Effect of Toxin Concentration on the Heat Inactivation of Staphylococcal Enterotoxin A in Beef Bouillon and in Phosphate Buffer

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The slope of the thermal inactivation curve of enterotoxin A in beef bouillon (initial pH 6.2) was found to be approximately 27.8°C (50°F) with three different concentrations of toxin. Inactivation by heat was dependent on the concentration of enterotoxin A heated. Enterotoxin A was inactivated by less heat in a pH 7.2 phosphate buffer than in beef bouillon as detected by serology. Results indicate that natural (usually low) levels of toxin rather than concentrated enterotoxin A should be used in heat inactivation studies.

The heat inactivation of enterotoxin A and of enterotoxin B in Veronal buffer has been discussed respectively by Hilker et al. (4) and Read and Bradshaw (8). The thermal inactivation of enterotoxin A in concentrated growth medium at pH 7.8 was the subject of a previous paper by Denny et al. (2). The slopes (z values) of the thermal inactivation curves found by the three sets of investigators were all about 27.8°C (50°F), regardless of the toxin concentration or medium in which the toxins were heated and regardless of animal or serological assay of the heated toxins. This standard z value is evident even though end points of heat destruction found by these authors at each of the five or six temperatures tested varied widely due to the factors just mentioned. Hilker et al. (4) noted an apparent correlation between high toxin concentration at the time of heating and high heat resistance of the toxin. The present authors report the investigation of initial enterotoxin A concentration on heat inactivation and also compare the thermal destruction of this toxin in beef bouillon and in phosphate buffer. The practical application of these findings is discussed.

MATERIALS AND METHODS

Production of enterotoxin A. Staphylococcus aureus strain 100 supplied by Merlin S. Bergdoll, Food Research Institute, University of Wisconsin, was used for enterotoxin A production. The procedure used was that of Denny et al. (2) in which the culture was grown on a shaker at 37°C (98°F) for 18 hr, centrifuged, and filtered. The exceptions were as follows:

Brain Heart Infusion (BHI) broth (Fisher) was used in six flasks, the pH was adjusted to 7.8 with 2 N NaOH after incubation, and 5.0 ml of 0.8% chloramphenicol (Calbiochem) in 95% ethanol was added to each flask as described by Morse et al. (6).

Concentration of enterotoxin A. The hydrophilic colloid procedure of Hilker et al. (4) was used except that the interior of each sac was rinsed with 5 to 10 ml of 0.15 M phosphate buffer at pH 7.4, the volume was reduced from an initial 3 liters to 240 ml, and the crude enterotoxin concentrate was dispensed in 10-ml portions in sterile screw-capped tubes and stored at -15°C (5°F).

Preparation of beef bouillon. A method for preparation of beef bouillon similar to that described by Campbell (1) was modified as follows. One 12-lb boneless chuck roast was cut into small cubes (about 2.5 to 5.0 cm). The cubes were placed in a 24-liter stainless-steel steam kettle, and 12 liters of tap water was added. The material was cooked at a simmer for 5 hr, and at intervals, fat and coagulated albumin were removed from the surface of the liquid. After cooking, the soup stock was separated from the beef cubes by siphon, and 93.0 g of NaCl and 4.6 g of ground white pepper (Crown Colony) were added to the liquid portion. To clarify the bouillon, 12 beaten egg whites and crushed egg shells were mixed with the liquid. The bouillon was boiled for 2 min, simmered for 20 min, and then filtered through four layers of cheese cloth. To the clarified filtrate, 27.2 g of beef extract (Difco) and 93.0 g of NaCl were added. The beef bouillon was boiled for 1 min, allowed to settle, and then respinked. The bouillon was made up to a final volume of 12 liters by the addition of tap water and was stored in 4-liter portions at -15°C (5°F). The final pH was 6.2.

Heat treatment of enterotoxin A. Crude enterotoxin
A was thawed under tap water and diluted in beef bouillon (pH 6.2) to concentrations of 60, 20, and 5 μg/ml as determined by serological assay. Samples of 1.0 ml of each enterotoxin concentration were dispensed into individual Pyrex thermal-death-time tubes (7 mm inner diameter, 9 mm outer diameter) 10 cm in length. Tubes were sealed with an oxygen flame, stored at −15 C (5 F), and thawed immediately before use. Thermal treatment was performed in an oil bath at temperatures of 100.0 C (212 F), 104.4 C (220 F), 110.0 C (230 F), 115.6 C (240 F), and 121.1 C (250 F) with a variation of ±0.1 C (±0.2 F). Crude enterotoxin A was also diluted in 0.15 M phosphate buffer at pH 7.2 to concentrations of 60, 20, and 5 μg/ml, and tubes handled in the same manner were heated at 121.1 C (250 F). Oil bath temperatures were regulated by a Thermocap relay (Niagara Electron Lab., Andover, N.Y.) adjusted for accuracy with a mercury-in-glass thermometer standardized by the National Bureau of Standards.

Heat inactivation time was determined for each enterotoxin A concentration. An average of seven different heating time periods was used for each toxin concentration at a given temperature. Intervals between heating times ranged from 1 min at 250 F to 10 min at 212 F. The longest time period at a particular oil bath temperature giving a positive serological reaction was considered the end point. At least three negative points were found above each end point. All tests were run in duplicate. Time lag in achieving the holding temperatures in the tubes was determined by thermocouple measurements, and the holding times used were corrected for this thermal lag.

