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Invited Review

The Mechanisms for Nitration and Nitrotyrosine Formation *in vitro* and *in vivo*: Impact of Diet

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The detection of 3-nitro-L-tyrosine residues associated with many disease states, including gastric cancer, has implicated a role for peroxynitrite *in vivo*, and thus endogenously produced nitric oxide and superoxide. Additionally, dietary nitrate has been suggested to be involved in the pathogenesis of gastric cancer through a mechanism involving reduction to nitrite and subsequent formation of potentially mutagenic nitroso-compounds. Studies have now demonstrated that a multitude of reactive nitrogen species other than peroxynitrite are capable of producing nitrotyrosine. Thus, we have reviewed the evidence that dietary nitrate, amongst other reactive nitrogen species, may contribute to the body burden of nitrotyrosine.

Keywords: Nitrotyrosine, nitrate, nitrite, peroxynitrite, nitric oxide, nitration, reactive nitrogen species

Abbreviations: Nitrotyrosine, 3-nitro-L-tyrosine; RNS, reactive nitrogen species; BSA, bovine serum albumin; LDL, low density lipoprotein; MCP-1, monocyte chemoattractant protein; EGC, epigallocatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; HNO_2, nitrous acid; Cl-NO_2, nitryl chloride; SIN-1, 3-morpholino-syndominine; $NO_2^{\bullet}$, nitrogen dioxide; NO-, Angeli's salt; NO_2BF_4, nitryl salt; iNOS, inducible nitric oxide synthase; NFL, low molecular weight neurofilament; MnSOD, managanese superoxide dismutase; SERCA, sarcoplasmic reticulum calcium ATPase; ALS, amyotrophic lateral sclerosis; SP-A, surfactant protein A; i.v., intravenous; NHPA, 3-nitro-4-hydroxyphenylacetic acid; NHPL, 3-nitro-4-hydroxyphenyllactic acid; NO_2^-, nitrite; NO_3^-, nitrate; N_2O_3, dinitrogen trioxide; $NO^{\bullet}$, nitric oxide; HOCl, hypochlorous acid; EDRF, endothelium derived relaxing factor; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNos, neuronal nitric oxide synthase; HbO_2, oxyhaemoglobin; Hb^{3+}, methaemoglobin; NH_3, ammonia; $O_2^{\bullet-}$, superoxide; XOD, xanthine oxidase; Mϕ, macrophage; N, nitrogen; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; HRPT, hypoxanthine-guanine phosphoribosyl transferase; DEN, diethylnitrosamine; DEA, diethylamine

INTRODUCTION

The presence of 3-nitro-L-tyrosine (Figure 1) has often been considered to be a "fingerprint" of peroxynitrite formation *in vivo*. However, it

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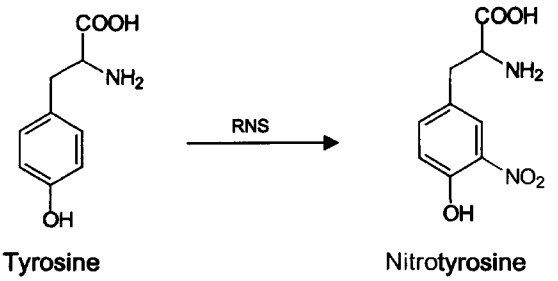

FIGURE 1 The structures of tyrosine and its nitrated product, 3-nitro-L-tyrosine.

has been demonstrated that, at physiological pH, a multitude of reactive nitrogen species (RNS) that may occur *in vivo* can nitrate both free and protein-bound tyrosine on the *ortho*-position *in vitro* (Table I). In addition, nitration of tyrosine residues by tetranitromethane has been used to elucidate the role of specific tyrosine residues in protein function.[24,25] Inhibition nitrotyrosine formation by RNS has been demonstrated *in vitro* by many compounds including dietary phenolics, such as the catechine polyphenols[3] and to a lesser extent hydroxycinnamates[2] (Table I). *In vivo*, nitrotyrosine formation has been prevented in humans by supplementation with ascorbic acid (1 g, twice daily)[26] and in a guinea pig model of ileitis by genistein.[27]

