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Lack of Influence of Dietary Nitrate/Nitrite on Plasma Nitrotyrosine Levels Measured Using a Competitive Inhibition of Binding ELISA Assay

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The action of peroxynitrite *in vivo* has been proposed to account for the involvement of nitrotyrosine in the pathogenesis of many diseases. However, it has been demonstrated that nitrite under acidic conditions, similar to those in the human stomach, also has the ability to nitrate tyrosine. Dietary nitrate is also implicated in the progression of gastritis and gastric cancer and elevated levels of nitrate are found in many disease states in which nitrotyrosine may play a role. Thus, we investigated whether the dietary nitrate intake might contribute towards the plasma protein-bound levels of nitrotyrosine.

Seven healthy, non-smokers participated in a two-day study consisting of a nitrate-low control day followed by a day during which three nitrate-rich meals were consumed. Maximal urinary excretion was attained 4–6 hours after consumption of a meal and the maximum was proportional to the dose. Plasma nitrate was elevated nine-fold, 1 hour after consumption of a meal containing 128.3 mg nitrate. Plasma nitrated protein levels did not appear to alter significantly from basal 1 hour after supplementation with a nitrate-rich meal. Thus dietary nitrate does not appear to contribute to the levels of plasma nitrated proteins, as determined using a competitive inhibition of binding ELISA assay, but this does not preclude any contribution it may make to the total body burden of nitrotyrosine.

Keywords: Nitrotyrosine, nitrate balance, urine, plasma, diet, nitrite

Abbreviations: Nitrotyrosine, 3-nitro-L-tyrosine; BSA, fatty acid free bovine serum albumin; Primary antibody, immuno affinity purified polyclonal anti-nitrotyrosine rabbit IgG antibody; Developing antibody, biotinylated donkey anti-rabbit IgG antibody; NaCl, sodium chloride; NaH_2PO_4, mono-sodium dihydrogen phosphate; BMI, body mass index; PBS, phosphate buffered saline; $NaNO_3$, sodium nitrate; $NaNO_2$, sodium nitrite

INTRODUCTION

3-Nitro-L-tyrosine (nitrotyrosine) is considered to be a marker of endogenous production of several reactive nitrogen species including peroxynitrite. Thus peroxynitrite has been implicated in the pathogenesis of several diseases

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including gastritis,[1] gastric cancer,[2] septic shock[3] and celiac disease[4] due to the detection of elevated levels of nitrotyrosine. However, it has also been demonstrated that tyrosine nitration by nitrite may occur in the normal acidic conditions (pH 1–2) of the stomach.[5,6] Thus the possible involvement of dietary nitrate cannot be discounted, especially as it has been postulated to be involved in the aetiology of cancer.[7–9] The involvement of nitrite in gastric cancer has been attributed to the ability of nitrite to react with amines and amides, in the acidic environment of the stomach, forming potentially carcinogenic *N*-nitroso compounds[10,11] or through the deamination of cellular DNA by nitrite.[12]

The major source of nitrate exposure in humans is from green leafy vegetables which contribute 75–80% of the total daily intake.[13] It has been estimated that 5% of ingested nitrate is reduced to nitrite by oral microflora.[14,15] Nitrate (and nitrite) can be absorbed from the stomach into the blood stream where it rapidly enters the erythrocytes.[16] Salivary glands concentrate and actively secrete nitrate from the circulation into the oral cavity, 25% of dietary nitrate undergoing enterosalivary re-circulation in this way.[14,15] The majority of nitrate (60–70%) is excreted unchanged in the urine within 24 hours of ingestion, reaching a maximum concentration 4–6 hours after the nitrate challenge.[14,17] Maximal plasma nitrate levels appear to be attained 40–60 minutes following challenge with nitrate.[16–18]

The purpose of this study was to investigate the effects of dietary nitrate on the levels of nitrate, nitrite and protein nitration in human body fluids.

