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Effect of Sodium Chloride and *p*H on Enterotoxin B Production

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Abstract

Genigeorgis, Constantin (University of California, Davis), and Walter W. Sadler. Effect of sodium chloride and *p*H on enterotoxin B production. J. Bacteriol. 92:1383–1387. 1966.—The growth and production of enterotoxin B by *Staphylococcus aureus* strain S-6 in Brain Heart Infusion broth with 2 to 16% sodium chloride and an initial *p*H of 5.1 to 6.9 was studied during a 10-day incubation period at 37 C. Growth was good at *p*H 6.9 and with a 16% concentration of salt, but no cells survived after 10 days of incubation at *p*H 5.1 and with a 16% concentration of salt. With geldiffusion technique, enterotoxin B was detected in broth with *p*H 6.9 and up to 10% salt or *p*H 5.1 and up to 4% salt. Growth and enterotoxin production were better when *p*H was increased and salt concentration was decreased. The dependence of toxin production on the interaction of these two factors was demonstrated.

The effect of sodium chloride and *p*H on the growth of food-poisoning staphylococci in culture media has been studied (8, 9, 10), but information is limited on the effect of these two factors on the production of enterotoxin. Such studies were considered to be quite difficult, because of the lack of an easy and expeditious assay for enterotoxin. Bergdoll (1) studied the effect of *p*H and Casman and Bennett (3) studied the effect of sodium chloride and *p*H on the production of enterotoxin B and A, respectively, so as to develop a culture medium giving higher yields of toxin than those used previously. Now that specific antisera against types A, B, and C enterotoxins have become available, and immunological techniques for identifying and measuring their concentrations have been worked out, studies on the conditions under which they are produced have become quite challenging. This paper reports results of a study of the combined effect of sodium chloride and *p*H on the growth and production of enterotoxin B by *Staphylococcus aureus* strain S-6 (producing both types A and B enterotoxins).

Materials and Methods

Purified enterotoxin B and specific rabbit antiserum were available through the courtesy of M. S. Bergdoll, University of Chicago.

Rather than the presently used semisynthetic media (1), Brain Heart Infusion (BHI) broth (Difco) was chosen as the culture medium for enterotoxin B production. BHI broth is a rich medium and closely resembles natural foods. In the BHI medium we were able to obtain yields of toxin of up to 180 μg/ml. This amount was considered satisfactory for the purpose of the present experiments.

A 150-ml amount of BHI broth was distributed among eight flasks. Sodium chloride was then added to obtain concentrations of 2, 4, 6, 8, 10, 12, 14, and 16% (w/v). The flasks were autoclaved, cooled, and then, from each concentration, five 25-ml samples were distributed to 125-ml Erlenmeyer flasks. Thus, five groups of flasks were prepared, each containing concentrations of sodium chloride ranging from 2 to 16%. The *p*H of each group was adjusted to 6.9, 6.5, 6.0, 5.5, or 5.1 by the addition of $NaH_2PO_4 \cdot H_2O$ (Table 1). The flasks were autoclaved again and inoculated within 2 hr. *S. aureus* strain S-6 was grown in BHI broth for 12 hr at 37 C, and the culture was washed three times with saline to remove residual enterotoxin. A suspension of washed cells was prepared after the last washing, and the number of cells was estimated by plating on BHI agar. A 0.25-ml amount of the suspension was inoculated into each flask to give 1.5×10^7 cells per milliliter. The flasks were placed on a rotary shaker (154 strokes per min) and were incubated aerobically for 10 days at 37 C. Samples (3 ml) were taken after 2 min, 12 and 24 hr, and 6 and 10 days, and optical density was measured at 660 mμ with a Spectronic-20 colorimeter (Bausch & Lomb, Inc.), and *p*H was determined with a Beckman H2 glass-electrode *p*H meter.

