Electron paramagnetic resonance spectroscopic investigation of the inhibition of the phosphoroclastic system of *Clostridium sporogenes* by nitrite

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The proposal that nitrite exerts its inhibitory effect on anaerobic bacteria by direct interaction with the iron–sulphur proteins of the phosphoroclastic system was investigated. The effects of nitrate, nitrite with or without ascorbate, and nitric oxide on the growth of *Clostridium sporogenes* in liquid cultures at pH 7.4, on the rates of hydrogen production, and on the activities of the enzymes pyruvate-ferredoxin oxidoreductase and hydrogenase, and of ferredoxin were investigated. In agreement with previous studies, nitrate was the least effective inhibitor of cell growth, and nitric oxide the most effective. Nitrite reductase activity was very low in *C. sporogenes*, indicating that the presence of external reducing agents would be necessary for the reduction of nitrite to nitric oxide. Inhibition by nitrite was enhanced by ascorbate; 0.5 mM-nitrite with 10 mM-ascorbate stopped growth completely. In partially-purified preparations 4.1 mM- NaNO_2 and equimolar ascorbate caused complete inactivation of hydrogenase activity but only partial (up to 78%) inactivation of pyruvate-ferredoxin oxidoreductase. This agreed with the loss of hydrogen production observed with nitrite *in vivo*. Inhibition occurred within 5 min, and was irreversible in each case. Electron paramagnetic resonance (EPR) spectroscopy showed that paramagnetic $[\text{Fe}(\text{NO})_2(\text{SR})_2]$ species were formed during growth in the presence of nitrite, and were associated with cells. However, the intensity of these EPR signals did not correlate with the inhibition of cell growth. The [4Fe–4S] clusters in ferredoxin were shown by EPR spectroscopy to be resistant to treatment with 3.6 mM- NaNO_2 and 3.6 mM-ascorbate. It is concluded that the effects of nitrite on pre-formed iron–sulphur proteins are not convincing as a basis for the lethal effects on bacterial cells.

Introduction

Nitrate and nitrite are preservatives used widely to inhibit the growth of clostridia in foods such as meat. The bacteriostatic effect of nitrate operates mainly through reduction to nitrite, which is further converted to other inhibitory species. The effects of these compounds are enhanced by reducing agents, such as ascorbate, which are added to meat products as stabilizers. The maximum NaNO_2 concentrations used in bacon, sausage, ham and other cured meats are around $0.03 \text{ mg (g food)}^{-1}$ (Knight *et al.*, 1987), which corresponds to approximately 0.4 mM-nitrite. Inhibition of clostridial growth by nitrate and nitrite has been widely studied over the past 20 years. These studies have involved a wide range of species and growth media ranging from fluid-broth to meats (Roberts & Ingram,

1966; Perigo *et al.*, 1967; Perigo & Roberts, 1968; Baird-Parker & Baillie, 1973; O'Leary & Solberg, 1976; Woods *et al.*, 1981; Woods & Wood, 1982). The bacteriostatic action of NaNO_2 in these systems is enhanced when it is reduced by ascorbate (Tompkin *et al.*, 1978*a, b*; Pierson & Smoot, 1981).

Perigo & Roberts (1968) found that when nitrite was heated with reinforced clostridial medium at pH 6–7, the nitrite disappeared and the medium became more inhibitory to clostridial growth. This is referred to as the 'Perigo effect', and is due to the heat-dependent formation by nitrite of more powerful inhibitors, termed 'Perigo-type factors' (Larkworthy *et al.*, 1977). Various inhibitory species, such as *S*-nitrosocysteine and Roussin's black salt, $[\text{Fe}_4\text{S}_3(\text{NO})_7]$ (Asan & Solberg, 1976), have been isolated from meats after nitrite addition. However, these have not been found in sufficient quantities to account for the levels of inhibition observed.

Abbreviations: DTT, dithiothreitol; $[\text{Fe-S-NO}]$, iron–thiol–nitrosyl complex.

Evidence has been presented that nitrite acts upon metalloproteins which are essential for bacterial growth. Tompkin *et al.* (1978c) noted that excess iron caused a decrease in the effectiveness of nitrite inhibition of cell growth, and suggested that nitric oxide formed complexes with iron-sulphur proteins such as the clostridial ferredoxins, which are essential for electron transport, enzyme activity and energy production. Iron-sulphur complexes are reactive with nitric oxide, forming a range of iron-thiol-nitrosyl ($[\text{Fe-S-NO}]$) complexes (Butler *et al.*, 1988). The phosphoroclastic system contains three iron-sulphur proteins: pyruvate-ferredoxin oxidoreductase, ferredoxin and hydrogenase. The inhibition of this system would be an effective way of inhibiting spore germination and cell growth.

Woods *et al.* (1981) found that when nitrite was added to a suspension of *C. sporogenes* cells in glucose medium, it resulted in a decrease in intracellular ATP and an accumulation of pyruvate in the medium. Similarly in *C. botulinum* cells, nitrite caused a decrease in intracellular ATP (Woods & Wood, 1982). It was concluded that the inhibition of the phosphoroclastic system of *C. sporogenes* by nitrite involved the reduction of nitrite to nitric oxide, and that inhibition was likely to occur by attachment to the non-haem iron centres present in ferredoxin and pyruvate-ferredoxin oxidoreductase. Pyruvate-ferredoxin oxidoreductase activity was inhibited to a greater extent than ferredoxin activity (Woods *et al.*, 1981). The activity of ferredoxin, in an NADH-linked assay, as electron acceptor from pyruvate-ferredoxin oxidoreductase was unaffected by preincubation with 6 mM-sodium nitrite. The inhibitory action of nitrite on the phosphoroclastic system was not as evident *in vitro* as *in vivo* (Woods & Wood, 1982).

