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Review article

Nitrates in the human diet – good or bad?

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Abstract — Although the nitrate and nitrite have been used for centuries, it has only recently been discovered that nitrate is manufactured in mammals by the oxidation of nitric oxide and that the nitrate formed also has the potential for disinfecting the food we eat. The mechanisms by which nitric oxide and other nitrogen oxides provide selective toxicity towards pathogens is not yet completely understood, and it is likely that the mechanisms will be different with different organisms. Whereas it is clear that acidified nitrite is produced on mucosal surfaces, and that this combination is effective in killing a variety of human gut and skin pathogens, there is no definite evidence as yet that this mechanism is truly protective in humans exposed to a contaminated environment. Further understanding of the complex chemistry of nitrogen oxides may also help develop new antimicrobial therapies based on augmenting what seems to be a simple and effective host defence system.

nitrates / nitrites / human diet / health

Résumé — Les nitrates dans l'alimentation humaine : une bonne ou une mauvaise chose ? Bien que les nitrates soient connus depuis des siècles, c'est seulement depuis peu qu'il a été découvert que les nitrates sont synthétisés chez les mammifères à partir du monoxyde d'azote, et que les nitrates formés ont la capacité de tuer les bactéries présentes dans les aliments consommés. Les mécanismes par lesquels le monoxyde d'azote et d'autres oxydes d'azote manifestent une toxicité sélective vis-à-vis de bactéries pathogènes ne sont pas encore totalement élucidés. Il est probable que ces mécanismes dépendent des organismes considérés. Alors qu'il est clair que les nitrites acidifiés sont produits à la surface des muqueuses, et que cela permet de tuer de nombreux agents pathogènes de la peau et de l'intestin de l'Homme, une incertitude demeure quant aux mécanismes de protection réels de l'Homme exposé à un environnement contaminé. Une meilleure compréhension de la chimie complexe des oxydes d'azote permettra d'aider au développement de nouvelles thérapies antibactériennes fondées sur l'accroissement de ce qui semble être un système de défense de l'hôte simple et efficace.

nitrates / nitrites / alimentation humaine / santé humaine

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1. INTRODUCTION

There has been considerable controversy concerning the possible harmful effects of nitrate in our diet and in the environment. Nitrate has, in general, been considered harmful, although the evidence for this has become less convincing with time. Recently, since the discovery of nitric oxide as an important biological molecule which regulates many bodily functions and provides host defence against numerous micro-organisms, the image of nitrate has, to some extent been rehabilitated. This is because of the discovery of a novel biochemical pathway which can lead to the formation of large, possibly protective, amounts of nitric oxide in mammals from the sequential reduction of inorganic nitrate. The purpose of this paper is to consider the possible harmful and beneficial properties of inorganic nitrates which are an essential component of biological systems and which are encountered in large amounts in our diet every day.

Inorganic nitrates (NO_3^-) have been added as a food preservative, especially pig meat, to make ham and bacon for centuries. As well as its beneficial effect to limit the growth of serious pathogens such as *Clostridium botulinum* [30], nitrate, or more specifically its reduction product nitrite (NO_2^-) has also the dubious benefit of rendering muscle tissue a bright pink colour, by the formation of nitrosomyoglobin. It has subsequently become clear that nitrate is generally non-reactive with organic molecules and has to be chemically or enzymatically reduced to nitrite to be effective as an antimicrobial agent [5]. Nitrate is also found in large quantities in green, leafy vegetables such as lettuce and spinach, particularly when grown under low light conditions (see below and Tab. I).

Despite its long use, there have been considerable concerns about the use of nitrate and nitrite as a preservative in food and about the content of these ions in vegetables and drinking water. There are two reasons for this.

Table I. Contribution (%) of various foodstuffs to dietary intake of nitrate and nitrite (after Committee on Nitrite and Alternative Curing Agents in Food [7]).

