It is rocket science - why dietary nitrate is hard to 'beet'! Part I: twists and turns in the realization of the nitrate-nitrite-NO pathway

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It is Rocket Science – Why Dietary Nitrate is Hard to Beet!
Part I: Twists and Turns in the Realisation of the Nitrate-Nitrite-NO Pathway

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Abstract

Dietary nitrate (found in green leafy vegetables such as rocket and in beetroot) is now recognised to be an important source of nitric oxide, via the nitrate-nitrite-NO pathway. Dietary nitrate confers several cardiovascular beneficial effects on blood pressure, platelets, endothelial function, mitochondrial efficiency and exercise. Whilst this pathway may now seem obvious, its realisation followed a rather tortuous course over two decades. Early steps included the discovery that nitrite was a source of NO in the ischaemic heart, but this appeared to have deleterious effects. In addition, nitrate-derived nitrite provided a gastric source of NO. However, residual nitrite was not thought to be absorbed systemically. Nitrite was also considered to be physiologically inert, but potentially carcinogenic, through *N*-nitrosamine formation. In Part I we describe key twists and turns in the elucidation of the pathway and the underlying mechanisms. This provides the critical foundation for the more recent developments in the nitrate-nitrite-NO pathway which are covered in Part II.

Introduction

Whilst the nitrate-nitrite-NO pathway has now largely been accepted as an alternative source of nitric oxide with an expanding range of beneficial effects, the characterisation of the kinetic processes leading to the realisation of the pathway was not straightforward. In Part I we describe key twists and turns in the establishment of the pathway over the last 2 decades.

Background to nitrate/nitrite's established properties?

Inorganic nitrate (NO_3^-) has a long history as an explosive agent, being a major component of gun powder, as described in a Chinese manual of war from 1044 AD [1]. It was also used in Chinese medicine for angina as described in an 8th century Chinese manuscript from the Dunhuang collection [1]. Inorganic nitrate was largely superseded when the organic (i.e., carbon-containing) nitrate compound, nitroglycerin, synthesised by Sobrero in 1812 [2], was stabilised and developed as dynamite by Alfred Nobel. Nitroglycerin was subsequently used medicinally (and renamed glyceryl trinitrate (GTN) to distinguish it from the explosive) to treat angina (including by Nobel). Although prior to the use of GTN, isoamyl nitrite was used, it was subsequently abandoned in favour of organic nitrates due to its shorter half-life, and along with isopropyl and isobutyl nitrite became drugs of abuse (poppers). As nitrovasodilators, organic nitrates and nitrites were considered to relax vascular smooth muscle via activation of soluble guanylate cyclase (sGC) resulting in elevation of cGMP either directly or through metabolism to NO, thus increasing coronary blood flow for example [3]. Although potassium nitrate was used as a weak diuretic in the early 20th Century [4] inorganic nitrite (NO_2^-) and nitrate have had minimal role therapeutically, and more recently as 'stable end products of NO metabolism' it was not thought that they could be metabolised back to NO in biological systems. The only current medicinal indication for sodium nitrite is as a treatment for cyanide poisoning.

Whilst they have had minimal therapeutic use, the inorganic nitrate and nitrite anions are common constituents of our diet. Relatively small amounts of nitrite occur naturally in food; however, nitrite (E249, E250) is commonly added in the curing process to preserve meats. By contrast, nitrate is found in large quantities in green leafy vegetables e.g. spinach and lettuce, as well as beetroot (red beet) and is also an EU-approved food additive (E251, E252). Despite these abundant sources, typically-ingested quantities of nitrate do not account for the majority of nitrate exposure. For example, Green *et al.* (1981) found that urinary nitrate excretion over 24 hours exceeded the amount of nitrate ingested (whether small or large quantities) by four-fold [5]. This suggested substantial endogenous nitrate production. Following the discovery of endothelium-derived relaxing factor (EDRF) as nitric oxide (NO), the pathway was elucidated by the same group, whereby Leaf *et al.* (1989) showed that consumption of [^{15}N] L-arginine led to $^{15}NO_3^-$ excretion in the urine, confirming L-arginine as the ultimate precursor for nitrate [6]. Thus L-arginine is the substrate for the NO synthases (NOS), resulting in NO production. Nitric oxide has multiple biological functions, including being one of the most potent vasodilators in man [7]. In addition to nitrate, spinach also contains high concentrations of L-arginine. However, L-arginine has rather poor oral bioavailability compared to L-citrulline, found in foods such as watermelon [8] and which is metabolised to L-arginine [9].

As a reactive free radical, NO possesses a short half-life of milliseconds in the circulation, being oxidised to nitrite and nitrate by oxygen and oxy-haemoglobin (oxyHb) [10]. In contrast to NO, nitrite and nitrate were thought to lack any useful physiological effect, being merely considered as “inert oxidative end products of endogenous NO metabolism” [10, 11], and as such were useful for measuring the activity of vasoactive NO [10]. Therefore, the metabolic sequence of L-arginine–NO–NO_2^-–NO_3^- became firmly established (though never described as a distinct “L-arginine-nitric oxide-nitrite-nitrate pathway”) with the focus on NO’s activity.

Besides being considered to lack any useful physiological effect, nitrite had been implicated as a potential carcinogen through the formation of *N*-nitrosamines [12], with concerns that it may lead to the development of oesophago-gastric cancers [13]. Coupled with reports of infantile methaemoglobinaemia, this led to the imposition of the maximum concentration of nitrate in tap water by authorities [14].

