

Acid Injury in *Staphylococcus aureus* For Official Use
 and Polyols Prevention by Sugars

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Cellular injury to *Staphylococcus aureus* produced by exposure to acidic acetate buffer (0.2 M, pH 4.6-4.7) was lessened by addition to the acidic medium of sugars (glucose, maltose, sucrose) or polyols (glycerol, mannitol, sorbitol). Injury was determined from duplicate plating of acid-treated cells on Tryptic Soy Agar containing either 1% sodium pyruvate (TSAP) or 7% added sodium chloride (TSAS). The differential growth on the two media indicates the amount of cellular injury during exposure to acid. Prevention of acid injury to *S. aureus* by added sugars or polyols was significantly correlated, negatively with water activity and positively with the molar hydroxy value (moles of solute multiplied by number of hydroxy groups per molecule). Results suggest that the combination of acid (either added or produced by microbial fermentation) with a low water activity produced by addition of sugars or polyols (conditions descriptive of high acid-intermediate moisture foods) may not produce complete killing of *S. aureus* and could constitute a food-poisoning hazard from subsequent outgrowth of injured cells if sufficient thermal processing is not carried out.

INTRODUCTION

Microorganisms may sustain cellular injury when subjected to sublethal environmental stresses. Such injured cells will not grow in media or under conditions satisfactory for unstressed cells and, consequently, are often considered to be dead. With transfer to a selected corrective, rich medium and with proper incubation conditions, these injured cells can repair the cellular damage and initiate growth (Witter 1981). Most previous research on injury in *Staphylococcus aureus* has been accomplished principally on heat injury (reviews by Busta 1976; Hurst 1977); little research has appeared concerning the effect of acids on injury in *S. aureus*. Minor and Marth (1972), using acidified culture media, showed injury and recovery in staphylococci. Smith and Palumbo (1978) demonstrated that cells of *S. aureus* added to meat sausage showed cellular injury when the sausages were fermented by a starter culture of lactic acid. Solutes such as sugars, polyols, or salts are known to confer a protective effect against both the injurious and lethal effects of heat toward *S. aureus* (Calhoun and Frazier 1966; Smith et al. 1981). These solutes, because of their ability to decrease water activity (A_w), have been used in the production of intermediate moisture foods. The lowered A_w of intermediate moisture foods contributes to their keeping qualities because most bacteria, including most food-borne pathogens, cannot grow at A_w values (<0.92). The *S. aureus*, however, is

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capable of growth at A_w values in the range of most intermediate moisture foods (0.86-0.90) (Troller and Christian 1978). Since *S. aureus* is sensitive to acidic conditions (Minor and Marth 1976), the presence of acid in intermediate moisture foods should serve to prevent or limit the growth of *S. aureus*. The present study was undertaken to determine whether non-ionic solutes that lower A_w will protect *S. aureus* from injurious effects of an acid environment.

MATERIALS AND METHODS

Preparation of cells. *Staphylococcus aureus* 196E was inoculated into 100 ml Tryptic Soy broth (Difco) and incubated on a reciprocating shaker (200 rpm) for 16 h at 37 C. After incubation, cells were harvested by centrifugation ($16,000 \times g$) and washed with three successive portions of sterile potassium phosphate buffer (0.1 M, pH 7.2). Washed cells were suspended in 10 ml of sterile distilled water.

Preparation of acetate buffer. Acetate buffer, 0.2 M, pH 4.6-4.7, was prepared from acetic acid and sodium acetate solutions (Gomori 1955). Sugars (glucose, sucrose, and maltose) and polyols (glycerol, mannitol, and sorbitol) were dissolved in the buffer on a wt/vol basis. All solutions were sterilized by filtration (Millipore type HA, 0.45 μ m).

Acid injury experiments. Both the control flask (no additions) and experimental flasks (containing sugar or polyol) contained 0.2 M acetate buffer (pH 4.6-4.7) in a final vol of 200 ml. Contents of the flask were temperature equilibrated in a constant temperature water bath (40 C) and were agitated by magnetic stirring. Temperature of the flask contents was monitored by a thermocouple probe. After equilibration to 40 C, 5 ml of washed cell suspension was added to each flask (approximately 5×10^8 cells/ml) with re-establishment of equilibration temperature in approximately 3 min. At intervals, 5-ml samples were removed aseptically from the flasks, placed in cold, sterile tubes, and cooled in an ice bath.

