Effect of Sodium Chloride and pH on Enterotoxin C Production

CONSTANTIN GENIGEORGIS, MOHAMED S. FODA, ANTONY MANTIS,1 AND WALTER W. SADLER

Department of Epidemiology and Preventive Medicine, School of Veterinary Medicine, University of California, Davis, California 95616

Received for publication 28 December 1970

Growth and production of enterotoxin C by Staphylococcus aureus strain 137 in 3% + 3% protein hydrolysate powder N-Z Amine NAK broths with 0 to 12% NaCl and an initial pH of 4.00 to 9.83 were studied during an 8-day incubation period at 37 C. Growth was initiated at pH values as low as 4.00 and as high as 9.83 at 0% salt level as long as the inoculum contained at least 10⁸ cells per ml. Rate of growth decreased as the NaCl concentration was increased gradually to 12%. Enterotoxin C was produced in broths inoculated with 10⁸ cells per ml and above and having initial pH ranges of 4.00 to 9.83, 4.40 to 9.43, 4.50 to 8.55 and respective NaCl concentrations of 0, 4, and 8%. In the presence of 10% NaCl, the pH range supporting enterotoxin C production was 4.54 to 7.30 for an inoculum level of 10⁸ cells per ml and 6.38 to 7.30 for 3.6 × 10⁷ cells per ml. In repeated experiments in which the inoculum contained 10⁸ cells per ml, we failed to demonstrate enterotoxin C production in broths with 12% NaCl and a pH range of 4.50 to 8.55 and concentrated up to 14 times. The effect of NaCl on enterotoxin C production followed the same pattern as its effect on enterotoxin B production. As the concentration of NaCl increased from 0 to 10%, yields of enterotoxin B and C decreased to undetectable amounts.

MATERIALS AND METHODS

Enterotoxin C production and purification. Enterotoxin C was produced by growing Staphylococcus aureus strain 137 (ATCC 19095) in a broth medium containing 3% + 3% protein hydrolysate powder (PHP) and N-Z Amine NAK (1). The enterotoxin C was later purified by the method of Borja and Bergdoll (2). A 32-mg amount of highly purified enterotoxin C was obtained from 10 liters of broth. The specific enterotoxin C antiserum to strain 137 and the crude enterotoxin C used during the purification steps were supplied by M. S. Bergdoll of the Food Research Institute of the University of Wisconsin.

Production of antisera. Two young New Zealand white rabbits (4 lb) were inoculated with the purified enterotoxin C. At weekly intervals each rabbit received in order, 11, 23, 112, and 575 μg of enterotoxin C dissolved in 1 ml of saline and later mixed, with a Vortex mixer, with 1 ml of complete Freund’s adjuvant. The antigenic preparation was injected intramuscularly and subcutaneously. Two months after the last injection, the immunity of the rabbits was challenged with 2.3 mg of enterotoxin C in saline injected intramuscularly. Beginning 1 week after the last injection, three 50-ml samples of blood were drawn from the heart over a 3-week period.

Additional challenges with 1.15 mg of enterotoxin

1 Present address: School of Veterinary Medicine, Aristotelian University, Thessaloniki, Greece.
C followed at 3-month intervals. The antiserum used in the present study came from the first blood sample and had a microslide titer (3) of 1:200 against 10 μg of enterotoxin C per ml. A single line of precipitation was obtained with as much as 2,500 μg of enterotoxin C per ml, treated by the microslide procedure, even against undiluted antiserum. These results indicated the high purity of the enterotoxin C preparation.

**Growth media.** The 3% + 3% PHP-NAK medium (1) was used throughout this study as the basic medium to which different amounts of NaCl were added and in which the pH was adjusted to various values. Thirty grams of N-Z Amine NAK (Sheffield Chemical, Norwich, N.Y.) and 30 g of protein hydrolysate powder (Mead Johnson and Co., Evansville, Ind.) were mixed with distilled water to make 600 ml. The powders were dissolved with mild heating. The medium was cooled and then distributed, in volumes of 30 ml, to 20 beakers. Sodium chloride in amounts of 0, 2, 4, 5, or 6 g was dissolved in each beaker, and the pH of each broth was adjusted to the desired value with 1 N NaOH or 1 N HCl. Next, each broth was transferred to a 50-ml volumetric flask, autoclaved for 15 min at 121 C, and cooled to room temperature. Then 0.5 ml of a solution containing 500 μg of both thiamine and niacin, and sterilized by filtration, was added. The volume of the broth in each flask was brought to 50 ml by adding sterile distilled water. A 9-ml sample of each broth was placed individually in a 25-ml Erlenmeyer flask (micro-Fernbach style). The flask was then covered with a glass cap, and later the broth was inoculated with the desired number of staphylococcal cells.