However, such concerns over nitrate are inconsistent with 'healthy' diets such as vegetarian diets and the Mediterranean diet, since these diets typically consist of fresh fruit and vegetables, particularly green leafy varieties, which are high in dietary nitrate, along with high amounts of unsaturated fats and low amounts of red meat. Vegetarian diets have been shown to reduce blood pressure when compared to omnivorous diets [15, 16]. The DASH (Dietary Approaches to Stop Hypertension) study showed that a 'combination-diet' high in fruit, vegetables and low fat dairy produce, and low in saturated fat, reduced blood pressure compared to a control diet (low in fruit, vegetables and dairy products, but high in saturated fat) [17]. Whilst the mechanisms for this may be multifactorial, the daily nitrate content has been estimated to be ~1200 mg (i.e., ~19 mmol) in the DASH diet [18]. Epidemiological evidence from the studies by Joshipura *et al.* suggested the importance of green leafy vegetables (high in dietary nitrate) in the protection against coronary heart disease and ischaemic stroke [19, 20].

Recent prospective interventional trial evidence supporting the Mediterranean diet is provided by a large multicentre parallel randomised controlled trial performed by Estruch et al., (2013). This was conducted in participants with high cardiovascular risk (without established cardiovascular disease) who were allocated to one of three groups: two groups that followed a Mediterranean diet with an additional supplement (either a litre of olive oil per week or a selection of mixed nuts), and one control diet (advice to reduce dietary fat) [21]. Over a median follow up of 4.8 years, the study demonstrated a ~30% reduction in major cardiovascular events (myocardial infarction, stroke, death from cardiovascular causes) with

the Mediterranean diets compared with the control diet. The potential for dietary nitrate to be an important factor in such healthy diets will be addressed by considering the following questions.

1. Is nitrite a source of NO?

It had long been acknowledged that nitrite is reduced to NO under acidic conditions. However, the potential physiological relevance had not been appreciated. NO production from nitrite was originally demonstrated in tissues under hypoxia/ischaemia, conditions associated with an acidic reducing environment. Zweier *et al.* measured NO production from nitrite using Electron Paramagnetic Resonance (EPR) spectroscopy in isolated rat hearts [22]. Under normal perfusion (control), NO production was absent. When subjected to 30 minutes of ischaemia, a strong signal was detected on EPR, indicating NO production. The intensity of this signal was 100-fold higher in the presence of added nitrite. Using $^{15}NO_2^-$ and observing the generation of ^{15}NO, it was confirmed that the NO was derived from nitrite. These effects were seen with a background of NOS inhibition i.e. this was a NOS-independent mechanism of NO generation. The quantity of NO produced was inversely associated with the pH of the heart: the lower the pH, the greater the NO production. From this, the authors concluded that NO is produced from nitrite under the reducing environment found in hypoxic/ischaemic/acidic conditions, in contrast to the conventional oxygen-dependent production of NO from NOS, which is inhibited by hypoxia and ischaemia [23].

2. What effect does nitrite have in IRI?

Twenty years ago, nitrite appeared to be predominantly harmful. Zweier *et al.* (1995) found that increasing NO production by adding 10 µM nitrite (enzyme independent) increased post-ischaemic myocardial injury in a model of ischaemia-reperfusion injury (IRI) in the isolated perfused Langendorff rat heart [22]. However, the percentage recovery of the rate pressure

product was <10% in control. Administration of the NOS inhibitor, *N*-nitro L-arginine methyl ester (L-NAME), decreased NO production by approximately 65% and led to a two-fold increase in recovery of contractility, suggesting that NO produced from nitrite could be damaging to myocardial tissue. Understandably, as a result of these findings of a deleterious effect of nitrite/NO in IRI, along with the notion that nitrite produced carcinogenic nitrosamines, and that NO was a pollutant in the atmosphere, interest in nitrite waned.

Almost a decade later, Webb *et al.* found that in a similar IRI model, nitrite (10 and 100 μM) substantially reduced infarct size and preserved left ventricular pressure following reperfusion [24]. This protective effect was lost in the presence of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (carboxy-PTIO), suggesting that nitrite-derived NO was beneficial. These findings were consistent with an emerging protective rather than damaging function of NO from NOS, as reviwed by Bolli et al., [25]. For example, infarct size had been found to larger in hearts of endothelial NOS (eNOS) knockout mice compared to wild type [26].

The majority of studies subsequently performed with nitrite supported a protective effect in IRI (Table 1). For example, Duranski *et al.* demonstrated protective effects of nitrite *in vivo* during IRI of both the liver and the heart in rats [27]. Nitrite limited the rise in liver enzymes during hepatocellular injury, and diminished hepatocellular necrosis, whilst intraventricular nitrite (48 nmol) prior to reperfusion of the ischaemic heart reduced infarct size by 67%. Administration of nitrite at the time of reperfusion was also effective in limiting infarct size in the brain in a rat model of stroke [28]. Table 1 summarises some of the early studies 1995-2008 which helped establish the role of nitrite IRI, up to the study in dogs by Gonzalez *et al*. [29].