Angermaier & Simon (1983) studied the hydrogenase and ferredoxin-mediated reduction of aliphatic and aromatic nitro-compounds in clostridia, and proposed that nitrite possibly reacted with cysteine residues with the formation of stable S-nitrosothiols, thereby releasing the iron and sulphur from the clusters. However, Reddy *et al.* (1983) showed that cells of *C. botulinum*, after treatment with nitrite, yielded EPR signals with *g*-values of 2.02–2.04. This type of spectrum is characteristic of low-spin nitrosyl-iron complexes of the general formula $[\text{Fe(NO)}_2(\text{SR})_2]$ (McDonald *et al.*, 1965), and has been observed in a variety of biological systems, including animal tissues, after treatment with nitrite (Vanin & Varich, 1981). Reddy *et al.* (1983) proposed that the formation of these complexes from iron-sulphur proteins and nitric oxide was the mechanism of botulin inhibition by nitrite in foods.

Carpenter *et al.* (1987) incubated *C. botulinum* cells with 14.5 mM nitrite, and estimated 94% inactivation of ferredoxin (assayed by monitoring the rate of reduction

of cytochrome *c*) and 70% inhibition of pyruvate-ferredoxin oxidoreductase. They stated that it was likely that nitrite exerted its antibotulin effect by the destruction of iron-sulphur enzymes within vegetative cells. In contrast to the earlier conclusions by Woods *et al.* (1981) they concluded that nitrite preferentially inhibited ferredoxin *in vitro*, although pyruvate-ferredoxin oxidoreductase activity was also inhibited.

We have investigated the mode of action of nitrite on the iron-sulphur proteins of the phosphoroclastic system of *C. sporogenes*. This organism is metabolically similar to the proteolytic strains of *C. botulinum* and *C. perfringens*, and therefore is useful as a model to investigate the inhibitory effect of nitrite and derivatives on food-spoilage bacteria. The direct effect of nitrite on the phosphoroclastic system in growing cells was studied by monitoring hydrogen production. The iron-sulphur proteins of the phosphoroclastic system, ferredoxin, pyruvate-ferredoxin oxidoreductase and hydrogenase, were studied in growing cells, in soluble cell extracts, and as the partially-purified proteins. They were incubated with nitrate, nitrite or nitric oxide, and changes in enzyme activities and the formation of EPR-detectable nitrosyl complexes were monitored.

Methods

Organism and growth conditions. Actively growing cultures of *C. sporogenes*, strain NCIB 10696, were inoculated (10 ml l^{-1}) into Oxoid nutrient broth no. 2, containing 1% (w/v) glucose and 5 ml l^{-1} of 10% (w/v) sodium thioglycollate, and incubated at 37°C . Cell growth was monitored as optical density at 550 nm. Cells were harvested by centrifugation after incubation for a further 20 h.

Inhibitors. Nitrite was autoclaved in 10 l of standard Oxoid nutrient broth no. 2, prior to the addition of glucose and thioglycollate. The nutrient medium was then inoculated with 10 ml actively growing clostridia l^{-1} . Cells were incubated at 37°C for 24 h before harvesting. Nitric oxide was added to cultures as a saturated solution. Water was thoroughly saturated with oxygen-free argon before bubbling with nitric oxide on ice under anaerobic conditions, to avoid oxidation of NO and consequent acidification.

Purification of proteins

Soluble cell extracts. Extracts were prepared in an anaerobic glovebox (Miller Howe Ltd) in an atmosphere of nitrogen ($<3 \text{ p.p.m. O}_2$). All buffers contained 1 mM-dithionite. Cells stored under liquid nitrogen (approx. 50 g) were thawed in 300 ml argon-saturated 100 mM-Tris/HCl pH 8.5 buffer, containing 1 mg lysosyme ml^{-1} and 0.1 mg DNAase ml^{-1} . The cells were incubated for 2 h at room temperature. The lysate was centrifuged under anaerobic conditions prior to centrifugation at $27600 g$ for 80 min at 4°C , and the supernatant (soluble extract) was collected.

Ferredoxin-free extract. Soluble cell extract (300 ml) was added to a DE52 (Whatman) column ($2 \text{ cm} \times 13 \text{ cm}$) equilibrated in 50 mM-Tris/HCl pH 8.0. The column-bound hydrogenase was washed with six column volumes of equilibration buffer, before adding 0.13 M-NaCl . At this salt concentration, the pale brown, hydrogenase-

active band that eluted from the column was collected in 15 ml fractions. Then buffer containing 0.28 M-NaCl was added, and a larger, more tightly bound brown band (containing the active pyruvate-ferredoxin oxidoreductase and hydrogenase) was collected in the same volume fractions. The ferredoxin, a dark brown band, was eluted with 1 M-NaCl.

Pyruvate-ferredoxin oxidoreductase and hydrogenase. The pyruvate-ferredoxin-oxidoreductase-active and hydrogenase-active fractions from the above preparation were pooled and diluted 5:1 in 20 mM-potassium phosphate pH 7.1, containing 1 mM-DTT and 0.05 mg ml⁻¹ glucose oxidase. This was added to a hydroxylapatite (Bio-Gel HTP) column (5.0 cm × 2.5 cm) equilibrated in the same buffer, and the column washed with 10 column volumes of the same buffer. Potassium phosphate buffer was then added as an increasing step-gradient, in 50 ml aliquots, up to 0.5 M in 0.1 M increments. Pyruvate-ferredoxin oxidoreductase and hydrogenase eluted at potassium phosphate concentrations of 0.1 M and 0.5 M respectively.

Ferredoxin. The ferredoxin fraction from the above preparation was diluted with equilibration buffer and reconstituted under oxygen-free nitrogen at 4 °C in a 200 ml Amicon pressure ultrafiltration vessel (10 kDa cut-off membrane). The resulting 30 ml sample was passed down a second DE52 column (1 cm × 15 cm), washed and eluted with a step-gradient (in 0.1 M increments) of 0.1–1.0 M-NaCl. A Sephadex G-75 column (0.8 cm × 50 cm), equilibrated in the same buffer, was used to remove high-molecular-mass proteins and nucleic acids. Ferredoxin was reconstituted by centrifugation at 2430 g in Amicon Centricon 10 microconcentrators, at 4 °C. The A_{390}/A_{280} ratio was between 0.5 and 0.7.

