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Effect of Available Water on Thermal Resistance of Three Nonsporeforming Species of Bacteria

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ABSTRACT

CALHOUN, C. L. (University of Wisconsin, Madison), AND W. C. FRAZIER. Effect of available water on thermal resistance of three nonsporeforming species of bacteria. Appl. Microbiol. **14**:416–420. 1966.—Cells of *Escherichia coli*, *Pseudomonas fluorescens*, and *Staphylococcus aureus*, previously grown in Trypticase Soy Broth (TSB) at a high level of available moisture (a_w 0.994) and at low levels produced by addition of NaCl or glucose, were heated in neutral phosphate buffer, and in this buffer adjusted to low levels of available moisture by means of NaCl or glucose. Glucose in the heating medium was more protective than NaCl for *E. coli* and *P. fluorescens*, but hastened the thermal destruction of *S. aureus*. Added protection was given *P. fluorescens* during heating in glucose-buffer solution at a_w 0.97 by previous growth in TSB adjusted to that a_w value with glucose. Added protection was given *E. coli* during heating in NaCl-buffer solution at a_w 0.98 by previous growth in TSB adjusted to that value with NaCl. With *S. aureus*, however, previous growth in TSB plus NaCl or glucose had little effect on heat resistance, but the solute in the heating medium had great influence, in that NaCl was very protective and glucose destructive. Opportunity may have been given during tempering of the cell suspension at 30 C in the heating medium prior to heating for the NaCl and glucose to diffuse into the staphylococcal cells.

Destruction by moist heat is believed to result from damage to proteins and protein complexes in the bacterial cell. Since the stability of proteins increases with decrease in moisture, the availability of water in the cell and outside it should have an influence on its heat resistance, and moisture content could be expressed in terms of available water (water activity or a_w). Present work attempted to determine: (i) whether growth of bacterial cells at low levels of available water causes a change in thermal resistance, and (ii) whether vegetative bacterial cells react similarly when heated in media of the same a_w, but with the a_w adjusted with an ionizing solute, sodium chloride, and a nonionizing solute, glucose.

Most work reported on the effect of salts and sugars in the heating menstruum has been with bacterial spores. In general, low concentrations of sodium chloride were protective according to Esty and Meyer (6), and Viljoen (10), but increasing levels decreased thermal resistance as shown by Weiss (11), and Anderson, Esselen, and Fellers (1). Baumgartner (2) found that protective levels of salt were higher for spores of a halophile. Protection of *Escherichia coli* by sugars has been demonstrated by Beavens (4), Baumgartner and Wallace (3), Baumgartner (2), and Fay (7), and of *Pseudomonas fluorescens* by Fay (7). Anderson, Esselen, and Fellers (1) reported hastened destruction of spores of *Bacillus coagulans* in the presence of 50% of sucrose or glucose. Kadan, Martin, and Mickelsen (8) showed that sucrose added to skim milk in concentrations up to 14% hastened the killing of *S. aureus* at 60 C, whereas from 25 to 57%, sucrose was increasingly protective. Glucose added with the sucrose increased the killing effect at low concentrations of sugar. Fay (7) reported that sugars differed in their protective effect.

MATERIALS AND METHODS

Cultures and media. Cultures employed were *Escherichia coli* strain H52, *Staphylococcus aureus* strain 196E, and a departmental stock culture of *Pseudomonas fluorescens*. Cultures for tests were grown to their maximal stationary growth phase in Trypticase Soy Broth (TSB; BBL), or in this medium adjusted to a desired a_w value with sodium chloride or glucose. Incubation was in a New Brunswick Gyratory Shaker

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at 37 C and 200 rev/min for *E. coli* and *S. aureus* and at 30 C and 400 rev/min for *P. fluorescens*. Three previous transfers had been grown under these conditions. Solutions of glucose to lower the a_w value were sterilized by passage through a Seitz filter. Sterile 0.1% peptone solution was employed for dilution blanks. It had been determined that it was not necessary to use dilution blanks with the a_w lowered to that of the original growth medium.

