

# Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans

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## Abstract

**Background/Aims**—It has been suggested that dietary nitrate, after concentration in the saliva and reduction to nitrite by tongue surface bacteria, is chemically reduced to nitric oxide (NO) in the acidic conditions of the stomach. This study aimed to quantify this in humans.

**Methods**—Ten healthy fasting volunteers were studied twice, after oral administration of 2 mmol of potassium nitrate or potassium chloride. Plasma, salivary and gastric nitrate, salivary and gastric nitrite, and gastric headspace NO concentrations were measured over six hours.

**Results**—On the control day the parameters measured varied little from basal values. Gastric nitrate concentration was 105.3 (13)  $\mu\text{mol/l}$  (mean (SEM)), plasma nitrate concentration was 17.9 (2.4)  $\mu\text{mol/l}$ , salivary nitrate concentration 92.6 (31.6)  $\mu\text{mol/l}$ , and nitrite concentration 53.9 (22.8)  $\mu\text{mol/l}$ . Gastric nitrite concentrations were minimal (<1  $\mu\text{mol/l}$ ). Gastric headspace gas NO concentration was 16.4 (5.8) parts per million (ppm). After nitrate ingestion, gastric nitrate peaked at 20 minutes at 3430 (832)  $\mu\text{mol/l}$ , plasma nitrate at 134 (7.2)  $\mu\text{mol/l}$ , salivary nitrate at 1516.7 (280.5)  $\mu\text{mol/l}$ , and salivary nitrite at 761.5 (187.7)  $\mu\text{mol/l}$  after 20–40 minutes. Gastric nitrite concentrations tended to be low, variable, and any rise was non-sustained. Gastric NO concentrations rose considerably from 14.8 (3.1) ppm to 89.4 (28.6) ppm ( $p < 0.0001$ ) after 60 minutes. All parameters remained increased significantly for the duration of the study.

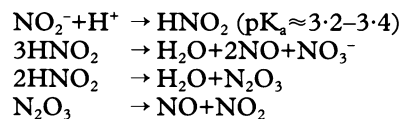
**Conclusions**—A very large and sustained increase in chemically derived gastric NO concentrations after an oral nitrate load was shown, which may be important both in host defence against swallowed pathogens and in gastric physiology.

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Keywords: nitric oxide, salivary nitrate and nitrite, stomach.

Nitric oxide (NO) is an important biological molecule that is responsible for cell signalling and host defence in a number of mammalian tissues.<sup>1,2</sup> It is now well established that mammalian cells produce NO from the amino acid L-arginine with a family of NO synthase enzymes.

We have recently proposed that NO may be synthesised by an alternative mechanism that relies on sequential reduction of nitrate.<sup>3</sup> Dietary nitrate (principally derived from green, leafy vegetables) is absorbed from the stomach and proximal small intestine into the plasma and subsequently concentrated in saliva.<sup>4,5</sup> Approximately 25% of dietary nitrate is recirculated. The dorsal surface of the tongue harbours large numbers of nitrate reducing facultative anaerobic bacteria, which rapidly reduce nitrate to nitrite under hypoxic conditions.<sup>6,7</sup> There is therefore a high concentration of nitrite in saliva, which increases with oral nitrate intake.<sup>4</sup> When swallowed, this nitrite is readily protonated under the acidic conditions of the stomach to form nitrous acid (acid dissociation constant  $\text{pK}_a \approx 3.2\text{--}3.4$ ), which in turn decomposes to various nitrogen oxides:



We have suggested that acidification of salivary nitrite is important in augmenting the antimicrobial effects of stomach acid and have shown that *Candida albicans* and *Escherichia coli* are much more susceptible to this combination than acid alone.<sup>3</sup> Although it is not clear how acidified nitrite acts to kill micro-organisms, it is possible that NO, or a product of NO, is responsible as formation of this molecule from L-arginine has been shown to be important in host defence.<sup>8</sup> The generation of NO in the human stomach has been demonstrated by measurement of NO in expelled gas after a carbonated drink in healthy subjects.<sup>9</sup> The purpose of this study was to more clearly quantify NO synthesis in the human stomach and determine the temporal relation of chemical NO production after nitrate ingestion in healthy volunteers.

