The Involvement of Nitric Oxide in the Inhibition of the Phosphoroclastic System in *Clostridium sporogenes* by Sodium Nitrite

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The phosphoroclastic system was demonstrated in cell-free extracts of *Clostridium sporogenes* by the production of carbon dioxide, acetyl phosphate, ATP and reduced NAD in the presence of pyruvate. The kinetics of acetyl phosphate production and NAD reduction were investigated. The addition of sodium nitrite to a suspension of *C. sporogenes* in glucose medium resulted in a rapid decrease in intracellular ATP concentration which was accompanied by an accumulation of pyruvate in the medium. This accumulation of pyruvate was caused by inhibition of the phosphoroclastic system by nitrite. Nitrite inhibits this system by reaction of nitric oxide, formed from nitrite, with the non-haem iron of pyruvate:ferredoxin oxidoreductase.

INTRODUCTION

The effect of sodium nitrite on the germination and outgrowth of bacterial spores and growth of food spoilage organisms in complex meat systems has been extensively investigated in relation to the use of nitrite in meat curing (Duncan & Foster, 1968). However, less attention has been given to the effect of nitrite on bacterial growth in simple media systems, particularly its effect on the physiology of the microbial cell.

Of particular interest in this context is the mechanism by which nitrite inhibits the growth of the food pathogen *Clostridium botulinum*. Recent work with aerobic bacteria has shown that nitrite inhibits oxidative phosphorylation causing a decrease in energy-dependent functions such as active transport (Rowe *et al.*, 1979; Yarbrough *et al.*, 1980). This suggested to us that the analogous system, i.e. substrate level phosphorylation, might be the site of action of nitrite in clostridia.

Some enzymes of glucose fermentation in *C. perfringens*, namely glyceraldehyde-3-phosphate dehydrogenase and aldolase, have been shown to be inhibited by nitrite (O'Leary & Solberg, 1976). However, the concentration of nitrite required (15 mM) was greater than that known to inhibit the growth of *C. sporogenes* (1.4 mM at pH 6.0) (Duncan & Foster, 1968). An important source of ATP in clostridia is the phosphoroclastic system in which pyruvate is oxidized to acetate, with carbon dioxide and hydrogen being formed as by-products. We report here the effects of nitrite on this system in *C. sporogenes* and propose a mechanism to explain these effects.

METHODS

Preparation of cell-free extracts. *Clostridium sporogenes* (mixed culture, NCIB 8053 and 10696) was grown in Oxoid nutrient broth no. 2 containing 1% (w/v) glucose autoclaved at 121 °C for 15 min. Prior to inoculation the medium was placed in a boiling-water bath for 1 h and then cooled rapidly. Anaerobic conditions were achieved by

the addition of 5 ml sterile 10% (w/v) sodium thioglycollate per litre medium. The medium was inoculated with 10 ml suspension of actively growing clostridia [grown for 21 h at 37 °C in 1% (w/v) glucose nutrient broth] per litre and incubated at 37 °C for 21 h. The cells were then harvested by centrifugation at 10000 g_{av} for 5 min and washed once in 0.1 M-potassium phosphate buffer, pH 6.5, to yield about 4 g cells (wet wt) per litre medium. The washed cells were suspended in 15 ml buffer and placed in an X-press (Biotec, Croydon, U.K.) previously cooled to -30 °C. The cells were disrupted by making three passages through the X-press and, once the frozen pellet had thawed, cellular debris was removed by centrifugation for 15 min at 10000 g_{av} at 4 °C. The clear brown supernatant was decanted, saturated with oxygen-free nitrogen and stored in a stoppered tube in ice. All manipulations were performed, as far as possible, under anaerobic conditions on ice; cell suspensions and buffers were saturated with oxygen-free nitrogen.

Analytical methods.

Gas production: Gas production was measured by standard manometric techniques using Warburg manometers (Umbreit *et al.*, 1957) incubated at 37 °C in a Series III Warburg apparatus (Townson & Mercer, Croydon, U.K.).

