

Effect of Water Activity on Enterotoxin B Production and Growth of *Staphylococcus aureus*

JOHN A. TROLLER

Winton Hill Technical Center, Procter & Gamble Company, Cincinnati, Ohio 45239

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Staphylococcus aureus C-243, an enterotoxin B-producing strain, was cultured on media adjusted to various water activity (a_w) levels by means of two different solute systems. Total numbers and rate of growth were diminished at low a_w levels, and enterotoxin synthesis was extremely sensitive to reduction in a_w . A reduction of a_w from 0.99 to 0.98 in one medium and from 0.99 to 0.97 in the other medium resulted in extremely low levels of enterotoxin in spent culture media despite the attainment of high numbers of staphylococci.

The importance of microbial moisture requirements in the spoilage of many types of foods has long been recognized. More recently, these requirements have been quantified, and the maximal, minimal, and optimal moisture levels, usually stated as water activity (a_w), for the growth of a number of bacterial, mold, and yeast species are known (13).

Staphylococci are particularly tolerant of low moisture levels, a characteristic frequently exploited by the addition of relatively high concentrations of NaCl to selective media. Scott (12) investigated the effect of a_w on the growth of 14 food-poisoning strains of staphylococci and found that the growth rates were uniformly reduced as a_w was lowered by the addition of solutes or combinations of solutes. It was further noted that the minimal a_w which would allow staphylococcal growth (0.86) was independent of the medium and of the solutes employed to adjust the a_w of the medium as well as the minimal water content which would allow growth. Scott thus concluded that the minimal water necessary for bacterial growth was best expressed as a_w rather than as relative water content or solute concentration. The relationships of a_w and nutrients, pH and temperature, and their effects on staphylococcal growth have been adequately reviewed elsewhere (3).

The relatively recent development of pure antisera (1) and subsequent serological techniques for the detection of staphylococcal enterotoxins (2) have resulted in numerous studies in which various factors affecting enterotoxin synthesis have been evaluated. Although the effect of NaCl solutions on enterotoxin production by staphylococci has been studied (4, 5, 7, 8), no

attempts have been made to relate the effects seen to more broadly applicable a_w values or to determine if the observed inhibition of enterotoxin was the result of a_w adjustment alone.

In the present study, the moisture requirements for enterotoxin B synthesis and staphylococcal growth are established, and the significance of these findings with regard to enterotoxigenesis in foods is discussed.

MATERIALS AND METHODS

Cultures and growth conditions. The *Staphylococcus aureus* C-243 strain used in this study was obtained from M. S. Bergdoll, Food Research Institute, Madison, Wis. This strain is the prototype, enterotoxin B-producing strain.

Stock cultures for storage were maintained at 5 C on porcelain beads, and working stock cultures were grown and subsequently maintained at 5 C on Plate Count Agar (Difco Laboratories, Inc., Detroit Mich.) slants which were transferred monthly. Stored stock cultures were used only when the levels of enterotoxin synthesis in control media declined. Plate counts were obtained in Plate Count Agar after 48 hr of incubation at 37 C. The medium used for the staphylococcal growth experiments was a modification of the medium of Rieser and Weiss (11) containing 3% partially hydrolyzed protein (PHP; Mead Johnson & Co., Evansville, Ind.) and 3% NZ Amine NAK (hydrolyzed protein; Sheffield Chemical, Div. of National Dairy Products Corp., Norwich, N.Y.), 0.001% nicotinic acid, and 0.00005% thiamine. The pH in all cases was adjusted to 6.8 before autoclaving; incubation was at 30 C on a shaker reciprocating at 108 excursions per min.

The basic medium described above was modified for the adjustment of a_w in two ways. In the first medium (medium A), a_w was adjusted by adding equal concentrations of additional PHP and NZ Amine NAK. This resulted in the a_w levels shown in Table 1 and the

TABLE 1. Relationship between solute content of basal medium and water activity (a_w)

Solute	a_w Adjustment % solute added	Per cent water ^a	a_w
Medium A (PHP + NZ Amine NAK ^b)	6 (Control)	94.5	.99
	30	77	.97
	50	68	.96
	75	57	.93
	100	50	.90
Medium B (glyc- erol)	6 (Control)	100	.99
	15	89	.98
	20	84	.96
	30	77	.93
	37.5	74	.90
	50	68	.87

^a Based on indicated solute content.

