WHO FOOD ADDITIVES SERIES: 50

NITRATE
(and potential endogenous formation of *N*-nitroso compounds)

First draft prepared by

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1. EXPLANATION

Nitrate occurs in the environment, in air, food (particularly in vegetables and fruits) and water, and is produced endogenously. It is also used as a food additive, mainly as a preservative and anti-microbial agent. It is used in foods such as cheese and cheese products, raw and processed meats, edible casings, processed fish and fish products and spirits and liqueurs.

Nitrate was reviewed by the Committee at its sixth, eighth, seventeenth and forty-fourth meetings (Annex 1, references 6, 8, 32 and 116). At its sixth meeting, the Committee allocated an ADI of 0–5 mg/kg bw to this substance, expressed as sodium nitrate. This ADI was based on a NOEL of 500 mg/kg bw per day for body-weight gain at a higher dose in a long-term study in rats and a short-term study of toxicity in dogs, with a safety factor of 100. This ADI was retained by the Committee at its eighth and seventeenth meetings. After the latter meeting, numerous toxicological and epidemiological data became available, which were considered by the Committee at its forty-fourth meeting.

At that meeting, the Committee concluded that nitrite should also be considered, because nitrate is readily converted in humans to nitrite. The rat was considered an unsuitable animal model for assessing the toxicity of nitrate, as it does not convert nitrate to nitrite in a quantitatively similar way to humans. Nevertheless, as the data on the toxicity of nitrate in other animal species were considered limited, the toxicity of nitrite in rats and the conversion rate of nitrate to nitrite were also evaluated.

The Committee at its forty-fourth meeting concluded that nitrate itself was not genotoxic, and the results of studies of carcinogenicity with nitrate were negative except when extremely high doses of both nitrate and nitrosatable precursors were administered. The available epidemiological data were considered to provide no evidence for an association between exposure of humans to nitrite and the risk for cancer. On the basis of this information, the NOEL of 370 mg/kg bw per day, expressed as nitrate ion, in a long-term study in rats was considered to be the most appropriate for the safety evaluation. When the proportion of nitrate converted to nitrite in humans was taken as 5% for the average individual and 20% for those with a high level of conversion, and when the NOEL for nitrite (6 mg/kg bw per day, expressed as nitrite ion) was used to calculate the 'transposed' NOEL for nitrate, expressed as nitrate ion, these values were estimated to be 160 and 40 mg/kg bw per day for average and high responders, respectively. As these figures were derived in part from data on human pharmacokinetics, use of a safety factor of less than 100 was considered to be justified.

On the basis of the NOEL of 370 mg/kg bw per day, expressed as nitrate ion, and a safety factor of 100, an ADI of 0–5 mg/kg bw expressed as sodium nitrate or 0–3.7 mg/kg bw expressed as nitrate ion was allocated. On the basis of the 'transposed' NOEL for nitrate of 160 mg/kg bw per day for normally responding persons (5% rate of conversion) and a safety factor of 50, an ADI of 0–3.2 mg/kg bw, expressed as nitrate ion, could be allocated. These two methods of deriving the ADI for nitrate thus resulted in similar figures, and the Committee at its forty-fourth meeting therefore retained the previously established ADI of 0–3.7 mg/kg bw, expressed as nitrate ion.

The Committee at its present meeting reviewed data relevant to the evaluation of nitrate which had become available since its forty-fourth meeting. Studies illustrating the relevance and severity of certain effects and other supporting data were also considered. New information on metabolism and toxicokinetics were included. Data on the putative health benefits of nitrate were not assessed, as this is not a safety issue and is therefore not within the purview of the Committee.

2. BIOLOGICAL DATA

2.1. Biochemical aspects

2.1.1 Absorption, distribution, metabolism and excretion

The following is based on a review of the literature (Walker, 1996), which gives a clear picture of the pharamacokinetics and metabolism of nitrate.

Ingested nitrate is readily absorbed from the proximal small intestine (Fritsch et al., 1979; Balish et al., 1981) and rapidly equilibrates with body fluids. In rats, about 50% of an oral dose was detected in the carcass within 1 h; in humans, peak levels were achieved in serum, saliva and urine within 1–3 h (Bartholomew & Hill, 1984). There is little absorption from the stomach in most species, although some has been reported from the rumen of cattle (Wright & Davison, 1964).

In humans and most laboratory animal species except the rat, nitrate is actively secreted in saliva in a dose-dependent manner (Cohen & Myant, 1959; Fritsch et al., 1985), but

Spiegelhalder et al. (1976) were unable to detect an increase in salivary nitrate concentration in humans after ingestion of up to 54 mg of nitrate.

The active transport mechanism is common to iodide, thiocyanate and nitrate, in that order of affinity, and smokers who secrete high levels of thiocyanate have lower salivary concentrations of nitrate (Forman et al., 1985). It has been estimated that, in humans, about 25% of an orally ingested dose of nitrate is secreted in saliva (Spiegelhalder et al., 1976; Tannenbaum et al ., 1976), but these estimates are confounded by the great interindividual and diurnal variability in endogenous synthesis and secretion of nitrite. Although rats are reported not to have the necessary mechanism for active salivary secretion of nitrate (hindering extrapolation of toxicological studies in rats to human), they do secrete circulating nitrate into other gastric and intestinal fluids by an active transport system (Witter & Balish, 1979), so that enterosystemic cycling of nitrate may occur in this species also. In dogs given nitrate intravenously, extensive salivary secretion and extensive biliary secreation were seen, confirming this pathway of excretion for both nitrite and nitrate. Nitrate appears in milk by a passive diffusion mechanism, and the concentrations in human and canine milk did not exceed the plasma levels after ingestion of a nitrate-containing meal (Green et al., 1982). After absorption and equilibrium in body fluids, nitrate is rapidly excreted in urine. In humans, about 65–70% of any orally administered dose of nitrate was excreted in urine. Excretion was maximal about 5 h after dosage and essentially complete within 18 h (Bartholomew & Hill, 1984). The excretion followed first-order kinetics, and the elimination half-life was estimated to be about 5 h (Green et al., 1981).

Some metabolic conversion of nitrate clearly occurs, as, in humans, about 3% of a dose of $^{15}NO_3^-$ appeared in the urine as urea and ammonia (Wagner et al., 1983); in rats, 1% of a dose appeared in urea, and ammonia was found in urine and faeces (Schultz et al., 1985). Nitrate can be reduced to nitrite by both enteric bacteria and mammalian nitrate reductase activity. Many species of microorganisms resident in the gastrointestinal tract have nitrate reductase activity (Hegesh & Shiloah, 1982), and this enzyme has been detected in rat liver and intestinal mucosa, although with much lower activity (Schultz et al., 1985).

Comparative studies with germ-free and conventional rats indicated that about half of the 40–50% reduction of a dose of nitrate to nitrite in conventional animals was effected by mammalian nitrate reductase (Ward et al., 1986). However, Fritsch et al. (1985) were unable to detect this pathway in dogs and reported that reduction by the oral and gastrointestinal microflora was the most important mechanism in mammals. The major site of conversion of nitrate into nitrite varies by species and depends on the sites of microbial colonization and absorption of nitrate. Interestingly, the presence of nitrite in human saliva was first reported more than 55 years ago (Varady & Szanto, 1940), but saliva taken directly from the salivary ducts of humans or dogs contains only nitrate, indicating that a significant amount of reduction occurs in the oral cavity (Muramatsu et al., 1979). This is attributed to a stable population of nitrate-reducing bacteria established at the base of the tongue. Stephany & Schuller (1978) suggested that the salivary concentration of nitrite was directly related to the orally ingested dose of nitrate, and other workers reached similar conclusions (Harada et al., 1975; Spiegelhalder et al., 1976). However, Tannenbaum et al. (1976) suggested that the reduction process is saturable at high intakes. On the basis of the 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). Oral reduction of nitrate is thus the most important source of nitrite for humans and most species that have an active salivary secretory mechanism.

The gastric pH, and hence bacterial populations, are low in the stomachs of rabbits, ferrets and healthy humans. Hence little further reduction of nitrate occurs at this site, and the nitrite concentrations 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 (Mirvish, 1975). In ruminants, the dense population of rumen microflora and the relatively high pH make this a major site of reduction of orally ingested nitrate, leading to well-documented methaemoglobinaemia induced by the nitrite produced. In humans subject to achlorhydria, bacterial colonization of the stomach can occur, and the situation then more closely resembles that in the rat and the dog. A strong correlation has been reported between gastric pH, bacterial colonization and gastric nitrite concentration in humans over a pH range of 1–7 (Müller et al., 1984), and concentrations as high as 5 mg/l have been reported in patients with achlorhydria associated with pernicious anaemia or hypogammaglobulinaemia (Ruddell et al., 1976; Dolby et al., 1984). The situation in human neonates is less clear. It is commonly asserted that infants under 3 months of age are highly susceptible to gastric nitrite production, because they produce little gastric acid, but Agunod et al. (1969), who examined infants aged between 12 h and 3 months, found only one with achlorhydria (Walker, 1996).

An open, randomized, cross-over pilot study was performed to investigate the toxicokinetics of nitrate and nitrite after an oral dose of sodium nitrate at 10 mg/kg bw in eight healthy volunteers (four men and four women) with normal gastric pH or gastric pH increased artificially with omeprazole (40 mg/day) starting 15 days before nitrate administration. Blood pressure was recorded for 3.5 h, and the per cent methaemoglobin was determined for 10 h after administration of sodium nitrate. Bacterial growth and gastric pH were determined just before nitrate administration, and the gastric juice pH was measured continuously for 3 h thereafter. Serial samples of gastric juice, plasma and saliva were collected for 24 h after administration of nitrate. The volume of all urine produced during 10 h after administration was determined; part of every sample was stored, and all samples were analysed for nitrate and nitrite.

None of the volunteers reported adverse effects attributable to intake of sodium nitrate. Blood pressure and per cent methaemoglobin were not affected. Gastric pH correlated positively with omeprazole treatment, but the number of nitrate-reducing bacteria did not. Nevertheless, the nitrite concentration in the gastric juice was approximately sixfold higher after nitrate administration combined with omeprazole treatment than after only nitrate. Nitrate was absorbed rapidly, the concentration in plasma increasing within 10 min. The half-life of nitrate in plasma was about 6.5 h. The concentration of nitrite in plasma did not change after nitrate administration. The cumulative salivary nitrate excretion over 24 h, expressed as a percentage of ingested nitrate dose, was 28%. The amount of nitrite formed in the saliva, expressed as a percentage of the nitrate dose ingested, was 8%. Approximately 70% of the nitrate dose was excreted in urine within 10 h of dosing. Omeprazole treatment did not affect the nitrate concentrations in plasma, saliva or gastric juice, the nitrite concentrations in plasma or saliva, the per cent methaemoglobin or blood pressure. Thus, increased gastric pH had no significant effect on systemic nitrate or nitrite after an oral dose of sodium nitrate at twice the ADI of nitrate (Colbers et al., 1996).

