Review article

Dietary nitrate in man: friend or foe?

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Based on the premise that dietary nitrate is detrimental to human health, increasingly stringent regulations are being instituted to lower nitrate levels in food and water. Not only does this pose a financial challenge to water boards and a threat to vegetable production in Northern Europe, but also may be eliminating an important non-immune mechanism for host defence. Until recently nitrate was perceived as a purely harmful dietary component which causes infantile methaemoglobinaemia, carcinogenesis and possibly even teratogenesis. Epidemiological studies have failed to substantiate this. It has been shown that dietary nitrate undergoes enterosalivary circulation. It is recirculated in the blood, concentrated by the salivary glands, secreted in the saliva and reduced to nitrite by facultative Gram-positive anaerobes (*Staphylococcus sciuri* and *S. intermedius*) on the tongue. Salivary nitrite is swallowed into the acidic stomach where it is reduced to large quantities of NO and other oxides of N and, conceivably, also contributes to the formation of systemic S-nitrosothiols. NO and solutions of acidified nitrite, mimicking gastric conditions, have been shown to have antimicrobial activity against a wide range of organisms. In particular, acidified nitrite is bactericidal for a variety of gastrointestinal pathogens such as *Yersinia* and *Salmonella*. NO is known to have vasodilator properties and to modulate platelet function, as are S-nitrosothiols. Thus, nitrate in the diet, which determines reactive nitrogen oxide species production in the stomach (McKnight *et al.* 1997), is emerging as an effective host defence against gastrointestinal pathogens, as a modulator of platelet activity and possibly even of gastrointestinal motility and microcirculation. Therefore dietary nitrate may have an important therapeutic role to play, not least in the immunocompromised and in refugees who are at particular risk of contracting gastroenteritides.

Nitrate: Nitrite: Nitric oxide: Vegetables

Governmental concern about and public fear of nitrate has, over the last 50 years, been the *bête noir* of farmers and water boards, with increasingly stringent regulations being enforced to lower nitrate concentrations in food and water (World Health Organization, 1970, 1984; Department of the Environment, 1984). Green vegetables such as lettuce and spinach, root vegetables such as beetroot, and water, both municipal and well water, are the main sources of nitrate in our diet (Ministry of Agriculture, Fisheries and Food, 1987). In cooler temperate zones where vegetables are grown in low-light-intensity high-temperature glass houses, nitrate levels are much higher than in vegetables grown outside under natural sunlight (Steingrover *et al.* 1993). Moreover,

the nitrate concentration of ground water is gradually increasing through leaching of fertilizer (Addiscot, 1996). The financial burden on water boards attempting to maintain the concentration below the present maximum allowable limit (50 mg/l; World Health Organization, 1984; Ministry of Agriculture, Fisheries and Food, 1987) is becoming onerous, and regulations to limit food nitrate concentrations threaten to stop production of some vegetables in Northern Europe. The regulations are based on the premise that dietary nitrate has only detrimental effects, such as increasing the risk of gastric cancer (Sen *et al.* 1969; Armijo & Coulson, 1975; Cuello *et al.* 1976; National Research Council, 1981; Ohshima & Bartsch, 1981; Correa *et al.* 1990; Rademacher

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et al. 1992; Xu *et al.* 1992) and infantile methaemoglobinaemia (Comly, 1945; Donahoe, 1949), and has no known beneficial effects. However, recent studies show that the postulated risks of nitrate to man, extrapolated from animal studies, have not been substantiated epidemiologically (Armijo *et al.* 1981; Forman *et al.* 1985; Knight *et al.* 1987, 1990; Boeing, 1991; Sobala *et al.* 1991; Pobel *et al.* 1995) and suggest that nitrate may have beneficial effects on the physiology of the intestinal tract (Pique *et al.* 1989; Desai *et al.* 1991; Benjamin *et al.* 1994; Bilski *et al.* 1994; Duncan *et al.* 1994; Lundberg *et al.* 1994; McKnight *et al.* 1997) and that it protects us against food and water-borne pathogens (Dykhuizen *et al.* 1996). From cohort mortality studies of healthy eaters (Key *et al.* 1996) and vegetarians (Burr & Sweetnam, 1982) who, by inference, have higher dietary nitrate levels, there appears to be a protective effect of such a diet on ischaemic heart disease.

