APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1976, p. 208-212
Vol. 31, No. 2

Printed in U.S.A.

Effect of Sodium Nitrite Inhibition on Intracellular Thiol Groups and on the Activity of Certain Glycolytic Enzymes in *Clostridium perfringens*[1]

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Received for publication 6 October 1975

Activities of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (GAP-DH) and aldolase (EC 4.1.2.13) in cells of *Clostridium perfringens* that had been inhibited with sodium nitrite were investigated. A complete loss in GAP-DH activity and a 67% decrease in aldolase activity were observed when growth of *C. perfringens* was inhibited. There was also a 91% decrease in the concentration of free sulfhydryl groups of soluble cellular components. Dithiothreitol restored some activity to inactive GAP-DH from sodium nitrite-inhibited cells, indicating that a loss of reduced sulfhydryl groups was involved in the inactivation of the enzyme. The evidence presented suggests that sodium nitrite inhibition of *C. perfringens* may involve an interaction of sodium nitrite as nitrous acid with sulfhydryl-containing constituents of the bacterial cell.

One of the important functions of sodium nitrite, an essential meat-curing ingredient, is the inhibition of microbial growth, especially that of the clostridia. Although sodium nitrite inhibition of clostridia is well documented (9, 17, 18, 20), the mechanism of inhibition has not yet been elucidated. Bacterial inhibition by sodium nitrite at acid pH was reported by Tarr (24) and has since been confirmed by a number of authors (3, 5, 20) who have shown that the inhibitory action of nitrite increases as pH is decreased. At acid pH levels, sodium nitrite exists as nitrous acid, an extremely reactive molecule capable of interaction with a wide variety of substances including myoglobin, ascorbic acid, phenols, secondary amines, amino groups, and thiol groups. Nitrous acid also has mutagenic effects (4). The concentrations of sodium nitrite used to produce the mutations vary from 0.5 M (15) to 2.0 M (4), which are at least 100 times greater than the levels that are generally considered inhibitory to growth of a variety of microorganisms (5, 17, 20). Mirna and Hofmann (16) reported that, although sodium nitrite reacts with both sulfhydryl (SH) groups and primary amino groups at pH 5.5, the reaction with SH groups is more rapid. The reactivity of sodium nitrite, as nitrous acid, suggests that inhibition of bacteria by sodium nitrite at acid pH may involve interaction of nitrous acid with some bacterial cellular constituent. Riha and Solberg (20) proposed that filter-sterilized sodium nitrite inhibition of *Clostridium perfringens* may be due to a reaction of sodium nitrite, as nitrous acid, with SH-containing constituents of the bacterial cell. Our studies were designed to explore the response of intracellular SH groups of *C. perfringens* to nitrite inhibition and to monitor the activity of certain SH-containing enzymes of the glycolytic pathway in sodium nitrite-inhibited cells.

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MATERIALS AND METHODS

Chemicals. Phosphoglycerate kinase (EC 2.7.2.3) (PGK), L-glycerolphosphate dehydrogenase (EC 1.1.99.5), triose phosphate isomerase (EC 5.3.1.1), fructose-1,6-diphosphate, and glycerate-3-phosphate were purchased from the Boehringer-Mannheim Corp. Adenosine 5'-triphosphate and triethanolamine-HCl were purchased from Sigma Chemical Co., and reduced nicotinamide adenine dinucleotide (NADH), 5,5-dithiobis-2-nitrobenzoic acid, and dithiothreitol (DTT) were purchased from the Nutritional Biochemicals Corp.

Microorganism. *C. perfringens* NCTC 8797 was the organism used for sodium nitrite inhibition studies. Stock cultures of the organism were maintained in cooked meat medium (Difco) at 4 C.

Media. R & S medium (19) adjusted to pH 6.3 and filter-sterilized using type GS 0.22-μm pore size micropore filters (Millipore Corp.) was the

basal medium used for growth and sodium nitrite inhibition of *C. perfringens*.

