

Protection Against Heat-Injury in *Staphylococcus aureus* by Solutes

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ABSTRACT

The effect of solutes on heat-injury in *Staphylococcus aureus* 196E was studied in 25% ground beef (GB) slurry or distilled water equilibrated at 49 C. Exposure to 49 C for 90 min resulted in a 3-4 log cycle increase in injured cells. The number of injured cells was the difference between bacterial counts on tryptic soy agar (TSA) + 1% pyruvate and TSA + 9% NaCl. Increasing levels of NaCl (1-9%) added to GB slurry gave increasing protection against heat-injury and resulted in a decrease in the number of injured *S. aureus*; glycerol and sucrose had a similar effect. At 0.85 M (equivalent to 5% NaCl), other compounds such as sodium citrate, KCl, NaNO₃, Na₂SO₄, Na₂HPO₄, NH₄Cl, CaCl₂, and LiCl were more effective than NaCl in protecting against heat injury; sodium acetate, MgSO₄, NaI, MnCl₂, MgCl₂, NaBr, NaH₂PO₄, and KI were less effective than NaCl. In the presence of 5% NaCl, it was necessary to raise the temperature from 49 to 55 C to obtain significant heat-injury to *S. aureus*. Addition of NaCl prevented the leakage of UV-absorbing materials and decreased the extent of magnesium ion leakage from heat-injured staphylococci.

In a study on the effect of food additives on heat injury in *Staphylococcus aureus*, we noted that addition of sodium chloride to the heating suspension protected the cells against heat-injury. Previously, only Lee and Goepfert (22), who indicated that high levels of sucrose protected *Salmonella typhimurium* from heat-injury, have reported on the protective effect of solutes toward heat-injury in microorganisms. Heating of *S. aureus* in bacteriological media, buffer or foods containing solutes which reduced water activity was ineffective for sterilization (3,6,16,20). In these studies, however, cell death but not non-lethal injury was examined. We undertook the present study to determine the effect of various solutes on the extent of heat-injury in *S. aureus*.

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²Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

MATERIALS AND METHODS

Growth of *S. aureus* and preparation of inoculum

S. aureus 196E was inoculated into 100 ml of Tryptic Soy Broth (Difco)² and incubated on a reciprocating shaker (200 rpm) at 35 C for 16 h. Cultures then were centrifuged in the cold (2-4 C) at 16,000 × g, washed three times with sterile phosphate buffer (0.1 M) and suspended in 6-7 ml of buffer.

Heat-injury procedure

The heating suspension consisted of 50 g of ground beef and 150 ml of distilled water with or without solute. The meat, water and water with added solute were sterilized separately by autoclave; after cooling, the meat and water were blended, under sterile conditions, into a smooth slurry. In some experiments, 200 ml of sterile distilled water with or without solute but without added meat were used as the heating suspension.

Meat slurries with and without added solute were equilibrated at 49 C in a constant-temperature water bath and with continuous agitation both during temperature equilibration and the experiment. Temperature was monitored with a thermocouple inserted below the surface of the heating suspension. Washed cells of *S. aureus* were added to each flask to a final concentration of approximately 10⁹ cells/ml. Approximately 3 min were required for temperature equilibration after addition of the cells. At selected times, 5-ml portions of the heated cell suspensions were removed, placed in cold sterile tubes and cooled in an ice bath.

Assay for injured cells

By use of a spiral plater (Spiral Systems Marketing, Bethesda, Md.), appropriate dilutions were plated on Tryptic Soy agar (TSA; Difco) plus 1% sodium pyruvate (TSAP) and on TSA plus 9% NaCl (TSAX). TSAP permits growth of both injured and non-injured cells; pyruvate acts as an exogenous H₂O₂ decomposer to remove H₂O₂ accumulated during cellular injury and thus allows the injured cells to grow (4,24). Only non-injured cells grow on TSAX. Plate counts were determined after 2 days of incubation at 35 C.