Metabolism of dietary nitrate

Understanding the metabolism of ingested nitrate is a prerequisite for evaluation of the hypothesized harmful and beneficial effects. Dietary nitrite is absorbed from the stomach and proximal small intestine into the plasma. It is actively concentrated, by a factor of ten, from the plasma into the saliva (Spiegelhalder *et al.* 1976; Tannenbaum *et al.* 1976; Duncan *et al.* 1995) and then re-secreted into the upper intestinal tract. Approximately 25 % of dietary nitrate is thus recirculated into this enterosalivary circulation (Spiegelhalder *et al.* 1976; Tannenbaum *et al.* 1976).

The dorsal surface of the tongue harbours a specialized flora of symbiotic nitrate-reducing facultative anaerobic bacteria which rapidly reduce nitrate to nitrite under hypoxic conditions (Sasaki & Matano, 1979; Duncan *et al.* 1995). Antibiotic inhibition studies show that of the species that reduce nitrite in culture (Li *et al.* 1997), *Staphylococcus sciuri* and *S. intermedius* are likely to be the only organisms of physiological importance *in situ* (C Duncan, C Leifert and M Golden, unpublished results). In rats reared in a germ-free environment, lingual nitrite production from nitrate is absent; this confirms that the nitrate reduction is bacterial rather than an intrinsic mammalian system (Duncan *et al.* 1995). This special flora is induced by a high-nitrate diet and is more abundant under unhygienic husbandry conditions (Duncan, 1998). Thus, from the salivary and dietary nitrate, a high concentration of nitrite is generated in the mouth; this increases after ingestion of nitrate (Lundberg *et al.* 1994; McKnight *et al.* 1997). When swallowed, on exposure to the acidic conditions of the stomach, the nitrite is rapidly protonated to form HNO_2 (acid dissociation constant pK_a 3·2–3·4). HNO_2, in turn, decomposes to various nitrogen oxides spontaneously:

$$NO_2^- + H^+ \rightarrow HNO_2 \qquad (pK_a\ 3·2–3·4)$$

$$3HNO_2 \rightarrow H_2O + 2NO + NO_3^-$$

$$2HNO_2 \rightarrow H_2O + N_2O_3$$

$$N_2O_3 \rightarrow NO + NO_2.$$
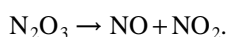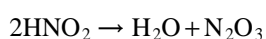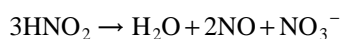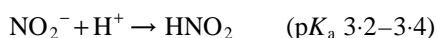
Additional reactions, with particularly rapid decomposition, occur in the presence of reducing equivalents, such as

ascorbic acid. Ascorbic acid is actively secreted by the gastric mucosa and is present in vegetables so that it will normally be present with nitrite in the stomach. The chemistry of this process is complex and the relative yields of the different products, under varying conditions of initial concentrations, pH and oxido-reductive potential, have not been determined.

This highly refined and specific mechanism results in the chemical production of $NO^\bullet$, and other oxides of N, under acid conditions, in the mouth and upper intestine. The process of recirculation into the saliva has been previously described (Spiegelhalder *et al.* 1976; Tannenbaum *et al.* 1976); however, it was the recognition that high levels of NO were chemically generated in the lumen of the stomach (Benjamin *et al.* 1994; Lundberg *et al.* 1994; McKnight *et al.* 1997) that sparked a resurgence of interest in the role of the enterosalivary circulation of nitrate and the effect of high or low dietary nitrate concentrations on mammalian physiology. NO is a small gaseous molecule which is responsible for cell signalling and host defence in a number of mammalian tissues (Moncada & Higgs, 1993; Angaard, 1994; Brunelli *et al.* 1995; DeGroote *et al.* 1995). It is well established that mammalian cells produce NO enzymically from the amino acid L-arginine with a family of NO synthase enzymes (*EC* 1.14.13.39) (Moncada & Higgs, 1993). However, the chemical reactions of the upper intestine produce up to 10 000 times the concentrations that occur in tissues from enzymic synthesis (McKnight *et al.* 1997). When the oxides of N are absorbed they react with haemoglobin to regenerate nitrate.

In evolutionary terms, it is unlikely that nitrate would be purposefully concentrated and secreted into the mouth, followed by chemical recycling, if this was actually harmful, for example by causing carcinoma. Such a sophisticated mechanism has surely evolved for a net beneficial purpose.