The formation of nitrotyrosine has been implicated in many inflammatory conditions, neurodegenerative diseases and cancer due to elevated levels observed both in humans (Table II) and in animal models of gut inflammation[27,30] and skin cancer[60] amongst others. However, there are some conflicting studies in which nitrotyrosine levels do not appear to change in disease.[61,62] The nitrotyrosine detected *in vivo* appeared to be localised in a variety of cells: epithelial cells,[26,31,33,57] polymorphonuclear leukocytes,[26] macrophages,[29–31,50] smooth muscle cells,[29,31] extracellular matrix,[26,57] neurons[40,42,48,49] and microglia.[42,51] Within cells it is often located close to inducible nitric oxide synthase (iNOS).[29,30,51,54] Nitric oxide itself is unable to nitrate tyrosine,[6,63–65] thus indicating the involvement of higher oxides of nitrogen.

Several purified proteins (Table I) have been nitrated *in vitro*, and some nitrated proteins have been identified in cell studies. These include prostacyclin synthase from rat mesangial cells treated with peroxynitrite or SIN-1,[66] the focal adhesion protein $p130^{cas}$ in human neuroblastomas SH-SY5Y cells treated with SIN-1[67] and multiple nitrated proteins in heart homogenates treated with peroxynitrite, or a mixture of myeloperoxidase, nitrite and hydrogen peroxide.[68] At present, however, nitrotyrosine residues have only been isolated from human serum albumin,[7] human low density lipoprotein,[7,9] low molecular weight neurofilament (NFL),[62] manganese superoxide dismutase (MnSOD),[69] rabbit β-VLDL apoproteins[70] and murine sarcoplasmic reticulum calcium-ATPase 2a (SERCA2a).[65]

Nitration of tyrosine residues in proteins induces the change of tyrosine into a negatively charged hydrophilic nitrotyrosine moiety that may alter enzyme activity. This could have deleterious effects due to mitochondrial respiratory dysfunction,[71] impaired lung function following inactivation of surfactant protein A (SP-A),[17] disrupted microtubule organisation that leads to altered cell morphology and epithelial-barrier dysfunction,[72] reduced antioxidant defence due to diminished activity of superoxide dismutase,[12,64,13] impaired cellular function due to reduced GTP binding,[15] inactivation of tyrosine kinases resulting in decreased phosphorylation[73] and thus affecting signalling cascades and inhibited neurofilament assembly weakens cell structure[74] and may reduce motor neuron survival. However, nitration of tyrosine may play a protective role by attenuating the thrombotic properties of tissue factor,[18] elimination of foreign bodies engulfed by phagocytes,[75] thymocyte negative-selection via apoptosis[76] and targeting proteins for degradation and elimination.[73] The majority of proteins appear to be unable to incorporate free nitrotyrosine upon their assembly,[72,77] the exception being α-tubulin that subsequently cannot function correctly.[72]