A feasibility study on the oral bioavailability of nitrate from vegetables was conducted in healthy volunteers. Preparations of beetroot, lettuce and spinach were fed to groups of six volunteers, and the rate of absorption of nitrate from the gastrointestinal tract and the concentrations of nitrate in plasma were determined. After an overnight fast, volunteers received a single dose of 300 g of vegetables, and plasma nitrate and nitrite concentrations

were determined frequently for 9 h after treatment. There was evidence that the vegetable was homogeneously mixed in the test diet. The beetroot portions administered to the volunteers contained an average of 570 mg of nitrate, the lettuce portions contained 740 mg and the spinach portions contained 540 mg. The nitrate in the vegetables was rapidly absorbed from the gastrointestinal tract, maximum plasma nitrate concentrations of 21–44 mg/l being observed 1–1.5 h after treatment. Small amounts of nitrite (0.1–0.2 mg/l) are detected under normal circumstances at the lower limit of quantification of nitrite in plasma, and intake of vegetables rich in nitrate did not increase the plasma nitrite concentration, which remained at < 0.3 mg/l in all volunteers (Kortboyer et al., 1998).

Another study was conducted to determine the oral bioavailability of nitrate from spinach, lettice and beetroot. In a cross-over design with four test periods, six men and six women were fasted and then given 500 mg of sodium nitrate (containing 360 mg of nitrate ion) intravenously, 300 g of spinach (containing 560 mg of nitrate ion), 300 g of fresh lettuce (containing 1000 mg of nitrate) or 300 g of cooked beetroot (containing 640 mg of nitrate ion). A wash-out period of at least 1 week was maintained between test periods. The bioavailability of nitrate from the vegetables was calculated with and without endogenous nitrate production by comparing nitrate concentrations in blood after vegetable administration and after intravenous administration of nitrate. The elimination half-life of nitrate from plasma was 6–7 h. The bioavailability of nitrate with correction for endogenous nitrate production was 98% ± 12% for spinach, 110% ± 14% for lettuce and 110% ± 15% for beetroot. The bioavailability without correction for endogenous nitrate production was 91% ± 10% for spinach, 89% ± 13% for lettuce and 93% ± 12% for beetroot. Thus, the bioavailability of nitrate from spinach, lettuce and beetroot was high and not significantly different from that of nitrate in drinking-water (Lambers et al., 2000a)

2.1.2 Physiologically based pharmacokinetic and mathematical models

The available assessment of risk to humans of exposure to nitrate was based on an evaluation of toxicity in rats. This requires reconsideration as, in humans, nitrite is formed by bacterial fermentation of nitrate secreted from blood into saliva, but this process is absent in the rat (Tannenbaum et al., 1979; Til et al., 1988). As nitrite is more toxic than nitrate, the toxicity of nitrite may supercede that of nitrate in humans. A nitrate–nitrite conversion in the order of 2% has already been seen to have this effect. The amount of nitrite formed from ingested nitrate should therefore be incorporated into the risk assessment of human exposure to nitrate.

The formation of nitrite in humans is complex, involving uptake of nitrate from food and or water, endogenous synthesis of nitrate, secretion of nitrate from blood into saliva, conversion of nitrate to nitrite by bacteria present in saliva and reconversion of nitrite to nitrate in blood. These processes were incorporated in a model of the toxicokinetics of nitrate and nitrite, which was calibrated with data on the toxicokinetics of nitrate and nitrite in volunteers (Wagner et al., 1983). According to the model, the average adult synthesizes nitrate at 120 mg/day, 32–60% of orally administered nitrate is secreted from blood into saliva, and 13–22% of the salivary nitrate is converted into nitrite. The nitrate–nitrite conversion rate in humans was determined to be 7–9%. The model was also used to estimate the formation of nitrite in an average adult after single and repeated (once every 24 h) doses of nitrate at its ADI (0–3.7 mg/kg bw). This calculation resulted in an exposure to nitrite of 0.27–0.36 mg/kg bw per day, which significantly exceeds the ADI for nitrite (0–0.06 mg/kg bw). Of this, 31–41% was estimated to have originated from endogenously synthesized nitrate. The calculated values for nitrite intake should be interpreted within the framework of the assumptions underlying the

model, as the effects of food intake (stimulation of salivary nitrite formation versus detrimental effects on nitrite in the stomach) were not included. Furthermore, in calculating human nitrite intake, a bioavailability of 100% was assumed for administered nitrate. The risk assessment of human nitrate intake should thus be based on a toxicological evaluation of the nitrite formed from ingested nitrate (Zeilmaker et al., 1996).

In order to quantify the kinetics of nitrate and nitrite in humans, a model was developed which comprises absorption of nitrate from drinking-water and vegetables, secretion of nitrate from blood into saliva, conversion of nitrate to nitrite, absorption of nitrite and interaction of nitrite with haemoglobin, yielding methaemoglobin and nitrate. The model contains 16 parameters, seven of which were obtained from the literature, while the remaining nine parameters (rate of endogenous nitrate formation, rate constant for the reaction of nitrite with haemoglobin, parameters of methaemoglobin repair in the blood, rate constants for the secretion of nitrate from blood into saliva, conversion of nitrate to nitrite in saliva, a stoichometric constant for the reaction of nitrite with haemoglobin, the volume of the central nitrite compartment and the absorption rate constant of nitrite) were obtained by fitting the model to various experimental data sets. The studies addressed nitrite-induced methaemoglobin formation in human haemolysates and intact human erythrocytes *in vitro* and various studies in which volunteers were given nitrate and nitrite orally or intravenously. According to the model, a single dose of nitrate from vegetables at up to 44 mg/kg bw would not induce clinical methaemoglobinaemia, whereas the average long-term intake of adults is < 3.7 mg/kg bw per day. Doses of 88–270 mg/kg bw progressively induced clinical methaemoglobinaemia and clinical hypoxia. In the model, lethal toxicity was predicted to occur at doses > 440 mg/kg bw. At doses up to 620 mg/kg bw, peak methaemoglobin concentrations were efficiently nullified by repair activity, so that the methaemoglobin concentration retruned to the background level within 24 h of administration of nitrate. Clear accumulation of methaemoglobin was found after repeated administration of a slightly higher dose of nitrate, 800 mg/kg bw, indicating rapid saturation of methaemoglobin repair activity at this dose.

The model was scaled from adults to neonates by adjusting the blood volume and the volumes of the central nitrate and nitrite compartments. In order to mimic the situation in neonates, partial methaemoglobin repair deficiency was simulated by assuming the methaemoglobin reductase activity to be 10% of that in adults. Repeated intake of nitrate in drinking-water at doses > 44 mg/kg bw was predicted to induce methaemoglobin accumulation. This value can be compared with the daily intake for a 4-kg neonate of 6.2 mg/kg bw from drinking 1 l of water containing nitrate at 25 mg/l. Exposure of patients with inflammatory disease to nitrite was simulated as continuous, i.e. 24-h, intravenous exposure to nitrite. In this case, saturation of methaemoglobin repair occurred at doses > 0.23 mmol/24 h (2.7 mg/kg bw), and exposure was up to 0.47 mmol/24 h (5.5 mg/kg bw). It was concluded that the intake by healthy adults of nitrate from food and/or drinking-water has negligible effects on methaemoglobin formation but may significantly affect methaemoglobin formation in neonates with inflammatory disease (Zeilmaker et al., 2002).

2.1.3 Biotransformation

(a) Humans

The effectiveness of several mouthwash solutions and toothpastes with antibacterial constituents in reducing the conversion of nitrate into nitrite in the oral cavity was tested in 15 male and female volunteers aged 22–40 years. The mean per cent salivary nitrate that was

reduced to nitrite after ingestion of 240 mg (3.8 mmol) of nitrate was 16 ± 6.2%. Use of an antiseptic mouthwash containing the active antibacterial constituent chlorhexidine resulted in a drastic decrease in the mean per cent reduced nitrate, to 0.9 ± 0.8%. Mouthwash solutions containing the antibacterial component triclosan or the antimicrobial enzymes amyloglucosidase and glucose oxidase did not affect the reduction of nitrate to nitrite. A toothpaste containing triclosan and zinc citrate with synergistic antiplaque activity also had no effect. Use of a pH-regulation chewing-gum raised the pH of the oral cavity from 6.8 to 7.3. By 30 min after nitrate ingestion, this rise was accompanied by a significant increase in the salivary nitrite concentration (van Maanen et al., 1996a).

(b) Methaemoglobin formation

Nitrite resulting from nitrate is oxidized in blood by a coupled oxidation reaction with oxyhaemoglobin (Smith & Beutler, 1966) in which methaemoglobin is produced, leading to the well-recognized acute toxicity of nitrite. The reaction rate between nitrite and haemoglobin is species-dependent: in humans, it is lower than that in ruminants but higher than that in pigs. In view of the methaemoglobinaemia induced by nitrite, many standards for nitrate in drinking-water have been drawn up on the basis of the levels (mainly in wells) associated with infantile methaemoglobinaemia and an assumed threshold below which the risk is minimal. This is apparently based on the assumption that infants are more likely than adults to have resident gastric microflora capable of reducing nitrate to nitrite. However, endogenous synthesis of nitric oxide 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 raised blood nitrate and methaemoglobin concentrations associated with acute diarrhoea (Hegesh & Shiloah, 1982). In another case, a dyspeptic child had 72% methaemoglobinaemia associated with a nitrate concentration in drinking-water of < 50 mg/l (Thal et al., 1961), while healthy infants tolerated intakes of up to 21 mg/kg bw (Kubler, 1958). In these cases at least, the endogenously formed nitrite appeared to have caused the methaemoglobinaemia, together with increased sensitivity of fetal-type haemoglobin to oxidation and the low levels of methaemo-globin reductase in neonates (Walker, 1996).

Two infants became ill after being fed formula that was reconstituted with water from private wells. Water samples were collected and analysed chemically and microbiologically. In the first case, the well water was considered bacteriologically safe and the nitrate intake was 9 mg/kg bw as nitrogen(equivalent to 78 mg/kg bw as nitrate ion). In the second case, the water was found to contain Escherichia coli, and the nitrate concentration was 27 mg/kg bw as nitrogen (equivalent to 93 mg/kg bw as nitrate ion). The second infant had a methaemoglobin concentration of 91% on arrival at hospital, whereas that of the first had returned to < 2%, which is within the normal range (Knobeloch et al., 2000).