Nitrate the foe: potential harmful effects of nitrate

Infantile methaemoglobinaemia

Initial concern about the safety of nitrate arose in about 1945 when it was recognized that infantile methaemoglobinaemia was associated with the feeding of reconstituted baby food in rural areas where water was drawn from local wells. Some well waters are high in nitrate and this was identified as the usual oxidant causing methaemoglobinaemia (Comly, 1945; Donahoe, 1949). More recently it has been appreciated that infantile methaemoglobinaemia is associated with bacterially contaminated high-nitrate well water (Johnson & Kross, 1990) in which nitrate is reduced to nitrite by bacterial nitrate reductase enzymes. The ingested nitrite load then oxidizes the Fe^{2+} of the young infant's oxyhaemoglobin to Fe^{3+} of methaemoglobin:

$$NO_2^- + oxyHb(Fe^{2+}) \rightarrow metHb(Fe^{3+}) + NO_3^-$$
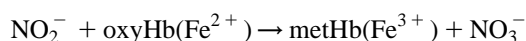
Any oxidants, such as aniline dyes used as colourants or sulfite, may produce methaemoglobinaemia (Howarth, 1951). The condition is now very rare in Europe; sporadic cases do occur, for example, where spinach with its high nitrate content has been cooked at home and then inappropriately stored so that bacterial contamination reduces the nitrate to nitrite (Simon, 1966; Philips, 1971). Nitrate is

much less hazardous than nitrite because the young infant does not develop the dense growth of symbiotic lingual bacteria that reduce nitrate to nitrite until after weaning. Breast-fed infants are not at risk as nitrate is not concentrated into human milk. The age at which the salivary nitrate concentrating mechanism develops has not yet been investigated.

There are several reasons why young infants are more susceptible (Jaffé & Hultquist, 1989) than those in the post-weaning period, to methaemoglobinaemia: (1) young infants have a higher gastric pH (pH > 4) and thus any ingested nitrite is less rapidly degraded in the stomach leaving more for absorption; (2) there are low concentrations of reducing agents such as ascorbic acid secreted by the immature stomach; these are derived in this age group from breast milk; (3) the intakes of water and food-energy are higher relative to body weight (about three times those of an adult); (4) the methaemoglobin reductase enzyme system does not mature until after weaning age; (5) in some races, congenital deficiency of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in the erythrocyte may lead to a secondary methaemoglobin reductase deficiency.

In particular, children and adults are far less susceptible to methaemoglobinaemia than young infants because of the induction of methaemoglobin reductase during the physiological post-weaning period. Furthermore, methaemoglobinaemia can be treated with reductants such as ascorbic acid (Carnrick et al. 1946); presumably a diet with a high ascorbate content will protect older infants and young children from methaemoglobinaemia.

No cases of methaemoglobinaemia have been reported in older children or adults from ingesting well water with a high nitrate concentration. Accidental and occasionally fatal poisoning has occurred where nitrite salts have been ingested (McQuiston & Belf, 1936; Gowans, 1990).

Carcinogenesis

The second main anxiety about dietary nitrate is that it may be causally associated with gastrointestinal cancer. It has been postulated that when salivary nitrite is swallowed into the stomach the HNO_2 may nitrosate secondary amines ingested in food to form nitrosamines (Sen et al. 1969) some of which are carcinogenic in animal studies (Low, 1974). The nitrosation may also occur exogenously, before food intake, between nitrite which has been formed from nitrate by contaminating bacteria and amines and amides present in the same food. Many studies have been undertaken in an attempt to establish categorically whether or not nitrate intake per se is directly linked to the development of gastrointestinal cancer (Sen et al. 1969; Correa et al. 1970, 1990; Hill et al. 1973; Armijo & Coulson, 1975; Cuello et al. 1976; Geleperin et al. 1976; Zaldivar, 1977; Amadori et al. 1980; Davies, 1980; Juhasz et al. 1980; Armijo et al. 1981; National Research Council, 1981; Ohshima & Bartsch, 1981; Jensen, 1982; Vincent et al. 1983; Beresford, 1985; Forman et al. 1985; Knight et al. 1987; Boeing, 1991; Sobala et al. 1991; Rademacher et al. 1992; Xu et al. 1992; Gonzalez et al. 1994; Pobel et al. 1995). The results are conflicting; on balance there does not appear to be a direct causative role of nitrate in gastric cancer (Table 1). On the other hand, the indirect evidence from nutritional and epidemiological studies shows that diets with a high salad or vegetable content, and thus high nitrate content, are protective against some types of cancer, particularly gastric cancer (Haenszel et al. 1976; Gonzalez et al. 1991, 1994; Corella et al. 1996; Key et al. 1996).

