the amount of isotope needed to enrich red blood cells in adults is prohibitive. Great care must be taken in the design of mineral bioavailability studies to ensure that all the above factors are taken into consideration.


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**The metabolism of dietary nitrates and nitrites**

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**Dietary exposure**

Diet constitutes an important source of exposure to nitrite and particularly nitrate. The major dietary source of nitrate is vegetables. Lettuce, spinach, celery and beetroot commonly contain more than 1 g of NO\textsubscript{3}/kg fresh weight and, depending on growing conditions (most notably temperature and light intensity, and to a lesser degree fertilizer use), may reach concentrations of 3–4 g/kg fresh weight [1]. Nitrate concentra-

tions in other vegetables, such as potatoes and cabbage, normally fall into the range 0.1–1 g/kg, but the amount consumed means that these make a substantial contribution to dietary intake. Nitrite occurs in plants at low concentrations, usually between 1 and 2 mg/kg fresh weight and rarely in excess of 10 mg/kg. However, potatoes have been reported to contain 2–60 mg/kg with a mean concentration of 19 mg/kg [2].

Estimates of mean nitrate intake range from
31 to 185 mg/day in various European countries, with vegetables supplying 80–85% [3]. In the U.K., the population mean intake of nitrate is 54 mg/day but in vegetarians this can be as high as 185–194 mg/day, emphasizing the importance of vegetables as the major source of nitrate [2]. The intake of nitrite is very much lower and averages 0.7–8.7 mg/day in various European countries, with both vegetables and cured meats being major sources.

**Endogenous synthesis**

In addition to dietary exposure there is considerable endogenous synthesis of nitrate in mammals, and, during rigorous exclusion of dietary nitrate, human volunteers excrete in urine about 1 mmol of NO₃⁻ per day [4], i.e. approximately the same amount as that provided by food. This endogenous nitrate arises from oxidation of NO, which is produced by a family of nitric oxide synthases, some constitutive and some inducible [5]. The constitutive enzyme produces NO for short periods (seconds) in response to intracellular messengers like bradykinin while the inducible forms produce much higher levels over periods of hours in response to immunostimulants. This synthesis of NO occurs in activated macrophages but has also been demonstrated in other cell types, including endothelial cells, neurons, neutrophils and hepatocytes [6–8], and is highly variable and much increased during infection.

The endogenous formation of nitrate independently of dietary sources has complicated the studies of the metabolism and pharmacokinetics of nitrate and nitrite, many of which can provide only quantitative or semi-quantitative data on their interconversion in vivo. The various pathways involved in the formation and metabolism of nitrate and nitrite are summarized in Figure 1. Clarification of the fate of orally administered nitrate/nitrite relative to endogenous synthesis may require the use of ¹⁵N-labelled sources but, although this may be feasible in mimicking exposure from drinking water, it is less so in relation to questions of relative bioavailability from vegetables.

**Pharmacokinetics and metabolism**

The pharmacokinetics and metabolism of nitrate and nitrite, and the potential formation of N-nitroso compounds in vivo, are obviously closely linked. Ingested nitrate is readily absorbed from the proximal small intestine [9,10] and rapidly equilibrates with body fluids. In rats, about 50% of an oral dose was detected in the carcass within 1 h, and in humans, peak levels in serum, saliva and urine were achieved within 1–3 h [11]. There is little absorption from the stomach in most species, although this has been reported from the rumen of cattle [12].

In humans and most laboratory animal species except the rat, nitrate is actively secreted in saliva in a dose-dependent manner [13,14], but Spiegelhalder et al. [15] were unable to detect an increase in salivary nitrate concentrations following ingestion of up to 54 mg of NO₃⁻. The active transport mechanism is common to iodide, thiocyanate and nitrate, in that order of affinity, and smokers who secrete elevated levels of thiocyanate have lower salivary concentrations of nitrate [16,17]. It has been estimated that, in humans, about 25% of an orally ingested dose of nitrate is secreted in saliva [15,18], but these estimates are confounded by the great inter-individual and diurnal variability in endogenous synthesis and secretion of nitrate.

Although the rat is reported not to possess the mechanism for active salivary secretion of NO₃⁻ (which has hindered extrapolation of experimental toxicological results to man), it does secrete circulating nitrate into other gastric and intestinal secretions by an active transport process [19], so that enterosystemic cycling of nitrate may occur in this species also. In the dog, after intravenous administration of NO₃⁻, in addition to strong salivary secretion, large amounts of NO₃⁻ were excreted in bile, confirming this pathway of excretion as well as oxidation of NO₃⁻. Nitrate appears in milk by a passive diffusion mechanism, and concentrations in human and canine milk did not exceed plasma levels after ingestion of a nitrate-containing meal [20].
After absorption and equilibrium in body fluids, nitrate is rapidly excreted in urine. In humans, independently of dose, about 65–70% of orally administered nitrate is excreted in urine. Excretion is maximal at about 5 h after dosage and essentially complete within 18 h [11]. The excretion follows first-order kinetics, and the elimination half-life has been estimated to be about 5 h [21]. Some metabolic conversion of nitrate clearly occurs (see Figure 1), since in humans about 3% of a dose of $^{15}$NO$_3$ appeared in urine as urea and ammonia [22]; in rats 11% of the dose appeared as urea and ammonia in urine and faeces [23].