TABLE I Reactive nitrogen species involved in tyrosine nitration *in vitro*

Nitrating species	Substrate	Inhibitors
Peroxynitrite ($OONO^-$)	Tyrosine[1–5]	Ascorbyl palmitate,[1] ascorbic acid,[5] caffeic acid,[2] catechin,[3] chlorogenic acid,[2] *m*-coumaric acid,[2] *o*-coumaric acid,[2] *p*-coumaric acid,[2] epicatechin,[3] EGC,[3] ECG,[3] ferulic acid,[2] gallic acid,[3] EGCG,[3] glutathione,[5] α-tocopherol,[3] trolox,[2,3] uric acid[5]
	Bovine serum albumin (BSA)[6–8]	Ascorbate,[6] uric acid,[6] sulfhydryls[6]
	Human serum albumin[7]	
	Low density lipoprotein (LDL)[7,9,10]	
	α_1-antiproteinase[7]	
	Angiotensin II[11]	
	Fibrinogen[7]	
	Manganese superoxide dismutase[12,13]	
	Monocyte chemoattractant Protein-1 (MCP-1)[14]	
	Pepsinogen[7]	
	Rac2[15]	
	Ribonucleotide reductase[16]	
	Surfactant protein A[17]	
	Tissue factor[18]	
Nitrous acid (HNO_2)	Tyrosine[19,20]	Catechin,[20] epicatechin,[20] EGC,[20] ECG,[20] EGCG,[20] ferulic acid,[20] rutin[20]
	BSA[8,19]	
	Angiotensin II[11]	
Nitryl chloride ($Cl\text{-}NO_2$) produced from: nitrite + HOCl	BSA[8]	
	LDL[10]	
3-Morpholino-syndominine (SIN-1): produces both $O_2^{\bullet -}$ and $NO^{\bullet}$	Tyrosine[4]	
	Bovine serum albumin[8]	
	Surfactant protein A[21]	Urate[21]
SpermineNONOate produces $NO^{\bullet}$	Tyrosine[4]	
	BSA[8]	
	Surfactant protein A[21]	
Oxidation products of nitrite produced by: peroxide + H_2O_2 + $NaNO_2$	Tyrosine[22]	
	BSA[22]	
Nitrogen dioxide ($NO_2^{\bullet}$)	Tyrosine[1,23]	
	BSA[23]	
	Bovine eye lens α-crystallin[23]	
Angeli's salt (NO^-)	BSA[8]	
Nitryl salt (NO_2BF_4): produces NO_2^+	Tyrosine[22]	

Proteins capable of removing the nitro-group from nitrotyrosine in proteins, in the absence of protein degradation, have been isolated from canine prostate[78] and and there appears to b ted proteins from rat

TABLE II Evidence of *in vivo* formation of nitrotyrosine, nitrate and nitrite in human disease. A selection of the diseases and the biological samples in which nitric oxide has been implicated due to the detection of elevated levels of nitrotyrosine, nitrate and nitrite

	Disease	Site of elevated nitrotyrosine	Site of elevated nitrate/nitrite
Inflammation	Atherosclerosis	Atherosclerotic lesions[9,28,29] Atherosclerotic plaques[30,31]	
	Gastritis	Gastric antral biopsy[26] Gastric fundus biopsy[31]	Gastric juice[30]
	Gastric ulcer	Ulcer margins[31]	Gastric juice[32]
	Ulcerative colitis	Colonic mucosa[33,34]	Urine[35]
	Crohn's disease	Colonic mucosa[34]	Urine[35]
	Celiac disease	Plasma[36] Duodenal biopsy[37]	Plasma[36] Urine[38]
	Septic shock	Plasma[39]	Plasma[39]
Neurodegenerative	Alzheimer's disease	Hippocampal tissue[40–42] Cerebrospinal fluid[41,44]	Cerebrospinal fluid[43]
	Amyotrophic lateral sclerosis (ALS)	Cerebrospinal fluid[45] Spinal cord[48,49]	Cerebrospinal fluid[46,47] Serum[46]
	Multiple sclerosis	Central nervous system tissue[50] Brain tissue[51]	Cerebrospinal fluid[50]
	Parkinson's disease	Subtantia nigra[40]	Cerebral spinal fluid[52] Neutrophils[53]
Cancer	Colon cancer	Tumour tissue[54,55]	Plasma[56]
	Gastric cancer	Gastric biopsy[57]	Serum[58] Gastric juice[59]

effect of nitrotyrosine. However, nitrotyrosine can be transported into cells[72] and absorbed from the diet by rats.[81] Intravenous (i.v.) administration of nitrotyrosine in rats inhibited the vasoconstrictive and hemodynamic responses to angiotensin II[82] and attenuated the hemodynamic responses produced by α- and β-adrenoceptor agonists (norepinephrine, epinephrine, phenylephrine and isoproterenol).[83] It has also been demonstrated that nitrotyrosine administered by i.v. injection to rats has a half-life in the plasma of 1.67 hours[84] and an oral dose (100 μg) was converted into two metabolites; 3-nitro-4-hydroxyphenylacetic acid (NHPA) and 3-nitro-4-hydroxyphenyllactic acid (NHPL) that were excreted in the urine (44% and 5% of oral dose, respectively).[81] These metabolites have been proposed as biomarkers of human exposure to nitrosating agents[81] such as nitrate and nitrite. This has been further corroborated as NHPA appears to be excreted in the urine of human volunteers, with a profile similar to that of nitrate, following consumption of a nitrate-rich meal.[85]

Thus, the origin of free nitrotyrosine detected *in vivo* probably arises from the nitration of free tyrosine, the breakdown of nitrated proteins and dietary intake and absorption. Uncertainty still remains as to whether nitrotyrosine formation initiates the onset of disease or is a consequence of the progression of disease.

