APPLIED MICROBIOLOGY, Feb. 1968, p. 308-310

Vol. 16, No. 2
Printed in U.S.A.

Heat Inactivation of Enterotoxin A from *Staphylococcus aureus* in Veronal Buffer

J. S. HILKER,[1] W. R. HEILMAN, P. L. TAN, C. B. DENNY, AND C. W. BOHRER
National Canners Association Research Foundation, Washington, D.C. 20036

Received for publication 13 October 1967

Serological tests were used to determine the slope of the thermal inactivation curve of crude enterotoxin A in Veronal buffer (*p*H 7.2), and the resulting *z* value was 27.8 C. (50 F). Serological assays also showed that the heat inactivation at each time-temperature depended on the original concentration of enterotoxin A. The usefulness of the Oudin tube serological test for determining end points of inactivation of naturally produced enterotoxin A (not concentrated) is discussed. We concluded that this test cannot be used to determine end points of heat inactivation for enterotoxin A in the minute quantities naturally produced in foods.

Time-temperature relationships for the thermal inactivation of staphylococcal enterotoxin B in Veronal buffer (*p*H 7.2) were recently reported by Read and Bradshaw by use of both biological (cats) and the Oudin tube serological tests (9). Recent studies by Denny et al. were conducted on heat inactivation of staphylococcal enterotoxin A, which is considered the most common of all enterotoxins associated with food poisoning (3). The end point results with enterotoxin A were determined biologically by use of testing with cats and monkeys. The research reported here established the slope of the thermal inactivation curve of enterotoxin A in Veronal buffer (*p*H 7.2) as well as the end points (less than 1 μg /ml) in the inactivation of 21 μg/ml of this enterotoxin by using the Oudin serological test.

MATERIALS AND METHODS

Production of crude enterotoxin A. The culture used throughout this study was *Staphylococcus aureus* strain 196-E, which was preserved by drying on porcelain beads (5). An 18-liter amount of Casamino Acids broth medium (1) was dispensed into 36 flasks (2 liter) in 500-ml samples, and these samples were inoculated with 0.5-ml quantities of an 18-hr Brain Heart Infusion (Difco) culture incubated at 35 C. The inoculated flasks were incubated at 35 C for 18 hr while being agitated at 270 strokes per min on a side arm (Burrell Corp., Pittsburgh, Pa.) mechanical shaker (3, 7).

Concentration of crude enterotoxin A. After incubation, pure cultures were centrifuged in 300-ml amounts at a relative centrifugal force (RCF) of 5,140 for 30 min in a refrigerated centrifuge (3.3 C). The supernatant fluid was passed through a 0.45 μ HA filter (Millipore Corp., Bedford, Mass.). The filtered liquid was placed in washed cellulose casein sacs (NoJax size 36, Union Carbide Corp., Chicago, Ill.), which were found to prevent passage of enterotoxin A by ultra-filtration under 12 inches of vacuum (2), and the sacs were knotted at both ends. The sacs were placed in six-liter plastic buckets, covered with a hydrophilic colloid (polyethylene glycol, 20,000), and stored at 3.3 C (usually 24 to 48 hr) until dialysis was complete, i.e., until the sacs contained no liquid (4). Following dialysis, the outer sides of the sacs were washed thoroughly with distilled water. The material remaining inside the sacs was removed by washing each sac with 10 to 15 ml of sterile distilled water and was measured in a 500-ml graduated cylinder. The initial volume of 18.0 liters was reduced to 0.325 liter, constituting a 55.38-fold concentration. This concentrated crude enterotoxin A was then preserved by freeze-drying.

The freeze-dried preparation of crude enterotoxin A was suspended in 108 ml of 0.04 M Veronal buffer (*p*H 7.2) and was centrifuged at an RCF of 5,140 for 10 min in a refrigerated centrifuge (at 3.3 C) to remove precipitate from the crude enterotoxin A suspension. About half (58 ml) of this preparation was diluted to a 55.38-fold concentration. The supernatant fluids were dispensed separately in 1-ml portions into Pyrex thermal-death-time tubes (7 mm inner diameter, 9 mm outer diameter) and were sealed in tube lengths of 4 inches (10 cm) with an oxygen flame. The sealed tubes containing the centrifuged suspension of crude enterotoxin A were then placed in a freezer and were kept frozen until used.

Thermal treatment of crude enterotoxin A. The thermal-death-time tubes of frozen crude concentrated enterotoxin A were thawed in a water bath at 23.3 C. Thermal treatment was accomplished in an oil bath at constant temperatures of 100, 104.4, 110, 115.5, 121.1, and 126.6 C (variation was ±0.1 C from the desired temperatures). The oil bath was

[1] Present address: Consultants and Designers, Inc., Goddard Space Flight Center, Greenbelt, Md. 20771.

controlled by a Thermocap relay adjusted for accuracy with a mercury-in-glass thermometer standardized by the National Bureau of Standards. 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. After heat treatment, the tubes were immersed in a water bath (23.3 C), after which toxin activity was tested.