## Methods

Ten healthy volunteers (six male; mean body weight 69 kg), who were not taking any medication, were invited to take part (21–43 years). Local Ethics Committee approval was given for this study. Volunteers were fully informed and written consent obtained. Subjects were studied after overnight fasting on two separate occasions at least one week apart. The experimental protocol on each day was identical, other than oral administration on the test day of a 50 ml solution containing 2 mmol of potassium nitrate BP (Thornton and Ross,

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Huddersfield, UK), or on the control day, potassium chloride (BDH chemicals, Merck Ltd, Poole, Dorset, UK). After three hours, a low nitrate (29  $\mu\text{mol}$  total), neutral pH drink (Complan) was given. The experimental order was randomly allocated.

After insertion of a fine bore nasogastric feeding tube (ET03 Medicina Ltd), blood, gastric headspace gas, gastric juice (5 ml), and unstimulated saliva<sup>10</sup> samples (1–3 ml) were taken at 20 minute intervals for two hours and 30 minute intervals thereafter. To facilitate the aspiration of gastric contents, five minutes before sampling 50 ml ambient air (<10 ppb) was introduced via the nasogastric tube. The pH of gastric juice samples was measured and then the juices alkalinised with 50  $\mu\text{l}$  5M NaOH to prevent further reduction of  $\text{NO}_2^-$ . Nitrate and nitrite assays were performed by a modified Griess reaction, nitrate being first reduced on a copper coated cadmium column as previously described.<sup>11</sup> Nitrite assays were performed in microwell plates and the diazo colour reaction detected at 540 nm. NO in aspirated headspace gas was analysed after dilution (10 ml gastric gas diluted to 100 ml total volume in NO impermeable container) with laboratory air to achieve final concentration less than 20 ppm using a chemiluminescence meter (Thermo Electron Instruments, model 42A, Warrington, Cheshire, UK), which is calibrated with standard NO/nitrogen mixtures (MG Gas Products Ltd, Reigate,

UK). The chemiluminescence analyser was connected to a Maclab and Macintosh data acquisition system.

#### Statistical analysis

The results on the control and test days were compared using two way analysis of variance with repeated measures and post-hoc analysis using matched pairs *t* tests; simple and Spearman rank correlation analysis. A value of  $p < 0.05$  was considered significant.

#### Results

On the control day basal concentrations of plasma nitrate (17.9 (2.4)  $\mu\text{mol/l}$ ), gastric nitrate (105.3 (23.1)  $\mu\text{mol/l}$ ), salivary nitrate (92.6 (31.6)  $\mu\text{mol/l}$ ), and salivary nitrite (53.9 (22.8)  $\mu\text{mol/l}$ ), gastric nitrite (<1  $\mu\text{mol/l}$ ) and headspace gas NO (16.4 (5.8) ppm) varied little over the six hour time course (Fig 1; Table).

In contrast, after oral administration of 2 mmol of potassium nitrate solution there were pronounced increases in plasma nitrate, gastric nitrate, salivary nitrate, salivary nitrite, and gastric headspace NO concentration. These remained considerably increased even at the end of the six hour study period (Fig 1; Table). Gastric nitrite concentrations tended to be low, variable, and any rise was not sustained although measurement was hampered by