Nevertheless, the toxicological data unequivocally show that preformed nitrosamines and nitrosamides cause carcinoma in animals (Magee & Barnes, 1967). The question thus arises whether carcinogenic nitrosamines are formed in the human stomach or food in doses sufficient to induce cancer when usual diets are taken. Whilst there is no direct human evidence to support the role of dietary carcinogenic nitrosamines in upper intestinal cancer where such nitrosamines are formed, we should assume that they are carcinogenic in man. This stricture, however, does not hold for nitrate which is not, in itself, carcinogenic. There is also no evidence of direct nitrite (other than through nitrosamine formation) carcinogenicity in animals (National Research Council, 1990).

Much of the recirculated nitrite is temporally separated from ingested amines. After ingestion of food the intragastric pH increases to a level that does not support nitrosamine formation for 0·5–2 h; nitrosation of secondary amines occurs most vigorously at acid pH (2·2–3·5; Bartsch et al. 1988). This may also explain why no increase in gastric cancer has been observed in patients on long-term acid suppressant therapy. Moreover, reducing substances such as

Table 1. Published studies giving evidence for and against the aetiological role of nitrate in gastric cancer

Studies suggesting a role for nitrate in the aetiology of gastric cancer		Studies giving evidence against a role for nitrate in gastric cancer	
Country	Reference	Country	Reference
UK	Hill et al. (1973)	USA	Geleperin et al. (1976)
Colombia	Correa et al. (1970)	UK	Davies (1980)
Colombia	Cuello et al. (1976)	UK	Beresford (1985)
Chile	Zaldivar (1977)	Chile	Armijo et al. (1981)
Hungary	Juhasz et al. (1980)	France	Vincent et al. (1983)
Italy	Amadori et al. (1980)	UK	Forman et al. (1985)
Denmark	Jensen (1982)	Italy	Knight et al. (1990)
		Spain	Gonzalez et al. (1994)
		France	Pobel et al. (1995)

ascorbic acid, and thiols, that are present in food prevent nitrosation and rapidly reduce nitrite and/or HNO_2 to NO. Indeed, the stomach itself secretes appreciable amounts of ascorbic acid in gastric juice (Sobala *et al.* 1989) which prevents endogenous nitrosamine formation. Even postprandially when the low gastric pH is re-established, nitrosamine formation is not particularly favoured as the reaction is second order with respect to nitrite concentration (Bartsch *et al.* 1988).

Physiologically, during digestion, there are no free amino acids liberated in the stomach so that their amino groups are generally protected within the peptide bond. Pepsin, the major gastric proteinase, only attacks peptide bonds where a tyrosine or phenylalanine provides the amino group; even in the small intestine less than 5 % of amino acid residues are in the free state (Nixon & Mawer, 1970).

The most compelling evidence that nitrate, in the concentrations likely to be ingested from a high-nitrate diet, is not causally associated with gastric cancer is the repeatedly confirmed observation that vegetarians, who have three times the nitrate intake of omnivores (Ministry of Agriculture, Fisheries and Food, 1992), and those who have a high intake of fresh fruit and vegetables (and hence nitrate) have lower standardized mortality ratios for gastric cancer (Gonzalez *et al.* 1991; Key *et al.* 1996). Furthermore, whilst the worldwide incidence of gastric cancer is steadily declining (Correa & Chen, 1994), water nitrate content and the intake of green vegetables are both increasing.

Teratogenesis

As nitrosating substances may affect DNA alkylation and transcription, several groups, primarily Australian, have examined the hypothesis that chronic high nitrate ingestion by a mother may affect the fetus either by germ-cell mutation or by transplacental transmission of tumourigenic nitroso compounds. In animal studies where pregnant hamsters have been fed with large doses of sodium nitrite, an increase of neurogenic tumours has been seen in the fetus; however, such effects are not seen with chronic ingestion of large doses of nitrate (Inui *et al.* 1979). Very large doses of nitrate could cause fetal anoxia where the mother has high levels of methaemoglobin (Geleperin *et al.* 1971). Epidemiological studies in human subjects are equivocal; some workers report an increased incidence of congenital malformations in areas where water nitrate levels are high (Scragg *et al.* 1982; Dorsch *et al.* 1984) and others report a lower incidence in such areas than in the background population (Arbuckle *et al.* 1988). One group has suggested that the incidence of spontaneous abortion is diminished in regions where nitrate is detectable in drinking water (Aschengrau *et al.* 1989). Thus, extrapolation from animal studies suggests that a large dose of unprocessed nitrite may have mutagenic potential in man, but nitrate *per se* appears not to be teratogenic