Measurements of phosphoroclastic activity

Measurement of hydrogen evolution in growing cell cultures. Actively growing cells (60 ml) were inoculated into 1 l of fresh medium, and the resultant culture was divided into 5 ml volumes in sterile septum-sealed 27 ml glass vials under nitrogen. Cultures, in triplicate, were incubated at 37 °C. After 4 h the inhibitor was added and the incubations continued. Samples of gas (0.1 ml) were withdrawn at regular time intervals before and after the addition of inhibitor, and injected into a gas chromatograph with a thermal conductivity detector, previously calibrated with hydrogen.

Assay of pyruvate-ferredoxin oxidoreductase and hydrogenase activity in growing cell cultures. Benzyl viologen was used instead of methyl viologen as terminal electron acceptor, because [benzyl viologen]⁺ is a more permeant cation than [methyl viologen]⁺ (Jones *et al.*, 1976). Triton X-100 was also added to remove the permeability barrier. The assay mixture, prepared in 1 ml septum-sealed cuvettes, contained 10 mM-pyruvate, 200 µM-coenzyme A, 2 mM-thiamin pyrophosphate, 2 mM-benzyl viologen and 2 mM-MgCl₂ in 50 mM-Tris/HCl, pH 8.0. Hydrogenase activity was also monitored by spectrophotometric assay, using reaction mixtures which contained 2 mM-benzyl viologen, 2 mM-DTT and 0.4% Triton X-100, in 50 mM-Tris/HCl, pH 7.0. The Triton X-100 was added to assays after they had been anaerobically prepared, as an argon-saturated concentrate. Other reagents for the assays of pyruvate-ferredoxin oxidoreductase and hydrogenase were bubbled for 20 min with oxygen-free argon or hydrogen respectively. All reactions were initiated by the addition of cells.

Determination of pyruvate-ferredoxin oxidoreductase and hydrogenase activity in cell extracts. These assays were the same as with whole cells, except that Triton X-100 was omitted, and 10 mM-methyl viologen was used as terminal electron acceptor. The rate of reduction of methyl viologen was monitored spectrophotometrically at 604 nm. One unit of enzyme activity (U) was calculated as one µmol of methyl viologen reduced min⁻¹, i.e. twice the rate of hydrogen or pyruvate consumption.

Assay of nitrite reductase activity. A series of 1 ml septum-sealed vials containing methyl viologen (0.5 mM), dithionite (5 mM) and nitrite (0.25 mM) in 50 mM-phosphate buffer pH 7.0 (in a total volume of 300 µl) were flushed under argon, prior to adding 50 µl of crude extract. The reduction of nitrite was monitored at room temperature under argon. Reactions were stopped at 5 min intervals up to 90 min, by adding 1 ml of buffer, and assayed for nitrite using the Griess-Llosvay Nitrite Assay kit (BDH).

Protein assay. The protein concentration was determined by means of the Bio-Rad dye reagent assay, using gamma-globulin as standard. Absorbance readings were made at 595 nm.

EPR spectroscopy. EPR samples were prepared of cell pellets, washed in argon-saturated 50 mM-Tris/HCl pH 8.0, and of supernatants. Spectra were recorded on a Varian E4 spectrometer with an Oxford Instruments ESR900 helium flow cryostat. Spin concentrations were determined by double integration with Cu-EDTA as standard. All EPR samples were studied at low temperature (below 20 K) to monitor the integrity of iron-sulphur clusters in proteins after nitrite addition, and at higher temperatures (above 50 K) to study [Fe-S-NO] formation.

Results

Effects of nitrate, nitrite and nitric oxide on whole cells of clostridia

Inhibition of exponential-phase growth. Oxoid nutrient broth no. 2 growth medium (pH 7.4) is widely used in the growth of many strains of clostridia and supports rapid cell growth. In our study, nitrite was added when cultures had reached a high density of cells (3×10^7 ml⁻¹) in exponential-phase growth. Experiments carried out under these conditions provided a stringent test of the ability of the antimicrobial agent used to inhibit active cell growth. When 1 mM-nitrate or 1 mM-nitrite (equivalent to concentrations permitted in foods) was included in the medium, growth of *C. sporogenes* was not

Table 1. Inhibition of exponential-phase cultures of *C. sporogenes* by nitrate, nitrite and nitric oxide

Cell yield was calculated from values of wet weights of harvested cells after growth in the presence or absence of inhibitors. Each inhibitor was added as a filter-sterilized solution after 4 h of cell growth. Yield of control cells was 3 g litre⁻¹. Each culture (in triplicate) was inoculated into fresh normal growth medium after inoculation with inhibitor, to see if growth recovery occurred.

Inhibitor	Cell yield (% of control)	Growth in fresh medium
1 mM-NaNO ₃	100	yes
1 mM-NaNO ₂	100	yes
4 mM-NaNO ₂	77	yes
0.5 mM-NaNO ₂ /4 mM-ascorbate	64	yes
0.5 mM-NaNO ₂ /10 mM-ascorbate*	<3	no
0.186 mM-Nitric oxide	<3	no

* Below this nitrate concentration, growth occurred.

Fig. 1. Ascorbate enhancement of nitrite inhibition of hydrogen production in growing cell cultures. Nitrite and ascorbate were added after cells had been incubated at 37 °C for 390 min. ■, Control cell cultures (water added); ▲, cultures + 0.5 mM- NaNO_2 ; ◆, cultures + 0.5 mM- NaNO_2 and 4 mM-ascorbate; ▼, cultures + 0.5 mM- NaNO_2 and 10 mM-ascorbate; ●, cultures + 10 mM-ascorbate.

inhibited. With 4 mM-nitrite in the medium, the cells continued to grow, with a yield of 77% of the control cultures but they formed agglutinated clumps when harvested, were difficult to resuspend, and were darker in colour than normal cultures. The increase in pigmentation appeared to be associated with insoluble cellular material. No residual nitrite was detected in the supernatant (growth medium) after growth with nitrite.