Demonstration of enterotoxin. After the measurement of optical density and *p*H, all samples were then centrifuged and the supernatant fluid was analyzed for enterotoxin by the single gel-diffusion tube test (7). The single gel-diffusion tube test was performed as

TABLE. 1 *Adjustment of pH by $NaH_2PO_4 \cdot H_2O$*

*p*H	Salt concn (w/v)							
	2%	4%	6%	8%	10%	12%	14%	16%
5.1	1.3452[a]	1.0290	1.0072	0.7847	0.7134	0.5547	0.4813	0.4059
5.5	0.5758	0.5176	0.4340	0.3697	0.2963	0.2390	0.2366	0.1912
6.0	0.2198	0.1816	0.1434	0.1099	0.1004	0.0860	0.0784	0.0741
6.5	0.0693	0.0527	0.0454	0.0382	0.0339	0.0325	0.0311	0.0292
6.9	0.0215	0.0167	0.0143	0.0110	0.0105	0.0096	0.0081	0.0072

[a] Amount (g) of $NaH_2PO_4 \cdot H_2O$ added to 25 ml of BHI broth with 2 to 16% (w/v) sodium chloride.

described by Hall et al. (7) except that each tube contained 0.3 ml of antiserum diluted 1:40 with 0.3% Oxoid Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.) in a standard buffer [0.02 M phosphate-buffered saline (*p*H 7.2) with 1:10,000 Merthiolate]. A 0.3-ml amount of the supernatant fluid was placed on the top of the agar column, and the tubes were sealed with plastelin and incubated 7 days at 30 C ± 1. The width (mm) of the specific precipitation zone was measured at the end of the incubation period. A standard enterotoxin B reference curve was available, based on data obtained when different amounts of highly purified toxin were added to BHI broth and were tested by single gel-diffusion tube test according to Hall et al. (7). This standard curve was not used for quantitative estimation of the concentration of toxin in the sodium chloride broth, because it has been demonstrated that the ionic strength and, therefore, the sodium chloride concentration of the broth affects to a considerable degree the width of the zone of the specific precipitation (6, 7). Thus, increasing the sodium chloride from 0 to 16% also increases the width. No concentration of the samples was applied for the detection of less than 0.45 to 0.9 μg of toxin per ml, which is the sensitivity limit of the single gel-diffusion system used (Genigeorgis, *unpublished data*). The identity of the band formed in the gel-diffusion tube with enterotoxin B antienterotoxin system was established by the use of the micro-diffusion slide test (5) with purified enterotoxin B as reference.

The number of viable cells was estimated in some of the samples by standard plate counts on BHI and Mannitol Salt Agar (Difco).

RESULTS

Tables 2, 3, 4, and 5 present the results on samples taken after different periods at 37 C and analyzed for optical density, final *p*H, enterotoxin B, and number of viable cells per milliliter. Table 2, however, does not accurately reflect the concentration of enterotoxin. It mainly demonstrates the presence or absence of enterotoxin. Also, considering the effect of ionic strength on migration of enterotoxin-antienterotoxin band, some comparative conclusion can be obtained regarding the effect of *p*H and sodium chloride concentration upon enterotoxin production. The results clearly demonstrate that both *p*H and salt concentration have an effect not only on growth, as demonstrated before (9, 10, 11), but also on toxin production. Growth rate decreased progressively with the gradual lowering of pH from 6.9 to 5.1 or with the gradual increase of sodium chloride concentration from 2 to 16%. An interaction of these two enviromental factors was demonstrated by the fact that the upper salt concentration and the lower *p*H limits for growth and enterotoxin production were dependent upon each other. Staphylococci grew well in 16% salt when initial *p*H was 6.9. At this *p*H, enterotoxin B was detecable in flasks with up to 10% salt. Detectable amounts of enterotoxin B were also produced at initial *p*H 5.5 with up to 8% salt, and at *p*H 5.1 with up to 4% salt. The growth of strain S-6 in BHI broth caused the *p*H of the medium to increase to values up to 8.6. The cells remained viable at this *p*H. At all *p*H values used, enterotoxin B concentration was always highest with the lowest sodium chloride concentrations. The best *p*H for enterotoxin B production was the highest of those used (6.9). After 10 days of incubation, there were 2×10^9 cells per milliliter of broth in the flask containing 16% salt, with an initial *p*H of 6.9 and a final pH of 7.4. Growth is demonstrated under these conditions in that the number of cells increased from 1.5×10^7 per milliliter to 2×10^9 per milliliter. No viable cells were demonstrated in samples taken after 10 days which contained 12, 14, and 16% salt with an initial *p*H of 5.1. In 32 of the 40 flasks, the optical density reached values above 1.44. The latter was the minimal optical density value in which enterotoxin was detected in 10 days. There were 11 flasks with optical densities above 1.44, ranging from 2.36 to 3.92 without any indication of toxin production. This means that enterotoxin production does not always correlate with good growth.