Within a decade, the original discovery of cardio-protection with nitrite in IRI had been translated into two clinical trials in patients with acute ST-elevation myocardial infarction (STEMI). In a double-blind placebo- controlled randomised parallel-group trial, Siddiqi *et al.* (2014) investigated the effects of intravenous sodium nitrite immediately prior to reperfusion in 229 patients with acute STEMI. Myocardial infarct size (the primary endpoint, measured in terms of the extent of gadolinium enhancement by cardiac MRI 6-8 days post-infarct) did not differ significantly between the groups [35]. It is possible that the dose given (70 µmol) was not sufficient to achieve a significant effect. Jones *et al.* used the intracoronary, rather than the intravenous route, delivering a high local dose of nitrite (1.8 µmol) prior to balloon dilatation during percutaneous coronary intervention (PCI) in 80 patients with acute STEMI [36]. There was no overall difference in the primary outcome - infarct size (versus placebo), measured by the area under the curve of creatine kinase (AUC CK) profile over 48 hours. However, there was an overall improvement (P=0.05) in myocardial salvage index (measured in a subgroup of 68 patients with cardiac MRI) and reduction in major adverse cardiac event at 1 year. In a sub-group analysis (n=66) in patients who had Thrombolysis In Myocardial Infarction (TIMI) flow grade score ≤1, there was a significant 19% reduction in AUC CK profile and cardiac MRI-determined infarct size. These findings are entirely compatible with the mechanism of action of nitrite - which requires an ischaemic environment, rather than one in which reperfusion has already partially occurred, and this deserves to be followed up in further trials.

3. Does nitrite dilate blood vessels?

Furchgott had shown in 1952 that high concentrations of sodium nitrite (100 and 1000 µM) relaxed strips of rabbit thoracic aorta [37]. However, these concentrations are >100-fold greater than physiological concentrations of nitrite in the circulation, which are usually ~0.1-0.4 µM. Cicinelli *et al.* (1999) postulated the existence of an arterial-to-venous (AV) gradient of NO, after measuring higher quantities of NO metabolites nitrite and nitrate, in arterial

samples than paired venous samples in healthy volunteers [38]. A year later, Gladwin *et al.* clearly demonstrated the presence of an AV nitrite gradient [39], which further suggested that nitrite is reduced to NO across the vascular bed, with a greater difference shown during handgrip exercise associated with oxygen extraction by the muscles from the vessels, and consistent with enhanced nitrite reduction to NO under condition s of lower oxygen tension.

However, whether nitrite could actually dilate vessels was not clear. In 2001, Lauer *et al.* showed that whilst regional nitrite concentration rose acutely following endothelial NOS stimulation with acetylcholine, reflecting changes in NO generation, the intrabrachial infusion of nitrite failed to produce any vasodilation [40]. In the same year, Modin *et al.* analysed the effects of nitrite in rat aortic rings at concentrations which resembled physiological states and its effect on aortic contractility upon reaching a "physiologically acidic milieu" (pH 6.6). The EC_{50} for nitrite-induced relaxation was decreased at low pH (6.66) relative to neutral pH (7.45), suggesting that nitrite may have an important vasodilatory role in acidic conditions. This effect was inhibited by the co-administration of the soluble guanylyl cyclase inhibitor -[1, 2, 4]-oxadiazolol-[4,3,-a]-quinoxalin-1-one (ODQ) suggesting that the vasorelaxatory effect of nitrite was dependent on its reduction to NO [41]. In further support of this requirement for reduction of nitrite, the reducing agent ascorbic acid potentiated nitrite's vasorelaxatory effect. Overall, nitrite appeared to have vasodilatory effects on blood vessels via reduction to NO.

Therefore, a hypoxic/acidic/ischaemic environment appeared to be necessary for nitrite reduction to bioactive NO. However, Cosby *et al.* (2003) found that forearm blood flow was increased by intra-brachial infusion of nitrite (36 and 0.36 µmol/min), resulting in supra- and near physiologic nitrite concentrations, respectively [42]. This vasodilatory effect was enhanced by handgrip exercise, which is associated with greater oxygen extraction from the circulation by the exercising forearm musculature, with a resultant greater formation of deoxyhaemoglobin (deoxyHb) possessing greater capacity for nitrite reduction, as evidenced by the formation of iron-nitrosylated haemoglobin (Hb). This requirement for hypoxia was

confirmed by Maher *et al.* who demonstrated that nitrite-induced vasodilatation with intrabrachial nitrite infusions (40 nmol/min to 7.84 μmol/min) was enhanced in hypoxic environments compared to normoxia in humans as assessed by forearm blood flow [43].

4. How is nitrite reduced to NO?

The description of enzyme-independent NO formation from nitrite by Zweier *et al*. referred to the lack of involvement of NOS [22]. Whether NO production was solely a result of the acidic disproportionation of nitrite, or other enzymes were involved in nitrite metabolism was not clear. However, identifying the mechanisms involved in controlling the reduction of nitrite to NO is crucial for understanding nitrite's physiological effects. As described above, Cosby *et al.* showed that NO production from nitrite, considered to be responsible for the vasodilatory effect, was confirmed by the rate of formation of iron-nitrosylated Hb during artery to vein transit [42]. The rate of NO formation increased as oxygen saturations fell, suggesting a hypoxia-regulated mechanism of nitrite bioactivation [11]. Maximal reduction of nitrite to NO occurred at an Hb oxygen saturation of ~50% (around the p50 of Hb), representing the optimum balance point for maximal availability of Hb in the deoxy- T-state for nitrite to bind to, coupled with maximal availability of Hb in the oxy- R-state for reduction to NO [44, 45] [46, 47]. This mechanism of NO formation is therefore dependent on three factors, as highlighted by equation 1: circulating nitrite as a substrate, the presence of hypoxia (as determined by the level of deoxyHb) and the presence of an acidic environment as a source of H^+.