Acetyl phosphate: Acetyl phosphate production from pyruvate was measured by a method adapted from that of Lipmann & Tuttle (1945). Cell-free extract, pyruvate and buffer were incubated together at room temperature in 1 ml total volume for 2 min. The production of acetyl phosphate was terminated by the addition of 1 ml of ice-cold neutral hydroxylamine hydrochloride (made by mixing equal volumes of 4 M-hydroxylamine hydrochloride and 3.5 M-NaOH). This mixture was incubated at room temperature for 10 min to allow the formation of acetylhydroxamic acid. The solution was then deproteinized and a purple complex was formed with Fe(III) by adding 1 ml each of 2.5 M-HCl, 0.74 M-trichloroacetic acid (TCA) and 0.19 M-ferric chloride in 0.1 M-HCl. Absorbance was measured at 500 nm (1 cm path length; Pye Unicam SP6-350 visible spectrophotometer). The assay was calibrated using a standard curve from 0 to 300 nmol acetyl phosphate ml⁻¹. Protein concentrations were determined by the Lowry method using bovine serum albumin as the standard.

ATP: ATP was measured by means of an LKB Luminometer 1250 using LKB ATP monitoring reagent consisting of standardized purified luciferin-luciferase (Myhrman *et al.*, 1978). To measure ATP production by the cell-free system, 0.1 ml ATP monitoring reagent was added to 0.6 ml 0.1 M-phosphate buffer, pH 6.5 and 0.2 ml cell-free extract. When a steady baseline was achieved (usually after 1–2 min) pyruvate was added and the increase in luminescence due to ATP production recorded.

To measure the ATP content of whole cells, the suspension was first diluted 10⁻³-fold with sterile water and then a sample was mixed with an equal volume of NRB reagent (Nucleotide Releasing Agent for Bacterial Cells; Hermitage Instrumentation, Wimborne Minster, Dorset), to release ATP from the cells. A sample of this solution (0.1 ml) was added to 0.2 ml ATP monitoring reagent and 0.7 ml 0.02 M-borate buffer, pH 7.4 and the luminescence was recorded. The system was calibrated using standards containing from 1.6 × 10⁻¹⁰ to 1.6 × 10⁻¹⁴ mol ATP ml⁻¹.

For the results presented in Fig. 1, the cells from 1 litre of medium were resuspended in 100 ml nutrient broth containing 1% (w/v) glucose (pH 6.0) and incubated at 37 °C. When ATP was being actively produced and pyruvic acid concentrations had stabilized, solid sodium nitrite was added to give a final concentration of 4.4 mM.

Pyruvic acid and glucose: Pyruvic acid was estimated by means of a pyruvic acid determination kit (Sigma) in which the decrease in NADH concentration catalysed by lactate dehydrogenase was measured at 340 nm. For the results presented in Fig. 1, pyruvic acid was measured by first adding 1 ml of cell suspension to 1 ml of ice-cold 12% (w/v) TCA (to prevent further accumulation of pyruvate) and then adding 0.5 ml of this solution to 2 ml 0.1 M-phosphate buffer, pH 6.5 and 0.5 ml 0.66 mM-NADH solution (0.11 mM final concentration). The amount of pyruvic acid was determined by recording the decrease in A_{340} when 50 units of lactic dehydrogenase was added. The system was calibrated using a standard solution containing 0.4 mg pyruvic acid ml⁻¹ (0.46 μmol ml⁻¹). The suspension contained $1.3 \pm 0.3 \times 10^9$ cells ml⁻¹ ($n = 18$) as determined using a haemocytometer (Weber Scientific Instruments, Lancing, U.K.). There was no increase in the number of cells during the course of the experiment. The glucose concentration of the TCA-treated samples was measured by means of glucose single assay vials (Sigma).

NAD reduction: The pyruvate-dependent reduction of NAD catalysed by the cell-free extract was measured by the increase in absorbance at 340 nm. A molar extinction coefficient of 6.22×10^3 l mol⁻¹ cm⁻¹ was used. The pyruvate concentration, unless otherwise stated, was 0.6 mM and 20–30 mg protein from the cell-free extract in a final volume of 2.5 ml were used. No additional cofactors were required; incubations were carried out at room temperature (20 °C).