EXPERIMENTAL SECTION

Bovine serum albumin (BSA) (fatty acid-free) was obtained from Boehringer Mannhein (Leicestershire, UK). Immuno-affinity purified polyclonal anti-nitrotyrosine rabbit IgG antibody raised to nitrated keyhole limpet haemocyanin (primary antibody) was purchased from TCS Biologicals (Buckinghamshire, UK). Biotinylated donkey anti-rabbit IgG antibody (developing antibody) was from Amersham Life Sciences (Buckinghamshire, UK). Both avidin and biotinylated horseradish peroxidase were from DAKO (Buckinghamshire, UK). Sodium chloride (NaCl) and mono-sodium dihydrogen phosphate (NaH_2PO_4) were obtained from BDH (Dorset, UK). Ultrapure water (18.2 MΩ) was used throughout. All other reagents were purchased from Sigma (Dorset, UK).

Subject Details

Seven (3 male, 4 female) healthy, non-smokers, who were not taking vitamin supplements at that time and were willing to comply with all aspects of this two-day study, were recruited. Ethical permission was granted by Guy's Research Ethics Committee. The volunteers, who gave informed consent, were aged between 23 and 48 years old with body mass indices (BMI) ranging from 17.8 to 26.3 kg/m^2. They were required to fast overnight prior to the first day of the study and void their first urine sample of the day.

Supplementation Protocol

This two-day study comprised a control day during which volunteers were restricted to a nitrite-low diet and a test day consisting of nitrate-rich meals. Bottled mineral water, containing minimal levels of both nitrate (0.3 mg) and nitrite (0.39 mg), (for preparation of drinks comprising water, tea and coffee) was supplied along with three meals on each day (nitrate-low the first day, nitrate-rich the second day) and an evening snack (Table I). Volunteers had a drink (approximately 250 ml) made from the bottled water every hour to a total of four litres per day. Samples of all meals and drink were taken for analysis. From 09:00 on the first day urine

TABLE I Composition and nitrate and nitrite content of the nitrate-low, control meals and the nitrate-rich, test meals supplied for the supplementation study. Samples were prepared and ion-exchange HPLC analysis carried out as described in the experimental section. Results are expressed as the mean NO_x^- of two separate experiments

Day	Meal	Meal content	Food g	NO_2^- mg	NO_3^- mg
Control	Breakfast	Toast (Granary bread)	70	0.00	3.4
	Lunch	Chicken and tomato sandwich, crisps, Cox apple, tangerine, nectarine	530	0.00	17.3
	Tea	Pasta with a tomato based sauce	410	0.00	16.5
	Supper	Chocolate chip cookies	22	0.00	1.2
	Drinks	Buxton mineral water, tea, coffee	4 litres	0.39	0.3
	Total		1032 + 41	0.39	38.7
Test	Breakfast	Bacon sandwich – grilled unsmoked bacon, white submarine roll, tomato, tomato ketchup	187	0.11	7.0
	Lunch	Ham salad – Wiltshire cured ham, cherry tomato, lettuce, cabbage, beetroot, potato salad, coleslaw, carrot, granary bread	515	0.11	128.3
	Tea	Bacon stir-fry – beansprouts, smoked bacon, white cabbage, haricot verts, carrot, broccoli, cauliflower	200	0.22	29.0
	Supper	Granary bread	140	0.00	6.8
	Drinks	Buxton mineral water, tea, coffee	4 litres	0.39	0.3
	Total		1042 + 41	0.83	171.3

samples were collected every 2 hours, for a total of 48 hours (time and volume were recorded). On the second day a qualified nurse took blood samples into heparinised tubes (20 ml) at 09:00 (fasted, basal) and 14:00 (1 hour post-prandial) and the plasma was obtained by centrifugation at 1250 × g, 5 °C for 20 minutes. All samples were stored at −70 °C. The order of events is depicted in Figure 1.

