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Effects of Meat-curing Salts and Temperature on Production of Staphylococcal Enterotoxin B[1]

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We investigated the effect of time, temperature, and the presence of sodium chloride, nitrates, and nitrites in the medium on the growth and production of enterotoxin B by *Staphylococcus aureus*. Assays by the double gel-diffusion method showed that maximal enterotoxin B production occurs at the beginning of the stationary phase of growth. Lowering the temperature of incubation decreased the amount of toxin produced without affecting the total amount of growth. Increases in concentration of curing salts reduced toxin production more rapidly than cell growth. The relationship of these observations to food-poisoning outbreaks is briefly discussed.

Cured meats, particularly hams, have often been implicated in outbreaks of staphylococcal food poisoning (11), and staphylococci are known to be capable of growth in the presence of permissible and palatable levels of curing salts (1). In 1941, Segalove and Dack (18) showed (by the use of monkey-feeding tests) that enterotoxin production was apparently reduced when the incubation temperature was decreased, but quantitative studies of the effects of temperature, nutritional conditions, and other factors on toxin production, as distinct from total cell growth, have become possible only recently. Purification of enterotoxin B (17) has increased the availability of specific antiserum and thus has permitted a more critical examination of these factors. Genigeorgis and Sadler (9) revealed that enterotoxin B production in Brain Heart Infusion was detectable in media containing 10% NaCl at *p*H 6.9. In their study, a quantitative measurement of the NaCl effect was impossible because NaCl affected the zone width in the single gel-diffusion tube test that they employed. Occasionally, they also observed that growth was influenced only slightly when toxin production had been eliminated. Also, other investigators have illustrated that nutritional factors (13), some inorganic salts (8), and streptomycin and chloramphenicol (8, 16) can reduce toxin production with only a slight effect on growth. However, all of these investigators carried out their experiments at 37 C, whereas foods implicated in food poisoning are rarely maintained at this temperature. The combined effects of nitrates, nitrites, and sodium chloride on the quantitative production of enterotoxin have not previously been investigated, nor has the appearance of enterotoxin in a culture been correlated with the growth curve of the organisms. The data presented in this paper indicate that temperature and length of incubation, as well as presence of curing salts, influence the appearance of toxin independently of the total growth of the culture.

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Materials and Methods

Bacterial strains and culture media. We used *Staphylococcus aureus* ATCC 14458 (U.S. Food and Drug Administration no. 243) throughout this study. Although this strain was originally isolated from a case of enteritis, it has been designated the prototype strain for enterotoxin B production (6), and its enterotoxin has produced an emetic response in cats (3). Also, this strain is serologically identical to the purified enterotoxin B produced by strain S-6, which causes vomiting and diarrhea in monkeys when fed orally (17). In our experience, this organism decreased in enterotoxin production after repeated transfer. Therefore, when a culture failed to produce the expected titer in control flasks, a new culture was started from the stock. Stock cultures were maintained on porcelain beads (10) and working stock cultures on Veal Infusion Agar (Difco) slants.

Production of enterotoxin. Growth from a 21- to 24-hr slant incubated at 37 C was suspended in 0.85% (w/v) NaCl, and its turbidity was adjusted to a reading of 47.5 units on a Klett-Summerson colorimeter (no. 47 filter, at a wavelength of 370 to 510

mμ). This suspension contained (by plate count) approximately 20 × 10^6 cells/ml and was used at the rate of 2% (v/v) as the inoculum for the various media. We employed Brain Heart Infusion (Fisher Scientific Co., Fairlawn, N.J.) for all studies on toxin production. Amounts of 100 ml in 1-liter Erlenmeyer flasks were incubated at appropriate temperatures in a reciprocating shaker bath at 90 rev/min with a 3-cm stroke (Aminco, Silver Spring, Md., model 4-8600) or at 74 rev/min with a 0.5 to 1.0-inch stroke (New Brunswick Scientific Co., New Brunswick, N.J., model 627). We obtained comparable results with the two shakers. Static broth cultures were incubated at 37 C in a Thelco (model 4) Incubator (Precision Scientific Co., Chicago, Ill.). NaCl and $NaNO_3$ in designated amounts were added, as required, to the media, the *p*H was adjusted to 7.0, and the flasks were sterilized by autoclaving at 121 C for 15 min. When nitrites were to be added, $NaNO_2$ solutions were sterilized by filtration and were added aseptically to the sterile media, adjusted to *p*H 7.0. All inorganic salts were reagent grade, and the concentrations described were in addition to any amount present in the medium. Except for experiments in which the effect of temperature on enterotoxin production and cell growth had been determined, all cultures were incubated at 37 C.

