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Nitrite Inhibition of *Clostridium botulinum*: Electron Spin Resonance Detection of Iron–Nitric Oxide Complexes

Abstract. *Vegetative cells of* Clostridium botulinum *were shown to contain iron-sulfur proteins that react with added nitrite to form iron–nitric oxide complexes, with resultant destruction of the iron-sulfur cluster. Inactivation of iron-sulfur enzymes (especially ferredoxin) by binding of nitric oxide would almost certainly inhibit growth, and thus is probably the mechanism of botulinal inhibition by nitrite in foods.*

Sodium nitrite is widely used as an additive in cured meats, where it has important antimicrobial (*1, 2*) and antibotulinal (*3, 4*) properties. The addition of nitrite to cured meat products has been a source of controversy, however, since nitrite may be a precursor to the carcinogenic nitrosamines, especially in fried, cured meats such as bacon (*5, 6*). A number of compounds have been tested as nitrite substitutes, but no compound having all the desirable properties of nitrite (including color, antioxidant, and antibotulinal properties) has been found (*7*). The search for an effective nitrite replacement has been complicated by the fact that the antibotulinal mechanism of nitrite remains unknown, although several mechanisms have been proposed (*8, 9*).

Mirna (*10*) and van Roon and Olsman (*11*) demonstrated that nitrite via nitrous acid may react with sulfur-containing amino acids. Tompkin *et al.* (*12*) suggested that nitrite may react with ferredoxin, an iron-containing enzyme necessary for energy production in some clostridial vegetative cells (*13*), thus inhibiting growth. Woods and Wood (*14*) showed that nitrite does inhibit the phosphoroclastic reaction, presumably catalyzed by ferredoxin, in *Clostridium sporogenes* and *Clostridium botulinum*, although *C. botulinum* has not been shown to contain ferredoxin. We report here the presence of iron-sulfur centers in *C. botulinum* and the disappearance of these centers with simultaneous formation of nondialyzable iron sulfur–nitric oxide complexes on treatment of vegetative botulinal cells with sodium nitrite and ascorbate.

Untreated, air-oxidized samples of *C. botulinum* exhibited an electron spin resonance (ESR) signal characteristic of a "HiPiP-type" iron-sulfur center (Fig. 1A) (*15*). This axial signal at $g = 2.02$ was not appreciably saturated by 20-mW power at 6.5 K and disappeared when the temperature was increased to 30 K, properties indicative of the presence of Fe_3-S_3* or Fe_4-S_4* centers. A crude sonicated cell suspension of *C. botulinum* would probably have other metal-protein complexes, but these would not exhibit a fast-relaxing axial signal at $g = 2.02$ at liquid helium temperature, which is characteristic for iron-sulfur centers.

Addition of sodium dithionite as a reductant to the preparation resulted in the disappearance of the signal at $g = 2.02$ and the appearance of features characteristic of "$g = 1.94$–type" iron-sulfur centers (Fig. 1B). The feature at $g = 1.94$ also relaxed very rapidly at 7 K and broadened significantly (although it was still present) at 30 K, further confirming that the signal was due to the presence of reduced iron-sulfur centers (*16*). These results demonstrate the presence of iron-sulfur centers in *C. botulinum*.