Plasma Protein Estimation

Plasma proteins were estimated by the Bradford assay.[19] The Bradford reagent was prepared by

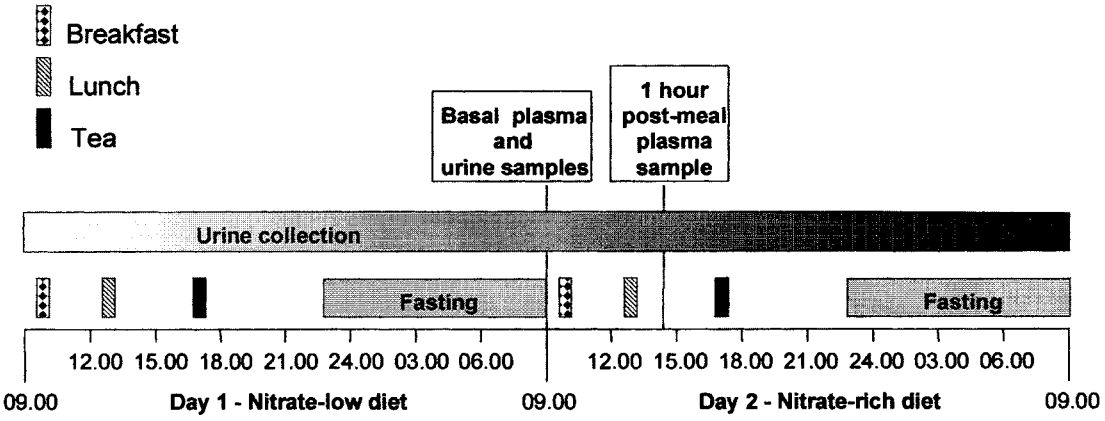

FIGURE 1 Timeline depicting the main events of this two-day human supplementation study comprising a low nitrate diet day and a nitrate-rich diet day. A total of four litres of water was drunk each day (250 ml every hour for 16 hours).

diluting the dye reagent concentrate five-fold with distilled water. Bradford reagent (200 µl) was added to 50 µl plasma (diluted by a factor of 1600) and the absorbance at 595 nm measured after 5 minutes by a Dynex MRX plate reader running Revelation analytical software (Dynex Technologies UK Ltd., Billingshurst, West Sussex, UK). Levels were quantified using standard curves generated from reciprocal dilutions of 400 µg/ml BSA.

Plasma Nitroprotein Determination

Nitrated protein levels were estimated by a competitive inhibition of binding ELISA assay.[20] The assay was performed in 96-well, flat-bottomed Nunc Maxisorp plates coated with 100 µl nitro-BSA (5 µg/ml peroxynitrite treated fatty acid free BSA in carbonate buffer (pH 9.2), 4.07:1.00 molar ratio of nitrotyrosine:BSA) which had been blocked with 300 µl ovalbumin (0.5% in 10 mM PBS) to prevent non-specific binding. Primary antibody was used at a dilution of 1:30000 of 100 µg immunoaffinity purified polyclonal anti-nitrotyrosine rabbit IgG in diluent (10 mM PBS, 0.05% (w/v) $Tween_{20}$, 0.5% ovalabumin) and 100 µl incubated with standard or sample (100 µl) for 2 hours at 37 °C on a plate shaker (Denley Wellwarm 1, Denley Instruments Limited, Billingshurst, Sussex), followed by four washes with PBS/Tween by a Dynex AM60 plate washer (Dynex Technologies UK Limited, Billingshurst, West Sussex, UK). The developing antibody (100 µl of biotinylated donkey anti-rabbit IgG used at a dilution of 1:1000) was then added and the solution incubated at 37 °C for 1 hour on a plate shaker, followed by four washes with PBS/Tween. To each well, 100 µl avidin-biotinylated horseradish peroxidase complex (2 µl avidin and 2 µl biotinylated horseradish peroxidase in 10 ml diluent) was added to all wells followed by incubation for a further hour at 37 °C on a plate shaker. Finally, the enzyme substrate (20 mg OPD tablet in 50 ml phosphate-citrate buffer (0.05 M, pH 5.0) containing 0.03% sodium perborate) was added and the colour allowed to develop for approximately 10 minutes at room temperature prior to termination by the addition of 4 M sulphuric acid (50 µl). The absorbance was read at 490 nm using a plate reader. The concentrations (expressed as nitro-BSA equivalents standardised to plasma protein levels) of nitrated proteins that inhibited anti-nitrotyrosine antibody binding were estimated from serial dilutions of 100 µg/ml nitro-BSA in diluent.

