# EVIDENCE ON DEVELOPMENTAL AND REPRODUCTIVE TOXICITY OF SODIUM NITRITE

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**DRAFT**

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## PREFACE

The Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65, California Health and Safety Code 25249.5 *et seq.*) requires that the Governor cause to be published a list of those chemicals "known to the state" to cause cancer or reproductive toxicity. The Act specifies that "a chemical is known to the state to cause cancer or reproductive toxicity … if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer or reproductive toxicity." The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency. The "state's qualified experts" regarding findings of reproductive toxicity are identified as the members of the Developmental and Reproductive Toxicant (DART) Identification Committee of OEHHA's Science Advisory Board (22 CCR 12301).

Sodium nitrite was identified as a candidate for consideration under the "authoritative bodies" provision of Proposition 65. Subsequent to publication of a notice of intent to list this chemical, it was determined that the data used by the authoritative body did not meet the criteria specified in 22 CCR 12306(g). Pursuant to 22 CCR 12306(i), sodium nitrite has been referred to the DART Identification Committee. This draft document provides the DART Identification Committee with information relevant to the reproductive toxicity of this chemical.

This document reviews all the available data on the reproductive and developmental toxicity of sodium nitrite, including studies not cited by U.S. EPA in the TRI process. The scope of the review here is broader than that previously reviewed by OEHHA during the data call-in and notice of intent to list stages for sodium nitrite under that authoritative bodies mechanism. This is due to the fact that 22 CCR 12306(i) specifies a different inquiry for the DART Identification Committee than that undertaken by OEHHA during its review of the chemical to determine if it met the "sufficient evidence" standard specified in 22 CCR 12306(g). The determination by the Committee is distinct from OEHHA's determination whether U.S. EPA had sufficient data for its formal identification of sodium nitrite as causing reproductive toxicity and calls for a review of all relevant information regarding sodium nitrite, not just that cited by U.S. EPA.

While this hazard identification document does not provide dose-response evaluation, exposure assessment or determination of allowable or safe exposure levels, the document does provide information which may be useful in such appraisals.

A public meeting of the Committee will be held on June 2, 2000, in Oakland, California. Following discussion and Committee deliberation, the Committee will determine whether sodium nitrite "has been clearly shown by scientifically valid testing according to generally accepted principles" to cause reproductive toxicity.

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# A. EXECUTIVE SUMMARY

Sodium nitrite is a water soluble, inorganic salt. Nitrite and nitrate are readily interconverted in the body by means of oxidation-reduction reactions. Hence the pharmacokinetics and metabolism of nitrite cannot appropriately be considered in complete isolation from nitrate. Nitrite and, by extension, nitrate, can also serve as precursors for the *in vivo* formation of carcinogenic N-nitroso compounds. Both nitrite and nitrate occur naturally in many foods, particularly vegetables. Both compounds also have food additive uses in the production of cured meat products. Both nitrite and nitrate can be found as contaminants of drinking water.

A major concern in considering the toxicology of nitrite is the induction of methemoglobinemia. Nitrite in the circulation leads to formation of methemoglobin (MetHb), which is incapable of transporting oxygen to the body's tissues and organs. Clinical symptoms of oxygen deficiency become apparent at MetHb levels of 10% and above, with coma and death resulting at levels of 60% or more. Neonatal methemoglobinemia, or "blue baby syndrome," is a serious, life-threatening, and well-documented toxic consequence of nitrite overexposure. Also of concern is the potential for nitrite, or the nitrosating species derived from it, to combine with secondary amines or N-alkylamides to form carcinogenic N-nitroso compounds (nitrosamines and nitrosamides). Many foods, beverages, and medicines contain the necessary constituents for formation of N-nitroso compounds.

Data relevant to the potential developmental toxicity of sodium nitrite come from both human epidemiological studies and studies in experimental animals. The available data address endpoints such as viability, growth, and morphological and functional parameters. Data also address the potential for transplacental carcinogenicity to result from exposure to sodium nitrite alone, or in combination with other precursors of N-nitroso formation.

No studies on humans were available which directly evaluated the potential for prenatal exposure to sodium nitrite to cause adverse effects on fetal viability, growth, morphology, or functional parameters. Relevant data do, however, include studies of prenatal exposure to nitrate, and a study of the effects of nitrosable drugs on human pregnancy. Two epidemiological studies conducted in Australia found statistically significant increases in risk for congenital malformations, particularly malformations of the central nervous system (CNS), associated with maternal consumption of water containing nitrate at levels in excess of 5 ppm. A third study, conducted in Canada, found a moderate, non-statistically significant increase in risk for CNS malformations associated with maternal consumption of well water having nitrate levels of 26 ppm or higher. In the Canadian study, when considering only consumption of tap or spring water during pregnancy (and excluding well water), increasing nitrate levels were not shown to be associated with an increased risk for birth defects.

Animal studies in which sodium nitrite exposure was restricted to the prenatal period have been conducted in guinea pigs, mice, and rats. Studies incorporating pre-and

postnatal exposure have been conducted in rats and mice; only one of these studies, which was conducted in rats, reported on endpoints at birth, which could thus be readily attributed to the prenatal portion of the exposure. Additional relevant information concerned the developmental toxicity of sodium or potassium nitrate in experimental animals, and studies of nitrite-induced maternal and fetal methemoglobinemia. The diversity of protocols and endpoints employed in these studies limits the extent to which general comparisons can be made between studies. There are, however, some areas where comparisons can be drawn.

Increases in fetal and/or maternal methemogolobin levels have been reported in sodium nitrite-treated guinea pigs, rats, and dairy cows, suggesting a potential for effects on the quantity of oxygen available to fetal tissues. In dairy cows, mean fetal $PO_2$ levels were found to be decreased following maternal infusion with nitrite. In two studies of guinea pigs exposed to sodium nitrite, and one in which guinea pigs were given potassium nitrite, treatment was associated with abortion of litters. At a given dose level of sodium nitrite, the effects were more severe if the guinea pig sows were ascorbic acid-deficient, rather than non-deficient.

Three behavioral and neurodevelopmental studies conducted in rats have indicated persistent effects of prenatal exposure to sodium nitrite that were detectable postnatally in animals assessed at various timepoints, including adulthood. These effects were attributed by the study authors to nitrite-induced prenatal hypoxia. The one additional study reporting on effects observable at birth in rats, did not evaluate behavioral or histopathological endpoints, but reported some indication of lower litter size in treated animals, and no significant effects of treatment on birth weight.

Of three studies conducted in mice, one found significant effects of sodium nitrite on fetal erythropoiesis. Another found significant effects on implantation size, the frequency of dead fetuses, and fetal weights. Due to the methodology and reporting of this study, however, it was questionable whether these effects could be attributed to treatment. A third mouse study was unable to identify any evidence of maternal or fetal toxicity in response to sodium nitrite.

Nine epidemiological studies examined maternal consumption of cured meats during pregnancy and risk for childhood brain tumors. A number of these studies showed a statistically significant increase in risk, including the two largest studies, which also found indications of a dose-response. Two studies, one relatively small and the other addressing only medullablastoma, did not find significant associations. Studies of maternal cured meat consumption and risk for childhood leukemia or other childhood cancers reported slightly elevated odds ratios in some cases, but none were statistically significant. A number of dietary studies in adults have found positive associations between consumption of nitrite-containing cured meats and brain tumor risk. Studies examining childhood cancer risk in relation to consumption of cured meats during childhood have seldom reported an association, although one study of childhood leukemia reported significantly increased risk in relation to childhood hot dog

consumption. Vitamin use was often found to modify the risks seen in these dietary studies.

While the epidemiological data do not implicate sodium nitrite in isolation from other dietary components, they are consistent with a general mechanism suggested by transplacental carcinogenicity studies conducted in experimental animals. For each of four studies reviewed in which sodium nitrite was given in combination with ethylurea, morpholine, citrulline, or cimetidine, tumors were observed, with the resulting tumor type dependent upon the specific precursor given. These results support the potential for toxicologically significant nitrosation to occur *in vivo*.

There were no available data on the potential of sodium nitrite to cause male or female reproductive toxicity in humans. Among the available animal studies, none were conducted under a standard multi-generation reproductive toxicity study protocol. Four studies included the treatment of both sexes during the mating period. None of the pair-based studies provided evidence for an effect of sodium nitrite on fertility, or on other reproductive parameters evaluated. A study on the effects of potassium nitrite in pregnant guinea pigs reported that fertility was retained, although fetal loss appeared to increase with increasing dose of potassium nitrite.

Data obtained from a general toxicity study provided some evidence of testicular changes at the histopathological level in male rats, but the observed effects could not be confidently attributed to sodium nitrite exposure. Two other studies found no histopathological changes in the male or female reproductive organs of sodium nitrite-treated animals. Experimental data have indicated that the pregnant animal is more susceptible to nitrite-induced hematological changes, as compared to the non-pregnant animal. Additionally, a continuous breeding study provided some suggestion that sodium nitrite might affect milk production, as postnatal weight gain was reduced in the offspring of dams given approximately 245 mg sodium nitrite/kg/day during pregnancy and lactation. Alternatively, this might have been the indirect result of poor palatability of sodium nitrite-treated water leading to reduced water consumption (and hence to reduced milk production), or due to a direct effect on the pups of sodium nitrite secreted in the dams' milk.

# B. INTRODUCTION

While the purpose of this document is to review the evidence specifically pertaining to the developmental and reproductive toxicity of sodium nitrite, it must be noted that nitrite and nitrate compounds are readily interconverted by oxidation-reduction reactions in biological systems (NAS, 1981a; Gangolli, et al., 1994; Walker, 1996; WHO, 1996a). Additionally, nitrite contributes to the formation of N-nitroso compounds *in vivo*. Thus, while the information presented here will focus on the consequences of administration of sodium nitrite, discussion of data derived from studies of related compounds, including sodium nitrate, potassium nitrite, and N-nitroso compounds will be introduced as appropriate.

## B.1. Chemical And Physical Properties

Sodium nitrite, or nitrous acid sodium salt, has a molecular weight of 69.00, and the formula of $NaNO_2$ (Merck Index, 1989). Like sodium nitrate ($NaNO_3$), potassium nitrate ($KNO_3$), and potassium nitrite ($KNO_2$), sodium nitrite is a water soluble, inorganic salt (U.S. FDA, 1972). All of these salts are stable in dry form at room temperature.

## B.2. Regulatory History

*B.2.1. Federal and State guidance levels*

U.S. Department of Agriculture (USDA) Meat Inspection Regulations (Title 9, Chapter 111, Subchapter A, Code of Federal Regulations, 1974) specify that the use of nitrites and nitrates, singly or in combination, cannot result in more than 200 ppm (calculated as sodium nitrite) in the finished product (Epley et al., 1992).

The drinking water standard set by the State of California (California Administrative Code Title 22) is a maximum contaminant level (MCL) of 45 ppm (or 45 mg/L) of nitrate, or 10 ppm of nitrate-nitrogen (nitrate-N) (Fan and Steinberg, 1996). As reported by Fan and Steinberg (1996), the U.S. Environmental Protection Agency (U.S. EPA) has established an MCL of 10 ppm for nitrate-N, and an MCL of 1 ppm for nitrite-N; the U.S. EPA MCL for total nitrate and nitrite, expressed as nitrogen, is 10 ppm.

*B.2.2. History under Proposition 65*

Sodium nitrite was formally identified by the U.S. EPA as causing reproductive toxicity in the course of implementation of the Toxic Release Inventory (TRI) program (*i.e.*, Section 313 of the Emergency Planning and Community Right-to-Know Act of 1986 (EPCRA)). Sodium nitrite was considered for listing under Proposition 65 because of this formal identification by an authoritative body.

The basis for U.S. EPA's identification of sodium nitrite as causing developmental toxicity under the TRI program (*Federal Register* 59:1788-1859, 1994) was documented by the Agency as follows:

> "230. Sodium nitrite (CAS No. 007632-00-0) (CERCLA) (Ref. 8). Sodium nitrite causes conversion (oxidation) of hemoglobin to methemoglobin. Methemoglobin cannot combine reversibly with oxygen and its formation can cause anemic hypoxia which may lead to intense cyanosis. Infants are particularly susceptible to this effect because of their higher stomach pH, immature enzyme systems, the reduced capacity of newborn erythrocytes to reduce MetHb to hemoglobin, and the increased rate of nitrite-induced oxidation of fetal hemoglobin to MetHb (approximately twice the rate of adult hemoglobin oxidation). Coma and methemoglobinemia/carboxyhemoglobinemia were reported in a human that received sodium nitrite (71 mg/kg) orally. In animal studies, methemoglobinemia was reported in dogs that received an intravenous dose of 30 mg/kg sodium nitrite and in rats administered a 10 mg/kg dose of sodium nitrite subcutaneously."
>
> "Fetotoxicity (fetal death) was reported following oral exposure of pregnant rats to sodium nitrite (30 mg/kg/day) during gestation days 1 through 22. In mice exposed orally to 80 mg/kg/day during gestation days 6 to 15 there was increased preimplantation loss and fetal death, and in mice exposed to a lower dose (20 mg/kg/day) during gestation days 1 to 14, abnormalities of the blood or lymphatic system were reported in offspring. In offspring of rats orally exposed to 26 to 256 mg/kg/day during pregnancy (gestation days 1 through 22) and/or during lactation (20 to 21 days after birth), effects on growth including biochemical and/or metabolic changes were noted."

Neonatal methemoglobinemia is a serious, life-threatening, and well-documented toxic consequence of nitrite exposure. It is also a toxic endpoint which is encompassed by U.S. EPA's definition of developmental toxicity as:

> "…adverse effects on the developing organism that may result from exposure prior to conception (either parent), during prenatal development, or postnatally to the time of sexual maturation."

However, as currently interpreted, the Proposition 65 statute precludes listing on the basis of developmental effects resulting solely from postnatal exposures. Therefore, in determining whether the criteria specified in 22 CCR 12306(g) have been met, only data that pertain to prenatal exposures are considered. Thus, methemoglobinemia in exposed human neonates resulting from postnatal exposures does not provide a basis for listing a chemical as causing reproductive toxicity under Proposition 65.

The four animal studies cited by U.S. EPA, in which exposures occurred that were entirely prenatal, all provide some indication of a potential hazard posed by sodium nitrite to the developing organism (*California Regulatory Notice Register*, May 21,

1999). These four studies were conducted by various protocols, focused on diverse endpoints, and do not support each other in identifying a particular target of sodium nitrite toxicity. Three of the cited studies (Shuval and Gruener, 1972; Globus and Samuel, 1978; Roth et al., 1987) have limitations in design and reporting such that they provided insufficient support for listing under the authoritative bodies provision of Proposition 65 (22 CCR 12306(g)).

With regard to the fourth study, which had been available to OEHHA in the Japanese language with English summary, comments received in response to publication of the Notice of Intent to List (*California Regulatory Notice Register*, December 4, 1998) included a full English translation of the publication (Shiobara, 1987). Detailed review of the complete text led to serious doubts as to the confidence with which conclusions can be drawn from the study. As treatment began on day six of gestation, the same day implantation occurs in mice, and as there was a significant increase in pre-implantation loss, the change in litter size may have been an artifact of non-treatment-related variation in implantation frequency. Thus, the body of evidence related to prenatal exposures cited by U.S. EPA was found to be insufficient to satisfy the regulatory criteria for listing under the authoritative bodies provision (i.e., 22 CCR 12306(g)).

Accordingly, as required by 22 CCR 12306(i), the chemical has been referred to the State's Qualified Experts so that they can render an opinion as to whether the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause reproductive toxicity. In preparing this Hazard Identification Document, studies which were cited by U.S. EPA in support of adding sodium nitrite to the TRI list as a developmental toxicant (*Federal Register* **59**:1788-1859, 1994 and **59**:61432-61485, 1994) have been reviewed in detail. Considerable additional information on the potential developmental and/or reproductive toxicity of sodium nitrite has also been included.

## B.3. Exposure

Due to the interconversion of nitrate and nitrite in biological systems (for discussion, see Section B.4., "Metabolism and Pharmacokinetics," below), exposure to both compounds is of significance to the potential toxicity of nitrite. Although they can be found in drinking water, the diet is generally the most significant source of human exposure to both compounds (WHO, 1985). Nitrites and nitrates are used as preservatives in products such as cured meats and some types of cheese, but the major dietary source of nitrate is vegetables (NAS, 1981a; Walker, 1996).

As reported in a review by Cassens (1997), nitrate concentrations in drinking water vary widely, and may be cause for concern in some areas. Among the European population, drinking water is not expected to contribute more than 30% of the total dietary nitrate intake (WHO, 1985). In regions where drinking water nitrate levels exceed 10 mg/L, however, the contribution from this source may be considerably higher. In the U.S., water from private wells frequently exceeds the standard of 10 mg/L nitrate-N or 45

mg/L nitrate (NIEHS, 1970). For bottle-fed infants, drinking water (given as plain water, or used to make up feeds) is considered to be the major source of dietary nitrate (WHO, 1985; Gangolli et al., 1994). Breast milk, on the other hand, tends to be low in nitrate, even when the lactating mother consumes nitrate-rich drinking water.

Nitrite is used as a preservative in cured meat products, to prevent the growth of the botulism toxin-producing organism, *Clostridium botulinum* (Sen and Baddoo, 1997). Additionally, formation of nitroso-compounds of myoglobin and hemoglobin enhance the red color of cured meats (NIEHS, 1970). USDA regulations allow addition of sodium nitrite to cured meats at a maximum concentration of 156 ppm (1/4 oz or 7 g/100 lbs of meat) (Cassens, 1997). Residual nitrite levels in retail cured meat products are considerably lower, with current assays showing a mean of approximately 10 ppm.

Nitrate levels can be relatively high (as much as 226 mg/kg and more) in foods such as lettuce, spinach, beets, and celery (NIEHS, 1970; WHO, 1985). According to a recent review (Walker, 1996), it is common for nitrate levels in these vegetables to exceed 1 g/kg fresh weight, and even attain levels as high as 3-4 g/kg.

Nitrite is also found in plant foodstuffs but, compared to nitrate, the concentrations are normally much lower (Walker, 1996). A typical amount for all vegetables would be 1-2 mg/kg fresh weight, but nitrite levels as high as 60 mg/kg have been reported in potatoes, with an average level of 19 mg/kg. There is a potential for nitrate to be converted to nitrite by microorganisms during storage of raw foods, as well as in prepared foods, once these are opened and exposed to ambient microbes (Am. Acad. Peds, 1970; NIEHS, 1970).

As might be expected, human nitrate and nitrite intakes vary widely, depending on the composition of the diet. Cassens (1997) reviewed reports of nitrite intake from different foods. According to the sources cited, 39% of dietary nitrite intake was from cured meat, 34% from baked goods and cereals, and 16% from vegetables. The remaining 11% was not accounted for. In the case of nitrate, 80 to 85% of dietary intake by European populations is considered to originate from vegetable sources (Walker, 1996).

The average daily intake of nitrates has been estimated at approximately 10-30 mg, with vegetarians having a two to 4-fold higher intake than non-vegetarians (WHO, 1985). Walker (1996) reported a somewhat higher range of 31-185 mg nitrate/day, for nitrate consumption among Europeans. In the UK, specifically, the mean nitrate intake was estimated at 54 mg/day, with a higher mean of 185-194 mg/day for vegetarians. Nitrite intake in European countries was reported to average around 0.7-8.7 mg/day, with both vegetables and cured meats being significant sources.

Fan et al. (1987) reviewed estimates of average daily nitrate and nitrite in the U.S. These estimates included both dietary and drinking water sources. For nitrate intake, published estimates ranged from 0.186-100 mg/person/day, with intakes for vegetarians estimated at 2.0-268 mg/person/day. Assuming that approximately 5% of an ingested dose of nitrate is reduced to nitrite in the oral cavity (NAS, 1981a; Walker, 1996; WHO, 1996a),

this level of nitrate intake would provide around 0.009-5.0 mg nitrite/person/day for nonvegetarians. Estimates of direct nitrite intake ranged from 0.01-0.77 mg/person/day, with vegetarians falling within this range (Fan et al., 1987).

## B.4. Metabolism And Pharmacokinetics

Nitrate and nitrite are readily interconverted by oxidation-reduction reactions in biological systems. Thus, the pharmacokinetics and metabolism of nitrate and nitrite cannot adequately be considered separately from one another; both are also intimately connected to the potential for *in vivo* formation of N-nitroso compounds (Gangolli et al., 1994; NAS, 1981a; Walker, 1996; WHO, 1996a).

### *B.4.1. Nitrate*

Ingested nitrate is readily absorbed from the proximal small intestine, reaching peak levels within 1-3 hours in human serum, urine, and saliva (Walker, 1996). In humans, about 25% of an ingested and absorbed dose of nitrate is actively secreted in the saliva, due to the functioning of an active transport mechanism common to iodide, thiocyanate, and nitrate (NAS, 1981a; Walker, 1996; WHO, 1996a). Salivary secretion of nitrate also occurs in most laboratory animals, apart from the rat.

The apparent lack of this particular transport mechanism in rats complicates extrapolation of experimental toxicology results obtained in that species to humans (NAS, 1981a; Walker, 1996; WHO, 1996a). Additionally, although nitrate reduction in the lower part of the gastrointestinal (GI) tract is relatively greater in rats than in humans, the nitrite formed there is less efficiently absorbed. These metabolic differences may be responsible for the observation that the no observed adverse effect level (NOAEL) for (sub)acute toxicity in humans is 10-60 times lower than the NOAEL in rats (WHO, 1996a).

