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Biochimica et Biophysica Acta 1411 (1999) 475–488

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Review

Nitrite and nitrosyl compounds in food preservation

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Received 27 August 1998; received in revised form 2 November 1998; accepted 16 December 1998

Abstract

Nitrite is consumed in the diet, through vegetables and drinking water. It is also added to meat products as a preservative. The potential risks of this practice are balanced against the unique protective effect against toxin-forming bacteria such as *Clostridium botulinum*. The chemistry of nitrite, and compounds derived from it, in food systems and bacterial cells are complex. It is known that the bactericidal species is not nitrite itself, but a compound or compounds derived from it during food preparation. Of a range of nitrosyl compounds tested, the anion of Roussin's black salt $[Fe_4S_3(NO)_7]^-$ was the most inhibitory to *C. sporogenes*. This compound is active against both anaerobic and aerobic food-spoilage bacteria, while some other compounds are selective, indicating multiple sites of action. There are numerous possible targets for inhibition in the bacterial cells, including respiratory chains, iron–sulfur proteins and other metalloproteins, membranes and the genetic apparatus.

Keywords: Roussin's salts; Nitrosothiol; Electron paramagnetic resonance spectroscopy; (*Clostridium botulinum*); (*Listeria monocytogenes*)

Contents

Abbreviations: DNIC, dinitrosyl iron–thiolate complex; EPR, electron paramagnetic resonance; RBS, Roussin's black salt; SNP, sodium nitroprusside

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0005-2728/99/$ – see front matter
PII: S0005-2728(99)00033-X

1. The nitrite controversy

Nitrate and nitrite have been used for centuries in curing and preserving meats and fish, and in the manufacture of certain cheeses [1]. For commercial purposes, salt mixtures were found to be more effective in curing processes if they contained saltpetre (potassium nitrate) [2]. During preparation, nitrate is reduced to nitrite which is the major active ingredient in these salt mixtures. Nitrate is reduced to nitrite by bacteria under anaerobic conditions, using the molybdopterin-containing nitrate reductase. Dietary nitrate may be reduced to nitrite by bacteria present in the mouth and sometimes in the stomach [3]. Doran [4] as cited in Binkerd and Kolari [1], was issued a U.S. patent in 1917 for replacement of nitrate with nitrite in curing brines.

When added to foods such as cured meats, nitrite has at least three functions [5]. Firstly, it contributes to the flavour; this may be due to the inhibition of development of rancid off-flavours [6]. Secondly, it reacts with myoglobin to give mononitrosylhaemochrome [7], which gives the characteristic pink colour of cured meat. Thirdly, it inhibits the growth of food spoilage bacteria, and most importantly, *Clostridium botulinum*. *C. botulinum* thrives under anaerobic conditions, and produces a neurotoxin which is one of the most lethal natural products known. Nitrite, together with cooking and the addition of salt, is a protection against food poisoning by this microorganism [2,8].

Nitrate and nitrite occur in the diet from numerous different sources [3,9,10]. Vegetables are a major source of nitrates, for example about 1000 mg/kg for leaf vegetables such as lettuce, and 200 mg/kg in root vegetables such as potatoes [11]. The average levels of nitrite (as $NaNO_2$) in cured meat products are in the range 10–40 mg/kg [12], with values in the U.S. being in the lower part of the range [13].

Although the preservatives which are permitted in foods are considered to be without potential adverse effects there have been concerns about the safety of nitrites. Nitrite, in high concentrations, is undoubtedly toxic to humans. Acute effects have been observed from accidental ingestion, for example in contaminated drinking water [14], sausages [15] and medicines [16]. The principal toxic effect is oxidation of oxyhemoglobin to ferrihemoglobin, leading to methemoglobinaemia. This can be fatal, particularly in newborn infants in which the methemoglobin-reducing capacity is low, leading to so-called 'blue baby syndrome' [17]. In Britain this condition is ex-

tremely rare. Other adverse effects of nitrite have been reported, including the inhibition of intestinal absorption in rats [18]. In 1985 the European Union set a limit of 50 mg/litre of drinking water.

Since the 1970s there has been concern about a possible link between nitrite and cancer. There is no conclusive evidence that nitrite is directly carcinogenic [19], but in high doses it has been implicated as a co-carcinogen [20]. It has been shown to induce mutations in some bacterial strains of *Salmonella typhimurium* used for detection of base-pair substitutions [21]. Some epidemiological studies have suggested a link between dietary nitrates and nitrites, and the incidence of cancer (cited in [22]). There is an unusually high incidence of oesophageal cancer in Henan province, China, and this has been associated with a diet of vegetables pickled in water containing high levels of nitrate and nitrite [23]. Recent studies have failed to show a correlation between dietary nitrite and gastric cancer [22,24].