The amount of phosphate added to control the *p*H varied from 0.007 to 1.3452 g per 25 ml of broth, and was increased as the *p*H decrease. To what extent the added phosphate affects the effect

TABLE 3. *Effect of initial pH and sodium chloride concentration on the growth of strain S-6 on BHI broth after 12 and 48 hr of incubation at 37 C*

Initial *p*H[a]	Time (hr)	Concn of sodium chloride (w/v)															
		2%		4%		6%		8%		10%		12%		14%		16%	
		OD[b]	*p*H	OD	*p*H	OD	*p*H	OD	*p*H	OD	*p*H	OD	*p*H	OD	*p*H	OD	*p*H
6.9	12	4.24	7.45	2.08	7.10	2.14	7.05	2.18	7.00	2.24	6.95	2.18	6.95	1.4	6.90	0.52	6.90
	48	4.88	8.15	4.88	8.15	4.88	8.00	4.80	7.92	4.45	7.78	3.04	7.70	1.88	7.35	0.36	6.90
6.5	12	3.66	7.10	2.60	6.75	1.98	6.75	1.93	6.75	1.92	6.70	1.92	6.70	1.40	6.68	0.50	6.50
	48	4.52	8.00	4.04	7.85	4.44	7.70	4.24	7.55	3.36	7.35	1.44	6.75	1.20	6.55	0.68	6.52
6.0	12	3.00	6.80	1.90	6.40	1.75	6.35	1.42	6.30	1.32	6.15	1.05	6.05	0.20	6.00	0.07	6.00
	48	4.40	7.35	4.40	6.98	3.16	6.85	3.16	6.60	2.84	6.50	1.24	6.10	1.00	6.00	0.38	6.05
5.5	12	1.65	5.90	1.44	5.80	1.35	5.75	1.32	5.65	1.24	5.60	0.79	5.55	0.03	5.50	0.025	5.50
	48	2.52	6.10	1.72	5.90	1.73	6.00	1.72	5.80	1.12	5.62	0.92	5.60	0.24	5.65	0.16	5.70
5.1	12	0.69	5.18	0.57	5.15	0.30	5.15	0.13	5.12	0.05	5.10	0.05	5.10	0.03	5.10	0.03	5.10
	48	1.48	5.25	1.08	5.15	1.12	5.28	0.32	5.18	0.18	5.20	0.18	5.15	0.18	5.15	0.08	5.15

[a] Initial optical density was 0.025 to 0.030.
[b] Final determination.

TABLE 4. *Effect of initial pH and sodium chloride concentration on the growth of strain S-6 on BHI broth after 6 and 10 days of incubation at 37 C*

Initial *p*H[a]	Time (hr)	Concn of sodium chloride (w/v)															
		2%		4%		6%		8%		10%		12%		14%		16%	
		OD[b]	*p*H	OD	*p*H	OD	*p*H	OD	*p*H	OD	*p*H	OD	*p*H	OD	*p*H	OD	*p*H
6.9	6	3.80	8.60	3.83	8.50	4.00	8.45	4.00	8.40	4.00	8.15	3.68	8.20	3.20	8.15	NT	NT
	10	NT[c]	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	2.2	7.4
6.5	6	3.85	8.15	3.84	8.35	3.84	8.20	3.92	8.20	3.92	8.15	3.92	7.95	NT	NT	NT	NT
	10	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4.2	7.4	2.0	7.04
6.0	6	3.36	7.85	2.8	7.8	3.04	7.65	2.60	7.25	3.24	6.90	NT	NT	NT	NT	NT	NT
	10	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	3.1	6.68	2.12	6.45	1.04	6.35
5.5	6	3.52	6.18	3.78	6.10	2.00	6.00	2.44	6.00	NT	NT	NT	NT	NT	NT	NT	NT
	10	NT	NT	NT	NT	NT	NT	NT	NT	3.08	5.9	2.04	5.92	0.24	5.72	0.10	5.62
5.1	6	3.40	5.75	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	10	NT	NT	2.68	5.58	2.36	5.50	0.13	5.25	0.13	5.24	0.07	5.15	0.07	5.15	0.01	5.20

[a] Initial optical density was 0.025 to 0.030.
[b] Final determination.
[c] Not tested.

of *p*H upon growth and enterotoxin production is not clear from the data obtained. Casman and Bennett (3) found that the yield of enterotoxin A in Edamin S (pancreatic digest of lactalbumin) increased when they increased the concentration of monosodium phosphate from 0 to 0.6%.