Reduction of nitrate to nitrite in vivo may be effected by both enteric bacteria and mammalian nitrate reductase activity. Many species of micro-organisms resident in the oro-gastrointestinal tract possess nitrate reductase activity [24], and this enzyme has been detected in rat liver and intestinal mucosa, although at much lower activity [23]. From comparative studies in germ-free and conventional rats in our laboratory, we concluded that of the 40–50% of a dose of nitrate reduced to nitrite in conventional animals approximately half was effected by mammalian nitrate reductase [25]. However, Fritsch et al. [13] were unable to detect such a pathway in dogs, and reduction by the oro-gastrointestinal microflora appears to be the most important mechanism in mammals. However, the major site of conversion of nitrate into nitrite varies with species and is dependent on the sites of microbial colonization and absorption of nitrate.

Interestingly, the presence of nitrite in human oral saliva was first reported more than 55 years ago [26], but saliva taken directly from the salivary ducts of man or dog contains only nitrate, indicating that a significant amount of reduction occurs in the oral cavity [27]. This is attributed to a stable population of nitrate-reducing bacteria established at the base of the tongue. Stephany and Schuller [28] suggested that the salivary concentration of nitrate was directly related to the orally ingested dose of nitrate, and other workers have reached similar conclusions [15,29], but Tannenbaum et al. [18] produced data that suggest that the reduction process may be saturable at high intakes. On the basis of the (highly variable) salivary levels of nitrate and nitrite after oral ingestion of nitrate by humans, it has been estimated that, of the 25% of ingested nitrate secreted in saliva, 20% is reduced to nitrite (i.e. about 5% of the oral dose) and it appears that oral reduction of nitrate is the most important source of nitrite for man and most species that possess an active salivary secretory mechanism.

While the interest in oral reduction of nitrate to nitrite has largely centred around the possible involvement in formation of carcinogenic N-nitroso compounds, more recently a physiological role has been postulated in which the generation of nitrite and resultant antimicrobial activity protects against ingress of pathogens by this portal [30].

Gastric pH and hence bacterial populations are low in the stomach of rabbits, ferrets and healthy humans, and hence little further reduction of nitrate occurs at this site, and nitrite levels in gastric contents are usually low. Conversely, rats and dogs have a higher gastric pH, and bacterial colonization can occur with consequent further reduction of nitrate at this site [31]. In ruminants, the dense population of rumen microflora and relatively high pH make this a major site of reduction of orally ingested nitrate, and this leads to the well-documented intoxication (methaemoglobinemia) by the nitrite produced [12].

In humans subject to achlorhydria, bacterial colonization of the stomach can occur and the situation then more closely resembles the rat and dog. A strong correlation has been reported between gastric pH, bacterial colonization and gastric nitrite concentrations in humans over a pH range of 1–7 [32], and elevated levels as high as 5 mg/l have been reported in achlorhydria associated with pernicious anaemia or hypogammaglobulinaemia [33,34]. The situation in human neonates is less clear. It is commonly asserted that infants under 3 months of age may be highly susceptible to gastric nitrite production because they have little gastric acid production [35], but Agunod et al. [36] examined 12 infants aged between 12 h and 3 months and found only one with achlorhydria.

With regard to the pharmacokinetics and metabolism of nitrite per se, studies of absorption of orally administered nitrite have been made difficult because of its reactivity with dietary constituents/stomach contents and instability at gastric pH levels. Under simulated gastric conditions in vitro, nitrite disappeared rapidly at pH < 5 and the loss was accelerated by food components [31].

Absorption of nitrite in the rat appears
slower than that of nitrate, but some gastric absorption has been reported [31]. Intestinal absorption of nitrite is more rapid in the mouse than the rat [19]. Although there is a dearth of information on the absorption of nitrite in man, it can be inferred that it occurs from reports of methaemoglobinemia after exposure [37]. Nitrite is not normally detectable in tissues and body fluids of animals after oral administration because of its rapid oxidation to nitrate; after intravenous administration in mice or rabbits, rapid equilibration occurs in tissues within 5 min and nitrite concentrations in body fluids fall rapidly to low levels within 30 min [38]. The plasma half-life in the distribution phase was reported to be 48, 12 and 5 min in dogs, sheep and ponies respectively [39]. Nitrite is oxidized in blood by a coupled oxidation reaction with oxyhaemoglobin [40] in which methaemoglobin is produced (Figure 2), leading to the well-recognized acute toxicity of nitrite. The reaction rate between nitrite and haemoglobin is species-dependent; in man it is lower than in ruminants but higher than in pigs.