Food [30]	Nitrate	Nitrite
Cured meats	1.6	39
Fresh meats	0.8	7.7
Vegetables	87	16
Fruit / juices	6	1.3
Baked foods / cereals	1.6	34
Milk / milk products	0.2	1.3
Water	2.6	1.3

Firstly, the theoretical possibility of forming carcinogenic N-nitroso compounds in food to which these ions are added and in humans in vivo, due to nitrosation of secondary amines also present in the diet or ingested as drug therapy [37]. Nitrosation of amines and other chemicals will occur rapidly under acidic conditions (such as in the human stomach) when nitrite is present, due to the formation of nitrous acid [42]. It will also occur in the stomach under more neutral pH when there are bacteria present. The mechanism of this presumably enzymatic nitrosation is not understood.

Nitrous acid is an effective nitrosating agent due to its ability to donate an NO^+ group. These nitrosation reactions are catalysed by halide ions (such as chloride) and thiocyanate due to the formation of nitrosohalide (such as NOCl) and nitrosothiocyanate (NOSCN) respectively, and these intermediates will more effectively donate NO^+ groups. Both chloride and thiocyanate ions are present in gastric juice in high concentrations, the latter is derived from the diet (particularly brassicas such as cabbage) and is concentrated in saliva. Nitrate in the diet is similarly concentrated in saliva following absorption and reduced to nitrite in the mouth. It therefore seems that the stomach is an ideal reaction chamber for the nitrosation of susceptible swallowed chemicals.

This scheme of human metabolism of nitrate and nitrite seems to be counterproductive for human health and has prompted a reconsideration of exactly how these ions are handled following ingestion. We have formulated a scheme whereby the formation of nitrogen oxides in the stomach and on the skin surface, derived from dietary nitrate and nitrite, may protect against bacterial, and possibly viral, pathogens.

A second potential mechanism for nitrite toxicity is the formation of methaemoglobin due to oxidation of the iron in haemoglobin. This is due to the reaction of nitrite with oxyhaemoglobin, where it is oxidised to form nitrate. It should also be noted that nitric oxide also reacts quickly with oxyhaemoglobin to produce methaemoglobin and nitrate. This may be more important than nitrite in causing this problem in infants fed on well water.

Methaemoglobinaemia is generally only a problem in young infants, but has been the main reason for statutory limitations on the concentrations of nitrate in drinking water. For nitrate to cause methaemoglobinaemia it has to be reduced to nitrite in food, water or in the body. The mechanisms by which this may occur will be considered in the next section.

2. NITRATE METABOLISM BY BACTERIA AND PLANTS

In nature, nitrogen is continually cycled between nitrogen gas, and the fully oxidised nitrogen molecule – nitrate and the fully reduced nitrogen molecule – ammonia. Plants and bacteria have somewhat different uses for nitrogen molecules. In general, plants need nitrogen as ammonia as a precursor to protein synthesis. They have the appropriate enzymes to reduce nitrate through to ammonia, a process requiring energy provided by photosynthesis. This occurs in at least two distinct steps catalysed by separate enzymes – nitrate reduction to nitrite (nitrate reductase) and then nitrite reduction to ammonia (nitrite reductase). Nitrate is present in the soil due to organic decomposition or the fixing of atmospheric nitrogen by nitrogen-fixing bacteria which may be associated with roots of certain leguminous plants. Nitrate is commonly stored in the leaves of vegetables such as lettuce and spinach, and accumulates to very high concentrations under low light conditions. This is presumably because of a reduction in the flux of nitrate through to ammonia when insufficient energy is available from photosynthesis. As a result, especially in cool climates, green vegetables contribute to our nitrate intake more than any other single source [6].

Although some micro-organisms will also reduce nitrate to ammonia for protein synthesis, many also use nitrate reduction for the purpose of anaerobic respiration. The enterobacteriacae such as *E. coli* can switch from using oxygen to burn available fuel to using nitrate as an electron acceptor (or oxidant) which is then converted to nitrite. The facultative anaerobes which inhabit the human mouth allow little further reduction of nitrite, presumably because they lack the nitrite reductase enzyme.