Sample Preparation for Nitrate and Nitrite Analysis

Plasma and drink samples were filtered through sterile, 0.22 µm Millex®-GP syringe filters prior to HPLC analysis. Urine samples (1 ml) were prepared by passing through a pre-prepared (1 ml methanol followed by 1 ml water) classic Sep-Pak C_{18} cartridge, followed by a wash with 1 ml water. Both fractions were collected and mixed thoroughly. To extract the nitrate and nitrite from meal samples for HPLC analysis, the method of Sen and Donaldson[21] was followed, with modifications. Freeze-dried samples (0.5 g) were suspended in 35 ml water. The suspension was heated in a water bath at 80 °C for 20 minutes with occasional stirring, cooled to room temperature and filtered through pre-wetted Whatman No. 1 filter paper. The supernatant was further filtered through a sterile, 0.22 µm Millex®-GP syringe filter. This standard procedure did not promote the oxidation of nitrite to nitrate as shown by the application of nitrite standards through the system.

HPLC Analysis of Nitrate and Nitrite

The isocratic anion exchange HPLC method of Radisavljevic *et al.*[22] for the measurement of nitrate and nitrite was used with modifications. The Waters HPLC system (Hertfordshire, UK) consisted of an autosampler with a peltier temperature controller (model 717), a model 626 pump with a model 600S controller, a photodiode array

detector (model 996) and a model 464 electrochemical detector. The equipment was controlled and the data processed by the Millenium software system. The column was a Hamilton PRP-X100 (4.1 × 150 mm) with a 10 μm particle size (Phenomenex, Macclesfield, Cheshire, UK) preceded by a PRP-X100 guard column with the temperature maintained at 30 °C. Injections (50 μl) were made by an autosampler with a 100 μl fixed loop. The mobile phase (pH 4) consisted of 20 mM NaCl with 50 mM NaH_2PO_4 that was pumped through the system at a flow rate of 2 ml/min^{-1}. Detection of nitrate and nitrite was by diode array at 220 nm and electrochemical detection (+0.8 V), respectively. Levels were quantified using standard curves generated from standard solutions of $NaNO_3$ and $NaNO_2$.

Statistical Analysis

Levels of nitrate and nitrite are expressed as mean ± SD whilst the nitroprotein data are expressed as mean ± S.E.M. Data was analysed by the two-tailed, paired student's *t*-test.

RESULTS

The composition and the nitrate and nitrite contents of the nitrate-rich and nitrate-low diets are given in Table I. The 24-hour intake of nitrate (38.7 mg) and nitrite (0.39 mg) on the control day increased to 171.3 mg and 0.83 mg, respectively on the test day. The drinks (tea, coffee and water) prepared from bottled mineral water contributed the entire amount of nitrite on the control day and 50% on the test day. The majority (75%) of the nitrate came from the mid-day meal on the test day but on the control day half was from the mid-day and half from the evening meal.

Plasma Nitrate and Nitrite

Post-supplementation, plasma samples were drawn 1 hour after consumption of a meal containing 128.3 mg nitrate and 0.11 mg nitrite. This time-point was chosen on the basis of the previous finding that the maximum elevation of plasma nitrate and nitrite occurs 40–60 minutes following challenge with nitrate.[16–18] Plasma nitrate levels rose in the seven supplemented volunteers by 39.5 ± 13 μM, from a fasted basal level of 4.6 ± 2.7 μM to 44.1 ± 13 μM. The levels of nitrite detected in the plasma (both basal and 1 hour post-prandial) were below the limit of detection of 0.1 μM for this method of analysis.

Plasma Nitroprotein

Overall, plasma protein-bound nitroprotein levels varied widely between the seven volunteers (basal 0.49–1.93 ng nitrated BSA equiv./mg protein, test 0.50–2.21 ng nitrated BSA equiv./mg protein). Basal fasted plasma nitroprotein (1.19 ± 0.16 ng nitrated BSA equiv./mg protein) did not alter significantly ($p \leq .1$) 1 hour after challenge with the meal (1.04 ± 0.21 ng nitrated BSA equiv./mg protein). The nitrate-rich meal did not alter the plasma protein-bound nitrotyrosine levels in a consistent manner in all volunteers, increasing in some and decreasing in others (Figure 2).