**Inoculation and incubation.** Applying the antiserum-agar plate technique of Sugiyama et al. (16), colonies of strain 137 producing large amounts of enterotoxin C were selected on PHP-NAK agar (pH 6.2) containing 1:40 enterotoxin C antiserum and lyophilized on porcelain beads (9). One bead was added to a test tube containing PHP-NAK broth (pH 6.2, 0% salt), and the broth was incubated for 24 hr. A flask containing 9 ml of the same medium was later inoculated with freshly grown cells and incubated overnight. The next morning the broth was centrifuged, and the supernatant fluid was kept for enterotoxin analysis. The cells were washed twice in saline and then suspended in saline to a desired optical density (OD) at 650 nm by using a Spectronic-20 colorimeter (Bausch & Lomb). Each experimental flask was inoculated with 0.1 ml of the cell suspension, placed in a water-bath shaker (Precision Scientific Co., Chicago, Ill., model 66900) at 37 C, and incubated for as long as 8 days at a speed of 160 rev/min. Control flasks were inoculated with 0.1 ml of distilled water. The number of cells in each flask was estimated by plating in duplicate on blood-agar. The initial OD and pH of the broth were determined and recorded at zero time. The pH was measured with a Beckman Expandomatic pH meter, by using a Q15 Thomas combination electrode.

**Analysis of broths.** Samples of broth, usually 2.5 ml, were taken at 2, 6, and 8 days of incubation. Of the 2.5 ml, 0.5 ml was used for determination of OD after dilution with similar broth. After its pH was determined, the remainder of the sample was placed in a cellophane tube and dialyzed at 2 to 4 C against 20 volumes of distilled water. The water was exchanged once. After dialysis, the samples were lyophilized in a VirTis lyophilizer (model 10-145-MRBA).

To test for the presence and amount of enterotoxin C, the lyophilized samples were first rehydrated with 0.6 ml of phosphate-buffered saline (pH 7.2, 0.02 m) plus 2% NaCl. Next the amount of enterotoxin C was measured by the single-gel, diffusion-tube test (6, 7), with an antiserum diluted 1:60 with gel. The gel diffusion tubes were incubated for 24 hr at 30 C, and diffusion band measurements were evaluated for the amount of enterotoxin C by using a previously constructed, standard enterotoxin C curve. The curve was prepared from data obtained by using various amounts of pure enterotoxin C dissolved in the phosphate-buffered saline. The specificity of the diffusion bands in the gel diffusion tubes as due to enterotoxin-antienterotoxin C precipitation was tested by the microslide, double-gel diffusion test (3).

Leftover sample material was heated at 100 C for 15 min and then tested for the presence of heat-resistant nuclease (Lachica and Genigeorgis, unpublished data).

**RESULTS**

Both NaCl and pH affected growth of staphylococci and production of enterotoxin C. The rate of growth of strain 137 decreased as the NaCl concentration was increased gradually to 12% (Fig. 1B). Optimum pH for growth of this strain appeared to be between 5.00 and 6.50.

In the presence of 0, 4, 8, and 12% NaCl, the respective pH limits for initiation of growth were 4.00 and 9.83, 4.20 and 9.43, 4.40 and 8.55 (highest pH tested), and 4.50 and 8.20 at the inoculum level of 10⁵ to 4 × 10⁸ cells per ml

---

**Fig. 1.** Effect of pH and NaCl concentration on enterotoxin C production (A) by *Staphylococcus aureus* strain 137 and on its growth (B) in PHP-NAK broth for 48 hr at 37 C.
(Table 1). When broths with 10% NaCl were inoculated with $3.5 \times 10^8$ cells per ml, growth was permitted, as this was demonstrated by plating when the pH range was 4.50 to 8.50.