The complex chemistry of nitrite, nitric oxide and related compounds makes it difficult to establish the level of associated risk [25]. It is known, for example, that *N*-nitroso compounds (nitrosamines) may be formed from nitrites [26]. Compounds such as *N*-nitrosodimethylamine have been shown to be carcinogenic in a wide range of animal species [23]. *N*-nitroso compounds have been detected in cured meats after cooking [27]. For example, concentrations of apparent total *N*-nitroso compounds of 2.9 μg/kg were measured in fried smoked bacon; of this, known volatile and non-volatile *N*-nitroso compounds accounted for only 10–20% [28]. There is also the possibility of formation of *N*-nitroso compounds by reaction with various compounds from peptides and other amino compounds in the acid conditions of the stomach. Nitrite continues to be used in meat products primarily due to its unique property of protecting against growth of the heat-resistant spores of *C. botulinum*, and subsequent toxin formation [29]

Much of the controversy about the possible toxicity and carcinogenicity of low levels of nitrite predates the discovery that nitric oxide and nitrite are normal human metabolites, being derived by nitric oxide synthases from arginine [30]. Nitric oxide is not a xenobiotic, but has many physiological functions, including, significantly, the inflammatory immune response to bacterial infection [31]. The amount of nitrite produced in this way is comparable to that ingested in the diet [3]. The human body has defences against the toxic effects of nitrite and nitric oxide which some bacteria, including *C. botulinum*, do not possess.

In view of the possible risk of toxicity and carcinogenesis, the amount of nitrite added to foods is progressively being restricted. Nitrite, rather than nitrate, tends to be added to cured meat products, and in the lowest concentrations consistent with food safety. The mechanism by which it inhibits bacterial growth is of considerable interest, and has been studied for more than 50 years [32], but is still not understood at the molecular level. The interactions of nitrite with various substrates, such as amino acids, peptides, metalloporphyrins and iron–sulfur clusters, are known [23]. It seems probable that if the mechanisms by which nitrite interferes with cell growth were understood, in terms of both the cellular target of nitrite action and the chemical events which lead to growth inhibition, other compounds which mimic the nitrite mechanism could be rationally designed or selected. Such compounds might be important as new food preservatives, antibiotics, or general bacteriostatic agents.

2. Chemistry of nitrite, nitric oxide and related nitrosyl compounds

Nitrite, when added to food systems and bacterial cells, undergoes complex chemical interconversions and metabolism. Some of the compounds formed are stable but kinetically reactive. Few of the metabolic products of nitrite, other than N_2 gas, can be considered inert. If nitrite is converted to forms that are undetectable by the analytical methods used, these may still act as a reservoir of NO-related species which can be reconverted to active forms. This has made it difficult to discover the fate of nitrite and other NO-related species, and to determine the reasons for their bacteriostatic action.

Many methods have been used to measure the compounds formed in meat from nitrite, but most have limitations; either they will only detect some chemical species, or they cannot distinguish different species. The Griess reaction, which produces a col-

oured azo compound, is a test for nitrite and related N(III) species, but under aerobic conditions NO can be converted to NO_2^-. Chromatographic methods are slow, and can lead to interconversion of species. Electrodes for detection of NO are sensitive to oxidising and reducing compounds present in biological materials, though more recent systems appear to overcome this difficulty. EPR spectroscopy can detect nitrosyl species such as nitrosyl iron complexes, only if they are paramagnetic, i.e., having an odd number of electrons. ^{15}N-nuclear magnetic resonance is selective for different species but relatively insensitive, and does not detect ^{15}N bound to macromolecules. It is not surprising that it has been difficult to account quantitatively for the nitrogen added as nitrite to food systems.

The chemistry of nitric oxide and its redox-related species NO^+ (the nitrosonium cation) and NO^- (the nitroxyl anion) [33] is central to an understanding of the biology of NO. Nitric oxide is an oxidant ($E = +1.18$ V for NO/N_2O), and a reducing agent ($E = +0.35$ V for NO_2^-/NO). NO contains N(II), the nitroxyl ion N(I) and the nitrosonium ion N(III), and each of them has a distinctive chemistry unique to itself. In biochemical systems, nitric oxide in solution has a half-life of a few seconds. Reaction with oxygen in aqueous solution is much slower than the loss of NO over this time period. It should be noted that, in oxygenated aqueous solution, autoxidation, which occurs through an unknown 'intermediate' and then to nitrite [34,35], is slow, compared with other reactions under physiological conditions. NO tends to react rapidly with other atoms or molecules that also contain unpaired electrons. Also important are the reactions with thiols to form *S*-nitrosothiols, which occurs in the presence of oxidants [36], and with metal ions to form nitrosyl complexes [37].