An epidemiological investigation was undertaken to assess the prevalence of methaemoglobinaemia in areas with a high nitrate concentration in drinking-water. Five areas were selected, with average nitrate concentrations of 26, 45, 95, 220 and 460 mg/l of drinking-water, as nitrate ion. A total of 178 persons (about 30 per group), matched for age and weight, who constituted 10% of the total population of each of these areas were selected and divided into five age groups. Detailed histories were taken of the selected populations, medical examinations were conducted, and blood samples taken to ascertain the per cent methaemoglobin. The data were analysed to verify any relationship between nitrate concentration and methaemoglo-binaemia. A high nitrate concentration caused severe methaemoglobinaemia (7–27%) in all groups, especially in the age groups < 1 year and > 18

years. The lower level in the age group 1–18 years is probably due to better reserves of cytochrome b_5 reductase activity and its adaptation to increasing nitrate concentration in water. High levels of methaemoglobin were seen with nitrate contents as low as 26 mg/l and as high as 460 mg/l. The highest per cent methaemoglobin was observed in infants < 1 year of age (Gupta et al., 2000)

In Finland, the Ministry of Social Affairs and Health has regulated nitrate in drinking-water, with an upper limit of 25 mg/l. About 18% of people use well water for drinking, and as many as one-fiftieth of the wells in some areas do not meet the national quality standard for nitrate. Nevertheless, the correlation between per cent methaemoglobin and nitrate concentrations in drinking-water of 25–50 mg/l was poor (Suvanto & Pohjola, 1995).

Although ingestion of nitrates in drinking-water has been considered to be the primary cause of acquired methaemoglobinaemia, a mechanism whereby gastrointestinal inflammation leads to methaemoglobinaemia has been established. It is against this background that recent research and a review of cases offer a more complex picture of the causes of infantile methaemoglobinaemia. If this is indeed so, current limits on allowable levels of nitrates in drinking-water, which are based solely on the threat of infantile methaemoglobinaemia, may be unnecessarily strict (Avery, 1999, 2001).

(c) Endogenous synthesis of nitrate

In addition to dietary intake, there is considerable endogenous synthesis of nitrate in mammals. Even with rigorous exclusion of dietary nitrate, human volunteers excreted about 1 mmol of nitrate per day in urine (Wishnok et al., 1995), i.e. approxi-mately the same amount as that provided by food. Endogenous nitrate arises from oxidation of nitric oxide, which is produced by a family of synthases, some constitutive and some inducible (Marletta, 1988). The constitutive enzyme produces nitric oxide 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 nitric oxide occurs in activated macrophages but has also been demonstrated in other cell types, including endothelial cells, neurones, neutrophils and hepatocytes (Ignarro, 1987; Lancaster, 1992), and is highly variable and much increased during infection. Endogenous formation of nitrate independently of dietary sources has complicated 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* (Walker, 1996).

2.1.4 Endogenous formation of *N*-nitroso compounds

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 derived nitrosating species, N_2O_3 and N_2O_4, and dietary amines. This reaction was first postulated more than 30 years ago and has since been studied extensively, by a number of approaches: incubation of precursors under simulated oral and gastric conditions *in vitro*, analysis of saliva and gastric contents after administration of precursors, determination of specific or total *N*-nitroso compounds in body fluids or excreta after treatment with precursors, and studies of carcinogenicity after co-administration of nitrate or nitrite and amines or amides. Many of the studies performed with the first approach involved unrealistically high concentrations of nitrite and, at best, provided no more than an indication of potential nitrosation. Low but measurable amounts of four volatile nitrosamines were found after incubation of luncheon

meat (containing nitrate at 30 mg/kg), egg and milk with human gastric juice containing thiocyanate at a concentration of 1.2 mmol/l at pH 2 (Walthers et al., 1979). However, other workers who incubated a wide range of foods under similar conditions (containing nitrate at 5–7 mg/l) were unable to detect any *N*-nitrosamines (Groenen et al., 1982). Analysis of gastric contents after consumption of meals containing nitrite indicated that volatile nitrosamines may be formed under similar conditions *in vivo* (Walters et al., 1979; Groenen et al., 1985).

Measurement of urinary *N*-nitrosoproline (NPRO) has been widely used as a surrogate for nitrosation *in vivo*, as this compound is non-carcinogenic, is excreted unchanged and occurs at low levels (2–7 μg/day) in persons on a low-nitrate diet (Ohshima & Bartsch, 1981). Although NPRO itself is not carcinogenic, the NPRO test measures the potential for intragastric formation of carcinogenic nitrosamines in humans. Nitrate and L-proline are administered to volunteers, and NPRO is produced by an acid-catalysed reaction of proline with nitrate-derived nitrite in the stomach. It is then absorbed and excreted in urine, which is analysed for NPRO. A number of reservations have been raised about the validity of this approach, since, in rats, a significant amount (40–90%) of urinary NPRO was not derived from ingested [^{15}N]nitrate, and there was no correlation in humans between nitrate intake and urinary excretion of NPRO (Tannenbaum, 1987). Furthermore, basal excretion of non-dietary NPRO was unaffected by ascorbic acid or *alpha*-tocopherol, whereas both these vitamins inhibited synthesis of NPRO from orally administered proline and [^{15}N]nitrate (Wagner & Tannenbaum, 1985), suggesting that there are at least two sites of nitrosation and that gastric nitrosation occurs in an acid-catalysed mechanism that is inaccessible to ascorbic acid. The last pathway probably involves nitrosating agents (N_2O_3 and N_2O_4) derived from nitric oxide (Walker, 1996).

The endogenous formation of volatile *N*-nitrosamines was studied after intake of nitrate at the ADI in combination with an amine-rich diet consisting of fishmeal rich in amines as nitrosatable precursors. Twenty-five women (mean age, 23 years; mean weight, 60 kg), who were non-smokers and who were not using medicines or vitamin preparations, ate the fishmeal with nitrate at the ADI for 7 consecutive days. They received a diet low in nitrate for 1 week before and 1 week after the test week. They agreed to donate saliva samples, collect 24-h urine samples and answer a questionnaire on food consumption and life style. Nitrate intake at the ADI (100 ml of a solution of sodium nitrate at 2800 mg/l) resulted in a significant increase in mean salivary nitrate and nitrite concentrations. The mean urinary nitrate excretion increased from 76 mg/24 h in the first week to 190 and 160 mg/24 h on days 1–3 and 4–7 during the test week, followed by a decline to 77 mg/24 h in the second control week. When the urine samples were analysed for volatile *N*-nitrosamines, both NDMA and *N*-nitrosopiperidine were detected. The mean urinary excretion increased significantly from 290 ng/24 h in the control week to 870 and 640 ng/24 h on days 1–3 and 4–7 during the test week and declined to 380 ng/24 h in the second control week. Excretion of *N*-nitrosopiperidine was not directly related to nitrate intake or to the composition of the diet. Nitrate excretion and NDMA excretion were significantly correlated, as were salivary nitrate and nitrite concentration and NDMA excretion (Vermeer et al., 1998).

The effect of certain dietary and other factors on the concentrations of urinary NPRO was studied in healthy adults (mostly men) given a diet low in preformed NPRO, nitrate and proline for 5 days and ascorbic acid on days 4 and 5, when tests were conducted. In the standard test, the volunteers took 400 mg of nitrate at 11:00, and at 12:00 they ate a standard 700-calorie meal containing 500 mg of proline. Urine was collected for 24 h, and samples were analysed for NPRO. This standard test yielded 26 ± 2 (mean ± SE) nmol of NPRO,

whereas 5 ± 1 nmol of NPRO were found when proline was taken alone. In variations on the standard test, the NPRO yield was not significantly affected by the sex of the volunteers, the time at which the standard meal was eaten, the size of the meal or drinking extra water after the meal. Doses of 100 and 200 mg of nitrate had lesser effects on NPRO yield than did the dose of 400 mg. One gram of ascorbic acid given 5 or 2 h before, with or 1 or 2 h after the meal with proline inhibited NPRO formation by 0, 71, 71, 67 and 19%, respectively. Chewing gum or tobacco for 2–3 h after the test meal did not increase NPRO formation or the salivary nitrate concentration, but the salivary nitrite concentration was reduced, especially when gum was chewed (Mirvish et al., 1995).

2.2 Toxicological studies

2.2.1 Acute toxicity

The acute toxic effects of nitrate were studied in groups of five goats aged 1.5–2.0 years, which received potassium nitrate at a dose of 1.3 g/kg bw, equivalent to 0.66 g/kg bw as nitrate ion. Partial anorexia, mild depression, frequent muscle tremor, incoordination, dyspnoea and brown discolouration of visible mucous membranes appeared from 2 h onwards, followed terminally by recumbency, salivation and colonic convulsions. The irreversible signs of toxicity appeared at about 5.8 h, when the animals were killed and organs and tissues sampled for histopathological examination. The haematological and biochemical alterations included a decrease in haemoglobin, a marked increase in the methaemoglobin, nitrate and nitrite concentrations of plasma, urine and cerebrospinal fluid and in blood glucose, with significant increases in ammonia nitrogen in rumen liquor, cholesterol, urea nitrogen, creatinine and aspartate aminotransferase activity in serum. A diphenylamine blue test on blood, plasma, urine, cerebrospinal fluid, rumen liquor and aqueous humour revealed strong positive reactions at the peak of toxicity. The cerebrospinal fluid nitrite appeared to be a dependable diagnostic value, as it truly reflected the toxicity of nitrate. Histopathological examination revealed changes in the kidneys (tubular changes with massive degeneration of the epithelium), liver (degenerative changes with congestion, focal haemorrhage and extensive empty dilatation of the central vein), intestine (infiltration onto mononuclear cells in mucosa and degenerative villi), lungs (congestion and haemorrhages of alveoli), heart (degenerative changes in endo- and myocardium with haemorrhage), lymph nodes (depletion of lymphocytes) and urinary bladder (lamina propria infiltrated by lymphocytes). Electrocardiography during induced acute toxicity showed marked tachycardia, an increased duration of the mean QRS complex and deviation in the configuration of the ST segment. These changes were a result of significant myocardial hypoxia due to marked methaemoglobinaemia (Mondal & Pandey, 1999; Mondal et al., 1999a, 2000).

2.2.2 Short-term studies of toxicity

In a 32–day experiment, groups of five goats aged 1.5–2.0 years were given potassium nitrate orally at a dose of 0 or 4 mg/kg bw per day, equivalent to 2.4 mg/kg bw as nitrate ion. The animals remained apparently normal, except for development of dullness and partial anorexia from day 22. The haematological and biochemical alterations included a significant decrease in haemoglobin concentration and increases in methaemoglobin, nitrate and nitrite concentrations in plasma and urine, in urea nitrogen and in aspartate aminotransferase activity in serum. The morphological changes seen in two animals killed on day 32 were mild

degeneration and congestion and haemorrhages in visceral organs. The authors concluded that there had been no cumulative effect of prolonged nitrate intake (Mondal et al., 1999b).