Reduction of nitrate to nitrite occurs *in vivo* as a result of mammalian nitrate reductase activity, as well as due to the nitrate reductase activity of microorganisms resident in the oral cavity and upper GI tract (NAS, 1981a; Walker, 1996; WHO, 1996a). In particular, the typically stable population of nitrate-reducing bacteria colonizing the base of the tongue is thought to be responsible for the significant level of nitrate reduction which takes place in the mouth. Salivary nitrite concentrations are considered to be directly related to the orally ingested dose of nitrate, although the reduction process may be saturable at high intakes. Allowing for considerable inter-individual variation, it has been estimated that 25% of total nitrate ingested by humans is secreted in the saliva. Of this 25%, 20% (or about 5% of the ingested dose) is reduced to nitrite. Hence, oral reduction of nitrate is considered to be the most important source of nitrite in humans, as well as in other species possessing an active salivary secretion mechanism.

In healthy adult humans, gastric pH is generally low. The low pH suppresses the growth of nitrate-reducing bacteria, and hence gastric nitrite levels are correspondingly low (NAS, 1981a; Walker, 1996; WHO, 1996a). Similar gastric conditions prevail in rabbits

and ferrets. Rats and dogs, on the other hand, have gastric pH sufficiently high as to allow bacterial colonization and *in situ* nitrite formation. In ruminants, which characteristically have a dense gastric population of microflora, the stomach is a major site of reduction of orally ingested nitrate.

With certain medical conditions, or even in healthy individuals, gastric pH in humans can rise sufficiently to allow bacterial colonization, and hence gastric nitrite formation (NAS, 1981a; Walker, 1996; WHO, 1996a). Neonates, who normally have a higher gastric pH than do adults, are also more likely to have a stomach colonized by nitrate-reducing bacteria (Am. Acad. Peds., 1970; U.S. FDA, 1972). This has been postulated as an underlying cause of the known greater propensity of infants for methemoglobin (MetHb) formation (Am. Acad. Peds., 1970; U.S. FDA, 1972).

At similar nitrate intake levels, however, healthy infants showed far lower percentages of MetHb than did infants suffering from gastroenteritis (Shearer et al., 1972; Goldsmith et al., 1975; Hegesh and Shiloah, 1982; Walker, 1996). Hence, bacterial contamination of the water supply has been implicated as a component, along with nitrate exposure, in risk for infant methemoglobinemia (Fan and Steinberg, 1996). Some authors (Hegesh and Shiloah, 1982; Avery, 1999) have taken this reasoning one step further, and postulated that gastrointestinal infection and inflammation lead to endogenous formation of excess nitrite or nitric oxide from nitrogen-containing compounds. Thus, in their view, gastroenterititis may be a more significant cause of infantile methemoglobinemia than high nitrate levels in drinking water.

*B.4.2. Nitrite*

While there is no direct information on the absorption of nitrite in humans, absorption is inferred from reports of methemoglobinemia following oral or percutaneous exposure (Walker, 1996; WHO, 1996b; Saito et al., 1996 and 1997). Following intravenous administration of nitrite, the plasma half-life in the distribution stage in dogs, sheep, and ponies was found to be 48, 12, and 5 minutes, respectively (Walker, 1996). Oral administration of nitrite to experimental animals is not usually followed by detectable levels in tissues and bodily fluids, presumably due to the rapidity with which nitrite is oxidized to nitrate (Walker, 1996; WHO, 1996b). Nitrite is not a normal constituent of urine in humans or animals; its presence is considered to indicate a bacterial infection of the urinary tract (NAS, 1981a; WHO, 1985).

In blood, nitrite undergoes a coupled oxidation reaction with oxyhemoglobin to produce methemoglobin. Unlike Hb, MetHb cannot exchange oxygen; hence, the presence of excess MetHb in the circulation proportionately reduces the ability of the blood to transfer oxygen. The rate of MetHb formation is species dependent, with the rate in humans being intermediate to the rates found in ruminants and pigs (Walker, 1996).

Nitrite can cross the placenta into the fetal circulation. This has been demonstrated in experimental animals following treatment by injection (Gruener et al., 1973; Shuval and Gruener, 1972; Kociba and Sleight, 1970). Lactating dams given nitrites in their drinking water showed high MetHb levels in their blood (Gruener et al., 1973). Their nursing

pups, however, showed no rise in MetHb, indicating that appreciable levels of nitrites were not transferred via the dams' milk.

Human infants have a high proportion of fetal hemoglobin in their blood, which has a greater propensity than adult hemoglobins for MetHb formation (Am. Acad. Peds., 1970). Possession of fetal hemoglobins, in combination with the low levels of MetHb reductase which are also characteristic of infants, is thus thought to contribute to the particular sensitivity of infants to nitrate/nitrite toxicity.

*B.4.3. N-Nitroso compounds*

Nitrite, or the nitrosating species derived from it ($N_2O_3$ and $N_2O_4$), can combine *in vitro* or *in vivo* with secondary amines (such as ethylurea) or N-alkylamides to form, respectively, carcinogenic nitrosamines ($R_1NNOR_2$) and nitrosamides ($R_1NNO.COR_2$) (U.S. FDA, 1972; Rustia and Schenken, 1976; Shank and Newberne, 1976; NAS, 1981a and 1981b; Alexandrov et al., 1990; Gangolli et al., 1994; Mirvish, 1995; Walker, 1996; WHO, 1996a and 1996b). Humans are exposed to a wide range of amines through endogenous production or ingestion of numerous foods, beverages and medicines (Maga, 1978).

Antioxidants, such as vitamin C, are good inhibitors of N-nitroso formation (Mirvish, 1995). Rats, mice, hamsters, and most other mammals synthesize vitamin C. Notable exceptions are humans, non-human primates, guinea pigs, and Indian fruit bats (Hardman *et al.*, 1996). These latter species must obtain vitamin C from the diet; hence, vitamin C intake may significantly impact the likelihood of endogenous N-nitroso formation.

Nitrosamines and nitrosamides must undergo further metabolism to generate the ultimately carcinogenic DNA-alkylating species (Mirvish, 1995). Nitrosamides are converted to alkylating species by chemical non-enzymic reactions, and often induce tumors at the site of administration. In the case of nitrosamines, on the other hand, the necessary reactions are dependent on the cytochrome *P*-450 enzyme system of the endoplasmic reticulum. Not only does the type and quantity of *P*-450 activity vary between organs and tissues, thus contributing to target organ specificity, but immature enzyme systems in the developing organism are also likely to be determinants of the degree of sensitivity to nitrosamine carcinogenicity (NAS, 1981b; Mirvish, 1995).

While the extent to which endogenous nitrosation occurs under actual food intake conditions is not entirely clear (Shank and Newberne, 1976; Gangolli et al., 1994; Walker, 1996), a rise in urinary excretion of N-nitrosodimethylamine (NMDA), a known carcinogen (Ivankovic, 1979), was observed in human volunteers ingesting potassium nitrate together with a fish meal containing nitrosable precursors (Vermeer et al., 1998). The total quantity of nitrate provided to each volunteer, by all sources, was equivalent to the current acceptable daily intake (ADI), or approximately 210 mg. The authors assumed that approximately 0.5% of the NMDA formed was secreted in the urine. On this basis, the volunteers were estimated to have produced about 174 µg NMDA, or 2.9 µg NMDA/kg bw/day, a value that can be compared to the 10 µg NMDA/kg bw/day

which is carcinogenic to rats. Nitrosamine formation was not prevented by the amount of vitamin C (17 mg/100g) and other antioxidants present in the vegetables which were also consumed by the volunteers. Observed correlations between urinary nitrate and salivary nitrate, and salivary nitrite versus urinary NDMA were taken to support a relationship between nitrate intake and endogenous NMDA formation.

## B.5. Toxicity: Methemoglobin Formation

*B.5.1. General*

A major concern in nitrite toxicology is induction of methemoglobinemia, especially in infants. The process by which hemoglobin (Hb) is oxidized to MetHb involves oxidation of the ferrous iron ($Fe^{2+}$) of the heme group to the ferric iron ($Fe^{3+}$) (WHO, 1996b; Shuval and Gruener, 1977). As part of this reaction, nitrate is eventually generated from nitrite. The ferric form is incapable of the normal physiological function of picking up and giving off oxygen in response to the partial pressure of the gas in the lung and tissue capillaries, respectively (NIEHS, 1970). Thus, transfer of oxygen from blood to the body's tissues and organs is inhibited.

Normal background MetHb levels in human blood average about 1-3% (WHO, 1996a; Fan and Steinberg, 1996). Clinically detectable reductions in oxygen transport become manifest at MetHb levels of about 10% and above (Fan and Steinberg, 1996). For example, oxygenation of muscle tissue, under working conditions, was found to be impaired at MetHb levels of 10-12% (Shuval and Gruener, 1977). Stupor, coma, and death result at 60% or more MetHb (Fan and Steinberg, 1996).

MetHb-formation is a reversible reaction. Reduction of MetHb to Hb is catalyzed by the enzyme system NADPH-MetHb reductase (WHO, 1996b), an enzyme active in red blood cells (Gruener et al., 1973). The rate of this reduction reaction varies between species, as well as with age of the organism. For example, MetHb levels in adult rats were found to be reduced by 50% at around 90 minutes after cessation of exposure to sodium nitrite in drinking water (Gruener et al., 1973). This rate was independent of the initial MetHb level.

NADPH-MetHb reductase activity has been measured in blood samples from pregnant rats, fetal rats, and pregnant women, and in human umbilical cord blood (Gruener et al., 1973). MetHb reductase activity was found to be ten times higher in rat fetuses than in pregnant rats or human cord blood. Pregnant women had MetHb reductase activity which was 1.5 times higher than that found in cord blood. At least part of the extreme sensitivity of human neonates to methemoglobinemia is thought to be due to their relatively low levels of MetHb reductase (NAS, 1981b; WHO, 1996b).

In addition to the very young, other populations thought to demonstrate particular sensitivity to MetHb formation are: pregnant women, individuals having genetically-determined deficiencies in MetHb reductase or glucose-6-phosphate dehydrogenase (an NADPH-generating enzyme), and possibly the elderly (Goldsmith et al., 1975; Fan et al.,

1987; NAS, 1981b; WHO, 1996a). The possibility of sex-based differences in MetHb reductase activity, or in other aspects of MetHb formation and degredation, is suggested by the sex difference in acute $LD_{50}$s found for mice of the ddy strain (Shiobara, 1987). The mean $LD_{50}$s reported for male and female ddy mice were 245 mg/kg and 194 mg/kg, respectively.

When the reductase system becomes saturated, the blood MetHb concentration increases, eventually resulting in ischemia, cyanosis, irreversible tissue damage, and death (WHO, 1996b). Methylene blue, ascorbic acid, and methionine all seem to have some protective effects against nitrite-induced MetHb formation. Methylene blue is used clinically as an antidote in cases of nitrite intoxication. Guinea pigs, like humans, must obtain ascorbic acid from the diet; ascorbic acid supplementation has been shown to influence the extent of MetHb formation in this experimental animal model (Kociba and Sleight, 1970).

*B.5.2. Infants and children*

Reported cases of methemoglobinemia in humans have generally occurred in infants of less than three months of age who have consumed water containing more than 22 mg /L nitrate-N (Craun et al., 1981). In the U.S., over 320 cases of infant methemoglobinemia have been reported to be associated with the use of nitrate-containing well water (NIEHS, 1970). Other cases, particular in Europe, have been associated with the consumption of high-nitrite containing vegetables, particularly spinach (Sander and Jacobi, 1967; NIEHS, 1970; Faivre et al., 1976; Hack and Dowes, 1983).

Apart from their greater total fluid intake per unit body weight, the particular susceptibility of human neonates to MetHb formation and toxicity has generally been attributed to three factors: the higher pH of the infant stomach, the lower levels of MetHb-reductase in infant erythrocytes, and the nature of fetal hemoglobins (Am. Acad. Peds., 1970; NIEHS, 1970; U.S. FDA, 1972; Walker, 1996; Shuval and Gruener, 1977; NAS, 1981b; WHO, 1996a and 1996b). The influence of gastric pH on the conversion of nitrate to nitrite, as well as the rate-limiting role of MetHb-reductase have been touched on in previous sections of this document. While there is no question that nitrite reacts directly with hemoglobin, it has been hypothesized that endogenous formation of nitrite, resulting from over production of nitric oxide by tissues inflamed due to bacterial infection, may be a more significant cause of infant methemoglobinemia than ingested nitrate (Hegesh and Shiloah, 1982; Avery, 1999).

Fetal hemoglobin is the third factor thought to be responsible for the enhanced sensitivity of infants to methemoglobinemia. Although the assumption has been challenged (Avery, 1999), it has generally been reported that, compared to adult Hb, fetal Hb converts more readily to MetHb (WHO, 1985). Fetal Hb binds oxygen more strongly than does adult Hb; after birth, fetal Hb is gradually replaced by adult Hb. In infants of less than three months of age, fetal Hb can comprise as much as 60-80% of total Hb (NAS, 1981b; WHO, 1996b). By around three months of age, fetal Hb has decreased to only 20-30% of total Hb, as the adult forms of Hb gradually come to predominate.

In a study of children aged 1-8 years, no relationship was found between MetHb levels and the nitrate concentration in their drinking water (Craun et al., 1981). The best predictor of MetHb levels identified in this study was a model incorporating gender, the presence of fecal coliform bacteria in water, and whether nitrate-preserved meats had been consumed in the 24 hours prior to testing. Individually, however, these "best predictors" did not show statistically significant associations with MetHb levels. For example, there was no trend associating higher MetHb levels in children with consumption of water from wells having greater levels of bacterial contamination.

Goldsmith et al. (1975) reported on the distribution of MetHb levels among populations in California. The study included groups of infants, children, adolescents, and adults. Other than for the infants, nitrate-nitrite intake, *per se*, was not evaluated in this study, but the populations studied resided in communities where water supplies were known to occasionally have elevated nitrite levels, and/or where photochemical air pollution was considered to provide exposure to oxides of nitrogen. The data obtained for infants appeared to support the influence of both nitrate-nitrite nitrogen intake, and health status as determining circulating MetHb levels. In all the Southern California populations evaluated in this study, the median MetHb values exceeded 2%, and ranged up to 5%. Such values are generally higher than normal background, and the authors expressed concern that the consequent interference with oxygen transport could be sufficient for some degree of circulatory impairment.

# C. DEVELOPMENTAL TOXICITY

## C.1. Developmental Toxicity; Non-Cancer Endpoints

### *C.1.1. Overview*

No human data were available which evaluated the potential effects of sodium nitrite intake during pregnancy on fetal viability, malformation frequency, measures of growth, or functional parameters. A number of studies conducted in experimental animals have evaluated non-cancer endpoints of developmental toxicity; these studies are discussed below. Data from animal studies in which exposure occurred only during the prenatal period (e.g. excluding lactational or other postnatal exposures), are summarized in tabular form at the end of the section C.1.2.2.

Studies which were cited by U.S. EPA in support of adding sodium nitrite to the TRI list as a developmental toxicant are marked with an asterisk (*). In preparing this Hazard Identification Document, additional studies addressing the potential developmental toxicity of sodium nitrite were identified, and have been included.

Further relevant information includes data pertaining to: the developmental toxicity of sodium nitrate in humans and experimental animals, the developmental toxicity of potassium nitrite in experimental animals, placental transport of nitrite, nitrite-induced

methemoglobinemia in the pregnant animal and her offspring, and the teratogenicity and transplacental carcinogenicity of N-nitroso compounds for which nitrite is a precursor.

*C.1.2. Animal developmental toxicity studies*

C.1.2.1. Exposure restricted to the prenatal period

C.1.2.1.1. Kociba and Sleight, 1970; nitrite treatment of ascorbic acid deficient guinea pigs

Several experiments were performed in an attempt to clarify the relationship between ascorbic acid deficiency and sensitivity to sodium nitrite toxicity. Guinea pigs were fed on a diet lacking in ascorbic acid, until their plasma ascorbic acid decreased to a level considered to be "subnormal." In the first experiment, administration of 50 mg/kg sodium nitrite by subcutaneous (s.c.) injection produced significantly higher MetHb levels in 12 ascorbic acid deficient guinea pigs than in 6 controls. Higher MetHb levels were found at all time points tested: 60, 90, and 120 min post injection. No control animals died, while mortality was 83.3% in treated/deficient animals.

In a second experiment, 11 pregnant guinea pigs were given 45 mg sodium nitrite/kg by s.c. injection during the last week of gestation. Treatment caused abortion in those pregnant guinea pigs having plasma ascorbic acid levels lower than 0.40 mg/100 ml. Females having plasma ascorbic acid levels higher than 0.40 mg/100 ml, did not abort their litters. Control sows having low ascorbic acid levels, but free of sodium nitrite treatment, carried their litters normally through parturition. All living and aborted fetuses were free of gross abnormalities.

In a third experiment, eight pregnant guinea pigs, five of which had low plasma ascorbic acid levels, were given 40 mg/kg sodium nitrite by s.c. injection. Fifty-five minutes after sodium nitrite treatment, maternal and fetal blood samples were taken, and determinations made of Hb, packed cell volume (PCV), and plasma ascorbic acid. Maternal Hb, PCV, and plasma ascorbic acid were lower in ascorbic acid deficient dams than in their offspring. MetHb levels in treated-deficient dams (35.2%) were significantly higher than in non-deficient dams (25.9%; $P = 0.05$). Fetal MetHb levels did not differ between the deficient and non-deficient group. Fetal MetHb levels for the two groups were 10.1% and 10.3%, respectively.

The authors attributed the more severe effects of sodium nitrite on ascorbic-acid deficient guinea pigs to: "(1) a relative decrease in ascorbic acid available as a reducing substance, and (2) decreased hemoglobin and PCV values, which increased the ratio of nitrite per erythrocyte."

C.1.2.1.2. Sinha and Sleight, 1971; nitrite treatment of pregnant guinea pigs

Several experiments were performed to investigate the effects of sodium nitrite on pregnant guinea pigs during the last 15 days of pregnancy. The reporting of this study was not exact, but it appears that single injections were given, sometime during days 47-

62 of pregnancy. Average gestation length in the guinea pig is 63-68 days, with litter sizes typically ranging from 1-6 pups (Trewin and Hutz, 1998).

In the first experiment, groups of 3-4 pregnant guinea pigs were given sodium nitrite by s.c. injection at doses of 50, 60, or 70 mg/kg bw. Two control animals were given 50 mg NaCl/kg bw, and one additional control was given 60 mg NaCl/kg bw. All control animals, as well as those given the low dose of sodium nitrite, gave birth to normal, live litters (Sinha and Sleight, 1971). Pregnant guinea pigs given a dose of 60 mg/kg all aborted their litters within 1-4 days of treatment. All pregnant guinea pigs given the high dose of 70 mg sodium nitrite/kg bw died within 60 minutes of treatment.

In a second experiment, two groups of nine guinea pigs were given s.c. injections of either $NaNO_2$ or NaCl at a dose of 60 mg/kg. Animals were sacrificed and examined at set intervals between 15 minutes and 56 hours post-injection. All fetuses of control animals survived, while 96% of fetuses from sodium nitrite treated sows died at three or more hours following the injection. Neither nitrite nor MetHb were detected in blood samples from control sows or their fetuses. MetHb was detectable in the blood of nitrite-treated sows and their fetuses for up to six hours following treatment. Maternal MetHb concentrations were higher than those of their offspring at all timepoints measured (20, 40, and 60 minutes post injection; $P<0.001$). Mean maternal MetHb levels ranged from 44-67%, and mean fetal levels from 8-22%. Nitrite was detected in maternal and fetal plasma of treated animals at 15 minutes and at 90 minutes following treatment, but not after three or more hours. Maternal plasma nitrite levels were significantly higher than fetal plasma nitrite levels ($P<0.01$).

Sequential pathological changes in the placental tissue were also noted as progressing with time after sodium nitrite treatment. At 18 hours and later timepoints, the placentas of treated animals were described as pale, compared to the ruddy placentas of control animals. Placentas from treated animals killed at 24 hours or more post-treatment were describes as "friable," and "dead and edematous."

In a third experiment, six pregnant guinea pigs were given an ip injection of 10 mg/kg methylene blue, a MetHb antagonist. Three of these animals were simultaneously given sodium nitrite by s.c. injection at a dose of 60 mg/kg bw; the other three were given sodium chloride. Pairs of animals were sacrificed at 3, 24, and 100 hours following treatment. Three additional animals were given both methylene blue and sodium nitrite, and allowed to carry their litters to term. Two animals were given sodium nitrite only (60 mg/kg), and a third animal was given methylene blue only (10 mg/kg); these animals were allowed to carry their litters to term. All fetuses from sows given 60 mg/kg sodium nitrite alone died, while all fetuses from sows given methylene blue (with or without sodium nitrite) survived.

In a fourth experiment, six guinea pigs at approximately day 60 of gestation were given a single s.c. injection of 60 mg sodium nitrite/kg. At 30, 60, and 90 minutes post-injection, maternal and fetal blood samples were drawn from uterine and umbilical blood vessels,

respectively. Methemoglobin levels and $PO_2$ values were determined for these samples. Maternal and fetal $PO_2$ dropped as MetHb levels rose.

C.1.2.1.3. *Globus and Samuel, 1978; study of erythropoiesis in fetal mice

Globus and Samuel (1978) conducted a study of erythropoiesis in fetal CD-1 mice following maternal exposure to sodium nitrite. The study was intended to test a working hypothesis that sodium nitrite-induced fetal methemoglobinemia would be expected to result in increased fetal erythropoietic activity.

CD-1 mice were time-mated, and the day of plug detection was considered to be day one of gestation. Treated animals were given 0.5 mg sodium nitrite per mouse, by intubation, daily throughout gestation, until sacrifice on gestation day 14, 16, or 18. For an approximate average body weight of 25 mg, this provided a dose of 20 mg/kg/day. Control animals were given distilled water, also by gavage. Twenty-three control and 23 treated litters were sacrificed for examination on gestation day 14; 12 control and 9 treated litters were examined on day 16; and four control and four treated litters were evaluated on gestation day 18.