$$DeoxyHb^{Fe(II)} + NO_2^- + H^+ \rightarrow Hb + HNO_2 \rightarrow Hb\text{-}NO + OH^- \rightarrow Hb^{Fe(III)} + NO + OH^-$$

(Equation 1)

The other ubiquitous globin, deoxymyoglobin (deoxyMb) reduces nitrite approximately 36-times faster than deoxyHb, because of its lower redox potential, and is therefore likely to be a very important nitrite reductase. The rate of reduction by deoxyMb is increased in lower pH environments. Myoglobin-dependent NO generation occurs as a large burst; whereas Hb-dependent NO generation is much more prolonged in comparison [48]. This may reflect different functions of nitrite reduction in these two molecules. Myoglobin (Mb) possesses a greater affinity for oxygen and hence nitrite reduction occurs more readily at very low oxygen tensions. Indeed, deoxyMb has been shown to be an effective nitrite reductase [46, 49], catalysing the production of NO ~36-fold faster than deoxyHb [49]. Therefore, the burst of nitrite reduction allows for better regulation of oxygen gradients under hypoxia, relating to the level of NO generation. In addition to Hb and Mb, deoxygenated neuroglobin (Ngb) and cytoglobin (Cgb) have been shown to reduce nitrite to NO [50, 51]; however, their low abundance questions their significance as nitrite reductases under physiological conditions [52, 53].

Besides the haem-containing globins, several studies have highlighted the nitrite reductive capacity of the molybdo-flavoproteins, in particular xanthine oxidoreductase (XOR) [24, 54]. Webb *et al.* showed that the production of NO from nitrite in rat and human heart homogenates was diminished in the presence of XOR inhibitors by ~50%, suggesting a role for XOR as a cardiac nitrite reductase [24]. Four years later, Webb *et al*. also showed XOR to be present on red blood cell membranes in addition to blood vessels (left internal mammary artery) offering readily-accessible sites for nitrite reduction within the circulation [55]. The activity of this XOR was upregulated with increasing acidosis and during hypoxia. [56, 57]. This provides a regulatory mechanism for nitrite's increased activity in ischaemic tissue. In addition to XOR, the related molybdo-flavoprotein, aldehyde oxidase appears to function as a nitrite reductase [58]. Aldehyde oxidase is unable to function as a dehydrogenase [58, 59], and is not to be confused with the aldehyde dehydrogenases.

Although, aldehyde dehydrogenase 2 (ALDH2), which when deficient is associated with oriental (alcohol) flush, may also have nitrite reductase properties [60].

The zinc-containing enzyme carbonic anhydrase (CA) has also been shown to catalyse the conversion of nitrite to NO [61]. However, since zinc in CA lacks redox activity, the enzyme is thought to function as a nitrous anhydrase, rather than a nitrite reductase, forming N_2O_3 which then rapidly breaks down to form NO_2 and NO, or forming nitrosothiols in the presence of thiols (e.g. glutathione), via zinc thiolate and nitrous acid (HNO_2) [62]. Definitive production of CA-catalysed NO from nitrite has been difficult to demonstrated, with some studies showing no evidence for this [63]. Finally, whilst initially NOS was thought to lack capacity to function as a nitrite reductase, work by Gautier et al., identified such activity for eNOS, located within the oxygenase domain [64]. Subsequently, biological relevance was shown in human erythrocytes, whereby inhibition with L-NAME attenuated the production of NO from nitrite [55].

5. Can nitrate generate nitrite-NO?

The potential of dietary nitrate as a source of gastric NO was first demonstrated by two independent groups in 1994. However, these were viewed as local processes specifically related to the acidic environment of the stomach, with relevance strictly restricted to local biological processes (and not as a systemic source of NO). Benjamin *et al.* demonstrated a 10-fold increase in salivary nitrite concentration 45 min after ingestion of potassium nitrate (200 mg (2 mmol nitrate)) in fasting individuals [65]. The entero-salivary circulation of nitrate to nitrite had been described previously, whereby ingestion of nitrate-containing vegetable juices of varying concentration correlated with salivary nitrite concentrations, due to concentration of ~25% of the nitrate load in the salivary glands, with subsequent secretion and reduction to nitrite [66]. However, the possibility that such nitrate-derived nitrite might be a source of NO had not been. Whilst stomach NO was not measured by Benjamin *et al.* in this study, concentrations of nitrite such as those found in saliva, e.g. 250 μM, were found to

kill Candida albicans and Escherichia coli in a pH-dependent manner, an effect that was likely to have been due to the production of NO.

Lundberg *et al.* did measure gastric NO production (in healthy and atopic individuals), with samples obtained through carbonated water-induced eructation and measured by ozone chemiluminescence [67]. Gastric NO production was found to be 100-times higher than exhaled NO. Ingestion of lettuce (50 g, ~1 mmol nitrate) just 5 minutes prior to measurement of gastric NO increased the concentration 5-fold. Moreover, gastric NO production was inhibited ~95% following ingestion of high dose proton pump inhibitor (PPI) omeprazole (three doses of 80 mg taken over the preceding 24 hour period) with or without lettuce ingestion. Therefore, nitrate was confirmed as a source of NO in the stomach, which was dependent on low pH (typical stomach pH 1-3, without PPI).