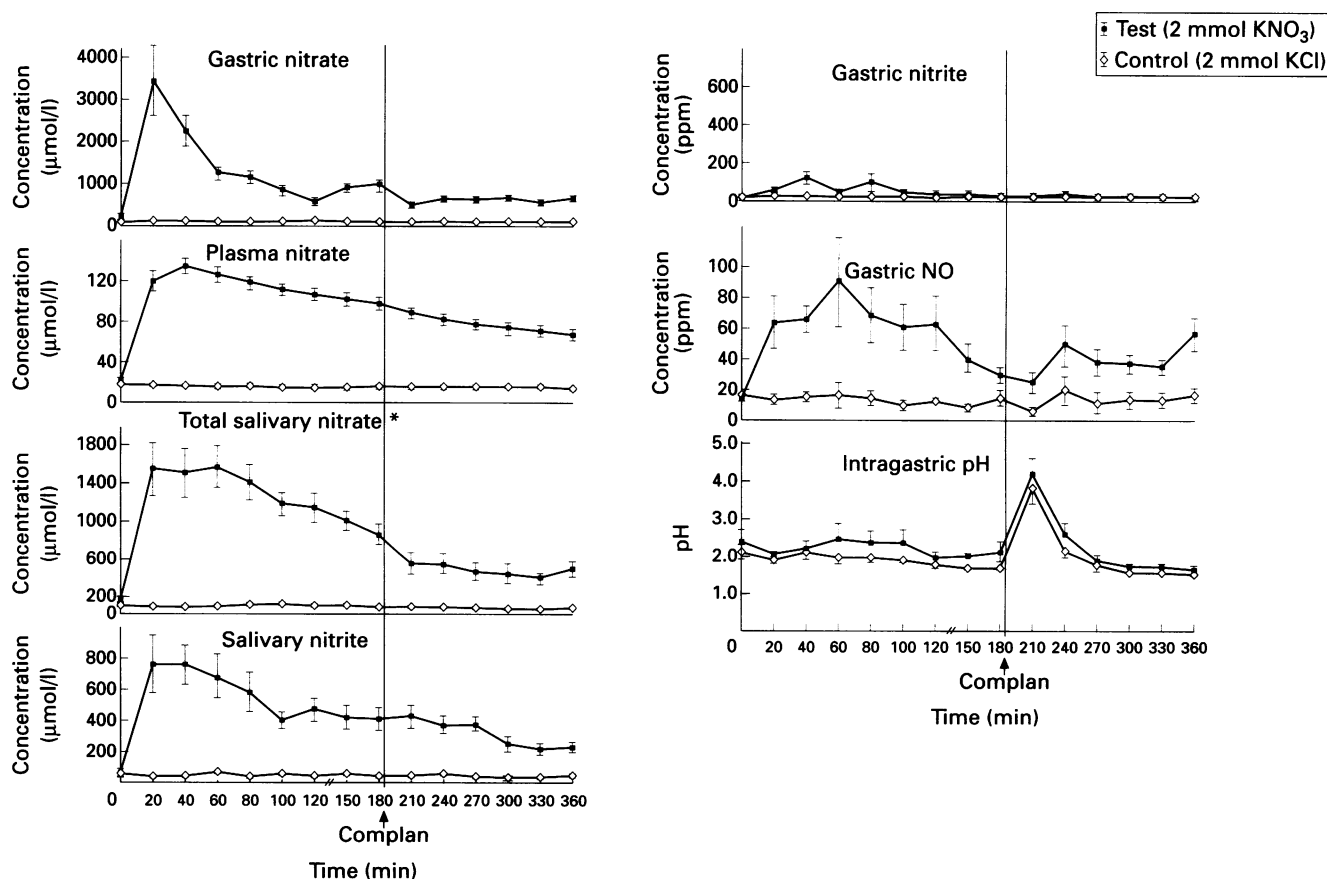


Figure 1: Effect of ingestion of nitrate on basal concentrations of plasma nitrate, gastric and salivary nitrates/nitrites, gastric NO (all  $p < 0.001$  ANOVA), and alteration of intraluminal pH with time and Complan ingestion, at 182 minutes represented by solid bar. \*Represents total salivary nitrate secreting by salivary glands before reduction on tongue (measured as nitrate plus nitrite). Data shown as mean (SEM).