Litter size, the number of resorption sites and dead implants, and embryo/fetal weights were recorded at the time of sacrifice. Embryos/fetuses were examined for gross anatomical defects, and, following removal of the livers for analysis, their carcasses were prepared for skeletal examinations. Parameters of erythropoiesis were determined for hepatic tissues, as the liver is the main site of erythrocyte production between gestation days 12 and 19 in the mouse.

No differences were found between treated and control animals, at any of the time points evaluated, for litter size, mean litter weight, mean embryo weight, mean number of resorption sites, or percentages of dead implants. There were no statistically significant differences between treated (n=42) and control (n=37) animals in the frequencies of skeletal abnormalities or variations, but the authors noted a tendency toward talipomanus and talipes in the nitrite-treated group. No data were provided to support this tendency.

Hepatic hematopoeisis was evaluated as percentages of total hepatic erythroblasts which were at various distinct stages of maturation. The overall frequency of red blood cells was significantly increased in treated animals at 14 and 16 days of gestation, but not at 18 days. The percentage of mature erythrocytes in hepatic tissue was reduced in treated animals on gestation days 14 and 16, but not on gestation day 18. The percentages of pro- and basophilic erythroblasts were significantly decreased in treated animals of 14 and 16 days gestation age, but not on gestation day 18. Polychromatophilic erythroblasts were significantly more frequent in treated than control animals on gestation day 14, and significantly less frequent on gestation day 16, with no difference between treated and control animals on gestation day 18. Orthochromatophilic erythroblasts were also found at a higher percentage in treated than control animals on gestation day 14, and to be unchanged from controls on gestation day 18. This cell type, however, was found to be more frequent in treated than in control animals on gestation day 16.

The overall frequency of white blood cells was slightly, but not significantly, reduced at all three time points. The proportion of neutrophil granulocytes was significantly increased in treated animals at day 18, but was unchanged from control values at the other time points.

The authors interpreted their results as indicating a treatment-dependent increase in embryonic hepatic production of erythroid cells. The lack of a sustained increase in mature red blood cells in the livers of day 18 fetuses was considered to be due to a normal developmental shift in the main site of erythropoesis from the liver to the bone marrow and spleen. The authors acknowledge, however, that no increase in red blood cell counts could be demonstrated in the peripheral circulation. Hence the functional significance of their findings remains unclear.

C.1.2.1.4 *Shiobara, 1987; Developmental toxicity study in mice.

Groups of 25 time-mated female Crj:CD-1 mice were exposed to sodium nitrite by gavage, at doses of 0, 20, 40, 80, or 120 mg/kg/day, on gestation days 6-15. On gestation day 17, 20 pregnant mice from each group were sacrificed for examination of their uterine contents. The five remaining mice in each group were allowed to deliver their offspring normally for postnatal observations.

One maternal death occurred in the 40 mg/kg/day group; no other dams died. No clinical symptoms of toxicity were reported during the course of the study. Maternal body weights on gestation day 17, and maternal body weight gains over the gestation period, were significantly reduced at the two highest doses of 80 and 120 mg/kg/day. Kidney and liver weights showed significant decrements from controls at some doses, but there was no evidence for a dose-response relationship. Furthermore, when considered relative to body weights, organ weights were found not to differ between groups. There were no differences between groups in hemoglobin or hematocrit values.

There were no differences between groups in the frequency of pregnancy among mated females, nor were there any incidents of total litter loss or premature delivery. The number of implantations per litter was significantly reduced in the high-dose group, as was mean litter size. As it is likely that implantation preceded or coincided with the beginning of treatment, the observed changes in implantation frequency are unlikely to have resulted from treatment. In turn, the change in litter size on gestation day 17 may have been an artifact of non-treatment related variation in implantation frequency.

The percentage of living fetuses at each dose was significantly reduced at both 20 and 120 mg sodium nitrite/kg bw, but not at the intervening doses of 40 and 80 mg/kg. The total number of dead fetuses, and the number of early fetal deaths was significantly increased at the high dose. At the low dose, the total number of dead fetuses was significantly decreased. Fetal weights were significantly increased over controls at all doses of sodium nitrite, with no apparent relationship to administered dose.

Isolated incidences of cleft palate were observed in all groups (treated and control), with no evidence for a treatment-related effect. No other external abnormalities were observed. There were some significant differences between treated and control fetuses in the pattern of ossification of skeletal elements, but there was no consistent treatment-related pattern of skeletal retardation or advancement. As compared to controls, the frequency of a 14th rib was significantly lower in the fetuses of animals given 20 or 80 mg sodium nitrite, but significantly higher in fetuses of the 40 or 120 mg/kg/day groups. No differences were observed between groups in the frequency of litters having at least one fetus with 14 ribs.

For the five females in each dose group who delivered their fetuses normally, only the high-dose group showed significant reductions in body weight, relative to controls, on gestation day 17. There were no significant differences between groups in the rate of body weight gain during gestation. When these dams were necropsied subsequent to weaning of their offspring, uterine weights were significantly reduced in the 20 and 120 mg/kg groups, but not in the two intervening dose groups. Hemoglobin was significantly increased in the 40 mg/kg dose group, and hematocrit was significantly decreased in the 120 mg/kg dose group.

Litter size at postnatal day 70 was significantly reduced from control levels in the 40 and 80 mg/kg groups. No offspring deaths were reported for the first 14 days following birth. During the interval between postnatal day 14 and postnatal day 70, one pup died in each of the control and low-dose groups, and two pups died in the high-dose group.

Pup body weights on postnatal day 14 were significantly reduced in the 20 and 120 mg/kg dose groups, but not in the 40 or 80 mg/kg/day dose groups. Hence, the dose-response pattern for body weight reductions mirrors that observed for litter size. In other words, the data suggest that pups in the smaller litters of the 40 and 80 mg/kg dose groups had less competition for milk, and thus were able to maintain a normal body weight.

By postnatal day 70, there were no differences between dose groups in the mean weights of male or female pups. Some significant differences in mean organ weights were found between dosed groups and the control group, but these differences were not apparent when organ weights were considered relative to body weights.

Compared to controls, hematocrits were significantly reduced in all groups of sodium nitrite-exposed female pups. Hemoglobin content was also reduced in treated animals, reaching statistical significance for males in the 20 and 80 mg/kg groups, and for females in the 40 mg/kg group.

C.1.2.1.5. Shimada, 1989; developmental toxicity study in mice

Groups of 16 to 19 pregnant ICR mice were given sodium nitrite in their drinking water at concentrations of 0, 100, or 1000 mg/L. Treated water was given between gestation days 7 and 18. At the low and high doses, the average sodium nitrite consumption was

determined to have been 0.81 and 7.3 mg/mouse/day (approximately 27 and 243 mg/kg/day), respectively. Females were sacrificed, and their uterine contents evaluated on gestation day 18.

There were no statistically significant changes in water consumption or gestational weight gain of treated dams. Nor were there any significant changes in the numbers of corpora lutea, live fetuses per litter, or resorbed or dead fetuses per litter. No significant differences were reported between groups for sex ratio, the frequency of runting, fetal body weights, or the frequencies of external or skeletal malformations. No significant changes were noted in the frequency of chromosomal gaps or breaks in maternal bone marrow cells, or in fetal liver cells.

C.1.2.1.6. Nyakas et al., 1990; developmental neurotoxicity of sodium nitrite

Pregnant Wistar rats were given 2 g/L sodium nitrite in drinking water, from gestation day 13 until parturition. Apart from untreated controls, other experimental groups were given either the calcium antagonist nimodipine, at a dose of 10 mg/kg bw, by gavage, from gestation day 11 through parturition, or both nimodipine and sodium nitrite. The number of animals assigned to each treatment group was not stated in the paper.

At two months postnatal age, male offspring were divided into test groups, and subjected to either discrimination or passive avoidance testing. Auditory discrimination for a drinking water reward (conducted following 23 hours of water deprivation) was tested, as well as visual discrimination in a (footshock) active avoidance situation. The passive avoidance test system consisted of avoiding footshock by learning not to enter a darkened compartment. Extinction and retention of these behaviors were also evaluated. Five to nine animals were subjected to each testing series.

Acquisition of the initial phase of all three tasks showed no significant differences between treatment groups. For all animals, performance of the auditory conditioned response was "almost errorless" within four training sessions. Acquisition of the visually cued, one-way avoidance response was also comparable between groups ($F_{2,13} = 0.1$; $P = 0.9$). More than 50% of the animals from all groups showed maximal passive avoidance following the first footshock experience.

Animals exposed prenatally to nitrite, however, were found to be impaired both in discrimination learning behavior (both auditory and visual), and in long-term retention of the passive-avoidance response. In the discrimination phase of the auditory test, the nitrite-treated rats made more errors than did either the controls or the nitrite plus nimodipine -treated group in response to the non-reinforced stimulus ($F_{1,12} = 21.26$; $P<0.001$ and $F_{1,16} = 10.68$; $P<0.01$, respectively). A similar pattern was found for the error rate in response to the reinforced stimulus ($F_{1,16} = 5.89$; $P<0.05$ and $F_{1,16} = 6.32$; $P<0.05$, for differences from the control and nimodipine plus nitrite groups, respectively).

Impairment of long-term retention of the passive avoidance response (intertrial intervals of 3 or 4 days) was shown for nitrite-exposed animals compared to the control group ($U = 33$; $P = 0.02$). The effect of nitrite was at least partially reversed by nimodipine, as no difference was found between the controls and the nitrite plus nimodipine group ($U = 75$; $P = 0.6$). However, there was no significant difference between the nitrite only group, and the nitrite plus nimodipine group ($U = 43$; $P = 0.09$). In the first two sessions of the retention test, significantly more individuals of the nitrite plus nimodipine group showed the avoidance response as compared to the nitrite treated group ($X^2 = 4.53$; $P<0.05$; and $X^2 = 4.11$; $P<0.05$, respectively). Differences between the nitrite-only group and the control group were also significant ($X^2$; $P<0.05$). By the third trial, the extinction process was too far advanced in all groups to reveal any between-group differences.

C.1.2.1.7. Nyakas et al., 1994a; effects of prenatal exposure to sodium nitrite on the postnatal development of hippocampal and neocortical cholinergic and serotonergic innervation

Pregnant Wistar rats were given 2 g/L sodium nitrite in drinking water, from gestation day 13 until parturition. Apart from untreated controls, other experimental groups were given either the calcium antagonist nimodipine, at a concentration of 1000 ppm in food pellets, from gestation day 11 through parturition, or both nimodipine and sodium nitrite. The number of animals assigned to each treatment group was not stated in the paper. Treatments were terminated at parturition, and the litters culled to eight pups. Brains of male pups were processed for histopathological staining of acetylcholinesterase (AchE) and serotonin (5-hydroxytryptamine: 5-HT)-positive fibers. Brains were sampled on each of post-natal days 1, 3, 5, 7, 10, and 20. From each litter group, only a single pup was assigned to one of the age-groups, to avoid possible litter-based confounding effects.

Prenatal nitrite exposure was shown to modulate the development of AchE and 5-HT positive fiber ingrowth into the hippocampal dentate gyrus (DG) and parietal neocortex during the first week of postnatal life. Fiber densities were evaluated from serial sections. Effects on both fiber types were region-specific in the hippocampus, and restricted to the DG. Based on an ANOVA test of treatment effects, nitrite was found to significantly influence cholinergic fiber density in the DG ($F_{1,39} = 3.92$, $P < 0.05$). The number of fiber crossings in the DG region was also decreased in nitrite-treated postnatal day 5 and 7 animals. Co-administration of nimodipine prevented these effects.

In the parietal cortex, the delay in cholinergic fiber ingrowth was more pronounced in deep than in superficial layers, while the serotonergic innervation was influenced more evenly. By postnatal day 10, the differences between nitrite-treated and control rats were no longer apparent. ANOVAs carried out for postnatal days 3-10, revealed a treatment effect on cholinergic fibers restricted to the deeper layers ($F_{2,66} = 3.71$, $P < 0.05$), with a specific effect of nitrite (without nimodipine) on lowering fiber density ($F_{1,39} = 5.67$, $P < 0.02$). On postnatal day 7, 5-HT fiber density was significantly lower in nitrite-exposed animals than in controls, in both layers of the parietal cortex. Again, the effect of nitrite was reversed in the presence of nimodipine.

The authors concluded that the impact of prenatal nitrite exposure was most evident during the early postnatal period, when rapid fiber proliferation normally takes place.

They attributed the effects of nitrite to prenatal hypoxia, and the mitigating effects of nimopidine to its neuroprotective or antihypoxic action. Nimopidine would be expected to block increases in intracellular $Ca^{2+}$ concentrations associated with perinatal brain damage of hypoxic/ischemic origin. They postulated that the transitory effects of prenatal hypoxia on fiber ingrowth could, in turn, result in long-lasting functional deficits in the rat brain.

C.1.2.1.8. Nyakas et al., 1994b; long-term behavioral effects of prenatal chronic anemic hypoxia

Pregnant Wistar rats were given 2 g/L sodium nitrite in drinking water, from gestation day 13 until parturition. Apart from untreated controls, other experimental groups were given either the calcium antagonist nimodipine, at a dose of 10 mg/kg bw, by gavage, from gestation day 11 through parturition, or both nimodipine and sodium nitrite. All treatments ceased at parturition. There were eight to ten dams in each treatment group, and no significant differences in litter size or pup birthweight were noted.

Behavioral testing was performed on adult male offspring at the ages of 5 months, 19 months, and 23-26 months. Spontaneous behaviors, such as open-field activity and social behavior, were measured repeatedly. Learning tests were applied only during old age, in order to avoid repeated use of footshock. Plasma corticosterone responses were measured at 28 months of age.

By 2-way ANOVA, both age and treatment-dependent effects were apparent on measures of open-field activity (latency to start locomotion and number of crossings of lines marking the test arena). A statistically significant interaction between these two factors ($F_{2,128} = 2.44$; $P<0.05$) indicated that the effect of prenatal nitrite exposure on start latency was more profound at advanced ages. Line-crossing activity also decreased with age ($F_{2,128} = 46.75$; $P<0.001$). Nitrite-only exposed rats were less active than controls at both 5 and 19 months of age ($P<0.05$), but not at 23 months.

Total duration of social interactions, as measured by recording the duration of activities such as inspection, mounting, grooming, and play fighting, showed a marked effect of treatment ($F_{2,43} = 76.31$; $P<0.001$). The number of social interactions occurring during the five minute observation periods also varied between treatment groups ($F_{2,43} = 3.42$; $P<0.05$); this was attributed primarily to the high activity levels of nitrite plus nimodipine-exposed animals at 23 months, as compared to the nitrite-only group.

At 24 months of age, rats exposed prenatally to nitrite showed no evidence of ability to discriminate between light and dark areas of the test cage, following avoidance training by administration of footshocks in the dark area. The vehicle controls, and nitrite plus nimodipine-exposed animals showed dark-avoidance behavior in initial trials, as well as extinction of this response in later trials, in response to withdrawal of the aversive stimulus. Nearly 60% of nitrite-exposed animals showed an extreme response to the footshock stimulus, and vocalized, jumped, and/or bit the grid floor. This was a significant increase over the frequency of such reactions in vehicle controls, or in animals prenatally exposed to nitrite plus nimodipine ($P<0.02$, by $X^2$).

At the age of 28 months, plasma corticosterone levels were measure 15, 30, and 90 minutes after test animals were subjected to a novel stress. Compared to basal corticosertone levels, determined from samples taken at one minute prior to application of the novel stressor, an increased corticosterone response was induced in all three groups ($F_{3,42} = 9.53$; $P<0.001$). The shape of the stress-response curve depended on the nature of prenatal treatment ($F_{3,42} = 2.71$; $P<0.02$). For vehicle control and nitrite plus nimodipine-exposed rats, corticosterone levels had returned to baseline levels by 90 minutes following the stress incident. In the nitrite-only exposed animals, plasma corticosterone was still significantly elevated above baseline levels at 90 minutes post-stress ($P<0.05$). Upon necropsy, adrenal weights, and adrenal weights relative to body weights were found to be significantly ($P<0.05$) higher in rats prenatally exposed to nitrite alone, as compared to control animals. Animals exposed to nitrite plus nimodipine had higher adrenal weights, but also higher body weights than controls, hence relative adrenal weights were comparable between the latter two groups.

## Table 1. Effects of Prenatal Exposure to Sodium Nitrite on Development in Guinea Pigs

| Reference | Study Design | Fetal Effects | Maternal Effects |
|---|---|---|---|
| **Kociba and Sleight, 1970** | 11 pregnant sows given 45 mg sodium nitrite/kg bw, s.c. Ascorbic acid status of animals varied. | No gross abnormalities.<br>All litters aborted in sodium nitrite treated, ascorbic acid-deficient animals.<br>No abortions in non-deficient, treated animals, or in deficient animals not given sodium nitrite. | No deaths in non-deficient group; 83.3% mortality in deficient group.<br>Higher MetHb levels in deficient than in non-deficient dams. |
| | 8 pregnant sows, 5 having low ascorbic acid status, were given 40 mg sodium nitrite/kg bw, s.c. | Fetal MetHb levels did not differ between deficient and non-deficient groups. | Maternal Hb, PCV, and plasma ascorbic acid lower in ascorbic acid-deficient dams than in their offspring.<br>MetHb levels significantly higher in deficient than in non-deficient dams. |
| **Sinha and Sleight, 1971** | 3-4 pregnant sows given 1 dose of 50, 60, or 70 mg sodium nitrite/kg bw, s.c. | At 60 mg/kg, all litters aborted.<br>No effects at lower dose, or in controls. | All dams died following 70 mg/kg. |
| | 2 groups of 9 sows given 60 mg sodium nitrite or sodium chloride/kg bw, s.c. | All control fetuses survived.<br>96% of exposed fetuses died.<br>Nitrite and MetHb were detectable in plasma of treated, but not control fetuses.<br>Gross pathological damage to placentas of treated animals. | No nitrite or MetHb detected in plasma of control sows.<br>In treated animals, nitrite and MetHb levels significantly higher in dams than fetuses. |
| | 6 pregnant sows were given 10 mg/kg methylene blue, i.p., and 60 mg sodium nitrite/kg bw, s.c.. 2 animals were given sodium nitrite only, and a 3rd was given methylene blue only. | All fetuses from animals given sodium nitrite alone died.<br>All but two fetuses from animals given methylene blue (with or without sodium nitrite) survived. | Not reported. |
| | 6 pregnant sows were given 1 dose of 60 mg sodium nitrite/kg bw, s.c.. Blood samples were drawn at 30, 60, and 90 minutes post injection | $PO_2$ levels dropped as MetHb levels rose | $PO_2$ levels dropped as MetHb levels rose. |

## Table 2. Effects of Prenatal Exposure to Sodium Nitrite on Development in Mice

| Reference | Study Design | Fetal Effects | Maternal Effects |
|---|---|---|---|
| **Globus and Samuel, 1978** | 20 mg sodium nitrite/kg/day by gavage, throughout gestation.<br>Sacrificed for evaluation on day 14 (n=23 each, control and treated), 16 (n=12 control, 9 treated), or 18 (n=4 each, control and treated). | No significant differences between groups for: litter size, weight, resorptions, fetal death, or skeletal abnormalities or variations.<br>Significant changes in hepatic erythropoiesis.<br>No sustained increase in hepatic mature red blood cells on day 18. | Not reported. |
| **Shiobara, 1987** | 0, 20, 40, 80, or 120 mg sodium nitrite given by gavage to groups of 25 pregnant mice on gestation days 6 - 15. | Significant decreases in: implantation frequency and mean litter size at 120 mg/kg/day; living fetuses at 20 and 120 (not 40) mg/kg/day; dead fetuses at 20 mg/kg/day.<br>Significant increases in: dead fetuses and early fetal deaths at 120 mg/kg/day; increased fetal weights at all doses, with no dose response. | 1 maternal death at 40 mg/kg<br>No clinical symptoms of toxicity.<br>Body weights on day 17, and gestational weight gain significantly reduced at 80 and 120 mg/kg/day.<br>Some decreases in absolute, but not relative, liver and kidney weights.<br>No effects on Hb or Ht. |
| **Shimada, 1989** | Sodium nitrite was given in drinking water to groups of 16-19 pregnant mice, during gestation days 7-18. Doses approximated 0, 27, and 243 mg/kg/day. | No significant changes in: live fetuses per litter, fetal weights, sex ratio; or in frequencies of resorptions, dead fetuses, runting, or external or internal malformations.<br>No significant changes in the frequency of chromosomal gaps or breaks in liver cells. | No significant changes in: water consumption, gestational weight gain, or frequency of chromosomal gaps or breaks in bone marrow cells. |

## Table 3. Effects of Prenatal Exposure to Sodium Nitrite on Development in Rats

| Reference | Study Design | Offspring Effects | Maternal Effects |
|---|---|---|---|
| **Nyakas et al., 1990** | 2 g sodium nitrite/liter drinking water from gestation day 13 through birth.<br>10 mg nimodipine/kg bw by gavage to some animals (with or without sodium nitrite), from gestation day 11 through birth.<br>Behavioral testing of male offspring at 2 months postnatal age.<br>Number animals/group not stated. | No changes in initial acquisition of learned responses.<br>Nitrite-exposed animals significantly impaired for discrimination learning behavior, and for long-term retention of passive-avoidance.<br>Co-administration of nimodipine prevented these effects. | Not reported. |
| **Nyakas et al., 1994a** | 2 g sodium nitrite/liter drinking water from gestation day 13 through birth.<br>1000 ppm nimodipine in food pellets to some animals (with or without sodium nitrite), from gestation day 11 through birth.<br>Number animals/group not stated.<br>Brain histology of male pups on post-natal days 1, 3, 7, 10, and 20. | Significant effects of sodium nitrite on ingrowth of cholinergic and serotonergic nerve fibers into the hippocampal dentate gyrus and the parietal neocortex.<br>Co-administration of nimodipine prevented these effects. | Not reported. |
| **Nyakas et al., 1994b** | 2 g sodium nitrite/liter drinking water from gestation day 13 through birth.<br>10 mg nimodipine/kg bw by gavage to some animals (with or without sodium nitrite), from gestation day 11 through birth.<br>8-10 dams/group.<br>Behavioral testing of male offspring at 5, 19, and 23-26 months. | Age and treatment had significant effects on measures of open field activity.<br>Impaired discrimination learning at 24 months of age (not tested at earlier time points). Hyperreactivity to footshock.<br>Significantly prolonged elevation in plasma corticosterone following a novel stressor in 28-month old nitrite-exposed animals.<br>Effects alleviated or prevented by nimodipine. | Not reported. |
| **Shuval and Gruener, 1972** | 0, 2000, or 3000 mg sodium nitrite/L drinking water to groups of 7-12 rats during gestation and lactation. | Mean litter sizes lower in treated than control animals; no statistical analysis.<br>Birthweights similar between groups. | Not reported. |

C.1.2.2. Exposure during both the pre- and postnatal periods

C.1.2.2.1. *Shuval and Gruener, 1972; postnatal growth and mortality

Groups of 12 pregnant rats were given sodium nitrite in drinking water at concentrations of either 2000 or 3000 mg/L. A group of seven controls was given plain tap water. While the experimental details are not provided, it appears that litters were delivered normally and remained with their dams until weaning. Treatment of the dams' drinking water continued during the lactation period. At weaning, the pups were put on plain drinking water.