Nitric oxide: Nitric oxide was measured by a chemiluminescence method (Glover, 1975) using a British Oxygen Luminox 101A chemiluminescence analyser. Samples were injected into boiling 0.5 M-NaOH to prevent non-enzymic production of nitric oxide.

DEAE-cellulose chromatography. The separation of the acidic protein, ferredoxin, from the remainder of the protein in the cell-free extract was achieved by DEAE-cellulose chromatography. This technique is well established

as the first step in the purification of clostridial ferredoxins (Mortenson *et al.*, 1962). The 10000 g_{av} supernatant (about 15 ml) from the disruption of the cells from 1 litre of medium was passed through a Whatman DE52 column (0.5 $\times$ 1 cm; volume approximately 0.5 ml) previously equilibrated with 0.1 M-phosphate buffer, pH 6.5. Only a small column was required since the ferredoxin was bound strongly by the column to form a dark brown band 2–3 mm deep; the remainder of the protein passed through the column and was collected. After washing with 0.1 M-phosphate buffer the ferredoxin was eluted from the column using 5 ml 0.75 M-phosphate buffer, pH 6.5. A typical recovery from the column, as measured by the total protein in the two fractions, was 70%.

The reconstituted system, capable of pyruvate-dependent NAD reduction, consisted of 1 ml 0.1 M-phosphate buffer, pH 6.5, 1 ml protein fraction (in the same buffer) and 0.2 ml ferredoxin fraction in 0.75 M-phosphate buffer, pH 6.5. The reaction was started by adding 0.2 ml 7.3 mM-sodium pyruvate in 0.1 M-phosphate buffer (final concentration of 0.6 mM-pyruvate).

Chemicals. Sodium pyruvate, hydroxylamine hydrochloride, acetyl phosphate, bovine serum albumin, Folin & Ciocalteu's reagent, NAD, NADP and ferredoxin purified from *Clostridium pasteurianum* were obtained from Sigma. Oxygen-free nitrogen and nitric oxide were obtained from BOC, Special Gases, Deer Park Road, London.

RESULTS

Effect of nitrite on whole cells

When sodium nitrite (4.4 mM final concentration) was added to cultures of *C. sporogenes* growing in nutrient broth containing glucose there was a rapid decrease in the intracellular concentration of ATP and a marked increase in the pyruvate concentration of the medium (Fig. 1). Gas chromatographic analysis of the medium showed that during a 2 h incubation nitrite also caused a decrease in the amount of acetate produced from 0.5 to 0.2 nmol per 10^6 cells. The accumulation of pyruvate shows that nitrite was inhibiting the phosphoroclastic system.

Carbon dioxide and hydrogen were produced by *C. sporogenes* at similar rates over a 60 min incubation. The presence of hydrogen as a product of glucose fermentation was confirmed by gas chromatography. Addition of 1.5 mM-sodium nitrite to the culture inhibited the rate of carbon dioxide production by 56% and hydrogen production by 48%, as determined by Warburg manometry.

In addition, nitrite inhibited the uptake of glucose by the clostridia. Organisms growing in nutrient broth containing 1% (w/v) glucose were harvested and resuspended in medium containing 0.1% (w/v) glucose and either 4.4 mM-sodium nitrite or 20 mM-sodium fluoride. Analysis of the amounts of glucose remaining in the medium showed that uptake was complete after 30 min in control cultures while the presence of nitrite caused a 50% inhibition of this rate. Complete inhibition of glucose uptake was obtained with fluoride, presumably as a consequence of the inhibition of enolase (Bücher, 1955).

Studies with cell-free extracts

Carbon dioxide production from pyruvate. The cell-free extract of *C. sporogenes* incubated at 37°C with 23 mM-pyruvate produced gas at a constant rate for over 30 min, after an initial lag phase of 5 min, as measured by Warburg manometry. This gas was completely absorbed by potassium hydroxide and was therefore tentatively identified as carbon dioxide. No hydrogen was produced indicating the absence of hydrogenase activity.