A variety of compounds that are formed under neutral physiological conditions can be conveniently viewed as NO^+ carriers [38,39]. Important examples of such compounds are metal nitrosyl complexes, *S*-nitrosothiols (RS-NO), *N*-nitroso compounds (for example, RNH-NO), and dinitrogen trioxide (N_2O_3).

2.1. Iron–sulfur–nitrosyl (Fe–S–NO) complexes

Roussin's salts are the best-known complexes of iron–sulfur–nitrosyl complexes, and could be considered as the first generation of metal nitrosyl complexes, being described by Roussin as early as 1858. Because of its inhibitory effect on bacteria at micromolar concentrations, Roussin's black salt was used to sterilise the water supply of Paris during the 19th century. Roussin's salts are now known to contain the anions $[Fe_4S_3(NO)_7]^-$ (Roussin's black salt, RBS) and $[Fe_2S_2(NO)_4]^{2-}$ (Roussin's red salt). RBS is unusual in being soluble in organic solvents such as diethyl ether, and so should be able to penetrate cell membranes easily. [Fe–S–NO] complexes are numerous and chemically reactive. Their formation and interconversions have been reviewed by Butler et al. [39]. Fig. 1 shows the structure of Roussin's salts, and some other bacteriostatic compounds which have been investigated.

2.2. EPR-detectable nitrosyl species

EPR spectroscopy has been used to study nitrosyl complexes of transition metals, to which the NO radical confers an unpaired electron. Nitric oxide itself, in frozen solutions, gives rise to a signal with $g \approx 1.95$, with a broad tail to higher field [40]. This is

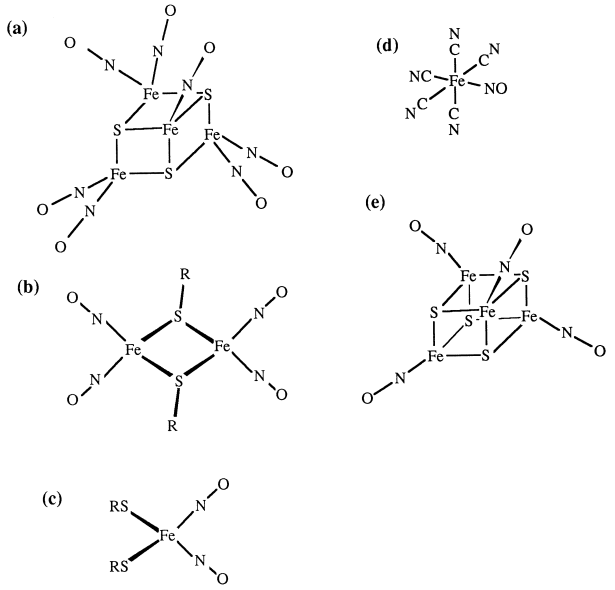

Fig. 1. Structures of some iron–sulfur clusters and nitrosyl complexes: (a) $[Fe_4S_3(NO)_7]^-$ (RBS); (b) Roussin's red salt; (c) dinitrosyl iron–thiolate complex (DNIC); (d) sodium nitroprusside (SNP); (e) $Fe_4S_4(NO)_4$.

only detectable at low temperatures (<20 K) and requires high concentrations of NO. Some complexes of non-heme iron with nitric oxide are more readily detectable, and can be used to observe molecular targets for NO in biological systems [41]. The dinitrosyl iron complexes of the type $[Fe(SR)_2(NO)_2]^-$ (DNIC) (Fig. 1a), which are low-spin ferrous–nitrosyl species ($S=1/2$), give narrow EPR signals with g-factors between 2.012 and 2.04 [42,43]. These signals are prominent due to their narrow width, and can be detected at ambient temperature. These paramagnetic iron–nitrosyl complexes have been observed in extracts of rat liver, following the administration of carcinogens [44]. In this case the nitrosyl groups were presumably derived by the action of macrophages on cancer cells. Dietary nitrite can also generate such compounds; extracts from organs of experimental animals on a diet supplemented with nitrite and ferrous sulfate, gave an anisotropic signal at $g=2.03$ [45]. Another EPR signal, at $g_\perp=1.999$ and $g_\parallel=1.927$, with resolved ^{14}N hyperfine splitting, is observed from nitroprusside during its reduction by thiols [46]. The nitrosyl groups exist in these complexes as nitrosonium cations, NO^+. There are also high-spin ferrous–nitrosyl species ($S=3/2$) characterised by g-values around 4.0 and 2.0; the archetypal complex of this type is the nitrosyl complex of Fe(II)–EDTA.

