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Effect of pH, Protein Concentration, and Ionic Strength on Heat Inactivation of Staphylococcal Enterotoxin B[1]

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Heat treatment of highly purified staphylococcal enterotoxin B causes a more rapid loss of immunological activity at 70 to 80 C than at 90 to 100 C. Toxicological results based on intravenous injection of dogs paralleled the results obtained by immunological means (single gel diffusion). The loss of immunological activity did not follow first-order kinetics. Results are given on the effects on heat inactivation of changing pH, ionic strength, and initial concentration of enterotoxin. Disc-gel electrophoresis of purified enterotoxin B showed a major and minor band. The minor band was a size isomer of the major band.

It is generally agreed that staphylococcal enterotoxin B is among the most heat-stable proteins. Whether or not the heat stability is influenced by the state of purity, pH, ionic strength, or concentration of the enterotoxin is not well known. Satterlee and Kraft (9) recently found that enterotoxin B was more rapidly inactivated by heat at 80 C than at 100 C. The enterotoxin they used was not well purified and, in some experiments, was mixed with other proteins to simulate conditions of cooking meat.

Schantz et al. (10) have prepared highly purified enterotoxin B and have reported that less than 50% activity was lost when the toxin was heated at 100 C at pH 7.3 for 5 min. Working with this highly purified toxin, Read and Bradshaw (7) found that loss of toxic activity by injection into cats paralleled the loss of immunological activity by a double gel diffusion assay. They reported an inactivation time of 87.1 min at 100 C at pH 2 for an enterotoxin solution containing 30 μg/ml.

We undertook this series of experiments to study in detail the heat denaturation of enterotoxin B. We were particularly interested in whether the anomaly found by Satterlee and Kraft (9) of more loss of activity at 80 C than at 100 C is a property of the enterotoxin or is due in some way to contaminating proteins. We also wanted to know what influences, if any, were exerted by changes in pH, ionic strength, and concentration of the enterotoxin.

MATERIALS AND METHODS

Enterotoxin production. *Staphylococcus aureus* strain S-6 was grown in 3% Protein Hydrolysate Powder (Mead Johnson and Co., Evansville, Ind.), 3% N-Z Amine NAK (Sheffield Chemical Co., Norwich, N.Y.), 0.001% niacin, and 0.00005% thiamine. The vitamins were sterilized by Seitz filtration and added to the autoclaved medium. The pH was adusted to 6.5 before sterilization. The inoculum was an 18-hr aerobic culture that had been stored frozen.

Fernbach flasks containing 600 ml of medium and 1% inoculum were incubated at 37 C on a rotary shaker (280 rev/min) for 24 hr. Cell-free culture fluid was obtained by centrifugation.

Enterotoxin assay. We used the single gel diffusion technique of Weirether et al. (12) to determine enterotoxin concentrations. M. S. Bergdoll of the Food Research Institute, Madison, Wis., kindly provided the antiserum to enterotoxin B and purified enterotoxin B for our standard curve. Measurements were made after incubation in a water bath at 30 C for 24 hr. A single batch of the antiserum and, therefore, one standard curve was used throughout the whole study.

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Enterotoxin purification. We followed the methods developed by Schantz et al. (10) with slight modifications. The cell-free culture fluid was diluted with eight volumes of water, and the *p*H was adjusted to 6.4 with dilute HCl. Approximately 9 to 10 liters of diluted supernatant fluid was purified at one time. The protein content of fractions during purification was determined by the biuret method.

Before each of the dialysis procedures, the pooled fractions were evaporated to half volume in a rotary evaporator at 30 C. The vacuum was adjusted to prevent foaming of the solution. The concentrated solution was dialyzed against distilled water to a phosphate content of approximately 0.01 M. After column chromatography, the pooled fractions were dialyzed, freeze-dried, and stored at −5 C.

Disc electrophoresis. Disc electrophoresis was used to determine the purity of the enterotoxin preparation and to detect changes in the heated enterotoxin solutions. The gel system of Reisfeld et al. (8) with a running gel *p*H of 4.3 and that of Williams and Reisfeld (13) with a running gel *p*H of 7.5 were used. Methyl green was used as tracking dye for the gel system at *p*H 4.3, and the electrophoresis run lasted for 50 min. For the gel system at *p*H 7.5, no suitable tracking dye was found, but an electrophoresis run of 2 hr was adequate for a good separation of the bands. The gels were stained with amido black.