^b PHP, protein hydrolysate powder; PHP and NZ Amine NAK are hydrolyzed and partially hydrolyzed protein materials.

sorption isotherm shown in Fig. 1. Although Scott (12) had shown that the lowest a_w supporting growth of *S. aureus* was independent of the solute used to adjust a_w , it was possible that observed effects could be due to inhibitory substances instead of, or in addition to, an isolated a_w effect. For this reason an alternate solute, glycerol, was used to adjust the a_w of a second medium (medium B). In addition to glycerol, this medium also contained 0.5% KH_2PO_4 , 0.2% K_2HPO_4 , and 0.1% $(\text{NH}_4)_2\text{SO}_4$. The a_w of both medium systems was measured after autoclaving, although autoclaving appeared to have little effect on a_w levels. Initial and final a_w values were, in all cases, equal, and corrections for the 1.0-ml inocula were applied to all listed a_w values.

Enterotoxin production. Ten-milliliter samples for enterotoxin analysis were withdrawn from growth flasks concurrently with removal of 1-ml samples for plate counts. The 10-ml samples were centrifuged at $45,000 \times g$ in a refrigerated centrifuge (model RC2-B, Ivan Sorvall, Inc., Norwalk, Conn.) for 15 min and the clear supernatant was directly analyzed for enterotoxin (medium B) or decanted into a short length of dialysis tubing (medium A). Dialysis against distilled water was at 5 C for 18 hr. The dialysis tube was then placed directly in a 50% solution of Carbowax 20 M (Union Carbide Corp., Chicago, Ill.) overnight at 5 C. The tube was subsequently opened and rinsed with 2.0 ml of 0.37% Brain Heart Infusion (BHI; Difco) broth. This material was stored at 5 C in screw-capped tubes. Enterotoxin solutions were found to be stable for relatively long periods at 5 C but were normally tested for enterotoxin content within 48 hr.

Enterotoxin concentrations were estimated by the single gel-diffusion tube method (10) as modified by Weirether et al. (14). Test solutions were placed on the surface of the agar column which normally contained a 1:64 dilution of specific antiserum. If very low levels of enterotoxin B were anticipated, a 1:128 antiserum dilution was employed to increase sensitivity. This

resulted in a decrease in minimal detectable concentrations from 2.0 to 0.5 μg of enterotoxin per ml. Reference plots for each antiserum dilution were established using crude enterotoxin B dissolved in 0.37% BHI. The antiserum and enterotoxin were obtained from M. S. Bergdoll.

Known enterotoxin controls. Known amounts of enterotoxin B (25 $\mu\text{g}/\text{ml}$) were mixed with uninoculated control media adjusted to various a_w levels. The concentration and analysis procedures were identical to those described for inoculated media except that the initial centrifugation and decantation steps were omitted. The enterotoxin-containing controls were maintained at 30 C for 24 to 26 hr before analysis for the toxin. Recovery of enterotoxin from these media ranged from 93 to 100%, suggesting that there was no direct action of low a_w on the enterotoxin itself and that the recoveries obtained were similar to those obtained from normal experimental media.

Humidity measurement. Estimations of a_w are expressed as a_w or the ratio of the vapor pressure of a given solution to that of pure water. The a_w can also be expressed as the equilibrium relative humidity, divided by 100, of an atmosphere above a given material in a closed chamber.

Humidity measurements were carried out with Hygrosensor elements (no. 4-4822; Hydrodynamics, Inc., Silver Spring, Md.) mounted in the lids of 4-oz jars. The sensors were attached to a Hygrometer Indicator (model 15-3000; Hydrodynamics, Inc.). All measurements were carried out at 30 C with approximately 15 ml of medium placed in a 4-oz jar. The samples were allowed to equilibrate for at least 4 hr prior to measurement. Sensors were always calibrated against a saturated KNO_3 solution prior to use.