Duncan C. *et al*. found that the salivary production of nitrite was due to bacteria on the posterior third of the tongue with nitrate reductase activity, which was absent in germ-free rats [68]. Following this, McKnight *et al.* found that ingestion of potassium nitrate (2 mmol) led to a sustained rise in salivary, gastric and plasma nitrate, salivary nitrite and gastric NO concentration for the duration of the study [69]. However, there was a small, non-significant rise in gastric nitrite, which suggested that the majority of the nitrite was rapidly converted to NO.

Therefore, the understanding of the entero-salivary circulation was that nitrate is readily taken up in the upper gastrointestinal (GI) tract, plasma levels rise and remain elevated. Once in the circulation, ~25% of the nitrate load is actively absorbed and concentrated by the salivary glands. Following secretion of nitrate-containing saliva into the oral cavity, commensal, facultative anaerobic bacteria reduce nitrate to nitrite via nitrate reductases – an important step since mammalian cells cannot effectively metabolise this nitrate [70]. Nitrite is then swallowed in the saliva to enter the low pH stomach milieu, where it is rapidly protonated to form nitrous acid (HNO_2) which then decomposes, via the reactions shown in Equations 2-5 to form NO [11].

$H^+ + NO_2^- \leftrightarrow HNO_2$ (Equation 2)

$HNO_2 \leftrightarrow NOOH$ (Equation 3)

$NOOH + NO_2^- \leftrightarrow N_2O_3 + OH^-$ (Equation 4)

$N_2O_3 \rightarrow NO_2 + NO$ (Equation 5)

6. Does nitrate inhibit platelets?

Platelet adhesion and aggregation are pivotal mechanisms in the pathogenesis of acute coronary syndrome and stroke [71]. McKnight *et al.* showed that an oral nitrate load in healthy volunteers resulted in significant inhibition of platelet activity when compared to the control (chloride solution) [72]. However, at that time, the mechanism by which nitrate resulted in platelet inhibition was not known. One possibility was the formation of S-nitrosothiols (RSNOs), from the S-nitrosylation of thiol-containing compounds in the presence of by nitrosating species derived from nitrite in the acid milieu of the stomach: HNO_2, N_2O_3 and NO^+. S-nitrosothiols were known to be potent inhibitors of platelet function *in vitro* and *in vivo* via cGMP-dependent and independent mechanisms [73].

7. Does nitrate inhibit platelets via SNOs?

Richardson et al., (2002) found that the ingestion of potassium nitrate (0.5 mmol and 2 mmol) by healthy volunteers increased gastric RSNO concentration and inhibited the platelet response to collagen. However, there was no increase in plasma or portal blood RSNO, the latter measured in patients with transjugular intrahepatic portosystemic shunts (TIPS) [74]. Although there was an association between a rise in gastric RSNO and inhibition of platelet function, it was unclear whether S-nitrosothiols exerted a direct effect on platelet function. There was also no increase in platelet cGMP levels. Additional studies, using electron paramagnetic spectrometry as well as ozone chemiluminescence, obtained similar findings – an increase in gastric RSNO concentration following ingestion of nitrate, with no significant

change in plasma RSNO concentration [75]. As plasma nitrite was not thought to increase following a nitrate load, plasma nitrite was not measured in these studies.

8. Does nitrate increase plasma nitrite?

Whilst ingestion of nitrate led to a large rise in plasma nitrate concentration, it was unclear whether nitrite levels increased in a similar way. Pannala *et al.* analysed the pharmacokinetic profile of nitrate and nitrite in plasma, urine and saliva following a high nitrate meal (120 g of lettuce - equivalent to 250 mg of nitrate, once daily for three days). They found that whilst salivary nitrite increased significantly, there was no significant increase in plasma nitrite (78 ± 4 nmol/L and 90 ± 3 nmol/L pre- and post-meal, respectively). On the other hand, plasma nitrate rose almost 7-fold [76]. Therefore, the explanation for the effects of dietary nitrate on platelet aggregation was not at all clear. As Figure 1 shows, nitrite did not appear to be absorbed from the gut into the circulation, and it was unclear whether RSNOs were absorbed. Besides this there was major doubt anyway whether nitrite had physiologically-relevant effects; even if it did, it seemed clear at the time that nitrate's effect were not mediated by nitrite.

In 2004, Lundberg and Govoni found that plasma nitrite concentration increased 4-fold in healthy participants following consumption of sodium nitrate (10 mg/kg), an effect that was prevented when volunteers avoided swallowing after the nitrate load [77]. The importance of lingual bacterial nitrate reduction was also shown in the same year in a study in germ-free sterile rats which showed that gastric NO production was negligible, even following a nitrate load [78]. Webb *et al.* also measured nitrite concentrations after ingestion of nitrate (500 mL beetroot juice) in healthy volunteers. They found that plasma nitrite concentrations increased significantly compared to control, peaking at ~3 hours after beetroot juice ingestion, returning to baseline levels by 24 hours [79]. A limitation in the study by Pannala *et al.* is that participant blood samples were measured only after 1 hour; whereas in the study by Webb

et al., a full kinetic profile of plasma nitrite levels was performed, which identified that a significant increase occurred around 2.5-3 hours post-ingestion. The delay in the peak of plasma nitrite, relative to plasma nitrate, is due to the time taken for nitrate to be absorbed through the enterosalivary circuit before reduction to nitrite, followed by accumulation in the circulation [80].

