Heat Injury and Recovery of Vegetative Cells of *Clostridium botulinum* Type E

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Vegetative cells of *Clostridium botulinum* type E (Tenno) were heated at 40.5 °C in a prereduced peptone-yeast extract broth (PY). During the heating period, cell numbers remained essentially constant for 3 h as indicated by roll tube counts in PY agar (PYA); however, injury and recovery from injury were observed when the cells were enumerated using PYA containing either 0.06 or 0.07% bile salts.

The heat injury of nonsporulating bacteria has been reviewed by Allwood and Russell (1). Recently the thermal injury and recovery of vegetative cells of the sporeformers *Bacillus subtilis* (5) and *Clostridium perfringens* (G. L. Ades and M. D. Pierson, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1973, p. 17) was reported. To our knowledge, no studies on the injury and recovery of vegetative cells of *Clostridium botulinum* type E have been reported.

All media and diluents used in this study were prereduced, pH 7.0 ± 0.1, and all transfers were made under oxygen-free CO₂ by the procedures described by Holdeman and Moore (3). The use of such procedures negated the effects of oxidized media and oxygen, often ignored factors in the study of anaerobic bacteria. Cultures of *C. botulinum* (Torry 4249 Tenno E obtained from the Virginia Polytechnic Institute and State University Anaerobe Laboratory) were maintained in a chopped meat medium. *C. botulinum* was grown in peptone-yeast extract broth (PY), dilutions were made in 0.1% peptone, and viable cells were assayed using roll tubes of PY containing 2.5% agar (PYA). Prior to use in growth and injury studies, the cells were serially transferred two times at 8-h intervals with incubation at 30 °C. A 500-ml round-bottom flask containing 350 ml of PY was used for growth and injury studies. The contents of the flask were mixed with a submersible magnetic stirrer. The temperature of the flask was controlled to within ± 0.02 °C by placing the flask in a covered, insulated water bath equipped with a Haake model E52 constant-temperature circulator. All roll tubes were incubated at 30 °C for 24 h prior to counting.

Heat injury is often demonstrated by the difference in plate counts between a stress medium and a non-stress medium (2). We therefore investigated the effect of NaCl and bile salts in PYA on the enumeration of *C. botulinum* type E. After 8 h of growth in PYA, viable cells were enumerated in PYA containing 0 to 4% NaCl and PYA containing 0 to 0.1% Difco bile salts. Concentrations of 1.5% NaCl

![Fig. 1. Growth curves for *Clostridium botulinum* type E in prereduced PY broth at 30 °C (○), 40 °C (△), 40.5 °C (△), and 42 °C (●). At various time intervals, the total viable count was estimated using PYA.](image-url)
and 0.06 and 0.07% bile salts in PYA (PYAS and PYAB, respectively) were chosen to estimate injury. These concentrations were found to give the least difference between PYA counts with and without these salts and yet indicate a maximum amount of injury.

C. botulinum type E grew readily in PY and attained maximum numbers after 8 h of growth at 30 C (Fig. 1). At 40 C in PY there was a 20-fold increase in cell numbers within 8 h. When the temperature of PY was raised to 40.5 C, the cell numbers remained constant for about 3 h and then they started to rapidly decline. At 42 C, cell numbers decreased from 10⁴ to 10⁵/ml during the first 7 h of heating.

Because a phosphate buffer is commonly used as the heating menstruum in heat injury studies (2, 5), attempts were made to demonstrate injury in prereduced 100 mM potassium phosphate buffer (pH 7.0). Death of C. botulinum type E was observed in this buffer at heating temperatures as low as 35 C. Within 3 h at 35 C in phosphate buffer, PYA counts dropped from 10⁴ to 10⁵/ml. The apparent rate of decline in numbers was somewhat greater when the cells were enumerated using roll tubes of PYA with either 1.5% NaCl or 0.06% bile salts.

The results from growth studies (Fig. 1) indicated that the growth medium (PY) was a possible heating menstruum in which maximum injury and minimum death could be attained. C. botulinum cells from the late log stage of growth (8 h at 30 C) were heated at 40.5 C in PY and assayed using roll tubes of PYA, PYAS, and 0.06 and 0.07% PYAB (Fig. 2). The spore count was also estimated throughout the heating period by the procedure described by Johnston et al. (4). Throughout the first 3 h of heating, the PYA counts remained essentially constant. There was a slight sensitivity to 1.5% NaCl demonstrated after 1 h of heating. However, there was a marked increase in sensitivity to both concentrations of bile salts during the first 30 min of heating. In the case of 0.06% PYAB, nearly 90% of the cells were sensitive, whereas about 99% were sensitive when samples were enumerated in 0.07% PYAB.

An unusual phenomenon occurred in that the cells that were sensitive to bile salts at 30 min of heating regained their bile salts tolerance after 2 h at 40.5 C in PY. Germination and outgrowth of spores apparently did not contribute to the observed recovery phenomenon since spore counts remained relatively stable (10⁴/ml) throughout the 7-h heating period (Fig. 2). It seems reasonable to assume that this phenomenon of change in bile salts sensitivity during heating can be attributed to injury followed by recovery. We found that, with C. perfringens, a similar type of phenomenon could be observed in PY at 50.5 C (G. L. Ades, and M. D. Pierson, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1973, p. 17).

LITERATURE CITED

1. Allwood, M. C., and A. D. Russell. 1970. Mechanisms of thermal injury in nonsporulating bacteria. Advan. Appl. Microbiol. 12:89-119.
2. Clark, C. W., and Z. J. Ordal. 1969. Thermal injury and recovery of Salmonella typhimurium and its effect on enumeration procedures. Appl. Microbiol. 18:332-336.
3. Holdeman, L. V., and W. E. C. Moore. Eds. 1972. Anaerobe laboratory manual. V.P.I. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va.
4. Johnston, R., S. Harmon, and D. Kautter. 1964. Method to facilitate the isolation of Clostridium botulinum type E. J. Bacteriol. 88:1521-1522.
5. Miller, L. L., and Z. J. Ordal. 1972. Thermal injury and recovery of Bacillus subtilis. Appl. Microbiol. 24:878-884.