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Chapter 4. Relationship of Environmental Nitrogen Metabolism to Human Health

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Chapter 4. Relationship of Environmental Nitrogen Metabolism to Human Health

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The need for humans to produce and consume food and other agricultural products is increasing. This need is directly related to increasing world populations, demands for goods and services, and expectations. Nitrogen (N) is contained in all of the amino acids and proteins in the foods consumed by humans. The use of N to produce food and other products is generally increasing as human needs increase. Proteins are an essential component of the human diet because, unlike plants, humans are unable to utilize more simple forms of N and rely on food sources for protein which can then be digested to amino acids and used for protein synthesis in the body. Estimation of protein requirements for humans depends both on the content of essential amino acids and digestibility of the protein.

Although humans may not be able to utilize simpler forms of N for normal physiological functions such as energy and synthesis of proteins, they are exposed to and capable of absorbing other forms of N such as nitrate (NO_3^-) and nitrite (NO_2^-) and even *N*-nitroso compounds (NOC). Nitrate can be obtained from plant sources as well as from contaminated drinking water and is easily absorbed by the intestine. Nitrate itself is not generally considered as a health risk but may become a concern due to its conversion to NO_2^-. The main health risk associated with NO_3^- consumption is methemoglobinemia due to the conversion of NO_3^- to NO_2^-, which in turn can interact with hemoglobin leading to formation of methemoglobin (MHb), leading to oxygen deprivation of the cells. Secondary and related deleterious effects of exposure to NO_3^- include increased respiratory infections, inhibition of iodine uptake by the thyroid, and possible reproductive problems. Although concerns about NO_3^- and NO_2^- have been focused mainly on harmful effects, there is growing acceptance for the beneficial effects of the related compound nitric oxide (NO). Nitric oxide is a free radical gas that acts as a messenger molecule for regulation of several systems including blood vessel dilation, hormonal and neurotransmission functions.

Nitrosamines and nitrosamides are in the group of N-containing substances identified as NOC substances. These compounds are important to consider in the

human diet since they are capable of participating in DNA alkylation and appear to be among the most potent and broadly acting carcinogens known. In addition to the effect of N-containing compounds on humans it is important to consider the impact of humans to N loading into the environment. We estimate the total intake and loss of N by humans in the United States to be in the range of 0.8–1.0 million metric ton per year indicating that N excretion by humans has a large potential to impact the environment. Such impacts on the environment by N from humans are just as important to consider as those from livestock wastes, inefficient fertilizer-N use, or from other N sources. It is especially important to recognize the potentially serious environmental effects that may occur as the human population continues to grow.

1. INTRODUCTION

The need for humans to produce and consume food and other agricultural products is increasing. This need is directly related to increasing world populations, demands for goods and services, and expectations. With increased human demands, the need and use of N to produce food and other products is increased because N is contained in all of the amino acids and proteins in the foods consumed by humans and is essential for their survival and health. Diets in many parts of the world are protein deficient, often having an imbalance of essential amino acids. In other cases, the consumption of certain forms of N or the endogenous formation of harmful N-containing compounds can be harmful to human health. Finally, the N consumed by humans is also excreted and must be absorbed back into the environment where it may have detrimental effects. Too often, problems associated with N in the environment are dismissed as primarily resulting from agricultural production systems and inefficiencies in properly managing N sources and their use. Numerous efforts continue to improve N-use efficiency, minimize losses and leakage of N into important water bodies, prevent natural resources degradation, and to understand the environmental impacts of N. However, it is also important to understand the potential for environmental impacts that can result from increasing amounts of excretory N as human populations grow. Amounts of N consumed and excreted by humans need to be quantified and acknowledged for their potential to impact the environment. Human numbers are increasing rapidly. The world's population reached a total of about 1.7 billion people in 1900 with an increase to a current estimate of just over 6.6 billion (U.S. Census Bureau, 2007). Within the United States, the population increased from 76 million in 1900 to over 275 million people in 2000 with the estimated population being 303 million in 2006 (U.S. Census Bureau, 2007). As will be discussed later in this chapter the Recommended Dietary Allowance (RDA) in the diet of a human adult weighing 70 kg is about 56 g of protein, or about 9 g N/day or about 3.25 kg N/year (Wildman and Medeiros, 2000; DRI, 2005). The demand for protein, and the N it contains, is increasing dramatically. It is highly important to understand N intake and metabolism, beneficial and harmful effects on N in the human body, and the implications that can be derived about human dietary needs for N. The objective of this chapter is to discuss sources

and forms of N in the diet, human requirements for N, metabolism, and potential harmful and/or beneficial effects of certain forms of N and N metabolites in the body on human health. In addition, estimates will be made of the amount of N consumed and excreted from food consumed by humans in the United States and perspectives provided about world dietary N needs.

2. THE NITROGEN CASCADE

Figure 1 demonstrates the balance observed in the N cascade as it relates to atmospheric, terrestrial, and aquatic ecosystems. This figure also reflects how human activities operate as external modifiers of the N cascade through production and use of fertilizer, consumption of N in the form of protein, and emission of atmospheric pollutants (Galloway et al., 1993; Cowling et al., 2001).

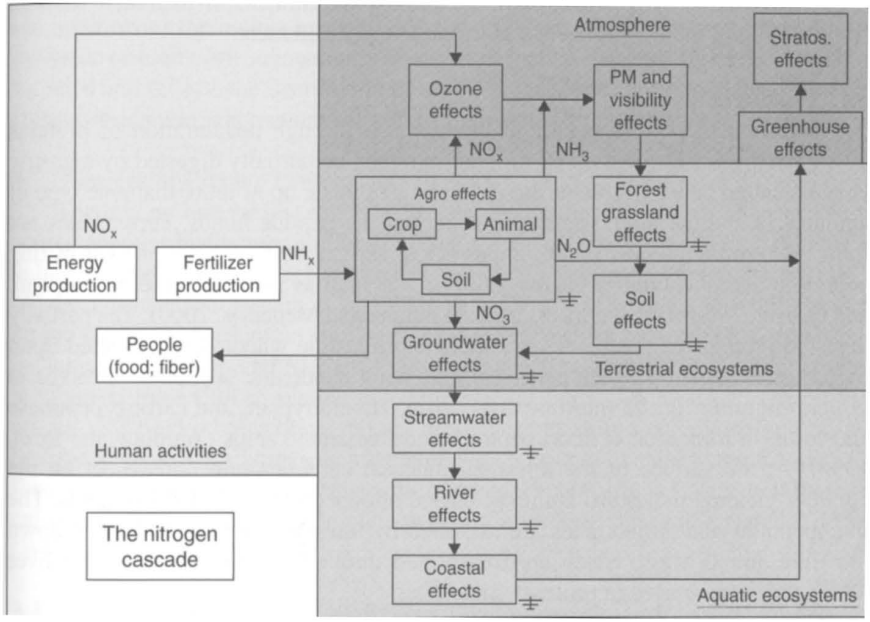

Figure 1. The nitrogen cascade.

Although clearly demonstrating the impact of humans on the environment, modification of this figure is required to include humans which, like other animals, consume N as protein and then excrete N that returns back into the environment. Secondly, compounds produced through natural processes in the N cycle and products created through human activities such as N oxides, although not consumed by humans can have a direct effect on both the environment and upon human health. Thus the N cascade must be considered from the perspective of the important relationship and balance between humans and their environment starting with basic

consumption and excretion and ending with more complex environmental impacts on human and environmental health from active nitrogenous compounds formed because of the balance between the humans and the environment.

3. CONSUMPTION AND EXCRETION OF PROTEIN IN THE HUMAN DIET

Plants can obtain N from the soil through uptake of nitrate (NO_3^-) and/or ammonium (NH_4^+) ions. The NO_3^- can then be reduced to the NH_4^+ form and utilized by the plant to synthesize N-containing compounds (Fowden, 1981). Unlike plants, animals and humans are mostly incapable of utilizing more simple forms of N and rely on food sources for that N compounds in the form of amino acids (Fowden, 1981). Amino acids are synthesized by plants and formed into proteins, which can then be ingested directly by animals and humans or indirectly through consumption of animal products such as milk, eggs, and meat.

3.1. Protein Digestion

Digestion of protein begins in the stomach through denaturation of proteins by stomach acid. The denatured proteins can then be partially digested by a gastric enzyme called pepsin. Proteins are in most cases made up of more than one type of amino acid and the amino acids are connected by peptide bonds. Pepsin does not fully break proteins into single amino acids, as it is only capable of hydrolyzing peptide bonds involving aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Wardlaw and Insel, 1996; Wildman and Medeiros, 2000). The partially digested proteins or peptones enter the small intestine where they are acted upon by proteases secreted by the pancreas. The major pancreatic peptidases involved in protein digestion in the intestine are trypsin, chymotrypsin, and carboxypeptidase and result in formation of short peptides and free amino acids (Wardlaw and Insel, 1996). At the surface of the intestinal mucosal cells, aminopeptidase act on the peptides yielding individual amino acids and oligopeptides of 2–4 amino acids. The oligopeptides and amino acids are absorbed by intestinal cells to be broken down into free amino acids, which are transported through the portal vein to the liver where they can be used in protein synthesis.