Assay for leakage from injured *S. aureus*

For the study of leakage of cellular constituents from injured cells, the control flasks for heat injury contained 200 ml of sterile distilled water and the experimental flasks contained 200 ml of sterile 5% saline solution. Flasks were equilibrated at 49 C before addition of cells. At timed intervals, 15 ml of sample were removed from each flask. Five ml were placed in cold sterile tubes, cooled in an ice bath and used for plate count determinations on TSAP and TSAX. The remaining 10 ml were centrifuged in the cold (2-4 C) at 17,000 × g to sediment cells. The supernatant fluids were analyzed for magnesium ions (Perkin-Elmer Atomic Absorption Spectrophotometer, model 306) and for ultraviolet-absorbing material (Beckman Spectrophotometer, model 25).

Determination of pH

At the end of each experiment, the pH values of the control and experimental solutions were determined (at approximately 25 C) with an Owens-Illinois 0-1 pH 2000 electrode and a Beckman pH meter, model 76.

RESULTS

On a plot of the \log_{10} viable cells/ml on the two media (TSAP and TSAX) versus time of heating at a particular temperature in a particular medium, the graphical difference (determined gravimetrically) between TSAP and TSAX represents an integration of the differential time-temperature rate equation for cellular injury under those conditions.

In Fig. 1, the area BDE represents the total differential content of heat-injured cells in the control meat slurry medium after 90 min at 49 C. Similarly, area ACE represents that found in the experimental meat slurry containing 5% sodium chloride; the data demonstrate an obvious protective effect against heat-injury by addition of salt. These integrated values can be used to determine the relative protective effect (RPE) toward heat-injury of a particular additive. RPE is defined as the ratio [control value (BDE) - experimental value (ACE)/control value (BDE)] with the range limits 0.0 to 1.0. An RPE of 1.0 indicates complete protection against heat injury in the presence of the experimental additive; an RPE of 0.0 indicates a complete lack of protection. The RPE calculated for 5.0% sodium chloride at 49 C for 90 min (Fig. 1) was 0.65.

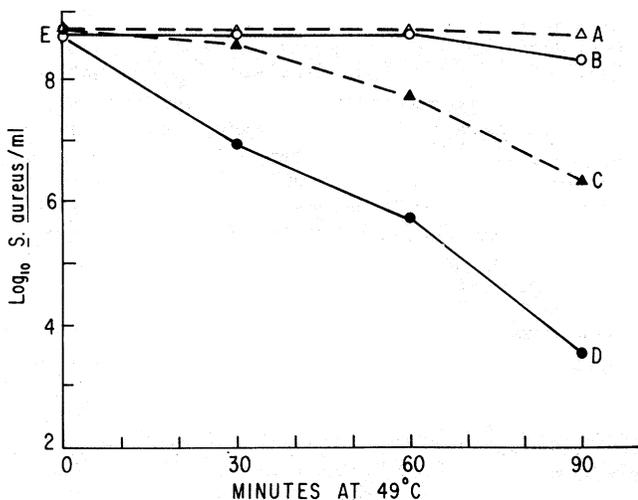


Figure 1. Effect of 5% NaCl in meat slurry on injury in *S. aureus* heated at 49 C.

For specific additives, the RPE may vary with the concentration of the additive and with the time of heating. With sodium chloride, the RPE values at 1, 3, 5, 7, and 9% added salt and 30, 60, and 90 min of heating are shown in Fig. 2. An increase in the salt concentration increased the RPE; there was some decrease in the protection at a given concentration of salt with the length of the period of heating. A high level of protection (RPE > 0.9) against heat-injury was observed at the 9% salt level at all time periods of heating. Slight differences

in the pH of the slurries were found; control meat slurries ranged from 5.8-6.2, salt-meat slurries ranged from 5.7-5.9. The ranges in pH do not appear to be responsible for the protective effects noted.

The non-ionic additive, sucrose, also protected *S. aureus* from heat-injury. Figure 3 presents the RPE values given by 20, 40 and 60% sucrose. Increase in protection occurred with increase in sucrose concentration, but more injury occurred with increased time of heating regardless of sucrose level. Interestingly, a 40% concentration of the non-ionic solute glycerol did not show decrease in protection with time of heating; for 30, 60 and 90 min, the RPE values were 0.93, 0.93, and 0.95, respectively.