Reported effects for pups included: increased mortality, decreased weight gain, and poor appearance of the fur. Mean litter size (presumably at birth) was 10 for controls, 9.5 at the low dose, and 8.5 at the high dose. No statistical analysis was provided. Mortality was reported as 6% for controls, 30% for the low-dose group, and 53% for the high-dose group. It is not stated, however, during what period that mortality occurred. Birthweights were similar for treated and control animals, but growth rates were substantially slower for treated animals. At weaning, the mean weight of control pups was 51.5 g, while the mean weights of low and high-dose group pups were 29.5 and 18.5 g, respectively. Subsequent to weaning, growth rates of the dosed groups improved. No evidence was found of abnormally high MetHb in pups, but mean Hb levels were approximately 20% lower in treated than control animals.

C.1.2.2.2. Gruener, 1974; effects of nitrite exposure on aggression in male mice

Five mated pairs of C57Bl6J mice were given sodium nitrite in their drinking water at a concentration of 1 g/L. Five additional pairs served as controls. When young were born, the adult males were removed. Litters were left with their dams for 21 days; treatment of the sodium nitrite exposed females was continued during this time. At weaning (21 days postnatal), 12 male offspring were chosen at random from each group and removed to isolation cages. Males from the sodium nitrite-treated group were continued on the 1 g sodium nitrite/L drinking water solution. Aggression testing was commenced following an eight week isolation period. Each animal was placed in a "fighting cage" with another animal for a period of ten minutes, once each week, for six weeks. Four of the sessions were with other males from the same group (treated or control), and two sessions were with males from the other group. The level of aggression was scored according to a predetermined scale having a maximum score of 20. Following this first round of aggression testing, the treated animals were switched to plain tap water, and after a break of two weeks, aggression testing was repeated.

For the first round of testing, aggression scores were significantly elevated for the treated animals. Aggression scores were higher for treated males paired with control males (mean=12.95), than for treated males paired with other treated males (mean=10.88). These differences disappeared following a two-week recovery period after withdrawal of sodium nitrite.

C.1.2.2.3. Vorhees et al., 1984; developmental toxicity and developmental neurotoxicity in rats

Groups of male and female Sprague-Dawley rats were given sodium nitrite in their feed at concentrations of 0, 0.0125, 0.025, or 0.05% (w/w). Treated diets were provided for 14 days prior to mating, throughout the breeding period (1-14 days), and throughout gestation and lactation. Weaned offspring were continued on the same diets for the duration of the study. Live litters were culled on postnatal day one to no more than 12 pups; litters of fewer than eight pups were discarded.

Feed consumption and body weight of parental animals were unaffected by treatment. Fecundity of sperm-positive females, gestation length, litter size, and sex ratio were all also unaffected by sodium nitrite. No external malformations were observed in newborns. Following culling, the numbers of litters in each group were 25, 15, 14, and 14; for the 0, 0.0125%, 0.025%, or 0.05% concentration groups, respectively.

While litter size and post-weaning pup mortality were both unaffected by sodium nitrite exposure, treatment was associated with increases in pre-weaning pup mortality. Statistically significant increases in pup mortality were reported for days 0 to 1 in the 0.025% group, and for days 2 to 24 in the 0.05% group.

Preweaning pup body weights were decreased in both the 0.025% and the 0.05% sodium nitrite groups, as compared to controls. These differences were statistically significant for both males and females, in both groups, on postnatal day 14. By weaning and thereafter, these differences were no longer apparent.

The preweaning appearances of surface righting, pivoting, negative geotaxis, and auditory startle behavior were all unaffected by exposure to sodium nitrite. Control of swimming direction and control of head height while swimming, however, were delayed in sodium nitrite exposed preweaning pups. These effects were more pronounced at the two higher sodium nitrite concentrations. Open-field activity was decreased in preweaning females of the middle concentration group ($P<0.05$).

In postweaning pups, sodium nitrite exposure was not associated with any delays or changes in: the timing of vaginal opening in females, mastery of a swimming maze, active or passive avoidance, running-wheels, or negotiating a rotating rod. At necropsy on postnatal day 90, there were no significant reductions in body or brain weights with sodium nitrite treatment. Females of the 0.0125% and 0.025% sodium nitrite groups showed a 4-5% reduction in eye weights ($P<0.05$).

Open field activity on postnatal days 40-45 was significantly reduced in both males and females of the high and low-sodium-nitrite concentration groups, but not in the mid-concentration group. As compared to controls, the low-concentration group also showed significantly longer response latencies on the first day of post-weaning testing. Due to the lack of a clear dose-response relationship, the authors considered that interpretation of responses at the low and mid-concentrations of sodium nitrite was problematical. They concluded, however, that the effects observed in the high-concentration group gave

evidence that sodium nitrite is capable of inducing moderate decreases in open-field locomotor activity.

C.1.2.2.4. Olsen et al., 1984; lifetime dietary study of sodium nitrite-treated meat in rats

A cohort of 70 male and 140 female rats was divided into groups and fed cured meat to which 0, 200, 1000, or 4000 mg sodium nitrite/kg had been added. One control group received casein as a protein source; another control group was given fresh chopped pork. Treated diets were provided to $F_0$ animals from ten weeks prior to mating, and continued until animals were terminated on the study. Subsequent to weaning, groups of 60-100 $F_1$ animals were continued on the respective $F_0$ diet.

Following canning, autoclaving, and storage prior to experimental use, the treated diets were determined to have nitrite contents of 6, 47, or 580 mg/kg. Data on feed consumption were not provided in the paper, but the doses of sodium nitrite consumed in the form of treated diets have been estimated at 5, 25, or 100 mg/kg bw (WHO, 1996b). No differences were noted between treated and control $F_0$ animals in appearance or behavior, nor were effects noted on reproductive parameters such as pregnancy rate, litter size, mean pup weight, or pup survival. No morphological abnormalities were observed in $F_1$ offspring. There were no significant differences in the rate of postnatal mortality between treated and control pups.

C.1.2.2.5. *Roth et al., 1987; postnatal endpoints in Long-Evans rats.

Postnatal endpoints were investigated in Long-Evans rats following exposure to sodium nitrite in drinking water during pregnancy and lactation. Three related experiments were reported in a single paper: I - pilot dose-response; II - dose-response; and III - cross-fostering.

In experiment I, three groups of 8-10 pregnant females were given 0, 2.0, or 3.0 g sodium nitrite/liter drinking water. According to the study authors, these concentrations resulted in total sodium nitrite consumption, over 18 days of gestation, of 3.94 and 5.40 mg/g bw (219 and 300 mg/kg bw/day, respectively). Lactating dams given the same drinking water solutions had higher sodium nitrite intakes of 420 and 514 mg/kg/day. Litters were delivered normally, and culled to eight pups on postnatal day 1. Hematological parameters were determined on postnatal days 9 and 16.

In experiment II, four groups of 5-8 pregnant animals were given 0, 0.5, 1.0, or 2.0 g sodium nitrite/liter drinking water. These concentrations were reported to result in total sodium nitrite consumption, over 18 days of gestation, of 1.3, 2.14, and 3.6 mg/g bw (72.2, 119, 200 mg/kg bw/day, respectively). Litters were culled to ten pups on postnatal day 2, and blood samples were taken on postnatal days 7, 9, 13, 16, and 20.

In experiment III, pregnant females were given either plain tap water, or tap water containing sodium nitrite at a concentration of 2.0 g/liter. All pups were fostered at birth, such that four groups were created: control (prenatal)/control (postnatal); control

(prenatal)/treated (postnatal); treated (prenatal)/control (postnatal); treated (prenatal)/treated (postnatal). Each litter consisted of ten pups, and there were 5-6 litters per group. Pups were sacrificed for hematological and histological assessment on postnatal days 7, 14, or 21.

No effects on maternal gestational weight gain were found in experiments I or II; data on maternal weight gain were not presented for experiment III. In some instances, maternal fluid consumption was significantly reduced in treated animals as compared to controls: at both doses in experiment I, and in the high concentration group in experiment II. Treatment had no effect on litter size, sex ratio, or mean pup weights in any of the three experiments.

Postnatally, adverse effects became manifest for both dams and pups. In experiment I, maternal water consumption and weight gain in the treated groups were significantly depressed during the lactation period. A dose-response trend for reduced maternal body weight was also apparent in experiment II, although the differences from controls did not reach statistical significance. Fluid consumption in experiment II was not significantly decreased by treatment.

In experiment I, pup growth was significantly depressed, and pup mortality was increased, both in a dose-dependent fashion. Affected pups were described as "pale, weak, and in generally poor condition with distended bellies." In experiment II, only the high-concentration group pups showed significant growth impairment. Pups from the lowest concentration group were significantly heavier than controls on postnatal days 3 and 6. There was no postnatal pup mortality in experiment II

In experiment III, the strongest effects on postnatal weight were seen for pups exposed to sodium nitrite during both gestation and lactation. Pups exposed during lactation alone were also affected, although to a lesser degree. Pups exposed only during the prenatal period showed no significant differences from controls in postnatal body weights, although there was an apparent influence of prenatal exposure on pup growth between postnatal days 1 and 8.

Hematological parameters were significantly affected in treated animals in all three experiments. In experiment I, hemoglobin content and red blood cell counts (RBCs) were significantly decreased in a dose-dependent manner on postpartum days 9 and 16. Maximum corpuscular volume (MCV) was significantly reduced at both concentrations of sodium nitrite, when measured on postnatal day 16. MVCs were also reduced in treated, relative to control, animals on postnatal day 9, but the differences were not statistically significant. Methemoglobin levels were not significantly different from controls at either sodium nitrite concentration, or on either day.

Methemoglobin levels were not determined in experiment II, but hemoglobin levels for the high concentration group (2.0 g $NaNO_2$/liter) were significantly lower than controls starting from postnatal day 7. RBCs for this group were significantly reduced relative to controls by postnatal day 9. Hematological effects were also seen in the lower

concentration groups, but only at later timepoints. The most sensitive endpoint was MCV, which was significantly reduced, in a concentration-dependent fashion, at all three concentrations on postnatal day 16.

The cross-fostering experiment (experiment III) demonstrated that the observed anemia was predominantly postnatal in origin. The prenatal-exposure-only group, however, did show a significant reduction in MCV on postnatal day 21. Methemoglobin was not measured in this experiment.

C.1.2.2.6. Roth and Smith, 1988; nitrite-induced iron deficiency in Long-Evans rats.

A series of three experiments was performed to evaluate the possible role of iron deficiency in the etiology of sodium nitrite-mediated developmental toxicity. In experiment I, pregnant Long-Evans rats were assigned to either control or treated conditions, with six animals in each group. Control animals were given plain tap water, while treated animals were given drinking water containing 3 g sodium nitrite/liter. Treatment began on gestation day 0, and continued throughout lactation. Litters were delivered normally, and culled to ten pups. Half the pups in each litter were given supplemental iron by i.p. injection, on postnatal days 0, 7, and 14. On each of postnatal days 7, 14, and 21, selected pups were sacrificed for hematological and histopathological analysis.

In experiment II, four groups of animals were delineated: ten pregnant controls, five virgin controls, 13 pregnant treated animals, and four virgin treated animals. Treated animals were given 2.0 g sodium nitrite/liter drinking water. All animals were sacrificed on postnatal day 15 for analysis of hematological parameters.

In experiment III, pregnant females were maintained throughout gestation and lactation on drinking water containing 0 (n=7) or 2.0 (n=8) g sodium nitrite/liter drinking water. All pups and dams were sacrificed for evaluation on postnatal day 15.

Results of experiment I included the finding that fluid consumption was significantly reduced in treated animals over the period of gestation, but maternal gestational weight gain was not significantly affected. At birth, pup sex ratio and litter size were unaffected by treatment, but pup weights were significantly lower in treated than control litters. Birth weights were analyzed by 2-way ANOVA, examining the contributions of sex and treatment.

By the second week postpartum, pups not supplemented with iron developed severe microcytic anemia. These pups showed reduced postnatal weight-gain, and significantly increased mortality before day 20 postpartum. Hemoglobin, RBC counts, and MCV values were all significantly lower in treated than in control pups; iron supplementation ameliorated or eliminated all of these effects.

As in experiment I, treated experiment II females (both pregnant and unmated) consumed significantly less water than their respective controls. At the same time, weight gains

over 22 days (the gestation period in the pregnant animals) were unaffected by treatment. Mean lactational weight gain in experiment II was significantly reduced in treated dams, relative to controls. As evaluated on postnatal day 15, treated dams showed significant reductions in MCV, hemoglobin, and plasma iron levels. RBCs were unchanged from control values. In unmated females, nitrite treatment did not affect hematological parameters or plasma iron levels.

Litter size and sex ratio were unaffected in this experiment. Birth weights were reduced in treated male and female pups, relative to controls, but these differences were not statistically significant. Postnatal pup growth was significantly depressed. By postnatal day 15, pups of treated dams were demonstrably anemic, and had significant reductions in MCV, RBC, and hemoglobin levels.

In experiment III, the pattern of results for maternal fluid consumption and weight gain during gestation and lactation was similar to that seen in the other two experiments. Litter size was significantly increased in sodium nitrite-exposed pups, and pup weights were significantly decreased. While the decrease in birth weight may have been an artifact of the larger mean litter size, the ANOVA used to evaluate these data is stated to have examined only sex and treatment as sources of variation in birth weight. Litter size was evidently not incorporated into the analysis.

As in the previous experiments, postnatal pup growth was adversely affected by sodium nitrite exposure, as were hematological variables. On gestation day 15, heart weights relative to body weights were found to be significantly increased in treated pups. Relative spleen weights of these same pups were significantly decreased. Liver iron content was significantly decreased, and liver copper content significantly increased following sodium nitrite exposure. Milk from sodium nitrite-treated dams was found to have a significantly lower iron content than the milk of control animals. The authors postulate that sodium nitrite exposed-dams suffered from iron deficiency, and hence produced iron-deficient milk, which in turn was responsible for the adverse effects observed on their offspring.

### *C.1.3. Other relevant information*

#### C.1.3.1. Studies of the developmental toxicity of compounds related to sodium nitrite

##### C.1.3.1.1. Human studies

Because nitrate is converted to nitrite *in vivo*, studies of pregnancy outcome following exposure to nitrate during pregnancy may also be considered of relevance to the developmental toxicity of sodium nitrite. A few studies have examined incidence of malformations in relation to maternal exposure to nitrate in drinking water (Scragg et al., 1982; Dorsch et al.,1984; Arbuckle et al., 1988). In addition, one study of the effects of nitrosatable drug use (drugs which are amines that can undergo endogenous or exogenous nitrosation reactions to form N-nitroso compounds) during pregnancy provides

information of some interest (Olshan and Faustman,1989). The study demonstrated adverse outcomes similar to those seen in women exposed to nitrites and nitrates during pregnancy.

Scragg et al. (1982) reported excess malformations (mainly neural tube defects and defects affecting multiple systems) in women living in South Australia, and found an association with the consumption of drinking water from specific sources which differed as to nitrate content. In a case-control analysis, women drinking from groundwater sources which had elevated levels of nitrate (over 5 ppm) had a statistically significant increased risk of delivering a child with congenital malformations (RR=2.8, 95% CI=1.6-5.1) compared to women who drank rainwater. For central nervous systems (CNS) defects, the risk was higher (RR=3.5, 95% CI=1.2-14.6). In a follow-up case-control study, Dorsch et al. (1984) matched women on hospital, maternal age, parity and date of birth of the child. Compared to women who drank only rainwater (lower nitrate source) during their pregnancy, women who consumed principally ground water had a statistically significant increased risk of bearing a malformed child (RR=2.8, 95% CI=1.6-5.1 or 0.8-11.08 for lake and bore water, respectively). Analysis by estimated nitrate concentration showed a gradient of risk with nitrate level; 5-15 ppm nitrate and >15 ppm nitrate (RR=2.6, 95% CI=1.6-4.1; RR=4.1, 95% CI=1.3-13.1) compared to those drinking water with <5 ppm nitrate. Risks were elevated for CNS abnormalities (RR=3.5, 95% CI=1.1-14.6) and for abnormalities of the musculoskeletal system (RR=2.9, 95% CI=1.2-8.0). These investigators mentioned the possibility that local industrial releases might also be contaminating the ground water used as drinking water, and that these potential exposures could be contributing to the malformations seen.

A case-control study conducted in Canada (Arbuckle et al., 1988) examined the relationship between maternal exposure to nitrates in drinking water and risk of delivering an infant with a CNS malformation. Cases (n=130) were matched with controls (n=264) based on maternal residence and date of birth. Water samples were collected from houses of study subjects (76% of controls and 80% of cases) and analyzed for nitrate and other substances; information on the drinking water source for some additional subjects for whom samples were not available was collected by questionnaire. In general, nitrate levels were not excessive: the 37.5, 62.5, and 87.5 percentile of nitrate levels for water sources sampled were: public water – 0.36, 0.39, 3.35 ppm; spring water – 0.23, 1.31, 16.67 ppm; well water – 1.25, 6.25, 26.03 ppm. Samples from two cases and three controls exceeded the national limit of 44 ppm. Using a conditional logistic regression model that included information on 96 cases, exposure to 26 ppm nitrate in well waters (compared to a baseline of 0.1 ppm) was associated with a moderate increase in risk of CNS malformation which was not statistically significant (OR=2.30, 95% CI=0.73-7.29). For other water sources, no increase in risk was seen with increases in nitrate exposure. Based on the sample size, the study had a fairly low (57%) probability of detecting an odds ratio of 2.5 for well water users. In the region under study which had a higher prevalence of CNS malformations, there was some suggestion of an increasing odds ratio with increasing nitrate in drinking water (for 0.36 ppm [67.5 percentile], OR=1.02, 95% CI=0.98-1.06; for 1.65 ppm [87.5 percentile], OR=1.14, 95% CI=0.90-1.44). These authors suggest that the difference in risk seen in their study,

as compared to the Australian studies reported above (Scragg et al., 1982; Dorsch et al., 1984), may be due to higher nitrate levels in the Australian regions studied, differences in susceptibility of the populations, or to misclassification bias in the earlier studies, which had a longer follow-up period.

Olshan and Faustman (1989) examined the possible effects on pregnancy outcome of exposure to nitrosatable drugs (drugs which are amines that can undergo endogenous or exogenous nitrosation reactions to form N-nitroso compounds) during pregnancy. They evaluated information on 6061 exposed women, as compared to 6921 randomly sampled pregnancies without such exposure. No significant increases in fetal, neonatal or infant death, or low birthweight were found. An increase in the risk for major malformations was observed, and this increase was greater when exposure during the first four months of pregnancy was examined separately (RR=1.33, 95% CI=1.11-1.58). Relative risks were also elevated for some subgroups of CNS malformations when these were examined individually (hydrocephaly, RR=2.48; craniosynostosis, RR=3.53; meningomyelocoele/meningcoele, RR=3.16), but confidence intervals were wide and all included 1.0.

C.1.3.1.2. Studies in experimental animals

C.1.3.1.2.1. Summary and review of regulatory data submitted to U.S. FDA (1972)
The U.S. Food and Drug Administration (U.S. FDA, 1972) summarized and reviewed submitted data on the developmental toxicity of sodium nitrate and potassium nitrate in mice, rats, hamsters, and rabbits. No statistical analysis is presented for any of the studies summarized. There were no apparent differences between high-dose group animals and controls (in any of the four species studied) for variables such as maternal survival and live litter frequency. Some parameters, such as "percent partial resorptions" in all four species and live litter size in rabbits, appear to show possible effects of treatment. However, in the absence of a statistical analysis, as well as no presentation of standard deviations or dose-response data, the significance of these possible effects can not be evaluated. The Agency concluded that neither potassium nor sodium nitrate was associated with any adverse effects on fetal survival, nor did either compound demonstrably increase the frequency of soft tissue or skeletal anomalies.

C.1.3.1.2.2. Developmental toxicity of potassium nitrite in guinea pigs
Groups of 3-6 female guinea pigs were housed with at least one male, and given drinking water which contained potassium nitrite at concentrations of 0, 300, 1000, 2000, 3000, 4000, 5000, or 10000 mg/L (Sleight and Atallah, 1968). These concentrations were estimated to provide doses of 0, 110, 270, 940, 1110, 1190, 1490, or 3520 mg/kg bw/day for 100-240 days (WHO, 1996b).