Analysis of the difference between major and minor bands. The two bands seen in disc electrophoresis of the enterotoxin were studied by the method proposed by Hedrick and Smith (4). The gel system of Reisfeld et al. (8) at *p*H 4.3 was used, except for the ratio of *N*,*N*′-methylene-bisacrylamide (Bis) to acrylamide in the running gel, which was kept constant at 1:30. The acrylamide concentration of the running gel was varied between 6 and 15%. At the end of the electrophoresis run, the dye front was marked by inserting a plastic bristle through the gel. The migration distances of the two bands were measured in millimeters relative to the dye front and expressed as the ratio of protein migration to dye migration. The logarithms of these values were plotted against gel concentration.

Heat inactivation. Enterotoxin B was dissolved in 0.08 M sodium phosphate buffer (ionic strength, 0.10) to give a concentration of 100 μg/ml. After filling capillary tubes (1.6 to 1.8 × 90 mm) about half full with the enterotoxin solution, the tubes were sealed with a propane torch without appreciably raising the temperature of the solution.

The tubes were heated to 60, 70, 80, 90, or 100 C by complete immersion in an oil bath. Come-up time was predetermined with the use of a thermocouple connected to a potentiometer. Duplicate tubes were taken out at the come-up time and at different times during the heating period, and the study at each temperature was done at least twice. The tubes were immediately plunged into ice water when removed from the oil bath. After cooling, the heated solutions were analyzed for enterotoxin activity. A sample was kept at 30 C during the entire heating time as a control.

Effect of pH. Enterotoxin solutions containing 100 μg/ml in sodium acetate buffer at *p*H 4.5 and in sodium phosphate buffer at *p*H 7.5 were prepared. In all *p*H experiments, the ionic strength was fixed at 0.10. The enterotoxin was subjected to heat inactivation at 70 and 100 C as described in the preceding section.

Effect of ionic strength. Phosphate buffers (*p*H 6.4) of various ionic strengths were prepared by adding NaCl to 0.015 M sodium phosphate solution (ionic strength, 0.02). The buffers were used to prepare enterotoxin solutions containing 100 μg/ml. These solutions were heated at 70 C for 8 min, cooled, and then analyzed for enterotoxin activity. Similarly, enterotoxin solutions (100 μg/ml) in sodium acetate buffers at *p*H 4.5 were subjected to heat treatment. The ionic strength of the sodium acetate buffer was varied by adding NaCl to 0.08 M acetate solution (ionic strength, 0.02).

Effect of initial concentration. Different amounts of enterotoxin were dissolved in sodium phosphate buffer at *p*H 7.5 (ionic strength, 0.10) and sodium acetate buffer at *p*H 4.5 (ionic strength, 0.02) to produce solutions of various concentrations. These solutions were heated at 70 C for 10 min and analyzed for enterotoxin activity after cooling.

Dog injections. Heated enterotoxin solutions were injected intravenously into dogs to determine the toxicity of the solutions. The emetic dose was first determined by use of unheated enterotoxin B. Three dogs were used per sample, and each dog was used only once. At 0.5 hr before injection, the dogs were fed with fresh hamburger meat. The enterotoxin solutions were injected through the cephalic vein, and vomiting within 3 hr was reported as a positive reaction. The enterotoxin was heat-treated about 1 hr before injection.

RESULTS

Enterotoxin production. Our average yield of enterotoxin B was 300 μg/ml of cell-free culture fluid. The inoculum gradually lost ability to produce enterotoxin during frozen storage. After a year in frozen storage, enterotoxin production decreased to an average of 50 μg/ml, but two successive transfers in double-strength Brain Heart Infusion restored the initial enterotoxin-producing ability of the bacterial culture.

Enterotoxin purification. During purification, dialysis of the pooled column fractions against distilled water caused loss of enterotoxin accompanied by the formation of a white precipitate. Attempts to recover the enterotoxin activity by dissolving the precipitate in urea and guanidine hydrochloride and then dialyzing out these reagents were unsuccessful. To decrease the enterotoxin loss during dialysis, the pooled fractions were concentrated in a rotary evaporator. Con-

centration of the protein seemed to increase the stability of the enterotoxin.