2.2.3 Reproductive toxicity

In an overview and evaluation of the available information on the health effects of nitrate and nitrite (Fan et al., 1987), an association was suggested between maternal ingestion of nitrate from drinking-water and developmental effects in their children, although no cause-and-effect relationship could be established. Studies in experimental animals have shown reproductive toxicity associated with intake of high levels of nitrate and nitrite, which are not likely to be encountered in drinking-water. No teratogenic effects were observed in rats, mice, rabbits or hamsters. Several cases of methaemoglobinaemis were reported among infants in the USA who drank water containing nitrate at concentrations higher than the current 'maximum contaminant level' of 45 mg/l. The uncertainties in the database have been discussed (Fan & Steinberg, 1996).

The effects of intake of nitrate on a number of biochemical and endocrine parameters and their impact on reproductive function were studied in groups of five feeder bulls aged 16–18 months given potassium nitrate or a control diet. The bulls were tested 30 days before administration of nitrate, for 30 days during administration and for 30 days afterwards. The initial dose of nitrate was 100 g/day (equivalent to 160 g/day as nitrate ion), which was increased at weekly intervals by 50 g up to 250 g (equivalent to 150 g/day as nitrate ion). Administration of nitrate resulted in a significant ($p < 0.01$) increase in methaemoglobin concentration, a significant ($p < 0.01$) increase in the serum concentration of bile acids and a prolonged biological half-life of progesterone, suggesting impairment of liver metabolism. Intake of nitrate also resulted in a significant ($p < 0.05$) increase in cortisol concentration during and after administration and depressed thyroid gland activity, as seen from a significant ($p = 0.05$) decrease in thyroxin concentration during administration. Non-detectable levels (< 0.001 µg/ml) of thyrotropin after administration indicated suppression of hypothalamic function. Effects of nitrate on the function of Leydig cells during and particularly after administration were apparent from weakened testicular responses to treatment with gonadotrophin. Analysis of seminal plasma revealed a significant ($p < 0.01$) increase in total acid phosphatase activity and a significant decrease in the concentration of fructose. Intake of nitrate also reduced sperm motility. While no difference was found in the frequency of primary morphological abnormalities, the number of secondary abnormalities rose by 115% in the post-administration period, suggesting damaged membrane integrity. Histological examination revealed degenerative lesions in cells of the spermiocyte and spermatid layers (Zraly et al., 1997).

The effect of nitrate on reproductive function was studied in groups of 23–24 sheep (aged 3.5–4.0 years) fed a diet containing potassium nitrate. In a preliminary experiment, a dose of 20 mg/kg bw (equivalent to 11 mg/kg bw as nitrate ion) induced a reliable per cent decrease (by 32%) in the number of sheep in estrus ($p < 0.05$). Concentrations in the diet resulting in a dose of 0.5, 1, 5 or 10 mg/kg bw per day for 48 days (equivalent to 0.27, 0.54, 2.7 and 5.4 mg/kg bw as nitrate ion) did not affect cyclic sexual behaviour; however, the fertility and gestation rates of sheep fed potassium nitrate were considerably lower (by 36% at 5 mg/kg bw per day and 33% at 10 mg/kg bw per day) than in the control group (Nestorova et al., 1997).

2.2.4 Special studies: Effects on adrenal and thyroid glands

Hypertrophy of the zona glomerulosa of the adrenals of rats was reported after administration of low doses of nitrite for 90 days; the effect was considered to be due to its conversion to nitrate. A study was therefore conducted to compare the effects of nitrate and nitrite on the zona glomerulosa. Three groups of 10 male Wistar rats were given drinking-water containing potassium chloride (control), potassium nitrite or potassium nitrate at a concentration of 36 mmol/l for 90 days. The body-weight gain of rats given nitrite or nitrate was slightly slower than that of controls, but no differences in food intake per kilogram body weight were observed between the three groups. The water intake of the group given nitrite was statistically significantly lower than that of the other two groups. The rats receiving nitrite appeared cyanotic during the first month of treatment but not thereafter, perhaps because the water intake was greater during the first month. At the end of the observation period, the concentrations of methaemoglobin and nitrite in blood were significantly increased; the nitrate concentration in plasma in the groups given nitrite or nitrate groups were similar but were higher than those of controls. Treatment with nitrite or nitrate had no consistent effect on the concentrations of thyroxin, free thyroxin, thyroid stimulating hormones, adrenocorticotrophic hormone, corticosterone or aldosterone in blood. Microscopic examination revealed slight hypertrophy of the adrenal zona glomerulosa in all rats given nitrite and minimal hypertrophy in 2/10 rats given nitrate. The results of morphometric analyses of the adrenals were in line with those of microscopic examination. In the rats given nitrite, the fraction of the surface area of the zona glomerulosa in median sections was significantly greater than that in the controls or rats given nitrate. The minimal hypertrophy of the adrenal zona glomerulosa observed occasionally in rats given nitrate was barely detectable by morphometric analysis. It was concluded that nitrate ion does not play a role in the etiology of hypertrophy of the zona glomerulosa of the adrenal glands in rats (Boink et al., 1996).

2.3 Observations in humans

2.3.1 Effects on the gastrointestinal tract

An epidemiological study was conducted to examine a possible correlation between the nitrate concentration of drinking-water, recurrent stomatitis and the activity of cytochrome b_5 reductase, which reduces methaemoglobin to haemoglobin. Five areas in the State of Rajasthan, India, were selected on the basis of a nitrate concentration (as nitrate ion) in drinking-water of 26, 45, 95, 220 and 460 mg/l; and 193 age- and weight-matched persons, representing 10% of the total population in each of these areas, were recruited. Detailed histories of recurrent stomatitis were recorded, a medical examination was conducted, and blood samples were taken to ascertain cytochrome b_5 reductase activity. The results of a multivariate regression analysis suggested a significant interdependence between the nitrate concentration of drinking-water, cytochrome b_5 reductase activity and recurrent stomatitis. It was concluded that increased cytochrome b_5 reductase activity, induced primarily by the presence of a high nitrate concentration in drinking-water, was the cause of recurrent stomatitis. The specific confounding variables that were taken into account were not described (Gupta et al., 1999).

A validated technique involving microdialysis probes was used to measure chemicals involved in nitrosation in the oesophagus, cardia and proximal and distal stomach of 15 healthy volunteers before and after ingestion of nitrate at a concentration of 2 mmol/l (equivalent to that in a portion of salad). Ingestion of nitrate ion increased the median concentration in saliva from 36 µmol/l to 250 µmol/l and that in the distal oesophagal from 29 µmol/l to 180 µmol/l (both $p < 0.01$). The concentration of nitrate in the stomach decreased

progressively but increased progressively with distance from the gastro-oesophageal junction, so that the highest ratio of nitrate:nitric oxide was at the junction. The authors concluded that nitrosation in the stomach is maximal at the gastro-oesophageal junction and that dietary nitrate is involved in mutagenesis and carcinogenesis at this site (Suzuki et al., 2002).

The anatomical distribution of nitric oxide generation in the lumen of the upper gastrointestinal tract was investigated under basal conditions and after ingestion of a quantity of nitrate equivalent to that in a portion of salad. Probes containing custom-made sensors that could detect nitric oxide and pH were installed in the oesophagus of 15 Helicobacter pylori-negative volunteers (10 men) with a mean age of 32 years. Nitric oxide and pH were measured for 2 min at 1-cm increments throughout the length of the stomach and distal oesophagus in persons who ingested potassium nitrate at 2 mmol/l and in controls. Serum nitrate and saliva nitrite concentrations were also recorded. Ingestion of nitrate increased the mean (range) serum nitrate from 30 µmol/l (18–49) to 95 µmol/l (32–150) and the mean salivary nitrite from 4.7 µmol/l (1.4–7.8) to 23 µmol/l (2.1–50) (both $p < 0.05$). After ingestion of nitrate, the peak concentration of nitric oxide was found within 1 cm distal to the gastro-oesophageal junction in 11 of the 15 (73%) persons. The pH step-up after nitrate was 7.5 µmol/l (0.5–31) and was significantly higher than at all other sites. Nitric oxide concentrations > 50 µmol/l were observed at the location at which the neutral oesophageal pH fell to acidic gastric pH. Generation of nitric oxide from dietary nitrate via salivary nitrite was found to be maximal at the gastro-oesophageal junction and cardia (Iijima et al., 2002).

2.3.2 Relationship between nitrate intake, subsequent endogenous formation of *N*-nitroso compounds and risk for stomach cancer

In vitro

To mimic physiological exposure to nitrite, nitrite was added gradually to the gastric compartment of a dynamic gastrointestinal model (based on toxicokinetics) simulating salivary nitrite mass flow after intake of nitrate at concentrations 0.1, 1, 5 and 10 times the ADI. Concomitantly, 100 g of cod were added as the source of primary amines for the nitrosation reaction. With a rapid decrease in the pH of the stomach, 2.3, 16 and 420 µg of NDMA were formed at 0.1, 1 and 10 times the ADI of nitrate, respectively; and with a slow decrease in pH in the compartment, 1.8. 5.1, 21 and 43 µg were formed at 0.1, 1, 5 and 10 times the ADI of nitrate, respectively. Thus, relatively large amounts of endogenous NDMA were formed under realistic physiological conditions (Krul et al., 2001a).

The accuracy of quantification of the endogenous formation of *N*-nitroso compounds in the human body is still a matter of debate. A dynamic gastrointestinal model simulating various physiological conditions in the gastric compartment was used to study the formation of NDMA after intake of various fish species as a source of amines, in combination with nitrite. Cod, herring, pollack and plaice induced NDMA formation in the gastric compartment, the amounts after intake of 100 g of fish ranging from a mean of 28 µg (23–29 µg) with plaice to 190 µg (160–240 µg) with cod. Mackerel and salmon samples did not increase NDMA formation under these conditions. The amounts of dimethylamine, trimethylamine and trimethylamine-N-oxide in the samples of cod and herring did not correlate with the amount of NDMA produced, indicating that the extent of NDMA formation cannot be predicted from amine concentrations. When cod was introduced into the model with 250 mg of ascorbic acid and nitrite was added gradually, a 66% reduction in the amount of NDMA formed was observed; addition of 1000 mg of ascorbic acid did not reduce the amount of NDMA further.

NDMA formation was also effectively inhibited, by > 90%, by the addition of green or black tea (2 g lyophilized tea dissolved in water). The addition of spinach (75 g) to food intake resulted in inhibition of NDMA formation with about the same efficacy as observed with ascorbic acid (Krul et al., 2001b).