3. NITRATE AND NITRITE METABOLISM IN MAN

It was first shown in 1916 that humans excrete more nitrate than they consume [27]. This was confirmed in careful studies by Tannenbaum's group in the 1970's and 1980's [16, 17] and is now known to be due, at least in part, to the formation of nitric oxide by mammalian cells from the amino acid L-arginine.

3.1. Nitric oxide synthesis via NO synthase

Following the demonstration in 1980 by Furchgott and Zawadski [15] that an intact

vascular endothelium was necessary for blood vessels to relax when exposed to the neurotransmitter acetylcholine, it was clear that endothelial cells were able to synthesise a short-lived vasodilator substance. It took seven years to identify this substance which turned out to be the simple molecule nitric oxide (NO) [29]. It is now known that there are three distinct nitric oxide synthase enzymes.

Two of these enzymes, the endothelial isoform and the neuronal isoform, continually synthesise NO whereas the inducible isoform produces this molecule from arginine only when the appropriate cell is exposed to bacterial cell wall products or pro-inflammatory cytokines such as interferon gamma and tumour necrosis factor alpha.

The function of the endothelial isoform is to provide continual vasodilatation. Inhibition of NO synthesis with arginine analogues such as methylarginine causes high blood pressure in animals and man, and intriguingly, it has recently been shown that nitric oxide synthesis is impaired in patients with hypertension [14]. Although many central (brain) neurones contain the neuronal form of NO synthase, the precise function of NO in central nervous function is yet unclear. It is likely that these two isoforms contribute to the majority of NO synthesis which is rapidly converted to nitrate when this molecule encounters oxidised haemoglobin or superoxide.

Inducible nitric oxide synthase is easily demonstrated within a few hours in mouse macrophages exposed to bacterial lipopolysaccharide (LPS). Indeed if rodents are exposed to LPS or made septicaemic there is a large rise in plasma and urinary nitrate concentration. It has been much more difficult to demonstrate synthesis of NO in human cells exposed to LPS, although it is clear that overwhelming infection will increase nitrate synthesis in man [28]. Merely injecting killed bacteria, as in a vaccine, is not effective in enhancing NO synthesis [23]. The one infection which will cause a large increase in NO synthesis is gastroenteritis. We do not yet know that this is due to induction of NO synthase. The large rise in plasma nitrate in this condition may, however, be effective in preventing the recirculation of pathogens through the stomach by mechanisms discussed below.

4. NITRATE AND NITRITE IN THE DIET

4.1. Nitrate

Because of the potential for toxicity, there have been many studies in the last 20 years which have studied the effect of nitrate in the human diet and estimated the intake of this ion in different populations. For those people who eat a large amount of vegetables, then the main source of nitrate will be the green leaves of plants such as lettuce and spinach. Significant amounts are also found in root vegetables such as beetroot and carrots. For those who eat few vegetables then the nitrate concentration of tap water becomes an important factor determining nitrate intake. The concentration of nitrate in drinking water has been limited to 50 $mg \cdot l^{-1}$ in Europe, mainly because of concern about methaemoglobinaemia in infants (although this is now extremely rare). As it is now known that nitrite is the effective antimicrobial product of nitrate, it is less common that nitrate itself is used as a preservative for meat products such as sausage and ham, and these foods generally have little nitrate content, although nitrate is available from pharmacies to use as a meat preservative.

4.2. Nitrite

The average intake of nitrite is considerably less than that of nitrate. The main source is again vegetables which will convert nitrate to nitrite on storage and when contaminated by nitrate-reducing bacteria. Occasionally this can result in very high

concentrations of nitrite in vegetable juices in particular. Nitrite is also a component of preserved meats such as sausage and ham. However much of this nitrite is chemically altered following addition to food (Tab. I). The amount of nitrite ingested from food, however, is only a fraction of that swallowed as a result of nitrate reduction in the mouth. The average concentration of nitrite in saliva is around 200 μM. Given that we swallow approximately 500 ml saliva each hour then we must ingest approximately 2.4 mmol of nitrite each day from this mechanism. For this reason, limitations on the nitrite content of food seem inappropriate.