Nitrate the friend: potential beneficial effects of nitrate

Nitrate and nitric oxide

NO appears to have insinuated itself into most spheres of current research. This small gaseous molecule which readily crosses membranes is involved in cell signalling (Moncada & Higgs, 1993), is a potent vasodilator (Furchgott & Zawadzki, 1980; Moncada & Higgs, 1993) and platelet aggregation inhibitor (Ignarro, 1989; Radomski *et al.* 1990, 1991), a non-specific acute-phase reactant (Geller *et al.* 1994) and has intrinsic antimicrobial activity (Brunelli *et al.* 1995; DeGroote *et al.* 1995). NO also reacts reversibly with thiols to form S-nitrosothiols (Stamler *et al.* 1992). S-nitrosothiols not only have a longer half-life and greater stability than NO but also have biological activity similar to that of NO (Radomski *et al.* 1992; deBelder *et al.* 1994). It is inherently difficult to separate their biological activities from those of NO *per se*. In general the roles of NO and S-nitrosothiols have only been studied in relation to enzymic synthesis at the cellular level. Our observation (McKnight *et al.* 1997) and that of others (Lundberg *et al.* 1994) that very much larger amounts of NO, which is readily diffusible through membranes, are generated chemically in the lumen of the gut after the oral ingestion of nitrate has led to a search for the physiological role of nitrate in the diet. The highly specialized mechanism of enterosalivary circulation and conservation of ingested nitrate may confer its advantage through the beneficial effects of upper intestinal NO formation which outweigh any theoretical harmful effect. It should be stressed that where nitrite, acid, O and NO coexist, other oxides of N will be present, such as peroxynitrite, that themselves have recognized biological roles, especially in their antimicrobial activity (Brunelli *et al.* 1995). The *in vivo* chemistry of nitrogen oxides is undoubtedly highly complex, even chemical modelling of relatively simple systems such as atmospheric nitrogen oxide reactions is far from being fully understood.

A novel mechanism for host defence?

A common perception of intestinal host defence to pathogens ingested in our food and water, is that it depends entirely on immune factors secreted in the saliva and gastric acidity itself. However, for many gastrointestinal pathogens such as *Campylobacter*, *Shigella* and *Salmonella*, even prolonged immersion in strong acid at pH 2, to emulate the normal fasting stomach, exerts only a bacteriostatic effect (Dykhuizen *et al.* 1996). Failure to kill these organisms means that viable pathogens would pass to the small intestine if the defence depended on acid alone. It has been presumed that for this reason man is susceptible to these organisms; however, for most pathogens the infecting dose is large and only a proportion of those people exposed develop illness. We suggest that acid alone is neither sufficient nor the only physiological mechanism for killing pathogenic bacteria in the upper intestinal tract. The addition of nitrite to acid, in concentrations found in the saliva after a meal containing green leafy vegetables, renders the gastric acid bacteriocidal (Dykhuizen *et al.* 1996). This synergistic enhancement of gastric acid's antibacterial activity by nitrite holds at any given pH, and, at a given pH, increases with the nitrite concentration and time of exposure. The concentrations of nitrite exerting these effects are within the physiological ranges of salivary nitrite (approximately $100 \mu M–1.5 mM$) and gastric acid concentrations (pH 1·5–6·0). For short exposure times stronger acid and increasing nitrite concentrations are

required to ensure that no viable bacteria remain. With *Salmonella typhi*, for example, to achieve a bactericidal effect with acid alone pH < 3 is required for several hours; as pH rises above this then nitrite in increasing concentrations is required to maintain the effect (0·6 mM at pH 3·5; 1·3 mM at pH 4·0). Above pH 5, bacterial kill is achieved only with unphysiologically high (> 10 mM) concentrations of nitrite within a reasonable gastric residence time; although they are killed by exposure to 1·0 mM for 24 h at this pH. Organisms exposed to gastric acid obtained endoscopically gave similar results with enhanced kill of organisms when nitrite-containing 'artificial' saliva was added. Alone, gastric acid exerted an antibacterial effect equal to that of acid of the same pH with 50 μM-nitrite added (Ndebele, 1996).