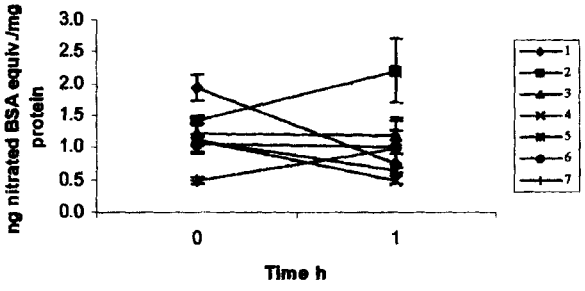

FIGURE 2 The changes observed in the plasma levels of nitrated protein (nitrated ng BSA equiv./mg protein) from fasted basal to one hour post-consumption of a meal containing 128.3 mg nitrate and 0.11 mg nitrite. Nitroprotein levels did not alter significantly ($p \leq .1$). Samples were prepared and analysis carried out by ELISA as described in the experimental section. Results are the mean ± S.E.M. of triplicate measurements from each of seven separate volunteers.

Urinary Excretion of Nitrate and Nitrite

On the test day, the urinary excretion profiles of both nitrate and nitrite obtained from each volunteer varied widely (Figure 3) ranging from two clearly defined peaks corresponding to excretion from each meal (representative excretion profile, Figure 3A) to less easily discernible peaks for nitrate (representative excretion profile, Figure 3B). Maximum excretion of nitrite occurred 4–6 hours after the evening meal in several volunteers, but only two volunteers appeared to have excreted measurable levels of nitrite corresponding to the other meals. Maximum urinary excretion of nitrate also appeared to occur 4–6 hours after consumption of a meal and the magnitude of the maximum correlated to the amount ingested. Large inter-individual variations were exhibited in the total urinary excretion of nitrite and nitrate (Table II), on both the control day ($0.5 \pm 1\,\mu g$/day and 50.4 ± 11 mg/day, respectively) and the test day ($15.2 \pm 33\,\mu g$/day and 133.1 ± 40 mg/day, respectively). A significantly ($p = 84 \times 10^{-5}$) greater amount of nitrate (82.7 ± 36 mg) and $14.7 \pm 35\,\mu g$ more nitrite were excreted on the test day than on the control day. A very low proportion of the ingested nitrite was recovered in the urine on either the control day ($0.1 \pm 0\%$) or the test day ($1.9 \pm 4\%$). A greater amount of nitrate ($130.1 \pm 29\%$) was eliminated in the urine over the 24-hour control period than was ingested (38.7 mg). However, in the majority of the volunteers there appeared to be a lower recovery in the urine ($74.4 \pm 7\%$) of ingested nitrate on the test day.

TABLE II The cumulative urinary excretion of nitrite and nitrate after supplementation with either a nitrate-low diet (0.39 mg nitrite and 38.7 mg nitrate) or a nitrate-rich diet (0.83 mg nitrite and 171.3 mg nitrate) for a day. Samples were prepared and analysis carried out by ion-exchange HPLC as described in the experimental section. Results are the mean of two separate experiments

Subject No.	$[NO_2^-]$ μg		$[NO_3^-]$ mg	
	Control	Test	Control	Test
1	0.0	90.8	36.0	108.8
2	0.0	2.5	41.2	132.2
3	0.0	0.0	52.6	129.9
4	3.7	1.6	68.0	213.1
5	0.0	9.4	42.5	124.6
6	0.0	0.0	57.7	81.5
7	0.0	2.3	54.5	141.6
Mean ± SD	0.5 ± 1.4	15.2 ± 33.5	50.4 ± 11.1	133.4 ± 40.4

DISCUSSION

The intake of nitrate (171.3 mg/day) and nitrite (0.83 mg/day) on the test day of this study were below the acceptable daily intake (ADI) of 3.65 mg/kg body weight nitrate ion (~219 mg/day) and 0.06 mg/kg body weight nitrite ion (~3.6 mg/day) recommended by the Commission of the European Communities Scientific Communities Scientific Committee for Food.[23,24] Intake of nitrite in this study was low (0.39–0.82 mg)