Nitrosylation by nitrite of synthetic iron–sulfur model complexes based on natural iron–sulfur proteins has also been shown under mild conditions [47]. The ready formation of paramagnetic mononuclear complexes $[Fe(SR)_2(NO)_2]^-$ from a range of diamagnetic precursors was observed. This suggests that nitrosylation of natural iron–sulfur clusters also proceeds via paramagnetic mononuclear complexes.

3. NO as a bacterial metabolite

The reduction of nitrate to nitrite has already been mentioned. Bacteria present in foodstuffs can carry out various further transformations. Many species of bacteria can catalyse some or all of the reactions of denitrification [48], in which nitric oxide is now recognised as an important intermediate [49,50]. Some of the relevant enzymes are:

Assimilatory and dissimilatory nitrate reductases:	$NO_3^- + 2e^-$	$\rightarrow NO_2^-$
Dissimilatory nitrite reductases:	$NO_2^- + e^-$	$\rightarrow NO$
Nitric oxide reductases:	$2\ NO + 2e^-$	$\rightarrow N_2O$
Nitrous oxide reductases:	$N_2O + 2e^-$	$\rightarrow N_2$
Assimilatory (and some dissimilatory) nitrite reductases:	$NO_2^- + 6e^-$	$\rightarrow NH_4^+$

The ability of bacteria to catalyse these reactions specifically is used in analysis, for example of nitrate which is reduced to nitrite (e.g., [22]). It is also exploited in the biological removal of nitrate from waste water [51].

Under aerobic conditions, NO may be produced by oxidation of ammonia and amino acids. A nitric oxide synthase which catalyses the formation of NO from arginine, like the eukaryotic enzyme, has been isolated from a *Nocardia* species [52]. Thus, for some bacteria, nitric oxide and nitrosyl species may be considered as normal metabolites. Different bacteria can express various of these enzymes, depending on the nitrogen sources present. NO can be released into the environment by some bacteria, to be consumed by others [53]. *Clostridium* and *Listeria*, to which NO is toxic, have in general low concentrations of the enzymes involved in nitrite metabolism, e.g., nitrite reductase [54].

4. Clostridium and Listeria

These micro-organisms belong to groups of bacteria that can be major health hazards through contamination of food [55]. *C. sporogenes* is a non-toxic analogue of *C. botulinum* (which causes botulism) and *C. perfringens*. Whereas the principal hazard from clostridia is the toxins that are produced in the food, *Listeria monocytogenes* causes an infection, listeriosis.

4.1. Clostridia

Clostridia are strictly anaerobic (except for a few aerotolerant species), proteolytic catalase-negative rod-shaped organisms, which produce heat-resistant spores [56]. The primary source of clostridia is soil. Some are free-living nitrogen-fixing organisms, such as *C. pasteurianum*; others, such as *C. acetobutylicum*, are used to produce commercial chemicals

such as butyric acid, butanol, acetone and enzymes. Other clostridia cause serious infections such as tetanus and gas gangrene.

Proteolytic clostridia are major causative agents of the spoilage of canned foods at neutral and slightly acidic pH, as they can form heat-resistant spores. The major species involved are toxin-producing strains of *C. botulinum,* and *C. perfringens*. Studies on *C. botulinum* can require stringent containment facilities, and so *C. sporogenes* is has been extensively studied as a model organisms. It has a similar metabolism to *C. botulinum*, but does not form toxins.

The clostridial toxins are water soluble, heat sensitive and acid stable proteins of 50 000 to 250 000 kDa. They affect the nervous system by preventing the secretion of neurotransmitter vesicles [57]. The early symptoms and signs of botulism are anxiety or agitation, drowsiness or blurred vision, and nausea or vomiting. Substernal burning or pain, abdominal distension and decreased bowel activity may occur. Death is usually the result of respiratory paralysis.

Clostridial endospores are more resistant than vegetative cells to heat, radiation or germicides, and cannot be easily destroyed. Spores survive heating at 60°C for several seconds, which kills vegetative cells of *C. sporogenes*. Germination of the dormant spore requires heat or specific activating substances. Germination of spores and subsequent vegetative cell growth require iron, high concentrations of glucose, cysteine and other essential amino acids.