After incubation, samples from the cultures were diluted on a Klett-Summerson colorimeter, as necessary, to give accurate turbidity readings. The cultures then were centrifuged, and the concentration of enterotoxin in the supernatant liquid was measured.

Toxin assay. Enterotoxin B was determined by the double gel-diffusion test of Ouchterlony (14, 15) modified by Wadsworth (19), Crowle (7), and Casman (5). This procedure gives a roughly quantitative measure of toxin; a line visible with the naked eye forms in the agar when about 1 μg/ml of toxin meets an optimal concentration of antibody (4). The addition of one or two drops of an 8% (w/v) solution of $HgCl_2$ a few minutes before the test was read accentuated the line and increased the sensitivity (W. G. Roessler, *personal communication*). When we used $HgCl_2$ for this purpose, it was important that we make the agar employed on slides for diffusion in accordance with the following arbitrary procedure. Sodium barbital (0.8 g) and NaCl (0.85 g) were mixed in 100 ml of distilled water and adjusted with HCl to *p*H 7.4. We added 1 g of agar (Difco) and dissolved it by direct heating, with constant stirring. As soon as the agar had melted completely, the preparation was transferred in 25-ml portions into bottles and autoclaved at 121 C for 15 min. Slight deviations from this routine (probably resulting in different final *p*H value) produced agar in which a heavy white precipitate appeared immediately upon addition of $HgCl_2$. Experiments in which varying concentrations of NaCl (up to 10%, w/v) were added to known concentrations of enterotoxin indicated that NaCl would not interfere with the sensitivity of the test.

Antiserum and reference enterotoxin. Rabbit antiserum, produced by injecting purified enterotoxin B from strain S-6 and yielding a single line with purified toxin (17), was lyophilized and was stored at 2 to 4 C. Reconstituted serum also was stored at 2 to 4 C, and it was used for periods up to 1 month. Reference enterotoxin for routine assays was a crude preparation produced by *S. aureus* ATCC 14458. We grew the culture in Brain Heart Infusion under aerated conditions at 37 C for 24 hr; the supernatant fluid was dispensed in 0.1-ml portions into screw-cap vials and was then lyophilized and stored.

Results

Enterotoxin stability under aerated conditions. Aerated cultures produced toxin more rapidly and in a larger amount than did static cultures (Fig. 1). To determine the stability of the enterotoxin in an aerated culture we divided the supernatant fluid from an 8-hr culture containing a high titer of enterotoxin into four portions and treated it as follows: (i) no further treatment, (ii) filtered through membrane filter (0.45-μ pore size), (iii) filtered through a Seitz filter (0.1-μ pore size), and (iv) added Merthiolate to make a final concentration of 0.01%. The samples were reincubated on the shaker at 37 C for 40 hr. In 18 hr, growth appeared in the untreated sample and it was not assayed because any toxin present could have resulted from new synthesis. In the other three samples, the toxin titer was unchanged during 40 hr on the shaker at 37 C.

Relationship of cell multiplication to enterotoxin production. Figure 2 shows that enterotoxin was first detectable after 5 hr of incubation—at about the end of the exponential growth phase and about the time the *p*H curve shifted. The enterotoxin titer increased steadily for 16 to 20 hr, although cell growth by 7 to 9 hr had reached the stationary phase.