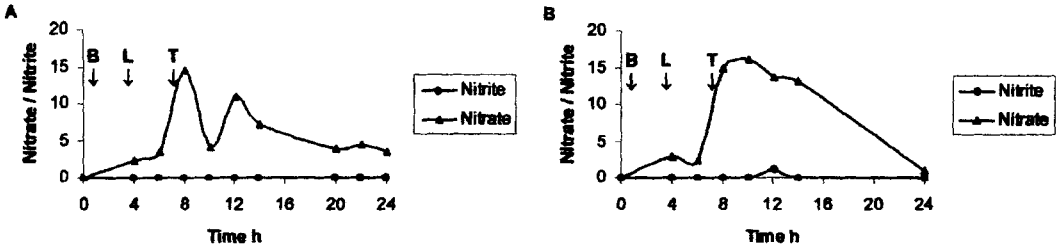

FIGURE 3 Two representative urinary excretion profiles of nitrite (μg/h) and nitrate (mg/h) after supplementation with three nitrate-rich meals (B = breakfast, L = lunch, T = tea). Breakfast, lunch and tea contained 0.11, 0.11 and 0.22 mg nitrite and 7.0, 128.3 and 29.0 mg nitrate, respectively. Samples were prepared and analysis carried out by ion-exchange HPLC as described in the experimental section. Results are the mean NO_x^- of two separate experiments.

but reflected the daily intake of nitrite (0.3–0.9 mg) in the UK.[25] A Total Diet study in 1997 found the average daily intake of nitrate by the UK population to be 88 mg, with an upper range of 136 mg.[26] It has been calculated that vegetarians are exposed to the greatest amount of nitrate (34.3–163.0 mg/day) in the UK.[27] Compared to these estimates, the amount of nitrate consumed in this study was low on the control day and around the highest vegetarian intake on the test day.

The fasted plasma level of nitrate (4.6 ± 2.7 μM), following a 24-hour low nitrate diet, was lower than the published normal range of 15–60 μM.[16–18,28–31] Presumably this reflects the nitrate-low diet on the day prior to sample collection as normal levels were attained (44.1 ± 13.3 μM) after dietary supplementation with 128.3 mg nitrate and 0.11 mg nitrite. The amount of nitrite in the plasma was predominantly less than 0.1 μM (the limit of detection), even after dietary supplementation, but this appears to be within the normal range of 0.02–2.84 μM.[16,29,30,32,33] Previously it has been found that maximum elevation of plasma nitrate and nitrite occurs 40–60 minutes following challenge with nitrate.[16–18] An increase in plasma levels of nitrate was revealed 1 hour after supplementation with an elevation of 39.5 ± 13 μM observed upon challenge with 128.3 mg nitrate. This ninefold elevation is in accordance with other studies where volunteers have ingested 124 mg,[18] 217 mg,[17] 307 mg[34] or 1779 mg[16] nitrate ion, as either a potassium or sodium salt, where maximum elevation of plasma nitrate levels of 7.5-, 6-, 11- and 49-fold were attained 40–45 minutes after the nitrate challenge; respectively.

There was no significant change in the plasma nitroprotein levels 1 hour after challenge despite the elevated levels of nitrate. The range observed in this study, both prior to and after the nitrate challenge (0.49–2.21 ng nitrated BSA equiv./mg protein) is lower than that previously detected (2.4 ± 0.3 ng nitrated BSA equiv./mg protein) using this method [Dr. N.J. Bradley, personal communication]. The wide range is probably partly accounted for by inter-individual variation. The levels of nitroprotein detected by this ELISA method, which is semi-quantitative, are not directly comparable to those of nitrotyrosine that were recently obtained by GC-MS.[35] There appear to be large inter-individual differences in the metabolism of dietary nitrate that is exemplified both by the very different urinary excretion profiles and the large variation in cumulative excretion observed. Despite this, as previously reported,[14] maximum urinary excretion which is proportional to the dose, occurs 4–6 hours after the challenge without exception and returns to basal within 24 hours.