Females in all groups became pregnant, indicating that fertility was retained by exposed males. Maternal weight gain was affected only at the highest potassium nitrite concentration; at that concentration, maternal weight gain was depressed. At the two

highest exposure levels, all fetuses, and one of the dams, died. Inflammation of the reproductive organs, and degeneration of the placenta were noted in females that aborted or carried mummified or resorbed fetuses. The percentage of fetal loss was higher in all treated litters than in controls, and appeared to generally increase with increasing dose, but no statistical analysis was provided.

C.1.3.2. Nitrite transport and toxicosis

C.1.3.2.1. Placental transport of nitrite

Kinetic experiments were performed in pregnant rats, using doses of 2.5 to 50 mg sodium nitrite/kg bw (Shuval and Gruener, 1972; Gruener et al., 1973). Doses were delivered either orally, or by injection (type of injection was not specified). Blood samples were taken, and fetuses removed from anesthetized females at regular intervals.

Nitrite levels in the fetal blood rose with a lag of about 20 minutes behind their mothers. The rise in nitrite was followed by a rise in MetHb. The kinetic pattern was the same in both dams and fetuses; different doses of sodium nitrite changed only timing and peak MetHb level.

For example, following an injected dose of 30 mg sodium nitrite/kg body weight, blood nitrite levels peaked at 32.5 μg/ml in the dams and 9.4 μg/ml in the fetuses (Gruener et al., 1973). Methemoglobin peaked at 60.2% of total Hb for dams, and at 27.2% of total Hb for fetuses. Lower doses gave lower levels, but similar kinetic patterns. The threshold for the "transplacental transfer effect" was a sodium nitrite dose of 2.5 mg/kg (Shuval and Gruener, 1972; Gruener et al., 1973).

C.1.3.2.2. Methemoglobinemia in pregnant and lactating rats and mice

Pregnant rats, as compared to nonpregnant rats, demonstrated higher susceptibility to nitrites in both chronic and acute experiments (Shuval and Gruener, 1972; Gruener et al., 1973). One hundred percent of five pregnant rats given 60 mg sodium nitrite/kg bw by injection died within one hour of treatment, while nonpregnant females survived this dose. In chronic studies where treated drinking water resulted in doses of approximately 200 to 250 mg/kg/day, pregnant rats became severely anemic, with a mean of $10.3 \pm 1.5$ g% Hb. Non-pregnant rats exposed to the same sodium nitrite levels had a mean Hb of $14.2 \pm 0.9$ g%, while control rats had a mean Hb of $14.3 \pm 0.8$ g% Hb.

Tarburton et al. (1985) studied the kinetics of MetHb formation *in vitro* using erythrocyte preparations from pregnant (n = 4) and non-pregnant (n = 6) Swiss-Webster mice. They found that the velocity of methemoglobinization was significantly higher ($P<0.05$) for erythrocytes originating from pregnant mice. Both the half-life and the lag phase of the reaction were significantly shorter for the pregnant animals.

C.1.3.2.3. Nitrite toxicosis in pregnant dairy cows and their fetuses

Six dairy cows in an advanced stage of pregnancy were given intravenous infusions of nitrite at doses of 7, 9.5, and 12 mg/kg bw (Van't Klooster et al., 1990). Maternal and

fetal blood vessels were catheterized. Infusions were conducted over a period of 30 minutes; each animal was subjected to three sessions, with the first session conducted no earlier than five days after the catheterization surgery.

Nitrite infusion caused a rapid, dose-related conversion of maternal Hb into MetHb, with the highest maternal MetHb levels recorded at 60 to 90 minutes following the start of infusion. Maternal blood pressure, both diastolic and systolic, fell almost immediately following nitrite infusion, with the lowest values recorded at 15 minutes following the cessation of infusion. The time lag to restoration of mean blood pressure increased with increasing nitrite dose, but the difference between the two highest doses was minimal. Maternal heart rates also tended to increase by 15 minutes post-infusion, with peak values occurring after 30 minutes. The rate increases were not dose-dependent, but the time lag to restoration of original heart rates increased with increasing dose. The pH and $PCO_2$ of maternal blood were not affected by nitrite infusion. Nitrite infusion caused maternal arterial $PO_2$ to decrease, but the decrease became only slightly more pronounced with increasing nitrite dose.

Following maternal infusions with nitrite, nitrite appeared in the fetal circulation, and fetal MetHb increased. An apparent dose-effect trend only reached statistical significance between doses of 7 and 12 mg nitrite/kg maternal bw ($P<0.05$). Responses of the fetal heart rate to maternal nitrite exposure were variable, with both bradycardia and tachycardia observed. Accelerated fetal heart rates were generally observed about 90 minutes following the start of maternal infusion with nitrite. Mean fetal blood pH was slightly decreased at around 90 minutes following maternal infusion with 9 or 12 mg nitrite/kg bw. At the high dose, the significance level of this change from pre-infusion values was $P<0.056$. The mean $PO_2$ of fetal blood was reported to be significantly (p value not provided) decreased within 15 minutes of the commencement of infusion, reaching its lowest level by about 45 minutes. The timing of the lowest fetal $PO_2$ levels coincided with the highest percentage of fetal MetHb. At the two lower nitrite doses, fetal $PO_2$ recovered gradually, returning to pre-infusion levels by 300 minutes post dosing. At the high dose of 12 mg/kg, mean fetal $PO_2$ remained significantly depressed ($P<0.05$ at 105 minutes; n=4).

The authors considered the effects of nitrite on maternal MetHb levels to be distinct from those on maternal cardiovascular parameters, and noted that both could independently influence fetal oxygenation. Regardless of the mechanism, the decrease in $PO_2$ of fetal arterial blood confirmed that impairment of utero-placental oxygen transfer occurred following maternal infusion with nitrite. At the doses and infusion rates used in this study, nitrite did not substantially increase the frequency of premature delivery or abortion; no other developmental parameters were evaluated.

C.1.3.2.4. Information from foreign language publications, available in English abstract only

Alexandrov and Janisch (1971) gave sodium nitrite to pregnant Sprague-Dawley rats by the oral route, with and without the additional application of ethylurea (ETU). Treatment was restricted to gestation days 9 and 10. Treatment-group sizes used in this study were

small, ranging from 3-15 animals. In the absence of a full translation, the doses used are unknown. Neither substance alone affected pregnancy outcome, but the combined treatment led to teratogenic effects. The authors concluded that sodium nitrite and ETU combined in the digestive tract of the pregnant animals to form ethyl nitrosourea, which in turn crossed the placenta, and disrupted development.

Kinoshita (1983) reported on the effects of prenatal sodium nitrite on "exercise function" in newborn mice. Sodium nitrite at a dose of 110 mg/kg bw was administered orally (probably by gavage) to pregnant JCL-ICR mice over one of 3, 7-day periods: early pregnancy, organogenesis, or fetal development. At one and four weeks postnatal age, mean MetHb contents were found to be high in weanlings born to dams exposed to sodium nitrite during organogenesis or fetal life. By 8 and 11 weeks postnatal age, MetHb contents did not differ between the different treatment groups, but were still somewhat higher than controls. Forced running distance of control animals at postnatal ages of 4 to 12 weeks increased over seven days. Exposed animals (any of the three treated groups) did not show this increase. Thus motor function of exposed weanlings was considered to have been inhibited for a period which was longer than the appearance of excess MetHb in their blood.

In a study of pigs published in the Romanian language (Neda et al., 1985), sodium nitrite was given to pregnant sows in drinking water and/or mixed in with feed. Initial concentrations used were 5 mg/L water and 10 mg/100 g feed; in later months these concentrations were raised to 10 mg/L water and 20 mg/100 g feed. Blood samples were evaluated after one week of treatment; at one, two, and three months of pregnancy; and at farrowing. As compared to controls, treated sows showed increases in serum nitrite and urea levels, and decreases in vitamin A levels. Offspring in the experimental group had lower Hb, Hct (hematocrit), and RBC counts, as well as higher serum nitrite levels.

In another study published in the Romanian language (Minciuna et al., 1985), pregnant heifers given 1-30 mg sodium nitrite/L drinking water showed no evidence of clinical disease or pregnancy problems. Parturition was normal, and calves showed no evidence of toxic insult. Maternal MetHb levels were found to exceed normal limits.

### *C.1.4. Integrative evaluation of developmental toxicity data (non-cancer endpoints)*

#### C.1.4.1. Overview

No studies on humans were available which directly evaluated the potential for prenatal exposure to sodium nitrite to cause adverse effects on fetal viability, growth, morphology, or functional parameters. Relevant, related information, however, included a study of the effects of nitrosable drugs on human pregnancy, and studies of human prenatal exposure to nitrate. Animal studies in which sodium nitrite exposure was restricted to the prenatal period have been conducted in guinea pigs, mice, and rats. Studies incorporating pre- and postnatal exposure have been conducted in rats and mice. Additional relevant information derived from studies in experimental animals included

developmental toxicity studies of sodium or potassium nitrate, and studies of nitrite-induced maternal and fetal methemoglobinemia.

Rather than employing standard study designs, the available data encompass a wide diversity of protocols and endpoints. This diversity limits the extent to which general comparisons can be made between studies. For example, one study in rats provided suggestive evidence of prenatal mortality associated with sodium nitrite exposure (Shuval and Gruener, 1972), but no other studies in rats provided data on comparable endpoints. Similarly, reports of prenatal effects on hematopoiesis have been derived from a study in mice, but potential functional consequences of the observed changes are unknown (Globus and Samuel, 1978).

There are, however, some areas where comparisons can be drawn. Increases in fetal and/or maternal methemoglobin levels have been reported in sodium nitrite-treated guinea pigs, rats, and dairy cows (Kociba and Sleight, 1970; Sinha and Sleight, 1971; Gruener et al., 1973; Van't Klooster et al., 1990), suggesting a potential for effects on the quantity of oxygen available to fetal tissues. In guinea pigs and dairy cows, fetal $PO_2$ levels were significantly decreased following maternal nitrite infusion (Sinha and Sleight, 1971; Van't Klooster et al., 1990). In the dairy cows, the timing of this decrease corresponded with peak fetal MetHb levels. In guinea pigs, levels of nitrite associated with rises in fetal and maternal MetHb were also associated with abortifacient and/or fetotoxic effects (Sinha and Sleight, 1971). It is also interesting to note that studies of neurobehavioral endpoints in rats (Nyakas et al., 1990, 1994a, and 1994b) found that adverse effects of sodium nitrite treatment could be prevented or alleviated by co-administration of nimodipine, a drug expected to have an anti-hypoxic effect.

C.1.4.2. Developmental toxicity: human data

Two epidemiological studies conducted in Australia examined the possible relationship between birth defects and high-nitrate concentrations in drinking water (Scragg et al., 1982; Dorsch et al., 1984). Statistically significant results from these studies indicated that elevated risk for congenital malformations, particularly malformations of the CNS, was associated with maternal consumption of drinking water containing sodium nitrate at concentrations in excess of 5 ppm. Findings from a case-control study conducted in Canada were far less striking than the Australian data (Arbuckle et al., 1988). Nitrate content of the water was generally associated with source (spring, tap, or well), with well water tending to have the highest nitrate content (26.03 ppm at the 87.5 percentile). For women who drank spring or tap water during pregnancy, the nitrate level, *per se*, was not shown to be associated with increased risk for birth defects. A moderate, non-statistically significant increase in risk for CNS malformations was associated with maternal consumption of well water having nitrate levels of 26 ppm or more.

N-nitroso compounds, which are known to be teratogenic and/or carcinogenic in experimental animals, have also been implicated as potentially responsible for the increased frequency of major malformations observed in offspring of women given nitrosatable drugs during pregnancy (Olshan and Faustman, 1989). Nitrite is a precursor

of N-nitroso compounds, but it has not been directly established that the necessary reactions occur in toxicologically significant quantities *in vivo*, under normal food intake conditions (Gangolli et al., 1994; Walker, 1996). However, the association between frequent consumption of hot dogs and/or other cured meat products during pregnancy, and an increased risk for childhood brain tumors in offspring (Bunin, 1998; see discussion of transplacental carcinogenesis in section C.2. below) suggests that such reactions might occur.

C.1.4.3. Developmental toxicity: experimental animal data

Data available in the published literature included studies of the effects of pre- and/or postnatal exposure to sodium nitrite on mice, guinea pigs, and rats. Additional relevant information on the developmental toxicity of compounds related to sodium nitrite is also discussed below.

Studies conducted in mice have not provided clear and consistent evidence for adverse effects of *in utero* exposure to sodium nitrite on measures of fetal viability, weight, sex ratio, or the frequencies of external or skeletal malformations (Globus and Samuel, 1978; Shiobara, 1987; Shimada, 1989). Nor did the Shiobara (1987) study provide clear, dose-dependent evidence for adverse effects of prenatal exposure on parameters of postnatal growth or viability. Treatment protocols used in all three of these studies covered at least a major portion of the organogenesis phase of development, and oral doses of sodium nitrite ranged from 20 mg/kg/day to 243 mg/kg/day.

The Globus and Samuel study (1978) was aimed primarily at detecting sodium nitrite-induced changes in fetal erythropoiesis in CD-1 mice. From the commencement of gestation until sacrifice, according to a time course schedule, pregnant animals were given a daily dose of 20 mg sodium nitrite/kg bw by gavage (Globus and Samuel, 1978). Treatment was associated with evidence for alterations in the proportions of hepatic erythroblasts at distinct stages of maturation. The authors interpreted the observed changes as indicative of a treatment-dependent increase in embryonic production of erythroid cells. No increase in peripheral red blood cell counts could be demonstrated, however, hence the functional significance of these findings remains unclear.

In pregnant guinea pigs, administration of 45 mg sodium nitrite/kg bw by s.c. injection during the last week of gestation resulted in spontaneous abortion of litters, but only in ascorbic acid-deficient females (Kociba and Sleight, 1970). Neither ascorbic acid deficiency alone, nor sodium nitrate in the presence of sufficient ascorbic acid, was associated with excess abortions. No gross abnormalities were noted in any living or aborted fetuses. In another study using guinea pigs (Sinha and Sleight, 1971), all pregnant sows given a dose of 70 mg sodium nitrite/kg bw by s.c. injection died within 60 minutes of treatment. All animals given a lower dose of 60 mg/kg bw aborted their litters. Co-administration of methylene blue, a MetHb antagonist, exerted a protective effect on fetuses.

Guinea pigs were also adversely affected by prenatal exposure to potassium nitrite in maternal drinking water (Sleight and Atallah, 1968). The percentage of fetal loss was higher in all treated litters than in controls, and appeared to generally increase with increasing dose (ranging from approximately 110 to 3520 mg potassium nitrite/kg bw/day), but no statistical analysis was provided. At the two highest doses, all fetuses and one dam died. Placental degeneration, and inflammation of the reproductive tract were noted in females having resorbed or mummified fetuses.

There were no studies available from the published literature which used rats in a standard developmental-toxicity protocol involving prenatal-only administration of sodium nitrite. There are, however, a series of studies which exposed pregnant rats to sodium nitrite during the latter half of gestation, and evaluated postnatal effects on behavior and neurological development (Nyakas, et al., 1990; Nyakas et al, 1994a and b). In other studies, sodium nitrite was administered to pregnant and lactating rats; in some cases, the weaned offspring were then exposed directly (Shuval and Gruener, 1972; Vorhees et al., 1984; Roth et al., 1987; Roth and Smith, 1988). Reported effects of such treatment have included increased postnatal mortality and depressed postnatal growth (Shuval and Gruener, 1972; Vorhees et al., 1984; Roth et al., 1987; Roth and Smith, 1988), as well as decreases in open-field locomotor activity (Vorhees et al., 1984), and effects on pup organ weights and iron status (Roth and Smith, 1988).

Simple learning in response to either a reward or an aversive stimulus was not affected in two month old male offspring of female rats exposed to sodium nitrite in the latter half of gestation (Nyakas et al., 1990). Discriminatory learning of both visual and auditory cues, however, was impaired in the nitrite-exposed animals, as was long-term retention of a conditioned passive-avoidance response. Effects of prenatal nitrite exposure did not diminish as the animals aged; at 24 months, nitrite-exposed animals were unable to discriminate between light and dark areas of the test cage in response to avoidance training (Nyakas et al., 1994b). Open-field activity levels, social behavior, and corticosterone levels in response to stress were also all affected in adult rats prenatally exposed to nitrite. Upon necropsy at 28 months of age, absolute and relative adrenal weights were found to be significantly higher in treated animals than in controls.

A study of cholinergic and serotonergic nerve fiber outgrowth in the hippocampus and neocortex of neonatal rats found significant delays associated with prenatal exposure to sodium nitrite (Nyakas et al., 1994b). The authors attributed their findings to nitrite-induced prenatal hypoxia, leading to retarded development of certain neurotransmitter pathways, in turn causing long-lasting dysfunctions in the developing rat brain. In the histopathological study, as in the behavioral studies (Nyakas, et al., 1990; and Nyakas et al, 1994b), co-adminsitration of the $Ca^{2+}$ channel-blocking drug, nimodipine, prevented the adverse effects associated with nitrite exposure alone. The protective effects of nimodipine were attributed to its antihypoxic activity, as it would be expected to block increases in intracellular $Ca^{2+}$ concentrations associated with perinatal brain damage of hypoxic/ischemic origin.

Pre- and postnatal exposure of rats to sodium nitrite has been reported to have adverse effects on hematological parameters, including dose-dependent decreases in Hb content, RBC counts, and MVC values (Roth et al., 1987). A later study by the same group (Roth and Smith, 1988) investigated the possible role of iron deficiency in producing the adverse postnatal effects associated with sodium nitrite exposure. In the absence of supplemental iron, sodium nitrite-treated pups developed severe microcytic anemia by the second week of postnatal life. The anemia was associated with significant depressions in hematological parameters, as well as with depressed growth and increased pup mortality. Iron supplementation mitigated or eliminated all of these adverse effects.

No adverse effects on pup weights or mortality were observed in a rat multigeneration study, in which animals were fed on a sodium nitrite-containing, cured-meat diet providing daily sodium nitrite doses of approximately 5, 20, or 100 mg/kg bw (Olsen et al., 1984; WHO, 1996b).

Two of the studies in which rats were exposed to sodium nitrite both pre- and post-natally presented data on parameters evaluated at birth. The Roth et al. (1987) paper described the results of a cross-fostering experiment, in which offspring were exposed during either, both, or neither the pre- and post-natal periods. Although data were not presented, the authors stated that prenatal-only exposure led to no statistically significant differences in body weights from control pups, but there was an apparent effect of prenatal exposure evident on postnatal days one through eight. Additionally, offspring exposed only prenatally to sodium nitrite showed a significant reduction in MCV on postnatal day 21. In a later study by the same group (Roth and Smith, 1988), pup sex ratio and litter size at birth were found to be unaffected by prenatal exposure to sodium nitrite, but birth weights were significantly lower (by an ANOVA test of sex and treatment as sources of variation) in treated than in control animals.

The U.S. FDA (1972) summarized the results of submitted data from teratogenicity studies on sodium and potassium nitrate, which were performed in rats, mice, hamsters, and rabbits. The Agency concluded that none of these studies demonstrated adverse effects of either compound on development. Conversely, the same N-nitroso compounds which can induce transplacental carcinogenicity in experimental animals when given late in pregnancy (see discussion in section C.2.3. below), may also induce a teratogenic response when given early in pregnancy (NAS, 1981b; Diwan et al., 1990; Donovan, 1999).

### C.1.4.4. Fetal and maternal methemoglobinemia

Administration of sodium nitrite to pregnant animals has been shown to result in fetal, as well as maternal, methemoglobinemia in guinea pigs and dairy cows (Kociba and Sleight, 1970; Sinha and Sleight, 1971; Van't Klooster et al., 1990). Experiments conducted in rats *in vivo*, and with mouse erythrocytes *in vitro*, have indicated that the pregnant animal is more susceptible to nitrite-induced hematological changes, as compared to the

nonpregnant animal (Shuval and Gruener, 1972; Gruener et al., 1973; Tarburton et al., 1985).

In guinea pigs, s.c. injections of 40 mg sodium nitrite/kg bw caused higher MetHb levels in ascorbic acid deficient dams than in non-deficient dams (Kociba and Sleight, 1970). Methemoglobin levels in the fetuses, however, were just over 10% regardless of the dams' ascorbic acid status. In a second study of methemoglobinemia in pregnant guinea pigs and their fetuses (Sinha and Sleight, 1971), nine animals were given 60 mg sodium nitrite/kg bw by s.c. injection, and evaluated at set intervals between 15 minutes and 56 hours post-injection. Nitrite and MetHb were undetectable in the blood of control sows and their fetuses, but were detectable in the blood of treated sows and fetuses for up to six hours following treatment. Mean MetHb levels ranged from 44-67% in dams, and 8-22% in the fetuses.

Nitrite infusion of pregnant, catheterized dairy cows caused a distinct sequence of responses in maternal animals and their fetuses (Van't Klooster et al., 1990). Nitrite appeared in both the maternal and fetal circulations, and both fetal and maternal MetHb levels rose in a dose and time dependent fashion. The authors considered the effects of nitrite on maternal MetHb levels to be distinct from nitrite's effects on maternal blood pressure and heart rates. Mean fetal $PO_2$ levels decreased following maternal infusion with nitrite, the lowest values coinciding with highest fetal MetHb values. The authors concluded that both the maternal cardiovascular changes, and the rise in maternal MetHb, could influence fetal oxygenation. Regardless of the mechanism, and despite the lack of substantial increases in the frequencies of premature delivery or abortion under the treatment conditions used in this study, the decrease in $PO_2$ of fetal arterial blood demonstrated that utero-placental oxygen transfer was impaired following maternal infusion with nitrite.