Disc electrophoresis of the purified enterotoxin showed a faster-moving major band and a slower-moving minor band at *p*H 4.3 (Fig. 1). The amount of minor band per unit weight of enterotoxin increased with storage time. With freshly prepared and highly purified enterotoxin, 278 μg could be applied to gels without visible contaminants. Since 0.7 μg of enterotoxin could be detected on disc electrophoresis, this indicates that contaminants are present at less than 1% of the enterotoxin, providing that any contaminants could be detected at the same levels as enterotoxin and that the electrophoresis conditions would separate contaminants from enterotoxin.

Electrophoresis of the purified enterotoxin by the system of Williams and Reisfeld (13) at *p*H 7.5 gave a faster-moving, major band and a slower-moving, minor band more intense and wider than the minor band seen in the gels at *p*H 4.3 (Fig. 1).

Analysis of the difference between major and minor band. The electrophoretic migration of the two bands was measured at *p*H 4.3 with increasing polyacrylamide concentration. A plot of the logarithms of the migration distances relative to the dye front (R_M) against gel concentration gave two lines that intersected near 0% concentration (Fig. 2). This pattern shows that the major and minor band proteins are size isomeric proteins (4) and the minor band has a higher molecular weight.

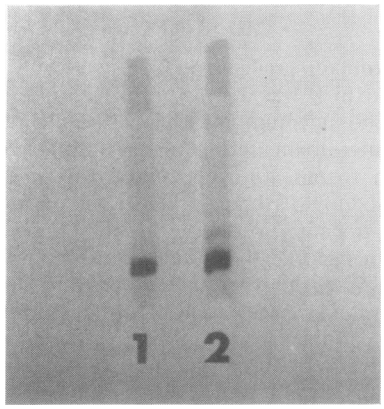

FIG. 1. *Disc-gel electrophoresis of enterotoxin B at pH 4.3. Running time, 50 min. (1) Gel containing 15 μg of enterotoxin (purified as described in Materials and Methods). (2) Gel containing 25 μg of enterotoxin (supplied by M. S. Bergdoll, Food Research Institute, University of Wisconsin).*

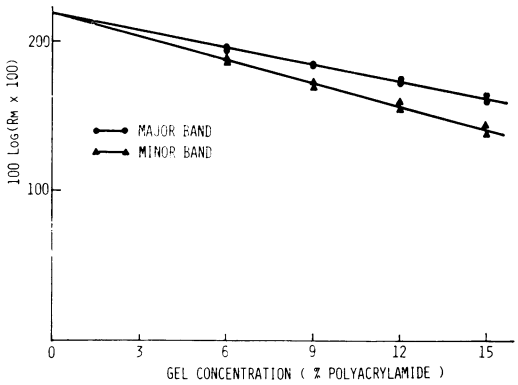

FIG. 2. *Plot of the relative migration rates (relative to the dye front) of major and minor bands in disc-gel electrophoresis at pH 4.3.*

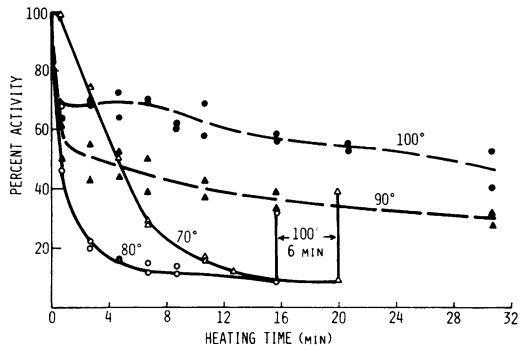

FIG. 3. *Inactivation of enterotoxin B with time at different temperatures. Conditions were: pH 6.4; initial concentration, 100 μg/ml; ionic strength, 0.10. Samples heated at 70 and 80 C were reactivated to the extent shown by heating at 100 C.*

Heat inactivation. Figure 3 shows the loss of immunological activity with time at different temperatures. The time of heating as shown excludes the come-up time for 100 C, which was 40 sec. Initial samples were taken at 40 sec at all temperatures. Activity as measured by gel-diffusion tests was almost completely gone after 20 min at 70 C and 15 min at 80 C, but it took 60 min at 100 C and 90 min at 90 C for the same amount of inactivation. The heated solutions at 90 and 80 C were visibly turbid. The last 10% of the activity took a relatively long time to be inactivated, and a residual activity still remained after 20 min at 70 C and 15 min at 80 C. Control samples kept at 30 C during the entire heating period did not show a decrease in activity. When the solutions that were inactivated at 70 and 80 C were heated at 100 C for 6 min, a recovery to 35 to 40% of the initial activity was obtained.