Inorganic salts and toxin production. Concen-

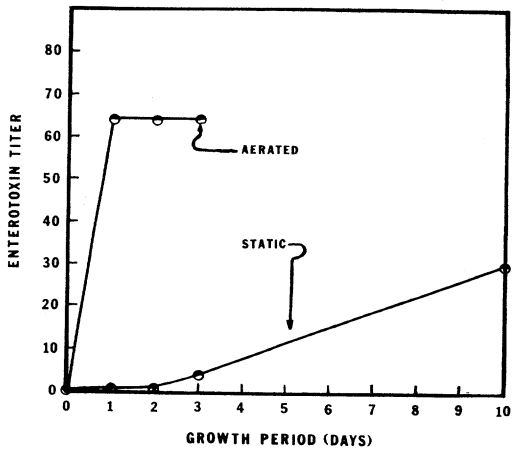

FIG. 1. *Comparison of aerated versus static incubation for enterotoxin production.*

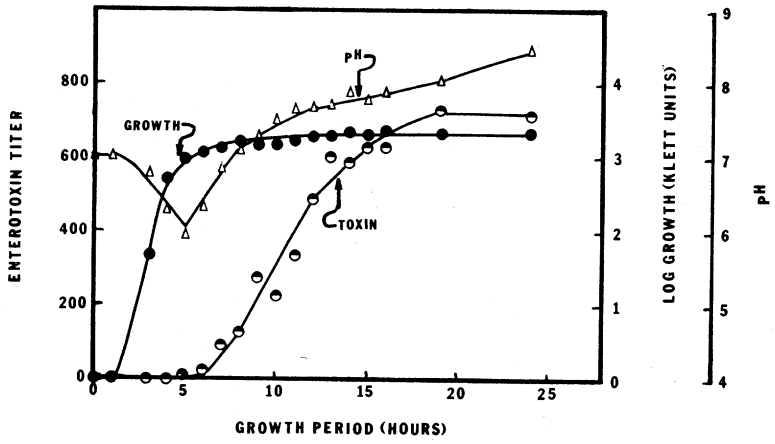

FIG. 2. *Enterotoxin production, growth, and pH changes in Staphylococcus aureus at 37 C.*

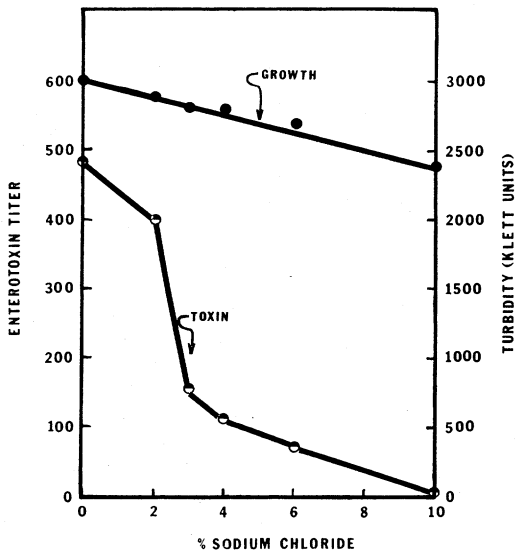

FIG. 3. *Effects of sodium chloride on total growth and enterotoxin production.*

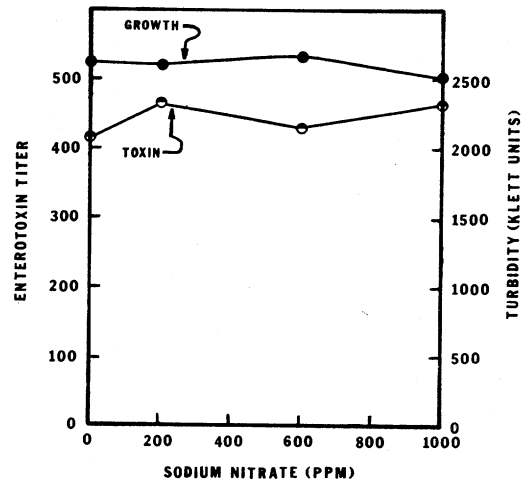

FIG. 4. *Effects of sodium nitrate on total growth and enterotoxin production.*

trations of NaCl up to 10% had a relatively slight effect on total growth, but they caused a definite decrease in toxin production above 3% NaCl (Fig. 3). Another medium that contained a casein hydrolysate as the nitrogen source was inoculated to determine whether the salt effect was related to the type of protein in the medium. Although enterotoxin levels were lower with the second medium, the rates of reduction in enterotoxin in relation to the NaCl concentration were comparable. Figures 4 and 5 suggest that neither $NaNO_3$ in concentrations up to 1,000 ppm nor $NaNO_2$ up to 200 ppm (maximum concentration permitted in cured meats) would affect either growth or enterotoxin production in broth. Table 1 indicates that combinations of the curing ingredients may have an effect at a lower salt concentration.