Epidemiological studies

Epidemiological studies can be ranked in increasing order, from ecological (or correlation) studies to cross-sectional studies, case–control studies, cohort studies and intervention trials. This classification of epidemiological study designs with respect to their potential for bias and, consequently, the strength of evidence they provide and the costs involved has been described in detail (e.g. van den Brandt et al., 2002). Intervention trials provide the strongest evidence for a causal relationship with risk and (because of the possibility to control for confounding and bias) have the least possible bias; however, they are usually the least feasible and the most expensive. Less expensive cohort studies allow assessment of exposure and selection of study participants before the health outcome of interest occurs and thus provide relatively strong evidence. Although the less expensive case–control studies generally involve assessment of exposure retrospectively in subjects with and without the health outcome, the resulting evidence is more debatable. This is particularly so in the case of dietary intake, because of the possibility of selection bias, recall bias and/or bias due to the presence of disease. Cross-sectional studies suffer from the additional problem that exposure and disease are measured at the same time, making it impossible to draw conclusions about cause and effect. Correlation studies cost the least, but they provide weak evidence and are much more susceptible to bias. In addition to the problem of extrapolating the data to the individual level (as the units of measurement are population groups), most such studies include limited data on exposure, rely on mortality (rather than incidence) rates and often do not include consideration of the induction period. Some investigators have stated that observational studies cannot, by definition, establish the causality of a relationship on the basis of a statistical association. However, when several studies of high quality, such as those in which the biases are shown to be minimal, are available and these consistently show a dose–response relationship, observational studies may well contribute to conclusions about causality. The power of observational epidemiological studies was established 50 years ago, when such studies revealed that smoking caused lung cancer.

(i) Ecological studies

The possible conversion of nitrates to nitrites under conditions of gastric achlorhydria and their transformation to nitrosamines in the presence of nitrosatable compounds led to a number of epidemiological studies of the possible relationship between high nitrate levels in drinking-water and mortality due to various cancers. One study was performed in the Province of Valencia, Spain, which has the highest concentration of nitrate in drinking-water in Europe, to compare mortality due to cancers of the stomach, urinary bladder, prostate and colon in the populations of 258 municipalities with different levels of nitrate. The mortality rates from gastric and prostate cancer increased with increasing intake of nitrates. In populations whose drinking-water contained nitrate at a concentration > 50 mg/l, the relative risk for gastric cancer was 1.9 for men and 1.8 for women aged 55–75 years ($p < 0.05$) when compared with groups with low nitrate intake, and the relative risk for prostate cancer was 1.9 for men aged 55–75 and 1.8 for those aged > 75 (Morales Suarez Varela et al., 1995). It was not clear from the text whether correction was made for confounding factors.

The possible association between risk for gastric cancer and the concentration of nitrate and the hardness of drinking-water from municipal supplies was investigated in a matched case–control study in Taiwan (China). Data on deaths from gastric cancer among eligible residents between 1987 and 1991 (6766 cases) were obtained from the Bureau of Vital Statistics. The 6766 controls had died from other causes and were matched individually to the cases by sex, year of birth and year of death. Data on nitrate, nitrogen and hardness in drinking-water were collected from the Taiwan Water Supply Corporation. The municipality of residence was assumed to be the source of intake of nitrate for cases and controls. No difference in mortality rates from gastric cancer were found between groups with different levels of nitrate intake. The odds ratios (with 95% confidence interval [CI]) for death from gastric cancer were 0.95 (0.87–1.0) for the group with nitrate intakes of 0.23–0.44 mg/l and 1.0 (0.93–1.1) for the group with nitrate intakes > 0.45 mg/l. In contrast, a significant negative relationship was found between drinking-water hardness and mortality from gastric cancer, with odds ratios of 1.2 (1.1–1.3) and 1.6 (1.5–1.8), respectively, for intake of moderately hard water and soft water compared with hard water (Yang et al., 1997).

In a study of a population whose drinking-water had a high nitrate content (mean, 98 mg/l), nitrate was assessed by regular checks of water quality by local public health authorities. Empirical Bayes estimates of standardized mortality rates from gastric cancer were calculated and were correlated to average nitrate concentrations in drinking-water, with control for the confounding effects of smoking, ethnicity and population size. The average nitrate concentrations proved to be significant predictors of gastric cancer mortality ($p = 0.05$). The most probable limit value for this role of nitrate was 95 mg/l. With this reference value, the Mantel-Haenszel odds ratio for the relationship between nitrate and mortality from gastric cancer was 1.5 (95% CI, 1.1–2.1). The results of this ecological study support the hypothesis that high levels of nitrate in drinking-water have a causal role in gastric cancer development, but only an abstract was available (Sandor et al., 1998).

An ecological study in Yorkshire, northern England, was conducted to examine the hypothesis that intake of high levels of nitrate in drinking-water increases the risks for cancers of the stomach, oesophagus and brain in adults. Nitrate concentra-tions for the period 1990–95 and the numbers of incident cancers in 1975–94 were available for 148 geographically defined zones, each supplying water of homogeneous chemical composition to an average population of around 20 000. No relationship was found between nitrate concentration and the incidence of stomach or oesophageal cancer, but the incidence of cancers of the brain and central nervous system was higher in areas with higher nitrate levels, with a relative risk of 1.2 (95% CI, 1.1–1.3) in the quartile of the population with the highest mean intake (30 mg/l) compared with the quartile with the lowest intake (2.4 mg/l). The increase in risk remained statistically significant ($p < 0.01$) after allowance for other covariates and for extra-Poisson variation in a regression model. The observed relationship with brain cancer requires confirmation (Barrett et al., 1998).

In another ecological study, data were obtained on the incidences of various cancers, contamination of drinking-water with atrazine and nitrate and related agricultural practices in 40 districts in the Province of Ontario, Canada. The data were merged for geographical and statistical analyses. Weighted (by population size) least-squares regression analyses were conducted with control for confounding socioeconomic and lifestyle factors. Maximum likelihood spatial error models were estimated when least-squares regression error terms were found to be spatially auto-correlated, with Maran’s I statistics. Nitrate levels ranging from 0 to 91 mg/l (maximum acceptable concentration, 10 mg/l) were negatively associated with the

incidence of stomach cancer. Although the negative association appears to have no direct biological explanation, such counterintuitive outcomes may be found in complex systems in which social and biological variables interact (Van Leeuwen et al., 1999).

Randomly selected 24-h urine samples from 39 populations in 24 countries were obtained in an international study of electrolyte excretion and blood pressure and used to measure sodium (5756 people) and nitrate (3303 people). The median sodium and nitrate concentrations were standardized by age (20–49 years) and sex and averaged per country. Ecological correlation regression analyses were done in relation to national rates of mortality from stomach cancer. The Pearson correlation for the 24 countries was 0.70 in men and 0.74 in women(both $p < 0.001$) for sodium concentration and 0.63 ($p = 0.001$) in men and 0.56 ($p < 0.005$) in women for nitrate. In multiple regression analysis of stomach cancer mortality, with sodium and nitrate as independent variables, the adjusted correlation coefficient, r^2, was 0.61 in men and 0.54 in women (both $p < 0.001$). Addition of the interaction term (sodium × nitrate) to this model increased the adjusted r^2 to 0.77 in men and to 0.63 in women. The model showed that the importance of nitrate as a risk factor for death from stomach cancer increased markedly with sodium concentration; however, the relationship with sodium was always stronger than that with nitrate. Salt intake, measured as sodium excretion in 24-h urine, is probably the rate-limiting factor for stomach cancer at the population level. No attempt was made to take cancer induction time into account: the urine samples were collected in 1986–87, whereas the data on gastric cancer mortality were for 1986–88 (Joossens et al., 1996).

(ii) Cross-sectional studies

The excretion of nitrate, nitrite, apparent total *N*-nitroso compounds and volatile nitrosamines was measured in 24-h urine from 61 Egyptians, divided into four groups: controls, Schistosoma haemotobium-infected patients and bladder cancer patients with and without a history of schistosomal infection. The urinary concentration of nitrate in S. haemotobium-infected patients was significantly higher than in the other three groups. The concentration of nitrite was below the limit of detection of the method (< 0.015 µg/mg creatinine) in all but one of the control samples. S. haemotobium infection significantly increased urinary nitrite to 0.9 ± 1.16 µg/mg creatinine (mean ± SD, $p = 0.001$), and the concentration of nitrite in both groups of patients with bladder cancer was about 20 times that in S. haemotobium-infected patients without bladder cancer. Excretion of apparent total *N*-nitroso compounds paralleled that of nitrite, and, overall, a good correlation was observed between these two variables ($r^2 = 0.71$, $p = 0.0001$). NDMA was present in all the samples analysed, and S. haemotobium infection significantly increased the urinary concentration over that of controls (4.0 ± 1.6 and 2.0 ± 3.0 ng/mg creatinine, respectively; $p = 0.01$). Among the cancer patients, the concentration of NDMA was higher than that in controls only in those with schistomal infection. The presence of *N*-nitroso compounds, including NDMA, in the urine of S haemotobium-infected patients both before and after the development of cancer and the observation that these compounds also occur in bladder cancer patients with no history of schistosomal infection suggest that these compounds might have a role not only in initiation of the carcinogenic process but also in its progression (Mohsen et al., 1999). As endogenous nitric oxide production can also result from schistosomiosis, cause and effect can be reversed.

A study was conducted in Egypt, an area with high environmental levels of nitrosating agents, to investigate whether salivary nitrate and nitrite and the activity of nitrate reductase in saliva affect the risk for oral cancer. The concentrations of salivary nitrite (8.3 ± 1.0 µg/ml) and nitrate (44 ± 3.7 µg/ml) and the activity of nitrate reductase (74 ± 10 nmol/ml per min) were

significantly ($p < 0.05$) higher in 42 oral cancer patients than in 40 healthy individuals (nitrite, 5.3 ± 0.3 µg/ml; nitrate, 27 ± 1.2 µg/ml; nitrate reductase activity, 46 ± 4 nmol/ml per min). The adjusted ORs and the 95% confidence intervals for oral cancer, categorized by the values for salivary nitrate and nitrite and nitrate reductase activity, showed a risk associated with a nitrite concentration > 7.5 µg/ml (OR, 3.0; 95% CI, 1.0–9.3), a nitrate concentration > 40 µg/ml (OR, 4.3; 95% CI, 1.4–13.3) and nitrate reductase activity > 50 nmol/ml per min (OR, 2.9; 95% CI, 1.1–7.4). The findings suggest that increased consumption of dietary nitrate and nitrite is associated with elevated levels of salivary nitrite. Together with the increased activity of salivary nitrate reductase, these observations may explain, at least in part, the role of nitrate and nitrite in the development of oral cancer in individuals in areas with a high burden of *N*-nitroso precursors (Badawi et al., 1998).