9. Does Nitrate lower blood pressure?

Given the finding of an increase in plasma nitrite following a nitrate load, coupled with the vasodilatory potential of nitrite as demonstrated by Cosby *et al.* [42], it was possible that dietary nitrate might lower blood pressure. Larsen *et al.* conducted a randomised, cross-over study in which healthy, non-smoking subjects were given sodium nitrate or sodium chloride (both 0.1 mmol/kg/day for 3 days). Whilst there was no change in systolic blood pressure, diastolic blood pressure was reduced by 3.7 mmHg by sodium nitrate, associated with almost a doubling in the plasma nitrite concentration [80].

In the study by Webb *et al*, systolic blood pressure was significantly lower following consumption of 500 mL of beetroot juice containing ~22 mmol dietary nitrate (and undetectable amounts of nitrite) compared to control (500 mL of water) with a peak change at 2.5 hours of 10.4±3.0 mmHg ($P<0.01$). Peak decreases in diastolic and MAP were seen at 3 hours after ingestion, with changes of 8.1±2.1 mmHg and 8.0±2.1 mmHg, respectively (both $P<0.01$) [79]. Since then, several studies have explored the effects of dietary nitrate on blood pressure, with 16 included in the meta-analysis of Siervo *et al.,* (2013) which showed a mean difference in SBP of -4.4 mmHg (95% CI -5.9 to 2.8) [81].

10. Is nitrate's BP lowering effect via nitrite?

In the first study by Webb *et al*, the peak reduction in blood pressure occurred at 2.5-3 hours corresponding to the peak rise in plasma nitrite concentration, suggesting that nitrite, rather than nitrate, accounted for the change in blood pressure. To provide more evidence that blood pressure reduction with nitrate was related to plasma nitrite concentration, the enterosalivary circulation was interrupted by asking subjects to spit out all of their saliva for 3 hours following beetroot juice consumption, or to swallow their saliva normally, in a further crossover study. Spitting saliva prevented the increase in plasma nitrite (plasma nitrate was unaffected) and prevented the decrease in systolic blood pressure seen with swallowing saliva normally.

Kapil *et al*. established a dose-response relationship existed between nitrate consumption and change in plasma nitrate, nitrite and blood pressure using potassium nitrate capsules containing 4, 12 and 24 mmol [82]. Also, using potassium chloride capsules as control, it was confirmed that the blood pressure lowering effect was independent of potassium. Furthermore, the peak of plasma nitrite concentration was associated with significantly higher levels of circulating cGMP, a sensitive marker of NO bioactivity [83]. This study also gave some insight into individual differences in blood pressure lowering responses to nitrate. Baseline blood pressure was inversely correlated with the reduction in blood pressure, i.e. the higher the baseline, the larger the reduction in blood pressure. This suggested the possibility of better blood pressure-lowering responses in hypertensive individuals. A *post-hoc* analysis also showed that females had a higher baseline concentration of nitrite than males, associated with the lack of a significant blood pressure reduction in response to nitrate. Therefore, baseline nitrite may predict the response of dietary nitrate-induced blood pressure reduction.

It was now clear that the blood-pressure lowering effects of dietary nitrate were explained through the "nitrate-nitrite-NO pathway", with nitrite being absorbed systemically. Therefore, nitrite held the key to the puzzle, as summarised in Figure 2.

Thus, nitrate is reduced to nitrite on the posterior surface of the tongue, which is then absorbed in the upper GI tract into the circulation. Nitrite then undergoes further reduction to produce the biologically active NO in the circulation.

Conclusion

We have described key twists and turns in the realisation of the nitrate-nitrite-NO pathway, highlighting some important physiological and therapeutic effects. These are summarised in Table 2. In Part 2 we explore more recently discovered properties and effects of nitrate and nitrite.

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Table 1. The early ischaemia-reperfusion injury (IRI) studies with nitrite from 1995 until the first study in the dog heart (2008). Nonstandard abbreviations used: rate pressure product (RPP); left ventricular developed pressure (LVDP); heart rate (HR); alanine aminotransferase (ALT); aspartate aminotransferase (AST); haematoxylin and eosin (H&E); myeloperoxidase (MPO); serum creatinine (SCr); creatinine clearance (CCL); fractional excretion of Na (FENa+); left ventricular ejection fraction (LVEF).

Study	**Organ/Route**	**Measures of cytoprotection**	**Outcome**
Zweier J. et al., 1995 [22]	Rat Heart IRI (Langendorff)	Recovery of RPP, LVDP, HR	Harmful
Webb A et al., 2004 [24]	Rat Heart IRI (Langendorff)	Infarct Size, Recovery of LVDP	Beneficial
Duranski et al., 2005 [27]	Rat Liver (intra-peritoneal) & Heart IRI (intraventricular)	Liver – AST/ALT; H&E/TUNEL Heart – Infarct Size	Beneficial
Lu P et al., 2005 [30]	Rat Liver IRI	ALT, MPO, ATP, 7d mortality, ATP	Beneficial
Jung et al., 2006 [28]	Rat Brain IRI	Infarct Volume, Doppler flowmetry; neurological tests	Beneficial
Basireddy M et al., 2006 [31]	Rat kidney IRI	Serum creatinine and blood urea nitrogen Histological scoring for loss of brush border, tubular necrosis, and red blood cell extravasation	Neutral
Tripatara P et al., 2007 [32]	Rat Kidney IRI	SCr, AST, CCL, Urine Flow, FE_{NA}^{+}, H&E	Beneficial
Bryan et al 2007 [33]	Mouse heart 7 days oral	Infarct size	Beneficial
Shiva et al 2007 [34]	Mouse IRI	Infarct size	Beneficial
Gonzalez et al., 2008 [29]	Dog Heart IRI	Infarct Size, LVEF, MRI, Microsphere Blood Flow, TUNEL	Beneficial