Cells in the human body can produce carbon skeletons to which amino groups from other amino acids can be added. The first step in catabolism (i.e., the breakdown) of amino acids is the removal of the alpha-amino group which can then be incorporated into other compounds or excreted. Removal of the alpha-amino group from most amino acids involves transfer to alpha-ketoglutarate and formation of an alpha-ketoacid (from the original amino acids), and the formation of glutamate. So alpha-ketoglutarate accepts the amino group and become glutamate. Glutamate can undergo transamination resulting in deamination for disposal of amino groups or it can donate its amino group to another carbon skeleton for the formation of nonessential amino acids. Transamination is catalyzed by several enzymes classified as

aminotransferases, a process important in excretion of amino acids brought into the urea cycle as glutamate. There are eight to nine essential amino acids that the body must obtain in the diet because the body either cannot make the carbon skeleton necessary for that specific amino acid, has no pathway for the addition of the amino group, or cannot process the amino acid in high enough quantity to meet the needs of the body (Wardlaw and Insel, 1996). These amino acids, referred to as essential amino acids, must be obtained from food sources and include isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Infants additionally require histidine for their growth and development (Wildman and Medeiros, 2000).

If a proper balance of the amino acids is not received protein synthesis is inhibited or can cease when the amino acid in the lowest concentration (limiting amino acid) is exhausted. This is known as the "all or none" law. If the limiting amino acid is depleted, then the remaining essential amino acids obtained from the diet will be broken down and be unavailable as a source for protein synthesis. Different types of food have different amounts of the amino acids needed for growth and maintenance. The biological value of food is the amount of N digested, absorbed, and used by the body but not excreted and reflects the distribution of amino acids found in the food and how well it meets the amino acid requirements of the individual (Wildman and Medeiros, 2000).

3.2. Sources of Dietary Protein

There is no one perfect food for protein requirements and just because a food source contains high concentrations of protein does not mean it has a high biological value. A protein with a high biological value, or a complete protein, is normally obtained from an animal source, such as meat and milk and would contain all essential amino acids in a distribution similar to what is required for growth and maintenance in humans (Wildman and Medeiros, 2000). Eggs show distribution of amino acids at mixtures most similar to what is required by the body and are used as the standard by which all other protein sources are measured. A protein source with a low biological value has all nine essential amino acids but not in a distribution necessary for normal growth and maintenance in humans. Protein sources with low biological values are also called incomplete proteins, a category that most plants fall into (Wildman and Medeiros, 2000).

3.3. Protein Quality

Plant protein sources are generally deficient in lysine, tryptophan, or methionine (Garrison and Somer, 1995). However, animal proteins do not need to be consumed to obtain a balance of all the essential amino acids. Incomplete protein sources can be combined to balance the limiting amino acids in another incomplete protein source. These two protein sources would be referred to as complementary proteins. For instance beans (low in methionine and tryptophan but high in lysine) can be combined with rice (low in lysine but high in methionine and tryptophan) to obtain a balance of all essential amino acids. Healthy adults require approximately 15% of the protein in their diets to be composed of essential amino acids

and a typical western diet supplies around 50%. When obtaining essential amino acids through incomplete proteins, healthy adults do not need to consume the complementary proteins in the same meal but can consume them throughout the day. Infants and children require a higher percentage of essential amino acids (around 35% of total protein) due to growth and development. If incomplete proteins are being consumed as the protein source in children, consumption of the complementary proteins in one meal is suggested (Wardlaw and Insel, 1996).

Protein is the second most plentiful substance in our bodies (with water being first), comprising one-fifth of our total body weight (Garrison and Somer, 1995). Proteins are a major constituent of all living cells and are important components of muscle, body organs, skin, hair, and nails as well as enzymes and immune system compounds (Garrison and Somer, 1995). Proteins also participate in regulation of fluid balance and regulation of blood pH and can serve as an energy source.

The liver is the main site of amino acid metabolism. In a normal adult human 75% of amino acids are utilized in protein synthesis by the continuous catabolism and synthesis of proteins (Garrison and Somer, 1995). An average adult male can synthesize 300 g of new protein a day (Garrison and Somer, 1995). Amino acids not used for protein synthesis can be converted to other important N-containing compounds including purines, pyrimidines, choline, creatine, niacin, and porphyrins (Garrison and Somer, 1995).

Proteins supply about 2–5% of the energy needs of the body. In comparing energy sources fat generates 9 cal/g of fat, carbohydrates generate 4 cal/g of carbohydrate, and proteins are similar to carbohydrates in that they can generate 4 cal/g of protein. However, considerable processing by the liver and kidneys is needed for utilization of energy from protein (Wardlaw and Insel, 1996). Proteins are first broken down into free amino acids and the amino group is removed for utilization as an energy source. Removal of N from amino acids results in the formation of carbon skeletons, which can be metabolized to acetyl Coenzyme A (acetyl CoA) and pyruvate and used in energy production through the citric acid cycle.

Catabolism of amino acids results in the formation of amino (NH_2) groups which are converted to the ammonia (NH_3) form. Ammonia must be excreted as increasing concentrations are toxic to the cells in the body. Excretion of NH_3 in humans occurs through the synthesis of urea. Urea is synthesized by the liver and secreted into the bloodstream where it is then taken up by the kidneys for excretion in the urine. An NH_3 and an NH_2 group react with carbon dioxide through a series of steps known as the urea cycle to form urea and water. Urea can then be excreted in the urine.

3.4. Protein Requirements by Humans

When humans hit the adult stage and are in a healthy state there is no net gain in body N stores (Fowden, 1981). In other words, protein intake beyond that required for maintenance and excretion in a normal healthy adult equals protein loss. This is known as "nitrogen balance" and can be determined by measuring the difference between protein-N intake and excretion of N in urine or urea-N excretion (Zeman, 1991). During growth or recovery from illness, protein requirements are

increased because of the need to build or repair new tissue. In this case a positive N balance would occur due to the intake of protein-N being higher than the output. If not enough protein is consumed and proteins are broken down faster than they are synthesized, then a negative N balance occurs and catabolism and excretion of protein is higher than its intake (Wildman and Medeiros, 2000).

If the amount of protein-N consumed is equal to the amount lost then a N balance exists. One equation for estimating the amount of protein that would need to be consumed to maintain N balance is:

$$\text{Nitrogen balance} = \frac{\text{24-h protein intake}}{6.25} - (\text{UUN} + 4)$$

In this equation, grams of protein ingested is converted to grams of N by dividing by 6.25 (protein contains about 16% N). Excretion of N over 24 h is equal to excreted urea in the urine (UUN) + 4 g/day nonurea-N loss. This amount includes nonurea-N in urine, fecal N loss from digestive enzymes and sloughed intestinal cells, and integumental loss or what is called obligatory loss (Zeman, 1991). A normal adult male (70 kg) loses approximately 54 ± 2 mg of N/kg body weight per day (Wildman and Medeiros, 2000).

It is important to consider that as ingestion of protein increases to levels approaching the individual requirement, there is a decrease in the utilization of dietary protein which can increase requirements from 0.45 to 0.57 g protein/kg body weight per day. In addition, source and quality of the protein can affect utilization and increase amounts that must be ingested. It has been determined that protein quality from western diets is about 75% of protein quality from eggs (Wildman and Medeiros, 2000). With this in mind dietary protein intake needed to maintain N balance would be 0.57 g protein/kg body weight per day × 100/75 = 0.8 g protein/kg body weight per day (Wildman and Medeiros, 2000).

In addition, recommendations for an individual as opposed to a population need to take into account the quality of the protein, age, and state of the individual as well as energy needs (Garrison and Somer, 1995). This value can vary as a result of growth and/or illness due to increased requirements. Children under two may need 1.2 g of protein/kg body weight for increased growth. Pregnant and lactating women can require up to 6 g of protein/kg body weight per day (Garrison & Somer, 1995). It is of interest to note differences between adults and infants. As stated above the dietary intake needed to maintain N balance in adults would be 0.8 g protein/kg body weight per day. However, due to increased needs for growth, requirements for very young infants can be as high as 1.98 g of protein/kg body weight per day during the first month of life but decreases to that of other age groups by about 4 months (Fomon, 1993).

Another method for estimation of N requirements is based on energy needs. If energy intake from carbohydrates and fats are too low, then the body uses protein as an energy source, which can result in degradation of muscle. Carbohydrates can act as N-sparing agents by prevention of amino acid catabolism for use as an energy source. A group that can be at risk of inadequate protein consumption in relation to

caloric intake are the elderly. The elderly population may have a higher protein need to prevent muscle loss and some studies suggest an increase to 1.25 g of protein/kg body weight per day in this population (Wardlaw and Insel, 1996). For maintenance in a normal healthy adult the ratio of kilocalories to N (from protein) suggested is around 300:1 or 300 kcal for every 1 g of N (or 6.25 g of protein) (Zeman, 1991). For anabolism the suggested ratio of calories to N is 150 kcal/g N.

In developing countries diets may be low in energy and protein leading to protein-energy malnutrition. It has been estimated that around 500 million children worldwide suffer from protein-energy malnutrition (Wardlaw and Insel, 1996). There are two major types of protein-energy malnutrition; marasmus and kwashiorkor. In marasmus or general starvation there is insufficient intake of both energy and protein resulting in overall wasting. There is little fat stored, muscle mass declines, and death occurs frequently from infections. Kwashiorkor is a word in Ghana meaning disease of the first child. This is due to the mother having another child and stopping breastfeeding of the first child thus changing the child's diet from the nutrient rich milk to roots and gruels significantly decreasing protein availability in the diet. In kwashiorkor, there is marginal but adequate energy consumption and extremely low protein intake. This results in fat stores being preserved as well as decreased catabolism of muscle protein. Turn over of amino acids in muscle is a vital function for synthesis of essential proteins such as albumin and immunoglobulins (Brody, 1994). In kwashiorkor infection, edema and poor growth are common with continuing decreases in proteins essential for proper function.

4. HUMAN NITROGEN CONTRIBUTIONS BASED ON INTAKE AND LOSSES

Nitrogen intake for US males and females can be calculated by assuming the N in protein as 16% and that the RDA recommendation is the same per unit of body mass for all age, gender, and social and cultural groups. Increased protein intake in healthy adults is not generally required because there are no identified benefits in exceeding 1.5 g protein consumption per kilogram body weight per day. However, most American adults meet or exceed the recommended allowance by consuming two to three times the recommended levels (Garrison and Somer, 1995).