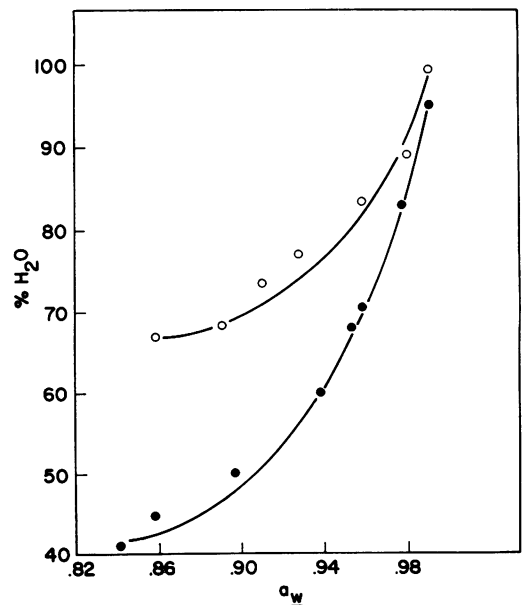


FIG. 1. Sorption isotherms of medium A (●) and medium B (○).

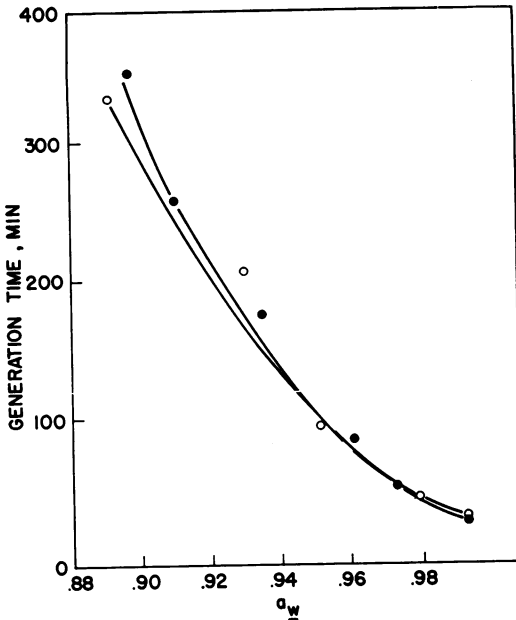


FIG. 2. Effect of a_w on generation times of *S. aureus* C-243 in medium A (●) and medium B (○).

RESULTS AND DISCUSSION

Effect of a_w on growth. The influence of a_w on growth rate of *S. aureus* C-243 can be seen in Fig. 2. Both control media ($a_w = 0.99$) supported rapid growth of the test organism; however, media poised at progressively lower a_w levels produced increasing generation times thus indicating that the observed generation times are a function of a_w . Maximal total numbers (Fig. 3 and 4) obtained at different a_w levels varied with the medium. In medium A, adjusted to a_w 0.90, plate counts were slightly greater than 10^7 staphylococci/ml, whereas medium B, adjusted to a similar a_w , produced counts in excess of 10^9 /ml. Control cultures of medium B also supported slightly higher total numbers than the medium A control. These data also show that at the lowest a_w levels tested (0.90 in medium A and 0.87 in medium B) total numbers of staphylococci exceeded 10^7 /g, a level frequently cited as being necessary to elicit a human emetic response in ingested foods. In view of the published (12) minimal a_w for growth of staphylococci (0.86), the level of growth in medium B adjusted to a_w 0.87 is somewhat unexpected; however, growth response to presumably threshold a_w levels could be great.