Nitrite in the presence of ascorbate was much more inhibitory than nitrite alone. Its concentration could be reduced to 0.5 mM if the concentration of ascorbate was raised to 10 mM (Table 1). Nitric oxide was also strongly inhibitory, even without added ascorbate, and 186 μM concentrations totally inhibited clostridial growth. This is consistent with previous proposals (Tompkin *et al.*, 1978*a, b*; Woods *et al.*, 1981; Reddy *et al.*, 1983), that the antimicrobial action of nitrite (in the absence of iron and cysteine) occurs via nitric oxide formation.

Hydrogen production

Nitrite inhibited hydrogen production far more efficiently than did nitrate. In the presence of increasing levels of ascorbate, nitrite inhibition was enhanced accordingly (Fig. 1).

Fig. 2. Inhibition of hydrogen production, hydrogenase activity and pyruvate-ferredoxin-oxidoreductase activity by 1 mM- NaNO_2 and 4 mM-ascorbate. Anaerobic nitrite solution was added to 5 ml cultures of cells grown in septum-sealed 27 ml vials, after 475 min of growth at 37 °C. Filter-sterilized water was added to control cultures. Samples of gas and of cells were withdrawn at regular time intervals under sterile conditions. Culture samples were assayed spectrophotometrically for pyruvate-ferredoxin oxidoreductase activity and hydrogenase activity, using Triton X-100 to lyse the cells. (a) Hydrogen production. (b) Hydrogenase activity. (c) Pyruvate-ferredoxin oxidoreductase activity. ■, Control cell cultures; ●, cell cultures + 1 mM- NaNO_2 and 4 mM-ascorbate.

Fig. 3. EPR signals associated with interactions of whole cells of *C. sporogenes* with nitrite and ascorbate. (a) Cells as prepared (gain $\times 150$). (b) Cells after incubation with 15 mM- NaNO_2 at room temperature for 15 min (gain $\times 200$). (c) Cells after incubation with 15 mM- NaNO_2 and 15 mM-ascorbate for 30 min at room temperature (gain $\times 100$). (d) Sample as (c), gain $\times 620$. Conditions of measurement: temperature 16 K, microwave power 20 mW, modulation amplitude 0.1 mT, microwave frequency 9.191 GHz.

Hydrogenase activity

When 1 mM- NaNO_2 and 4 mM-ascorbate were added to growing cultures of *C. sporogenes*, hydrogenase activity was inhibited in the same time scale as hydrogen production (Fig. 2a, b). This indicated that the loss of hydrogen production could be accounted for by inhibition of hydrogenase.

Pyruvate-ferredoxin oxidoreductase activity

The addition of 1 mM- NaNO_2 and 4 mM-ascorbate to growing cultures of *C. sporogenes* caused pyruvate-ferredoxin oxidoreductase activity to decrease, in a period of 1 h, to a constant rate which was 22% of the control rate (Fig. 2c).

Nitrite reductase activity

Nitrite reductase activity in the crude cell extract was very low ($52 \text{ nmol min}^{-1} \text{ mg}^{-1}$). This suggested that, if the antimicrobial action of nitrite was through reduction to nitric oxide, then nitrite reductase would not readily

form such a product. Hence inhibition of the growth of *C. sporogenes* was dependent on the addition of reducing agents.

EPR spectroscopy

EPR spectra, recorded at 16 K, of cells grown under normal conditions showed signals with g -values of around 1.94, characteristic of proteins with reduced [4Fe-4S] clusters, such as ferredoxin. These signals are apparent in the wide-scan spectrum shown in Fig. 3(a). They were not observed at temperatures above 40 K, owing to rapid electron-spin relaxation. When cells were incubated at room temperature with 15 mM-nitrite for 15 min, the EPR spectra showed that the iron-sulphur proteins remained in a reduced state (Fig. 3b). In some instances the $g = 1.94$ signal decreased in size. There was also an increase in a signal at $g = 4.3$ due to high-spin Fe^{3+} ions, which is a further indication of a more oxidized state. In general, it was observed that as long as the spectra indicated the presence of reduced 'ferredoxin-type' proteins ($g = 1.94$), the cells remained viable.

If cells were incubated with both 15 mM-nitrite and 15 mM-ascorbate they were no longer viable. The EPR spectra of these cells no longer showed the $g = 1.94$ signal due to [4Fe-4S] clusters (Fig. 3c), indicating that either oxidation or destruction of the clusters had occurred. A $g = 2.03$ signal was also prominent. Another signal appeared at $g = 4.0$; this is indicative of high-spin Fe-NO formation and is associated with a weak downward feature at $g = 2.0$. In addition an unidentified broad signal appeared around $g = 1.65$ (Fig. 3d). The signal was only detectable at temperatures of measurement below 40 K.

EPR signals around $g = 2.03$, induced by addition of nitrite to *C. sporogenes* cells, were larger in resting cells incubated with nitrite *in vitro* (such as in the experiments performed by Reddy *et al.*, 1983), than in growing cells. In the absence of ascorbate, 3 mM- NaNO_2 induced nitrosyl formation in cells resuspended in buffer. Ascorbate stimulated the production of the nitrosyl complexes. EPR signals were observed after incubation of *C. sporogenes* with nitrite and ascorbate at 37 °C for 1 h, with nitrite concentrations ranging from 0.5 mM (which permits cell growth) to 5 mM (Fig. 4). However, if 3 mM-nitrite was introduced *in vivo* into fluid growth medium during cell growth, cells harvested after further overnight growth were devoid of EPR-detectable nitrosyl signals. $g = 2.03$ signals were not detected in the supernatants from these cultures, even where 40 mM-nitrite and 40 mM-ascorbate had been present. However, signals at $g = 4.0$ and 2.0 were detected at 16 K in supernatants from cells grown in the presence of 10 mM-nitrite and equimolar ascorbate, which caused total growth inhibition. Signals of this type are character-

Fig. 4. EPR spectra showing nitrosyl formation in cells after incubation with nitrite and ascorbate. Cells (a) as prepared, (b) with 0.5 mM- NaNO_2 /ascorbate added, (c) with 3 mM- NaNO_2 /ascorbate added, (d) with 5 mM- NaNO_2 /ascorbate added. Conditions of measurement: temperature 90 K, microwave power 20 mW, modulation amplitude 0.02 mT, microwave frequency 9.233 GHz, gain $\times 1000$.

istic of high-spin ferrous-nitrosyl complexes, in contrast to the low-spin $g = 2.03$ species (Bonner *et al.*, 1978; Rich *et al.*, 1978).