Inclusion of the current meta-analysis takes into consideration changes in national reporting of recommendations with the inclusion of both Estimated Average Requirements (EAR) (median) and an RDA's (meeting the 97.5th percentile for healthy adults of 0.65 and 0.83 g of good-quality protein per kilogram per day for maintenance of a normal, healthy, individual) (Rand et al., 2003) (Table 1)[1]. Most of this loss is as N in urine (37 mg N/kg body weight) and feces (12 mg N/kg body weight), with the remaining 5 mg lost as a result of cutaneous and other miscellaneous N routes.

[1]Rand et al. (2003) study published offers more specific data on the most solid data but maintains a similar range of obligatory loss as indicated in (Table 1)

Table 1.
(Rand et al., 2003).

	Sex	Age*	No. of subjects	Nitrogen intake (mg/kg/day)	Urinary nitrogen (mg/kg/day)	Fecal nitrogen (mg/kg/day)	Nitrogen balance** (mg/kg/day)
Individual substudies							
Atinmo et al., 1985 (30)	M	Y	15	14.8	44.8	20.2	−59.43 ± 5.7
Bodwell et al., 1979 (33)	F	Y	11	1.8	30.7	7.7	−41.4 ± 6.1
Bodwell et al., 1979 (33)	M	Y	13	1.8	30.9	8.8	−42.7 ± 6.8
Bricker and Smith, 1951 (36)	F	Y	25	3	25.2	8.7	−35.7 ± 4.1
Calloway and Margen, 1971 (37)	M	Y	13	0	38	14	−55.59 ± 7.6
Huang et al., 1972 (49)	M	Y	50	5	33.4	13.1	−52.5 ± 5.3
Inoue et al., 1974 (53)	M	Y	9	2	33.3	12.7	−52 ± 3.7
Nicol and Phillips, 1976 (59)	M	Y	9	14.7	34	23	−53.3 ± 6.4
Scrimshaw et al., 1972 (64)	M	Y	83	11	37.2	8.8	−39.8 ± 6
Scrimshaw et al., 1976 (65)	F	O	11	10	24.4	9.8	−29 ± 6.3
Tontisirin et al., 1981 (69)	M	Y	4	0	34.9	12.6	−58.5 ± 4.2
Uauy et al., 1978 (72)	M	O	8	0	34.5	12.2	−51.5 ± 11.2
Uauy et al., 1982 (73)	M	Y	8	6.7	36.2	16.1	−50.4 ± 9.9
Young and Scrimshaw, 1968 (79)	M	Y	8	6	36.6	9	−44.4 ± 3.2
Zanni et al., 1979 (83)	M	O	6	0.9	27.3	9.5	−40.32 ± 3.4
Substudy comparisons							
Twelve substudies in males	–	–	–	–	–	–	−50.0 ± 6.6
Three substudies in females	–	–	–	–	–	–	−35.4 ± 5.6***
Twelve substudies in the young	–	–	–	–	–	–	−48.8 ± 6.0
Three substudies in the old	–	–	–	–	–	–	−40.3 ± 7.7
All 15 substudies	–	–	–	–	–	–	−47.1 ± 6.4

Summary of data for obligatory nitrogen losses in healthy adults
*Y, young; O, old.
**$X \pm$ SD.
***Significantly different from the 12 substudies in males; $P = 0.005$.

Table 2 shows calculations based on the RDA recommendations and not the exceeded amount discussed above and are therefore expected to result in conservative estimates of the actual dietary N intake by the US population. These estimates were made based on obligatory losses of protein for a carbohydrate-containing protein-free diet. Such losses are reported as 54 mg N/kg body weight for a 70 kg male (Wildman and Medieros, 2000). Estimated N intake per person, annual US N intake, and estimated N loss (obligatory excretion) per person are shown.

Table 2.
Population, average weight, estimated N intake per person and annual US N intake, and estimated N loss (obligatory excretion) per person and annual US N loss as a function of age ranges for males and females in the United States.

Range	Population (1000s)	Mean weight (kg)	Estimated annual N-intake (kg N/ person)	Estimated annual US N-intake (Metric ton N)	Estimated annual N-loss (kg N/ person)	Estimated annual N-loss (Metric ton N)
Male age						
<5	9,683	12	0.56	5,429	0.24	2,290
5–13	18,303	32	1.50	27,465	0.63	11,587
14–17	8,094	54	2.52	20,392	1.06	8,603
18–24	13,579	72	3.36	45,678	1.42	19,270
25–49	50,578	79	3.69	186,677	1.56	78,754
50+	34,398	77	3.60	123,745	1.52	52,205
Total	134,635					
Total				409,385		172,709
Female age						
<5	9,264	12	0.56	5,194	0.24	2,191
5–13	17,458	31	1.45	25,285	0.61	10,667
14–17	7,636	57	2.67	20,369	1.13	8,593
18–24	13,015	60	2.80	36,484	1.18	15,392
25–49	51,535	63	2.94	151,686	1.24	63,993
50+	41,834	65	3.04	127,041	1.28	53,596
Total	140,742					
Total				366,059		154,431
US Total	275,377			775,444		327,140

The average weights for US age groups in Table 2 were adapted from those from the US RDA for protein (Wildman and Medieros, 2000; DRI, 2005). Population statistics report did not use the 2006 Population Clock estimations and were restricted to the more accurate data reported by the most current U.S. Census Bureau (2007).

We consider our estimates for both total N intake and N loss (excretion) conservative. In the case of N intake, US diets in many sectors of the population exceed the RDA for protein intake and thus the N-intake estimate of about 775,000 t N shown in Table 2 is also likely exceeded. It could be readily visualized that N intake approaches 1×10^6 t of dietary N in the human population and that dietary N intake ranges from 0.8 to 1×10^6 t/year. Just as estimates of intake are likely low in Table 2, so too the estimate of obligatory N loss is less than the actual N loss. The obligatory N loss is measured on individuals on a carbohydrate containing protein-free diet. The base amount of N loss (54 mg/kg body weight) will be exceeded once protein is added back to the diet. Factors affecting the increased amount of N loss are protein quality, amount of protein consumed, including that in excess of the RDA, and because for adults N loss should about equal N intake. Consequently the estimate of N loss for the US population is likely be near the estimate of N intake, that is, somewhere in the range of 0.8–1.0 million metric tons of N.

In the above discussion of N intake and N loss, the United States has been used as a case study because a base of data was readily available. As a developed country, it is likely that the data from the United States can be extrapolated to other developed countries with similar age, weight, and dietary N-intake patterns. We considered it inadvisable to extrapolate this data to less-developed countries or to the world, because of the unlike dietary, age and weight distribution, income, and cultural differences that exist. Irrespective, it can be noted that the US population (about 275 million) is less than 5% of the world population of about 6.1 billion people. It can certainly be stated that humans excrete huge amounts of N in both the United States and in the world. The amount can only increase when it is considered that the world population is projected to increase to about 8 billion people during the next 25 years. The N excreted by humans has just as much potential to impact the environment as does N from livestock wastes, inefficient fertilizer-N use or from any other N source. As a comparison in the United States, where approximately 1×10^6 t N is excreted by humans, the amount of commercial fertilizer-N consumed was about 10.9×10^6 t in 2002 (FAO, 2004), and N excreted in animal manure was estimated as 4.1 and 7.8×10^6 t for collectable and all animal manure in 1997, respectively (see Chapter 16). Consequently, the need exists to recognize the highly significance role of N in the human diet, as protein, and also the very significant potential impact of increasing human excretion on N as it enters the environment and that this impact will increase as the human population grows. Although no worldwide estimates were made of the amounts of N excreted by humans or animals were made for this study, world fertilizer-N consumption in 2002 was 84.7×10^6 t (FAO, 2004).

5. EFFECTS OF NITROGENOUS COMPOUNDS ON HUMAN HEALTH

We have thus far considered the N cascade from the perspective of human use, metabolism, and excretion into the environment. As stated above in Figure 2 there can be added environmental impacts on human and environmental health from active nitrogenous compounds formed through human/environmental interactions and these compounds may impact human health.

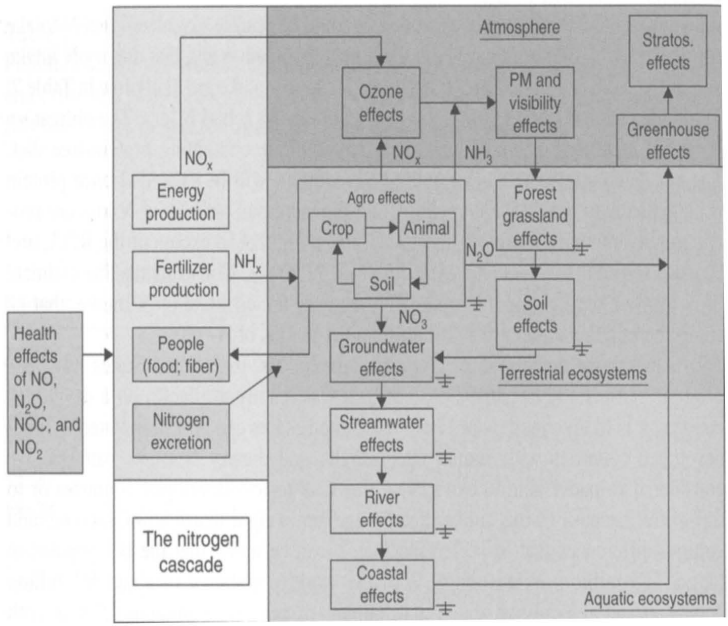

Figure 2. Modified nitrogen cascade.