Determination of pH. The pH of the control and experimental flasks was determined at the end of each experiment utilizing a Beckman pH Meter model 76 with an Owens-Illinois O-I pH 2000 electrode.

Detection of injured cells. Appropriate dilutions of the flask contents were made in peptone water (0.1% Difco peptone) and were surface-plated both onto Tryptic Soy agar (TSA; Difco) plus 1% sodium pyruvate (TSAP) and TSA plus 7% NaCl (TSAS) utilizing a spiral plater (Spiral Systems Marketing, Bethesda, MD). Plates were counted after 48 h incubation at 37 C.

RESULTS

The conditions of temperature and pH for producing maximum injury and minimum cellular death were determined in preliminary experiments, and a

subsequent experiment was conducted at 40 C and in a 0.2 M acetate buffer, pH 4.5-4.7. The injurious effect of 0.2 M acetate buffer (pH 4.7) on *S. aureus* and the ability of glucose to protect the cells against acid are shown in Fig. 1. Both acid injured and noninjured cells produce colonies on TSAP medium, whereas only noninjured cells produce colonies on TSAS. The difference in count between TSAP and TSAS is, therefore, a measure of the amount of injury to *S. aureus*. The difference in bacterial count between the control buffer plated on TSAP (curve EB) and TSAS (curve ED) is the extent of acid injury