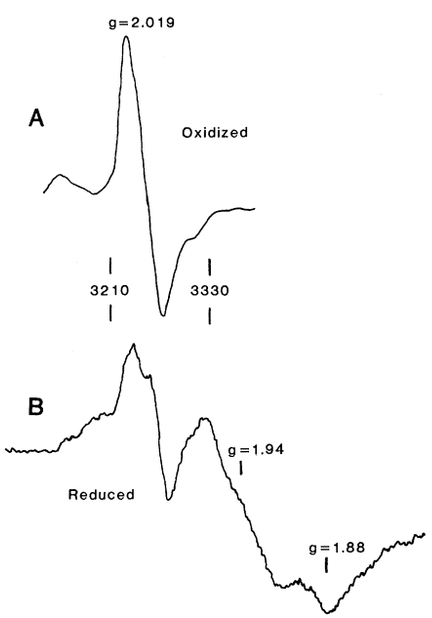

Fig. 1. Electron spin resonance spectra for untreated preparations of *C. botulinum* Type A (American Type Culture Collection). The cells were grown in chopped liver broth (*25*) and then propagated in anaerobic growth medium (*26*) for 1 week at 37°C. Actively growing vegetative cells were centrifuged at 7000*g* for 20 minutes, resuspended in 0.1 percent peptone in water, and centrifuged and suspended twice again to obtain washed, packed cells. Next the cells were sonicated with a microtip probe (Heat Systems–Ultrasonics, Inc.), transferred to ESR tubes, frozen in liquid nitrogen, and stored at 77 K. Instrument settings: microwave frequency, 9.136 GHz; modulation frequency, 100 kHz; and modulation amplitude, 12.5 G (6.5 K). The instrument gain is 5×10^2 in (A) (oxidized) and 8×10^2 in (B) (reduced with excess sodium dithionite). The *x*-axis represents magnetic field in gauss.

Neither untreated (Fig. 1) nor nitrite-treated samples of *C. botulinum* exhibited an ESR signal characteristic of heme centers (high or low spin), consistent with the reported absence of cytochromes in Clostridia (*17, 18*). Figure 2A shows the ESR signal that appeared on addition of 200 ppm sodium nitrite to cell suspensions. This axial signal has a principal upward feature at $g = 2.035$ and is characteristic for nitrosyl complexes of a variety of iron-containing proteins, peptides, and chelate complexes (*19*), including purified succinate dehydrogenase (*20*). Thus, addition of nitrite to preparations of *C. botulinum* vegetative cells results in the formation of iron-nitrosyl complexes.

Figure 2B shows a spectrum for a preparation that was exposed to 500 ppm ascorbic acid in addition to nitrite. The signal is identical in shape to that in Fig. 2A but is approximately eight times more intense (note the difference in instrument gain). This signal did not disappear or decrease in intensity on dialysis with 0.075*M* EDTA in 0.1*M* phosphate buffer (*p*H 7.0). The increase in signal intensity in the presence of ascorbate is in agreement with the observation that nitrite is more inhibitory to microorganisms in the presence of reducing agents (*21*). Thus ascorbate enhances the formation of iron-nitrosyl complexes, probably by accelerating the reduction of nitrite to nitric oxide.

The iron-sulfur signal at $g = 1.94$ observed in untreated samples (upper trace in Fig. 2C) decreased significantly in intensity on treatment with nitrite plus ascorbate (lower trace in Fig. 2C). The loss of the iron-sulfur signal at $g = 1.94$ (Fig. 2C) and the appearance of the iron-nitrosyl signal at $g = 2.035$ (Fig. 2B) on the addition of nitrite and ascorbate suggests that iron-sulfur complexes in botulinal cells react with nitrite to form iron-nitrosyl complexes. This effect is inhibitory, if not lethal, to the organism. Destruction of the iron-sulfur centers of purified succinate dehydrogenase under similar conditions has been reported (*20*).

These findings demonstrate that vegetative cells of *C. botulinum* contain iron-sulfur proteins that react with added nitrite to form iron–nitric oxide complexes, with resultant destruction of the iron-sulfur cluster. Woods *et al.* (*22*) reported findings suggesting such a mechanism, including the formation of small but detectable amounts of nitric oxide on the addition of nitrite to cell-free systems. Inhibition of the phosphoroclastic system was observed on the addition of nitric oxide, but such a result would be expected for any process dependent on iron-sulfur centers (*20*).

Although iron-sulfur center destruction would certainly result in antibotulinal effects, nitrite addition may have other inhibitory actions. In a study by O'Leary and Solberg (*23*), nitrite greatly reduced the concentration of free sulfhydryl groups of soluble cellular components of *Clostridium perfringens* cells, glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.1.12) activity was abolished, and aldolase activity decreased 67 percent.

Yarbrough *et al.* (*24*) showed that nitrite inhibits a variety of biological functions in aerobic bacteria, including active transport, oxygen uptake, and oxidative phosphorylation, consistent with an inhibition of electron transport processes in general by destruction of iron-sulfur centers. Because of the function of iron-sulfur proteins in electron transport and adenosine triphosphate generation in aerobic bacteria, inactivation by binding of nitric oxide would almost certainly inhibit growth, and we conclude that this may be the most important site of nitrite reaction and the basis of the antibotulinal activity of nitrite in foods.