Heating procedures. Thermal death time (TDT) tubes of Pyrex tubing (9-mm outside diameter, 1-mm thickness) were 12.5 cm long, sealed at one end and constricted near the other end, and plugged with a sterile rubber stopper after introduction of 2 ml of inoculated test medium. Tubes in bundles of three were tied with twine so as to keep the tubes well separated.

The basal medium in which the cells were heated was sterile, double-strength 0.067 M phosphate buffer at *p*H 7.0, prepared with Na_2HPO_4, mixed with an equal volume of sterile distilled water. The a_w of this menstruum was 0.994. Heating media of low a_w were a mixture of equal volumes of double-strength sterile buffer and double-strength sterile solutions of NaCl or glucose at *p*H 7.0. A 10-ml amount of culture in the maximal stationary phase was centrifuged. The pellet was suspended in the same medium as that to be used in heating, and was shaken on a Vortex Junior mixer for 5 min, followed by straining through a sterile cotton and gauze pad to break up and remove cell clumps. Enough of this cell suspension was added to the heating medium to give a viable count of about 10^7 cells per milliliter. Then the tubes were tempered in a water bath at 30 C. Total time for filling vials and holding in the bath was exactly 1.5 hr. Twelve replicate TDT tubes were provided for each sampling time, and several replicate runs were made.

Heating of the completely immersed vials was in a circulating water bath (Sargent S-84805) rated as sensitive to within ±0.01 C. The temperature of the heating medium within a vial was measured by means of a carefully positioned Yellow Springs model 42SC Telethermometer and a model 80 laboratory recorder. Come-up time was gauged from the telethermometer and recorder, and timing of the heating began when the contents of the vial reached the desired temperature. Immediately after the heat treatment, the vials were cooled in a water bath at 15 C.

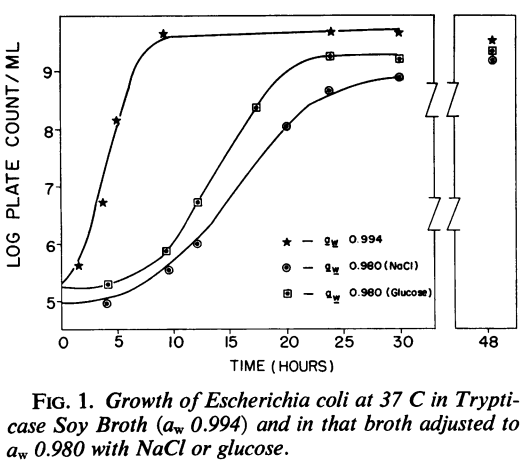

FIG. 1. *Growth of Escherichia coli at 37 C in Trypticase Soy Broth (a_w 0.994) and in that broth adjusted to a_w 0.980 with NaCl or glucose.*

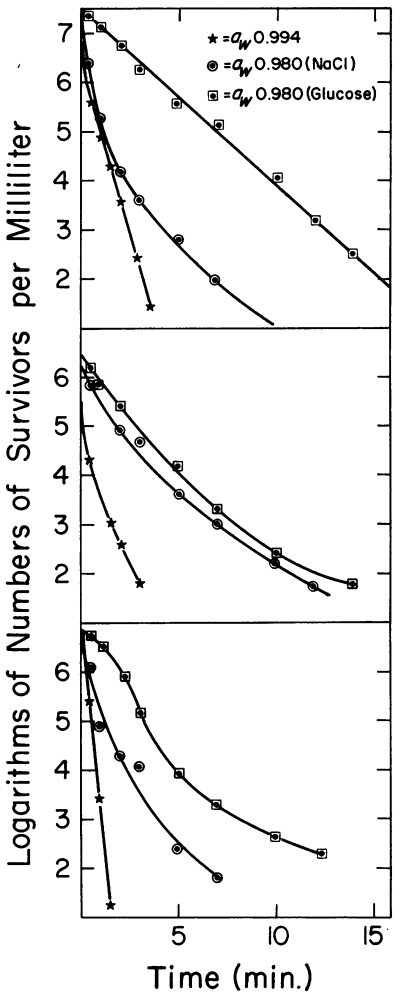

FIG. 2. *Survival of Escherichia coli at 60 C in phosphate buffer (pH 7.0) at a_w 0.994, 0.980 (NaCl), and 0.980 (glucose), after growth at a_w 0.994 in Trypticase Soy Broth (top part), a_w 0.980 (NaCl; middle part), and a_w 0.980 (glucose; bottom part).*