NITRATE/NITRITE

Nitrate (NO_3^-) and nitrite (NO_2^-) are naturally occurring, water soluble anions. The nitrate ion is the conjugate base of the strong acid nitric acid ($pK_a = -1.37$). The nitrite ion is the conjugate base of the weak acid nitrous acid ($pK_a = 3.37$) which decomposes readily to give water and dinitrogen trioxide (N_2O_3) or nitric acid, nitric oxide ($NO^\bullet$) and water. Salts of both acids are readily soluble in water with nitrites being much more stable than the acid itself.[86]

I. The Fate of Nitrate and Nitrite in the Human Body

The fate of nitrate in the human body is represented schematically in Figure 2. Ingested nitrate first encounters specialised symbiotic nitrate-reducing bacteria, that reside on the dorsal surface of the tongue and which reduce some (5%) of the nitrate to nitrite.[87–91] The nitrite thus formed is then chemically reduced to nitric oxide ($NO^{\bullet}$) under the acidic conditions of the stomach (Figure 3A).[89] Nitrate is absorbed from the stomach into the blood stream where it rapidly enters erythrocytes.[88] Salivary glands concentrate and actively secrete nitrate from the circulation into the oral cavity,[87–89] 25% of dietary nitrate undergoes enterosalivary recirculation in this way.[87,90,91] The majority of nitrate (60–70%) is excreted unchanged in the urine within 24 hours of ingestion[85,87,92] and appears to be predominantly tubular.[88] Maximal urinary excretion occurs 4–6 hours following challenge with potassium nitrate[87] or as it occurs naturally as part of a meal.[85] A minor amount is also excreted unchanged in the sweat[87] and faeces (0.1–0.5 %).[87,92] In radiolabel tracer studies in humans using ^{15}N-labeled nitrate, the nitrogen from administered $^{15}NO_3^-$ was recovered as ammonia and urea in the urine (3%) and faeces (0.2%).[92] The half-life of nitrate in the body has been determined to be approximately 5 hours.[92]

After administration of nitrite in meat, urinary excretion of nitrate but not nitrite was elevated[93] indicating that nitrite is oxidised during its passage through the body possibly in the stomach (Figure 3A). Oxidation of nitrite to nitrate can also occur via reactions with oxyhaemoglobin,[94] HOCl[10,95] and peroxidases.[22,96]

II. Endogenous Sources

Nitrate balance studies conducted in humans consistently conclude that a greater amount of nitrate is excreted than can be accounted for by

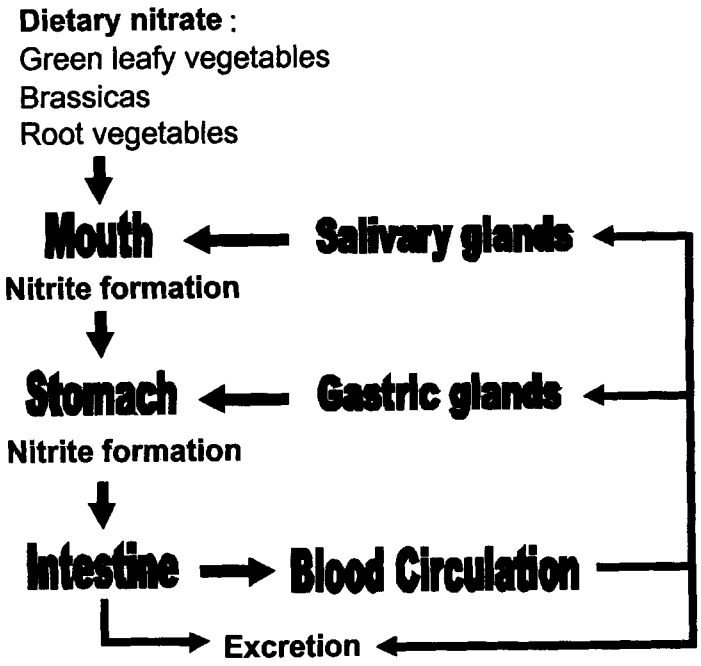

FIGURE 2 Diagrammatic scheme representing the fate of nitrate following ingestion by humans.