DISCUSSION

Bergdoll (1), using gel diffusion, studied the effect of the initial *p*H of a medium containing enzyme-hydrolyzed casein supplemented with niacin and thiamine upon the production of enterotoxin B by strain S-6. Under his experimental

TABLE 5. *Effect of pH and sodium chloride concentration on the survival of cells of strain S-6 in BHI broth after 10 days of incubation at 37 C*

Initial *p*H	Concn of sodium chloride (w/v)[a]						
	4%	6%	8%	10%	12%	14%	16%
6.9	NT[b]	NT	NT	NT	NT	NT	2×10^9
6.5	NT	NT	NT	NT	NT	3.6×10^8	3.5×10^7
6.0	NT	NT	NT	NT	5×10^8	1.4×10^8	1×10^7
5.5	NT	NT	NT	2.3×10^8	2×10^8	6×10^4	8×10^1
5.1	1.6×10^8[c]	5.5×10^7	5×10^3	4×10^3	0[d]	0	0

[a] Concentrations at 2% (w/v) were not tested.
[b] Not tested.
[c] Results of plate counts expressed as cells per milliliter.
[d] Zero-time plate counts were 1.5×10^7 cells per milliliter.

conditions, more toxin was produced at *p*H 6.0 than at 5.0, 7.0, or 8.0. Our results with the sodium chloride-supplemented media differ in that yields of toxin were higher with more alkaline broth, but they agree in demonstrating that enterotoxin B can be produced in media with a *p*H of 5. Lechowich et al. (9) found that aerobic growth in Tryptose-Yeast Extract broth (Difco) (with 5% sodium chloride) was prevented when the initial *p*H was 4.8. However, the rate of growth increased as the initial *p*H was increased from 4.8 to 6.9. Casman and Bennett (3) found that a semisolid Brain Heart Infusion agar at *p*H 5.3 to 5.5 gave the highest yield of enterotoxin A. Christian (4) reported that staphylococci had a wide *p*H range for growth in normal rich bacteriological medium. However, when he added approximately 3.5 M sodium chloride, the *p*H range that permitted growth was narrowed to between 5.0 and 6.0. Our results indicate that as sodium chloride concentration was increased, a higher *p*H was necessary for growth and toxin production to be comparable to those at the previous lower salt concentration.

Hucker and Haynes (8) found that the growth rate of food-poisoning staphylococci in veal broth decreased as the sodium chloride concentration was increased from 6 to 12%. Growth was definitely faster in the absence of salt. Our results agree with those observations, also demonstrating that toxin production followed a similar pattern. Nunheimer and Fabian (10) reported that the growth of enterotoxigenic staphylococci was inhibited by concentrations of salt from 15 to 17.5%, and a germicidal effect was demonstrated with concentrations over 20%. Our results do not agree completely with those reports, for they demonstrate that even 12% salt had a germicidal effect when initial *p*H was 5.1, whereas 16% salt supported good growth when initial *p*H was 6.9. Buttiaux and Moriamez (2) reported that *S. aureus* survived in meat brines with 23.5% salt and various amounts of nitrite and nitrate. The percentage of survival was higher with incubation at 6 C than at 15 C. Parfentjev and Catelli (11) reported that *S. aureus* grew at 37 C in Tryptose Broth saturated with sodium chloride. Disagreements on the upper salt concentration limits that permit growth can be explained as a result of the interaction of the different environmental factors such as nutrients, *p*H, or temperature. Enterotoxin B was produced in detectable amounts in BHI broth containing up to 10% salt and having a *p*H of 6.9. The possibility that, with an increase of the initial *p*H of broth, enterotoxin may be produced at even higher salt concentration cannot be excluded. On the basis of the present results, we cannot also eliminate the possibility that enterotoxin can be produced in meat brines with high salt concentration.

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