Recovery of heated cells. The heated cell suspension was plated in triplicate by means of spread-plates of Yeast Dextrose Agar (Difco), which had been dried for 24 hr at 37 C. This nutritionally complex and rich medium was chosen to encourage growth of heat-damaged cells. Trials had shown that there was no advantage in adjusting the a_w of the recovery medium to values as low as those in which the organisms had been grown or heated.

RESULTS

In TSB, *E. coli*, *P. fluorescens*, and *S. aureus* had minimal a_w values for growth of 0.970, 0.960,

and 0.90, respectively, when the a_w had been adjusted with NaCl. At these low levels of available water, however, the lag period was for several days. Therefore the a_w values selected to represent low levels of available moisture for the three species in the present work were 0.980, 0.970, and 0.950, respectively. The value a_w 0.950 was chosen for *S. aureus* to avoid a thick sirup when adjustment was with glucose.

Effect of a_w on heat resistance of E. coli. Figure 1 compares growth of *E. coli* at 37 C in TSB at a_w 0.994 and in that broth adjusted to a_w 0.980 with NaCl or glucose. Both solutes were inhibitory, but the salt was more so. The maximal population after 24 hr, however, was not much less at a_w 0.980 than at 0.994.

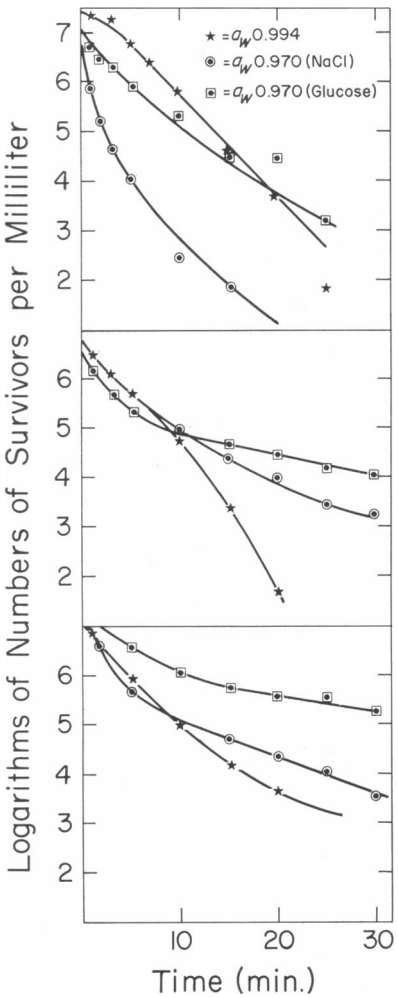

FIG. 3. *Survival of Pseudomonas fluorescens at 53 C in phosphate buffer (pH 7.0) at a_w 0.994, 0.970 (NaCl), and 0.970 (glucose), after growth at a_w 0.994 in Trypticase Soy Broth (top part), a_w 0.970 (NaCl; middle part), and a_w 0.970 (glucose; bottom part).*