Production of enterotoxin A antiserum. Antiserum specific for enterotoxin A was produced by injections of 95% purified preparations of enterotoxin A into a male white New Zealand strain rabbit. This purified toxin was obtained from M. S. Bergdoll, Food Research Institute, University of Wisconsin. Immunization consisted of subcutaneous (10) successive flank injections of 5, 10, 10, 50, 200, and 1,000 μg of toxin dissolved in 0.85% physiological saline and administered at intervals of 0, 9, 15, 14, 14, and 35 days, respectively (M. S. Bergdoll, *personal communication*). All immunization suspensions were prepared in equal volumes of Freund's adjuvant. One week after the last injection, the rabbit was bled by cardiac puncture (6). Serum was obtained, preserved with Merthiolate (1:10,000), freeze-dried (8), and found to have a titer of 1:140.

Preparation of assay tubes for enterotoxin A. Pyrex glass tubes (100 by 6 mm) were used as single diffusion assay tubes for enterotoxin A. The tubes were coated with 0.5% Ionagar (Oxoid), placed over a desiccant, and vacuum-dried under 25 inches (63.5 cm) of vacuum. After drying, the coated tubes were half filled with 0.5 ml of antiserum-agar mixture. The mixture was made by preparing a heated 0.6% Ionagar suspension in 0.04 M Veronal buffer (*p*H 7.2) containing 0.85% sodium chloride and Merthiolate (1:10,000). An equal volume of 1:20 antiserum, previously diluted in the same diluent without the agar, was then added to this mixture. This procedure gave the antiserum a final concentration of 1:40. A similar mixture was prepared containing antiserum for enterotoxin A at a final dilution of 1:120. (The antiserum used in this study was specific for enterotoxin A when a dilution greater than 1:10 was used.) Following solidification of the antiserum agar mixtures, 0.5-ml quantities of the heated crude enterotoxin A in 0.04 M Veronal buffer (*p*H 7.2) were added over the antiserum-agar, and the tubes were sealed with parafilm and incubated at 30 C for 7 days (7).

Detection of antigen-antibody complexes. Antigen-antibody complexes were viewed with a shielded light at an oblique angle. As diffusion of the complexes occurred, measurements were made with a caliper (Vernier) and were recorded. Tests were deemed negative when no antigen-antibody complexes were formed at both 1:40 and 1:120 dilutions of antiserum.

RESULTS AND DISCUSSION

Single gel-diffusion assays. The sensitivity of the single gel-diffusion technique, as determined by assaying known amounts of 95% purified enterotoxin A in Veronal buffer, was found to be 1.0 μg/ml, i.e., below this concentration no visible antigen-antibody complexes were formed. Typical standard reference curves were plotted from the data and were utilized to assay the unknown amount of enterotoxin A in the 1.0-ml portions of crude enterotoxin A suspended in Veronal buffer. Assays revealed the presence of 90 μg of enterotoxin A per ml in the original reconstituted suspension, and 21 μg per ml was found to be present in a diluted portion of this original reconstituted enterotoxin A.

Heat inactivation of enterotoxin A. End points for the heat inactivation of 90 μg per ml of enterotoxin A were not obtained after heating for 18 min at 121.1 C, 29 min at 115.5 C, 47 min at 110 C, 67 min at 104.4 C, or 106 min at 100 C, and at least 3.25 μg of toxin per ml remained after each of these time-temperature treatments. Only barely detectable amounts (1.0 μg/ml) were found at the same time-temperatures when 21 μg/ml was used, and this indicates that the larger initial concentration of enterotoxin A had a much higher heat tolerance than did the lower concentration. However, equal remaining amounts of enterotoxin, when plotted for the higher toxin concentration, indicated that the slope of the inactivation curve (*z* value) was about 27.8 C (50 F).

End points for the heat inactivation of en-

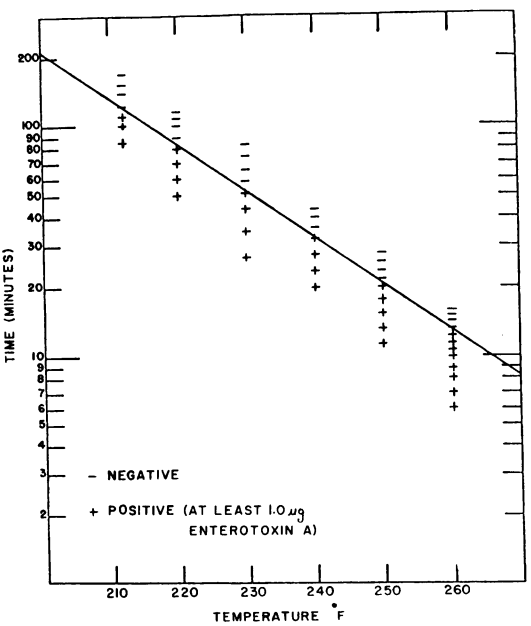

FIG. 1. *Serological detection after heating 21 μg of enterotoxin A per ml. The temperatures used were equivalent to 100, 104.4, 110, 115.5, 121.1, and 126.6 C.*

terotoxin A were obtained for 21 μg per ml of crude enterotoxin A in Veronal buffer. Figure 1 shows results after heat treatment of the enterotoxin, as indicated by single gel-diffusion assays. The end points represent the disappearance of a positive reaction which occurred when the concentration of enterotoxin A was reduced to less than 1.0 μg per ml by the heat treatment. The end points for exposure temperatures of 100, 104.4, 110, 115.5, 121.1, and 126.6 C were 130, 90, 59, 36, 22, and 14 min, respectively.