Heating at 60 C caused a slow inactivation. The most rapid decrease was during the first 2 hr, wherein about 30% of the activity was lost. After 24 hr, the solution was still 50% active.

Failure to immerse the tubes immediately in ice water after the heating time at 100 and 90 C caused an increased inactivation of 20 to 30%. Disc electrophoresis at *p*H 4.3 of the enterotoxin solutions heated at 60 C for 24 hr, 70 C for 10 min, and 100 C for 10 min showed only the major band at a decreased intensity.

With pure enterotoxin, a dose of 0.1 μg/kg was sufficient to cause emesis when injected into dogs, and a dose of 0.3 μg/kg gave a positive reaction in all of three dogs. Heating the enterotoxin solution at 70 C for 15 min at *p*H 6.4 eliminated emetic toxicity when 0.3 μg/kg was injected. The enterotoxin inactivated at 70 C for 15 min and then reheated at 100 C for 6 min gave a positive reaction in two of three dogs. The heat-activated solution was given at a dose of 0.5 μg/kg of body weight based on the enterotoxin activity before heating.

Effect of pH. When the *p*H was changed from 6.4 to 4.5 or to 7.5, no large change was seen in the inactivation curves at 70 C as long as the ionic strength was maintained at 0.10. Figure 4 shows data for enterotoxin inactivation at *p*H 4.5. The data for *p*H 7.5 were similar. At 100 C, the enterotoxin was more stable at *p*H 6.4 than at *p*H 4.5 or 7.5 (Fig. 3 and 4).

When the enterotoxin solution heated at 100 C for 10 min at *p*H 7.5 was analyzed by disc electrophoresis at *p*H 4.3, only one band corresponding to the major band of the unheated toxin was found. The solution heated at 100 C for 15 min was injected into a dog. A dose of 0.3 μg/kg based on initial activity was used. The dog reacted with vomiting and diarrhea in 1.5 hr after the injection.

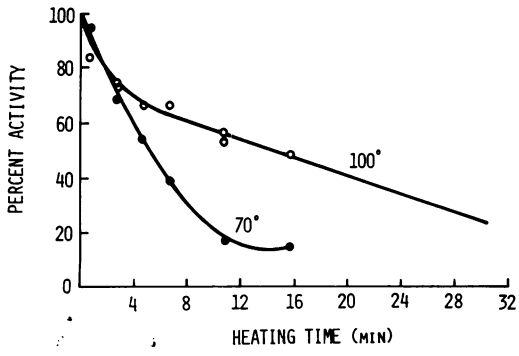

FIG. 4. *Heat inactivation of enterotoxin B at pH 4.5. Initial concentration, 100 μg/ml; ionic strength, 0.10.*

Effect of ionic strength. The effect of increasing the ionic strength from 0.10 to 1.0 at *p*H 6.4 was a gradual increase in the amount of remaining activity in solution after 8 min of heating at 70 C (Fig. 5). Below an ionic strength of 0.10, there is indication of a maximum of activity retention near an ionic strength of 0.05.

At *p*H 4.5, the amount of remaining activity after 8 min of heating at 70 C was decreased from 62 to 10% as the ionic strength was increased from 0.05 to 1.0. Between 0.02 and 0.05, there was a maximum of activity retention, with 50 to 60% of the activity still remaining after 8 min of heating.

The inactivation curves at 70 and 100 C of an enterotoxin solution at *p*H 4.5 and ionic strength 0.02 are shown in Fig. 6. The 70 C curve showed a pattern very different from those at *p*H 6.4 and 7.5. After 30 min of heating, 45% of the activity still remained, and there was no visible aggregation.

Effect of initial concentration. At *p*H 4.5, an initial concentration of 80 to 100 μg/ml showed a maximum of activity retention when heated at 70 C for 10 min. As shown in Fig. 7, a drop in remaining activity accompanied the increase in initial concentration.

At *p*H 7.5 and at a higher ionic strength, the remaining activity also decreased with increasing initial concentration.

DISCUSSION

The two components found in gel electrophoresis of enterotoxin B were attributed by Schantz et al. (10) to a low ionic strength in the gel. Both components were reported toxic to monkeys. According to Joseph and Baird-Parker (6), however, the major band protein was three times as toxic as the minor band protein and the two proteins differed only in charge and toxicity. Chu (2) mentioned the presence of a small amount of dimer with an isoelectric point of 6.8 in his purified enterotoxin B prepared according to the method of Schantz et al. (10). Our minor band is still positively charged at *p*H 7.5, migrating toward the cathode in disc electrophoresis, and does not seem to be identical to his dimer. Our results, however, indicate that the minor band protein has a higher molecular weight than does the major band and that the differences in the isoelectric points may be due to ion binding by this protein.