Effect of a_w on enterotoxin synthesis. The synthesis of enterotoxin at various a_w levels in media A and B is shown in Fig. 5 and 6. Both control cultures ($a_w = 0.99$) produced appreciable

levels of enterotoxin B; however, the medium B control produced a considerably greater amount of enterotoxin. This difference between the two control media could be a reflection of losses resulting from the separation techniques required prior to enterotoxin analysis in medium A or to differences in the basic formula of the control media. A comparison of enterotoxin synthesis (Fig. 5 and 6) with growth curves (Fig. 3 and 4) shows good agreement with published data (6, 8) indicating that the greater portion of enterotoxin synthesis occurs during the late logarithmic phase of growth. More significantly, however, these data show low enterotoxin levels with decreases in a_w despite virtually no change in numbers of staphylococci. In the case of medium A, which utilized the N-containing components of the medium (PHP and NZ Amine NAK) to adjust a_w , a small quantity of enterotoxin appeared in the medium at a_w 0.97 commencing at 40 hr. No detectable levels ($> 0.5 \mu\text{g}/\text{ml}$) of enterotoxin B were found in media poised at a_w 0.96, 0.93, or 0.90 despite numbers of viable staphylococci in excess of those ($> 10^7/\text{g}$) normally believed to be required to induce enterotoxin poisoning in humans. A similar result was noted

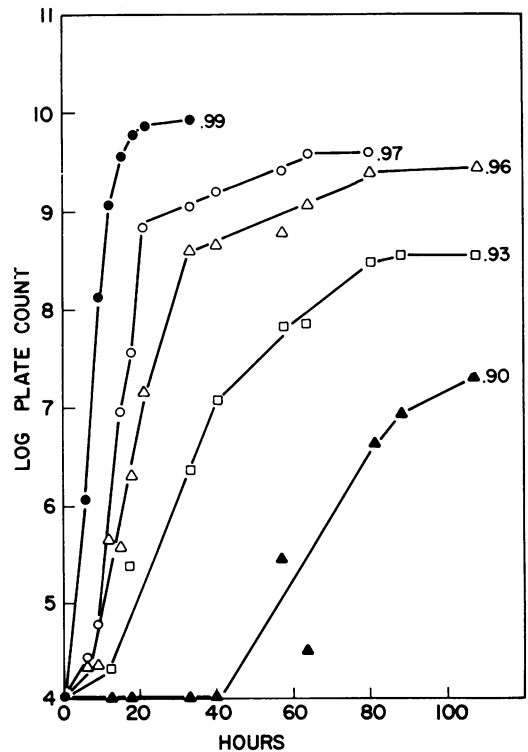


FIG. 3. Effect of a_w on growth of *S. aureus* C-243 in medium A.

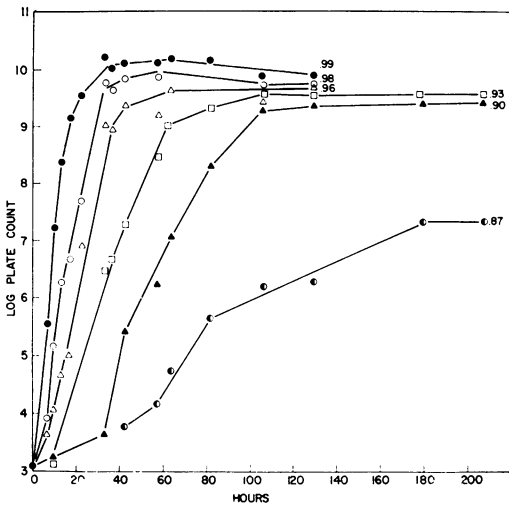


FIG. 4. Effect of a_w on growth of *S. aureus* C-243 in medium B.

with medium B. The control medium (a_w 0.99) supported the synthesis of high levels of enterotoxin B; however, the only other medium in which enterotoxin could be found was that adjusted to 0.98 in which only 4.2 μg of enterotoxin per ml could be found after 33 hr of incubation despite the presence of 6.8×10^9 staphylococci per ml. At progressively lower a_w levels, 0.96, 0.93, 0.90, and 0.87, enterotoxin B could not be detected, although, as in medium A, maximal cell numbers exceeded $10^7/\text{g}$.

Both experimental systems (glycerol and hydrolyzed protein medium constituents) produced virtually identical total plate counts, rates of growth, and extent of a_w effect on enterotoxin synthesis, thus strongly suggesting that this effect is due to a_w adjustment rather than the potential presence of unknown materials deleterious to enterotoxin synthesis in the medium components. Enterotoxin synthesis is reported to be catabolite repressed by glucose (9); however, media used in these studies were glucose-free and any contribution of glucose to the inhibition of enterotoxin synthesis must be discounted.