Shuval and Gruener (1972) and Gruener et al. (1973) reported on experiments comparing hematological parameters of pregnant and nonpregnant rats following administration of sodium nitrite. Compared to treated nonpregnant animals, treated pregnant animals had lower Hb levels, higher MetHb levels, and became severely anemic. An *in vitro* study using erythrocyte preparations from pregnant and nonpregnant mice, found that pregnancy significantly influenced the kinetics of MetHb formation (Tarburton et al., 1985).

## C.2. Transplacental Carcinogenesis

### *C.2.1. Overview*

While human data on sodium nitrite exposure, *per se,* were not available, there is a considerable body of human epidemiological data pertaining to the consumption of (nitrite-containing) cured meats and hot dogs during pregnancy, and risk for childhood cancer. Studies performed in experimental animals have looked at the potential

transplacental carcinogenicity of sodium nitrite alone, or in combination with nitrosable amines or amides.

Amines and amides (secondary amines, such as ethylurea) can react with nitrite *in vivo* to form N-nitroso compounds (NAS, 1981a and 1981b; Mirvish, 1995; WHO, 1996a and 1996b) (Figure 1). The resulting nitrosamines and nitrosamides can, in turn, be activated *in vivo* to carcinogenic DNA-alkylating species (Mirvish, 1995). A large number of N-nitroso compounds have been shown to have carcinogenic activity in experimental animals, with tumor type and target organ(s) varying according to the specific compound (NAS, 1981a and 1981b; Mirvish, 1995; WHO, 1996a and 1996b). The specificity appears to be similar whether the ultimate nitroso compound itself, or its requisite precursors, are administered.

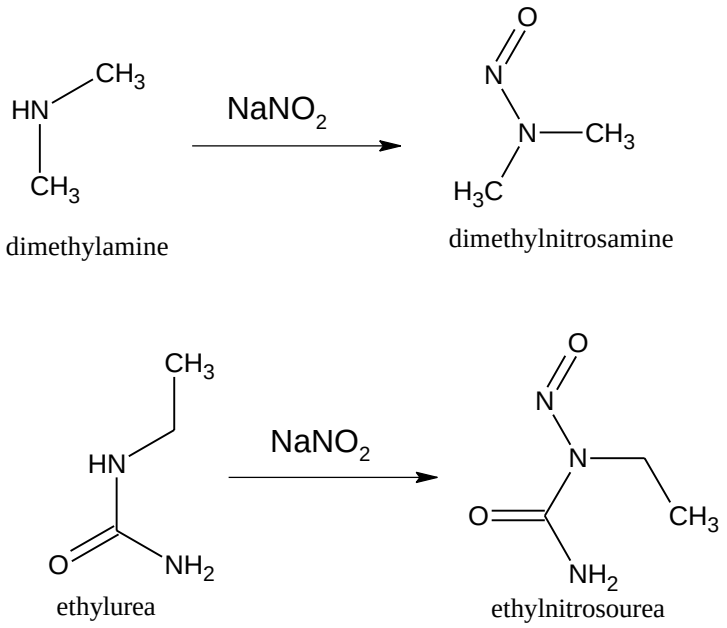


**Figure 1. Formation of N-nitroso compounds**

*C.2.2. Human data*

C.2.2.1. Brain tumors

Nine studies of childhood brain tumors and maternal diet during pregnancy have been conducted (Bunin et al., 1993 and 1994; Cordier et al., 1994; Kuijten et al., 1990; McCredie et al., 1994; Preston-Martin et al., 1982 and 1996; Sarasua and Savitz, 1994; Schymura et al., 1996). Bunin (1998) reviewed these studies and found the associations of maternal consumption of cured meat with risk of childhood brain tumors were quite consistent across studies, particularly for hot dog consumption (see Table 4). Frequent consumption was associated with increased risk of brain tumor in most of the studies, with statistically significant increased risks in four of the studies (odds ratios ranging from 1.4 to 2.5).

The largest study (Preston-Martin et al., 1996), a population-based case-control study of 19 counties on the U.S. West coast, found risks increased with increasing frequency of consumption in a dose-response manner (4-7 times/week, OR=1.4, $P<0.05$; >7 times/week, OR=2.1, $P<0.05$). Results of another large study (reported only in abstract form) are also suggestive of a dose-response: Schymura et al. (1996), in a population-based case-control study conducted in upstate New York, reported the odds ratio for hot dog consumption once per week compared to less often was 1.33 (95% CI=1.00-1.76), and for 2 to 3 times per week increased to 2.01 (95% CI=1.10-3.65); maternal consumption of other cured meats once a week was also associated with an increased risk of childhood brain tumors (OR=6.04, 95% CI=1.89-19.31). The two smallest studies (Cordier et al., 1994; Sarasua and Savitz, 1994) and one study limited to a particular tumor type (Bunin et al., 1994) did not find elevated risks with all cured meats, although one of the small studies (Sarasua and Savitz, 1994) did find an association with hot dog consumption.

All but one of the nine studies reviewed by Bunin (1998) with relevant data found frequent consumption of hot dogs was associated with higher risk of childhood brain tumors. Bunin (1998) notes that further research is needed to resolve the discrepancy between studies in terms of an association of cured meat consumption with specific tumor types. Bunin (1990) also notes that while the studies reviewed provide support for the hypothesis that transplacental exposure to N-nitroso compounds (or their precursors) increases the risk of brain tumors in childhood, it is possible that some aspect of diet correlated with cured meat consumption is the risk factor, such as fat or total caloric intake.

On the other hand, the potential protective role of maternal consumption of vegetables and fruits during pregnancy was examined in some of the studies reviewed by Bunin (1998). Vegetables are a source of nitrate, and they are also sources of known inhibitors of nitrosation, vitamin C and vitamin E, as are fruits. Animal studies indicate that being fed an inhibitor along with N-nitroso compound precursors (e.g., nitrite or nitrate) leads to fewer animals developing tumors (as discussed in Bunin, 1998); large doses of vitamin C completely prevented tumor induction in some studies (Mirvish, 1994). In studies of childhood brain tumors that looked at vegetable consumption, ORs less than 1.0 were found for those consuming vegetables (Bunin et al., 1993 and 1994; Cordier et al., 1994; McCredie et al., 1994), suggesting a protective effect of such consumption, although not all were statistically significant findings. Preston-Martin et al. (1996) found that risk increased with increasing average daily milligrams of nitrite from cured meats ($P<0.005$), but not with nitrate from vegetables. Preston-Martin et al. (1996) also examined prenatal vitamin use, which decreased risk when used throughout pregnancy (OR=0.54, 95% CI=0.39-0.75); for those mothers who consumed above the median level of nitrite from cured meat, risk was greater if vitamins were not taken (OR=2.4, 95%CI=1.4-3.6) than if they were (OR=1.3).

**TABLE 4. Studies of Brain Tumors in Childhood and Maternal Diet During Pregnancy: Results for Cured-Meat Consumption**

| Study | Sample Size (Cases /Controls) | Age (years) | Tumor Type | Maternal Consumption of Cured Meat | |
|---|---|---|---|---|---|
| | | | | All Cured Meats | Hot Dogs |
| Preston-Martin *et al*. (1982) | 209 / 209 | 0-14 | Tumor of brain or cranial meninges | OR = 2.3** high intake | OR = 1.7 ≥ twice/week |
| Kuijten *et al*. (1990) | 163 / 163 | 0-14 | Astrocytic glioma | OR = 2.0** ≥ 9 times/week | ___ |
| Bunin *et al*. (1993) | 166 / 166 | 0-6 | Medulloblastoma/ primitive neuro-ectodermal tumor of brain | OR = 1.1 ≥ 5 times/week | OR = 1.0 ≥ once/week |
| Bunin *et al*. (1994) | 155 / 155 | 0-6 | Astrocytic glioma | OR = 1.7* ≥ 5 times/week | OR = 1.9** ≥ once/week |
| Cordier *et al.* (1994) | 75 / 113 | 0-15 | Brain tumor | No association | ___ |
| McCredie *et al.* (1994) | 82 / 164 | 0-14 | Tumor of brain or cranial nerves | OR = 2.5** ≥ 2.4 times/week | ___ |
| Sarasua and Savitz (1994) | 45 / 206 | 0-14 | Brain tumor | No association | OR = 2.3** > 0 times/week |
| Preston-Martin *et al.* (1996) | 540 / 801 | 0-19 | Brain tumor | OR = 1.4** 4-7 times/week OR = 2.1** ≥7 times/week | OR = 1.4** ≥ once/week |
| Schymura *et al.* (1996) | 338 / 676 | 0-14 | Brain tumor | ___ | OR = 1.3 ≥ once/week OR = 2.0** 2-3 times/week |

OR. Odds ratio: *$p = 0.10$; ** $P<0.05$
Source: Bunin (1998)

C.2.2.2. Leukemia

Three studies provide information on consumption of cured meats during pregnancy and childhood leukemia risk (Sarasua and Savitz, 1994; Peters et al., 1994; Ross et al., 1996). Study results are summarized in Table 5. Two of the studies (Peters et al., 1994; Sarasua and Savitz, 1994) also collected information on childhood consumption of cured meats, and looked separately at risks associated with maternal and childhood dietary components. None of the odds ratios for maternal consumption of cured meats were statistically significant in these studies, two of which had rather small study sizes.

The study by Ross et al. (1996), which focussed on risk in infants, found nonsignificant elevated risks for acute lymphoblastic leukemia (ALL) cases, in contrast to the results for acute myeloid leukemia (AML) cases. Vitamin intake by the mothers of the ALL cases studied by Sarasua and Savitz (1994) appeared to modify the odds ratios for at least some of the cured meats categories studied. For example, for women who took vitamins during pregnancy, consumption of ham, bacon, or sausage one or more times per week had an OR=1.4 (95% CI=0.7-2.9); for women who did not take vitamins during pregnancy, the odds ratio for consumption of these meats less than once per week was 1.7 (95% CI=0.3-9.1) and for consumption one or more times per week was 2.9 (95% CI=1.1-7.9). Peters et al. (1994), with the largest study size, had greater detail on intake levels, and detected an increase for maternal hot dog consumption suggestive of a dose-response (see Table 5).

An elevated risk of leukemia with children's consumption of hot dogs in the study by Peters et al. (1994) was the strongest finding in these studies. Peters et al. (1994) found risk increased by servings per month, from 1.4 for low intake, to 1.7 for medium, and 5.8 (95% CI=2.1-16.2; $P<0.001$) for high intake by the child, and persisted after adjustment for other risk factors. The study by Ross et al. (1996), which examined risk in infants, provided no information on childhood consumption of cured meats. Sarasua and Savitz (1994) found an increased risk of ALL only when examining childhood cured meat intake in relation to vitamin use (e.g., children who ate hot dogs and took no vitamins: OR=2.9; 95% CI=1.0-8.6). Of interest here is the report by Preston-Martin et al. (1996) that hot dogs are the cured meat that has the greatest average serving size (by weight) in children's diets.

Overall these results indicate the need for further studies, with analyses by leukemia type and detailed information on maternal and childhood cured meat consumption.

**Table 5. Studies of Childhood Leukemia and Maternal Cured Meat Consumption During Pregnancy**

| Study | Sample Size (Cases /Controls) | Age (years) | Leukemia Type | Maternal Consumption of Cured Meat Odds Ratios (CI[1]) |
|---|---|---|---|---|
| Sarasua and Savitz (1994) | 56 / 206 | 0-14 | ALL | Ham, bacon, sausage 1+/week<br>OR[2]=1.5 (0.7-3.0)<br>No association seen with consumption of other cured meats. |
| Peters et al. (1994) | 232 / 232 | 0-10 | ALL and ANLL (# each was not provided) | Hot dogs<br>Amount OR CI<br>1-3.9/mth 0.9 not reported<br>4-11.9/mth 1.8 not reported<br>12+/mth 2.4 (0.7-8.1) *P*=0.1<br><br>No association seen with consumption of other cured meats. |
| Ross et al. (1996) | 84 / 97 | 0-1 (<12.5 mths) | 54 ALL 30 AML (analyzed in matched sets) | All cured meats<br>Amount OR CI<br>*ALL cases*<br>1-3/mth 1.7 (0.5-5.3)<br>≥4/mth 1.7 (0.6-4.6)<br>*AML cases*<br>1-3/mth 0.6 (0.1-3.6)<br>≥4/mth 0.3 (0.1-1.5) |

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; ANLL, acute non-lymphocytic leukemia; CI, confidence interval; OR, odds ratio.

1. All confidence intervals are 95% unless otherwise indicated.
2. OR adjusted for consumption of other types of cured meat, age at diagnosis, per capita income of family.

C.2.2.3. Other tumors, and all tumors

Two studies with small numbers of cases examined cured meat intake in relation to childhood cancers other than brain tumors and leukemia (Grufferman et al., 1982; Sarasua and Savitz, 1994). Grufferman et al. (1982) collected dietary information for the family generally and not for the mother's intake during pregnancy, and reported no significant findings for cured meats intake and rhabdomyosarcoma (a major subset of soft tissue sarcomas), with a slight elevation for bacon (OR=1.5, 95% CI=0.7-3.5). Sarasua and Savitz (1994) found a rather strong but not statistically significant increased risk for lymphomas in relation to maternal consumption of lunch meat during pregnancy (OR=2.3, 95% CI=0.9-6.0) and a slight elevation for ham, bacon or sausage consumption (1.3, CI not reported); these authors found soft tissue sarcoma risk was not increased in relation to cured meat intake (data not provided).

Two review articles have considered groupings of the available studies on childhood cancer and cured meat and/or dietary nitrites (Eichholzer and Gutzwiller, 1998; Blot et al., 1999). Eichholzer and Gutzwiller (1998) reviewed studies on several cancer sites in relation to dietary nitrates, nitrites and N-nitroso compounds, including brain tumors (but not any other childhood cancers). They consider the results from some of the studies of brain tumors in children (Preston-Martin et al., 1982; Howe et al., 1989; Kuijten et al., 1990; Bunin et al., 1993; Bunin et al., 1994) as well as two of the many existing studies in adults, and conclude that these data provide little support for the hypothesis that N-nitroso compounds are involved in the etiology of brain tumors.

In a more comprehensive review of childhood cancers commissioned by concerned meat producers, Blot et al. (1999) examine a diverse set of fourteen studies: the nine studies of childhood brain tumors reviewed by Bunin (1998); studies of childhood leukemia (Peters et al., 1994; Ross et al., 1996) and rhabdomyosarcoma (Grufferman et al., 1982); and two studies, a case-control study (Howe et al., 1989) and an ecologic study (Murphy et al., 1998), which had no data on maternal intake of cured meats during pregnancy. Blot et al. (1999) consider the available case-control studies to have methodological shortcomings that can only be remedied by conducting a cohort study of a large population of women of child-bearing age, which they recommend as a next step in research in this area.

*C.2.3. Animal studies*

C.2.3.1. Druckery, et al. 1963, as summarized by U.S. FDA, 1972; teratogenicity and transplacental carcinogenesis in rats

Groups of 30 to 37 rats were given sodium nitrite in drinking water to achieve doses of 0 or 100 mg/kg bw. The study was continued for three generations, with $F_1$ and $F_2$ animals being fed 500 mg diethylamine/kg bw, as well as sodium nitrite. Mean lifespan was lower for treated than control animals, for all three generations (730 days for controls; and 630, 625, and 610 days for nitrate exposed animals of successive generations). Hb levels and erythrocyte counts were within normal limits for all groups. The few tumors observed during histopathological examination were considered to be

spontaneous, and not treatment related. The authors concluded that nitrite alone, or in combination with diethylamine, had no carcinogenic or teratogenic effect at the doses used.

C.2.3.2. Rustia, 1975; transplacental carcinogenicity of ethylurea plus sodium nitrite in hamsters; effects of sodium ascorbate

Groups of 4-6 pregnant hamsters were subjected to one of the following treatments: group 1 - 200 mg ethylurea (EU)/kg bw, plus 100 mg sodium nitrite/kg by intragastric intubation; group 2 - same as group one, with the addition of 200 mg sodium ascorbate (NaASC)/kg bw; group 3 - same as group 1, except that mixture was placed directly into the cecum of the intestine; group 4 - 100 mg EU/kg bw, intragastrically; group 5 - 50 mg $NaNO_2$/kg bw, intragastrically; group 6 - untreated controls. Treatments were given one time only, on gestation day 15. Offspring were delivered normally, and observed throughout their natural lifespan. Offspring and maternal animals were necropsied upon spontaneous death, or following sacrifice of moribund individuals. Histopathology was performed on all tumors, all organs and tissues showing gross pathological alterations, and on selected sections of the CNS, lungs, liver, kidneys, spleen, stomach, and urogenital organs.

Progeny of group 1 females (18 male and 18 female young) developed significant incidences of neurogenic tumors of the peripheral nervous system (PNS). Such tumors occurred in 50% of the females and 33% of the males from this group. The average latency period for tumors was 48 weeks in females and 56 weeks in males; this was a non-significant difference. No PNS tumors were observed in control animals, animals prenatally exposed to either ethynitrosourea precursor alone, animals exposed to sodium nitrite and EU via the maternal intestine rather than the stomach, or animals prenatally exposed to EU precursors with the addition of NaASC. Non-neurogenic neoplasms observed in treated groups were similar to the spontaneously occurring tumor types observed in controls. Two group 3 progeny, one male and one female from the same litter, had malformations of both hind legs. The authors attributed these malformations to mechanical trauma sustained during introduction of test compounds to the maternal intestine.

C.2.3.3. Rustia and Schenken, 1976; transplacental effects of ethylurea plus sodium nitrite in hamsters

Pregnant hamsters were given a combined treatment of the ethylnitrosourea (ENU) precursors, ethylurea (EU; 100 mg/kg) and sodium nitrite (50 mg/kg), by gavage, on each of gestation days 12-15 (day 1 = vaginal smear positive for sperm). Eighty-three newborn offspring, from eight treated litters were weaned and separated by sex (39 females and 32 males). Twelve non-surviving offspring were reported as "cannibalized or missing within the first 2 post-delivery days." In addition, forty-seven female, and 46 male pups served as untreated controls. The animals were maintained until they died spontaneously, or were sacrificed moribund.

Prenatal exposure to ethylurea and sodium nitrite was associated with significantly higher percentages and multiplicities of tumors than were found in untreated controls. The

frequencies of peripheral nervous system (PNS) tumors did not differ between treated males and females (56.3 and 69.2%, respectively). Treated females, however, had shorter latencies for all tumors, as well as greater muliplicities of neoplasms than treated males, or control females or males.

The observed PNS tumors were considered to be consistent with those induced by ENU in hamsters. Therefore, the data were taken to indicate that administration of EU plus sodium nitrite led to *in vivo* formation of ENU, which crossed the placenta to initiate a carcinogenic response in the offspring. The greater sensitivity of female offspring was taken to suggest that estrogenic hormones had some influential role on the action of ENU.

C.2.3.4. Shank and Newberne, 1976; transplacental carcinogenicity of sodium nitrite and morpholine in rats and hamsters

From the day of conception, pregnant Sprague Dawley CD rats and pregnant Syrian golden hamsters were fed on an agar-gel diet containing various concentrations of nitrite and/or morpholine. Dietary levels of sodium nitrite or morpholine were 0, 5, 50, or 1000 ppm; the two compounds were given in various dosage combinations.
N-Nitrosomorpholine (NNM) served as a positive control. Dietary levels of NNM were 0, 5, or 50 ppm. Male and female $F_1$ offspring of both species were selected at random for long-term carcinogenicity studies. For the rats only, an $F_2$ generation was derived from $F_1$ mothers. The studies were terminated when the number of survivors fell to 20% of the original population, or at week 125 for $F_2$ rats, or week 110 for $F_1$ hamsters.

For $F_1$ and $F_2$ rats combined, the median age at death was approximately the same for untreated controls as for animals given 1000 ppm of either sodium nitrite or morpholine (109, 108, and 117 weeks, respectively). The combination of 1000 ppm sodium nitrite with 5, 50, or 1000 ppm morpholine, demonstrated a clear, dose-dependent effect of morpholine on the median age at death (105, 90, and 38 weeks, respectively). The reverse was not true, nor were low concentrations (5 or 50 ppm) of both compounds associated with clear decreases in median age at death.

Hepatocellular carcinoma was the most frequent tumor associated either with nitrite plus morpholine, or with NNM. Angiosarcomas affecting various organs were also observed, but did not show a clear dose-response as compared to the liver tumors. There were no morphological differences between tumors induced by NNM and those induced by the combined administration of morpholine and sodium nitrite. When the dietary levels of both sodium nitrite and morpholine were high (1000 ppm of each), the incidence of tumors was essentially 100%. With the sodium nitrite concentration held at 1000 ppm, and the morpholine concentration reduced (from 1000, to 50, to 5 ppm) the liver-cell carcinoma rate decreased in a linear, dose-related fashion. The converse titration, decreasing the nitrite concentration while maintaining a high morpholine level, also resulted in decreasing frequencies of hepatocellular carcinoma and angiosarcomas. This latter result was attributed to the quantitative dependence of nitrosation of secondary amines (such as morpholine) on the square of the nitrite concentration.

In the sodium nitrite control group (1000 ppm sodium nitrite with 0 ppm morpholine), one out of 44 $F_2$ generation rats developed a hepatocellular carcinoma. A subcutaneous angiosarcoma was observed in one out of 52 $F_1$ sodium nitrite controls. These tumors were regarded as insignificant findings. On the other hand, the incidence of lymphoreticular tumors was considered to be high in the high nitrite concentration group (27%, compared to 6% in the negative controls). The total number of animals developing tumors other than hepatomas and angiosarcomas was also high in this group (61% as compared to 18% among negative controls).