Since the calculated RPE values may be a reflection of experimental conditions rather than the actual decrease in numbers of injured *S. aureus*, this factor was examined statistically (Fig. 4). The linear correlation coefficient by regression analysis was -0.922 when the RPE values were plotted against \log_{10} in the difference in

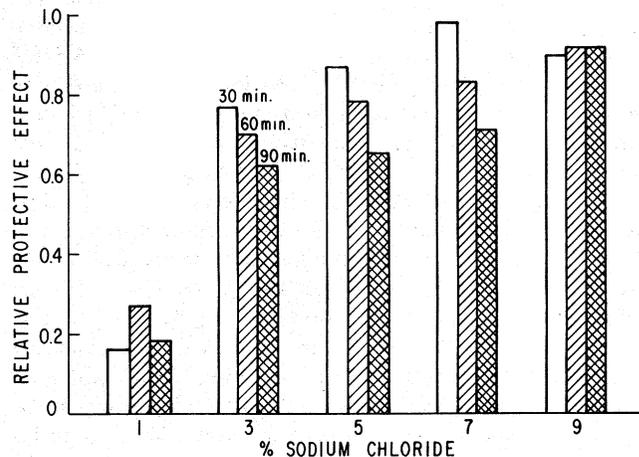


Figure 2. Protection of *S. aureus* against heat-injury at 49 C in meat slurry containing various concentrations of NaCl.

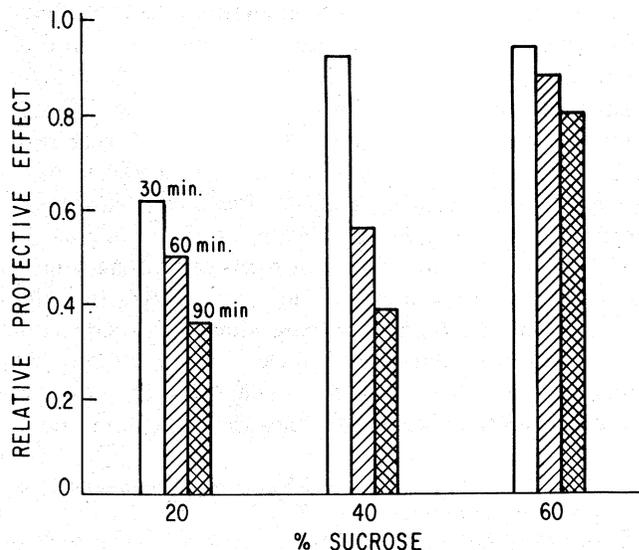


Figure 3. Protection of *S. aureus* against heat-injury at 49 C in meat slurry containing various concentrations of sucrose.

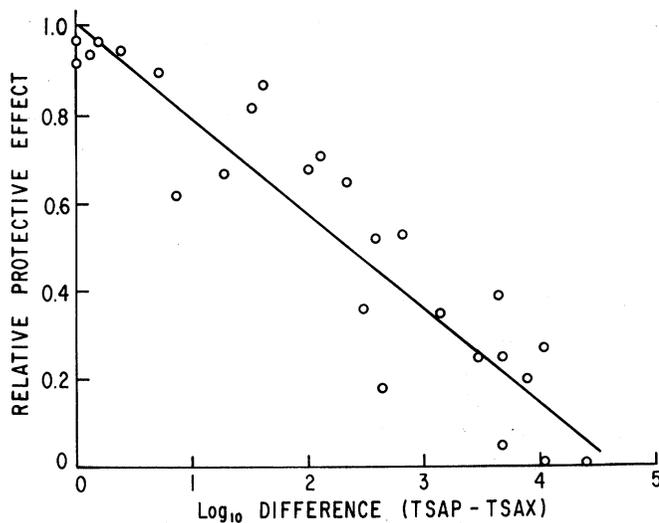


Figure 4. Relationship between relative protective effect and number of heat-injured *S. aureus*.

TABLE 1. Effect of ionic salts on the prevention of injury to *S. aureus* heated in meat slurry at 49 C for 90 min.