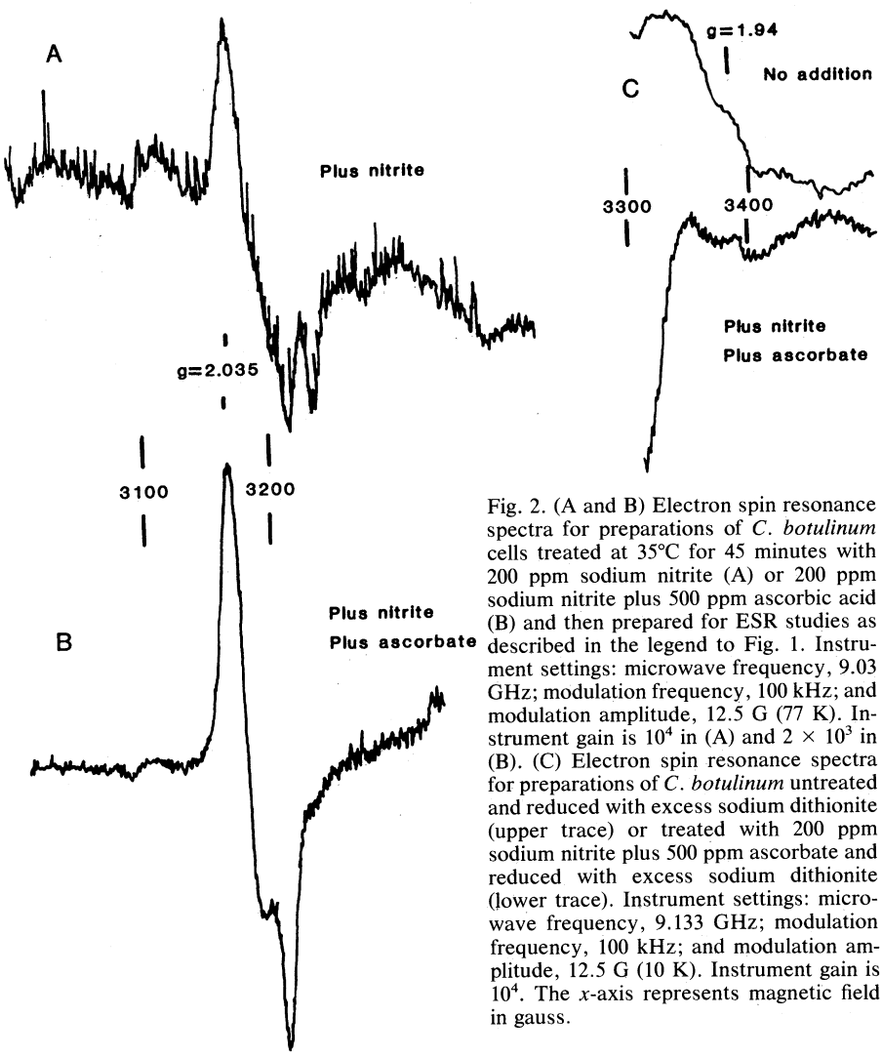

Fig. 2. (A and B) Electron spin resonance spectra for preparations of *C. botulinum* cells treated at 35°C for 45 minutes with 200 ppm sodium nitrite (A) or 200 ppm sodium nitrite plus 500 ppm ascorbic acid (B) and then prepared for ESR studies as described in the legend to Fig. 1. Instrument settings: microwave frequency, 9.03 GHz; modulation frequency, 100 kHz; and modulation amplitude, 12.5 G (77 K). Instrument gain is 10^4 in (A) and 2×10^3 in (B). (C) Electron spin resonance spectra for preparations of *C. botulinum* untreated and reduced with excess sodium dithionite (upper trace) or treated with 200 ppm sodium nitrite plus 500 ppm ascorbate and reduced with excess sodium dithionite (lower trace). Instrument settings: microwave frequency, 9.133 GHz; modulation frequency, 100 kHz; and modulation amplitude, 12.5 G (10 K). Instrument gain is 10^4. The *x*-axis represents magnetic field in gauss.

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