After an overnight fast and consumption of a nitrate-low diet (0.39 mg NO_2^-, 38.7 mg NO_3^-), the urinary excretion of nitrate (50.4 ± 11.1 mg/day) was higher than that observed after a 14 hour fast (≤ 20 mg/day), a low-nitrate, solid food diet for 1–2 days (10–40 mg/day) or the Cambridge nitrate-free liquid diet for 3 days (20 mg/day).[14] The discrepancy may be explained by the greater amount of nitrate and nitrite in the diet of our volunteers. Previous studies have either not quantified nitrite or their method was not sensitive enough to detect the low levels of nitrite present in urine.[16,28,32] In this study, the urinary nitrite levels were low and generally below the limit of detection (< 0.1 μM). In support of this is the finding that variation in plasma nitrite levels may be attributed to a genetic component as there are different alleles for eNOS that appear to alter the amount of $NO^\bullet$ produced by this isoform of nitric oxide synthase.[36]

The low recoveries of nitrite (0–11%) are consistent with its oxidation to, and thus recovery as, nitrate.[32] The amount of nitrate excreted (36–68 mg/day) after dietary consumption of nitrate within the UK daily intake (control) was similar to the average UK excretion levels of 27–96 mg/day detected in a large collaborative study.[8,37] On the test day, during which the nitrate intake was higher than the UK average,

a greater amount of nitrate was excreted (81.5–333.0 mg/day). Recovery of ingested nitrate in the urine (48–176%) is similar that previously reported.[9,14,17] It has been suggested that endogenous production is the source of any excess nitrate recovered, which has been calculated to be 37–136 mg/day.[17,32,38] However, endogenous synthesis is hard to assess when the nitrate load is high, resulting in loss of dietary nitrate by its interactions,[14,17] which can be greater than endogenous formation.[38] This is consistent with the data presented here, in which excess nitrate is recovered almost exclusively when the nitrate intake was $\leq$107 mg and amounts to an endogenous production of 2.5–41.8 mg. Whilst this is in agreement with published reports it has to be borne in mind that some of the excess nitrate could have arisen from oxidation of dietary nitrite to nitrate and underestimation of the amount ingested. Also, the calculation has not taken into account the loss of dietary nitrate due to degradation[14] or storage within the body[16,17] as evidenced by studies showing the recovery of only 54% of radiolabelled dietary in the urine within 24 hours.[17]

Differences in the resident oral and gastrointestinal bacteria, gastric pH, endogenous production and speed of consumption can all contribute to the inter-individual variations in the plasma levels of nitrate, nitrite and nitroprotein along with the urinary excretion profiles and recoveries observed. In particular, the large urinary nitrite peaks observed after breakfast in one volunteer may have arisen from the stimulation of nitrate-reducing bacteria in the buccal cavity. From the unaltered concentrations of nitroprotein detected, it appears that any nitroamino acids (e.g. nitrotyrosine) that may be formed are not incorporated into plasma proteins. Many factors could account for this observation such as nitrotyrosine may not have been formed, this may be an inappropriate timepoint for sample collection for maximal nitroprotein or the small amount formed is not detectable above the relatively large basal levels. Other possibilities are that free, rather than protein-bound nitro-tyrosine may form or that nitrotyrosine may form in the stomach but not enter the circulation. It has been shown that nitrotyrosine can be taken up by cells.[39] In rats an oral dose is absorbed and detected as metabolites in the urine.[40] However, in support of free rather than plama protein-bound nitrotyrosine formation, it appears that α-tubulin is the only protein in which incorporation of free nitrotyrosine within cells has been shown.[39]

In summary, a dose-dependent elevation of urinary nitrate was observed on consumption of nitrate-rich meals with maximal excretion 4–6 hours after ingestion. Plasma nitroprotein was unaltered 1 hour after ingestion of a nitrate-rich meal whereas plasma nitrate increased after the nitrate challenge. If tyrosine is nitrated in the stomach, it is more likely to be the free amino acid, formed as such or after digestion of nitrated proteins, i.e. it might be predicted that nitrite is more likely to raise free nitrotyrosine.

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