The effects of NaCl and pH on the production of enterotoxin C by strain 137 are indicated in Fig. 1A. Enterotoxin C was produced in broths inoculated with more than $10^8$ cells per ml and having an initial pH range of 4.00 to 9.83, 4.20 to 9.43, 4.50 to 8.55 and NaCl concentrations of 0, 4, and 8%, respectively (Fig. 2). In the presence of 10% NaCl, the pH range supporting enterotoxin C production was 5.45 to 7.30 for the inoculum level of $10^6$ cells per ml and 6.38 to 7.30 for the level of $3.5 \times 10^6$ cells per ml. In repeated experiments in which the inoculum contained $10^6$ cells per ml, we failed to demonstrate enterotoxin C production in broths with 12% NaCl and a pH range of 4.50 to 8.55 and concentrated up to 14 times. The optimum pH for the production of enterotoxin C appeared to be between 5.50 and 6.5 (Fig. 1A).

When the OD of the broth and the yield of

![Figure 2](image-url)

**Fig. 2. Initial combinations of pH and NaCl which supported (shaded area) or did not support (●) enterotoxin C production.**

| Initial pH | Time of incubation (days) | Concentration of sodium chloride (w/v) | OD | pH | OD | pH | OD | pH | OD | pH |
|------------|---------------------------|----------------------------------------|----|----|----|----|----|----|----|----|
| 4.00a      | 2                         | 0.91                                   | 4.3| 0.1 | 4.0| 0.1 | 4.0| NT | NT |
|            | 6                         | 15.6                                   | 8.6| 0.1 | 4.0| 0.1 | 4.0| NT | NT |
| 4.20a      | 2                         | 1.9                                    | 4.9| 0.1 | 4.2| 0.1 | 4.2| NT | NT |
|            | 8                         | 14.7                                   | 8.5| 0.7 | 4.7| 0.7 | 4.2| NT | NT |
| 4.40a      | 6                         | NT                                     | NT | 10.2| 7.4| 0.6 | 4.6| NT | NT |
|            | 8                         | NT                                     | NT | 10.2| 8.4| 8.3 | 7.7| NT | NT |
| 4.50a      | 2                         | 10.3                                   | 7.4| 1.6 | 6.0| 0.5 | 4.8| 0.1 | 4.7 |
|            | 6                         | 11.7                                   | 8.8| 4.1 | 7.0| 3.7 | 6.7| 0.2 | 4.8 |
| 5.45a      | 2                         | 14.0                                   | 8.8| 7.7 | 7.1| 6.7 | 7.0| 1.4 | 5.9 |
|            | 6                         | 18.9                                   | 8.4| 11.2| 7.4| 8.3 | 8.0| 8.2 | 7.7 |
| 6.40a      | 2                         | 12.6                                   | 7.6| 7.3 | 7.5| 5.7 | 7.2| 1.8 | 6.3 |
|            | 6                         | 15.0                                   | 8.8| 10.0| 8.4| 6.0 | 7.8| 5.6 | 7.2 |
| 7.20a      | 2                         | 12.1                                   | 8.1| 6.1 | 7.0| 3.9 | 7.4| 1.8 | 6.9 |
|            | 6                         | 14.1                                   | 8.9| 8.0 | 7.6| 8.4 | 7.9| 7.0 | 7.4 |
| 8.55a      | 2                         | 9.8                                    | 7.7| 5.1 | 8.0| 0.5 | 8.4| 0.2 | 8.5 |
|            | 6                         | 13.5                                   | 8.9| 4.9 | 8.5| 1.4 | 8.4| 0.1 | 8.5 |
| 8.80a      | 2                         | 14.2                                   | 8.8| 4.4 | 8.6| NT  | NT | NT | NT |
|            | 8                         | 14.7                                   | 8.8| 7.1 | 8.4| NT  | NT | NT | NT |
| 9.43a      | 2                         | 7.0                                    | 9.1| 3.0 | 9.2| NT  | NT | NT | NT |
|            | 6                         | 12.6                                   | 8.8| 2.7 | 8.8| NT  | NT | NT | NT |
| 9.83a      | 2                         | 0.4                                    | 9.6| 0.1 | 9.8| NT  | NT | NT | NT |
|            | 6                         | 1.7                                    | 9.0| 0.1 | 9.1| NT  | NT | NT | NT |

* Initial OD 0.100 or 3.3 $\times 10^6$ cells/ml.

b Not tested.

* Initial OD 0.045 or 10$^6$ cells/ml.