Inhibition of isolated iron-sulphur proteins

Extract and purified enzyme samples were treated with nitrite in two ways: either extract was added directly to assay medium in which nitrite was present, or extract was incubated with nitrite prior to withdrawal of a sample for assay. In the latter case, nitrite was diluted into the assay medium by a factor of between 100- and 1000-fold. By comparing the two conditions it was possible to determine whether inhibition was an immediate process, and whether it could readily be reversed.

The effects of 10 mM- NaNO_3 were observed on soluble extracts of *C. sporogenes* which were devoid of ferredoxin. Inhibition of pyruvate-ferredoxin oxidoreductase and hydrogenase activities only occurred in the presence of 10 mM-ascorbate, indicating that reducing

Table 2. Inhibition of hydrogenase and pyruvate-ferredoxin oxidoreductase by nitrite derivatives

Soluble extract from *C. sporogenes* was incubated with each inhibitor at room temperature for 15 min prior to assay. Each assay was performed a minimum of 3 times. Control specific activity of pyruvate-ferredoxin oxidoreductase and hydrogenase was 0.20 units mg^{-1} and 0.06 units mg^{-1} respectively, where 1 unit was equivalent to 1 μmol of methyl viologen reduced min^{-1} .

Addition(s)	Specific activity (% of control)	
	Pyruvate-ferredoxin oxidoreductase	Hydrogenase
1.64 mM- NaNO_3	100	100
4.1 mM- NaNO_3	100	ND
10 mM- NaNO_3	100	96
4.1 mM- NaNO_3 + 4.1 mM-ascorbate	74	ND
10 mM- NaNO_3 + 10 mM-ascorbate	68	64
0.5 mM- NaNO_2	90	ND
1.64 mM- NaNO_2	79	68
4.1 mM- NaNO_2	32	28
4.1 mM- NaNO_2 + 4.1 mM-ascorbate	29	0
1.64 mM-Nitric oxide	39	21

ND, Not determined.

conditions were necessary. NaNO_2 at 0.5 mM concentration, which is comparable with the amounts used in foodstuffs, caused 10% inhibition of pyruvate-ferredoxin oxidoreductase activity (Table 2).

At higher concentrations of NaNO_2 , inhibition of pyruvate-ferredoxin oxidoreductase activity was rapid and irreversible, but incomplete. After NaNO_2 was incubated with pyruvate-ferredoxin oxidoreductase, and then diluted into the assay medium, activity did not recover, despite the 100-fold dilution of the inhibitor. However, prolonged incubation of the enzyme with NaNO_2 did not increase the degree of inhibition. NaNO_3 , NaNO_2 and NO had a greater effect on hydrogenase activity than on pyruvate-ferredoxin oxidoreductase activity (Table 2). Hydrogenase activity when measured in the presence of DTT (which protected the enzyme) was severely but not totally (92%) inhibited by 5 mM-nitrite and 5 mM-ascorbate (Table 3), both in the presence and absence of ferredoxin (0.02 mg ml^{-1} assay). Nitric oxide at 1.64 mM concentration did not totally inhibit pyruvate-ferredoxin oxidoreductase or hydrogenase activity. Growth of *C. sporogenes* was totally inhibited by 0.5 mM-nitrite and 10 mM-ascorbate, or by 0.186 mM-nitric oxide (Table 1). Therefore the antimicrobial effect of nitrite or nitric oxide cannot be explained by direct inhibition of preformed pyruvate-ferredoxin oxidoreductase or hydrogenase.

Table 3. *Enhancement of pyruvate-ferredoxin oxidoreductase or hydrogenase activity by normal and nitrite-treated ferredoxin*

Soluble extract devoid of ferredoxin was used in these assays. Pyruvate-ferredoxin oxidoreductase and hydrogenase assay mixtures had various combinations of 0.02 mM-ferredoxin (Fd), 5 mM-DTT, and 5 mM- NaNO_2 with 5 mM-ascorbate added to them. Assays were performed at 30 °C.

Reagent(s) added to assay	Mean sp. activity of hydrogenase [U mg ⁻¹ $\pm$ SD (3)]	Percentage inhibition of activity
None (control)	0.387 $\pm$ 0.025	
DTT	0.478 $\pm$ 0.049	
DTT + Fd	0.515 $\pm$ 0.072	
DTT + NaNO_2 + ascorbate	0.039 $\pm$ 0.007	92
DTT + Fd + NaNO_2 + ascorbate	0.043 $\pm$ 0.004	92
Mean sp. act. of pyruvate-ferredoxin oxidoreductase (U mg ⁻¹ $\pm$ SD)		
None (control)	2.70 $\pm$ 0.15	
Fd	4.50 $\pm$ 0.007	
NaNO_2 + ascorbate	1.70 $\pm$ 0.03	37
Fd + NaNO_2		
Ascorbate	2.90 $\pm$ 0.05	36

Ferredoxin enhanced the pyruvate-methyl-viologen oxidoreductase activity of pyruvate-ferredoxin oxidoreductase. Preincubation of ferredoxin (prior to assay) with 5 mM- NaNO_2 (Table 3) had no significant effect on this enhancement.