Kinetics of acetyl phosphate production. The kinetics of acetyl phosphate production from pyruvate by cell-free extracts from *C. sporogenes* was investigated without the addition of cofactors such as thiamin pyrophosphate, coenzyme A or NAD (Wolfe & O'Kane, 1953). The Michaelis constant for pyruvate was determined as 2.4 mM and the maximum rate was 37 nmol acetyl phosphate min^{-1} (mg protein) $^{-1}$. The rate of acetyl phosphate production was not inhibited by sodium nitrite up to 24 mM.

ATP production. The rate of ATP production in a cell-free system was 0.14×10^{-12} mol ATP min^{-1} (mg protein) $^{-1}$ at a pyruvate concentration of 0.7 mM. Sodium nitrite (16 mM) caused 30% inhibition of ATP production.

Fig. 1. Effect of 4.4 mM-nitrite added (at time 0) to a culture of *C. sporogenes* incubated at 37 °C: ○, intracellular ATP concentration; ●, pyruvate concentration in the medium.

Kinetics of NAD reduction. The addition of pyruvate to the cell-free system resulted in the reduction of endogenous NAD. There was an initial delay in NAD reduction presumably due to a requirement to accumulate reduced intermediates before reduction could occur. The reaction ceased after about 4 min. It could not be stimulated by the addition of further pyruvate, whereas addition of NAD resulted in a rapid increase in the amount of reduced NAD. Subsequent addition of NADP caused a rapid decrease in absorbance at 340 nm.

The enzyme system responsible for NAD reduction was very labile: 50% of the activity disappeared after 4.5 h at 0 °C even under anaerobic conditions and overnight storage at -20 °C resulted in complete loss of activity. The ability to produce ATP was also lost after storage at -20 °C. This is in contrast to the acetyl phosphate-producing activity which was stable to storage under these conditions. NAD reduction was also sensitive to oxygen: bubbling air through the system caused both a decrease in the rate and an increase in the delay before NAD reduction.

The maximum rate of NAD reduction by the cell-free extract [$4 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] was obtained at a pyruvate concentration of 0.4 mM. A plot of rate against pyruvate concentration was sigmoidal indicating that the rate-determining enzyme in NAD reduction is an allosteric enzyme.

The pyruvate-dependent reduction of NAD was inhibited by sodium nitrite: 8 mM-nitrite caused a 50% inhibition of rate while the lag period increased with increasing nitrite concentration.

Site of action of nitrite and nitric oxide

Ferredoxin was separated from other proteins in the cell-free extract by DEAE-cellulose chromatography. Subsequently, the pyruvate-dependent NAD-reducing activity could be reconstituted. Increasing the amount of ferredoxin in the reconstituted system caused an increase in the rate of NAD reduction and a decrease in the length of the lag period. The system could also be reconstituted using a commercially available ferredoxin purified from *C. pasteurianum*.

Sodium nitrite inhibited the reconstituted system to the same extent as the cell-free system. Nitrite did not react directly with ferredoxin since preincubation of this fraction with 6 mM-nitrite, which would produce 40% inhibition in the whole system, caused less than 20% inhibition. This low inhibition was due to the nitrite carried over during subsequent reconstitution resulting in a final nitrite concentration of 0.6 mM.

Table 1. *Effect of incubating cells with nitrite on the reconstituted phosphoroclastic system*

Cells were incubated with 4.4 mM-nitrite at 37 °C for 1 h (or left untreated) before disruption and separation of the ferredoxin and protein fractions. The phosphoroclastic system was reconstituted from the fractions indicated.