In preliminary *in vivo* experiments in healthy volunteers, we have confirmed the *in vitro* results. Live attenuated oral *Salmonella typhi* Ty21a vaccine was enclosed in a short segment of dialysis tubing, impermeable to high-molecular-mass molecules such as immunoglobulins and interferon; the capsule was then swallowed into the stomach on a length of soft dental floss (Fig. 1). After exposure times of 5–20 min the capsules were retrieved, the pH was checked to ensure that the capsule had in fact been exposed to gastric contents, and the contents of the dialysis capsule were cultured to assess bacterial survival. The procedure was repeated after administration of a drink containing the amount of nitrate typically found in half a British lettuce, approximately 2 mmol, which caused the expected rise in salivary nitrate and nitrite. In five of six subjects, bacterial survival was shorter after the nitrate drink (Ndebele, 1996).

If these results are confirmed, dietary nitrate will have an important physiological role in augmenting salivary and gastric nitrite to concentrations that reduce the time required to kill ingested pathogens to within the normal residence time of food in the stomach. For food-borne, rather than water-borne pathogens this mechanism is particularly pertinent because gastric pH rises after food ingestion to levels that are not bactericidal unless there is nitrite present. Whilst the pH may rise above the critical level for bacterial kill with nitrite present, in fact gastric pH recovers to about the pK_a of HNO_2 within 30 min at which point the mechanism we propose for bacteriostasis, and subsequently kill, will come into effect. Food- and water-borne pathogens still represent a significant hazard to health, even in affluent societies, whereas in the developing world they are a major cause of mortality. *Escherichia coli* 0157 is more susceptible to acid plus nitrite than to acid alone, although it is more resistant than the other pathogens that we have tested (Dykhuizen *et al.* 1996). It is of interest that *E. coli* 0157 only emerged as a significant human pathogen after the introduction of regulations to prevent the addition of nitrate to processed meats.

The precise chemical species responsible for microbial damage by acidified nitrite remains unclear. It is clearly a product of protonated nitrite that is formed naturally in the stomach after a nitrate load has been ingested and converted to nitrite on the tongue. The variables of nitrite concentration, acidity, exposure time, presence of reductants, nitroso-thiol formation and other anti-bacterial oxidants that are present in saliva (thiocyanate and iodide) will all affect the chemical species formed and the way in which pathogens are affected. It is known that NO complexes with the Fe–S centres of bacterial respiratory enzymes and also directly damages DNA (Wink *et al.* 1991). NO may also combine with superoxide to form peroxynitrite and the hydroxyl radical (Hogg *et al.* 1992) which may be involved in bacterial kill, however, whether such species are formed in the lumen of the intestine is unknown. Moreover, HNO_2 which is an intermediate in the production of NO, may nitrosate N-, S-, and O-groups on bacterial surface molecules, thus exerting a toxic effect.

Evidence against a direct antimicrobial role of NO in the stomach is our finding that ascorbic acid, a powerful reducing agent which greatly enhances NO production, in fact reduces the antimicrobial activity of acidified nitrite against *Yersinia enterocolitica* (Fite, 1996). Iodide, a naturally-occurring abundant oxidant in saliva, augments the antimicrobial effect of nitrite and acid synergistically. Thiocyanate, which catalyses nitrosation at acidic pH, is concentrated in the saliva (Tenuovo & Makinen, 1976), particularly in smokers and those subsisting on plants such as yam and cassava, and dramatically increases the anti-microbial activity of acidified nitrite (Fite, 1996). This synergism may be of particular importance in developing countries where cassava (*Manihot* spp.) is the staple food and hygienic conditions are extremely poor. In such societies the rapid development of a symbiotic nitrate-reducing flora on the tongue and the avoidance of antibiotics at the time of weaning may be critical to prevent 'weanling diarrhoea'.

We suggest that the increased incidence of gastroenteritis associated with the use of gastric acid suppressant agents (Neal *et al.* 1994) is not simply due to the increased gastric pH *per se* but more to the effect of the pH alteration on the equilibrium of HNO_2 and the oxides of N which will form far less readily above the pK_a of HNO_2 and will not be available to halt the growth of pathogenic organisms. This provides a physiological explanation for the observation
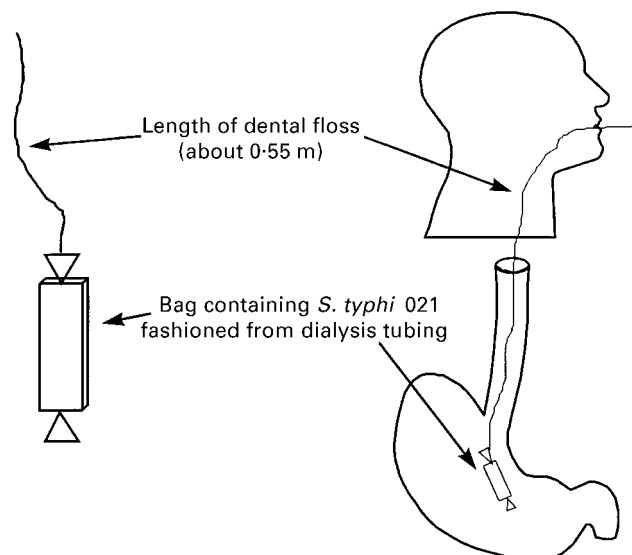