C. sporogenes has a complex fermentative metabolism [58]. It is capable of growth in media without carbohydrates by using the Stickland reaction. This reaction involves the oxidation of some amino acids such as alanine, valine, leucine and isoleucine, coupled to the reduction of others, such as glycine and proline. The addition of glucose to such a medium causes significant enhancement of growth. Major end products of glucose metabolism are acetate, propionate, butyrate and isobutyrate, hydrogen and carbon dioxide [58].

4.2. Listeria

L. monocytogenes is a catalase-positive bacillus which can grow in milk products such as soft cheeses, and in meat and fish [59,60]. There are haemolytic (pathogenic) and non-haemolytic (non-toxic) strains. They are able to grow aerobically or anaerobically, over a range of temperatures between −4°C and 50°C, and survive for long periods of time. This may lead to the contraction of listeriosis from products stored in domestic refrigerators. *L. monocytogenes* grows at pH values between 4.7 and 9.2. pH, temperature and concentration of preservatives are the most important factors in the inhibition of *L. monocytogenes* growth in food.

Although *L. monocytogenes* is able to ferment carbohydrates yielding lactic acid it does not produce gas. Like *C. sporogenes*, it does not reduce nitrate.

5. The nature of the bacteriostatic species

Many types of cured meats are subjected to a heating process which destroys vegetative cells. The heating of nitrite in bacteriological medium can result in the production of many different compounds. Some of these are more toxic to bacteria than nitrite itself. Perigo et al. [61] showed that nitrite when heated in a bacteriological medium was more inhibitory towards growth of *C. sporogenes* than nitrite added aseptically to the medium after autoclaving. This effect was found to occur in the temperature range 95–125°C at pH values about 6.0; the unidentified inhibitory substance(s) became known as the *Perigo factor*(*s*). A later study by Perigo and Roberts [62] showed an enhanced inhibitory effect of nitrite heated in laboratory media against 30 strains of clostridia. It was reported that a reducing agent such as thioglycollate, ascorbate or cysteine, as well as a protein hydrolysate, were necessary components of the laboratory medium in order to produce the effect.

The situation in meats was found to be more complex than in liquid media. Johnston et al. [63] compared the effect of heating nitrite in medium and in meat systems, and showed that heating at 110°C for 20 min enhanced the inhibition of *C. botulinum* in a medium containing nitrite at a concentration of 20 mg/kg but not in a meat system containing nitrite at 150 mg/kg or greater. The addition of meat to a nitrite-containing bacteriological medium was found to neutralise the inhibitory factor. Ashworth and Spencer [64] demonstrated an increased inhibition by nitrite when heated in minced pork; the inhibitor

produced did not show a pH-dependence, as was the case with unheated nitrite. The magnitude of the increase in inhibition on heating was small compared with the effect seen in a medium system. Tompkin et al. [65] showed that supplementation of perishable canned cured pork with iron, as a result of incorporating an iron salt or beef heart and beef liver, caused a *decrease* in the antibotulinal properties of nitrite. Thus there are uncertainties about the importance of Perigo factors in preservations of canned meats. Nevertheless, these factors are of great interest due to their high toxicity to clostridia, compared with nitrite.

Hansen and Levin [66] used the incorporation of [^{14}C]uracil into ribonucleic acid by *Bacillus cereus* as a system to test the antimicrobial properties of a number of compounds derived from nitrite. They showed that nitrosothiols of thioglycollate and β-mercaptoethanol, and a heat-induced inhibitor from nitrite-containing medium (i.e., a Perigo factor) were effective inhibitors of uracil incorporation during spore outgrowth, and other stages in the life cycle of *Bacillus cereus*, probably as a result of the inactivation of several sensitive metabolic steps or systems.

6. Bacteriostatic compounds derived from nitrite

6.1. Nitrous acid

In early experiments, Tarr [67] showed that the preservative action of nitrite in fish was greatly increased by acidification, suggesting that nitrous acid (HONO) was the active form. In bacteriological media the inhibitory action of nitrite on several species of bacteria was shown to increase with decreasing pH, particularly at pH 6.0 and below. This effect was confirmed in other bacteria, including vegetative cells of *C. sporogenes* [61] and spores of *C. botulinum* [68]. Nitrite, which could be produced in the saliva, was found to be bactericidal to the gastric pathogen *Helicobacter pylori* at acid pH [69]. The effect of acidification is presumably because nitrous acid is a much more active species than the anion, but present in very low concentrations at neutral pH (the pK_a of nitrous acid is 3.4). Nitrous acid and *N*-nitroso compounds can diazotise and deaminate amino groups in, for example, nucleotides.