## Effect of an inorganic nitrate load (2 mmol) on plasma salivary and gastric nitrate, nitrite and nitric oxide concentrations

Sample	Control (mean (SEM)) ( $\mu\text{mol/l}$ )	Test (mean (SEM)) ( $\mu\text{mol/l}$ )	Peak time (mins)
Gastric nitrate	133.4 (21.2)	3430.1 (832.1)	20
Plasma nitrate	17.9 (2.4)	134 (7.2)	40
Total salivary nitrate	92.6 (31.6)	1516.7 (280.5)	20–40
Salivary nitrite	53.9 (22.8)	761.5 (187.7)	20–40
Gastric nitrite	0.63 (0.63)	105.3 (32.3)	40
Gastric NO (ppm)	16.4 (5.8)	89.4 (28.6)	60

Gastric NO values are parts per million (ppm). Nitrate and nitrite values are  $\mu\text{mol/l}$  as denoted.

difficulty in achieving a clear supernatant that would not interfere with absorption at 540 nm. Due to the turbidity of some gastric juice samples even after repeated centrifugation, absorption readings were inappropriately high although there was no visibly detectable pink colour of the diazo reaction.

Peak gastric nitrate concentration occurred at the first measurement after intake (20 min), plasma values peaked at 40 minutes and gastric headspace NO concentration reached a peak at 60 minutes (Table). All remained significantly increased for the duration of the study ( $p < 10^{-8}$  ANOVA; apart from gastric nitrite). The highest measured gastric headspace gas NO value was 291 ppm 60 minutes after intake of the nitrate solution.

Gastric acidity was not significantly affected by nitrate intake but dropped dramatically after the ingestion of Complian recovering by the next sampling time and thereafter decreasing (Fig 1). Although gastric NO was falling by the time the neutral drink was ingested, presumably parallel to the decline in salivary nitrate and nitrite concentrations, there was nevertheless a significant reduction in NO production when gastric pH rose after the drink and a significant recovery when gastric pH returned to basal conditions. This negative relation between gastric juice pH and stomach headspace gas NO concentration is shown in Figure 2 ( $r_s = -0.55$ ;  $p < 0.01$ ). There was no significant relation between stomach NO and salivary nitrite concentrations (control day ( $r_s = 0.32$ ;  $p = 0.024$ ); test day ( $r_2 = 0.46$ ;  $p < 0.001$ )), nor were any of the measured variables different between sexes.

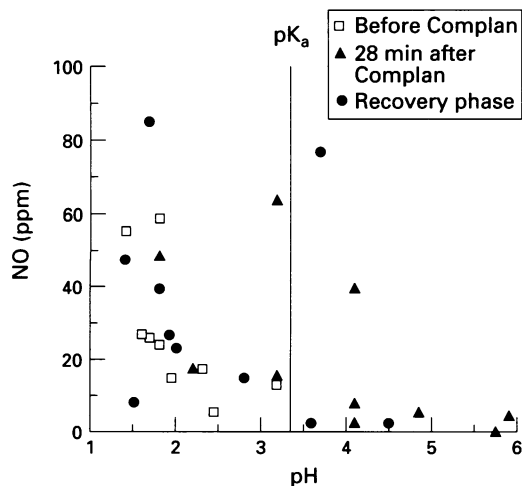


Figure 2: pH dependency of gastric NO production. Spearman rank correlation  $-0.55$ ,  $p < 0.01$ .

## Discussion

This study shows that very large concentrations of NO are generated in the stomach after an oral nitrate intake. The maximum concentration of NO was seen approximately 60 minutes after a dose of nitrate, which represents the average intake of an adult over one to two days in the UK.<sup>12</sup> The mean concentration of NO at this time averaged 90 ppm; this is about 7000 times that found in exhaled breath.<sup>9</sup> The concentrations of stomach headspace gas NO were considerably higher in our study than those previously reported (basal expelled gastric NO 800–6000 parts per billion).<sup>9</sup> This may be due to dilution of gastric gas in the previous study with carbon dioxide generated from the carbonated drink used to induce belching. The higher values measured in this study may be an underestimate of true NO concentrations in the stomach as NO at this concentration will readily combine with oxygen to form  $\text{NO}_2$ , albeit by second order kinetics, which is not measured by the chemiluminescence analyser we used. Furthermore, as we had to inject air into the stomach five minutes before sampling the concentration of NO in headspace gas may not have reached equilibrium with the gastric juice. In previous studies in vitro we found that headspace NO reaches equilibrium with the aqueous phase in a shaken closed vessel at about one hour.<sup>7</sup>