Fig. 1. Schematic representation of the method used to investigate the effect of dietary manipulation on the vaccine strain of *S. typhi in vivo*.

Length of dental floss (about 0·55 m)

Bag containing *S. typhi* 021 fashioned from dialysis tubing

that antibiotic treatment predisposes treated individuals to Salmonella gastroenteritis (Neal *et al.* 1994), even when the Salmonella is sensitive to the antibiotic used, because the antibiotic will kill the symbiotic nitrate-reducing bacteria of the tongue and hence reduce salivary nitrite concentration. Apart from bacteria, yeasts such as *Candida albicans* are also sensitive to acidified nitrite (Dougall *et al.* 1995) and antibiotic treatment greatly increases the incidence of oral thrush (Walker *et al.* 1979); again we suggest that this is due to diminution of salivary nitrite concentration. Furthermore, nitrite-containing toothpaste delays tooth decay (Miller *et al.* 1994) whereas medications inhibiting salivary secretion accelerate tooth decay (McMahon *et al.* 1993; Dasanyake *et al.* 1995).

Many lines of evidence are now coming together to support a beneficial physiological role of dietary nitrate as a potent and essential component of host defence against many of the common pathogens of the gastrointestinal tract.

Cardiovascular protection

Many theories have been proffered over the last few decades in an effort to explain the evident cardioprotective effect of diets with a high content of fruit and vegetables. Undoubtedly, the antioxidant vitamins and folic acid that such a diet contains have important effects on the health of the cardiovascular system. However, they fail to explain completely the protection afforded by vegetables (Gaziano *et al.* 1995; Hennekens *et al.* 1995), and more recently phyto-oestrogens have emerged as an additional factor that remains to be fully investigated (Goldin & Gorbach, 1996). Platelet adhesion and aggregation are important in the pathogenesis of vascular occlusive disease (Davies & Thomas, 1981). Both NO and the S-nitrosothiol, S-nitroso-glutathione, are known to be potent inhibitors of platelet aggregation when administered systemically (Radomski *et al.* 1990, 1992; deBelder *et al.* 1994). However, we have shown (McKnight *et al.* 1997) that large quantities of easily diffusible NO gas are generated in the confined lumen of the stomach: a monoepithelialized, vascular viscus. Furthermore, the diet contains thiols which will be readily S-nitrosated in this acidic environment; thus, we would anticipate that absorption of these products would have systemic effects. An attractive hypothesis is that (1) locally, NO gas maintains the gastric microvasculature and (2) systemically, by nitrosating thiol groups which in the acidic stomach will be mainly in the reduced form, it will be absorbed and exert an inhibitory effect on platelet aggregation. Whilst the effect of systemically administered nitrosothiols has been shown (Radomski *et al.* 1992; deBelder *et al.* 1994), until now similar effects have not been shown after administration of oral nitrate. Recently in a small number of individuals (*n* 6) we have shown by aggregometry Born (1967) that there is inhibition of platelet aggregation following an oral nitrate load which is not seen after taking control anions such as chloride (Fig. 2; G McKnight, N Benjamin and MH Golden, unpublished results). The mechanism of this inhibition has still to be clarified. Nevertheless, these data show that oral nitrate has a rapid and pronounced systemic effect on platelet function which has the potential to prevent thrombotic episodes. We hypothesize that the nitrate content of
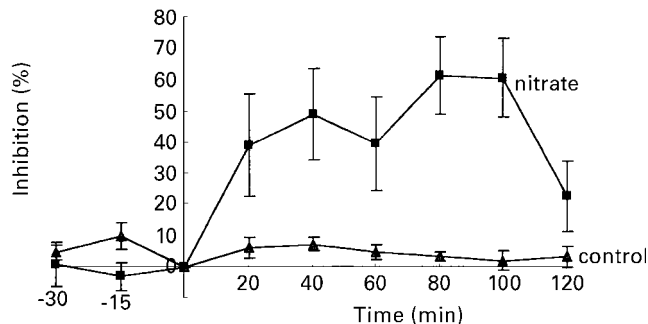

Fig. 2. Inhibition of platelet aggregation by 2 mM-KNO$_3$ (■) compared with 2 mM-KCl (▲) over a period of 2 h after dose administration.

vegetables actively contributes to their demonstrated cardio-protective properties.