6.2. Peroxynitrite

Under aerobic conditions the toxic effects of nitric oxide on bacterial cells are influenced by the presence of oxygen, and of oxidising species derived from it such as peroxide [70] and superoxide. Thus in aerobic conditions the effect of NO itself may be diminished [71] while the more reactive species, peroxynitrite, is believed to become involved [72–74]. This can be formed from the reaction of nitric oxide with superoxide, or nitroxyl ion with oxygen [38].

Table 1 shows the effects of various compounds on the growth of *C. sporogenes* and *L. monocytogenes*.

6.3. Fe–S–NO complexes

Other likely candidates for compounds derived from nitrite in medium systems during the cooking process, are those related to the Roussin's salts and iron nitrosothiols [2,5]. Studies were directed at the types of compound that are formed in growth media and in meat systems, by heating nitrite with various components of the growth media, and testing their effects on bacterial growth. Some very effective inhibitors were found using heated mixtures of nitrite, cysteine and ferrous ions [54,75]. Butler et al. [76] showed by quantitative FTIR that RBS was formed in good yield. This suggested that iron–sulfur–nitrosyl complexes may be responsible for the inhibition of cell growth. These and similar results indicated that bacteriostatic compound(s) are derived from nitrite.

Moran et al. [77] demonstrated that RBS inhibited vegetative cells of *C. perfringens* at very low concentrations, approximately 0.5 μM. Higher concentrations (50 μM) were found to be needed to inhibit the germination of spores of *C. sporogenes*. However, this is remarkably more effective than nitrite itself, which is only inhibitory at concentrations of the order of 10 mM (Table 1).

6.4. Nitrosothiols and N-nitroso compounds

The effects of the nitrosylcysteinylferrate anion

Table 1
Minimum inhibitory concentrations for nitrite and related species for growth of *C. sporogenes* and *L. monocytogenes*

Complex	K_i (μmol dm^{-3})	
	L. monocytogenes	*C. sporogenes*
Nitrite		5000
Nitric oxide[a]		40
$NH_4[Fe_4S_3(NO)_7]$ (RBS)	3	1.3
$[Fe_2(SCH_2CH_2OH)_2(NO)_4]$ (Roussin's red salt ester)	45[b]	5.0
$Fe_4S_4(NO)_4$		10
$Na_2[Fe(CN)_5NO]$ (SNP)	Non-toxic	28
$[Mo(acac)_2(NO)_2]$	600	Non-toxic
$[Ru(acac)_2(NO)Cl]$	500	Non-toxic
$[Ru(bipy)_2(NO)Cl](PF_6)_2$	300	400
$[Ru(phen)_2(NO)Cl](PF_6)_2$	200	700
Trioxodinitrate ($Na_2N_2O_3$)		200
SIN-1		1000

The concentrations are those required for 50% inhibition of growth in liquid culture under anaerobic conditions. Data are as presented [80].
[a]Added as NO in solution.
[b]Unpublished observations.

were tested on the growth of *Salmonella*, *Streptococcus faecium* and *C. sporogenes*, and found to be very effective inhibitors [78]. On the other hand the *N*-nitroso compounds, which are carcinogenic in animals, were not found to have anticlostridial activity [79].

An approach to understanding the mode of action of nitrogenous compounds has been to test the effects on bacterial growth and enzymic activities of compounds, for which the chemistry is well understood. Cui et al. [80] showed that sodium nitroprusside (nitrosylpentacyanoferrate(II), $[Fe(CN)_5NO]_2^-$) is highly toxic to *C. sporogenes*. Nitroprusside contains NO in the formal oxidation state NO^+, and is a good nitrosating agent. Related transition-metal nitrosyl complexes were less bactericidal, and there was a relationship between the toxic action of the complexes and their nitrosating ability.

SIN-1 (3-Morpholinosydnonimine-*N*-ethylcarbamide) is used as a slow releaser of nitric oxide. It was found to have a similar type of toxicity to nitric oxide in aqueous solution, and both were considerably more toxic than nitrite itself. It seems likely that many of these species are short-lived in growth media, and are converted to longer-lasting forms which continue to inhibit cell growth. Examples of such compounds are nitrosothiols, which show long-term inhibitory effects [78].