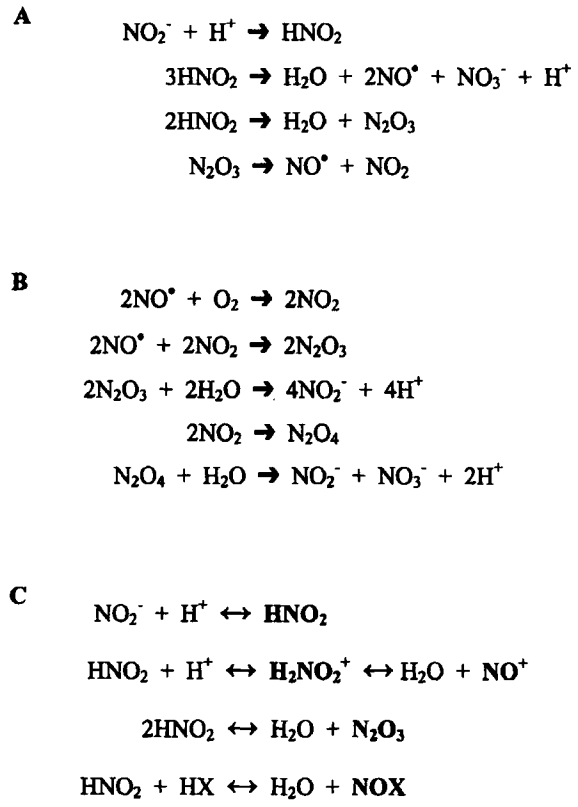

FIGURE 3 The steps involved in the chemical reduction of nitrite to nitric oxide ($NO^\bullet$) in the acidic conditions of the human stomach (A); the autoxidation of $NO^\bullet$ to nitrite in aqueous media (B); the interconversion of various proposed nitrosating species (bold) in acidic media (C).

ingestion, it therefore appears that humans can synthesise 600–2,200 μmol nitrate per day.[92,93,97] Additionally, cells appear to contain an NO_3^--H^+-cotransporter that has been suggested to eliminate the products of $NO^\bullet$ metabolism from cells.[98] The sources of this endogenous production, namely nitric oxide and bacteria resident in the gastrointestinal tract are discussed below.

Nitrogen Monoxide

Nitrogen monoxide or nitric oxide ($NO^\bullet$) is a free radical whose biological role began to emerge in the early 1980s. This small molecule produced by the endothelium, with vasorelaxant properties, was named endothelium derived relaxing factor (EDRF).[99–101] Its production and multiple roles have since been extensively investigated. Several isoforms of nitric oxide synthase (NOS) the enzyme responsible for the production of $NO^\bullet$ from arginine with the concomitant formation of citrulline have been identified.[102–105] The main isoforms are inducible NOS (iNOS), constitutive endothelial NOS (eNOS) found in vascular endothelial cells[106] and constitutive neuronal NOS (nNOS) found in human brain,[107] skeletal muscle[107] and both the peripheral and central nervous systems.[106,108] iNOS resides in many different cell types including endothelial cells,[109,110] macrophages,[109–111] chondrocytes,[112] neutrophils[26,75] and gastric mucosal cells.[113] Additionally, bacteria such as *Helicobacter pylori* also contain NOS[114] and nitrite can be reduced to $NO^\bullet$ in the ischaemic heart.[115,116] $NO^\bullet$ serves many useful functions such as regulation of blood pressure,[117,118] inhibition of platelet aggregation,[119–121] inhibition of phagocyte adhesion to the endothelium[120] and neurotransmission.[122–124] It is also thought to act as an antioxidant and an anti-atherosclerotic agent due to its ability to induce glutathione synthesis[125] but too much $NO^\bullet$ can be toxic.[126,127]