Effects of nitrite on EPR spectroscopic properties of iron-sulphur proteins

When soluble extract from *C. sporogenes* cells was incubated with 14 mM- NaNO_2 and 14 mM-ascorbate, iron-nitrosyl signals were detected at 16 K with *g*-values of 2.03 (Fig. 5). An additional unidentified signal was observed at *g* = 1.65. Both signals were detected under these conditions, but there was no correlation in their amplitudes in different samples, indicating that they are from different species. The *g* = 1.65 signal was not observed in cells after growth with concentrations of equimolar nitrite and ascorbate below 3 mM, but was of particular interest because in our studies (Payne *et al.*, 1990), similar signals have been observed in cells grown in the presence of [Fe-S-NO] complexes, where only micromolar levels of nitrite were available. If the signal is related to a cellular interaction, this may suggest that very high nitrite levels are necessary to form this signal when the Fe and S source (for [Fe-S-NO] formation) is a protein. Therefore, total extraction of these iron-sulphur centres by nitrite may be more difficult than anticipated

Fig. 5. EPR-detectable nitrosyl formation in nitrite-treated extract of *C. sporogenes*. (a) Soluble extract as prepared. (b) Incubated with 14 mM- NaNO_2 and 14 mM-ascorbate at room temperature for 15 min. Conditions of measurement: temperature 16.5 K, microwave power 20 mW, modulation amplitude 0.1 mT, microwave frequency 9.193 GHz, gain $\times$ 200.

by Angermaier & Simon (1983). The signals appear to have a close association with cell damage and pigmentation. After an extract devoid of ferredoxin was incubated with 1.1 mM-NO, the same signal was observed, indicating that it was independent of the presence of ferredoxin.

A cell extract, containing pyruvate-ferredoxin oxidoreductase and hydrogenase activity, but devoid of ferredoxin, was incubated with 4 mM-nitrite and 4 mM-ascorbate, and yielded EPR spectra with *g* = 2.03 signals, indicative of [Fe-S-NO] species. At these concentrations of nitrite and ascorbate, pyruvate-ferredoxin oxidoreductase and hydrogenase activities were severely inhibited (Table 2), and therefore nitrite interaction with [4Fe-4S] centres in these proteins could have been responsible for nitrosyl formation.

After incubating ferredoxin isolated from *C. sporogenes* with 3.6 mM- NaNO_2 and equimolar ascorbate for 1 h at room temperature, EPR spectra of the reduced protein at 15 K (data not shown) still showed the presence of normal amounts of reduced [4Fe-4S] centres at *g* = 1.94. Therefore reduced ferredoxin appears to be relatively stable to attack by nitrite.

Discussion

Relevance of nitrite concentrations used

Concentrations of nitrite added to meats are mainly written as p.p.m. quantities, or mg nitrite (kg liver)⁻¹, etc. For the sake of this study the literature values were calculated into millimolar amounts, assuming that 1 kg of meat was equal in volume to 1 kg of water. The values calculated were therefore somewhat underestimated, compared with nitrite concentrations added to meat. Concentrations of nitrite previously found necessary to inhibit clostridial growth in liquid growth medium have ranged from 4.4 mM at pH 7.0 to 7.0 mM at pH 7.5 (Roberts & Ingram, 1966; Perigo *et al.*, 1967; Perigo & Roberts, 1968; Baird-Parker & Baillie, 1973; O'Leary & Solberg, 1976; Woods *et al.*, 1981). We have similarly shown that *C. sporogenes* readily grow in liquid cultures in the presence of nitrite, at concentrations above those used in food preservation (Pierson & Smoot, 1981; Knight *et al.*, 1987), i.e. >2.3 mM.

Effectiveness of ascorbate with nitrite

Ascorbate acted synergistically with nitrite to inhibit cell growth. Baird-Parker & Baillie (1973) were able to decrease the minimum inhibitory concentration of nitrite for all strains of clostridia to around 1.5 mM using ascorbate at concentrations between 5.7 mM and 57 mM. In the present study, 0.5 mM nitrite was adequate, with 10 mM ascorbate, despite high cell density. Direct interaction of nitric oxide with iron-sulphur centres in proteins may have been responsible for inhibition of cell growth (resulting in [Fe-S-NO] formation). Nitric oxide was far more efficient at inhibiting growth than nitrite and ascorbate. This suggests that the formation of nitric oxide by reduction of nitrite by ascorbate is not very efficient.

How are cells killed?

The pigmentation of insoluble cell components after cell growth with nitrite is in agreement with observations made by O'Leary & Solberg (1976), who found that *C. perfringens* cells inhibited by 14 mM- NaNO_2 were dark grey/brown in colour, exhibited a different mass consistency and were harder to disperse in buffer. The pigmentation appeared to be associated with cell walls or membranes, and it was proposed that such damage could be a primary event in cell death.

EPR-detectable Fe-NO complexes

Reddy *et al.* (1983) observed that iron-nitrosyl ($g = 2.03$) signal formation in *C. botulinum*, after treatment with

2.9 mM- NaNO_2 and equimolar ascorbate, occurred simultaneously with the disappearance of EPR signals ($g = 1.94$ at 16 K) indicative of reduced [4Fe-4S] centres. They suggested that the antimicrobial activity of nitrite depended on 'inhibition of electron-transfer processes in general, by destruction of iron-sulphur centres'. However, we have observed that iron-nitrosyl signal formation does not correlate with growth inhibition in *C. sporogenes*. We have also observed that, in our cell preparations, $g = 1.94$ signals at 16 K are present after incubation of 3 mM- NaNO_2 with equimolar ascorbate. These signals were smaller than those found in uninhibited cell samples, but this may have been partially due to sample oxidation by nitrite. Generally, actively growing *C. sporogenes* exhibited EPR spectra indicative of reduced iron-sulphur proteins, and strongly inhibited cells showed spectra characteristic of oxidized iron-sulphur proteins. However, low-spin ferrous-nitrosyl signals were often present in both cases.