Sodium nitrite inhibition of C. perfringens and preparation of cell extracts. *C. perfringens* NCTC 8797 was grown at least twice in fluid thioglycolate medium (Difco) for 24 h at 37 C and then for 6 h at 43 C. The 6-h culture was centrifuged at 12,000 × *g* for 5 min at 22 C using an MSE high-speed 25 centrifuge. The packed cells were washed twice with sterile 0.1% peptone water and resuspended in 10 ml of the same medium. A 1-ml volume of this suspension was used to inoculate 100 ml of R & S medium. The inoculated flasks were incubated in a 43 C water bath until the exponential phase of growth was reached at 10 to 12 h. The contents of the flasks were centrifuged at 12,000 × *g* for 5 min at 22 C. The packed cells were resuspended in sterile 0.1% peptone water using 10 ml of peptone water for every 100 ml of growth medium that had been centrifuged. A 10-ml volume of this suspension was used to inoculate 1 liter of R & S medium containing 1,000 μg of sodium nitrite per ml. The initial cell concentration in the inoculated flasks was about 10^7 cells/ml. The inoculated flasks were incubated at 43 C for 12 to 14 h, and then the cells were harvested by continuous centrifugation at 15,000 × *g* at room temperature in a Sorvall super-speed centrifuge (type SS-1) adapted with a Szent-Gyorgyi and Blum continuous-flow system. The harvested cells were washed two to three times with 0.2 M triethanolamine-HCl buffer (pH 7.6) containing 10^{-3} M $MgCl_2$ and then frozen at −20 C. Confirmation that growth of *C. perfringens* had been inhibited by the sodium nitrite was made by plating of the cultures on SPS agar (Difco) prior to harvesting the cells. SPS plates were incubated under nitrogen at 37 C and examined for absence of growth at 18 to 24 h. Nitrite-inhibited cultures were also analyzed for the presence of survivors by incubating 1-ml aliquots of the culture in fluid thioglycolate medium at 37 C and visually inspecting for growth at 24 to 72 h.

Control cells of *C. perfringens* were prepared by growing the organism for 12 to 14 h in R & S medium containing no nitrite, and the harvesting was carried out as described for nitrite-inhibited cells.

Cell extracts of control and nitrite-inhibited cells were prepared as follows. Frozen cells were suspended in 0.2 M triethanolamine-HCl buffer (pH 7.6) containing 10^{-3} M $MgCl_2$ under a nitrogen atmosphere in a Sta-Safe glove box chamber (GF Supply Division Standard Safety) that had been evacuated and flushed three times with nitrogen gas. The cell suspension (50 mg of cells [wet weight] per ml) inside the chamber was flushed with nitrogen gas for 5 min, and then the cells were disintegrated with a Branson Sonifier (model W140/W155) using a sealed stainless-steel water-jacketed processing vessel that was water-cooled at 4.0 C during sonic treatment. Sonic oscillation was for 6 min at 120 W. The solution containing the disintegrated cells was transferred to a centrifuge tube in the nitrogen-filled chamber and flushed with nitrogen gas for 3 min, and the tube was then sealed. The cell contents were centrifuged at 40,000 × *g* at 6 C for 20 min in an MSE high-speed centrifuge to remove whole cells and cellular debris. The supernatant containing the soluble cell fraction was transferred to a test tube in the nitrogen-filled chamber and flushed with nitrogen gas for 3 min, and the tube was then sealed with a rubber septum. The soluble extracts were stored overnight at 4 C prior to the assay for enzyme activity to minimize interference from a very active NADH oxidase that was present in the freshly prepared cell extracts but not in stored extracts. Storage overnight had no effect on the activity of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; GAP-DH) and aldolase (EC 4.1.2.13).

Enzymes assays. Enzyme activity of GAP-DH in cell extracts of *C. perfringens* was determined by using an NADH-coupled reaction (2) modified so that ethylenediaminetetraacetate was omitted from the assay medium and the amount of phosphoglycerate kinase was doubled.

NADH oxidase in cell extracts of *C. perfringens* was determined by following the progress of NADH oxidation at 340 nm in a Beckman DBG spectrophotometer (2). Aldolase was assayed by the method of Groves and Gronlund (11), except that triethanolamine-HCl buffer adjusted to pH 7.6 was substituted for tris(hydroxymethyl)aminomethane and cysteine-HCl was omitted from the assay.

In all assays of enzyme activities in the cell extracts of *C. perfringens*, the assay media were purged with nitrogen prior to the addition of cell extract and the assays were carried out in sealed spectrophotometer cells. This was done to reduce the interference from NADH oxidase, which was active in the presence of oxygen (11). Overnight storage of the extracts under nitrogen also helped to minimize the endogenous NADH oxidase activity. NADH oxidase activity in cell extracts of *C. perfringens* was subtracted from the GAP-DH and aldolase activities of the extracts to correct for endogenous NADH oxidation.