* Initial OD 0.140 or 4.0 $\times 10^6$ cells/ml.
enterotoxin C after 48 hr of incubation were plotted as ordnates on semilog paper and the NaCl concentration was plotted as the abscissa, an essentially linear relationship was evident between concentration of NaCl in the broth and both OD and yield of enterotoxin C. Both OD and yields decreased with increases in the NaCl concentration. This relationship was true at least for pH 5.50, 6.50, and 7.50 and 0 to 8% NaCl. Data for higher NaCl concentrations and pH values outside the range of 5.50 to 7.50 were not sufficient to permit evaluation.

**DISCUSSION**

At first glance the present findings indicate that production of enterotoxin C in foods heavily contaminated with staphylococci cannot be prevented by the proper manipulation of pH, NaCl concentration, or combinations of the two within levels acceptable by the consumer. Yet in view of the ideal laboratory conditions used, it is difficult to extrapolate the findings in broths to food products or environmental situations that might exist in food processing plants. The medium used for these experiments was favorable for the growth of staphylococci and free from natural inhibitors and competing microorganisms that may be present in food. The broths were heavily inoculated with staphylococci and incubated at or near optimal temperature with heavy aeration. Consequently the limiting pH values and NaCl concentrations represent the minima and maxima below or above which there is no enterotoxin C production. Therefore, a processor could feel safe in the knowledge that no enterotoxin is produced in a given product if it is more acid, alkaline, or salty than the limiting pH and NaCl concentration values presented above.

Initiation of staphylococcal growth at a pH as low as 4 has been observed for the first time. This is not surprising in view of the heavy inoculum used and the recent reports on the probability of initiating staphylococcal growth in various environments (4; Genigeorgis, Abstr. 131, 3rd Int. Congr. Food Sci. Technol.). These studies indicated that, for each combination of pH and NaCl, there is a minimum number of cells required for growth in a new population. The inocula with greater numbers of cells thus have a higher probability of initiating growth.

Whether the growth at such a low pH was due to inadvertent selection of a few acid-tolerant cells, or to the shifting of the pH to higher values by metabolic products of nonreproductive living cells or decomposition products of dead cells, has not been determined. Shifting of the pH to higher values will eventually initiate growth and production of enterotoxin. Markus and Silverman (13) have demonstrated release of about 600 μg of protein per ml, including enterotoxin B, by $3 \times 10^{10}$ nonreplicating staphylococcal cells per ml in a nitrogen-free medium incubated for 10 hr at 37°C.

In the present study, there was an inverse linear relationship between growth of staphylococci and concentration of NaCl at pH levels of 5.50, 6.50, and 7.50. In other studies, statistical analysis of the data indicated a direct linear relationship between concentration of NaCl and the probability of initiating growth by five staphylococcal strains in broths with different pH. When statistically untested data for each strain were plotted, the relationship varied from linear to sigmoid, depending on the pH of the broth (4; Genigeorgis, et al., unpublished data). The inhibitory effect of NaCl on the total viable population, regardless of the pH and temperature of incubation of the medium, has been reported previously (10).

Production of any of the enterotoxins has not been reported before in media, including foods, with pH values as low as 4 or as high as 9.83. Tatini et al. (Bacteriol. Proc., p. 10, 1969) reported production of enterotoxin A in sterile, reconstituted, nonfat milk solids with an initial pH of 4.50. Detectable amounts of enterotoxin A were associated with 3 to 5 million cells per ml regardless of the initial inoculum (10⁹ to 10⁸ cells per ml). We have demonstrated enterotoxin B production in Brain Heart Infusions at pH 5.05 (6). Reiser and Weiss (14) recently reported on the production of enterotoxins A, B, and C in four media at three pH levels (5.30, 6.00, and 6.80). With strains 137 and 483, they obtained maximum yields of enterotoxin C at pH 6.00 and 6.80, respectively, in the medium we used in this study. They found that neither the medium nor the pH in the range used materially affected production of enterotoxin A by strains 100 and 196 but that there was considerable effect of pH and medium on production of enterotoxins B and C. Kato et al. (11) studied the effects of pH, composition of broth (various protein hydrolysates), and aeration on production of enterotoxin A. The pH of their broth ranged from 5.0 to 8.0 and the toxin yields ranged from 2.9 to 4.2 μg/ml. In contrast to its effect on production of enterotoxins B and C, pH did not appreciably affect production of enterotoxin A.