Table 2. Twists and turns in the realisation of the nitrate-nitrite-NO pathway

	Negative/Neutral	Positive
Background to nitrate's (NO_3^-) & nitrite's (NO_2^-) properties?	Physiologically inert metabolites of NO [10]	
1. Is nitrite a source of NO?	No – nitrite is a physiologically inert metabolite of NO [10]	Yes – in the ischaemic heart: Zweier et al.,1995 [22]
2. What effect does nitrite have in IRI?	Deleterious - Zweier et al., 1995 [22]	Protective - Webb et al., 2004
3. Does nitrite dilate blood vessels?	No – Lauer et al., 2001 [40]	Yes - Modin et al., 2001 [41], Cosby et al., 2003
4. Are there nitrite reductases?	Non-enzymatic/non-NOS Zweier et al.,1995 [22]	Hb [42], Mb [85], XOR [24], AO [58], eNOS [64]
5. Is nitrate a source of NO?		Benjamin et al., 1994 [65], Lundberg et al.,1994 [67]
6. Does nitrate inhibit platelets?		Yes – McKnight et al., 1999 [72]
7. Does nitrate inhibit platelets via SNOs?	No? – Richardson et al., 2002 [74]	
8. Does nitrate increase plasma nitrite?	No - Pannala et al., 2003 [76]	Yes – Lundberg et al., 2004 [77], Webb et al., 2008 [79]
9. Does nitrate lower BP?		Yes – Larsen et al., 2006 [80], Webb et al., 2008 [79]
10. Does nitrate lower BP via nitrite?		Yes – Webb et al., 2008 [79]