Salt ^a	RPE ^b	μ^c	a_w^d	pH ^e
Na ₃ citrate • 2H ₂ O	0.97	5.10	-	6.8
KCl	0.97	0.85	0.971	6.0
NaNO ₃	0.94	0.85	-	5.9
Na ₂ SO ₄	0.90	2.55	0.969	6.1
Na ₂ HPO ₄ • 7H ₂ O	0.87	2.55	-	8.1
NH ₄ Cl	0.82	0.85	0.971	5.9
CaCl ₂	0.68	2.55	0.952	4.5
LiCl	0.67	0.85	0.968	5.7
NaCl	0.65	0.85	0.968	5.8
Na acetate	0.53	0.85	0.968	6.4
MgSO ₄ • 7H ₂ O	0.52	3.40	0.983	5.6
NaI	0.35	0.85	0.969	5.8
MnCl ₂ • 4H ₂ O	0.27	2.55	-	3.8
MgCl ₂ • 6H ₂ O	0.20	2.55	0.949	5.1
NaBr	0.0	0.85	0.970	6.1
NaH ₂ PO ₄ • H ₂ O	0.0	2.55	0.979	4.9
KI	0.0	0.85	0.971	6.1

^aAll salts were tested at 0.85 M.

^bValues represent relative protective effect.

^cValues represent ionic strength at 25 C.

^dValues represent water activity; calculated by use of the formula

$$\ln a_w = \frac{-(18.016) v m \phi}{1000}$$

where v equals number of ions, m equals 0.9 molal, and ϕ equals molal osmotic coefficient (15,26).

^eValues represent pH of meat slurry with added salt; pH of control meat slurry ranged from 5.9-6.2.

bacterial count between TSAP and TSAX of solute containing meat slurry heated for 90 min.

The effect of selected anions and cations on protection was also determined (Table 1). Salts were added to meat slurry at a concentration of 0.85 M, equivalent to 5% sodium chloride. Although molar concentrations were identical, the ionic strengths (μ) of the various salt

solutions varied widely. This factor of ionic strength appears not to be critical, as shown by the data presented in Table 1 and from the effect of altering salt concentration on the RPE values (Fig. 2). When the RPE data (Table 1) are examined on the basis of the anion (with Na⁺ as common ion) or the cation (with Cl⁻ as the common anion), the relative effectiveness of the ions in protecting *S. aureus* from heat-injury, ignoring differences in ionic strength or pH, have a strong similarity to the classical lyotropic or Hofmeister series.

Little injury was apparent when cells of *S. aureus* were heated at 52 C in distilled water containing 5% NaCl; however, cells heated in the absence of salt showed an extensive amount of death as demonstrated by the precipitate decrease in count on TSAP (Fig. 5). Comparison of cells heated in distilled water for 90 min at 49, 52 or 55 C demonstrated a large increase in killed cells (cumulative cell death) and a decrease in the number of injured cells (cumulative cell injury) as the temperature of heating increased (Fig. 6). With addition of 5% sodium chloride to distilled water, only a small amount of cell death was noted at all three temperatures; extensive cumulative cell injury occurred only at 55 C (Fig. 6). Cumulative cell injury was defined as the area (determined gravimetrically) between TSAP and TSAX. In Fig. 5, the area ABD defined cumulative cell injury for *S. aureus* heated at 52 C in the absence of NaCl. Cumulative cell death was defined as the area between a horizontal line drawn from zero time TSAP and experimental TSAP; in Fig. 5, the area A'AC defined cumulative cell death for cells heated at 52 C in absence of salt.

An examination of the heating suspension (distilled water) after removal of the injured cells revealed the presence of an unknown substance(s) absorbing at 260 nm. No ultraviolet-absorbing material was demonstrated in the supernatant fluid after cells were heated in the presence of 5% NaCl in distilled water. The A_{260} values after *S. aureus* was heated for 0, 30, 60 and 90 min in distilled water at 49 C were 0.0, 0.185, 0.225 and 0.230, respectively. Additionally, increases in magnesium concentration of the supernatant fluid were noted: approximately 6-fold increase after cells were heated 60 min in the absence of salt, and approximately 2-fold in the presence of salt.