The effect of NaCl on production of enterotoxin C aerobically appears to follow the same pattern as with enterotoxin B (6, 8, 12, 15). As the concentration of the salt increases from 0 to 10%, the yields of enterotoxins B and C decrease.
to undetectable amounts. Enterotoxin C was also produced aerobically in cured meats having up to 10% NaCl in the brine (Genigeorgis et al., unpublished data). We have obtained similar results with enterotoxin B (5).

The minimum final OD of a culture positive for enterotoxin C was 1.35. Although high yields of toxin are always accompanied by high OD values, there were broths containing 10 and 12% NaCl with OD values as high as 8.7 but no enterotoxin. Such results indicate that the mechanism of enterotoxin production is more sensitive to osmotic changes than the mechanism controlling cell multiplication. Similar findings have been reported for enterotoxin B (5, 6, 8, 12). Adjustment of pH and addition of NaCl after sterilization of the basic broth did not significantly affect the growth of staphylococci and the production of enterotoxin C. Therefore, for practical reasons this adjustment was done before sterilization.

ACKNOWLEDGMENTS

This investigation was sponsored by the Food Protection and Toxicology Center of the University of California and supported by Public Health Service Research grant FD 00103 from the Food and Drug Administration.

The authors acknowledge the cooperation of M. S. Bergdoll of the University of Wisconsin in providing enterotoxin C and its specific antiserum.

LITERATURE CITED

1. Bergdoll, M. S., C. R. Borja, and R. M. Avena. 1965. Identification of a new enterotoxin as enterotoxin C. J. Bacteriol. 90:1481-1485.

2. Borja, C. R., and M. S. Bergdoll. 1967. Purification and partial characterization of enterotoxin C produced by Staphylococcus aureus strain 137. Biochemistry 6:1467-1473.

3. Casman, E. P., R. W. Bennett, A. E. Dorsey, and J. E. Stone. 1969. The microslide gel double diffusion test for the detection and assay of staphylococcal enterotoxins. Health Lab. Sci. 6:185-198.

4. Genigeorgis, C. 1969. Interaction between curing factors and staphylococci in culture media and food. Proc. 5th Symp. World Ass. Vet. Food Hygienists, Yugoslavia.

5. Genigeorgis, C., H. Riemann, and W. W. Sadler. 1969. Production of enterotoxin B in cured meats. J. Food Sci. 34:62-68.

6. Genigeorgis, C., and W. W. Sadler. 1966. Effect of sodium chloride and pH on enterotoxin B production. J. Bacteriol. 92:1383-1387. (Erratum, vol. 93, p. 772.)

7. Hall, H. E., R. Angelotti, and K. H. Lewis. 1965. Detection of staphylococcal enterotoxin in foods. Health Lab. Sci. 2:179-191.

8. Hojvat, S. A., and H. Jackson. 1969. Effects of sodium chloride and temperature on the growth and production of enterotoxin B by Staphylococcus aureus. Can. Inst. Food Technol. J. 2:56-59.

9. Hunt, G. A., A. Gourevitch, and J. Lein. 1958. Preservation of cultures by drying on porcelain beads. J. Bacteriol. 76:453-454.

10. Iandolo, J. J., L. J. Ordal, and L. D. Witter. 1964. The effect of incubation temperature and controlled pH on the growth of Staphylococcus aureus MF 31 at various concentrations of NaCl. Can. J. Microbiol. 10:808-811.

11. Kato, E., M. Khan, L. Kupoviesh, and M. S. Bergdoll. 1966. Production of enterotoxin A. Appl. Microbiol. 14:966-972.

12. McLean, R. A., H. D. Lilly, and J. A. Alford. 1968. Effects of meat-curing salts and temperature on production of staphylococcal enterotoxin B. J. Bacteriol. 95:1207-1211.

13. Markus, Z., and G. J. Silverman. 1969. Enterotoxin B synthesis by replicating and nonreplicating cells of Staphylococcus aureus. J. Bacteriol. 97:506-512.

14. Reiser, R. F., and K. F. Weiss. 1969. Production of staphylococcal enterotoxins A, B, and C in various media. Appl. Microbiol. 18:1041-1043.

15. Stark, R. L., and P. R. Middaugh. 1969. Immunofluorescent detection of enterotoxin B in food and a culture medium. Appl. Microbiol. 18:631-635.

16. Sugiyama, H., M. S. Bergdoll, and G. M. Dack. 1960. In vitro studies on staphylococcal enterotoxin production. J. Bacteriol. 80:265-270.