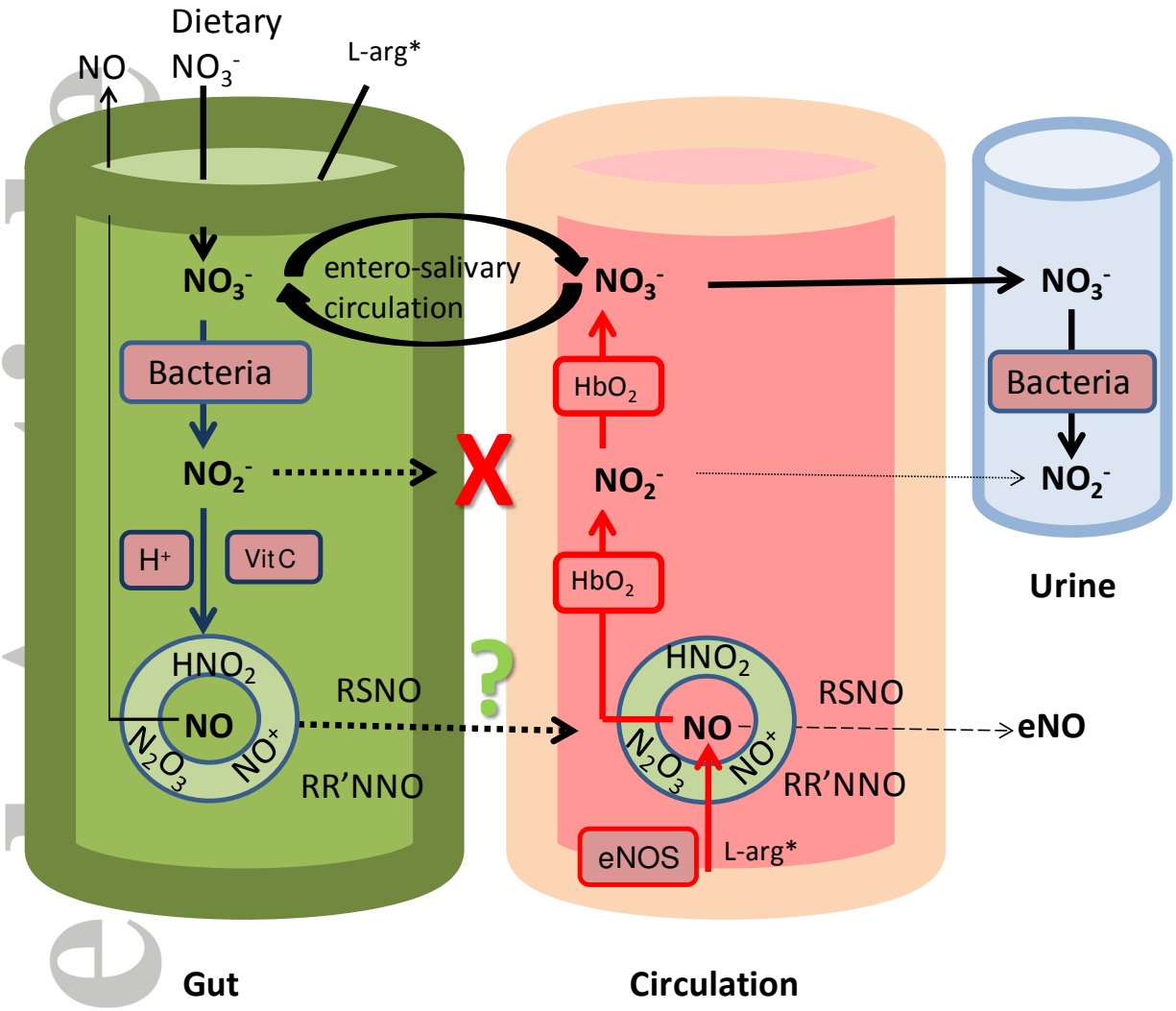

Figure 1 – Compartmentalised kinetic model (involving the gut, circulation and urinary tract) of the kinetic processes understood to occur up to a decade ago in the handling of dietary nitrate (NO_3^-), with the focus on S-nitrosothiol production from the nitrosating species (HNO_2, N_2O_3, NO^+) generated in the stomach from acidic disproportionation (in addition to liberation of free NO gas), but uncertainty over systemic S-nitrosothiol absorption. The small amounts of residual nitrite (NO_2^-) were not thought to be absorbed systemically.

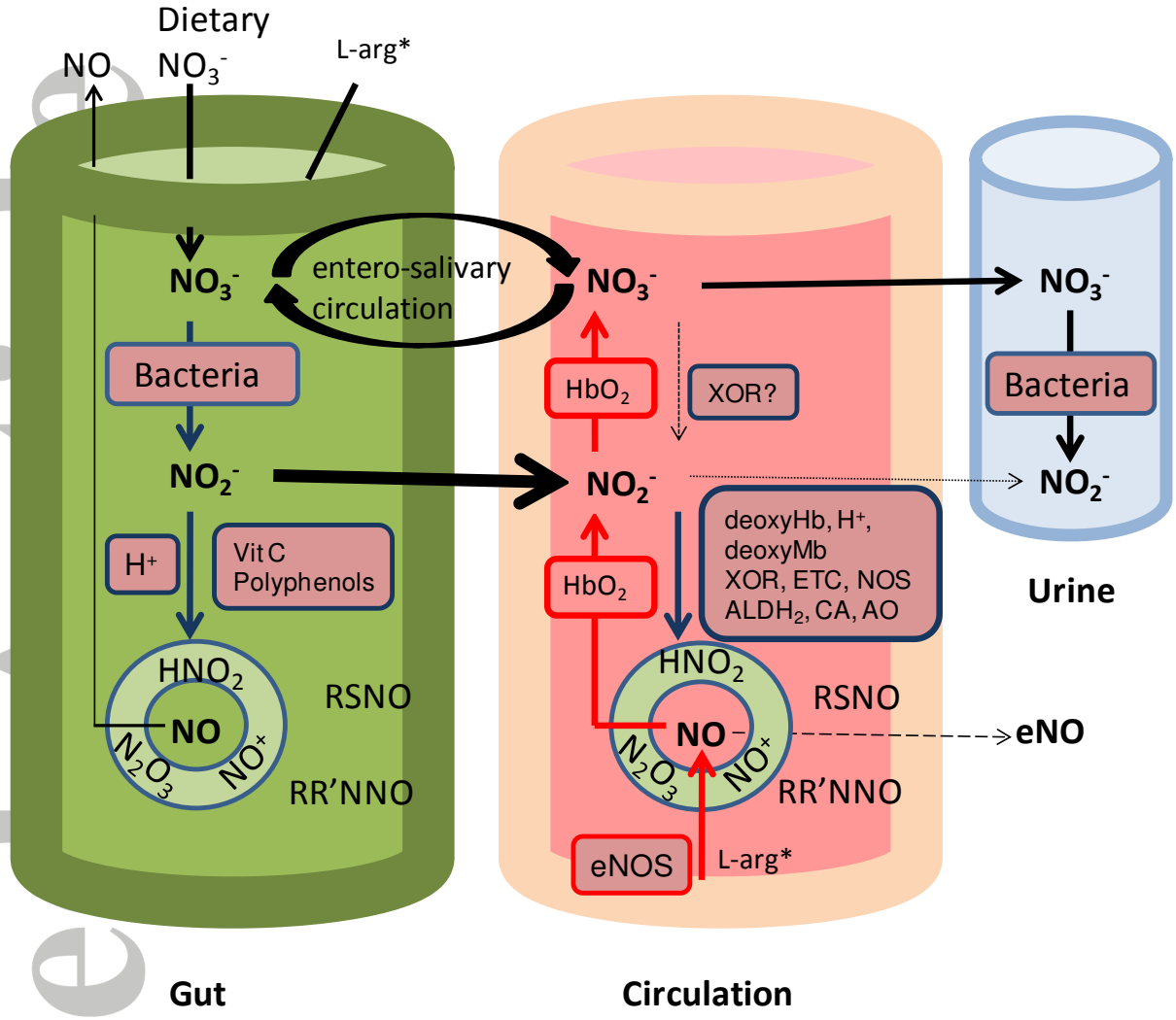

Figure 2. Compartmentalised Model (involving the gut, circulation and urinary tract) of the fully realised 'nitrate-nitrite-NO' pathway. Red and blue arrows represent pathways that are favoured under oxygenated and deoxygenated conditions, respectively [84].