Although humans may not be able to utilize more simple forms of N for normal physiologic functions such as energy and synthesis of proteins, they are exposed to and capable of absorbing other forms of N in the diet such as NO_3^-, which must be considered for its effect on human health. The natural presence of NO_3^- in plants is due to the N cycle in which organic forms of N are converted to the mineral forms of N; that is, NO_3^-, NH_4^+, and nitrite (NO_2^-) by microorganisms and taken up by plants. Because NO_3^- can be accumulated in the tissues of a number of edible plants, vegetables can be a major dietary source of NO_3^-. Humans can also be exposed to NO_3^- that is present in rural drinking water supplies due to contamination from agricultural or other sources. Under normal conditions NO_3^- is found only in small amounts in drinking water and is mainly ingested in the diet from cured meats and green vegetables such as spinach and lettuce and roots such as beets and

carrots. Nitrate is easily absorbed by the intestine and in general rapidly metabolized and excreted in the urine. In general NO_3^- is not usually considered a health risk but may become a concern due to its conversion to NO_2^- and NOC. Because of these concerns NO_3^- metabolism in humans must be examined.

5.1. Methemoglobinemia

The main health risk associated with NO_3^- consumption (specifically from water sources) in humans is development of methemoglobinemia due to NO_2^- derived from NO_3^-. The iron contained within hemoglobin, in red blood cells, is normally in the ferrous (reduced) state. Through oxidation, ferrous iron can be converted to ferric iron and this conversion of the iron to the oxidized state prevents transport of oxygen throughout the body. When the ferrous iron in heme is converted to ferric iron the resulting hemoglobin is referred to as MHb. Methemoglobin does not have the capacity to transport enough oxygen to supply the cells with an adequate amount of oxygen.

The presence of a small amount of MHb is usually normal, making up approximately 1% of the total hemoglobin (Hb) in adults and around 2% in infants (Wright et al., 1999). Red blood cells do contain mechanisms to protect against oxidation and are capable of converting MHb back to hemoglobin. However, the red blood cell has a finite life span and over time loses its ability to protect against oxidation. Continued oxidative stress resulting in the formation of MHb is considered to be a normal mechanism for the identification of aging cells that the body needs to remove from circulation (Coleman and Coleman, 1996; Faivre-Fiorina et al., 1998; Wright et al., 1999). Although MHb is a normal product of metabolism in the body, methemoglobinemia occurs under abnormal conditions when levels of MHb become too high for adequate transport of oxygen to cells in the body.

One of the most apparent manifestations of methemoglobinemia is cyanosis in which the skin can appear blue in color, because of the lack of oxygen in the blood. Consequently, methemoglobinemia in infants is referred to as "blue baby syndrome." Cyanosis has been shown to be evident at levels of only 5–10% of Hb in the form of MHb especially in the appearance of the bluish color in the lips and nails (Bruning-Fann and Kaneene, 1993a). Blood taken from an individual with methemoglobinemia exhibits a characteristic chocolate brown color due to oxidation. Other manifestations include drowsiness and lethargy as well as diarrhea and vomiting and, due to oxygen deprivation to body cells, can lead to death.

5.1.1. Contribution of ingested nitrate

Development of methemoglobinemia from the ingestion of water containing high NO_3^- is well known with the most common cases associated with contaminated well water. However, some municipal water contamination has been reported as well (Bruning-Fann and Kaneene, 1993a). Most cases of methemoglobinemia result from a water source with NO_3^- levels above 45 ppm (i.e., 10 ppm NO_3^--N); however, some reports indicate increased concentrations of NO_3^- in the water

boiled for sterilization due to evaporation (Bruning-Fann and Kaneene, 1993a). The ingested NO_3^- can be reduced to NO_2^- in the gut by nitrate-reducing bacteria. In addition, bacterial contamination of water high in NO_3^- is common and contributes to increased toxicity by reducing NO_3^- before consumption.

In humans, NO_3^- in food does not in general contribute significantly to methemoglobinemia. This may be due to the presence of compounds in foods such as ascorbic acid that can chelate (bind) and thus inhibit NO_2^- formation in the gut. The most common food-related toxicity is the ingestion by infants of infant formula mixed with contaminated well water (Bruning-Fann and Kaneene, 1993a). Increased concentration of NO_3^- in breast milk has not been substantiated by the literature suggesting that it is not concentrated in human milk and reflects the plasma levels of the mother (Bruning-Fann and Kaneene, 1993a; Dusdieker et al., 1996).

There is some evidence to support improper storage or handling of foods as contributors to methemoglobinemia. Many vegetables such as lettuce, spinach, and root vegetables such as carrots contain high levels of NO_3^-. Plant NO_3^- converted to NO_2^- prior to ingestion has been implicated as a cause of contamination in home-prepared spinach, stored at room temperature (Fomon, 1993). Similar conditions were seen in the preparation and improper storage of carrots.

5.1.2. Factors affecting susceptibility to methemoglobinemia

Several factors contribute to increased sensitivity to methemoglobinemia. Age is considered a contributor to increased sensitivity for several reasons. Infants under 6 months are more sensitive to methemoglobinemia than children and adults, since their stomach pH is greater than 4. This higher pH decreases the effectiveness of the stomach in prevention of the growth of nitrate-reducing bacteria. Older adults can also experience higher stomach pH and be at risk for methemoglobinemia for this same reason.

Infants also have increased risk because they consume more water per unit of body weight than an adult and as mentioned above infants who's only food source is home-prepared formula are more at risk than breast-fed infants if the formula is made with tap water high in NO_3^- (Bruning-Fann and Kaneene, 1993a). Pre-natal methemoglobinemia has also been shown to occur in pregnant women consuming water containing high concentrations of NO_3^-. Nitrite has clearly been shown to cross the placenta and cause methemoglobinemia in the developing fetus (Wright et al., 1999). In addition, pregnancy in rats has long been associated with naturally higher levels of MHb which may exacerbate any contribution from nitrate ingestion and may need to be considered in humans as well (Tarburton et al., 1985). Another factor which might play a role in the sensitivity of the fetus and in the newborn infant is the presence of fetal hemoglobin. Fetal red blood cells have higher oxygen affinity than adult red blood cells due to differences in the amino acid sequence of the globin chains. This causes fetal Hb to be more easily oxidized than the predominant form of Hb in adults (Wright et al., 1999). Although fetal Hb decreases after birth, it is still present in the newborn infant and may contribute to sensitivity to

MHb formation during the first months of life. Under conditions of diarrhea and dehydration metabolic acidosis can occur. Although the mechanism is not completely understood, condition of acidosis can lead to methemoglobinemia even without known exposure to nitrates and may contribute to increased sensitivity in the presence of nitrates.

Over 140,000 people receive dialysis treatment each year. Dialysis patients are also at risk and must be careful to use water known to contain less than 2 ppm N in the NO_3^- form (i.e., NO_3^--N) (Fan et al., 1987).

Although not related to increased formation of MHb, anemic patients might experience more deleterious effects due to less hemoglobin bioavailability. A normal person with a hemoglobin concentration of 15 g/dL with 20% being composed of MHb would still have 12 g/dL of normal hemoglobin, whereas an anemic individual with a hemoglobin value of 8 g/dL would only have 6.4 g/dL of normal hemoglobin available (Wright et al., 1999).

5.1.3. Metabolic pathways and protective mechanisms

Since the red blood cell is constantly exposed to high concentrations of oxygen and is thus exposed to free radicals of oxygen, the body has developed protective mechanisms for reduction of iron in MHb back to the ferrous state and if nitrates are removed minor cases of methemoglobinemia can resolve on their own. In a normal adult human approximately 15% of the MHb can be reduced back to hemoglobin per hour (Wright et al., 1999). The cytochrome-b_5-MHb reductase pathway is the main form of reduction protection in the body with 99% of MHb reduction occurring through this pathway (Wright et al., 1999). The two enzymes involved in this pathway are cytosolic cytochrome-b_5 and cytochrome-b_5 reductase. Nicotinamide adenine dinucleotide (NADH) produced through the glycolytic pathway is also required for the reaction to occur. This pathway helps to control normal endogenous formation of MHb; however, factors related to decreases in enzymatic activity can contribute to formation of higher concentrations of MHb.

Under normal conditions infants under 6 months have lower levels of cytochrome-b_5 reductase with concentrations at birth only being half that of an adult (Hjelt et al., 1995; Wright et al., 1999). In addition some congenital factors related to this pathway may aggravate conditions brought on by NO_3^- ingestion in adults. Cytochrome-b_5 reductase and cytochrome b_5 deficiencies can occur. Both deficiencies are autosomal recessive and result in increased levels of MHb due to decreased reduction capacity of this main pathway.

Another pathway to consider in the treatment of MHb is nicotinamide adenine dinucleotide phosphate (NADPH)-MHb reductase. This enzyme is not specific for the reduction of MHb but instead functions in the metabolism of xenobiotics. Methylene blue is commonly used in the treatment of methemoglobinemia. However, methylene blue itself is an oxidizing agent. NADPH-MHb reductase has an affinity for methylene blue and reduces the dye to leukomethylene blue, which has an affinity for MHb and can act as a reducing agent to reduce the iron to the

ferrous state. The reaction occurs quickly and significant amounts of MHb can be reduced within 30 min. In a person with a congenital NADPH-MHb reductase deficiency the methylene blue will not be converted as efficiently leading to more methylene blue acting as an oxidizing agent than a reducing agent which can in turn cause hemolysis of the red blood cells (Wright et al., 1999).