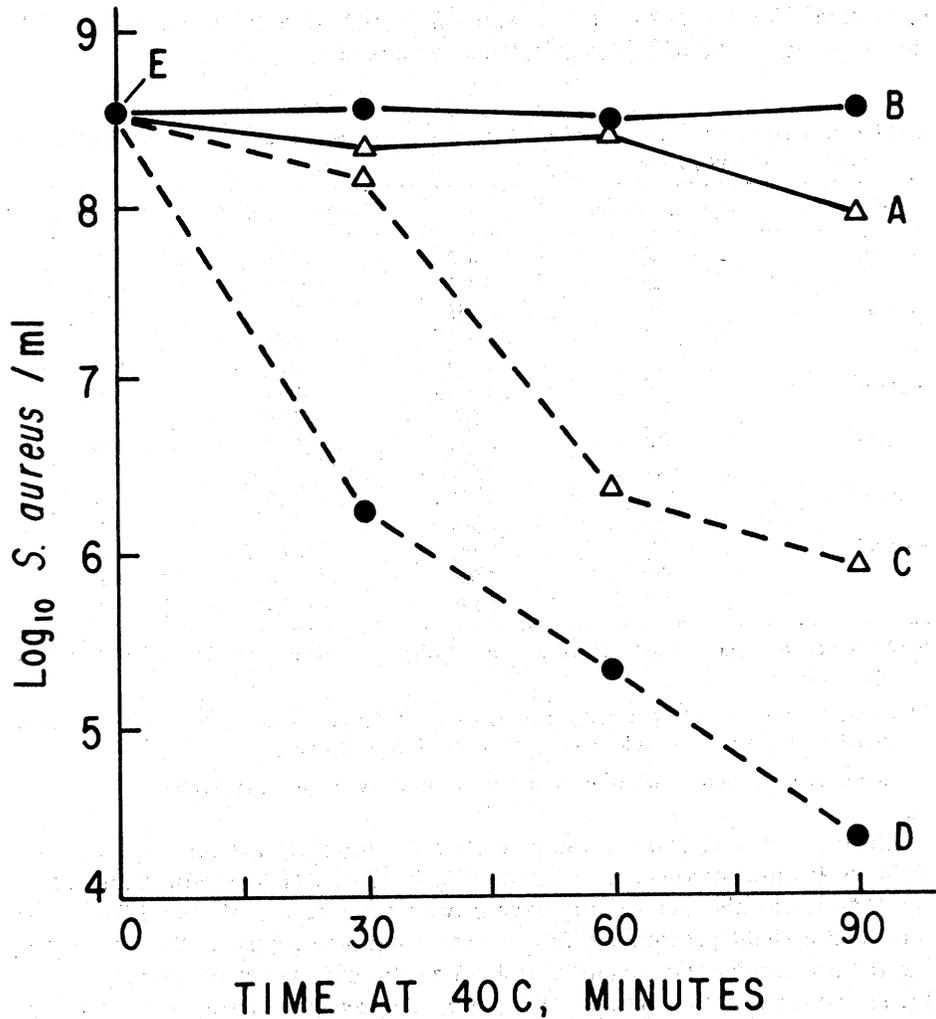


FIG. 1. Protection of *S. aureus* against acid injury by 50% glucose. (B) - *S. aureus* in 0.2 M acetate buffer, pH 4.7; plated on TSAP. (D) - *S. aureus* in 0.2 M acetate buffer, pH 4.7; plated on TSAS. (A) - *S. aureus* in 0.2 M acetate buffer, pH 4.5 plus 50% glucose; plated on TSAP. (C) - *S. aureus* in 0.2 M acetate buffer, pH 4.5 plus 50% glucose; plated on TSAS. Point (E) is the initial log₁₀ plate count.

sustained by *S. aureus*; the difference in counts between the buffer containing glucose plated on TSAP (curve EA) and TSAS (curve EC) is the extent of injury sustained in the presence of 50% glucose. Approximately 20-fold fewer cells were injured in the presence of glucose.

Other sugars and polyols exerted a similar protective effect (Table 1). The protective effect is expressed by Relative Protective Effect (RPE). In Fig. 1, RPE may be defined by the ratio: control lacking glucose (area BDE) minus experimental containing glucose (area ACE) divided by control lacking glucose (area BDE). An RPE of 1.0 indicates complete protection against acid injury in the presence of sugar or polyol; an RPE of 0.0 indicates complete lack of protection. For the 50% glucose solution in 0.2 M acetate buffer, pH 4.5 shown in Fig. 1, the RPE was calculated to be 0.56.

TABLE 1. Water activity (A_w), Molar (OH) value, and Relative Protective Effect (RPE) of various sugars and polyols

Compound	%Concentration	A_w^a	Molar (OH) ^b	RPE at 90 min
Glucose	50	0.892	13.89	0.56
	25	0.965	6.94	0.09
Sucrose	50	0.935	11.70	0.55
	25	0.981	5.85	0.42
	10	0.994	2.34	0.0
Maltose	50	0.943	11.11	0.74
	25	0.982	5.56	0.0
Glycerol	50	0.812	16.30	0.49
	25	0.935	8.15	0.0
Mannitol	20	0.975	6.59	0.28
	10	0.989	3.30	0.11
Sorbitol	50	0.898	16.48	0.96
	25	0.966	8.24	0.61
	10	0.989	3.30	0.04

Flasks containing sugars or polyols had a final pH of 4.5-4.8; final pH in control flasks ranged from 4.6-4.8.

^a Calculated by the use of the formula (Chirife et al. 1980): $A_w = S_1 e^{-KS_2^2}$ where S_1 is mole fraction of water, S_2 is mole fraction of solute, K is a constant.

^b Moles of sugar or polyol multiplied by number of hydroxy groups in molecule.

Glucose, maltose, and sucrose protected *S. aureus* against acid injury at the 50% level; only sucrose was effective at 25% (Table 1). Glycerol protected at the 50% level only, but sorbitol gave protection at both 50% and 25%. Mannitol had a slight protective effect in preventing acid injury at the 20% level, but the low solubility of mannitol in buffer solutions prevented examination of higher concentrations. These data were analyzed for correlation with the calculated A_w value (Table 1) and to the molar hydroxy values (molar hydroxy value equals moles of sugar or polyol multiplied by the number of hydroxy groups on the molecule). There was a negative correlation between protection (RPE) and A_w ($r = -0.53$; $p < 0.05$) and positive correlation between the molar hydroxy value and RPE ($r = +0.79$; $p < 0.001$).