In aqueous solutions, $NO^\bullet$ autoxidises to nitrite[128,129] (Figure 3B) and addition of $NO^\bullet$ to blood results in the formation of both nitrate and nitrite.[130] It is thought that in the vascular system, $NO^\bullet$ is rapidly oxidised by reaction with oxyhaemoglobin (HbO_2) resulting in the formation of methaemoglobin (Hb^{3+}) and nitrate (NO_3^-).[130] $NO^\bullet$ also reacts with Hb^{3+} forming a complex (Hb-NO) that can hydrolyse to Hb^{2+} and nitrite.[131] That $NO^\bullet$ is a source of endogenous nitrite is confirmed by the incorporation of radiolabelled ammonia ($^{15}NH_3$)[132] and arginine (^{15}N-arginine)[133] into urinary nitrate and nitrite.

Peroxynitrite

Peroxynitrite ($ONOO^-$) has been implicated in the pathogenesis of many disease states encompassing cancers and autoimmune, inflammatory,

and neurodegenerative diseases via detection of increased levels of nitrotyrosine, despite evidence that peroxynitrite is not the sole species capable of nitrating tyrosine (Table I). Additional evidence that peroxynitrite plays a role in atherosclerosis is its ability to initiate the peroxidation of low density lipoprotein[134] that can result in the formation of F_2-isoprostanes, lipid oxidation products that may contribute to the atherosclerotic process.[135]

A requirement for the formation of peroxynitrite from the near diffusion limited reaction between superoxide and nitric oxide ($6.7 \times 10^9\,M^{-1}s^{-1}$)[136] is that both $O_2^{\bullet-}$ and $NO^{\bullet}$ are formed in close proximity at high local concentrations. Thus production is dependent on the rates of production of these two species[4,137] d peroxynitrite is most likely to form near macrophages,[138,139] neutrophils[140,141] and endothelial cells[142,143] which produce both $O_2^{\bullet-}$ and $NO^{\bullet}$.

The function of peroxynitrite is not well established as it may be an important detoxification mechanism for reactive oxygen species[144,145] yet it exhibits cytotoxic properties itself.[144,146] The conflicting effects are further emphasised by the demonstration that peroxynitrite exerts both damaging and cytoprotective effects towards platelets.[147] It has also been postulated to be an important microbicidal/bacteriocidal compound.[148]

The reaction of peroxynitrite or its conjugate acid (ONOOH), with a wide variety of biomolecules results in production of nitrite.[149] Peroxynitrite exists in two different conformations *cis-* and *trans-*. The *trans*-conformation forms upon protonation of the *cis*-form[150] and rearranges to nitrate.[150,151] However, the majority (80%) of peroxynitrite exists in the stable *cis*-conformation *in vivo*.[151]

III. Intake from Exogenous Sources

Nitrogen (N) is required for the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins in both plants and animals.[152] Humans need to acquire compounds containing nitrogen from food and therefore a minimum quantity of animal or plant protein is needed. It is estimated that one third of human dietary protein now derives from synthetic nitrogen fertilisers via the Nitrogen cycle.[152]

A large collaborative study (ECP-INTERSALT) determined that intake of nitrate in Britain is in the lower tertile of Europe and around the median of the 30 countries studied worldwide.[153,154] A Total Diet study in 1997 found the daily intake of nitrate by the UK population to be 88 mg, with an upper range of 136 mg.[155] It has been calculated that vegetarians are exposed to the greatest amount of nitrate (34.3–163.0 mg/day) in the UK.[156] It appears that the total dietary intake of nitrate in the UK was lower in 1979 being 61 mg/person (ranging from 24 to 102 mg/person) with 75% derived from vegetables, 8 mg from water used to make non-alcoholic drinks and 53 mg from food alone.[157] This is similar to the amount calculated for the UK population in 1973 (63 mg/person)[158] with 50% contributed by vegetables and water and meat contributing 25% each. Higher daily nitrate consumption (215 mg/person) from vegetables, fruit and meat in Singapore has been reported, but this differs between the Chinese (250 mg/person) and the Malay and Indian (113 mg/person) populations.[159] The contribution from water with different nitrate contents was highlighted in a sample of 50 people from the Netherlands who consumed 145–231 mg nitrate/day with the amount contributed by water varying from 0 to 25 mg in tap water and 34–132 mg in well water.[160] Daily intake of nitrite in 1979 in the UK was low (0.3–0.9 mg/person) having been detected only at low concentrations (< 2.4 mg/kg) in cereals, meats and vegetables.[157]