Compared to the rat, hamsters were relatively resistant to the tumorigenic activities of NNM or morpholine plus sodium nitrite. The untreated controls and the 50 ppm NNM group had one case each of hepatocellular carcinoma. In comparison, five cases (31%) were observed in the group receiving 1000 ppm each of sodium nitrite and morpholine, and no cases were observed in any other group. Four cases of angiosarcoma occurred in untreated controls, with one case each found after treatment with 1000 ppm sodium nitrite, 50 ppm sodium nitrite plus 1000 ppm morpholine, and 50 ppm each of sodium nitrite and morpholine. A number of other tumor types were observed, but these were distributed evenly between groups, and showed no particular pattern in relation to treatment. Apart from the one subcutaneous angiosarcoma noted above, no tumors were observed in the group given 1000 ppm sodium nitrite alone.

C.2.3.5. Ivankovic, 1979; study of the transplacental carcinogenicity of sodium nitrite and L-citrulline, singly or in combination

In a sketchily-reported experiment, 50 mg sodium nitrite/kg bw and/or 100 mg L-citrulline/kg bw was (were) given by gastric intubation to pregnant BD rats from "day 13 to day 23 of pregnancy." Malignant tumors of the kidney (Wilm's type) resulted in 6 of 22 surviving offspring prenatally exposed to both compounds. Neither compound alone was associated with tumor development.

C.2.3.6. Anderson et al., 1985; mouse study of transplacental and chronic carcinogencity of sodium nitrite alone, or in combination with cimetidine

Treatment was initiated in seven to eight-week old C57BL/6 female, and BALB/c male mice. Sodium nitrite was provided in drinking water, at concentrations of 0, 0.184, or 1.84 g/L. Mice were bred after two weeks of treatment, producing a generation of $B6CF_1$ progeny. These progeny were weaned at four weeks postnatal age, and continued on treated drinking water until their natural deaths. Treatment of the dams was also continued. The concentrations of sodium nitrite used were determined to have provided doses of 0, 30.7 mg/kg, or 310 mg/kg, respectively. The authors state that these doses were chosen as equivalent to common human exposure levels (the low dose), and to a level ten times higher than that amount (the high dose). Other treatments investigated included: cimetidine (CM), nitrocimetidine (NCM), and CM plus nitrite.

The authors state that data were collected on: the number of females becoming pregnant, the average time from introduction of the male until birth of a litter, average gestational weight gain, number of stillborn litters, mean litter sizes at birth and at weaning, and the numbers of male and female offspring. While the data are not provided in the paper, it is

stated there were no significant differences between groups for these parameters. Twenty litters were born to the control group, and 14 and 15 litters to the low and high-dose sodium nitrite-treated groups, respectively.

Sodium nitrite treatment did not affect survival times or body weights in this experiment. Common non-neoplastic lesions included cystic seminal vesicles and preputial glands in males, and cystic uteri, ovaries, and mammary glands in females. Incidences of these lesions were not correlated with chemical treatment. There was a small increase in the incidence of lymphoma in males exposed to the lower (31%), but not the higher (10%), dose of sodium nitrite (13% in controls; $P$=0.020 by one-tailed exact test). There was a significant, dose-related effect on the numbers of large lung tumors in males treated with sodium nitrite either alone or in combination with CM (significance of dose-response effect for sodium nitrite alone: $P$=0.0094; for sodium nitrite and CM: $P$=0.014). The authors concluded that this correlation implied a direct or indirect effect of sodium nitrite on the growth rate of some lung tumors.

C.2.3.7. Hawkes et al., 1992; study of glioma frequency in mice

VM mice, a strain known for susceptibility to spontaneous glioma formation, were given 0.2% sodium nitrite in their drinking water. One hundred mice were exposed *in utero*, as well as throughout postnatal life. Two hundred mice received the same concentration of sodium nitrite beginning at weaning. An additional 200 mice served as untreated controls. The animals were group housed, and drinking water consumption was not monitored. Sodium nitrite exposure had no demonstrable effect on the frequency of tumor formation, nor was any systemic toxicity observed.

## Table 6. Transplacental Carcinogenicity of Sodium Nitrite (part 1 of 2)

| Reference | Study Design | Tumors | Other Effects |
|---|---|---|---|
| Druckery et al., 1963, summarized by U.S. FDA, 1972 | Sodium nitrite in drinking water to doses of 0 or 100 mg/kg bw for 3 generations. Groups of 30-37 rats.<br>$F_1$ and $F_2$ also fed 500 mg diethylamine/kg bw. | Observed tumors were considered to be spontaneous, and not treatment related. | Reduced mean lifespan (no statistics).<br>Normal Hb levels and erythrocyte counts.<br>No teratogenic effects observed. |
| Rustia, 1975 | Groups of 4-6 pregnant hamsters: 1) 200 mg EU + 100 mg sodium nitrite/kg bw by gavage, 2) 200 mg each EU and sodium ascorbate, + 100 mg sodium nitrite/kg bw by gavage, 3) same as 1, but directly into the cecum, 4) 100 mg EU/kg bw by gavage, 5) 50 mg sodium nitrite/kg bw by gavage, 6) untreated controls.<br>Treatment on gestation day 15 only. | Significant increase in neurogenic tumors of the PNS in Group 1 offspring.<br>No PNS tumors observed in other groups.<br>Types and frequencies of non-neurogenic neoplasms were similar in treated and control groups. | 2 progeny from the same group 3 litter had malformation of both hind legs. Not considered to be treatment related. |
| Shank and Newberne, 1976 | Pregnant rats or hamsters given nitrite and/or morpholine in the diet. Each compound was given at 0, 5, 50, or 1000 ppm, in various combinations.<br>Positive control: NNM (0, 5, or 50 ppm). | Increased frequency of hepatocellular carcinoma was associated with sodium nitrite plus morpholine, or with NNM in rats. Nearly 100% at high doses of both compounds.<br>Angiosarcomas were also observed, but with no clear dose-dependency.<br>Lymphoreticular tumors, as well as all tumors other than the above, were found at increased frequencies in the 1000 ppm nitrite-only group.<br>Hamsters were relatively resistant to the tumorigenic activities of NNM or morpholine plus sodium nitrite. | Median age at death for F1 and F2 rats combined, was affected only by the combination of 1000 ppm sodium nitrite with 5, 50, or 1000 ppm morpholine. |

EU = ethylurea
PNS = peripheral nervous system
NNM = N-nitrosomorpholine

## Table 6. Transplacental Carcinogenicity of Sodium Nitrite (part 2 of 2)

| Reference | Study Design | Tumors | Other Effects |
|---|---|---|---|
| **Ivankovic, 1979** | 50 mg sodium nitrite/kg bw and/or 100 mg L-citrulline/kg/bw to pregnant rats by gavage on days 13-23 of pregnancy. Number animals not stated. | 6/22 surviving offspring prenatally exposed to sodium nitrite + L-citrulline developed Wilm's type kidney tumors.<br>Neither compound alone was associated with tumors. | Not discussed. |
| **Anderson et al., 1985** | Sodium nitrite doses of 0, 30.7 or 310 mg/kg bw were delivered in drinking water to young mice prior to mating.<br>Treatment continued through lifetime of dams and their offspring.<br>20 control litters; 14 low-dose litters; 15 high-dose litters.<br>Other treatments included cimetidine (CM) and CM + sodium nitrite. | Lymphoma was increased in low-dose, but not high dose, sodium nitrite-exposed males.<br>"Large lung tumors" significantly increased in males treated with sodium nitrite alone, or in combination with CM | Reproductive and litter parameters (fertility, gestational weight gain, litter size, sex ratio) stated to not differ between groups.<br>Sodium nitrite did not affect survival times or body weights.<br>Cystic lesions were observed in male and female reproductive organs, but incidences were not correlated with chemical treatment. |
| **Hawkes et al., 1992** | 0.2% sodium nitrite in drinking water of VM mice (a strain known for susceptibility to spontaneous glioma formation).<br>100 mice exposed *in utero*, as well as postnatally. 200 mice exposed from the time of weaning. 200 untreated controls. | No effect of treatment on tumor frequency. | Reported lack of systemic toxicity. |

*C.2.4. Other relevant information*

C.2.4.1. Consumption of cured meats by children and adults, other dietary factors, and risk for brain tumors

Several studies collected data on childhood consumption of cured meats and childhood brain tumor risk, with mixed results. In the study by Preston-Martin et al. (1982), ORs for low, moderate, and high consumption during childhood of all cured meats combined were 1.0, 1.3, and 2.3 (p [linear trend] = 0.01); these authors note that a child's consumption was highly correlated with the mother's consumption of these foods, and childhood consumption had little effect after maternal consumption was taken into account. A case-control study conducted by Howe et al. (1989), who did not report any data on maternal consumption of cured meats and was not reviewed by Bunin (1998), found no increased risk associated with childhood consumption of more than one versus one or less serving per week. Bunin et al. (1993), in a study focussed on brain tumors in young children, found no association with child's diet in the first year of life, with the exception of nonsignificant effects of multivitamin, fruit and juice intake. Sarasua and Savitz (1994) found that children's consumption of hot dogs two or more times per week was associated with an elevated, though not statistically significant, risk of brain tumor (OR=2.8, 95% CI=0.9-8.7). These investigators found higher risks among those children who ate cured meats and did not take vitamins: for example, among those children not taking vitamins, more than zero servings per week of hot dogs led to an OR=6.8 (95% CI=2.5-18.5) compared to vitamin-using children who did not eat hot dogs.

A number of studies of dietary factors in adult brain cancer have been conducted. Lee et al. (1997) summarize seven studies conducted prior to their own, and note that while comparisons are difficult because of differences in study design, cases were more likely than controls in most studies to consume cured foods, especially cured meats, and less likely to consume vegetables or fruits. According to Schwartzbaum et al. (1999), seven of ten published case-control studies in adults find a relationship between consumption of at least one type of nitrite-containing cured meat and adult brain tumor risk.

In recent studies in California, Lee et al. (1997) found that men with brain cancer were twice as likely as controls to have high nitrite and low vitamin C consumption (OR=2.0, 95% CI=1.2-3.5); for women this association was less pronounced and not statistically significant (OR=1.5, 95% CI=0.8-2.7). Blowers et al. (1997) found risk increased with increasing consumption of cured meats, most notably bacon (OR for third tertile=6.6, 95% CI=1.9-11.5); risk was reduced for those with increasing intake of vegetables such as bell peppers (OR for third tertile=0.2, 95% CI=0.1-0.7). Lee et al. (1997) suggest that total body burden of oxidants may play a role in brain cancer causation.

The relation of cured meat intake to that of vegetables and vitamins was examined by Murphy et al. (1998) using dietary information from the National Household Food Consumption Survey (NFCS) of 1977-78 and the Continuing Survey of Food Intakes by Individuals (CSFII) of 1989-91. They found that cured meat intake was inversely

correlated with intake of nitrate-containing vegetables and vitamin A and C (although not significantly so for vitamin C), and positively correlated with total caloric intake. This trend was based on data that combined information on both sexes and all ages, and was examined in a sample of the general population.

One study designed to assess the effects of various dietary factors in the development of adult brain tumors (Kaplan et al., 1997), found a significant positive association with high protein intake (OR=1.94, 95% CI=1.03-3.63). The association was stronger for high consumption of N-nitroso compounds than for low consumption. Consumption of total fat appeared to be inversely related to brain tumor risk, and no association was seen for intake of nitrate, nitrite or total N-nitroso compounds.

In a pilot study, Schwartzbaum et al. (1999) examined glioma risk in adults in relation to diet and found that only energy consumption continued to be strongly and positively associated with glioma risk after adjustment of fat, protein, and cured meat intake. They also found that energy consumption above the median increased risk only when serum antioxidant levels (vitamin C and alpha-tocopherol) levels were below the median. Because of the small sample size, Schwartzbaum et al. (1999) were not able to determine whether energy intake confounds the relationship between nitrite-containing cured meat consumption and glioma risk, but they suggest that future studies control for energy consumption to avoid bias.

### C.2.4.2. Transplacental carcinogenesis and teratogenesis of N-nitroso compounds

Data on the transplacental carcinogenesis and teratogenesis of N-nitroso compounds have been reviewed by the National Academy of Sciences (NAS, 1981b). The Academy notes the many differences between the developing organism and adults that may influence susceptibility to cancer-inducing chemicals. The fetus differs from the adult in metabolic capability, rate of cell proliferation, cell-type composition of certain tissues, hormonal balance, and immunological capacity.

A number of studies have demonstrated the transplacental carcinogenicity of N-nitroso compounds, particularly N-nitrosoethylurea (NEU; also known as N-ethylnitrosourea or ENU), in rats, mice, rabbits, and hamsters (Druckrey et al., 1966; Ivankovic, 1979; NAS, 1981b; Diwan et al., 1996). The live offspring in these various studies were observed to have tumors affecting brain, spinal cord, peripheral nervous system, blood, kidney, liver, and lung. Other N-nitroso compounds tested and found to be active transplacental carcinogens include nitrosomethylurea, nitrosoethylbiuret, and nitrosomethylurethane (NAS, 1981b).

While nitrosamides, nitrosoureas, nitrosocarbamates, and nitrosguanidines are believed to spontaneously degenerate to their ultimately carcinogenic form, fetal metabolic capability may be of particular significance when considering the transplacental carcinogenicity of nitrosamines (NAS, 1981b). Unlike other N-nitroso compounds, nitrosamines are believed to require a hydroxylation step for production of the ultimate carcinogen. As

summarized by the NAS (1981b), nitrosamines administered during pregnancy were found to have low tumor yields in dams and their offspring. The reviewers concluded that the fetus did not necessarily possess the enzymes required for metabolic activation of nitrosamines.

The NAS (1981b) review also summarizes studies of the teratogenicity of N-nitroso compounds. When given during the first half of pregnancy, a number of these compounds have been shown to elicit brain and/or bone abnormalities in rats, hamsters, and minipigs (Givelber and DiPaolo, 1969; Koyama et al., 1970; Ivankovic, 1979). The same N-nitroso compounds producing terata when given to rats or hamsters early in pregnancy, may act as transplacental carcinogens when given later in pregnancy (Druckrey et al., 1966; NAS, 1981b; Diwan et al., 1990; Donovan, 1999). The apparent decreased sensitivity of young embryos to carcinogenesis, however, may be more a function of the number of available target cells, than of the intrinsic sensitivity of those cells (Donovan, 1999).

### *C.2.5. Integrative evaluation*

#### C.2.5.1. Human data

Epidemiological studies have consistently identified an association between frequent consumption of hot dogs and/or other cured meat products during pregnancy, and an increased risk for childhood tumors, particularly brain tumors, in offspring. These findings parallel results from studies of cured meat consumption and adult glioma; in both types of studies, vitamin intake (either as supplements or in vegetables) appears to have a protective or risk-reducing effect. In interpreting these data, however, possible alternative explanations for the results also need to be considered. Possible confounding factors include other dietary components correlated with cured meat consumption, and biased recall of dietary intakes.

Studies of dietary factors in adult brain cancer generally provide evidence supportive of the hypothesis of transplacental carcinogenesis, with the majority of the published studies finding a relationship between consumption of at least one type of nitrite-containing cured meat and adult brain tumor risk. As more recent studies in California (Lee et al., 1997; Blowers et al., 1997) have shown, cured meat intake appears to increase risk, especially for those who do not consume vegetables or other sources of antioxidants; indications are that dietary factors such as total body burden of oxidants may play a role in brain cancer causation.

The analysis of dietary information conducted by Murphy et al. (1998) suggests that, in the general population, several dietary factors co-vary. Cured meat intake may be inversely correlated with intake of nitrate-containing vegetables and vitamins A and C, but positively correlated with total caloric intake. Studies in adults which have examined the role of dietary factors in brain cancer causation (Kaplan et al., 1997; Schwartzbaum et al., 1999), indicate that glioma risk in adults may be related to factors such as high

protein intake (Kaplan et al., 1997) and energy consumption (Schwartzbaum et al., 1999). The results of Schwartzbaum et al. (1999) suggest an interaction of energy consumption with antioxidant intake. As of yet these studies have not been able to determine whether such factors confound the relationship between consumption of nitrite-containing cured meats, and risk for glioma.

Co-ingestion of antioxidants appears to be an ameliorating factor in tumor production, as has been shown for experimental animals fed N-nitroso precursors. No increased risk for tumors in children was associated with maternal consumption of nitrate-containing vegetables (which are also high in known inhibitors of nitrosation) (Preston-Martin et al., 1996). Similarly, taking prenatal vitamins was associated with lowered risk for childhood tumors among the offspring of mothers who consumed greater than the median level of nitrite from cured meats.

Blot et al. (1999) finds the declining trend in cured meat consumption identified in the ecologic study conducted by Murphy et al. (1998), paired with declining levels of residual nitrite in cured meats over the same period, to be incompatible with the hypothesis that cured meat intake may be causally associated with brain tumor incidence, which has been increasing. Such overall trends do not provide information about the association of cured meat consumption with risk in individuals whose children get cancer, a question which is directly addressed by the case-control studies. In addition, Preston-Martin et al. (1996) cite a survey they conducted (unpublished) which found that although the amount of nitrite per gram has declined for most cured meats since 1970, it has increased dramatically for hot dogs.

A potential for biased recall of diet could have occurred if mothers of children with cancer more fully reported consumption of foods, such as cured meats, which were viewed as unhealthy. In some studies that looked at childhood diet, reported intake of foods that may be considered unhealthy (e.g., hamburgers) led to associations of similar magnitude as those found for cured meats, a possible indication of biased reporting. Diet in pregnancy, however, showed a much more limited array of associations in most studies (e.g., Sarasua and Savitz, 1994). Furthermore, increased risk for childhood tumors was not always shown to be associated with other foods that may also be considered unhealthy (e.g., alcohol consumption, in the study by Preston-Martin et al., 1996).

Some have argued (Blot et al., 1999) that the structure of the diet questionnaires in some studies, with only a limited list of items, enhanced the prospects of recall bias. Nevertheless, in studies of adult glioma, which used more comprehensive dietary surveys (e.g., Lee et al., 1997; Blowers et al., 1997), similar associations of cured meat intake with cancer risk have been found. Additionally, the association of childhood brain tumors with maternal consumption of cured meats during pregnancy is remarkably consistent, with the results of some studies even indicating a dose-related effect. The association is also apparently specific; associations between childhood cancers at other sites and maternal consumption of cured meats are not nearly as strong or consistent as those for brain tumors.

C.2.5.2. Transplacental carcinogenesis in experimental animals

A number of studies have demonstrated the transplacental carcinogenicity of N-nitroso compounds, such as N-nitrosoethylurea (NEU; also known as N-ethylnitrosourea or ENU), in rats, mice, rabbits, and hamsters (Druckrey et al., 1966; Ivankovic, 1979; NAS, 1981b; Diwan et al., 1996). The live offspring in these studies were observed to have tumors affecting brain, spinal cord, peripheral nervous system, blood, kidney, liver, and lung. Other N-nitroso compounds tested in experimental animals, and found to be active transplacental carcinogens, include nitrosomethylurea, nitrosoethylbiuret, and nitrosomethylurethane. In humans, an increased risk for childhood tumors was found to be associated with maternal exposure to nitrosatable drugs during pregnancy (Olshan and Faustman, 1989).

Alexandrov et al. (1990) noted the potential for endogenous formation of carcinogenic nitroso compounds following oral intake of precursors (amines or amides, plus nitrate or nitrite). Data from animal experiments have indicated that the necessary reactions can occur *in vivo*, and that excess tumor formation can be associated with such exposures (Rustia, 1975; Rustia and Schenken, 1976; Shank and Newberne, 1976; Ivankovic, 1979). Endogenous N-nitroso formation can be inhibited by co-ingestion of antioxidants such as ascorbic acid, and the relevance of this inhibitor to carcinogenesis and teratogenesis *in vivo* has been supported by animal experiments (Alexandrov et al., 1990).

In contrast to the results for N-nitroso compounds or simultaneous administration of N-nitroso precursors, administration of sodium nitrite on its own has not been consistently associated with tumors of prenatal origin (Druckery et al., 1963 [as summarized by U.S. FDA]; Rustia, 1975; Shank and Newberne, 1976; Ivankovic, 1979; Alexandrov et al., 1990; Hawkes et al., 1992). Shank and Newberne (1976), reported increased frequencies of lymphoreticular and other tumors in rats, but not in hamsters, exposed pre- and post-natally to sodium nitrite at a concentration of 1000 ppm in the diet. Anderson et al. (1985) found a small increase in the frequency of lymphoma in male mice following pre- and postnatal exposure to 30.7 mg sodium nitrite/kg bw, but observed no increase over control values at the higher dose of 310 mg sodium nitrite/kg. In this same study (Anderson et al., 1985), there was a significant dose-response effect for the numbers of large lung tumors in sodium nitrite-treated male mice. The authors interpreted this result as implying an effect of treatment on the growth rate of some lung tumors.

# D. REPRODUCTIVE TOXICITY

## D.1. Overview

No data were identified pertaining to the potential reproductive toxicity of sodium nitrite in humans. Nor were any animal studies identified which fully conformed to U.S. EPA guidelines for a multi-generation reproductive toxicity study (U.S. EPA, 1998). Available relevant data included a continuous breeding study conducted in mice, as well as chronic, subacute, and developmental toxicity studies conducted in laboratory animal species.

## D.2. Pair-based Studies

*D.2.1. Continuous breeding study*

Swiss CD-1 mice were subjected to the National Toxicology Program's (NTP) Reproductive Assessment by Continuous Breeding (RACB) protocol (NTP, 1990; Chapin, 1997). Forty mating pairs served as controls, and 20 mating pairs were assigned to each dose group. Sodium nitrite was given in the drinking water at concentrations of 0, 0.06, 0.12, and 0.24% w/v. As calculated from water consumption and body weight data, these concentrations resulted in approximate doses of 125, 260, and 425 mg/kg/day.