DISCUSSION

The mechanism is not understood by which solutes such as sugars, polyols, and salt protect microbial cells from the deleterious effects of environmental stress. Most studies have dealt with heat stress. In those studies addition of the protective substances lowered the A_w of the stress medium, but several reports in the literature indicated the alleviative effect appears not to be solely from the lowered A_w value. When either glucose or NaCl was added to phosphate buffer to give similar A_w values, the heat resistance of *Escherichia coli* and *Pseudomonas fluorescens* increased to a greater degree in the medium containing glucose than in that containing NaCl. However, NaCl was more effective than glucose for increasing heat resistance of *S. aureus* in solutions of equal A_w (Calhoun and Frazier 1966). Sucrose was more effective than either NaCl or glycerol in increasing the heat resistance of *Salmonella seftenberg* and *S. bedford* even though all of the substances were of similar A_w values (Baird-Parker et al. 1970). Using various solutes to produce solutions with an A_w of 0.96, Goepfert et al. (1970) found that sucrose increased effectively the resistance of *S. montevideo* against heat lethality, whereas sorbitol had only about one-third of the protective effect of sucrose; fructose or glycerol were without effect. Smith et al. (1981) demonstrated that the chemical nature of the salts used for decreasing the A_w was more important than the A_w value in determining the magnitude of the protective effect of salts against heat injury in *S. aureus*. When the neutral salts were arranged in descending order of RPE values according to common anion (Cl^-), K^+ was the most effective cation in protecting *S. aureus* from heat injury followed by $\text{NH}_4^+ > \text{Ca}^{++} = \text{Li}^+ > \text{Na}^+ > \text{Mn}^{++} > \text{Mg}^{++}$. When the salts were arranged according to common cation (Na^+), the order of protection (in descending order) was citrate⁻⁻⁻ > nitrate⁻⁻ > sulfate⁻⁻ > $\text{HPO}_4^{\text{---}}$ > Cl^- > acetate⁻ > I^- . Such an order resembles the classical Hofmeister series or lyotropic series. In lyotropic action, neutral salts stabilize native macromolecules with respect to thermal transition to a more disordered form. Concentration of solute and electrostatic effects also contribute to the stability of macromolecules in the presence of the salt (von Hippel and Schleich 1969).

Acid injury may occur by a mechanism distinctly different from heat injury. Leakage of nucleotides (indicated by increased UV absorption by the supernatant media at 260 nm) occurs in heat-stressed cells, but is absent in acid-injured cells (Blankenship 1981; Smith et al. 1981). The leakage of cytoplasmic components generally indicates injury to the membrane and a breakdown of the permeability barrier. The *S. aureus* cells have associated with their membrane a peptidoglycan, composed of repeating units of 1,4 linked N-acetylglucosamine and -muramic acid residues, linked to a cross-linked peptide configuration (principally lysyl and D- and L-alanyl residues). Teichoic acids containing ribitol and glucosamine also are present, either attached directly to the peptidoglycan or in close proximity (Buchanan and Gibbons 1974). A lowered pH would be expected to increase the electrostatic charge positively, leading to electrostatic repulsion within the peptidoglycan. Such alterations could presumably limit transport of materials both in and out of the cell. High con-

centrations of salt in the medium might be expected to either increase or decrease the electrostatic effect depending upon the anion and cation present. Uncharged solutes, however, may stabilize the charged polymers through hydrogen bonding to the water molecules and separating the charges. Statistical analysis of these data indicates that polyhydroxy substances (sugars and polyols) show a significant effect in protection which was related partially to molar concentration and partially to the content of hydroxy groups present on the solute.

Corry (1976), using *Salmonella typhimurium* as well as two osmophilic species of yeast, showed that the increase in plasmolysis (cell shrinkage) caused by sucrose, glucose, fructose, sorbitol, or glycerol correlated with the degree of protection given by these solutes against heat lethality. Thus, heat resistance induced by the presence of polyols or sugars appeared to be associated with the degree of dehydration of the cell given by these substances. Therefore, dehydration of the staphylococcal cell by sugars or polyols may provide an alternative or additional protection against the injurious effects of an acid environment.

Intermediate moisture foods are becoming an important food commodity and probably will continue to do so as energy sources become increasingly more scarce and expensive. Thus *S. aureus* can grow readily in intermediate moisture foods because they can tolerate A_w conditions that will not permit the growth of other food-poisoning bacteria; however, staphylococci are quite sensitive to acidic conditions (Minor and Marth 1976).

Hansemann et al. (1980) showed that the growth of *S. aureus* occurred in a medium poised at A_w of 0.87 (or lower) and pH 4.7 (or lower). Our experimental conditions are analogous to that found in a high acid-intermediate moisture food and our data indicate that even if growth of *S. aureus* would not occur at low A_w and pH, the protective effect exerted by polyols and sugars will not lead to injury or death of the cells. Therefore, the combination of acid — either added or produced by microbial fermentation — and A_w lowering sugars or polyols in such intermediate moisture food may not prevent potential *S. aureus* enterotoxin formation especially if the level of *S. aureus* is high (10^6 - 10^7 /g). If the A_w and the pH values are above the limits defined by Hansemann et al. (1980), the protected cells might well be expected to grow and produce toxin.

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