The inactivation times, corrected for thermal lag, were plotted on semilogarithmic paper, and a straight-line curve was formed (Fig. 1). The inactivation of 21 μg of enterotoxin A per ml in Veronal buffer (*p*H 7.2) can be expressed as a straight-line semilogarithmic curve with a slope (*z*) of 27.8 C (50 F) to traverse one log cycle of time and a heat resistance, at 121.1 C (250 F), of 22 min ($F_{250}^{50} = 22$).

All heat inactivation determinations were conducted with two tubes per interval, and the tests at 21 μg per ml were repeated. All assays were in agreement, and the end points determined for the heat inactivation of enterotoxin A were reproducible.

The 27.8 C (50 F) slope (*z* value) for the serologically determined heat inactivation compares quite well with the 26.7 C (48 F); cat emetic reaction) and 25.5 C (46 F) slopes (monkey emetic reaction) previously described by Denny et al. (3) for crude enterotoxin A. This finding indicates that the rate of inactivation should be about the same for different concentrations of enterotoxin A. Read and Bradshaw (9) showed a slope of 30.6 C (55 F) for crude enterotoxin B. This suggests that enterotoxin A (the main food-poisoning type) is inactivated at the higher temperatures (above 121.1 C) more rapidly than is enterotoxin B.

Previous tests (3), using 7 μg per ml of enterotoxin A, gave heat inactivation end points of 8 to 11 min at 121.1 C, as compared to the present 22 min at 121.1 C at an enterotoxin concentration of 21 μg/ml. All of these tests were conducted with concentrated enterotoxin. However, under ideal laboratory conditions, enterotoxin A from strain 196-E was produced only in low concentrations, i.e., about 1 μg per ml. It is doubtful that enterotoxin A would be produced in foods in a concentration higher than this, and higher amounts would occur only as a result of concentrating the food. Therefore, our results with high enterotoxin levels indicate that enterotoxin A produced naturally in foods has lower heat inactivation end points than those found in other investigations. Since the sensitivity of the Oudin tube test with enterotoxin is about 1 μg/ml and the naturally produced enterotoxin A (not concentrated) probably never exceeds this value, the Oudin tube technique is considered unsatisfactory for determining end points of heat inactivation in normal concentrations of enterotoxin in foods. Feeding monkeys low concentrations of heated enterotoxin A appears to provide meaningful end point determinations in terms of application to illness induced in man. Human subjects would have to be used for the ultimate determination of heat inactivation.

ACKNOWLEDGMENTS

We thank Merlin S. Bergdoll, Food Research Institute, University of Wisconsin, Madison, for the 95% purified enterotoxin A used as a control in this project. We also thank R. W. Sternberg and G. R. Bee for their technical assistance in the heating portion of the research.

This investigation was supported by Public Health Service research grant UI-00097 from the National Center for Urban and Industrial Health.

LITERATURE CITED

1. CASMAN, E. P. 1958. Serological studies of staphylococcal enterotoxin. Public Health Rept. (U.S.) **73**:599–609.
2. DENNY, C. B., AND C. W. BOHRER. 1963. Improved cat test for enterotoxin. J. Bacteriol. **86**:347–348.
3. DENNY, C. B., P. L. TAN, AND C. W. BOHRER. 1966. Heat inactivation of staphylococcal enterotoxin A. J. Food Sci. **31**:762–767.
4. DENNY, C. B., P. L. TAN, AND C. W. BOHRER. 1966. Isolation and purification of enterotoxin A by polyacrylamide gel electrophoresis. J. Env. Health **29**:222–230.
5. HUNT, G. A., A. GOUREVITCH, AND J. LEIN. 1958. Preservation of cultures by drying on porcelain beads. J. Bacteriol. **76**:453–454.
6. KABAT, E. A., AND M. M. MAYER. 1964. Experimental immunochemistry, 2nd ed., p. 874–875. Charles C Thomas Publisher, Springfield, Ill.
7. KATO, E., M. KHAN, L. KUJOVICH, AND M. S. BERGDOLL. 1966. Production of enterotoxin A. Appl. Microbiol. **14**:966–972.
8. KWAPINSKI, J. B. 1965. Methods of serological research, 1st ed., p. 114. John Wiley and Sons, Inc., New York.
9. READ, R. B., JR., AND J. G. BRADSHAW. 1966. Thermal inactivation of staphylococcal enterotoxin B in Veronal buffer. Appl. Microbiol. **14**:130–132.
10. READ, R. B., JR., W. L. PRITCHARD, J. G. BRADSHAW, AND L. A. BLACK. 1965. In vitro assay of staphylococcal enterotoxins A and B from milk. J. Dairy Sci. **48**:411–419.