DISCUSSION

Control of water activity by addition of solutes such as salts or sugars has been used to limit microbial growth in preserved foods, particularly those with intermediate moisture levels (10). Various workers have studied the effect of the addition of solutes to buffers, bacteriological media or foods on the resistance of *S. aureus* and other microorganisms to the bactericidal effects of heat. Although *S. aureus* heated at 60 C in phosphate buffer poised at a_w 0.950 with sodium chloride showed increased heat resistance as compared to the organism in

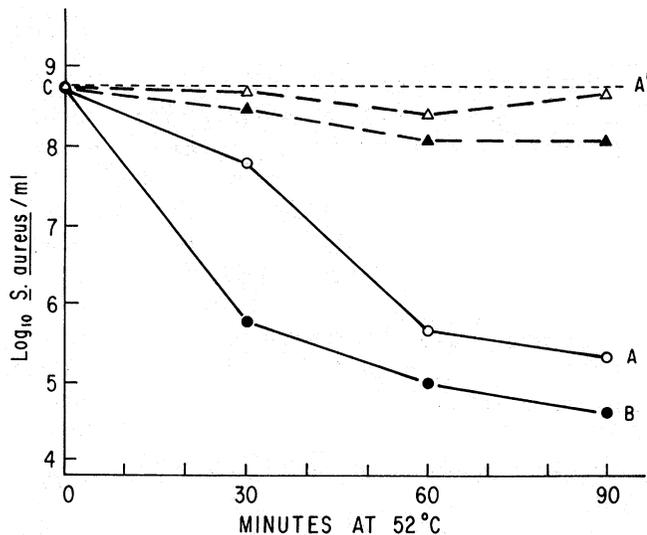


Figure 5. Effect of 5% NaCl in distilled water on injury in *S. aureus* heated at 52 C.

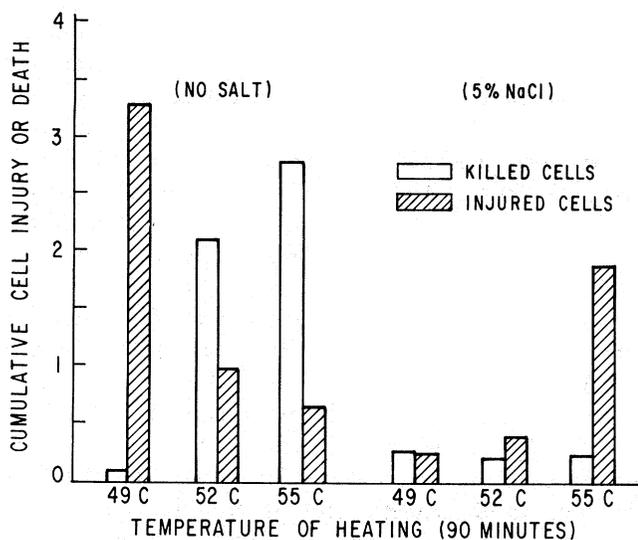


Figure 6. Influence of temperature on injury and killing of *S. aureus* heated in distilled water with and without NaCl.

control buffer at a_w 0.994, those heated in buffer poised at 0.950 with glucose showed no protection (6). With Tris-maleate buffer at 60 C, addition of 4 or 8% sodium chloride increased the heat-resistance of *S. aureus* (3). Hsieh et al. (16) noted that the heat-resistance of *S. aureus* increased as the a_w of the bacteriological media was decreased by addition of glycerol. At the temperature range of 55-60 C, the maximum heat resistance was in the range of 0.76-0.85. Increased resistance of staphylococci to killing at 60 C was noted also in skim milk containing 26-57% sucrose (20) and in pork macerate containing 8.4-8.5% NaCl (3). Recently, Hughes and Hurst (17) and Hurst et al. (19) have shown that addition of high levels of $MgCl_2$, NaCl, KCl, glucose, or sucrose to the growth medium of *S. aureus* permitted growth and enterotoxin formation at temperatures at least 2 C higher than that in the unsupplemented growth medium. The authors suggested that the protective effect of the solutes were due to the osmotic effect of the

non-penetrating Cl^- or sugar molecules. Glucose, but not sodium chloride, when used to adjust the a_w of phosphate buffer, increased the heat-resistance of *Escherichia coli* and *Pseudomonas fluorescens* (6). Addition of sucrose increased the heat-resistance of *Salmonella montevideo* 13-fold, and addition of sorbitol increased the resistance 4-fold as compared to glycerol or fructose at the same a_w (12). By adjusting the a_w of bacteriological media to 0.90, Baird-Parker et al. (2) found that the heat resistance of *Salmonella senftenberg* 775W increased 30-fold when sucrose was used in comparison to NaCl or glycerol.