At six hours after nitrate intake the concentration remained considerably higher than baseline, suggesting a prolonged effect of dietary nitrate on stomach NO synthesis. The increased NO generation was associated with the expected rise in salivary nitrate and nitrite and the timecourse suggests that stomach NO synthesis derives from acidification of salivary nitrite, even though the correlation between stomach NO and salivary nitrite in individual subjects did not reach statistical significance. The concentration of stomach headspace NO was, however, associated with the degree of acidity in the stomach. During the course of the experiment, on both study days, stomach acidity increased initially, presumably due to the presence of a nasogastric tube. After ingestion of Complian there was a short lived reduction in acidity accompanied by reduction in nitric oxide generation even allowing for the expected fall in NO by this stage in the study. As gastric acidity recovered so NO generation followed and reached pre-meal levels and at the end of the study day NO levels were still significantly higher than on the control day. This is probably because of the persisting increase in plasma nitrate and thus assimilation into saliva even at the end of the study period.

Previous studies in vitro have shown that NO is generated when nitrite is acidified. The amount of NO generated in the stomach in vivo, however, greatly exceeds that expected from in vitro studies, suggesting another reducing agent such as ascorbate may be present in saliva or gastric juice to increase nitrite reduction to NO.<sup>7</sup>

We have previously suggested that salivary concentration of nitrate and subsequent rapid

reduction to nitrite by lingual bacteria is a symbiotic process designed to serve a useful function. The very high and sustained concentrations of stomach NO generated by chemical reduction of salivary nitrite are likely to destroy or inhibit swallowed microbial pathogens. Several studies suggest that a variety of organisms are sensitive to this substance. *Helicobacter pylori*, which inhabits the mucus layer covering the mucosa (only a few microns thick) will be exposed to these high concentrations of NO that rapidly diffused through both lipid and aqueous mediums; the sensitivity of this organism to NO is as yet unknown.

In addition NO is known to be involved in the regulation of gastric mucosal blood flow<sup>13</sup> and hence the preservation of mucosal integrity, gastric motility,<sup>14</sup> and mucus production. Previous studies have suggested a role for NO in stimulated gastrin and gastric acid secretion<sup>15 16</sup> although the high NO concentrations after nitrate intake did not reduce gastric pH in this study.

Although there has been continuing concern as regards the nitrosating potential of nitrate containing dietary constituents, epidemiological studies have failed to establish any causal association between gastrointestinal malignancy and dietary nitrate.<sup>17</sup> While we have not attempted in this initial study to quantify nitrosamine production, in the normal acidic stomach the large amounts of NO that are generated and the very low gastric nitrite concentrations resulting from a nitrate load would suggest that nitrosamine formation is not favoured.

In summary, this study shows that dietary nitrate, by bacterial, as previously described,<sup>7</sup> and chemical reduction, may generate very large concentrations of NO in the stomach, much greater than those generated by intrinsic NO synthase.<sup>18</sup> The mechanism we describe may be extremely important in augmenting the antiseptic properties of gastric acid and modifying physiological gastric function. Additionally, the formation of NO in the stomach from acidified nitrate may be one of the mechanisms by which the nitrosating

potential of nitrite and nitrate containing constituents of the diet is minimised. We believe that the mechanism of enterosalivary circulation of nitrate is designed to produce large concentrations of NO in the mouth and stomach and thus may have beneficial as well as possibly deleterious effects on human health.

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