The frequency of variants of hypoxanthine-guanine phosphoribosyltransferase in peripheral blood lymphocytes and endogenous nitrosation in human populations with various nitrate levels in their drinking-water were investigated in four groups of women volunteers. The groups with low and medium intake of nitrate from tap water (14 and 21 women) had concentrations of 0.02 and 18 mg/l, respectively, from public water supplies, whereas those with medium and high intakes of nitrate from well water (6 and 9 women) had mean concentrations of nitrate of 25 and 140 mg/l, respectively. Higher nitrate intake resulted in a dose-dependent increase in 24-h urinary nitrate excretion and in increased salivary nitrate and nitrite levels. The mean log frequency of variants in peripheral lymphocytes was significantly higher in the women with medium intake from well water than in those with low or medium intake from tap water. An inverse correlation was found between the peripheral lymphocyte labelling index and the nitrate concentration of drinking-water. Analysis for *N*-nitrosamines in the urine of 22 women by gas chromatography–mass spectrometry revealed the presence of *N*-nitrosopyrrolidine in 18. Analysis of the mutagenicity of well water samples showed that a small number caused reverse mutation in *Salmonella typhimurium* after concentration on a XAD-2 resin column. Thus, consumption of drinking-water with high concentrations of nitrate can confer a genotoxic risk, as indicated by increased hypoxanthine-guanine phosphoribosyl transferase variant frequencies and endogenous formation of *N*-nitroso compounds from nitrate-derived nitrite (van Maanen et al., 1996b).

(iii) Case–control studies

The increasing incidence of non-Hodgkin lymphoma (NHL) in the USA is only partially explained by known risk factors. In a study of 156 cases and 527 controls in Nebraska, USA, the average intake of nitrate from community water supplies was estimated for 1947–79. Long-term consumption of community water with average nitrate levels in the highest quartile (> 4 mg/l as nitrate nitrogen, equivalent to 18 mg/l) was positively associated with risk for NHL (OR, 2.0; 95% CI, 1.1–3.6). Dietary nitrate, mainly from vegetable, was not associated with risk after adjustment for vitamin C and carotene intakes, as persons with a lower intake of vitamin C were at slightly greater risk for NHL than those whose daily intake was > 130 mg, for all levels of intake of nitrate in drinking-water. Similar findings were made for the combined effect of nitrate in drinking-water and carotene. The nitrate levels in private wells, measured at the time of interview for 51 cases and 150 controls, were not associated with the risk for NHL after adjustment for pesticide use. These findings indicate that long-term exposure to nitrate in drinking-water at levels > 18 mg/l may contribute to the risk for NHL (Ward et al., 1996).

In another study of the association between NHL and nitrate in drinking-water, a population-based case–control study was conducted among white men in Minnesota, USA. By linkage of residential history with community water records, the average exposure to nitrate in drinking-water in 1947–75 was estimated for 73 cases diagnosed between 1980 and 1982 and for 147 controls who had used community water supplies. No association was found between nitrate in community water supplies and NHL within the range of concentrations studied (median of highest exposure category, 2.4 mg/l; range, 0.1–7.2 mg/l) (Freedman et al., 2000).

A population-based case–control study in Denmark addressed the hypothesis that parental occupation in agriculture increased the risk for testicular cancer in the offspring. The factors investigated were childhood residence on a farm, in the country or in an area with a high nitrate concentration in groundwater and the parents' occupation in agriculture. The only association that emerged was with childhood residence in an area with a high nitrate concentration. The excess risk was, however, confined to men who had not grown up on a farm or in the country, making it unlikely that nitrate per se was responsible (Møller, 1997).

A study of risk factors for childhood brain tumours was conducted to investigate the association between source of residential drinking-water during pregnancy and the occurrence of brain tumours among offspring. Dipstick measurements were made of nitrates and nitrites in tap water in the houses of a subset of women who were living in the same house in which they had lived during their pregnancy. A total of 540 patients with childhood brain tumours and 801 controls were identified in Los Angeles County and the San Francisco Bay Area of California and western Washington State, USA. Overall, no increased risk for childhood brain tumours was found in offspring of women for whom wells were the source of water; however, an increased risk (OR, 2.6; 95% CI, 1.3–5.2] was observed among offspring of women in Washington State and a decreased risk (OR, 0.2; 95% CI, 0.1–0.8) among those in Los Angeles County who relied exclusively on well water. Among the small subset of participants for whom dipstick measurements of tap water were available, the risk for childhood brain tumours associated with the presence of measurable nitrite and/or nitrate was 1.1 (95% CI, 0.7–2.0). Given the crude measurement method used and the fact that measures were often obtained years after the pregnancy, the relevance of the dipstick findings is unclear. The lack of consistency in the findings for residential water source does not support the hypothesis of an increased risk with consumption of well water; however, regional differences in the well water content of nitrite may exist, and the increased risk observed in western Washington State deserves further evaluation (Mueller et al., 2001).

A case–control study on gastric cancer and diet conducted in Marseille (France) included 92 patients with histologically confirmed adenocarcinoma and 128 controls undergoing functional reduction for injuries or trauma. The participants were interviewed by a trained dietician who administered a questionnaire about usual diet during the year preceding the first symptoms for cases or preceding the interview for controls. The intakes of nitrite, nitrite and pre-formed NDMA from food were estimated from a food composition table compiled ad hoc. Odds ratios were calculated after adjustment for age, sex, occupation and calorie intake. A high intake of preformed NDMA was associated with an increased risk for gastric cancer, the ORs for the second and third tertile of NDMA intake being: $OR_2 = 4.1$ (95% CI, 0.93–18) and $OR_3 = 7.0$ (95% Cl, 1.8–26). Intake of nitrate and nitrite was not associated with an increased risk for stomach cancer. Consumption of vegetables was protective in general, independently of their estimated nitrate content (Pobel et al., 1995).

A study was conducted to investigate whether consumption of foods and beverages containing nitrosamines, nitrite and nitrates affects the risks for laryngeal, oesophageal and oral cancer. In a population-based case–control study in western Washington State, USA, dietary consumption of these substances was measured for 645 cases (169 laryngeal, 125 oesophageal and 351 oral cancer) and 458 controls. After adjustment for tobacco and alcohol use and other known risk factors, the risk for upper aerodigestive tract cancer was found to be 52% lower in persons who consumed larger amounts of nitrate (upper tertile) as compared with the lowest tertile ($p < 0.001$ for trend). Nitrate intake was associated with a reduction in the risk for cancers at all three sites. The reduction in risk for oesophageal cancer with increasing nitrate consumption was more evident in frequent tea drinkers than in other persons. The ORs for oesophageal cancer per category of nitrite intake were: 1.0 (reference) for < 1.1 mg/day, 1.2 for intakes between 1.1 and 1.6 mg/day and 1.6 (95% CI, 0.73–3.4) for intakes > 1.6 mg/day (p for trend = 0.20). There was no significant association between nitrite consumption and the risk for laryngeal or oral cancer; however, for individuals with a history of canker sores (an indicator of possible endogenous nitrosation), the risk for oesophageal cancer was seven times greater in those with a high nitrite intake than in those with a low intake. Consumption of foods with high concentrations of NDMA was associated with a 79% increase in the risk for upper aerodigestive tract cancer ($p = 0.037$ for trend). Daily consumption of beer and of nitrite-containing meats was associated with increased risks for oesophageal cancer (OR, 2.5 and 1.8, respectively). The OR for cancer of the oral cavity was also increased for people who drank beer daily (OR, 1.8). Persons who consumed large amounts of ascorbic acid from foods and from supplements were less likely to develop upper areodigestive tract cancer than were individuals with lower ascorbic acid intake (p for trend = 0.003) (Rogers et al., 1995).

In a study to evaluate the roles of maternal nutrition during gestation and subsequently in the etiology of childhood brain tumours, all 300 incident cases of nervous system tumours diagnosed in children under 18 in Israel between 1984 and 1993 were identified. Two matched population controls per case were selected (n = 574). Personal interviews were conducted with a semi-quantified three-step food frequency questionnaire. Univariate analysis showed that increased consumption of vegetable fat (p for trend = 0.01; 95% CI, 1.1–3.2), carbohydrates (p for trend = 0.05; 95% CI, 1.0–5.9) and vitamin E (p for trend = 0.05; 95% CI, 1.0–3.3) during childhood was significantly associated with risk for brain tumour. No associations were found with nitrate, nitrite or vitamin C. A significant positive association with potassium consumption during gestation (p for trend = 0.01; 95% CI, 1.1–3.7) was noted. In a multivariate analysis, the only persistent associations were with vegetable fat (OR, 1.4; 95% CI, 1.1–1.7) in the diet during childhood and potassium intake during gestation (OR, 1.4; 95% CI, 1.0–2.0) (Lubin et al., 2000).

(iv) Cohort studies

The association between intake of nitrate or nitrite and gastric cancer risk was investigated in a prospective cohort study begun in 1986 in the Netherlands, of 120 852 men and women aged 55–69 years. At baseline, data on dietary intake, smoking habits and other covariates were collected by means of a self-administered questionnaire. For data analysis, a case–cohort approach was used, in which the person–years at risk were estimated for a randomly selected subcohort of 1688 men and 1812 women. After 6.3 years of follow-up, 282 microscopically confirmed incident cases of stomach cancer were detected, with 219 in men and 63 in women. The rate ratios for gastric cancer with increasing quintiles of mean nitrite intake were: 1.0 (intake, 0.01 mg/day), 1.2 (0.04 mg/day), 1.2 (0.09 mg/day), 0.88 (0.16 mg/day) and 1.4 (0.35

mg/day). The 95% confidence interval for the rate ratio derived by contrasting the highest versus the lowest intake quintile was 0.95–2.2. A test for trend in rate ratios across quintiles gave a *p* value of 0.24. The authors concluded that the study did not support a strong positive association between nitrite intake and gastric cancer risk (van Loon et al., 1998).

A cohort study was conducted to investigate the relationship between intake of nitrates, nitrites and NDMA and the risk for cancers of the gastrointestinal tract in 9985 adult Finnish men and women who were initially free of cancer. During a follow-up period of up to 24 years, 189 gastrointestinal cancer cases were diagnosed in the cohort. The intakes of nitrate, nitrite and NDMA were estimated from data on food consumption obtained during an interview about the total diet of the participants over the previous year. The mean daily intake of nitrates was 77 mg, and that of nitrites was 5.3 mg. Nitrates were provided mainly by vegetables (92%), whereas nitrites were derived mainly from cured meats and sausages (94%); dietary NDMA was provided by smoked and salted fish (52%) and cured meats and sausages (48%). The mean daily intake of NDMA from the diet was 0.052 µg, and that from beer was 0.071 µg. A significant positive association was observed between intake of NDMA and subsequent occurrence of colorectal cancer, with a relative risk between the highest and lowest quartiles of intake of 2.1 (95% CI, 1.0–4.3). Of the various sources of *N*-nitroso compounds, smoked and salted fish were found to be significantly associated with the risk for colorectal cancer (relative risk, 2.6; 95% CI, 1.2–5.5), and intake of cured meat was nonsignificantly associated (relative risk, 1.8; 95 % CI, 0.98–3.5). No such association was observed with intake of other fish or other meat. No significant association were observed between NDMA intake and cancers of the head and neck or of the stomach or between nitrate or nitrite intake and the risk for cancers of the gastrointestinal tract (Dich et al., 1996; Knekt et al., 1999).