5. METABOLISM OF NITRATE IN HUMANS

It was found in the mid 1970s that this anion was handled in a peculiar way in the human body [35, 38]. When swallowed it is rapidly absorbed and at least 25% is concentrated in the salivary glands by an as yet uncharacterised mechanism, so that the nitrate concentration of saliva is at least 10 times that found in plasma. The nitrate is then rapidly reduced to nitrite (NO_2^-) in the mouth by mechanisms which will be discussed below. Saliva containing large amounts of nitrite will be acidified in the normal stomach to produce nitrous acid which could potentially nitrosate amines to form N-nitrosamines, which experimentally are powerful carcinogens [8]. From this theoretical understanding of nitrate metabolism a number of studies have been performed which looked at the relationship between nitrate intake and cancer (particularly gastric cancer) in humans. In general it was found that there was either no relationship or an inverse relationship, so that those individuals who had a high nitrate intake had a lower rate of cancer [1, 13, 20, 39]. Similarly, in animal studies, it has been generally impossible to demonstrate an increased risk of cancer (or any other adverse effect) when nitrate intake is increased [7].

It is now thought that endogenous nitrate synthesis derives from constitutive NO synthetase (NOS) enzymes acting on L-arginine [18]. The NO formed is rapidly oxidised to nitrate when it encounters superoxide or oxidised haemoglobin. It is still not clear whether all endogenous nitrate synthesis derives from this route as, following prolonged infusion of ^{15}N-labelled arginine, the enrichment of urinary nitrate with this heavy isotope is only about one half of the steady state of ^{15}N arginine enrichment [21]. This may mean that nitrate also derives from another source, or that the intracellular enrichment of labelled arginine is less than that in the plasma due to transamination reactions. This means that even on a nitrate-free diet, there are considerable concentrations of nitrate in plasma (around 30 μM) and in the urine (around 800 μmol per 24 h). Although it is not protein bound, nitrate has a long half-life of 5–8 hours [40], which seems to be because it is about 80% reabsorbed from the renal tubule by an active transport mechanism [19].

This peculiar metabolism of nitrate – renal salvage, salivary concentration and conversion to nitrite in the mouth made us consider that this may be a purposeful mechanism to provide oxides of nitrogen in the mouth and stomach to provide host defence against swallowed pathogens [3]. The first studies we performed were to investigate the mechanism of nitrate reduction to nitrite in the mouth.

5.1. Oral nitrate reduction

Although Tannenbaum et al. [38] had considered that salivary bacteria may be reducing nitrate to nitrite, Sasaki and Matano [31] showed that in humans this activity is present almost entirely on the surface of the tongue. They considered that the nitrate reductase enzyme was most likely to be a mammalian nitrate reductase. Using a rat tongue preparation, we also found that the dorsal surface of the tongue in this animal had very high nitrate reductase activity,

which was confined to the posterior two-thirds [11]. Microscopic analysis of the tongue surface revealed a dense population of gram negative and gram-positive bacteria, 80% of which, in vitro, showed marked nitrate reducing activity.

Our suspicion that the nitrate reduction was being accomplished by bacteria was strengthened by the observation that the tongues of rats bred in a germ-free environment, which had no colonisation of bacteria, demonstrated no nitrate reducing activity on the tongue. Furthermore, treatment of healthy volunteers with the broad spectrum antibiotic amoxycillin results in reduced salivary nitrite concentrations [10]. Although we have not been able to characterise the organisms in normal human tongues (this would require a deep biopsy as the majority of the bacteria are at the bottom of the papillary clefts of the tongue surface), the most commonly found nitrite-producing organisms in the rat were *Staphylococcus sciuri*, followed by *Staphylococcus intermedius*, *Pasteurella* spp. and finally *Streptococcus* spp. [21]. Both morphometric quantification of bacteria on tongue sections and enumeration of culturable bacteria showed an increase in the density of bacteria towards the posterior tongue.