7. Molecular mechanisms of nitrite inhibition of anaerobic bacteria

Banwart [81] has defined four basic sites of bacteriostatic action of preservatives: enzymes and other proteins; the genetic system; cell walls or membranes; and the binding of essential nutrients.

7.1. Iron–sulfur proteins and energy metabolism

The inhibition of respiration has long been considered a possible mechanism of the bacteriostatic action of nitrite, at least for aerobic organisms (reviewed by Davidson and Juneja [82]). This has been used to explain the cytotoxic action of nitric oxide produced by macrophages, which has been shown to inhibit mitochondrial cytochrome *c* oxidase [40,83], and bacterial terminal oxidases [84].

Tompkin et al. [65] suggested that nitric oxide, formed via nitrous acid from nitrite, reacts with the iron–sulfur proteins of bacteria. Iron–sulfur proteins are important in energy metabolism of both aerobic and anaerobic bacteria and thus are likely targets for the bacteriostatic action of nitrite, nitric oxide and related compounds. Synthetic model compounds based on the [2Fe–2S] and [4Fe–4S] clusters in natural iron–sulfur proteins, have been shown to under-

go nitrosylation under mild conditions, producing EPR-detectable iron–sulfur nitrosyl species [47].

Woods et al. [85,86] studied the effect of nitrite on the metabolism, particularly the glucose metabolism, of cells of *C. botulinum*. They found that when nitrite was added to a suspension of cells of *C. sporogenes* incubated in medium containing glucose, there was a large and rapid decrease in the intracellular concentration of ATP and an excretion of pyruvate from the cells. The increase in pyruvate implied that the inhibitory action of nitrite was on the phosphoroclastic system, which is an important source of ATP in the clostridia. The phosphoroclastic system converts pyruvate to CO_2, hydrogen and acetyl phosphate which is further converted to acetate by acetate kinase+ADP. The system comprises three iron–sulfur proteins: pyruvate:ferredoxin reductase, ferredoxin and hydrogenase. Wood et al. postulated that for *C. sporogenes* an important mechanism of nitrite inhibition is by formation of nitric oxide complexes with the non-haem iron of pyruvate-ferredoxin oxidoreductase (PFR).

The effects of nitrite and nitrosyl complexes on pyruvate:ferredoxin reductase were examined by Carpenter et al. [87], who reported that nitrite caused the production of EPR-detectable Fe–S–NO complexes in *C. botulinum* [88]. McMindes and Siedler [89] observed an inhibition of pyruvate:ferredoxin reductase by nitrite, which was attributed to the action on sulfhydryl groups. Payne et al. [54,90] studied the action of various bactericidal nitrosyl complexes on the iron–sulfur proteins of clostridia, and the activity of ferredoxin, pyruvate:ferredoxin reductase and hydrogenase. They found no correlation between the bactericidal action of the complexes, and the inhibition of the isolated proteins. For example, RBS had little effect on the iron–sulfur clusters in ferredoxin, and yet cells grown in the presence of RBS had low levels of iron–sulfur proteins. It was suggested that the Fe–S–NO complexes might inhibit the synthesis of iron–sulfur clusters, a process which is poorly understood.

Nitric oxide has been proposed to inactivate other iron–sulfur proteins. Iron–sulfur clusters in proteins in which the iron atoms are fully coordinated by sulfur, as in ferredoxins, are not very sensitive to NO [91]. However aconitase, an enzyme of the citric acid cycle, which is involved in the interconversion of citrate and isocitrate, contains a [4Fe–4S] cluster which is particularly vulnerable to attack by NO [92,93]. This is because one iron atom of the cluster is not ligated by cysteine, but binds water or citrate instead. Another iron–sulfur protein which is sensitive to nitric oxide is ferrochelatase, the enzyme which inserts iron into haem [94,95].

7.2. Other proteins

Riha and Solberg [96] proposed that nitrite inhibition of *C. perfringens* may be due to reaction of nitrite, as nitrous acid, with SH-containing constituents of the bacterial cells. Reaction of nitrous acid with thiols can produce nitrosothiols, which can prevent the action of enzymes such as glyceraldehyde-3-phosphate dehydrogenase [97]. Protein-bound nitrosothiols and compounds such as nitrosocysteine can also serve to store NO and release it again for further reactions [98,99], or transfer NO^+ to receptor groups of high nucleophilicity.

Other amino acids in proteins can show the effects of exposure to NO-derived species. Nitrotyrosine has been used as a marker for the presence of peroxynitrite in mammalian systems [100]. Deamination of lysine by nitrite has been suggested as a marker for exposure of proteins in food systems to nitrite [101].