Woods *et al.* (1981) detected an accumulation of pyruvate in the medium after incubation of resuspended cells with 4.4 mM- NaNO_2 , and attributed this to direct inhibition of pyruvate-ferredoxin oxidoreductase by nitrite. Our results agree with Woods *et al.* (1981), in the finding that pyruvate-ferredoxin oxidoreductase activity is more significantly inhibited than ferredoxin by nitrite, and that the oxidoreductase is more readily inhibited by nitrite *in vivo* than *in vitro*. We have shown that nitrite and ascorbate, at concentrations higher than those used in the food industry, do not cause total destruction of [4Fe-4S] clusters in preformed proteins. Carpenter *et al.* (1987) reported that ferredoxin activity was damaged to a greater extent than pyruvate-ferredoxin oxidoreductase. However, they used high concentrations of nitrite (14.5 mM), and did not achieve total inhibition in either case. Our EPR spectroscopic studies indicate that the purified protein, if isolated under strictly anaerobic conditions, is remarkably resistant to nitrite.

Nitrite has been proposed to specifically inhibit these ferredoxin-type enzymes as part of its antimicrobial action (Tompkin *et al.*, 1978a; Woods *et al.*, 1981; Reddy *et al.*, 1983; Carpenter *et al.*, 1987). This study has provided evidence that EPR-detectable iron-nitrosyls are indeed formed when nitrite is incubated with extract at high concentration. However, the importance of this process in the antimicrobial activity of nitrite is in dispute. The concentration of nitrite necessary to achieve inhibition is higher than would be present in conditions of food preservation.

Inhibition by nitrite of pyruvate-ferredoxin oxidoreductase was found to be only partial; while 78% of the activity was readily destroyed, the remaining activity was stable. Similarly, Carpenter *et al.* (1987) reported only 70% inhibition of pyruvate-ferredoxin oxido-

reductase activity after incubating the enzyme with 14.5 mM- NaNO_2 . This effect could be explained if there existed two forms of pyruvate-ferredoxin oxidoreductase, one resistant and the other susceptible to nitrite attack. One form may be totally inactivated while the other is less susceptible to inhibition. Uyeda & Rabinowitz (1971), during purification of pyruvate-ferredoxin oxidoreductase from *C. acidi-urici*, found that activity was separated into a 70% unstable fraction and a 30% stable fraction.

It is not clear if the inhibition of pyruvate-ferredoxin oxidoreductase activity by nitrite would be significant *in vivo*. We have previously shown that pyruvate-ferredoxin oxidoreductase activity is protected from attack by nitrite by its substrate, pyruvate (Payne & Cammack, 1986).

Clostridia are able to generate various 2-oxoacids from exogenous fatty acid homologues containing one carbon atom less. The fatty acids appear to be activated by a kinase, and the fatty acyl-phosphates formed are metabolized to their CoA derivatives, which are the substrate for the ferredoxin-linked reductive carboxylation system (cf. James *et al.*, 1988). During our purification of pyruvate-ferredoxin oxidoreductase, phenyl pyruvate, 2-oxopentanoate, 2-oxobutyrate, 2-oxoisovalerate, 2-oxoisocaproate, 2-oxooctanoate and oxaloacetate-oxidizing ability were found to be present in cell extracts. It is not known if any of these activities are associated with iron-sulphur proteins. However, the activities were found to be inhibited to varying degrees (none of them totally) by 10 mM- NaNO_2 and equimolar ascorbate.

Inhibition of hydrogen production *in vivo* occurred in parallel with inhibition of hydrogenase activity. We have shown that hydrogenase is the most sensitive iron-sulphur enzyme in the phosphoroclastic system to nitrite attack. Inhibition of hydrogen production during cell growth might therefore occur through interaction of either nitrite or nitric oxide with the hydrogenase enzyme. Recent studies on the hydrogenases of *C. pasteurianum* have shown that there are two different enzymes, one specifically catalysing hydrogen uptake and the other bidirectional. Each contains an unusual, highly oxygen-sensitive iron-sulphur cluster, possibly containing six iron atoms, which is probably the site of hydrogen binding (Adams *et al.*, 1989). Studies on the hydrogenases of *Desulfovibrio* spp. have shown that the enzymes that contain only iron-sulphur clusters are more sensitive to inhibition by nitric oxide than are those that contain nickel as well as iron-sulphur clusters (Berlier *et al.* 1987).

Hydrogenase activity may not be absolutely essential to cell growth. Even total inhibition of hydrogen production may only result in a decrease in the rate of cell

growth, and an alteration of the products of metabolism. Meyer *et al.* (1986) found that when cultures of *C. acetobutylicum* were gassed with carbon monoxide, hydrogen production was totally inhibited. The alteration in electron transport triggered solvent (butanol and ethanol) formation and inhibited acetate and butyrate production. The authors concluded that electrons normally used for hydrogen formation were used for solvent production instead. Perhaps nitrite inhibition of hydrogenase *in vivo* may trigger a similar alteration in electron transport.