Glucose-6-phosphate dehydrogenase (G6PDH) deficiency may also contribute to methemoglobinemia. This enzyme is part of the hexose monophosphate shunt and is therefore involved in the synthesis of NADPH, which is utilized in the reduction of methylene blue and may therefore decrease the effectiveness of the treatment (Wright et al., 1999). Although cytochrome-b_5 reductase is the main pathway for the reduction of MHb and methylene blue is the most common clinical treatment used, other compounds can play minor roles. Both ascorbic acid and glutathione can act indirectly as cellular anti-oxidants and in some individuals with congenital deficiencies up-regulation of reduction is thought to occur through these compounds. However, deficiencies in these compounds do not lead to methemoglobinemia (Wright et al., 1999).

Dextrose can also act indirectly by contributing glucose for glycolysis and for the hexose monophosphate. The reduced NADH created through glycolysis is utilized in the cytochrome-b_5 reductase pathway and the NADPH produced in the hexose monophosphate shunt is involved in the reduction of methylene blue. Therefore, high enough concentrations of glucose for these pathways should be considered for increased efficiency (Wright et al., 1999).

5.2. Secondary and Related Effects

5.2.1. Acute respiratory infection

A possible secondary effect of methemoglobinemia from exposure to NO_3^- in water is the increased risk of respiratory tract infections. Animal studies have suggested changes in bronchi and lung cells along with increased presence of lymphocyte in the lung (suggesting increased infection) in animals consuming high NO_3^- diets. The damage to the tissue increased with higher NO_3^- levels in the ingested water. A study in 8-year-old children in India demonstrated a strong positive correlation between methemoglobinemia due to well water NO_3^- and the incidence of respiratory tract infections. It is thought that the high NO_3^- leads first to methemoglobinemia, causing hypoxia and increased free radicals of NO and oxygen. Since NO can act as a vasodilator this may cause changes in pulmonary circulation and alveoli providing high-risk conditions for respiratory tract infections (Gupta et al., 2000).

5.2.2. Thyroid

Although methemoglobinemia has been identified as a major human health risk from exposure to NO_3^-, there are other health risks from NO_3^- in drinking water including deleterious interactions with the thyroid gland. The thyroid gland synthesizes two iodoamino acid hormones which play a role in general metabolism and

developmental regulation, as well as regulation of tissue differentiation. Thyroid hormones are distinctive in that they require iodine to be active.

Nitrate appears to inhibit both uptake and retention of iodine by the thyroid. Nitrate is of similar in size and charge to the iodide ion and appears to compete with the iodide-binding site in the thyroid (Zralý et al., 1997). The effect on the thyroid is similar to that seen with the administration of thiocyanate and perchlorate anions, which also inhibit accumulation of iodide in the thyroid (Jahreis et al., 1986).

The effect of NO_3^- on the thyroid was first observed in 1952 in rats (Bruning-Fann and Kaneene, 1993a). Further studies in rats and similar studies in chickens confirmed altered thyroid metabolism and decreased iodide uptake along with increased thyroid size, which is indicative of an attempt of the thyroid to compensate for decreased hormone synthesis (Bruning-Fann and Kaneene, 1993a).

Ruminant animals such as sheep also demonstrate decreased uptake of iodine by the thyroid. However, there is some evidence to support an increased ability of ruminants to adapt to increased NO_3^- consumption over time (Bruning-Fann and Kaneene, 1993a). On the other hand, administration of potassium nitrate to bulls resulted in decreased thyroxin levels, indicative of depressed thyroid gland activity. It is thought that this decrease in hormone level leads to an observed effect on the libido and delayed onset of erection and mounting, suggesting that the effect of NO_3^- on ruminant animals should not be overlooked (Zralý et al., 1997).

It is also apparent that human populations exposed to high NO_3^- levels in drinking water show a similar increase in thyroid volume and decreased levels of thyroid stimulating hormone. The effect is dose dependent with differences in thyroid volume occurring above 50 mg/L (van Maanen et al., 1994). Guidelines for concentrations of NO_3^- in water have been developed for the prevention of methemoglobinemia. As summarized by Fraser and Chilvers (1981), the current WHO European Standards for drinking water recommends levels of NO_3^- of less than 50 mg/L, while the standard in the United States is 45 ppm (USEPA, 1973, 2001). It is important to note that at around 50 mg/L alteration in thyroid metabolism might be manifested in humans (van Maanen et al., 1994).

Alteration in thyroid metabolism must be seriously evaluated in both the monogastric and ruminant animal in that it may partially explain some other effects seen from NO_3^- consumption including immune function, reproduction, and fetal developmental problems.

5.2.3. Birth defects and reproduction

The potential effect of NO_3^- on reproduction and normal fetal development in humans remains a topic of controversy.

It has been clearly shown that NO_2^- can cross the placental barrier in animals (Bruning-Fann and Kaneene, 1993b), and research suggests that transfer may occur in humans as well due to the presence of fetal methemoglobinemia when high NO_3^- was consumed by the mother during pregnancy (Bruning-Fann and Kaneene, 1993b). Due to placental transfer of NO_3^- the risk of spontaneous abortion associated with

fetal methemoglobinemia has been studied. A clear relationship between NO_3^- intake and abortion has been established in ruminants. In cows, fetal death and abortion have been linked to ingestion of high NO_3^- by the mother. (Bruning-Fann and Kaneene, 1993a; R.F. Follett, unpublished). Similar results are seen in sheep (Bruning-Fann and Kaneene, 1993a). The cause of the abortion is thought to be due to fetal methemoglobinemia, which results in death due to hypoxia and is supported by demonstration of decreased oxygen saturation of umbilical cord blood and elevated nitrate in the dead calves (Bruning-Fann and Kaneene, 1993a).

The effect of NO_3^- ingestion in monogastric animals during pregnancy is less clear and in general is less likely to occur in the presence of NO_3^- since higher levels of NO_2^- is required than in ruminants for detrimental outcomes to occur (Bruning-Fann and Kaneene, 1993a; Fan and Steinberg, 1996). Ground water NO_3^- and NO_2^- as causes of fetal methemoglobinemia have also been identified as risk factors for spontaneous abortions in humans (Bruning-Fann and Kaneene, 1993b). However, other studies have shown no association between increased water NO_3^- consumption and abortion at levels higher than 40 ppm NO_3^- (Bruning-Fann and Kaneene, 1993b).

Past research has also suggested nitrosating substances may influence DNA alkylation and transcription. Therefore the effects of NO_3^- and NO_2^- during pregnancy need to be considered in the proper development of the fetus (van Maanen et al., 1996a). A review by Fan and Steinberg (1996) suggests that studies in animals such as mice, rats, and guinea pigs do not appear to fully support an increase in congenital malformations from ingestion of NO_3^- and NO_2^- at levels that might be encountered in drinking water (Fan et al., 1987; Bruning-Fann and Kaneene, 1993b).

Although links with NO_3^- and NO_2^- consumption and birth defects of the central nervous and musculoskeletal systems have been alluded to in humans, epidemiological studies and historical literature did not support a clear connection but encouraged further studies (Bruning-Fann and Kaneene, 1993b).

Although this past research may demonstrate some controversy, the most current epidemiological research available shows a summary of the negative health impacts of high NO_3^- water to the occurrence of pregnancy complications and infant development. (Manassram et al., 2006) (Table 3). The most current references provide strong evidence for more fetal development and maternal health concerns from NO_3^- than previously expected.

6. NITRIC OXIDE

The role of NO_3^- and NO_2^- in the diet has mainly focused on their deleterious effects. However, there is growing acceptance for the beneficial importance of these compounds in the synthesis of NO. Nitric oxide is a free radical gas important in normal physiological function where it acts as a messenger molecule for regulation of several systems including blood vessel dilation, hormonal and neurotransmission functions. Nitric oxide can be produced endogenously with the main source

Table 3.
Summary of epidemiologic studies that evaluated the exposure to nitrate in drinking water and reproductive and developmental effects (Manassram et al., 2006).

Reference	Infant subject number	Exposure	Outcome
Brender et al., 2004	185 cases; 225 controls	Drinking water and, dietary nitrate and nitrosatable drugs	Neural tube defects
Cedergren et al., 2002	71,978 infants	Drinking water nitrate during early pregnancy	Congenital cardiac defects
Bukowski et al., 2001	546 cases; 4,098 controls	Drinking water nitrate during early pregnancy	Premature birth, low birth weight
Croen et al., 2001	538 cases; 539 controls	Drinking water nitrate during early pregnancy	Neural tube defect
Tabacova et al., 1997	61 pregnancies	Drinking water nitrate and NO_2 ambient air during pregnancy	Pregnancy complications
Tabacova et al., 1998	51 infant–mother pairs	Drinking water nitrate and NO_2 ambient air during pregnancy	Neonatal health status
Arbuckle et al., 1988	130 cases; 260 controls	Drinking water nitrate	Central nervous system defects
Aschengrau et al., 1989	286 cases; 1,391 controls	Drinking water nitrate during pregnancy	Spontaneous abortion
Dorsch et al., 1984	218 infant case–control pairs	Drinking water in nitrate	Congenital malformations
Scragg et al., 1982	699 perinatal deaths	Drinking water nitrate	Deaths from congenital malformations
Super et al., 1981	486 infants	Dinking water nitrate	Premature birth, low birth weight

being from the amino acid arginine. The enzyme NO synthase (NOS) catalyzes a five-electron oxidation of an amide nitrogen of arginine resulting in the synthesis of citrulline and NO (Murray and Clark, 1994; Ellis et al., 1998). The NOS enzyme contains a tightly bound heme and is similar in structure to the cytochrome P-450 reductase enzyme (Pufahl and Marletta, 1993). Nitric oxide is a small molecule and is lipophilic allowing for rapid diffusion through cell membranes for interaction with intracellular target compounds (Ignarro, 1999). Nitric oxide has a biological half-life of approximately 5 s allowing it to act exceptionally well as a local mediator of physiological function (Moncada and Higgs, 1991). Nitric oxide reacts in the body with water to form NO_2^-, which is an unstable compound in blood and quickly converts to NO_3^- (Ellis et al., 1998). Both NO_3^- and NO_2^- can then be excreted in the urine.