7.3. DNA and gene expression

Nitrite does not appear to be reactive with the bases of DNA at neutral pH, but NO and nitrosothiols have been implicated in strand breakage in the presence of superoxide and hydrogen peroxide, respectively [102,103]. Such reactions might have a bearing on the bacteriostatic effects on organisms such as *Listeria* and *E. coli* under aerobic conditions. Ribonucleotide reductase, which is essential for the formation of DNA, is another target for nitric oxide and related species [104,105]. Kroncke et al. [106] have shown that NO can attack zinc finger-type DNA-binding proteins and could affect gene regulation.

7.4. Cell walls and membranes

The cell wall of *C. sporogenes* contains DL-diaminopimelate and galactose. Viewed under the electron

microscope, it is typical of Gram-positive bacteria [107], with an inner, amorphous, electron-dense layer and an ordered outer layer composed of cylindrical subunits, commonly referred to as the S layer. S-Layer proteins range from 40 to 200 kDa [108,109]. From *Clostridium difficile* two proteins, of 32 kDa and 45 kDa, were isolated [110].

Some bacteriostatic agents interfere with cell wall production, by inhibiting synthesis of monomer units or their polymerisation. Others act without entering the cells. A reaction on the cell wall or membranes may alter the permeability of the cell, impairing the passage of nutrients into the cell, or allowing leakage of cellular constituents. Damage of the cell wall alone does not usually kill the microbial cell, but the increased permeability may allow entry of toxic species.

O'Leary and Solberg [111] found that cells of *C. perfringens* inhibited by 14 mM nitrite were dark grey or brown in colour, had an altered consistency and were harder to disperse in buffer. They postulated that this pigmentation was associated with cell walls and membranes and it was suggested that damage could be a primary event. Likewise, Payne et al. [90] found that cells of *C. sporogenes* were darker in colour and clumped together when they were grown in the presence of mixtures of ferrous sulfate, cysteine and nitrite. Buchman and Hansen [112], who examined the mechanism by which nitrite and *S*-nitrosothiols inhibit outgrowing spores of *Bacillus cereus* T, presented evidence that nitrite-induced bacteriostasis in an aerobe is associated with inactivation of membrane sulfhydryl groups and that these sulfhydryl groups are critical for cell viability. Most importantly, the results correlated sulfhydryl modification with the actual inhibitory event. Possible targets include essential proteins involved in the uptake of nutrients.

When SNP was added to cultures of *C. sporogenes* and *L. monocytogenes*, paramagnetic reduced species were observed, which decayed over a few minutes [113]. This is indicative of the reaction of nitroprusside with thiols, a reaction which ultimately leads to the release of NO [114,115]. A rapid initial reaction was taking place, which was correlated with the disappearance of thiol groups from the cell membrane. However, it was found that whereas *C. sporogenes* was damaged by this treatment, *L. monocytogenes* was insensitive (Table 1). By contrast *L. monocytogenes* was very sensitive to RBS, when grown both aerobically and anaerobically, which indicates that the mechanism of inhibition is different for the two compounds.

There is also evidence for cell lysis in the presence of some nitrosyl complexes. *C. sporogenes* cells grown in the presence of RBS or SNP, when examined by electron microscopy, showed blistering of the cell surface at low concentrations of the inhibitors, and lysis at higher concentrations [113,116]. By contrast, no evidence of lysis was observed with bactericidal concentrations of nitrite, nitric oxide or SIN-1. Thus cell lysis may be a secondary effect of a primary lesion, or there are several bactericidal mechanisms.

8. Concluding remarks

The use of nitrite as a food preservative represents a problem of balancing risks. It is difficult to assess these risks until the mechanisms of action are better understood. Despite over 50 years of research, the actions of this compound are not clear. Much further work is needed to understand the speciation of nitrite and nitrosyl species in food systems, and their effects on metabolism of bacteria and humans. This is a subject which deserves re-investigation using modern techniques of analysis. Molecular biology also has much to offer. The exact form of any genetic damage caused by nitrite may be examined. Mutants that are more or less sensitive to nitrite could be isolated and characterised. In this way it should be possible to identify the sites of action of nitrite, and cellular mechanisms for resistance.

Acknowledgements

The work was supported by the Biotechnology and Biological Sciences Research Council, and a K.C. Wong studentship to X.-Y.C. R.C. and M.N.H. are members of the Centre for the Study of Metals in Biology and Medicine, King's College, London.

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