The amount of the minor band protein increased during storage of our freeze-dried entero-

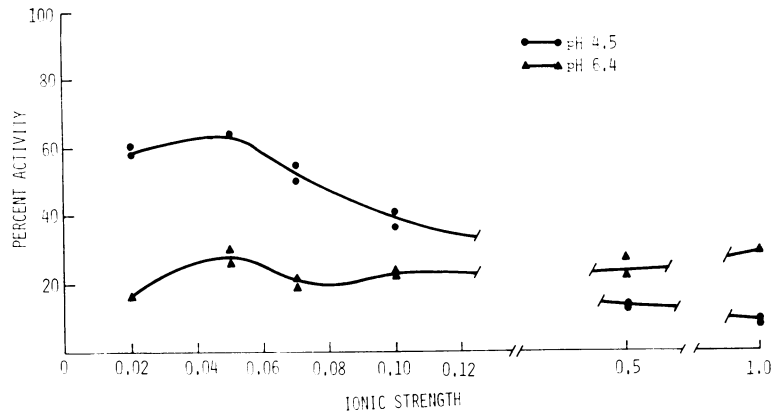

FIG. 5. *Effect of ionic strength on the percentage activity remaining in solution after 8 min of heating at 70 C. Initial concentration was 100 μg/ml.*

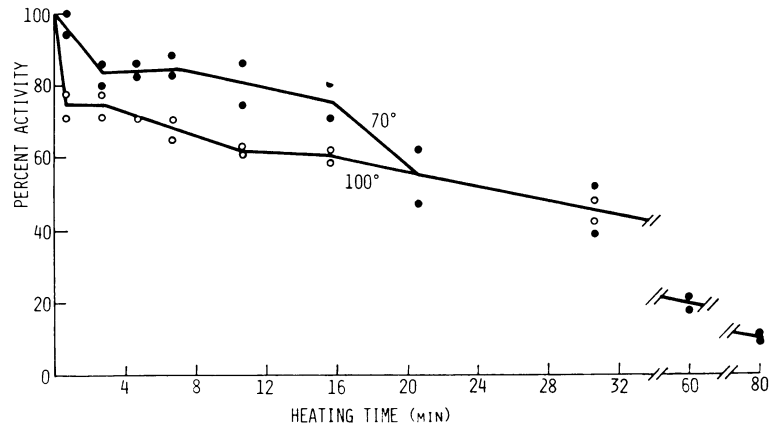

FIG. 6. *Heat inactivation of enterotoxin B at pH 4.5. Initial concentration, 100 μg/ml; ionic strength, 0.02.*

toxin. The purified enterotoxin B obtained from M. S. Bergdoll must contain a considerable amount of the minor band protein because electrophoresis of 25 μg of the toxin gave a dark minor band (Fig. 1). Since the toxin was prepared according to the method of Schantz et al. (10) and initially contained at least 95% of the major toxin band as analyzed by electrophoresis at *p*H 4.5, the increase in the percentage of the minor band probably developed during storage.

Disc electrophoresis of the undenatured toxin at *p*H 7.5 gave a broader and more intense minor band than at *p*H 4.3. Storage of the toxin for 2 days at *p*H 7.5, however, did not noticeably increase the amount of minor band. Since the ionic strength of the gel system at *p*H 7.5 (0.0003) is lower than that of the gel system at *p*H 4.3 (0.012), the enhanced polymerization might be the effect of low ionic strength rather than *p*H. This minor band was still visible in electrophoresis at *p*H 7.5 after the toxin had been heated at 100 C for 10 min, indicating that 100 C does not dissociate it into 30,000 molecular weight protein. Gel electrophoresis at *p*H 4.3 of the solutions heated at 100 C for 10 min, 70 C for 10 min, and 60 C for 24 hr showed only a decreasing enterotoxin B band and did not reveal any intermediates in the heat denaturation process.