The role of NO in the body can be divided into two groups. First is its action as a messenger molecule where it plays a role in vascular tone, platelet activation, immune response, and as a neurotransmitter. The second category is related to its function as a cytotoxic molecule important in host defense but also as a harmful compound related to autoimmune diseases (Moilanen and Vapaatalo, 1995).

6.1. Target Compounds

Nitric oxide has five main molecular targets in the body; heme proteins, enzymes, DNA, thiols, and superoxide (O_2^-) (Radomski, 1995). One of the most important heme proteins that NO binds is guanylate cyclase (GC). Binding of NO results in the activation of GC leading to increased synthesis of cyclic guanosine monophosphate (cGMP), a compound involved in the mediation of ion flux, modulation of cyclic adenosine monophosphate (cAMP)-mediated responses, and increases in protein kinase phosphorylation reactions (Radomski, 1995). Nitric oxide also interacts with hemoglobin and myoglobin resulting in the degredation of NO and thus a decrease in the biological activity of NO (Radomski, 1995). Nitric oxide can act on other enzymes such as complex I and II of the mitochondrial electron transport chain and aconitase in the TCA cycle. Interaction with these enzymes has been linked to some of the cytotoxic effect of NO (Radomski, 1995). Inhibition of DNA synthesis can occur from macrophage NO through the inhibition of ribonucleotide reductase; the rate-limiting enzyme in DNA synthesis (Lepoivre et al., 1991). Interaction with thiols through the nitrosylation of sulfhydryl groups may offer a storage mechanism for readily available NO (Stamler et al., 1992; Radomski, 1995). Interaction of NO with superoxide results in the formation of peroxynitrite, a highly reactive molecule, which can participate in the oxidation of many compounds and may relate to some of the more detrimental effects of NO.

6.2. Relaxation of Smooth Muscle

One of the best-documented functions of NO in the body is its action as a relaxing factor on smooth muscle of blood vessel walls leading to vasodilatation and a decrease in blood pressure (Ellis et al., 1998). The interaction of NO_3^- with

hemoglobin in methemoglobinemia allows for an understanding of the role of NO in vasodilatation. Nitric oxide first diffuses out of the cell that it was synthesized in and acts on neighboring cells. The intracellular enzyme GC contains a heme prosthetic group to which the NO can bind resulting in a conformational change causing the activation of the enzyme. This results in the production of cGMP, which causes relaxation of the vessel walls, which leads to vasodilatation and hypotension or a decrease in blood pressure. In the heart, cGMP will act to relax the muscle and to decrease the force of the contractions by stimulating ion pumps that maintain low cytosolic Ca^{2+} concentrations.

Although the primary synthesis of NO in the body occurs through arginine metabolism, it has been suggested that dietary NO_3^- and NO_2^- can be converted to NO and contribute to endogenous NO synthesis as well (Gruetter et al., 1981). Vasodilator therapy is commonly used for management of congestive heart failure. Nitrate compounds, such as nitroglycerin (glyceryl trinitrate), are among the oldest and most utilized compounds in vasodilator therapy with a well-documented decrease in intramyocardial pressure leading to improved perfusion of the heart. Although NO_3^- is generally considered an inorganic molecule, within the medical field it is common to refer to a number of compounds, including nitroglycerin, as "organic nitrates." While it has been known for over 100 years that ingestion of nitroglycerin resulted in dilation of veins and arteries, it was not until 20 years ago that a connection with NO was truly recognized (Ignarro, 1999). Nitroglycerin interacts with thiols such as cysteine and glutathione in the cell such that chemically unstable nitrosothiols are formed, upon which NO is released (Ignarro, 1999). The formation of NO is slow for nitroglycerin allowing for the effects of nitroglycerin to be long-lasting. Pharmacological administration of nitroglycerin along with many other organic nitrates has been clearly shown to result in the formation of NO. The formation of NO from inorganic NO_3^- and NO_2^- is important in considering the role diet might play in regulation of NO formation.

The role of orally administered sodium nitrite is well documented and is known to increase GC activity leading to vasodilatation (Classen et al., 1990). Inorganic NO_2^- is a weak activator on its own but similar to nitroglycerin it is slightly enhanced in the presence of thiols which decrease its chemical stability resulting in increased release of NO (Ignarro, 1999). Although a strong connection has not yet been established, some discussion has centered on the contribution of dietary NO_3^- to blood pressure. Hypertension appears to be lower in vegetarians and the major contribution of NO_3^- in the diet is from vegetables. Therefore the potential contribution of dietary NO_3^- to decreased blood pressure has been considered but not fully evaluated (Classen et al., 1990). However, research suggests "inorganic nitrates" may have little consequence on vasodilatation unless converted to NO_2^- (Classen et al., 1990).

Ingestion of NO_3^- and NO_2^- from water has also been studied in relation to hypertension but the studies are conflicting (Bruning-Fann and Kaneene, 1993b). With higher incidences of hypertension in communities with nitrate-free water than in communities with water levels averaging 45 ppm and reported increases in

hypertension in communities consuming water with nitrate when compared with communities consuming nitrate-free drinking water suggesting the need for further investigation on the effect of dietary nitrates and well water nitrates on hypertension (Bruning-Fann and Kaneene, 1993b).

The importance of NO in relaxation of smooth muscle also appears to contribute to the regulation of other physiological functions. Nitric oxide has been identified in exhaled air and is thought to contribute to the moderation of normal respiration through bronchial dilation (Ward et al., 1993). Nitric oxide mediated relaxation of smooth muscle is also of benefit to the gastrointestinal tract serving as a regulator of gut motility (Calignano et al., 1992). Interestingly NO may be extremely important in the development of the infant gut as well. Infants appear to show high production of NO shortly after birth (Honold et al., 2000). In addition, NO_3^- and NO_2^- present in human breast milk may also result in the formation of NO. As discussed earlier, NO_3^- and NO_2^- are present in breast milk at levels reflecting maternal plasma. At normal physiological levels NO_3^- and NO_2^- may be converted to NO and play a critical role in regulating the infant's developing mucosal blood flow and gastric motility. Nitric oxide may also play a role in the gut that is similar to adults through the development of bacteriostasis (Iizuka et al., 1999).

Nitric oxide has been shown to increase during pregnancy with a concomitant rise in cGMP and is important in the prevention of pre-eclampsia. Pre-eclampsia is a multi-system disorder seen in 4–5% of all pregnancies and is a leading cause of both maternal and neonatal death. Symptoms include increased blood pressure and hypertension. Long-term inhibition of NO synthesis results in manifestation of the symptoms and an NO deficiency has been linked to women exhibiting pregnancy-induced hypertension (Weiner et al., 1994).

6.3. Platelet Aggregation

Endothelial cell release of NO not only results in vasorelaxation but also is a potent inhibitor of platelet adhesion and aggregation (Radomski et al., 1993). Nitric oxide is an inhibitor of platelet aggregation by stimulation of platelet cGMP, which appears to interfere with binding of platelets to endothelial cells.

6.4. Neurotransmission

Increased cGMP synthesis due to the presence of NO may also be important in neuronal transmission and may be involved in regulation of memory, learning, and pain perception (Radomski, 1995). Although the function is not completely elucidated, it is clear that NOS if found in high concentrations in the myenteric plexus of nerves and that inhibitors of NO synthesis impair proper neurotransmission (Ignarro, 1996). Cyclic GMP may act as an intracellular messenger in the target cell and elicits a response such as increased synaptic connections (Ignarro, 1996). Nitric oxide may also act on nonneuronal cells in a vasodilatory function leading to increased blood flow.

6.5. Cytotoxic Effects

6.5.1. Food treatment

Nitrate and NO_2^- salts are commonly added to cured meats to give color and to increase product stability through anti-oxidant and cytotoxic mechanisms. The anti-oxidant and cytotoxic effects are in fact due to the interaction of iron with NO formed from the reduction of the added NO_3^- and NO_2^-. The first recognition of NO in the curing of meat was in 1908 when it was determined that the red pigment of cooked cured meats such as ham was due to the presence of a heat-denatured NO–myoglobin complex. The flavor of rancid meat is due to the oxidation of unsaturated fats and is catalyzed by iron-containing compounds such as heme. Addition of NO_2^- stabilizes heme compounds by formation of a NO–heme complex. In addition, NO inhibits oxidation through conversion of heme to a ferrous heme-NO radical, which can act as an anti-oxidant, as well as the binding of NO to free ionic iron to form such compounds as low molecular weight cysteine-iron-NO radicals which also have anti-oxidant properties (Cornforth, 1996).

6.5.2. Prevention of infection

The anti-microbial action of NO is varied. Nitric oxide can inhibit and form complexes with heme, iron–sulfur, and copper proteins thus impairing function as well as interfering with incorporation into key enzymes. Inhibition of clostridia appears to be due in part to interaction of NO with pyruvate-ferredoxin oxidoreductase, an iron–sulfur enzyme involved in regeneration of adenosine triphosphate (ATP) (Cornforth, 1996). Inactivation of enzymes containing no redox metals such as glyceraldehyde-3-phosphate dehydrogenase, part of the glycolytic pathway, can occur as well. Nitric oxide can also inhibit ribonucleotide reductase, a crucial enzyme in the formation of DNA through quenching of a tyrosine radical. Interference with DNA also occurs through NO by promoting deamination of N-terminal and other amino groups of proteins and through this, process can be mutagenic such as seen in *Salmonella typhimurium* and *Pseudomonas stutzeri* where mutations in the nucleotide sequence occur that prove lethal to the bacteria. The common practice of addition of ascorbate accelerates the formation of NO and decreases formation of nitrosamines thus aiding in the anti-bacterial effects NO_3^- and NO_2^- salts (Cornforth, 1996).