These data tend to confirm the work of McLean et al. (8) who found that concentrations of NaCl greater than 3% ($a_w < 0.98$) severely limited enterotoxin B production by a similar strain of *S. aureus*. These workers also noted that this inhibition was produced without appreciably affecting total growth of the staphylococcus. Genigeorgis and Sadler (4), however, observed enterotoxin B production from *S. aureus* S-6 in BHI broth containing 10% NaCl. Experiments in our laboratory indicate that BHI plus 10% NaCl possesses an a_w of 0.92. This medium inoculated

with the S-6 strain has failed to produce enterotoxin under the described (4) experimental conditions despite the presence of $> 10^9$ staphylococci/ml. Control cultures of *S. aureus* S-6 in BHI ($a_w = 0.99$) readily produced demonstrable

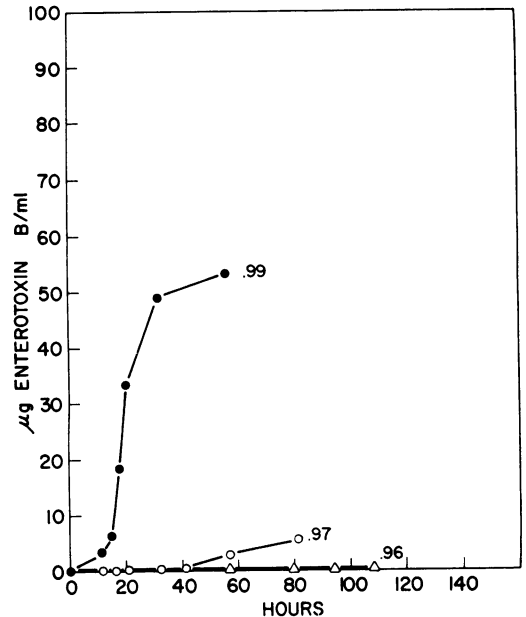


FIG. 5. Effect of a_w on enterotoxin B synthesis in medium A.

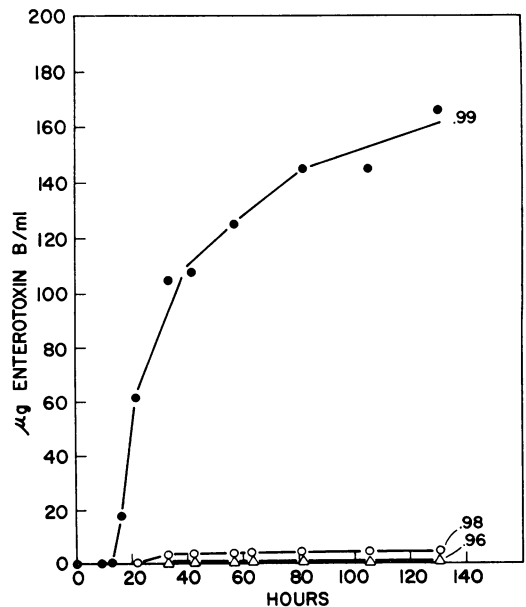


FIG. 6. Effect of a_w on enterotoxin B synthesis in medium B.

levels of enterotoxin B. An explanation of these differences is not readily apparent.

Markus and Silverman (7), working with an *S. aureus* strain which produced enterotoxin A, found that toxin production relative to growth was not influenced by NaCl. These authors pointed out that the synthesis of enterotoxin A differed from enterotoxin B synthesis in that the former appeared to be a primary metabolite and enterotoxin B a secondary metabolite. It is not surprising, therefore, that salt concentrations might affect differently the synthesis of these toxins.

The extrapolation of these data to actual foods would suggest that only very minor differences in critical solute concentrations could prevent or limit the production of enterotoxin. It would also suggest that the presence of rapid growth and high numbers of a potentially enterotoxigenic strain of *S. aureus*, although undesirable, would not necessarily indicate the presence of enterotoxin. Confirmatory data await the outcome of current studies on food materials adjusted to various a_w levels.

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