Several factors may account for the large variation in the daily intake including the amount and type of foods consumed by individuals. The levels of nitrate in a plant differ depending

on the site within the plant, application of fertiliser,[161,162] season,[163,164] sunshine,[163,164] maturity at harvest,[162] storage[162,165] and cooking.[161,166] Negligible nitrite has been detected in the majority of fruit and vegetables.[85,159] Nitrate levels are also negligible in fruit[85,159] but are wide ranging in vegetables of different types[85,159,163] and within a specific variety.[159,163] Green leafy vegetables consistently have high nitrate contents[85,159,163] and along with water contribute the majority of nitrate intake by humans.[159] Nitrate and nitrite as their sodium and potassium salts are used as additives (E249–252) in certain foods.[167] They are added to meat products (cured meat, bacon, ham, sausages, pork pies) due to their ability to inhibit the growth of *Clostridium botulinum*[157] and although not used in cheese manufactured in the UK, nitrate can be used in cheesemaking in Continental Europe.[157]

IV. Nitrate/Nitrite and Illness/Disease

Nitrite can cause a condition called methaemoglobinaemia through its interaction with haemoglobin so that the blood is less efficient in transporting oxygen.[157] The majority of cases occur in infants and areas where drinking water is obtained from wells with high nitrate content.[168,169] It has also been suggested that exposure of pregnant mothers to high levels of nitrate in drinking water can lead to neural tube defects in their babies.[170] This reaction with haemoglobin to form methaemoglobin has been utilised in the use of sodium nitrite as an antidote against cyanide poisoning as methaemoglobin can extract the cyanide anion from cytochrome oxidase.[171]

Nitrate intake has been linked to human cancers of the stomach due to the effect of nitrite produced from it, but a lot of controversy remains.[154,159,172–174] Nitrate intake has also been implicated in non-Hodkgin's lymphoma[175] and type I diabetes[176] whilst dietary nitrite may play a role in glioma (brain cancer)[177] and colorectal cancer.[178] The putative role of nitrate in cancer is supported by the finding that nitrate in drinking water can elicit a dose dependent increase in hypoxanthine-guanine phosphoribosyl transferase (HPRT) variant frequency in peripheral lymphocytes (a marker of DNA damage) which is a prerequisite for tumour formation.[160] Nitrate itself (117 mM) does not appear to mediate any toxic effects in mammalian cells[98] but nitrous acid (HNO_2) has been shown to be mutagenic in bacterial systems such as *Escherichia coli*[179,180] and *Salmonella typhimurium*[181] and also in the yeast *Saccromyces cerevisiae*.[182]

Administration of sodium nitrite (100–3,000 mg/L) in the drinking water of rats raised their methaemoglobin levels, led to some changes in the liver and spleen, fibrosis in the heart, thin and dilated arteries, and atrophied and dilated bronchi accompanied by lung emphysema.[183]

Whilst the presence of nitrite in the stomach is thought to have adverse health effects due to its ability to form nitroso-compounds which can be carcinogenic,[184–188] it has also been proposed to serve a protective role in augmenting the antimicrobial effects of stomach acid, a possible reason for the recirculation of nitrate into the salivary glands.[189,190] Levels of nitrate and nitrite in biological fluids are enhanced in several diseases (Table II) in which elevated nitrotyrosine has been detected indicating that either they or their precursors nitric oxide and peroxynitrite play role in the pathogenesis of these diseases.