The body is capable of utilizing dietary NO_3^- and NO_2^- as a source of NO for use as an anti-microbial agent. Dietary NO_3^- mainly from foods high in NO_3^- such as green leafy vegetables is absorbed from the stomach and the small intestine into the plasma. Nitrate is then concentrated in saliva such that the more dietary NO_3^- consumed the more NO_3^- and NO_2^- found in the saliva (McKnight et al., 1999). Approximately 25% of NO_3^- ingested in the diet is re-secreted into the saliva. Salivary NO_3^- can be converted to NO_2^- by the action of lactoperoxidase, lysozymes, and lactoferrin which are involved in cleaning the oral mucosal cells. Conversion of NO_3^- to NO_2^- in the saliva can also occur due to the presence

of NO_3^- reducing bacteria on the tongue (van Maanen et al., 1996b; McKnight et al., 1999). Salivary $NO_2^-$is then swallowed, where in the acidic conditions of the stomach the NO_2^- can be protonated to form nitrous acid. Nitrous acid can then decompose into N oxides including NO which is believed to act in the stomach as an anti-microbial agent for swallowed bacteria (McKnight et al., 1999), who also showed that large concentrations of stomach NO formed after intake of oral NO_3^- (potassium nitrate). The importance of NO formation due to protonation under acidic conditions is supported by increased bacterial colonization in patients with achlorhydria, in which conversion of NO_2^- to NO would be impaired.

6.6. Immune System Effects

Another location of NO in relation to infection is macrophage from the immune system. (Ellis et al., 1998). The cytotoxic activity of macrophage is partially due to the endogenous production of NO from arginine which is catalyzed by cytokine inducible NOS. Cytotoxicity is due to at least a 100-fold increase in local NO concentrations in comparison to levels generated by other cells in the body such as endothelial cells. If macrophage is arginine-deficient or if inhibition of the enzyme is induced by administration of arginine analogs, there is a decrease in bactericidal effectiveness. Nitric oxide production relates to their cytotoxic effect and has been shown to act on bacteria, tumor cells, viruses, fungi, protozoans, and helminths (Moilanen and Vapaatalo, 1995). Although NO is the main oxide produced, synthesis of peroxynitrite ($ONOO^-$) occurs in macrophage due to the spontaneous reactions of NO with O_2^- and H_2O_2 also produced by the macrophage as represented in the following equation:

$$NO + O_2^- \rightarrow ONOO^-$$
$$2ONOO^- + H_2O_2 \rightarrow 2OH^- + NO_2 + 2O_2$$

This more reactive oxide leads to the formation of a highly reactive hydroxide radical OH and nitrogen dioxide (NO_2) which kill bacteria through oxidative damage.

6.7. Autoimmune Disease

Nitric oxide production is not always beneficial in its role in free radical production as it can be cytotoxic to the host cells as well. Nitric oxide can act as a cytotoxic molecule in autoimmune disease such as seen in diabetes. Increased NO synthesis from an autoimmune response results in decreased insulin secretion and damage to the islet cells. Similarly tissue damage during arthritis has been linked to toxic levels of NO due to an autoimmune response (Moilanen and Vapaatalo, 1995). Nitrate concentrations are higher in the sinovial fluid of patients with rheumatoid arthritis and levels are increased in urine as well suggesting increased endogenous formation of NO (Moilanen and Vapaatalo, 1995). The origin of NO in the joints is not completely known but stimulation of chondrocytes by IL-2 appears to increase

NO production by the chondrocytes (Moilanen and Vapaatalo, 1995). Nitric oxide plays more of a harmful effect in arthritis appearing to contribute to inflammation and destruction of tissue.

The destructive effect is supported by evidence showing alleviation of symptoms when inhibitors of NO synthesis are given (Moilanen and Vapaatalo, 1995). The role of NO in inflammation has not been fully elucidated with both pro-inflammatory and anti-inflammatory effects. However, dual function of mediators is common in the inflammatory response (Moilanen and Vapaatalo, 1995).

Although this review touches upon only a few established roles of NO, research continues to expand as the significance of NO in many other physiological functions becomes more apparent and more in-depth reviews of the literature are available (Moilanen and Vapaatalo, 1995; Radomski, 1995; McKnight et al., 1999).

7. CANCER

Many epidemiological studies have demonstrated a relationship between diet and cancer risk and it has been suggested that diet is associated with as much as 35% of all deaths from cancer in the United States (Doll and Peto, 1981; Howe et al., 1986; Ferguson, 1999). Nitrate itself is not considered to be carcinogenic, however, NO_3^- can be reduced under a variety of conditions to NO_2^-. Nitrite is a more reactive compound and can participate in the nitrosation of many substrates in the diet including secondary and tertiary amines and amides resulting in the formation of NOC.

7.1. The Role of *N*-Nitroso Compounds

In general, NOC include both nitrosamines and nitrosamides that can participate in DNA alkylation which can lead to tumor formation. Animal experiments have demonstrated NOC to be the most potent and broadly acting carcinogen known (Ferguson, 1999). *N*-Nitroso compounds in the hundreds have been tested as carcinogens and over 80% have been found to be carcinogenic in at least 40 animal models (Eichholzer and Gutzwiller, 1998).

Of the NOC that have been tested in the laboratory for carcinogenic activity, humans are exposed to only a small percentage and almost all are nitrosamines (Lijinsky, 1999). In animal models nitrosamines have been linked to bladder, esophagus, kidney, liver, lung, nasopharyngeal, and thyroid cancer (Mirvish, 1995). In general, nitrosamines are stable compounds and require metabolic activation to have any carcinogenic effect. The activated compounds are unstable and have a relatively short half-life. Therefore, it is thought that the sensitivity of the organ to the nitrosamine might be influenced by the nitrosamine activating systems within the specific organ capable of producing reactive alkylating compounds (Magee, 1989). Metabolic activation of nitrosamines is thought to occur due to the presence of a class of enzymes with overlapping substrate specificity known as cytochromes P-450; more specifically a subfamily of cytochromes P-450 involved in ethanol detoxification (Magee, 1989).

Humans are also exposed to nitrosamides, which are compounds that do not need to be activated and if directly ingested in animal models will cause tumors in the stomach and duodenum. Nitrosamides can also act distantly and are primarily linked in animal models to cancer of the lymphatic and nervous systems (Mirvish, 1995). The sensitivity of the brain and possibly some of the other target organs has been hypothesized to be due to a deficiency or lack of the enzyme alkyltransferase, which can facilitate the repair of alklylated DNA (Mirvish, 1995).

7.1.1. Dietary exposure

Dietary related exposure to NOC can occur through both exogenous and endogenous sources. Few "western style" foods contain detectable amounts of pre-formed NOC (Hotchkiss, 1989). Asian-style foods show a slightly higher content of NOC due to preparations involving smoking (Hotchkiss, 1989). A main source of pre-formed NOC in western diets that has been of concern is from NO_2^- containing foods such as cured meats, especially bacon (Hotchkiss, 1989). Foods exposed to N oxides such as beer are another substantial source of pre-formed NOC in the western diet (Ferguson, 1999). Malt-based beverages such as beers and scotch whiskey contain NOC due to reaction of amines in the barley with N oxides produced during drying of the malted barley in natural gas kilns (Mirvish, 1995). An average beer drinker is exposed to twice the amount of nitrosamines as an average bacon eater (Whitney et al., 1994). Concentrations of NOC have been reduced by decreasing the kiln temperature or indirect heating of the barley (Mirvish, 1995). Nitrate-containing foods may also contribute to exogenous NOC if contaminated with nitrate-reducing bacteria, resulting in the formation of NO_2^- which can then interact with amines and amides found in the food (McKnight et al., 1999).

In the past, fried bacon was found to contain up to 100 ppb of nitrosamines (mainly in the form of *N*-nitrosopyrrolidine and dimethylnitrosamine). After addition of NO_2^- it was lowered and the inhibitory effect of ascorbic acid on nitrosamine formation was discovered, levels have decreased significantly (17 ppb *N*-nitrosopyrrolidine, 9 ppb *N*-nitrosothiazolidine, 4 ppb dimethylnitrosamine, 0.7 ppb *N*-nitrosopiperidine) (Mirvish, 1995). Presently, the average intake of exogenously produced NOC from a western style diet has dropped significantly and has been estimated to be on average 0.5–1.0 μgu/day (Hotchkiss, 1989).