Protein fraction	Ferredoxin fraction	Maximum rate of NAD reduction [nmol min ⁻¹ (mg protein) ⁻¹]	Total protein (mg)	Ferredoxin protein (mg)
Control	Control	0.86	71.5	1.0
Control	Nitrite-treated	0.16	71.0	0.5
Nitrite-treated	Control	0.00	58.0	1.0
Nitrite-treated	Nitrite-treated	0.00	57.5	0.5

The effect of incubating *C. sporogenes* cells in medium containing 4.4 mM-sodium nitrite on the two parts of the reconstituted system was investigated. Cells grown in the normal way were resuspended in fresh medium adjusted to pH 6.0 containing nitrite and incubated at 37 °C for 1 h. They were then disrupted and the ferredoxin and protein fractions were separated by DEAE-cellulose chromatography. The nitrite-treated 10000 *g*_{av} supernatant was much paler than the control and when the systems were reconstituted no NAD-reducing activity could be found with the nitrite-treated protein fraction even with control ferredoxin (Table 1). The nitrite-treated ferredoxin fraction contained less protein than did the control but retained some activity.

Nitric oxide caused an inhibition of NAD reduction in the reconstituted system. When nitric oxide was passed through the ferredoxin fraction prior to reconstitution there was a 60% decrease in the rate of NAD reduction. Passing nitric oxide through the protein fraction caused a 40% decrease in NAD reduction.

Nitric oxide is produced by the cell-free system in the presence of sodium nitrite. When cell-free extract (24 mg protein) was incubated with 0.6 mM-pyruvate and 40 mM-sodium nitrite at room temperature, 420 nmol nitric oxide were produced in 3 min. The total amount of nitrite present was 120 µmol, and so total conversion was 0.4%.

Nitric oxide production from sodium nitrite is increased by the presence of ascorbic acid (Evans & McAuliffe, 1956; Riha & Solberg, 1973). In an experiment in which 1 mM-ascorbate was included in a cell-free system containing 5 mM-sodium nitrite, the inhibition of NAD reduction increased from 31 to 53%.

DISCUSSION

The pyruvate-dependent production of carbon dioxide, acetyl phosphate and ATP with the concomitant reduction of NAD demonstrates the presence of the phosphoroclastic system in cell-free extracts of *C. sporogenes*. Hydrogenase activity appeared, however, to be absent from this system. The production of carbon dioxide and hydrogen by cell-free extracts in the presence of pyruvate has been measured in several clostridial species including *C. butylicum* (Koepsell & Johnson, 1942), *C. butyricum* (Wolfe & O'Kane, 1953) and *C. botulinum* (Simmons & Costilow, 1962). Hydrogenase activity has been measured by production of hydrogen from dithionite-reduced ferredoxin (Valentine & Wolfe, 1963). In the present study, however, an acidic gas, absorbed by potassium hydroxide, was produced by cell-free extracts in the presence of sodium dithionite. This gas was tentatively identified as sulphur dioxide.

In cultures, however, *C. sporogenes* produced hydrogen and carbon dioxide at an equal rate and volume which suggests that hydrogenase is present *in vivo*. Holdeman *et al.* (1977) also demonstrated production of hydrogen from glucose by *C. sporogenes*. In view of the sensitivity of hydrogenase to oxygen and its instability (Orme-Johnson & Orme-Johnson,

1978; Klivanov *et al.*, 1979) we suggest that this enzyme is present in *C. sporogenes* but is destroyed during preparation of the cell-free extract.

In the present work with cell-free extracts, NAD reduction was used as a measure of the activity of the phosphoroclastic system. However, this reduction of NAD may also represent an alternative physiological fate for the reducing power liberated by the phosphoroclastic system, other than hydrogen. Thauer *et al.* (1969) demonstrated the production of hydrogen from reduced NAD by hydrogenase via ferredoxin in *C. kluyveri* which suggests that reduced NAD and hydrogen are alternative products from reduced ferredoxin.

In stationary phase cultures, fermentative organisms normally produce surplus reducing power and the evolution of hydrogen would represent disposal of this surplus in clostridia. Alternatively, reduction of pyruvate to lactate, butyrate, succinate, propionate, butanol and ethanol, known products of clostridial glucose fermentation (Brooks & Moore, 1969), may also be a means for regeneration of NAD. During outgrowth of the germinated spores, however, reducing power may be required for several purposes, such as adjustment of the redox potential of the medium, detoxication of oxygen by NADH oxidase (O'Brien & Morris, 1971) and in the synthesis of various cell constituents. In these circumstances the phosphoroclastic system (and glycolysis) would supply two components essential for the development of the cell, i.e. ATP and reducing power, and thus most of the cell's reducing power may be used via NAD.