Cancer incidence was studied in a cohort of 21 977 women in Iowa, USA, who were 55–69 years of age at baseline in 1986 and had used the same water supply for > 10 years (87% for > 20 years); 16 541 of these women were on a municipal water supply, and the remainder used private wells. Nitrate intake in 1955–88 was assessed from public databases for municipal water supplies (quartile cut-off points, 0.36, 1.0 and 2.4 mg/l for nitrate nitrogen, equivalent to 1.6, 4.5 and 11 mg/l expressed as nitrate ion). As no data on individual water consumption were available, each women was assigned an average intake calculated on a community basis; no data on intake were available for women using private wells. Cancer incidence in 1986–98 was determined by linkage to the Iowa Cancer Registry. A total of 3150 cases were identified. No consistent association was found with increasing nitrate concentration in drinking-water and NHL, leukaemia, melanoma or cancers of the colon, breast, lung, pancreas or kidney, but positive associations were found with bladder cancer (relative risks across nitrate quartiles: 1, 1.7, 1.1 and 2.8) and ovarian cancer (1, 1.5, 1.8 and 1.8) and inverse associations with uterine (1, 0.86, 0.86 and 0. 55) and rectal cancer (1, 0.72, 0.95 and 0.47) after adjustment for a variety of risk and protective factors, agents that affect nitrosation (smoking, vitamin C and vitamin E intake), dietary nitrate and water source. Similar results were obtained when the analyses were restricted to nitrate concentrations in drinking-water in 1955–64. The positive association with bladder cancer is consistent with some previous findings, but the association with ovarian cancer and the inverse associations with uterine and rectal cancer were unexpected (Weyer et al., 2001).

2.3.3 Relationship between nitrate intake and genotoxic effects

A study was conducted to investigate the potential genotoxicity of nitrates and nitrites in drinking-water. The frequencies of sister chromatid exchange and chromatid or chromosomal aberrations were studied in peripheral blood lymphocytes from 70 children aged 12–15 who were permanent residents of areas of Greece with high concentrations of nitrates in drinking-water (56– 88 mg/l). The control group comprised 20 healthy children living in areas with a low nitrate concentration in drinking-water (0.7 mg/l). No increase in the mean number of chromatid or chromosomal breaks was observed in children with nitrate concentrations > 55 mg/l, but the frequency increased with intakes > 70 mg/l ($p < 0.01$). The mean number of sister chromatid exchanges per cell was not increased significantly (Tsezou et al., 1996).

2.3.4 Putative relationship between nitrate intake and effects on the endocrine system

In a mechanistic study on gonadotrophin-stimulated steroidogenesis in mouse Leydig tumour cells *in vitro*, the effects of a number of chemicals, including nitrate, were examined. Nitrate completely abolished the effect of 1 IU/l of human chorionic gonadotrophin on androgen secretion. The inhibition could be partially overcome by increasing the gonadotrophin concentration. Inorganic nitrate probably inhibited steroidogenesis via conversion to nitric oxide without involving the guanylate cyclase-cyclic guanosine monophosphate pathway (Panesar, 1999).

It has been suggested that increased nitrate intake affects the function of the thyroid gland in humans, as was observed in a study in pigs (Annex 1, reference 117), by competitive inhibition of iodide transport leading to decreased thyroid hormone secretion, followed by an increase in thyroid-stimulating hormone. To test this hypothesis, a 4-week study was conducted in which 10 volunteers received sodium nitrate in 200 ml of distilled water at a dose of 15 mg/kg bw once a day for 28 days (three times the current ADI of 0–3.7 mg/kg bw expressed as nitrate ion), and 10 volunteers received 200 ml of distilled water and served as the control group. Both groups received an iodine-restricted, low-nitrate diet, and compliance was checked by measuring the urinary iodide and plasma nitrate concentrations. Before and after the 28-day treatment period, the per cent uptake of radioactive iodine was measured 5 h and 24 h after administration to investigate competition of nitrate in iodide transport. Before and 2, 3 and 4 weeks after treatment with nitrate, blood samples were taken for measurement of thyroid hormones and insulin-like growth factor I, as measures of thyroid gland function. Nitrate had no effect on hormone concentrations during the 4-week treatment but increased the 24-h per cent uptake of radioactive iodine by 1.5 times (20–29%) over that before treatment, whereas a decrease had been expected. The authors concluded that intake of nitrate at three times the ADI would not cause changes in thyroid gland function in a healthy population (Lambers et al., 2000b).

2.3.5 Putative relationship between nitrate intake and insulin-dependent diabetes mellitus

Although a number of studies performed before 1991 showed no association between nitrate levels in drinking-water and insulin-dependent diabetes mellitus (IDDM), an ecological study in the USA (Kostraba et al., 1992) showed a significant positive correlation, and a study in the United Kingdom (McKinney et al., 1996) showed a clear excess of childhood IDDM in rural areas, which were presumed to have higher concentrations of nitrate in drinking-water than urban areas. Further epidemiological studies were therefore performed to test the hypothesis

that the incidence of childhood IDDM is increased in areas with higher levels of nitrate in domestic drinking-water.

A population-based study performed in conjunction with the Yorkshire Register of Childhood Diabetes included 1797 children aged 0–16 years in whom IDDM was diagnosed while they were resident in the geographical area of the former Yorkshire Regional Health Authority, which covered the period 1978–94 and was about 97% complete. Three categories of nitrate intake were defined on the basis of population density, with equal populations. In 9330 water samples taken between 1990 and 1995, the nitrate concentration exceeded 25 mg/l in 2775 (30%) and exceeded 50 mg/l in 31 of these (0.1%). A positive association was found between the nitrate content of domestic water and the incidence of IDDM (McKinney et al., 1999).

An ecological study addressed the possible relation between nitrate levels in drinking-water in the Netherlands and the incidence of type 1 IDDM, to determine whether the standard of the European Commission and WHO for nitrate in drinking-water (37 mg expressed as nitrate ion) is adequate to prevent this disease. During 1993–95 in the Netherlands, 1104 cases of type 1 diabetes were diagnosed among 2 829 020 children aged 0–14, and 1064 of these cases were used in the analysis. The mean concentrations of nitrate in drinking-water in 3932 postal code areas in 1991–95 were divided into two categories, one based on equal numbers of children with various nitrate levels (0.25–2.1, 2.1–6.4 and 6.4–41 mg/l) and the other based on cut-off values of 10 and 25 mg/l for nitrate. Standardized incidence ratios for type 1 diabetes were determined for subgroups of the 2 829 020 children with respect to nitrate category, sex and age and as compared in univariate analysis with a chi^2 test for trends. Incidence rate ratios were calculated by multivariate analysis in a Poisson regression model. An effect of increasing age on the incidence of type 1 diabetes was seen, but there was no effect of sex or of nitrate concentration in drinking-water for the two categories. For a nitrate concentration > 25 mg/l, both the standardized incidence ratio and the incidence rate ratio were increased (1.5); however, the increase was not statistically significant, probably because of the small number of cases (15 of 1064). The authors concluded that there is no convincing evidence that nitrate in drinking-water at current levels is a risk factor for childhood type 1 diabetes mellitus in the Netherlands, although a threshold > 25 mg/l could not be excluded as higher concentrations were not studied (van Maanen et al., 2000).

2.3.6 Putative relationship between nitrate intake and neural tube defects

A population-based case–control study was conducted in California, USA, between June 1989 and May 1991 to investigate a possible association between periconceptional exposure of mothers to nitrate in drinking-water and the diet and the risk for neural tube defects in their offspring. The mothers of 538 cases and 539 normal controls were interviewed regarding residential history, consumption of tap water at home and dietary intake during the periconceptional period. Dietary nitrate intake was not associated with an increased risk for neural tube defects; concentrations > 45 mg/l in drinking-water as nitrate ion were associated with an increased risk for anencephaly (OR, 4.0; 95% CI, 1.0–15) but not for spina bifida. Increased risks for anencephaly were also observed among the offspring of mothers who drank groundwater only with nitrate concentrations below the maximum level (OR, 2.1; 95% CI, 1.1–4.5 for 16– 35 mg/l; and OR, 6.9; 95% CI, 1.9–25 for 36–67 mg/l compared with < 5 mg/l, all as nitrate ion). Adjustment for identified risk factors for anencephaly did not substantially alter these associations, nor did control for maternal dietary intake, total vitamin C intake or quantity of tap water consumed (Croen et al., 1997, 2001).

2.3.7 Putative relationship between nitrate intake and sudden infant death syndrome

It has been shown (Wiklund et al., 1998) that enteric bacterial urease is inhibited in victims of sudden infant death syndrome (SIDS). One possible inhibitor of this bacterial activity is nitrate. If ambient pollution by nitrate is involved in the etiology of SIDS, only a fraction of the nitrate concentration often found in drinking-water would be enough to cause such inhibition. The 636 cases of SIDS that occurred in Sweden during the period 1990–96 were analysed with regard to geographical and seasonal distribution, the nitrate concentration in drinking-water and changes in groundwater level. Both the birth rate and the incidence of SIDS decreased during the study period. One-quarter of the municipalities, constituting 11% of the population, had no cases, the maximum incidence being 6.5 per 1000 live births. The northernmost parts of the country had the highest incidence, and individual deaths were associated with recharging of groundwater, which increases its nitrate content. The local incidence of SIDS was correlated (r^2 = 0.34–0.87) to the maximal recorded concentration of nitrate in drinking-water. The seasonal distribution of SIDS was very different from the south to the north of the country and appeared to be associated with differences in groundwater level subsequent to precipitation, frost penetration and melting of snow. Consumption of drinking-water with high peak concentrations or wide variations in nitrate concentration was correlated to the incidence of SIDS. The regression plots showed that the incidence of SIDS would exceed 1.5% at nitrate concentrations of 40–60 mg/l (George et al., 2001).