SH determinations. Free SH group determinations in cell extracts of *C. perfringens* were made by the method of Ellman (8).

Protein determinations. Protein determinations were made by the method of Lowry et al. (14), with bovine serum albumin as a standard.

Effect of DTT on C. perfringens inhibition by sodium nitrite. Aliquots (10 ml each) of R & S medium, containing 0, 100, 150, 160, 170, 180, 200, or 250 μg of sodium nitrite per ml, were pipetted into sterile test tubes, with six replicates for each nitrite concentration. Half of the tubes contained 0.1 ml of micropore filter-sterilized DTT solution. Each tube was inoculated with 0.1 ml of a *C. perfringens* suspension to give an inoculum level of approximately 10^2 cells/ml. The tubes were incubated at 43 C and examined visually for growth from 24 to 72 h.

RESULTS

C. perfringens cultures that were incubated with sodium nitrite and were subsequently unable to form colonies on sodium nitrite-free

SPS agar were able to grow in fluid thioglycolate medium at 37 C in a few cases.

When sodium nitrite-inhibited *C. perfringens* cells were harvested, it was observed that these cells were different in certain physical characteristics from control cells. The inhibited cells were dark gray to brown in color, whereas control cells were light tan. The darker color of the nitrite-inhibited cells appeared to be associated with the cell wall or membrane since the color was localized in the insoluble particulate fraction when the cells were fractionated. In addition, inhibited cells exhibited a different cellular mass consistency and were harder to disperse in buffer. When these cells were examined with a microscope at ×1,000, no apparent cellular changes were detected.

The results for GAP-DH and fructose-1,6-diphosphate aldolase activities in soluble extracts of fractionated control and sodium nitrite-inhibited *C. perfringens* cells are presented in Table 1. No GAP-DH activity could be detected in extracts of inhibited cells, and aldolase activity was only 33% of that in the control cells. When 10 mM DTT was included in the assay mixture for GAP-DH, in stored samples (5 C, 30 days), the extracts from inhibited cells demonstrated recovery up to a level equivalent to approximately 18% of the activity in the control samples.

Results of the SH group determinations made on the soluble extracts from fractionated control and sodium nitrite-inhibited *C. perfringens* cells are presented in Table 1. There was a decrease of 91% in the SH concentration of soluble cellular constituents in the inhibited cells.

Since there was a massive loss of free SH groups in the soluble cellular constituents of sodium nitrite-inhibited *C. perfringens* cells and there was a slight recovery of GAP-DH activity when the SH-protecting reagent DTT was added to the assay medium, the effect of DTT as a protector of *C. perfringens* against nitrite inhibition was investigated. The minimum concentrations of sodium nitrite required to inhibit growth of the cells in the presence and absence of DTT were determined. The results show that the concentrations of sodium nitrite required for the inhibition of 10^2 cells of *C. perfringens* per ml in the absence of DTT ranged from 160 to 180 μg/ml. In the presence of DTT, the minimum concentrations of sodium nitrite required for inhibition were in the range of 100 to 160 μg/ml. Thus, 10 mM DTT did not provide any protection against nitrite inhibition.

TABLE 1. *Effect of growth inhibition by sodium nitrite upon various components in the soluble extracts of C. perfringens cells*

Cells	GAP-DH activity (U/mg of protein)	Aldolase activity (U/mg of protein)	SH groups (mmol/g of protein)
Control	1.94	0.227	0.290
$NaNO_2$ inhibited	0.00	0.074	0.0026

DISCUSSION

Damage to the cell wall or membrane indicated by the graying or browning of *C. perfringens* cells incubated with inhibiting concentrations of sodium nitrite may be a primary event that would permit nitrite, in some form, to enter the cell and interact with soluble cellular components or to cause cell lesions. It is also possible that the discoloration could have no influence upon the cell wall or membrane but could be attributable to adsorbed compounds formed as a result of sodium nitrite being present in the medium. However, in a model system employing talcum as the adsorbent, no difference in color was observed between the sodium nitrite-containing and control media. It seems reasonable, however, that since sodium nitrite or nitrous acid, which may be the active form (20), is a small molecule, it could cross the cell membrane and enter the cell. The observation by Riha and Solberg (21) that sodium nitrite disappears during the exponential phase of *C. perfringens* growth when noninhibitory sodium nitrite concentrations are present in a glucose-containing medium suggests that the sodium nitrite enters the cell and is metabolized.