With regard to the methaemoglobinemia produced by nitrite, drinking water standards for nitrate have been drawn up on the basis of levels in drinking water (mainly from wells) associated with infantile methaemoglobinemia and an assumed threshold below which the risk is minimal. This was apparently based on the assumption that infants are more likely than adults to have a resident gastric microflora capable of reducing nitrate to nitrite and that the phenomenon of infantile methaemoglobinemia is, in part, a consequence of this. However, this must be questioned, as the endogenous synthesis of NO and subsequently of nitrite can rise dramatically during infantile gastroenteritis. In one study, hospitalized infants with a low nitrate intake (2–7 mg/day) had elevated blood nitrate and methaemoglobin levels associated with acute diarrhoea [24], and, in another case, a dyspeptic child had 72% methaemoglobinemia associated with a nitrate level in drinking water below 50 mg/l [41] while healthy babies tolerated intakes of up to 21 mg/kg body weight [42]. In these cases at least, it appears that nitrite of endogenous rather than dietary origin was involved in causing the methaemoglobinemia, together with the increased sensitivity of fetal-type haemoglobin to oxidation and immature low levels of methaemoglobin reductase in neonates.

A further concern relating to the metabolism of dietary nitrate and nitrite is the potential formation in vivo of carcinogenic N-nitroso compounds from nitrite, or the nitrosating species derived from this, N₂O₃ and N₂O₄, and dietary amines. This was first postulated more than 30 years ago and since then has been extensively studied using a number of approaches: (i) in vitro incubation of precursors under simulated oral and gastric conditions; (ii) analysis of saliva/gastric contents after administration of precursors; (iii) determination of specific or total N-nitroso compounds in body fluids or excreta after treatment with precursors; (iv) carcinogenicity studies after co-administration of nitrate/nitrite and amines or amides. These different approaches have been reviewed previously [1].

With regard to the first approach, many of the studies performed have used unrealistically high concentrations of nitrite and at best provide no more than an indication of a potential for nitrosation to occur. We found low but measurable amounts of four volatile nitrosamines after incubating luncheon meat (containing 30 mg of NO₃⁻/kg), egg and milk with human gastric juice containing 1.2 mM thiocyanate at pH 2 [43], but other workers incubating a wide range of foods under similar conditions (5–7 mg of NO₃⁻/l) were unable to detect any N-nitrosamines [44]. Analysis of gastric contents after consumption of meals containing nitrite have confirmed that volatile nitrosamines may be formed under similar conditions in vivo [43,45].

The measurement of urinary N-nitrosoproline has been widely used as a surrogate for total in vivo nitrosation, as it is non-carcinogenic, is excreted unchanged and occurs at low levels (2–7 μg per day) in subjects on a low-nitrate diet [46]. However, there are a number of reservations about the validity of this approach,
since, in the rat, a significant amount (40–90%) of urinary nitrosoproline was not derived from ingested [15N]nitrate [47] and in humans there was no correlation between nitrate intake and urinary excretion of N-nitrosoproline [48]. Furthermore, the basal excretion of non-diety N-nitrosoproline was unaffected by ascorbic acid or α-tocopherol whereas both these vitamins inhibited the synthesis of N-nitrosoproline from orally administered proline and [15N]nitrate [47], suggesting that there are at least two sites of nitrosation, i.e. gastric nitrosation under acidic conditions and nitrosation at other sites by a non-acid-catalysed mechanism inaccessible to ascorbic acid. The latter pathway probably involved nitrosating agents (N2O3 and N2O4) derived from NO; these are good nitrosating agents at or near neutral pH, and nitrosation by this route is higher in animals treated with *Escherichia coli* lipopolysaccharide [49].

Neither nitrate nor nitrite per se were carcinogenic in 2-year studies in rats and mice (although the absence of salivary secretion in rats must be taken into consideration), and epidemiological studies have failed consistently to demonstrate a correlation between nitrate exposure and cancer incidence in humans. Indeed vegetarians tend to have a lower cancer incidence than the general population, and, in one carcinogenicity study in rats, a lower incidence of mononuclear cell leukaemias was observed.

So, after more than 30 years of intensive research in this area, it has to be concluded that whether or not nitrosation occurs *in vivo* in high enough yields to pose a relevant risk to human health is still a controversial question [50].

Role of dietary flavonoids in protection against cancer and coronary heart disease
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Introduction
The weight of the epidemiological evidence for a protective effect of vegetables and fruits against cancer is impressive [1,2]. Various hypotheses have been proposed to explain this very consistent beneficial effect of an increased consumption of vegetables and fruits. An attractive hypothesis is that vegetables and fruits contain compounds that have a protective effect independent of that of known nutrients and micronutrients. This is supported by in vitro and in vivo studies which show that naturally occurring plant compounds may inhibit various stages in the cancer process [3]. In these studies flavonoids have also been studied extensively.

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. The basic structure of flavonoids allows a multitude of substitution patterns in the benzene rings A and B: phenolic hydroxyls, O-sugars, methoxy groups and sulphates. Variations can also occur in the heterocyclic ring C, giving rise to flavonols, flavones, catechins, flavanones, anthocyanidins.

Abbreviation used: LDL, low-density lipoprotein.
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