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References

- ADAMS, M. W. W., ECCLESTON, E. & HOWARD, J. B. (1989). Iron-sulphur clusters of hydrogenase I and hydrogenase II of *Clostridium pasteurianum*. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 4932-4936.
- ANGERMAIER, L. & SIMON, H. (1983). On the reduction of aliphatic and aromatic nitro compounds by clostridia; the role of ferredoxin in its stabilisation. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **364S**, 961-975.
- ASAN, T. & SOLBERG, M. (1976). Inhibition of *Clostridium perfringens* by heated combinations of nitrite, sulfur and ferrous or ferric ions. *Applied and Environmental Microbiology* **31**, 49-52.
- BAIRD-PARKER, A. C. & BAILLIE, M. A. H. (1973). The inhibition of *Clostridium botulinum* by nitrite and sodium chloride. *Proceedings of the International Symposium on Nitrite Meat Products II*, 77-91.
- BERLIER, Y., FAUQUE, G. D., LE GALL, J., CHOI, E. S., PECK, H. D. & LESPINAT, P. A. (1987). Inhibition studies of three classes of *Desulfovibrio* hydrogenase - application to the further characterisation of the multiple hydrogenases found in *D. vulgaris* Hildenborough. *Biochemical and Biophysical Research Communications* **146**, 147-154.
- BONNER, W. D., JR, BLUM, H., RICH, P. R. & SALERNO, J. C. (1978). Further observation on a mitochondrial metallocompound and on a ferrous-EDTA-NO model compound. In *Frontiers of Biological Energetics*, pp. 997-1001. London: Academic Press.
- BUTLER, A. R., GLIDEWELL, C. & LI, M.-H. (1988). Nitrosyl complexes of iron-sulphur clusters. In *Advances in Inorganic Chemistry*, pp. 335-393. Edited by A. G. Sykes. London: Academic Press.
- CARPENTER, C. E., REDDY, D. S. & CORNFORTH, D. P. (1987). Inactivation of clostridial ferredoxin and PFR by sodium nitrite. *Applied and Environmental Microbiology* **53**, 549-552.
- JAMES, E. W., KELL, D. B., LOVITT, R. W. & MORRIS, J. G. (1988). Electrosynthesis and electroanalysis using *Clostridium sporogenes*. *Bioelectrochemistry and Bioenergetics* **20**, 21-32.
- JONES, R. W., GRAY, T. A. & GARLAND, P. B. (1976). A study on the permeability of the cytoplasmic membrane of *E. coli* to reduced and oxidised benzyl viologen and methyl viologen cations; complications in the use of viologens as redox mediators for membrane bound enzymes. *Biochemical Society Transactions* **4**, 671-673.
- KNIGHT, T. M., FORMAN, D., AL-DABBAGH, S. A. & DOLL, R. (1987). Estimation of dietary intake of nitrate and nitrite in Great Britain. *Food and Chemical Toxicology* **25**, 277-285.
- LARKWORTHY, L. F., TURNBULL, M. H. & YAVARI, A. (1977). Possible role of iron II amino acid complexes in nitrite depletion and inhibitor formation in cured meats. *Chemistry and Industry*, May 21st, 401-402.

- MCDONALD, C. C., PHILLIPS, W. D. & MOWER, H. F. (1965). An electron spin resonance study of some complexes of iron, nitric oxide and anionic ligands. *Journal of the American Chemical Society* **87**, 3319–3326.
- MEYER, C. L., ROOS, J. W. & PAPOUTSAKIS, L. (1986). CO gassing leads to alcohol production and butyrate uptake without acetone formation in continuous cultures of *Clostridium acetobutylicum*. *Applied Microbiology and Biotechnology* **24**, 159–167.
- O'LEARY, V. & SOLBERG, M. (1976). Effect of sodium nitrite inhibition on intracellular thiol groups and on activity of certain glycolytic enzymes in *Clostridium perfringens*. *Applied and Environmental Microbiology* **31**, 208–212.
- PERIGO, J. A. & CAMMACK, R. (1986). Inhibition by nitrite of the phosphoroclastic system of *Clostridium sporogenes*. *Biochemical Society Transactions* **14**, 1283–1284.
- PAYNE, M. J., GLIDEWELL, C. & CAMMACK, R. (1990). Interaction of iron–thiol–nitrosyl compounds with the phosphoroclastic system of *Clostridium sporogenes*. *Journal of General Microbiology* **136**, 2077–2087.
- PERIGO, J. A. & ROBERTS, T. A. (1968). Inhibition of clostridia by nitrite. *Journal of Food Technology* **3**, 91–94.
- PERIGO, J. A., WHITING, E. & BASHFORD, T. E. (1967). Observations on the inhibition of vegetative cells of *Clostridium sporogenes* by nitrite which has been autoclaved in a laboratory medium, discussed in the context of sub-lethally processed cured meats. *Journal of Food Technology* **2**, 377–397.
- PIERSON, M. D. & SMOOT, L. A. (1981). Nitrite, nitrite alternatives and the control of *Clostridium botulinum* in cured meats. *CRC Critical Reviews in Food Science and Nutrition* **17**, 141–187.
- REDDY, D., LANCASTER, J. R., JR & CORNFORTH, D. P. (1983). Nitrite inhibition of *Clostridium botulinum*; Electron spin resonance detection of iron-nitric oxide complexes. *Science* **221**, 769–770.
- RICH, P. R., SALERNO, J. C., LEIGH, J. S. & BONNER, W. D., JR (1978). A spin 3/2 ferrous-NO derivative of an iron containing moiety associated with *Neurospora crassa* and higher plant mitochondria. *FEBS Letters* **93**, 323–326.
- ROBERTS, T. A. & INGRAM, M. (1966). The effect of sodium chloride, potassium nitrate and sodium nitrite on the recovery of heated bacterial spores. *Journal of Food Technology* **1**, 147–163.
- TOMPKIN, R. B., CHRISTIANSEN, L. N. & SHAPARIS, A. B. (1978a). Antibotulinal role of isoascorbate in cured meats. *Journal of Food Science* **43**, 1368–1370.
- TOMPKIN, R. B., CHRISTIANSEN, L. N. & SHAPARIS, A. B. (1978b). Enhancing nitrite inhibition of *Clostridium botulinum* with isoascorbate in perishable canned cured meat. *Applied and Environmental Microbiology* **35**, 59–61.
- TOMPKIN, R. B., CHRISTIANSEN, L. N. & SHAPARIS, A. B. (1978c). The effect of iron on botulinal inhibition in perishable canned cured meat. *Journal of Food Technology* **13**, 521–527.
- UYEDA, K. & RABINOWITZ, J. C. (1971). Pyruvate:ferredoxin oxidoreductase. IV. Studies on the reaction mechanism. *Journal of Biological Chemistry* **246**, 3120–3125.
- VANIN, A. F. & VARICH, V. YA. (1981). Nitrosyl non-heme iron complexes in animal tissues. *Studia Biophysica* **86**, 177–185.
- WOODS, L. F. J. & WOOD, J. M. (1982). A note on the effect of nitrite inhibition on the metabolism of *Clostridium botulinum*. *Journal of Applied Bacteriology* **52**, 109–110.
- WOODS, L. F. J., WOOD, J. M. & GIBBS, P. A. (1981). The involvement of nitric oxide in the inhibition of the phosphoroclastic system in *Clostridium sporogenes* by sodium nitrite. *Journal of General Microbiology* **125**, 399–406.