Studies on increased resistance to the bactericidal effects of heat in the presence of solutes suggest that the chemical nature of the solute is more important than the a_w . The data presented in Table 1 also indicate that chemical structure of the solute is more important than either a_w or ionic strength in protecting *S. aureus* against heat-injury. When the salts listed in Table 1 are arranged in descending order of RPE values according to common cation (Na^+) or common anion (Cl^-), they form a lyotropic or Hofmeister series. The effect of salts on the temperature of gel-sol transitions, thermal denaturation of nucleic acids proteins, solubility of proteins, critical micelle concentration of phospholipids and hydration of proteins follows the lyotropic series (14,15).

One consequence of heat-injury in *S. aureus* is leakage of cellular components, apparently from damage to the cytoplasmic membrane. Busta (5) and Hurst (18) indicated that cellular components isolated from the medium following heating include lipids; phospholipids; amino acids; various sodium, potassium and magnesium salts; and unidentified components absorbing at 260 and 280 nm. In the present study, addition of 5% sodium chloride to the heating suspension prevented leakage of ultraviolet-absorbing material and reduced leakage of magnesium ions. Allwood and Russell (1) showed that addition of 34% sucrose prevented leakage of 260-nm absorbing material during heating of staphylococci; Lee and Goepfert (22) obtained similar results with *S. typhimurium* and suggested that sucrose stabilized the bacterial membrane against heat damage. With *S. aureus* protoplasts lacking the cell wall but possessing a cytoplasmic membrane, structural integrity can be maintained by addition of solutes such as sucrose, potassium chloride or sodium chloride to the suspending medium (23,25).

The biochemical basis for the interaction between solute concentration and temperature in preventing cellular injury has not yet been determined. In these experiments, neither ionic strength nor pH of the salt solutions appears to be the prime factor involved in protection (Table 1). Although no linear relationship between the relative protective effect and calculated water activity was seen (Table 1), another parameter of water may be involved in this protection. Osmotic pressure may be a factor as it has a direct negative

correlation to the natural logarithm of the water activity and to the absolute temperature, whereas temperature has only a slight effect on water activity (27). With non-electrolytes, significant deviations in actual water activity and osmotic pressure values from those calculated by Raoult's law appear to be related partially to the number of hydroxyl groups or uncharged amino groups present on the molecule and to the degree of hydrogen bonding with water molecules (7). Such interactions decrease the fugacity of the water molecule and the water activity. With electrolytes, the relative protective effect appears to follow the Hofmeister or lyotropic series, which is generally related to the hydration ability of the ion (15). Thus solutes which form bonded complexes with water molecules may produce a structuring of the water about and within the microbial cell to allow a more stable form of cellular macromolecules at a particular temperature in the presence of solute (9). Baird-Parker et al. (2) showed that addition of sucrose to a test culture of salmonellae required a 10-C increase (from 60 to 70 C) in the temperature to produce a bactericidal effect. In the present study, a similar effect was noted for *S. aureus* where a 6-C increase (49 to 55 C) in the presence of 5% sodium chloride was necessary to produce injury.

The site of action for protection against temperature injury and death afforded by solutes has not been ascertained. Microbial membranes appear to be involved at least in part because of the decreased loss in internal components into the external medium in the presence of sucrose (1,22) and sodium chloride (present study). Since membranes are composed principally of proteins and lipids in approximately a 4:1 ratio (13), the added solutes may stabilize the membrane protein structure against thermal denaturation. Alternatively, the membrane phospholipids may be involved by an increase in the melting temperature with increased solute concentrations. Additionally, cellular nucleic acids and nucleotides could be stabilized by salts, and limited damage could be repaired by cellular mechanisms more readily (21).

The increase of temperature required for the injury or death of microorganisms in the presence of certain solutes has important bearing on the safety of food products, particularly those of intermediate moisture content. If such foods were to receive a marginal heat treatment (sufficient for foods of low solute content), organisms present might be injured rather than killed. Such injured staphylococci have been shown to repair, grow and produce toxin (8,11). Consequently, food with such characteristics should be processed sufficiently to ensure that all organisms have been killed rather than injured.

ACKNOWLEDGMENTS

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