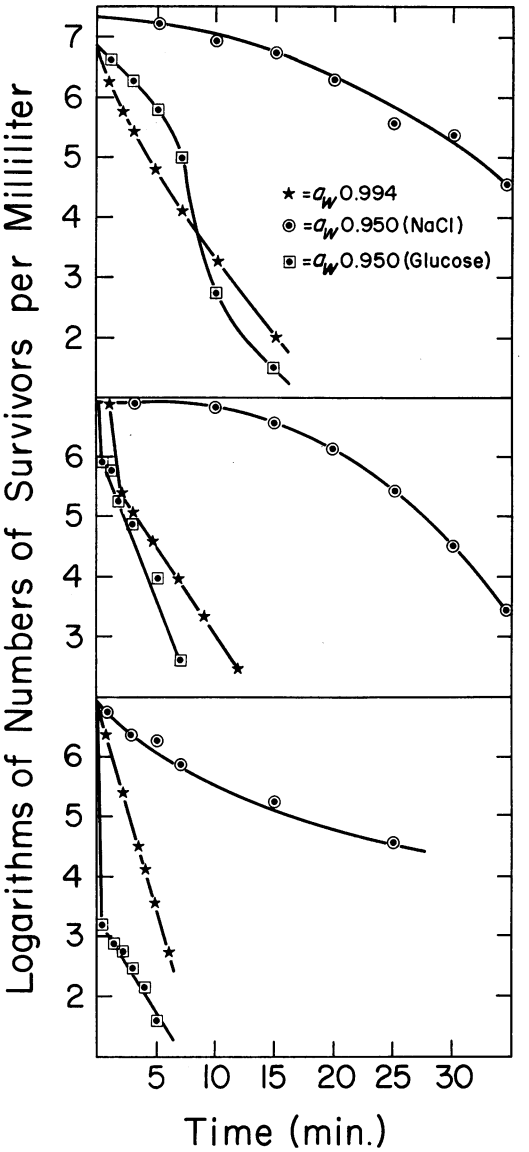

FIG. 4. *Survival o, Staplylococcus aureus at 60 C in phosphate buffer (pH 7.0) at a_w 0.994, 0.950 (NaCl), and 0.950 (glucose), after growth at a_w 0.994 in Trypticase Soy Broth (top part), a_w 0.950 (NaCl; middle part), and a_w 0.950 (glucose; bottom part).*

Figure 2 shows survivor curves of *E. coli* heated at 60 C in phosphate buffer at a_w 0.994, and at a_w 0.980, adjusted with NaCl and with glucose. In the top part of the figure, cells grown in TSB at a_w 0.994 were protected by glucose in

the heating medium, but comparatively little by NaCl. The middle part of Fig. 2 shows that cells grown in TSB at a_w 0.980 produced by means of NaCl were protected about equally by glucose or NaCl in the heating menstruum. But when *E. coli* had been grown in TSB at a_w 0.980 produced by means of glucose (bottom part of Fig. 2), the organism was protected more by glucose in the heating medium than by NaCl.

Effect of a_w on heat resistance of P. fluorescens. Growth curves of *P. fluorescens* at 30 C in TSB resembled those for *E. coli* shown in Fig. 1, with NaCl more inhibitory than glucose, although the sugar did not inhibit the pseudomonad as much.

Figure 3 shows survivor curves of *P. fluorescens* heated at 53 C in phosphate buffer at a_w 0.994, and at a_w 0.970 adjusted with NaCl and with glucose. In the top part of the figure, cells grown in TSB at a_w 0.994 were killed at almost the same rate in buffer at a_w 0.994 and at 0.970, adjusted with glucose, although the latter gave a little protection. In buffer at a_w 0.970, adjusted with NaCl, however, the death rate was greatly accelerated. The middle part of Fig. 3 shows that cells grown in TSB at a_w 0.970 produced by means of NaCl were protected some by glucose or NaCl in the buffer, a little more by glucose. In the bottom part of Fig. 3, *P. fluorescens* grown at a_w 0.970 produced by means of glucose was protected considerably by glucose in the heating medium, and much less by NaCl.

Effect of a_w on heat resistance of S. aureus. As might be expected on the basis of the ability of *S. aureus* to grow at a_w values of 0.90 or lower, growth of this species at 37 C at a_w 0.950 was delayed compared with that at a_w 0.994, but inhibition was not great, and maximal numbers after 48 hr were almost the same at a_w 0.994 and at a_w 0.950, whether produced by glucose or by NaCl. Again, inhibition was greater during active growth when the a_w was lowered by means of NaCl than when decreased by means of glucose.

The survivor curves in Fig. 4, however, show the great protective effect of NaCl in the heating medium, and the marked killing effect when the a_w had been adjusted downward by means of glucose, resulting in faster destruction than in the buffer at a_w 0.994.