Denaturation is usually a monomolecular reaction with first-order kinetics (5). The heat inactivation of enterotoxin B at 70 C did not follow first-order kinetics. A persistent residual activity was noticed during the heating. To determine whether this activity was due to precipitate redissolving during the assay, the precipitate was centrifuged before the gel-diffusion test. The re-

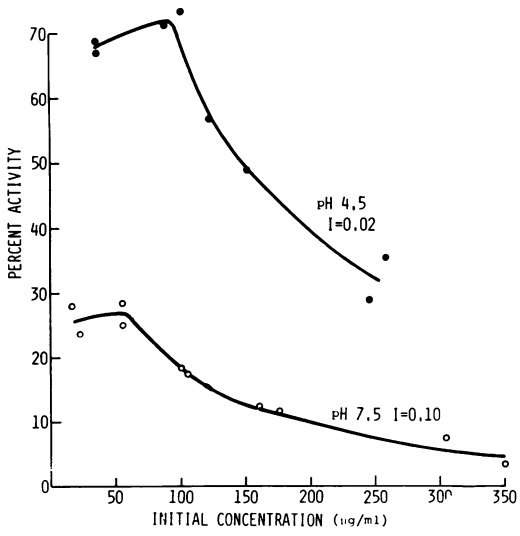

FIG. 7. *Effect of initial concentration of enterotoxin B on the percentage activity remaining in solution after 10 min of heating at 70 C.*

sidual activity remained at the same level as in the heated sample that still contained the precipitate.

It was important to use capillary tubes to shorten the time needed for toxin solution to come up to the denaturing temperature. The rate of cooling also had an effect on the activity of the solutions heated at 90 and 100 C. When the capillary tubes were not immersed immediately in ice water, the inactivation curve at 100 C was displaced 20 to 30% lower than the curve in Fig. 3.

The rapid loss of activity and accompanying visible aggregation when the enterotoxin was heated at 70 and 80 C seem due to a heat aggregation. There may be some retention of the native state since heating at 100 C can redissolve the aggregate, causing a 35 to 40% recovery of immunological activity. This heat aggregation of molecules in their native state or only slightly modified occurs with a number of proteins (1, 3, 5, 11) and can be reversed by higher temperatures because of the low bond energy of the coagulation sites. Perhaps an investigation of the disassociation of the aggregate by various reagents would help identify the nature of these bonds. The inactivation that occurs at higher temperatures, for enterotoxin B at 90 and 100 C, involves an aggregation generally irreversible since intermolecular hydrophobic, ionic, and disulfide bonds could be involved (5).

During isolation of enterotoxin B, the toxin at low protein concentrations was stable at high ionic strength, but precipitated upon dialysis. This could be analogous to the slight prevention of aggregation at 70 C seen in the effect of low ionic strength on heat denaturation. The two curves are different for *p*H 4.5 and 7.5, but, since ionic strength does have an effect, it shows that ionic bonds play a part in this 70 C heat denaturation.

As expected for an aggregation phenomenon, an increase in the concentration of protein and, therefore, aggregation sites increases the amount of denaturation at 70 C.

The enterotoxin aggregated at 70 C and *p*H 6.4 had no toxicity when injected into dogs and did not react with the antibody. The dose given was such that, if the aggregated toxin had retained its initial toxicity, a 100% positive reaction would be obtained, but if the activity were equal to the amount of immunological activity (10% of the initial), the reaction would be negative. The recovery of immunological activity upon reheating at 100 C seemed to parallel the recovery of the toxic activity when injected into dogs. With 40% remaining activity as measured by gel diffusion, the effective dose in the dogs was 0.2 µg/kg, sufficient to obtain a positive reaction.

Satterlee and Kraft (9) have shown that, in an impure system, enterotoxin B is immunologically inactivated more rapidly at 70 to 80 C than at 90 to 100 C. We have repeated this under various conditions on a pure system, showing that the 70 to 80 C denaturation can be explained by a reversible heat aggregation of the pure toxin. What slight effects other proteins would have on the pure enterotoxin denaturation remains to be seen, but from previous work (9) it seems that the 70 to 80 C heat-inactivated aggregate can be formed in a food environment. This aggregate was biologically inactive when tested by intravenous injection into dogs; however, oral administration of this aggregated enterotoxin might give a positive reaction since the various conditions such as the *p*H of the digestive tract might be capable of releasing the toxin in an active form. On the other hand, the toxin might be more labile to proteolytic attack after having been heated at 70 C.

In general, the enterotoxin is very heat-stable, losing activity very fast initially and then leveling off. This decrease in activation rate might be due to a stabilizing effect of the denatured toxin.

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