Possible gastroprotective effect of high nitrate and/or vegetable intake

Whilst is it clear that NO may damage DNA and cause mutations in human cells *in vitro*, the epidemiological evidence suggests that a high-nitrate diet confers a gastro-intestinal protective effect. We speculate that this effect may be partly explained by an enhanced gastric emptying and rapid recovery of gastric acidity associated with the enhanced nitrate content and gastric NO production with high-vegetable diets. However the preservation of the gastric microcirculation by chemically synthesized intra-gastric NO, and hence possible suppression of atrophic gastritis, may also play a part. The possibility that nitrite in the acidic stomach may contribute to the natural defence against *Helicobacter pylori* is under investigation *in vivo*; we have shown that *H. pylori* is susceptible to acidified nitrite *in vitro* (Dykhuizen *et al.* 1998), even in undisturbed biopsies with the mucus layer intact (A Fraser, C Leifert and M Golden, unpublished results). It is intriguing that the incidence of *H. pylori* in the developed world is reducing whilst dietary nitrate and standards of hygiene are increasing (Cave, 1996). Doubtless, gastric cancer is of multifactorial aetiology but a protective, rather than a detrimental, role for nitrate, by suppression of *H. pylori*, is possible. Ironically, as *H. pylori* is the most potent gastric carcinogen commonly present in man, even in terms of gastric cancer, nitrate may turn out to be more friend than foe, despite the theoretical risk of nitrosamine formation.

Other gastrointestinal effects

The potential physiological roles of this chemical, rather than enzymic, generation of luminal nitrogen oxides are now open for investigation and speculation. For example, the advice given to patients with irritable bowel syndrome includes instructions to eat more fibre. We speculate that, if this was in the form of green plant leaves with their nitrate-containing matrix capable of generating luminal NO, some amelioration of symptoms of spasm may be experienced. Certainly anecdotal evidence suggests that frequently these

patients' diets are as lacking in fruit and vegetables as in other forms of fibre.

Conclusion

With the advent of interest in NO, dietary nitrate by way of its sophisticated enterosalivary recirculation and formation of NO and other oxides of N has recently been the subject of renewed scrutiny. Until recently, nitrate in the diet was stigmatized by its chemical relationship to nitrosamines, through nitrite, and there was no appreciation that nitrate in food and the nitrite subsequently formed may be qualitatively different from directly administered nitrite or pre-formed contaminating nitrite in stored foods. With increasing recognition that dietary nitrate and its recirculation may have evolved to confer an evolutionary advantage by way of provision of a non-immune mechanism of host defence against ingested gastrointestinal pathogens, modulation of microcirculation, platelet activity and possibly even gastrointestinal motility, nitrate should be liberated from the stigma of association with nitrosamine chemistry.

We envisage a future where antibiotic therapy may not be the treatment of choice for intestinal infection and where increased understanding of the luminal metabolism of components of the diet and of saliva will lead to interventions that will prevent illness of the mouth, stomach, intestine and cardiovascular system, and probiotic therapy with organisms active in the upper intestinal lumen will assume a therapeutic role. This would have implications for the control of antibiotic resistance and, at least initially, would be most useful in animal husbandry where antibiotic resistance is a major problem. In man the therapeutic importance of this mechanism in preventing oral and gastrointestinal infection may be particularly important in the immunocompromised and those who are unavoidably exposed to contaminated food and water. For example, the diets used to sustain refugees are devoid of nitrate and thiocyanate whilst epidemic gastrointestinal infection devastates the camps and there are no current affordable solutions being investigated. Before nitrate and probiotic therapy can become reality, the understanding of basic physiological N chemistry needs to be more profound, the active transporter at the salivary gland must be further characterized, along with the kinetics of the microbial nitrate reductase enzyme. The role of this system in the weanling child and the safety of nitrate at this age also have to be established. This knowledge should then provide the basis for entirely new therapeutic interventions and very cheap preventative strategies easily transferred to the impoverished areas of the Third World where intestinal infection is a common cause of death. This knowledge may also lead to a much needed re-evaluation of the limitations on nitrate intake and hence increase agricultural production and redirect funds into water management.

Acknowledgements

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