We now believe that these organisms are true symbionts, and that the mammalian host actively encourages the growth of nitrite-forming organisms on the surface of the tongue. The bacteria are facultative anaerobes which, under hypoxic conditions, use nitrate instead of oxygen as an electron acceptor for oxidation of carbon compounds to derive energy. For the bacteria, nitrite is an undesirable waste product of this process, but is, we believe, used by the mammalian host for its antimicrobial potential elsewhere.

5.2. Acidification of nitrite – production of NO in the mouth and stomach

Nitrite formed on the tongue surface can be acidified in two ways. It can be swallowed into the acidic stomach, or it may encounter the acid environment around the teeth provided by organisms such as *Lactobacillus* or *Streptococcus mutans* which are thought to be important in caries production.

Acidification of nitrite produces nitrous acid (HNO_2) which has an acid dissociation constant of 3.2, so that in the normal fasting stomach (pH 1–2) complete conversion will occur. Nitrous acid is unstable and will spontaneously decompose to NO and nitrogen dioxide (NO_2). Under reducing conditions more NO will be formed than NO_2. Lundberg et al. [22] were the first to show that there was a very high concentration of NO in gas expelled from the stomach in healthy volunteers, which increased when nitrate intake was increased and reduced when stomach acidification was impaired with the proton pump inhibitor omeprazole. We have conducted further studies on the amount of NO produced following ingestion of inorganic nitrate, measured more directly using nasogastric intubation of healthy human volunteers. Following 1 mmol of inorganic nitrate, the amount of nitrate found in a large helping of lettuce, there follows a pronounced increase in stomach headspace gas NO which peaks at about 1 hour and continues to be elevated above the control for at least 6 hours [26]. The concentration of NO measured in the headspace gas of the stomach in these experiments would be lethal after about 20 minutes if breathed continuously.

The concentration of NO in the stomach is much higher than would be expected from the concentration of nitrite in saliva and the measured pH in the gastric lumen. In vitro studies suggest that these concentrations of nitrite and acid would generate about one tenth of the NO that is actually measured (McKnight, Smith and Benjamin, unpublished data). It is likely that a reducing substance such as ascorbic acid [32–34], which is actively secreted into the stomach, or reduced thiols (which are in high

concentrations in the gastric mucosa) are responsible for the enhanced NO production. We were surprised to find that NO is also generated in the oral cavity from salivary nitrite [11] as saliva is generally neutral or slightly alkaline.

The most likely mechanism for this production is acidification at the gingival margins as noted above. It will be important to determine if this is the case as the nitric oxide formed in this way may be able to inhibit the growth of organisms which generate acid. Such a mechanism for local NO synthesis from nitrite may in part explain the importance of saliva in protection from caries. As in the stomach, acidification of saliva results in larger amounts of nitric oxide production than would be expected from the concentration of nitrite present.

5.3. Nitric oxide synthesis from the skin

Generation of nitric oxide from normal human skin can also readily be detected using a simple apparatus such as a glass jar sealed around the hand, with nitric oxide-free gas passed through it to a chemiluminescence detector [41]. As nitric oxide has the ability to diffuse readily across membranes, we first considered it most likely that we were measuring nitric oxide which had escaped from vascular endothelium to the skin surface, manufactured by constitutive NOS. However, when the NOS antagonist monomethyl arginine was infused into the brachial artery of healthy volunteers in amounts sufficient to maximally reduce forearm blood flow, we found that the release of NO from the hand was not affected. Furthermore, application of inorganic nitrite substantially elevated skin NO synthesis. This coupled with the observations that NO release was enhanced by acidity, and reduced by antibiotic therapy makes it likely that again NO is being formed by nitrite reduction. Normal human sweat contains nitrite at a concentration of about 5 μM, and this concentration is precisely in line with that which we would predict would be necessary to generate the amount of nitric oxide release we observed from the skin. The source of nitrite is not clear, but is likely to be from bacterial reduction of sweat nitrate by skin commensal organisms which are known to elaborate the nitrate reductase enzyme.

