the control weeks.

As expected, nitrate intake at the ADI level resulted in a significant increase in mean 24-hr nitrate excretion in urine compared with the control weeks. In the test week, 88% and 75% of the nitrate dose was excreted in the urine. Previous reports have shown that upon nitrate ingestion, 65–70% of the nitrate dose is excreted in the urine within 24 hr, while about 5% is converted to nitrite, and the rest is secreted in sweat (± 10%), in colonic or vaginal secretions, and in tears (32,33).

Additionally, the urine samples were screened for nitrite content. Nitrite is not present in the urine of healthy individuals, but urinary tract infections (frequently present in young women) can lead to nitrite generation in the urinary tract. This implies the risk of nitrosamine formation in the urinary tract and confounding of the results. Nitrite was not detected in the urine samples of the female volunteers in this study.

Analyses of the urine samples for five volatile N-nitrosamines showed that two volatile nitrosamines were present in the urine: NDMA and NPIP. Two other studies have been performed with human volunteers consuming test meals containing nitrate and fish, in which urine samples were analyzed for nitrosamine content (28,34). Lakritz et al. (34) concluded that nitrosamine levels in urine, blood, and gastric juice were not significantly affected by ingestion of nitrosamine precursors. Maanen et al. (28) reported a significant increase in NDMA excretion in urine after consumption of food rich in nitrate and amines. Yamamoto et al. (35) measured nitrosamines only in blood, not in urine, and observed no effect after feeding eight individuals a Japanese diet rich in nitrate and amines. Additionally, Tricker et al. (36) reported that dimethylamine and piperidine were the most abundant (volatile) nitrosamine precursors present in gastric juice. They observed NDMA, NPIP, and NPYR in the urine of spine-injured paraplegics (37), and the amounts of NDMA excreted are comparable with our results (650 versus 871 and 640 ng/24 hr), although they found higher NPIP levels (250 versus 86 and 94 ng/24 hr); we did not analyze the urine samples for NPYR. However, the nitrosamines excreted in urine of spine-injured paraplegics were predominantly formed in the urinary tract as a result of bacterial infections, while in our study the urinary nitrosamines were probably formed in the stomach. Thus, urinary nitrosamine levels in healthy volunteers consuming nitrosamine precursors in combination with nitrate intake at the ADI level are comparable with levels found in a group at high risk for nitrosamine formation. In the control weeks, mean NDMA excretion in urine was 287 and 383 ng/24 hr, and these results are in agreement with the 270 ng/24 hr detected in the urine of an Egyptian control population (38).

Absolute levels of volatile nitrosamines formed in the stomach are probably much higher than the amounts excreted in urine. Spiegelhalder and co-workers (39,40) showed that between 0.5 and 2.4% of an ingested NDMA dose was excreted unmetabolized in urine if ethanol was administered simultaneously. Without concomitant ethanol intake, less than 0.5% of the ingested NDMA was excreted unmetabolized in the urine. Assuming that 0.5% of the NDMA is excreted in urine, the volunteers in this study may have formed 174 µg of NDMA per day or 2.9 µg per kg body weight per day during days 1–3 of the test week. This exposure can be compared with the 10 µg NDMA per kg per day that is carcinogenic in rats (41). The results clearly demonstrate that nitrate intake in combination with fish consumption resulted in an increase in NDMA excretion in urine of about 200%. NPIP excretion, on the other hand, was not directly related to nitrate intake and the composition of the diet consumed during the study. However, a significant correlation was found between the cumulative nitrate excretion in urine and the cumulative NPIP excretion in urine, suggesting that NPIP formation, in contrast with NDMA formation, slowly increased after repeated high nitrate exposure; although subsequent metabolism seems to be comparable with NDMA metabolism (42–44).

Vegetables contain vitamin C and other antioxidants that might prevent nitrosamine formation (45–47). In the present study, nitrosamine formation did increase during nitrate intake in combination with a fish meal, even though the volunteers consumed vegetables. These vegetables (cauliflower, peas, carrots, green beans) were low in nitrate, and their mean vitamin C content was approximately 17 mg of vitamin C per 100 g vegetables (48). Thus, the amount of vitamin C and other antioxidants in these vegetables appeared insufficient to prevent nitrosamine formation.

Results of linear regression analyses showed a good correlation between nitrate excretion in urine and NDMA excretion in urine. Additionally, correlations were found between the log of the salivary nitrate concentration and nitrate excretion in urine, between log salivary nitrate concentration

and NDMA excretion in urine, and between log nitrite concentration in saliva and NDMA excretion in urine. The correlations between urinary nitrate and salivary nitrate and nitrite versus urinary NDMA support a relationship between nitrate intake and endogenous NDMA formation.

In conclusion, nitrate intake at the ADI level in combination with a fish meal containing nitrosatable precursors increased NDMA excretion in urine and therefore implies the risk of increased formation of carcinogenic N-nitrosamines. The results of this study suggest that the risk of formation of carcinogenic N-nitrosamines should be taken into account in the currently used ADI value for nitrate and in the drinking water guideline for nitrate.

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