The magnitude of the decrease in the free-SH content of the soluble cellular extract from inhibited *C. perfringens* cells would result in massive disruptions of cell functions. Included would be impairment of those enzymes which contain SH groups essential for their activity. Both GAP-DH and aldolase are such enzymes (1, 7, 12, 22).

Evidence that nitrite disrupts metabolic processes dependent upon enzymatic activity has been presented in the literature. Hiller and Bassham (13) reported that CO_2 fixation in *Chlorella pyrenoidosa* was inhibited by nitrous acid and that the principal site of inhibition appeared to be in the carbon cycle between fructose and sedoheptulose diphosphates and the corresponding monophosphates. The enzyme involved, fructose-1,6-diphosphatase, appears to have SH groups that are important to its conformational integrity. Dainty and Meredith (6) reported that nitrite, at pH 5.5, inhibited the glucose-, pyruvate-, and NADH-dependent O_2 uptakes of washed-cell suspen-

sions of *Microbacterium thermosphactum*, the glucose system being the most sensitive. They speculated that the nitrite effect may be due to the inhibition of one or more of the enzymes involved in glucose catabolism. The ability of DTT to restore some GAP-DH activity suggests that SH groups were involved in inactivation of the enzyme. The reaction between sodium nitrite and SH groups at acid pH, when the nitrite is present as nitrous acid, results in the formation of nitrosothiols and is the basis of the Saville (23) method for the quantitative determination of thiols. Mirna and Hofmann (16) reported that, in model systems containing equimolar amounts of glutathione and sodium nitrite, little loss in glutathione SH groups took place at pH 7.4 even after heating at 100 C for 15 min. At pH 5.0 there was a small loss of SH groups after heating. However, in strong acid solution (pH 2.3), almost all of the thiol groups of glutathione had disappeared, most of them even before the heating. These reports indicate that little nitrite-SH interaction might be expected in R & S medium at pH 6.3. However, in model cured-meat systems, a loss of SH groups in the presence of sodium nitrite occurred at pH levels between 5.6 and 5.8 (16). This could indicate a reaction other than the formation of nitrosothiols. Glazer (10), in a discussion concerning chemical modification of protein groups, noted that, as a consequence of the microenvironment within the folded structure of proteins, functional groups may exhibit reactivities extremely different than those observed with model compounds. For example, the essential SH group of papain is about 30,000 times more reactive with chloroacetate than is the SH group of cysteine at the same pH (25). The interaction of sodium nitrite with thiol groups of proteins at pH values higher than might be expected from work done with model systems may be the result of an increase reactivity of protein thiol groups. Thus, the entry of nitrite as nitrous acid into the bacterial cell could result in interactions between the nitrous acid and protein SH groups. Groves and Gronlund (11) reported that *C. perfringens* metabolizes glucose via the Embden-Meyerhof pathway of glycolysis. Therefore, inactivation of GAP-DH would result in severe restrictions upon the energy metabolism of this organism. The results of the present study suggest that an interaction between nitrite and SH groups is involved in the inhibition of *C. perfringens* growth and that differing reactivities of protein SH groups with nitrite may account for selective inactivation of particular enzyme systems.

The tremendous loss in SH groups of soluble cellular components of *C. perfringens* observed during sodium nitrite inhibition and the partial reactivation of DTT-treated GAP-DH suggested the possibility that an SH-protecting reagent such as DTT might protect the whole cell against sodium nitrite inhibition. The inability of DTT to protect *C. perfringens* cells from nitrite inhibition may have been due to an inability of the DTT to cross the cell membrane.

The occasional outgrowth of sodium nitrite-inhibited cells may be the result of a few surviving cells that, although possibly damaged by the sodium nitrite, as shown by their inability to reproduce on SPS agar, are able to recover and multiply when placed in a sodium nitrite-free maximal growth medium.

It should be pointed out that the cells studied in these experiments were in contact with high levels of sodium nitrite for periods up to 12 h. Thus, there is the possibility that the effects observed were secondary responses and occurred only after the cell had been irreversibly damaged in some other manner.

The elucidation of the mechanism of sodium nitrite inhibition of *C. perfringens* appears to require further study of the effects of sodium nitrite on biochemical processes, including metabolic processes, protein synthesis, and membrane permeability.

ACKNOWLEDGMENT

This study was supported by Hatch Act Funds from the U.S. Department of Agriculture.

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