The effects produced in cultures of *C. sporogenes* by 4.4 mM-nitrite with respect to the accumulation of pyruvate during continued utilization of glucose indicate that the major site of action of nitrite, at this concentration, is the phosphoroclastic system. O'Leary and Solberg (1976) have shown that nitrite at a concentration of 15 mM will inhibit enzymes of glucose fermentation, namely glyceraldehyde-3-phosphate dehydrogenase and aldolase, in *C. perfringens*. Yarbrough *et al.* (1980) found that aldolase from aerobic organisms such as *Escherichia coli* and *Pseudomonas aeruginosa* was inhibited by nitrite at concentrations in the range 10 to 100 mM. It therefore seems likely that the effect of nitrite on glucose utilization which we have observed is not due to a direct effect of nitrite on the enzymes of glycolysis and is more likely to be due to a feedback inhibition of these enzymes by the accumulated pyruvate. An inhibitory effect of pyruvate on glucose utilization has been demonstrated in a number of micro-organisms including *Escherichia coli* (Morgan & Kornberg, 1967), *Neurospora crassa* (Mattoo & Rao, 1974) and *Aspergillus nidulans* (Desai & Modi, 1977).

The rapid decrease in intracellular ATP concentrations after addition of nitrite (Fig. 1) may appear out of proportion to the theoretical 50% contribution to ATP production made by the phosphoroclastic system. However, the ATP pool within the cell is undergoing rapid turnover in maintaining ionic composition (Stouthamer, 1973), flagellar movement (Holwill, 1977) and in synthetic pathways within the cell; once ATP production drops below a certain threshold value the ATP pool would be rapidly depleted.

The experiments with cell-free extracts indicated that the observed inhibition of the phosphoroclastic system occurred by means of nitric oxide. Nitric oxide, a potent iron-ligand that forms co-ordination complexes with iron (McDonald *et al.*, 1965; Woolum *et al.*, 1968), could inhibit the phosphoroclastic system at either of the two non-haem iron moieties present in this system. The first of these moieties is present in ferredoxin, but we have shown that nitrite does not react directly with ferredoxin although there is a decreased activity in ferredoxin from cells preincubated with nitrite (Table 1). The second non-haem iron forms part of pyruvate:ferredoxin oxidoreductase, an enzyme which has been purified and characterized from *C. acidithiobacillus* by Uyeda & Rabinowitz (1971). This enzyme consists of a single protein molecule containing thiamin pyrophosphate and a non-haem iron chromophore. Reaction of nitric oxide with this non-haem iron would cause inhibition of the phosphoroclastic system especially the electron transport involved in NAD reduction and hydrogen production.

In cell suspensions nitrite inhibits acetate and ATP production from glucose but in cell-free extracts acetyl phosphate production is not inhibited although there is an effect on ATP production *in vitro*. It would therefore appear that in short-term *in vitro* experiments the inhibitory action of nitrite on the phosphoroclastic system is not as evident as *in vivo*.

From studies of the inhibition of clostridia in nitrite-cured meat systems Tompkin *et al.* (1978) hypothesized that nitrite inhibited *C. botulinum* by the reaction of nitric oxide, known to be produced in cured meats, with an iron-containing protein in the cell, probably ferredoxin. Our results suggest that, while ferredoxin can react with nitric oxide *in vitro*, the main site of inhibition *in vivo* is pyruvate:ferredoxin oxidoreductase.

In conclusion, nitrite is a potent inhibitor of many species of micro-organisms, both aerobic and anaerobic, and whilst it appears that the active inhibitory agent outside the cell is closely correlated with nitrous acid (Castellani & Niven, 1955), the mechanism of action may vary for different physiological types of micro-organism (Yarborough *et al.*, 1980). The results presented in this paper suggest that for *C. sporogenes* an important mechanism of nitrite inhibition is by formation of a nitric oxide complex with the non-haem iron of pyruvate:ferredoxin oxidoreductase. Further work is currently underway to extend our studies to *C. botulinum*.

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