Nitrites and secondary amines are present in a variety of foods and may be able to contribute to the endogenous formation of NOC. *N*-Nitroso compound synthesis in the body is thought to take place through acid catalyzation and bacterial nitrosation in the stomach and by NO formation and involves the presence of NOC precursors (Mirvish, 1995). Nitrite, participating in acid catalyzed nitrosation in the stomach, can be supplied through food, water, and salivary re-secretion (Bruning-Fann and Kaneene, 1993a). It has been suggested that approximately 80% of NO_2^- found in the stomach is due to the reduction of ingested or endogenous NO_3^- re-secreted in saliva and 20% is due to the ingested NO_2^- from preserved meats and other foods as well as water (Mirvish, 1995). To participate in nitrosation, nitrite

must first be converted to nitrous acid (HNO_2) through acidification in the stomach (Mirvish, 1995). It is thought (HNO_2) can then be protonated to form $H_2NO_2^+$ which may interact with amines and amides from food to form NOC (Mirvish, 1995; McKnight et al., 1999).

$$NaNO_2 + H^+ \rightarrow HNO_2 + Na^+$$
$$2HNO_2 \Leftrightarrow N_2O_3 + H_2O$$
$$2N_2O_3 + O_2 \rightarrow 2N_2O_4$$
$$HNO_2 + H^+ \Leftrightarrow H_2NO_2^+$$
$$H_2NO_2 + RNHCOR \rightarrow RN(NO)COR \text{ (nitrosamide)} + H_2O + H^+$$

Or

$$N_2O_3 + R_2NH \rightarrow R_2N\text{-}NO \text{ (nitrosamine)} + HNO_2$$

Nitrosation can still occur in the stomach at higher pH such as seen in achlorhydria due to the increased presence of bacteria. Bacteria such as *Escherichia coli* are capable of reducing NO_2^- to NO and in the presence of oxygen can form N_2O_3 which can participate in the formation of nitrosamines (Mirvish, 1995). This reaction occurs at neutral pH.

$$2NO + O_2 \rightarrow 2NO_2$$
$$NO + NO_2 \Leftrightarrow N_2O_3$$
$$N_2O_3 + R_2NH \rightarrow R_2N.NO \text{ (nitrosamine)} + HNO_2$$

7.1.2. Inflammatory and immune responses

Conditions in the stomach and other organs may also contribute to NO and NOC formation. Ulcerative colitis, parasitic infection of the bladder and liver, hepatitis B, and colonization of the stomach by *Helicobacter pylori* are all examples of conditions which may result in localized formation of NO during the inflammatory response due to immune stimulation of macrophage and neutrophils utilizing arginine for the synthesis of NO (Leaf et al., 1989). It has been hypothesized that NO can react with oxygen in other locations in the body in a similar reaction to that seen in the stomach during achlorhydria. This results in formation of N_2O_3 which is capable of interacting with endogenous amines to form NOC (Mirvish, 1995) (Leaf et al., 1989).

In animal experiments NOC have been shown to be the most broadly acting and potent group of carcinogens known (Eichholzer and Gutzwiller, 1998; Lijinsky, 1999; McKnight et al., 1999). The NOC have been shown to induce cancer in over 40 animal species including monkeys (Magee, 1989; Dayal and Ertel, 1997). In addition, no animal species has been found to be resistant to NOC-induced carcinogenesis

(Magee, 1989). Humans are exposed to a variety of exogenous and endogenous NOC as well and exposure to NOC in humans has been linked to several cancers including increased risk of stomach, esophageal, nasopharyngeal, bladder, and colon cancer (Bartsch et al., 1990). Research in humans however is fairly limited to epidemiological case-control studies and the compiled data remains inconclusive (Eichholzer and Gutzwiller, 1998). Although the epidemiological data may be unclear, the substantial evidence from animal studies suggests serious consideration be taken as to the potential risk of these compounds in humans.

7.2. Stomach and Gastric Cancer

Incidences of stomach cancer vary geographically with the highest occurrence seen in Japan and China and the lowest in North America and Greece (Forman, 1989). Stomach cancer is the second cause of death from cancer worldwide and in the United States ranks seventh (Yamaguchi and Abe, 1999). If dietary NO_3^- is to be considered a risk for stomach cancer through endogenous formation of NOC then vegetarians should be at a greater risk in that they can consume three times the concentration of NO_3^- when compared to omnivores (McKnight et al., 1999). In fact diets high in vegetables and thus also high in NO_3^- have been shown to be protective against some types of cancer including gastric cancer (McKnight et al., 1999). This is thought to result from the inhibition of nitrosation by compounds such as ascorbic acid, alpha-tocopheral, and polyphenols that are present in vegetables (Mirvish, 1995; Yamaguchi and Abe, 1999). Polyphenols are also present in green and black tea both of which have been shown to inhibit nitrosation (Sobala et al., 1989; Mirvish, 1995).

Most human studies on gastric cancer risk and dietary NO_2^- intake showed no significant relationship. However, a potential association was found in the presence of high amounts of the free amino acid methionine and high amounts of NO_2^- suggesting the occurrence of endogenous NOC formation (Eichholzer and Gutzwiller, 1998). Substantial endogenous nitrosamine formation in the stomach from NO_2^- when consuming an average diet might be inhibited by availability of free amino acids because they are not generally present in the stomach in high concentrations. Pepsin, an enzyme in the stomach involved in proteolytic digestion, cannot cut all peptide bonds into free amino acids as it only cleaves peptide bonds involving aromatic amino acids such as tyrosine, tryptophan, or phenylalanine. Therefore after gastric peptide digestion less than 5% of amino acids found in the upper small intestine are in a free state (Nixon and Mawer, 1970). In fact even at the intestinal level, absorption of amino acids by the enterocytes occurs mainly through absorption of dipeptides.

Another consideration in the endogenous formation of NOC is related to pathological conditions of the stomach. Patients with gastric achlorhydria have been shown to be at higher risk for gastric cancer (Yamaguchi and Abe, 1999). Chronic atrophic gastritis results in decreased gastric juice and achlorhydria (Yamaguchi and Abe, 1999). It is thought that achlorhydria leads to increased stomach pH and more optimal conditions for colonization by bacteria. These bacteria might participate in NO_3^- reduction or induce NO production through the immune system leading to formation

of NOC. This hypothesis is supported by the strong link between *Helicobacter pylori* infection and increased gastric cancer (Eichholzer and Gutzwiller, 1998). *Helicobacter pylori* is a gram negative bacteria most commonly located in the stomach and detection has been associated with the presence of atrophic gastritis (Yamaguchi and Abe, 1999). While this is considered to be a major hypothesis linking increased cancer rates seen in patients with achlorhydria and in patients with *H. pylori* infection, research still needs to be performed to validate the model.

Although NOC formation from dietary NO_3^- and NO_2^- might not show a strong link to gastric cancer, the contribution of pre-formed NOC must be considered. Induction of gastric tumors by pre-formed NOC has been well established in animal models (Eichholzer and Gutzwiller, 1998). It is also clear that pre-formed NOC can occur due to the interaction of NO_2^- with secondary amines in food and that certain foods and methods for food preservation increase the formation of free amines and amides. For example, high-protein foods that are dried and stored for an extended period, such as fish, show increased concentration of amines and amides (Hotchkiss 1989). In addition extended storage of uncured meat prior to processing has been demonstrated to increase NOC content after the meat had been cured and fried (Hotchkiss, 1989). Human studies provide support for increased gastric cancer risk with increased consumption of cured and smoked meats and fish and salted fish; foods potentially containing substantial amounts of NOC (Yamaguchi and Abe, 1999). A review by Eichholzer and Gutzwiller (1998) of human epidemiological studies on the risk of gastric cancer from ingestion of pre-formed NOC suggest that although the epidemiological evidence does lean toward the contribution of preformed NOC in foods to gastric cancer, the evidence may not be strong enough yet to fully support the hypothesis.

7.3. Esophageal and Nasopharyngeal Cancer

The strongest connection between NOC and human cancer can be seen in its potential role in esophageal and nasopharyngeal cancer (Eichholzer and Gutzwiller, 1998). Esophageal cancer incidences are found to be highest along a geographical area that has been termed the "Asian esophageal cancer belt" and extends from Russia and Turkey to Eastern China (Eichholzer and Gutzwiller, 1998). Many possible reasons have been suggested for the prevalence of esophageal cancer and one connection may be due to nutrient deficiencies such as low consumption of ascorbic acid combined with the consumption of nitrosamines from mold-infected cereals eaten in some of the areas along the Asian esophageal cancer belt (Mirvish, 1995). Nitrosamine formation may occur in corn due to nitrosation of methylalkylamines present in the mold *Fusarium moniliforme* (Mirvish, 1995). Other types of food with suspected NOC content are pickled foods and beer (Hothckiss, 1989; Mirvish, 1995; Eichholzer and Gutzwiller, 1998).

Nasopharyngeal cancer is rare in most countries but is high in areas of China, Greenland, and Tunisia (Mirvish, 1995). Dietary causes are thought to be associated with consumption of salted dried products such as fish, which contain volatile nitrosamines

such as dimethylnitrosamine, clearly shown to induce nasopharyngeal cancer in rats (Mirvish, 1995; Eichholzer and Gutzwiller, 1998). The salted and dried fish also contains amines which might participate in endogenous nitrosation (Magee, 1989).

Studies have also suggested a link between increased consumption of foods containing NOC and childhood cancers; specifically brain tumors. Nitrosoureas which may be produced in bacon and salted dried fish have been shown to cause brain tumors and leukemia in rats (Mirvish, 1995). Consumption of foods containing pre-formed NOC such as hot dogs by pregnant mothers or by the child have been studied as an increased risk factor for childhood brain tumors (Kuijten et al., 1990; Sarasua and Savitz, 1994; Mirvish, 1995). While the potential contribution of NOC to childhood cancers must be carefully examined due to the evidence from animal studies, the compiled literature in humans does not demonstrate a definitive connection (Eichholzer and Gutzwiller, 1998). In summary, even though solid confirmation of a connection between cancer and NOC may not be apparent from human epidemiological studies, animal models offer strong support for cancer risk from NOC and therefore the link between human NOC exposure and cancer should continue to be investigated.

8. SUMMARY AND CONCLUSIONS

The need by humans to produce and consume food and other agricultural products is increasing with a growing population leading to increased human demands for N to produce food and other products. Therefore it is important to consider the impact of human intake, metabolism, and excretion of N-containing compounds will have on both humans and the environment. Protein was shown to be an essential source of N for humans. Other N-containing compounds were shown to impact human health as well, in both potentially deleterious and beneficial ways. Therefore it is important to continue to analyze and recognize the impact that N-containing compounds have on humans and the impact that will occur on the environment as the human population continues to grow.

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