This observation has led us to the hypothesis that skin nitric oxide synthesis may also be designed as a host defence mechanism to protect against pathogenic skin infections, especially against fungi. The release of nitric oxide is inevitably increased following licking of the skin (due to the large amount of nitrite in saliva), which may explain why animals and humans have an instinctive urge to lick their wounds [4].

6. IMPORTANCE OF NITROGEN OXIDES IN HOST DEFENCE

Much of the evidence for the importance of the arginine-nitric oxide system in host defence comes from the observation that inhibition of nitric oxide synthesis using arginine analogues impairs the ability of inflammatory cells (such as macrophages) or whole animals to kill invading pathogens [9]. This is particularly the case for intracellular organisms such as *Leishmania* major and *M. tuberculosis* [24, 25, 36], where there is particular evidence that the ability of the host to synthesise nitric oxide may be important in containing latent infections. The ability to synthesise adequate amounts of nitric oxide may also be important in reducing the severity of falciparum malaria infections in humans [2].

6.1. Mechanisms of nitric oxide-mediated microbial killing

This subject has been extensively reviewed recently by Fang [12]. It is clear that many organisms are not killed by nitric oxide alone, but require the synthesis of

other, more reactive nitrogen oxide species. The reaction of nitric oxide with superoxide anion (which is also produced by activated inflammatory cells) to produce peroxynitrite has received most attention [12]:

$$NO + O_2^- = ONOO^-.$$

This reaction is very rapid, indeed more rapid than the reaction of superoxide with the enzyme superoxide dismutase. Peroxynitrite is a very reactive species, which can easily be protonated to form peroxynitrous acid (ONOOH) which may then cleave to produce nitrogen dioxide and hydroxyl.

6.2. Antimicrobial activity of acidified nitrite

Following the observation that nitrite is manufactured in the mouth and then acidified in the stomach, we went on to determine the susceptibility of common food pathogens to acidified nitrite solutions. We found that the susceptibility varied as follows: *Y. enterocolitica* > *S. enteriditis* > *S. typhymurium* = *Shig. sonnei* ($P < 0.05$). *E. coli* 0157 and *Shig. sonnei* are most resistant to acid; they survive exposure at pH 2.1 for 30 minutes which kills the other microorganisms. However, *E. coli* 0157 shows inhibition of growth up to pH 4.2 when the other organisms apart from *Y. enterocolitica*, manage to maintain growth unless nitrite is present in the solution. It seems that *E. coli* 0157 manages to survive a relatively acid environment by slowing down growth activity. Its ability to survive this way is undone by the addition of nitrite to the medium.

Helicobacter pylori clearly must be able to tolerate the nitrosative stress found in the stomach, and indeed, this organism is more resistant than some other organisms to the combination of nitrite and acid [24]. The reason for this is not evident. It may be that it can withstand the effect of nitrogen oxides as it is protected against acid stress by the generation of ammonia from urea via urease enzyme. Alternatively it may have developed specific biochemical mechanisms for protection. If this is the case, such mechanisms would be an attractive target for eradication of this important pathogen.

7. CONCLUSIONS

Although nitrate and nitrite have been used for centuries, it has only recently been discovered that nitrate is manufactured in mammals by the oxidation of nitric oxide and that the nitrate formed also has the potential for disinfecting the food we eat.

We do not yet completely understand the mechanisms by which nitric oxide and other nitrogen oxides provide selective toxicity towards pathogens, and it is likely that the mechanisms will be different with different organisms. Whereas it is clear that acidified nitrite is produced on mucosal surfaces, and that this combination is effective in killing a variety of human gut and skin pathogens, we have no definite evidence as yet that this mechanism is truly protective in humans exposed to a contaminated environment.

Further understanding of the complex chemistry of nitrogen oxides may also help develop new antimicrobial therapies based on augmenting what seems to be a simple and effective host defence system.

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