

A Research Note

NITROSAMINES AND THE INHIBITION OF *Clostridia* IN MEDIUM HEATED WITH SODIUM NITRITE

INTRODUCTION

PERIGO et al. (1967) and Perigo and Roberts (1968) described the formation of an inhibitor for the growth of several species of *Clostridium* during the sterilization of a culture medium in the presence of NaNO_2 . They concluded that nitrite reacted with a component of the medium and the inhibitory activity differed from that of nitrite alone. The nature of the inhibitor is not known. It was further shown by Johnston et al. (1969) that in meat suspensions heated with NaNO_2 the inhibitory action was due to NO_2^- , although meat heated for 4 days at 80°C required less NO_2^- to inhibit *Cl. botulinum* than suspensions of meat not receiving the additional heat treatment. The extended heat treatment of the meat may have caused decomposition of the proteins with liberation of amino acids, peptides, and possibly, amines. Furthermore, the medium used by Perigo et al. contains yeast extract, beef extract and enzymic digests of meat thus making it rich in amines and amino acids. Nitrite reacts with amines and amino acids forming N-nitroso compounds, either N-nitrosamines or N-nitrosamides which are toxic and carcinogenic to animals and mutagenic to various species of microorganisms (Adelberg et al., 1965; Marquardt et al., 1963). We investigated the possibility that the "Perigo inhibitor" might be certain volatile N-nitroso compounds.

MATERIALS & METHODS

THE MEDIUM of Perigo et al. (1967) was used at pH 7.0 to reduce possibility of interference by HNO_2 at lower pH levels. Tryptone, peptone, yeast extract, and beef extract were obtained from Difco Labs. Sodium nitrite was added to tubes of medium so that, by doubled dilution, levels ranging from 640 to 1.25 ppm were obtained. In one set of conditions the NO_2^- was autoclaved in the medium at 15 psig for 15 min; in a second set of conditions the medium and NO_2^- solutions were autoclaved separately, then mixed aseptically. *Cl. botulinum* B1218 (USDA culture) was grown at 35°C for 24 hr in thioglycollate medium (Difco), diluted 10-fold with water, and one drop added to each tube containing 4 ml of the Perigo medium. The medium was overlaid with Vaspar and gentle heating used to drive out excess air. The tubes were incubated at 35°C

for 18 hr and the minimum inhibitory concentration (MIC) taken as the tube in which there was no visible growth or gas production.

To analyze for the presence of nitroso compounds a batch of medium was autoclaved at 15 psig for 20 min, NO_2^- added at 640 ppm and the whole re-autoclaved at 6 psig for 20 min. 200 ml of heated culture medium was extracted 2x with 200 ml each time of CH_2Cl_2 after saturation with NaCl. The combined extracts were treated with anhydrous Na_2SO_4 and concentrated to 5 ml in a Kuderna-Danish concentrator using a steam bath. The solution was washed 2x with 5 ml each 0.1M HCl to remove some interfering compounds, dried with anhydrous Na_2SO_4 and concentrated to 0.5 ml. The concentrate was analyzed for certain volatile nitrosamines by gas chromatography, using a Varian Model 1740 instrument equipped with two 9 ft x 1/8 in. OD stainless steel columns packed with 15% Carbowax 20M-TPA on 60-80 Gas-Chrom P. The standard flame ionization detector was modified for use as an alkali flame ionization detector as described by Howard et al. (1970). Flow conditions were: helium, 58; hydrogen, 45; and air, 188 ml/min. Injector port and detector temperatures were 190° and 250°C , respectively. Column temperature was programmed from $115-185^\circ\text{C}$ at $4^\circ/\text{min}$.

Confirmation of nitrosamines was carried out with the same gas chromatograph connected to a DuPont Model 21-492 mass spectrometer. The GC column was maintained at 115° and injection port and detector temperatures at 200° and 230° respectively. The column effluent was split 1:1 and passed into the mass spectrometer via an inlet line heated to 200°C . The mass spectra were obtained at an ionizing voltage of 70 ev and an ion source temperature of 200°C .

RESULTS & DISCUSSION

THE RESULTS OBSERVED on growing the *Cl. botulinum* in the nitrite-treated media confirmed the observations of Perigo et al. (1967) and Perigo and Roberts (1968). The MIC of NO_2^- in the medium autoclaved with NO_2^- was 40 ppm; in the medium to which NO_2^- had been added aseptically, the MIC was 640 ppm. An inhibitor of the growth of clostridia was formed under the conditions described.

An extract of a batch of medium prepared with 640 ppm NO_2^- (subsequent testing with *Cl. botulinum* showed the MIC was 10 ppm) was examined for the presence of nitrosamines. The chromatogram in Figure 1 shows peaks with

retention times similar to those for N-nitrosodimethylamine, N-nitrosomethylethylamine, and N-nitrosomethylpropylamine. However, structure confirmation with the mass spectrometer showed that none of these components contained a nitrosamine.

The inhibitory activity of a number of nitrosamines against *Cl. botulinum* was examined. The following nitroso compounds were placed into solution (200 mg in 10 ml water) and sterilized by Seitz filtration: N-nitrosodimethylamine (DMNA), N-nitrosodiethylamine, N-nitrosopropylamine, N-nitrosomorpholine, N-methyl-N-nitrosoethylamine, N-nitrosodiethanolamine, 1-nitrosopiperidine, N-nitrosodiphenylamine, N-methyl-N-nitrosoaniline and N-nitrosomethylurea. These nitroso compounds were prepared and purified in the laboratory. All preparations except the last three were added to the culture medium, at pH 7.0, to give solutions containing 10, 100 and 1000 ppm nitroso compound. The last three compounds were too insoluble so saturated solutions were prepared and two \log_{10} dilutions made. Inoculation

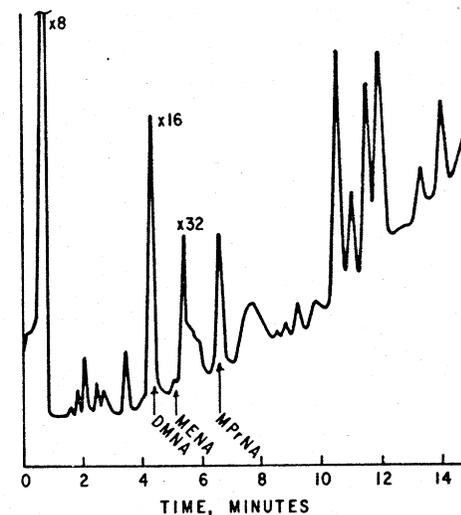


Fig. 1—Gas chromatogram of the extract of culture medium. Arrows indicate retention times for N-nitrosodimethylamine (DMNA), N-nitrosomethylethylamine (MENA), and N-nitrosomethylpropylamine (MPNA).

and incubation were as described. None of the nitroso compounds showed inhibitory action against *Cl. botulinum* even at the highest concentration tested.

The "Perigo inhibitor" does not appear to be a nitrosamine detectable under the experimental conditions used, nor do the several nitrosamines tested appear to be inhibitory to the growth of clostridia.

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