NITROSO-COMPOUND FORMATION

Nitration and nitrosation constitute the majority of chemistry associated with reactive nitrogen species. Many different reactive nitrogen species have been proposed to be responsible for the nitration and nitrosation of compounds. Examples include N_2O_3,[191–193] NO_2,[192] N_2O_4,[191,193] amides by nitracidium ion $H_2NO_2^+$,[194] NOX (where X is a base such as a chloride anion),[195,196] NO_2^+[95] and NO_2-Cl,[95] (whereas

nitric oxide *per se* is unable to participate directly in nitration and nitrosation reactions[192]). These species can undergo interconversion under acidic conditions[128,194,196] (Figure 3C). Elevated levels of *N*-nitroso compounds are found in the gastric juice of patients with gastric cancer and its precursor states.[59] In animal models *N*-nitroso-compounds have been shown to be carcinogenic.[184–188] In addition, a nitrosated extract of Japanese fish (*sanma hiroki*) induced tumours in the forestomach and glandular stomach of rats.[197] Specifically, tyrosine has been identified as one of the precursors of nitroso-compounds in food that exhibit direct acting mutagenicity towards *Salmonella* strains after treatment with nitrite.[198,199]

Human urine contains several *N*-nitrosamino acids[160,200] that could originate either from the diet[157] or be endogenously produced and which increase in amount after ingestion of nitrate.[201] However, there appeared to be no difference in the amount of *N*-nitroso compounds excreted between people consuming different amounts of nitrite in their drinking water[160] but the total amount of nitrate ingested was not that different.

Secondary amines, amino acids and amides react optimally at $pH < 4$,[194,202] nitrosation being enhanced by halides[194,195,202] and thiocyanate.[195,202] This suggests that the majority of nitrosation by nitrite occurs in the acidic conditions of the stomach. This is probable, especially for tyrosine, as its nitration appears to be highly dependent upon pH with little nitrotyrosine formed at $pH \geq 2.2$.[22,81,85] Additionally, it has been shown that DEN (diethylnitrosamine) is formed upon incubation of diethylamine (DEA) and nitrite in gastric juice from rat, rabbit, cat, dog and humans and is also detected in stomach of cats and rabbits fed DEA and nitrite.[203,204] Some *N*-nitroso compounds may arise from the action of certain bacteria that may colonise the stomach during disease states which at elevated pH are capable of catalysing nitrosation reactions[205] and activated macrophages also have the ability to nitrosate amines *in vitro*.[206]

Dietary phenolics appear to inhibit nitrosation *in vivo*[207,208] and nitration of tyrosine *in vitro*.[85] Nitration and nitrosation reactions can occur under acidic conditions such as that of the stomach with such dietary agents. Paradoxically, however, *C*-nitrosophenols formed from phenolic compounds can act as catalysts for the reaction between amines and nitrite in mildly acidic media.[209] This is possibly the mechanism by which chlorogenic acid[210] and gallic acid[211] catalyse nitrosation of amines but we have seen no evidence for this as, in our systems, phenolic compounds inhibited rather than catalysed the nitration of tyrosine by nitrous acid.[85] Nitrosation of the flavonoid constituents of food can also result in compounds that are mutagenic in *Salmonella Typhimurium* TA100.[212]

CONCLUSIONS

In conclusion, nitrotyrosine detected *in vivo* may arise from peroxynitrite or nitryl chloride. However, little attention has been paid to sources such as dietary nitrate in the acidic environment of the stomach. The evidence implicating dietary nitrate/nitrite in human disease has been inconclusive with the exception of nitrite and methaemoglobinaemia (blue baby syndrome) Thus, the impact of diet-derived reactive nitrogen species on the potential formation of nitrotyrosine *in vivo* and its subsequent incorporation into proteins has been little explored. The formation of nitrotyrosine either in the stomach or during inflammation is probably dependent upon a multitude of factors such as the pH, bacterial colonisation, catalytic factors (thiocyanate), inhibitory factors (phenolics), competing substrates and the inflammatory mediators (HOCl, peroxidases) produced. The contribution of nitrotyrosine from the diet needs to be assessed, along with its general pharmacology in humans, namely, absorption, incorporation into proteins,

toxicity and excretion. Finally, the formation of nitrated proteins *in vivo* whether via their interaction with reactive nitrogen species or as a consequence of the incorporation of nitrotyrosine into proteins remains to be elucidated, as well as the precise role, if any, of nitrotyrosine in the pathogenesis of human disease.

Acknowledgments

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