2.4 Miscellaneous aspects

2.4.1 Claimed beneficial effects of nitrate

In a critical review of studies of the effect of nitrate, it was claimed that nitrate can have beneficial or protective effects on health. The implication of dietary nitrate (via conversion to nitrite) in the formation of methaemoglobin and carcinogenic nitrosamines in humans has led to restriction of nitrate and nitrite levels in food and drinking-water; however, the authors considered that there is no epidemiological evidence for increased risks for gastric or intestinal cancer in population groups with high dietary intake of vegetables or nitrate. A re-evaluation of the negative perception of dietary nitrate was conducted on the basis of studies on the metabolism and enterosalivary circulation of nitrate in mammals, which showed that nitrate is converted to nitrite in the oral cavity, which then ‘fuels’ mammalian resistance to infectious disease. In the acid environment of the stomach, nitrite is reduced to nitric and other nitrogen oxides and, conceivably, also contributes to the formation of systemic S-nitrosothiols. Nitric oxide 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 was bactericidal for a variety of gastrointestinal pathogens, such as Yersinia and Salmonella. Nitric oxide is also known to have vasodilatory properties and to modulate platelet function, as do S-nitrosothiols. Thus, the authors concluded that nitrate in the diet determines the production of reactive nitrogen oxide species in the stomach and acts as a host defence against gastrointestinal pathogens, as a modulator of platelet activity and is possibly involved in gastrointestinal motility and microcirculation. The authors concluded that dietary nitrate may have an important therapeutic role to play, for instance in immunocompromised persons and in persons at particular risk of contracting gastroenteritis (Duncan et al., 1995; Dykhuizen et al., 1996; McKnight et al., 1997; Vallance, 1997;

McKnight et al., 1999). The authors also reported that the conversion of nitrate into oxides of nitrogen prevents the formation of carcinogenic nitrosamines (Duncan et al., 1997).

2.4.2 Other opinions on hazard and risk of nitrate

Other international committees, such as those convened by the European Commission and IARC, have assessed the safety of nitrate and nitrite, and some criticisms have been made of the safety evaluation performed by the Committee at its forty-fourth meeting. For instance, L'Hirondel & L'Hirondel (1997) noted that the drinking-water standard for nitrate, which is based mainly on methaemoglobin formation, was established by another WHO committee and not by the Expert Committee. The limit of 50 mg/l for sodium nitrate was considered to be conservative, as the enteritis that occurs in cases of gastrointestinal tract infection has a greater influence on methaemoglobin formation than nitrite derived from conversion of nitrate. The authors claimed that most cases of methaemoglobinaemia have been associated with intake of well water containing nitrate at > 100 mg/l and concomitant microbiological contamination.

The authors also criticized establishment of an ADI for both nitrate and nitrite and the use of a safety factor of 500 for nitrate. They further criticized use of the NOEL for nitrite to transpose the ADI to nitrate.

They considered that two of the studies with nitrite should be re-evaluated. They stated that the one showing hypertrophy of the zona glomerulosa in the adrenal cortex in rats (Til et al., 1988) should be reconsidered, as other studies (Vleeming et al., 1997; Boink et al., 1999) demonstrate that the minimal hypertrophy can be considered a physiological adaptation to the slight oscillation in blood pressure caused by nitrite via the renin–angiotensin system. They stated that it should therefore not be considered a toxic effect relevant for the situation in humans. They considered that the study of Shuval & Gruener (1972, 1977) should also be re-evaluated. Although differences were found in the hearts, lungs and coronary arteries of treated and control animals at concentrations of sodium nitrite $\geq$ 200 mg/l, the Environmental Protection Agency (1990) and L'Hirondel & L'Hirondel (1997) stated that these changes were due at least partly to an absence of the coronary thickening and narrowing that normally occurs in aged rats. Hence, these changes may not be inherently adverse.

3. COMMENTS

The few new studies on the toxicokinetics and metabolism of nitrate in animals that have become available since the forty-fourth meeting of the Committee confirm that the rat is not a good surrogate species for humans in this respect, as it does not show salivary transport of nitrate and therefore has limited conversion of nitrate to nitrite.

In a study of the conversion of nitrate to nitrite in humans, in which sodium nitrate was administered in drinking-water at a single dose of 7.3 mg/kg bw, expressed as nitrate ion, neither blood pressure nor methaemoglobin concentration was affected. The nitrite concentration of the gastric juice was approximately six times higher after administration of nitrate in combination with pretreatment with omeprazole at 40 mg/day (which increased the gastric pH) than after nitrate alone. Nitrate was absorbed rapidly, the concentration in plasma increasing within 10 min, and the half-life of nitrate in plasma was about 6.5 h; about 70% of the dose was excreted in urine within 10 h of dosing. The plasma concentration of nitrite did not change after nitrate administration. About 8% of the total nitrate administered was

converted to nitrite in saliva, as found in other studies. The Committee at its forty-fourth meeting concluded that the range of nitrate conversion is 5–7% for normal individuals and 20% for individuals with a high rate of conversion.

The results of studies in humans on the potential of a high nitrate intake to cause methaemoglobinaemia were equivocal. Some of the studies showed an association between a high nitrate concentration in drinking-water and methaemoglobinaemia, and others indicated that gastrointestinal infections, inflammation and the ensuing overproduction of nitric oxide are major factors in infantile methaemoglobinaemia. No increase in methaemoglobin concentration was seen in volunteers after a single administration of sodium nitrate in drinking-water providing a dose of 7.3 mg/kg bw, expressed as nitrate ion.

A study in humans showed that nitrate in vegetable matrices and from other sources, such as drinking-water, is almost totally bioavailable.

As nitrate shares a common transport mechanism with iodide, studies were conducted to determine whether nitrate affects thyroid function. A 28-day study with volunteers given sodium nitrate in drinking-water at a concentration equivalent to 15 mg/kg bw per day (11 mg/kg bw per day expressed as nitrate ion) showed no effects on thyroid function and no increase in the per cent of methaemoglobinaemia. A 90-day study of toxicity in rats showed that sodium nitrate at a dose of 50 mg/kg bw per day did not affect the thyroid or the zona glomerulosa of the adrenals.

In studies in humans, consumption of drinking-water containing sodium nitrate at a concentration of 2800 mg/l concomitantly with volatile *N*-nitrosatable amines in the diet (in cod, salmon or shrimp) led to a two- to threefold increase in urinary excretion of *N*-nitrosodimethylamine and *N*-nitrosopiperidine.

Several studies were reviewed on the effect of administration of nitrate on the release of nitric oxide at the junction of the oesophagus and the stomach in humans, which, it was speculated, might be associated with an increased incidence of cancer at this site. However, no such association has been observed in epidemiological studies.

A number of epidemiological studies have been published since the forty-fourth meeting of the Committee on the relationship between nitrate intake and cancer risk. At its present meeting, the Committee ranked the study designs according to their capacity to provide evidence of a relationship. In the descriptions below, relative risk estimates are given for those studies in which levels of intake of nitrate were provided.

Six ecological (correlation) studies were reported on nitrate in drinking-water and mortality from or incidence of cancer. Elevated risks were found for prostate cancer and for brain tumours (each in one study), but the results of six studies on gastric cancer were conflicting. The results of ecological studies (in which populations are the units of measurement) cannot be extrapolated to the individual level. Furthermore, most of the ecological studies were based on limited data on nitrate concentrations and on cancer mortality rates (rather than incidence rates), and none took an induction period for cancer into account.

Three of the studies were cross-sectional, involving measurement of, e.g., salivary nitrate in cancer patients and healthy subjects. Because cross-sectional studies do not take into account the time between exposure and disease, any observed differences in biomarkers of exposure

might also be a consequence of the disease; therefore these studies cannot contribute to a causal interpretation of the results of studies of nitrate intake and cancer risk.

Seven case–control studies on nitrate in drinking-water and/or food and cancers at various sites were reviewed. In the studies on nitrate in drinking-water, conflicting results were reported with regard to an association with non-Hodgkin lymphoma, and no association was found with brain tumours. In the studies on dietary nitrate, no association was found with oral, oesophageal, gastric or testicular cancer. No other cancer sites have been studied.

Three prospective cohort studies have been conducted on nitrate intake and cancer risk. A cohort study in the Netherlands, with 6 years of follow-up, found no significant association between the incidence of gastric cancer and intake of nitrate from food or drinking-water, with relative risks for increasing quintiles of total nitrate intake of 1.0 (reference quintile), 1.2, 0.7, 0.9 and 0.9 for mean intakes of 60, 85, 100, 120 and 180 mg/day, respectively. Neither the relative risks nor the trend across relative risks was significant. A further analysis of the effect of nitrate within tertiles of vitamin C intake also did not reveal a positive association between nitrate intake and gastric cancer. A Finnish cohort study on dietary nitrate, with 24 years of follow-up, reported no association with the risks for tumours of the stomach, colorectum or head and neck. The average nitrate intake in this cohort was reported to be 77 mg/day. A cohort study in Iowa, USA, with 11 years of follow-up, revealed no consistent association between intake of nitrate from drinking-water and the risks for cancers at many sites, and an inverse association was reported with cancers of the uterus and rectum. Positive associations with nitrate intake were observed only for cancers of the ovary and urinary bladder, although it was not possible to determine whether other factors in drinking-water were responsible for these associations. In addition, no evidence of a dose–response relationship was found for any of the cancer sites addressed in the study in Iowa. The cohort studies included control for various potential confounders, such as intake of vegetables, age and smoking.

Overall, the epidemiological studies showed no consistently increased risk for cancer with increasing consumption of nitrate. These data, combined with the results of the epidemiological studies considered by the Committee at its forty-fourth meeting, do not provide evidence that nitrate is carcinogenic to humans.

A number of studies were performed to determine whether there are associations between nitrate intake in drinking-water and insulin-dependent diabetes mellitus, neural tube defects or sudden infant death syndrome. In none of these studies was a hypothesis proposed for the mechanism of an association. Two studies were conducted on the incidence of insulin-dependent diabetes mellitus and nitrate intake via drinking-water. One study in Yorkshire, United Kingdom, suggested a positive association, but the authors considered that the finding required confirmation. A study in the Netherlands with a larger number of subjects did not show a positive association. The two studies on nitrate intake and neural tube defects also showed no association. In a recent ecological study in Sweden, a correlation was reported between the nitrate concentration in drinking-water and the occurrence of sudden infant death syndrome; however, no confounding factors were taken into account. The Committee considered that it would be premature to include these observations in its safety assessment.

4. EVALUATION

The Committee concluded that the pivotal observed toxic effects of nitrate are consequent on its conversion to nitrite *in vivo*. The Committee at its present meeting established an ADI of 0–0.07 mg/kg bw for nitrite (see earlier part of this section). As the new data on nitrite would not provide a basis for a significant change in the previous ADI for nitrate, the Committee retained the ADI of 0–5 mg/kg bw expressed as sodium nitrate, or 0–3.7 mg/kg bw, expressed as nitrate ion, established at its forty-fourth meeting.

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