DISCUSSION

Scott (9) concluded that the minimal a_w for growth of his organisms, which included *S. aureus*, was largely independent of the solute used to tie up the water. Wodzinski (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1960) had similar results with *P. fluorescens*, *Aerobacter aerogenes*, and *Lactobacillus viridescens*. Present results indicate, however, that the solute employed to lower the a_w value of either the medium for growing bacterial cells or the substrate in which the cells are heated may affect the survival of cells during heating. With *E. coli* and *P. fluorescens*, glucose was more protective than NaCl, the ions of which may be damaging. With *S. aureus*, however, the reverse was true, for NaCl was much more protective than glucose, which even hastened destruction.

Christian and Waltho (5) showed that bacteria tested by them had little control over their intracellular a_w and rapidly equilibrated to the a_w of their environment. This may have occurred in the present work when cells were held at 30 C in the presence of NaCl or glucose prior to heating, and the solute taken up probably affected the thermal resistance of the cells. Diffusion of solute into gram-positive cells might be more rapid than into gram-negative cells with their higher lipid content in the cell wall. Results with *S. aureus* support this supposition, for this organism, whether grown at a_w 0.994 or at a_w 9.50 produced by means of NaCl or glucose, was killed rapidly and at about the same rate in glucose plus phosphate buffer at a_w 0.950. With the two gram-negative bacteria, however, previous growth at low a_w values produced by means of the two solutes also had an effect, as is shown in Fig. 2 and 3. *P. fluorescens*, for example, was protected much more by glucose after having been grown previously in the same concentration of the sugar, and *E. coli* was protected more by NaCl after having been grown in NaCl solution at a_w 0.980.

Hastened killing of *S. aureus* at 60 C in skim milk to which up to 14% of sucrose had been added was reported by Kadan, Martin, and Mickelsen (8), and protection increased as the sucrose was increased to 25 to 57%, the molar equivalent of which is about 13 to 30% of glucose. Therefore, it would be expected that the 36% of glucose used in the present experiments would be protective rather than markedly destructive. Skim milk, however, contains more protective substances than does the phosphate buffer.

Protection against heat by means of sugar is not limited to gram-negative bacteria, as was demonstrated by Fay (7), who reported that sucrose protected *Bacillus subtilis*. Also gram-negative *Salmonella pullorum* was not protected.

ACKNOWLEDGMENT

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LITERATURE CITED

1. ANDERSON, E. E., W. B. ESSELEN, JR., AND C. R. FELLERS. 1949. Effects of acids, salt, sugar and other food ingredients on thermal resistance of *Bacillus thermoacidurans*. Food Res. **14**:499–510.
2. BAUMGARTNER, J. G. 1937. The salt limits and thermostability of a new species of anaerobic halophile. Food Res. **2**:321–329.
3. BAUMGARTNER, J. G., AND M. D. WALLACE. 1934. The destruction of micro-organisms in the presence of sugars. I. The role of sucrose in the commercial processing of canned fruits. J. Soc. Chem. Ind. **53**:294–297T.
4. BEAVENS, E. A. 1930. The effect of lactose on the survival of *Escherichia coli* when heated to 145°F for 30 minutes. J. Dairy Sci. **13**:91–93.
5. CHRISTIAN, J. H. B., AND J. A. WALTHO. 1962. The water relations of staphylococci and micrococci. J. Appl. Bacteriol. **25**:369–377.
6. ESTY, J. R., AND K. F. MEYER. 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes. XI. J. Infect. Diseases **31**: 650–663.
7. FAY, A. C. 1934. The effect of hypertonic sugar solutions on the thermal resistance of bacteria. J. Agr. Res. **48**:452–468.
8. KADAN, R. S., W. H. MARTIN, AND R. MICKELSEN. 1963. Effects of ingredients used in condensed and frozen dairy products on thermal resistance of potentially pathogenic staphylococci. Appl. Microbiol. **11**:45–49.
9. SCOTT, W. J. 1957. Water relations of food spoilage microorganisms. Advan. Food Res. **7**:83–127.
10. VILJOEN, J. A. 1926. Heat resistance studies. 2. The protective effect of sodium chloride on bacterial spores heated in pea liquor. J. Infect. Diseases **39**:286–290.
11. WEISS, H. 1921. The heat resistance of spores with special reference to spores of *Bacillus botulinus*. J. Infect. Diseases **28**:70–92.