Nine animals died during the 14-week breeding period: 3, 4, 0, and 1, in the control through high dose groups, respectively. These deaths were not considered to be treatment related. The experiment revealed no treatment-related effects on the mean number of litters per pair, cumulative days to deliver each litter, mean litter size, or pup birth-weight or viability.

The males were removed after 14 weeks of continuous breeding, and the females allowed to deliver and rear their last litters. Exposure of dams was continued through the lactation period. Postnatal mortality was not affected by treatment, but weights of the high-dose group pups were reduced by 12-17% from postnatal day 7 to postnatal day 21. The authors were unsure whether this was a direct effect of sodium nitrite exposure, or an indirect result of reduced maternal water consumption, and hence reduced milk production.

Only the high-dose and control group pups were carried through to breed a second generation. Following weaning, these ($F_1$) animals were exposed to sodium nitrite in their drinking water, at the same dose their parents had received. Non-siblings were mated within their treatment group at approximately 74 days of age, and the females allowed to carry and deliver their first litters. No effects of treatment on fertility or reproductive success were noted. Post-delivery estrous cycle and sperm parameters were not altered by treatment. The number, weight and viability of $F_2$ young were also unaffected, as were $F_1$ terminal body and organ weights.

*D.2.2. Developmental toxicity study*

In a study of developmental toxicity and developmental neurotoxicity, Vorhees et al. (1984) fed groups of male and female Sprague-Dawley rats diets containing sodium nitrite at concentrations of 0, 0.0125, 0.025, or 0.05% (w/w). The numbers of sperm-positive females were 35, 22, 24, and 28 for the 0, 0.0125%, 0.025%, or 0.05% concentration groups, respectively. Treated diets were provided for 14 days prior to mating, throughout the breeding period (1-14 days), and throughout gestation and lactation. Weaned offspring were continued on the same diets as their parents for the duration of the study.

Feed consumption and body weight of parental animals were unaffected by treatment. As no data were provided on the number of animals caged together for mating, fertility cannot be assessed from this study, other than noting that sufficient pregnancies occurred in each group for the experiment to be continued. The percentages of sperm-positive females producing litters was unchanged by treatment. Gestation length, litter size, and sex ratio were all also unaffected by sodium nitrite. No external malformations were observed in newborns. In female offspring produced in this study, sodium nitrite exposure throughout pre- and postnatal developmental was not associated with any change in the timing of vaginal opening.

*D.2.3. Lifetime dietary and drinking water studies*

A cohort of 70 male and 140 female rats was divided into groups and fed cured meat to which 0, 200, 1000, or 4000 mg sodium nitrite/kg had been added (Olsen et al., 1984). One control group received casein as a protein source; another control group was given fresh chopped pork. Treated diets were provided to $F_0$ animals from ten weeks prior to mating until animals were terminated on the study. Subsequent to weaning, groups of 60-100 $F_1$ animals were continued on the respective $F_0$ diet.

Following canning, autoclaving, and storage prior to experimental use, the treated diets were determined to have nitrite contents of 6, 47, 580 mg/kg. Data on feed consumption were not provided in the paper, but the doses of sodium nitrite consumed in the form of treated diets have been estimated at 5, 25, or 100 mg/kg bw (WHO, 1996b). No differences were noted between treated and control $F_0$ animals in appearance or behavior (Olsen et al., 1984). Nor were effects noted on reproductive parameters such as pregnancy rate, litter size, mean pup weight, or pup survival. No morphological abnormalities were observed in $F_1$ offspring. There were no significant differences in the rate of postnatal mortality between treated and control pups.

In a multigeneration study of teratogenesis and transplacental carcinogenesis, groups of 30 to 37 rats were given sodium nitrite in drinking water to achieve doses of 0 or 100 mg/kg bw (Druckery, et al. 1963, as summarized by U.S. FDA, 1972). The study was continued for three generations, with $F_1$ and $F_2$ animals being fed 500 mg diethyamine/kg bw, as well as sodium nitrite. Mean lifespan was lower for treated than control animals, for all three generations (730 days for controls; and 630, 625, and 610 days for nitrate exposed animals of successive generations), but the authors

concluded that nitrite alone, or in combination with diethylamine, at the doses used, had no carcinogenic or teratogenic effects. Reproductive parameters were only noted in passing, but it is noted that 15 matings of the $P_0$ generation resulted in 94 offspring (an average of 6.3 pups/litter), and that 22 matings of the $F_1$ generation resulted in 149 offspring (an average of 6.7 pups/litter).

In a study of transplacental and chronic carcinogencity (Anderson et al., 1985), treatment was initiated in 7 to 8-week old C57BL/6 female, and BALB/c male mice. Sodium nitrite was provided in drinking water, at concentrations of 0, 0.184, or 1.84 g/L. Mice were bred after two weeks of treatment, producing a generation of $B6CF_1$ progeny. These progeny were weaned at four weeks postnatal age, and continued on treated drinking water until their natural deaths. Treatment of the dams was also continued. The concentrations of sodium nitrite used were determined to have provided doses of 0, 30.7 mg/kg, or 0.31 g/kg, respectively. The authors state that these doses were chosen as equivalent to common human exposure levels (the low dose), and to a level ten times higher than that amount (the high dose). Other treatments investigated included: cimetidine (CM), nitrocimetidine (NCM), and CM plus nitrite.

The authors state that data were collected on: the number of females becoming pregnant, the average time from introduction of the male until birth of a litter, average gestational weight gain, number of stillborn litters, mean litter sizes at birth and at weaning, and the numbers of male and female offspring. While the data are not provided in the paper, it is stated there were no significant differences between groups for these parameters. Twenty litters were born to the control group, and 14 and 15 litters to the low and high-dose sodium nitrite-treated groups, respectively. Sodium nitrite treatment did not affect survival times or body weights in this experiment. Common non-neoplastic lesions included cystic seminal vesicles and preputial glands in males, and cystic uteri, ovaries, and mammary glands in females. Incidences of these lesions were not correlated with chemical treatment.

*D.2.4. Subchronic dietary studies*

The U.S. Food and Drug Administration (U.S. FDA, 1972) reviewed and summarized toxicity studies of sodium nitrate, sodium nitrite, and nitrosamines. In one study discussed by the FDA, rats were fed on fish containing added sodium nitrite. Doses were adjusted to correspond to the sodium nitrite intake of an average-sized man consuming 1 lb. of fish containing 908 mg of sodium nitrite per day, six days per week. Five pairs of brothers and four pairs of sisters were used on the study: one member of each pair serving as a control, while the other was treated. There were no deaths during the study, and no differences in weight gain between treated and control animals. No histological examinations were performed, nor were reproductive organs weighed. No data on reproductive endpoints were provided, but the reproductive performance was said to have been unaffected at an average daily intake of 0.167 g sodium nitrite/rat over 121 days.

## D.3. Male Reproductive Toxicity

*D.3.1. Chronic toxicity studies*

Grant and Butler (1989) studied the chronic toxicity of sodium nitrite in the male rat. Groups of 5-6-week old, male, F344 rats were fed reduced-protein diets contain sodium nitrate at concentrations of 0.2 or 0.5% (w/w). Fifty rats were assigned to each treatment group, and 20 control rats were given the reduced-protein diet without added sodium nitrite. Animals were continued on their diets until they were sacrificed as moribund, or after 115 weeks of treatment.

Sodium nitrite exposure was associated with RBC (red blood corpuscle) count reduction over the first eight weeks of treatment. RBC's remained low until at least week 28 of the study, and then gradually rose to reach normal levels by approximately week 52. The reductions in RBC's seen in the high-concentration group were associated with reductions in the MCV (mean red cell volume), Hct (hematocrit), and Hb (hemoglobin concentration).

Body weight gain in treated rats showed a dose-related reduction, which was statistically significant at the higher sodium nitrite concentration. The body weight reductions relative to the pattern of feed intake, indicated that feed utilization was impaired by sodium nitrite exposure. Mortality data, on the other hand, showed a more favorable survival rate for treated animals, as compared to controls. This difference, however, was not statistically significant.

Compared to controls, treated animals suffered significantly *lower* incidences of lymphomas, leukemias, and testicular interstitial cell tumors. The authors suggested that the reduced weight gain of treated animals might have reduced susceptibility to tumor formation, and exerted a positive influence on life span.

On the other hand, findings of focal or diffuse testicular interstitial cell hypoplasia were more frequent among treated animals. While these changes appeared to increase with increasing dose, statistical significance was not reported. The study authors postulate that the hyperplasia might have been a function of hormonal imbalance related to geriatric changes in older animals.

Van Logten et al. (1972; and as reviewed by U.S. FDA, 1972 and WHO, 1996b), fed groups of 30 male and 30 female rats on a cured meat diet containing 0, 200, or 5000 mg/sodium nitrite/kg. The meat was mixed in a 40% weight ratio with a standard diet, thus giving the animals daily sodium nitrite doses of approximately 0, 4, or 100 mg/kg body weight. The study was terminated after 29 months. The 40% meat diet, in the absence of sodium nitrite, resulted in mean body weights which were higher than those of standard-diet controls. Meat with added sodium nitrite resulted in lower body weights. These changes appear to have been dose-related, and were statistically significant in many cases. Mean feed consumption, however, was not reported to be different between any of the meat-fed groups. There were no differences between groups in mortality. Organs evaluated at the gross and histopathological levels included the testes and prostate. No treatment-related changes were found.

*D.3.2. Subacute and subchronic toxicity studies*

In a study of the effects of nitrobenzene (NB) exposure on male Fischer-344 rats, sodium nitrite was used as a positive control for induction of methemoglobinemia (Bond et al., 1981). Animals exposed to a single dose of NB developed hepatic and testicular lesions, as well as elevated levels of methemoglobin. In order to distinguish the effects of methemoglobinemia from other potential mechanisms of NB toxicity, five additional male rats were given sodium nitrite by i.p. injection, at a dose of 15 mg/kg, every three hours, for three days. This regimen supported a steady MetHb level of 2-40%. Sodium nitrite-treated animals were sacrificed one hour following the final injection, and histopathological examinations were made of the brain, liver, and testes. No evidence of pathology was found.

The U.S. Food and Drug Administration (U.S. FDA, 1972) reviewed and summarized toxicity studies of sodium nitrate, sodium nitrite, and nitrosamines. In one 18-week study conducted in male rats, sodium nitrite was added to a meat diet. Over the course of the study, the amounts of sodium nitrite given varied between 0.2 and 0.5 g/rat/day. At study termination, histopathological changes were observed in the heart, lung, brain, kidney, and testis. The changes were attributed to stagnant hypoxemia, which occurred as a result of hypotensive vasodilation caused by the nitrite. However, the number of animals used in the experiment was so small (6 total) that no meaningful statistical analysis could be performed.

## D.4. Female Reproductive Toxicity

Van Logten et al. (1972; and as reviewed by U.S. FDA, 1972 and WHO, 1996b), fed groups of 30 male and 30 female rats on a cured meat diet containing 0, 200, or 5000 mg/sodium nitrite/kg. As consumed in a weight ratio of 40% with a standard diet, daily doses of sodium nitrite were approximately 0, 4, or 100 mg/kg body weight. The study was terminated after 29 months. The 40% meat diet, in the absence of sodium nitrite, resulted in mean body weights which were higher than those of standard-diet controls. Meat with added sodium nitrite resulted in lower body weights. These changes appear to have been dose-related, and were statistically significant in many cases. Mean feed consumption, however, was not reported to be different between any of the meat-fed groups. There were no differences between groups in mortality. Organs evaluated at the gross and histopathological levels included the ovaries and uterus. No treatment-related changes were found.

## Table 7. Effects of Sodium Nitrite on Reproduction (page 1 of 2)

| Reference | Study Design | Reproductive Endpoints | Other Effects |
|---|---|---|---|
| **NTP, 1990** | Continuous breeding protocol.<br>Swiss CD-1 mice.<br>20 mated pairs/dose group; 40 mated pairs as controls.<br>Sodium nitrite in drinking water to doses of 0, 125, 260, and 425 mg/kg/day.<br>High dose and control pups were continued on to breed a 2d generation. | No treatment-related effects on: mean number of litters/pair, cumulative days to deliver each litter, mean litter size, birth weight, or pup viability.<br>No effect on postnatal mortality of pups exposed during lactation.<br>Reduced body weights of nursing pups.<br>No effects on reproduction of high-dose $F_1$ animals, or on viability and weight of $F_2$ offspring.<br>No effects on post-delivery estrous cycle or sperm parameters. | 9 deaths during the study period were not considered to be treatment related.<br>No effect on $F_1$ terminal body and organ weights. |
| **Vorhees et al., 1984** | Male and female rats fed on a diet containing 0, 0.0125, 0.025, or 0.05% sodium nitrite (w/w).<br>Treatment from 2 weeks prior to mating, throughout gestation and lactation, and to weaned $F_1$ offspring.<br>22-35 plug-positive females/group. | Mating data not provided, but no changes in % of litters from plug-positive females.<br>No effects on: gestation length, litter size, or sex ratio of offspring.<br>No external malformations.<br>No change in timing of vaginal opening in female offspring. | No effect on feed consumption or body weight of parental animals. |
| **Olsen et al., 1984** | 70 male and 140 female rats (total) fed on meat containing sodium nitrite to doses of 0, 5, 25, or 100 mg/kg bw.<br>Diets given from 10 weeks prior to mating, throughout gestation and lactation, and to weaned $F_1$ offspring. | No effects of treatment noted on pregnancy rate, litter size, mean pup weight, pup survival, or malformation frequency. | No differences noted in appearance of behavior of treated $F_0$ animals. |
| **Anderson et al., 1985** | 7-8 week old male and female mice given sodium nitrite in drinking water, to doses of 30.7 mg or 0.31 g/kg/day.<br>Animals were bred after 2 weeks; $P_0$ dams and $F_1$ progeny continued on treatment. | No significant changes in measures of fertility, or offspring viability or sex ratio. | No effect on survival times, body weights, or gestational weight gain.<br>Incidences of non-neoplastic lesions were not correlated with chemical treatment. |

## Effects of Sodium Nitrite on Reproduction (page 2 of 2)

| Reference | Study Design | Reproductive Endpoints | Other Effects |
|---|---|---|---|
| **Grant and Butler, 1989** | 5-6 week old male rats fed low-protein diets containing sodium nitrite at 0, 0.2, or 0.5% (w/w).<br>50 animals/treatment group. 20 controls.<br>Continued on diets for 115 weeks, or until sacrifice moribund. | Increased frequency of focal or diffuse interstitial cell hypoplasia. Appeared to increase with increasing dose, but not statistically significant. | Significant reductions in RBCs, resolved by 52 weeks.<br>Reduced MCV, Hct, and Hb in high-concentration group.<br>Concentration-related decrease in body weight gain, statistically significant at the high concentration.<br>Evidence of impaired feed utilization.<br>Better survival rate for treated than control animals, but not statistically significant.<br>Significantly lower incidences of lymphomas, leukemias, and testicular interstitial cell tumors. |
| **Van Logten, 1972** | Groups of 30 male and 30 female rats fed meat containing sodium nitrite to doses of 0, 4, or 100 mg/kg bw/day. Diet continued for 29 months. | No treatment-related changes in gross or histological appearance of testes and prostate, or ovaries and uterus. | Reductions in mean body weights appeared to be dose-related. |
| **Bond, et al., 1981** | 5 male rats given 15 mg sodium nitrite/kg bw, i.p., every 3 hours for 3 days. | At sacrifice 1 hr following final injection, no evidence of histopathological damage to testes. | Treatment supported a steady MetHb level of 2-40%.<br>At sacrifice 1 hr following final injection, no evidence of histopathological damage to brain or liver. |

## D.5. Other Relevant Data: Effects On Reproduction Of Potassium Nitrite Exposure In Guinea Pigs

Groups of 3-6 female guinea pigs were housed with at least one male, and given drinking water which contained potassium nitrite at concentrations of 0, 300, 1000, 2000, 3000, 4000, 5000, or 10000 mg/L (Sleight and Atallah, 1968). These concentrations were estimated to provide doses of 0, 110, 270, 940, 1110, 1190, 1490, or 3520 mg/kg/bw/day for 100-240 days (WHO, 1996b).

Females in all groups became pregnant, indicating that fertility was retained by exposed males. Maternal weight gain was affected only at the highest potassium nitrite concentration; at that concentration, maternal weight gain was depressed. At the two highest exposure levels, all fetuses, and one of the dams, died. Inflammation of the reproductive organs, and degeneration of the placenta were noted in females that aborted or carried mummified or resorbed fetuses. The percentage of fetal loss was higher in all treated litters than in controls, and appeared to generally increase with increasing dose, but no statistical analysis was provided.

## D.6. Integrative Evaluation

There were no available data on the potential of sodium nitrite to cause reproductive toxicity in humans. Among the available animal studies, none were conducted under a standard multi-generation reproductive toxicity study protocol (U.S. EPA, 1998), and only a limited number included the treatment of both sexes during the mating period. None of the pair-based studies (NTP, 1990; Chapin, 1997; Vorhees et al., 1984; Olsen, 1984; Anderson et al., 1985; U.S. FDA, 1972) provided evidence for an effect of sodium nitrite on fertility, or on other reproductive parameters evaluated. A study of the effects of potassium nitrite in guinea pigs also reported no effect of treatment on fertility (Sleight and Atallah, 1968).

Two studies (Grant and Butler, 1989; U.S. FDA, 1972) have provided some evidence of testicular changes at the histopathological level in male rats, but the observed effects could not be confidently attributed to sodium nitrite exposure. No evidence of testicular pathology was identified in animals subjected to a 3-day regimen of sodium nitrite injections (Bond et al., 1981).

No evidence for adverse effects of sodium nitrite on female reproduction was obtained from the studies reviewed (NTP, 1990; Chapin, 1997; Vorhees et al., 1984; Olsen, et al., 1984; Van Logten et al., 1972; U.S. FDA, 1972). In addition to fertility, relevant endpoints addressed by at least one of these studies included: mean live litter size, pup birthweight and viability, post-delivery estrous cycle parameters, gross and histopathological evaluation of the ovaries and uterus, and timing of vaginal opening in exposed female offspring. There is some suggestion that sodium nitrite might affect milk

production, as postnatal weight gain was reduced in the offspring of dams given approximately 245 mg sodium nitrite/kg/day during pregnancy and lactation (NTP, 1990; Chapin, 1997). Alternatively, this might have been the indirect result of poor palatability of sodium nitrite-treated water leading to reduced water consumption (and hence to reduced milk production), or due to a direct effect on the pups of sodium nitrite secreted in the dams' milk.

A study on the effects of potassium nitrite in pregnant guinea pigs reported that fertility was retained, although fetal loss appeared to increase with increasing dose of potassium nitrite (Sleight and Atallah, 1968). It is not known whether the difference in results from the studies using sodium nitrite is due to the use of the potassium salt, or to the generally higher doses given, or to a greater sensitivity of the guinea pig as compared to the rat and mouse.

# E. SUMMARY

## E.1. Developmental Toxicity

Studies conducted in mice have not provided clear and consistent evidence for adverse effects of *in utero* exposure to sodium nitrite on standard parameters of developmental toxicity. Administration of sodium nitrite to pregnant guinea pigs, particularly ascorbic acid deficient guinea pigs, has resulted in abortion of litters. Behavioral and neurodevelopmental studies conducted in rats have indicated life-long consequences of prenatal exposure to sodium nitrite. These effects have been attributed to nitrite-induced prenatal hypoxia. Fetal, as well as maternal, methemoglobinemia has been reported in guinea pigs and dairy cows, following administration of sodium nitrite to the maternal animal. In dairy cows, mean fetal $PO_2$ levels were found to be decreased following maternal infusion with sodium nitrite.

Epidemiological studies support an association between maternal consumption of nitrite-containing cured meats during pregnancy and childhood cancers, particularly brain tumors. While these data cannot implicate sodium nitrite in isolation from other dietary components, they are consistent with the results of transplacental carcinogenicity studies in which sodium nitrite, in combination with amine or amide precursors of N-nitroso compounds, were given to experimental animals.

## E.2. Male Reproductive Toxicity

There were no available data on the potential of sodium nitrite to cause male reproductive toxicity in humans. Among the available animal studies, none were conducted under a standard multi-generation reproductive toxicity study protocol, and only a limited number included the treatment of both sexes during the mating period. None of the pair-based studies provided evidence for an effect of sodium nitrite on fertility, or on other reproductive parameters evaluated. Two studies provided some evidence of testicular

changes at the histopathological level in male rats, but the observed effects could not be confidently attributed to sodium nitrite exposure. No evidence of testicular pathology was identified in animals subjected to a 3-day regimen of sodium nitrite injections.

### E.3. Female Reproductive Toxicity

There were no available data on the potential of sodium nitrite to cause female reproductive toxicity in humans. With regard to the available animal toxicity studies, no evidence for adverse effects of sodium nitrite on female reproduction was obtained. In addition to fertility, relevant endpoints addressed by at least one study included: mean live litter size, pup birthweight and viability, post-delivery estrous cycle parameters, gross and histopathological evaluation of the ovaries and uterus, and timing of vaginal opening in exposed female offspring. There is some suggestion that sodium nitrite might affect milk production, as postnatal weight gain was reduced in the offspring of dams given approximately 245 mg sodium nitrite/kg/day during pregnancy and lactation. Alternatively, this might have been the indirect result of poor palatability of sodium nitrite-treated water leading to reduced water consumption (and hence to reduced milk production), or due to a direct effect on the pups of sodium nitrite secreted in the dams' milk.

A study on the effects of potassium nitrite in pregnant guinea pigs reported that fertility was retained, although fetal loss appeared to increase with increasing dose of potassium nitrite. It is not known whether the difference from the studies using sodium nitrite is due to the use of the potassium salt, to the generally higher doses given, or to a greater sensitivity of the guinea pig as compared to the rat and mouse.

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