Effect of temperature. In an experiment in which frequent determinations were established during the first 85 hr of incubation at 20 C, we observed that the culture had reached the stationary phase between 55 and 60 hr, and toxin production had reached its maximal titer of 100 by 65 hr. The 37 C control had a titer of over 500. From another experiment (Table 2), we showed that with prolonged incubation at 16 C and 20 C, total growth approached that observed

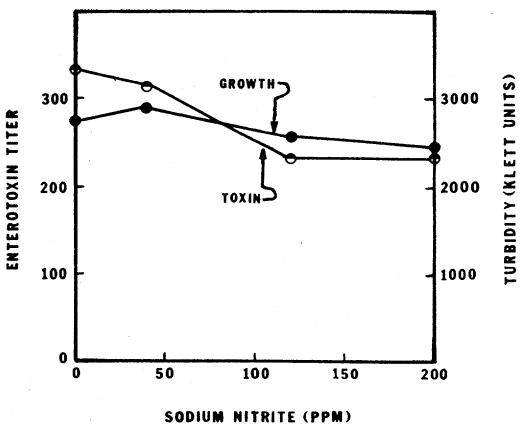

FIG. 5. *Effects of sodium nitrite on total growth and enterotoxin production.*

TABLE 1. *Effects of combination of curing ingredients on growth and enterotoxin production*

Medium	Klett reading[a]	Titer[a]
BHI control	2,650	400
NaCl, 2%	2,400	300
$NaNO_2$, 120 ppm	2,700	400
$NaNO_3$, 200 ppm	2,600	400
$NaNO_3$, 600 ppm	2,900	400
Combination A[b]	2,350	150
Combination B[c]	2,250	150

[a] Average of four trials.
[b] BHI broth, plus 2% NaCl, 120 ppm $NaNO_2$, and 200 ppm $NaNO_3$.
[c] Same as footnote *b* except that the amount for $NaNO_3$ was 600 ppm.

TABLE 2. *Effect of different incubation temperatures on growth and enterotoxin production by Staphylococcus aureus ATCC 14458*

Incubation time (hr)	Log of Klett reading			Enterotoxin titer		
	16 C	20 C	37 C	16 C	20 C	37 C
18	—[a]	—	3.4	—	—	150
112	3.0	3.0	3.2	8	20	340
170	2.9	2.8	3.3	10	25	260
207	2.7	—	—	8	—	—

[a] Determination not made.

at 37 C, but the production of enterotoxin remained low.

DISCUSSION

The ability of staphylococci to grow in the presence of curing salts (12) and at temperatures near 20 C to a cell density essentially equivalent to that of 37 C (18) has been known for many years. However, the data given in this paper emphasize that it is impossible to extrapolate the effect of these parameters on growth to a similar effect on enterotoxin B production. We recognized that the double gel-diffusion method for the detection of enterotoxin is only semiquantitative. Variations between experiments are commonly observed even though replicate determinations within a given experiment usually agree quite closely. Nevertheless, it is apparent that, in relatively low concentrations, curing salts will reduce toxin production. Whether the effect of combinations of curing salts on toxin production is more than an additive effect is not clear. Likewise, when we lowered the temperature of incubation of a culture, the amount of toxin produced decreased more rapidly than did its growth rate. Future studies will establish whether the temperature effect is on enterotoxin synthesis or on its release from the cell.

The absence of detectable enterotoxin until the end of the log phase and its accumulation during the stationary phase of growth further indicate that enterotoxin production is not inseparably bound to cell multiplication. This observation, plus the appearance of enterotoxin after the shift in *p*H and the association of enterotoxin with only a relatively few strains of *S. aureus*, suggest that it is a secondary metabolite, like many antibiotics (2).

Applicability of the data we obtained with this strain of *Staphylococcus* isolated from a nonfood source to known food-poisoning strains producing enterotoxin B might be questioned. However, the ability of the enterotoxin B from strain 243 to cause an emetic response in cats and its serological identity to the enterotoxin that produces the typical food-poisoning syndrome in monkeys implies that it is typical enterotoxin B. Obviously, other strains that produce the same chemical entity might respond differently to the environment. Nonetheless, the effects shown in this investigation could have served to limit food-poisoning outbreaks, even though large numbers of staphylococci were involved.

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