**Seralogical assay.** Rabbit antiserum specific for pure enterotoxin A was prepared by the method of Hilker et al. (4). The single diffusion procedure described by Oudin (7) was the serological method used for detection of enterotoxin. Gel diffusion tubes were incubated for 7 days at 34 C (93.2 F) and measured at 24-hr intervals for precipitation. A standard curve was prepared with purified enterotoxin A at concentrations between 1 and 60 μg of beef bouillon per ml at 0.15 M phosphate buffer. Length of the precipitation band was recorded every 24 hr for 7 days. Band length was plotted against enterotoxin concentration on semilogarithmic graph paper. All standard curve assays were run in triplicate. Heated enterotoxin was assayed for serological activity immediately after thermal treatment. Absence of a visible precipitin band after 7 days of incubation was considered a negative reaction. The sensitivity of the test was found to be about 1 μg/ml.

**Production of enterotoxin A in canned beef bouillon.** Two porcelain beads coated with *S. aureus* strain 100 dried by the method of Hunt et al. (5) were placed in 7.0 ml of Brain Heart Infusion broth (Fisher) and incubated for 18 hr at 37 C (98.6 F). The number of viable cells in the culture was estimated by plate count. Before inoculation, 100 ml of beef bouillon was placed in a 202 × 204 can (total capacity 103 ml), sealed, and then sterilized at 121 C (250 F) for 15 min. A small hole punched in the top surface of this nonenameled sealed can was soldered closed after addition of a 0.5-ml portion of the culture (1.4 × 10⁶ organisms/ml). All operations were performed aseptically. The beef bouillon was incubated for 14 days at 37 C (98.6 F) and checked microscopically for a pure culture of cocci. After incubation, the bouillon was centrifuged for 30 min at 3,000 × g. The supernatant fluid was assayed for enterotoxin A by the serological method described earlier. The supernatant fluid was also concentrated by dialysis against polyethylene glycol 20,000 at 4 C (39.2 F), and the concentrate was serologically assayed for enterotoxin A.

**RESULTS**

**Heat inactivation in beef bouillon.** The effect of concentration on the heat inactivation curves of enterotoxin A in beef bouillon at pH 6.2 is shown in Fig. 1. The curves were drawn through as many end points as possible for slope comparison. The curves appeared as straight lines with slopes (s values) ranging from 27.5 to 29.1 C (49.4 to 52.5 F). (Temperature conversion from F to C is

![Fig. 1. Heat inactivation curves of enterotoxin A in beef bouillon. Enterotoxin concentrations: ■, 60 μg/ml; ●, 20 μg/ml; ▲, 5 μg/ml. Symbols represent the longest heating period at each temperature giving a positive serological reaction.](image-url)
accomplished by multiplying by %. Usual temperature conversion tables do not apply to slope conversion on semi-log paper.) The highest initial concentration, 60 \mu g/ml, had the highest end points, and the lowest toxin concentration, 5 \mu g/ml, showed the lowest tolerance to heating. The inactivation of enterotoxin A was not directly proportional to concentration, since the difference in the end points at each of the five temperatures was greater between 5 and 20 \mu g/ml than between 20 and 60 \mu g/ml. Heat inactivation was more effective at the lowest initial concentration than at the higher initial concentrations. There was a protective effect of concentration between 20 and 60 \mu g/ml, but this effect was self-limiting or near its maximum at these levels.

**Heat inactivation in phosphate buffer.** The end points of heat destruction of enterotoxin A in phosphate buffer and in beef bouillon at 121 C (250 F) are compared in Table 1. The heat resistance of the toxin in beef bouillon is greater by a factor of three to five times than in 0.15 M phosphate buffer. Again, the highest initial toxin concentration in phosphate buffer had the highest heat resistance. Heat inactivation of enterotoxin A was more effective at the lowest initial concentration than at higher initial concentrations in both heating menstrua.

**Production of enterotoxin A in canned beef bouillon.** Strain 100 inoculated into canned beef bouillon and incubated for 14 days showed visible growth. The odor was like that of pus from a staphylococcal infection. A pure culture of cocci was seen on microscopic observation. The total plate count in the can was 300,000, indicating that growth was complete and the death stage had been reached during the long incubation period. Serological assay showed that only about 0.2 \mu g of enterotoxin A per ml was produced.

**DISCUSSION**

One of the most interesting findings is that the slope of the thermal-inactivation-time curve for enterotoxin A in beef bouillon was about 27.8 C (50 F), which is very close to that reported previously by us (2, 4) and by other authors (8) in other substrates. This indicates that if the heat inactivation time required for a given concentration of enterotoxin is known at any specific temperature, the inactivation time might be estimated accurately at other temperatures by constructing a curve with this slope.

The results of the experiments reported here show that the heat inactivation of enterotoxin A in beef bouillon or phosphate buffer was related to but not directly proportional to initial concentration. Therefore, the results indicate that \( D \) values (90% destruction values) would not be identical for different concentrations of toxin. Reiser and Weiss (9) have shown that the maximum amount of enterotoxin A that could be produced under ideal conditions is about 5 or 6 \mu g/ml. In beef bouillon, only about 0.2 \mu g/ml was produced. Because the effect of concentration is so important on the survival of enterotoxin A during heating, the true picture can be found only if enterotoxin A is heated at a concentration which is near the limit of that normally produced. Tests with enterotoxin A heated at concentrations above about 5 \mu g/ml should be unnecessary because the toxin is not created or found in nature at this level. Experimental enterotoxin production in the beef bouillon of about 0.2 \mu g/ml indicates that the heat resistance in this food would be far less than that reported for 5 \mu g/ml in Table 1.

The increase in heat resistance of enterotoxin A which was observed as the toxin concentration was increased (Fig. 1) may be explained in part by a protective effect afforded one protein molecule by other protein molecules since enterotoxin A is a simple protein. Haurowitz (3) explains that, unless space exists between the peptide chains of a closely folded native protein in solution, denaturation by heat may be difficult.

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