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Mechanism of Thermal Injury in *Staphylococcus aureus*

I. Relationship Between Viability and Leakage

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The viability of and leakage from suspensions of *Staphylococcus aureus* stored in the temperature range 3 to 60 C were determined. There was a direct relationship, up to 50 C, between death and leakage, both of which increased with increasing temperature. At temperatures above 50 C, there was a different pattern of response, the total leakage being less at 60 C than at 50 C, although there was greater membrane damage at the higher temperature. Sucrose, 1 M, almost completely prevented leakage at 37 and 50 C but not at 60 C.

In an earlier publication from this laboratory (1) it was reported that aqueous suspensions of *Staphylococcus aureus*, strain NCTC 3761, showed decreasing viability with increasing temperature of storage. Attempts were made to explain this phenomenon.

This paper describes observations on changes in viability and total counts of, and leakage constituents from, suspensions of *S. aureus* stored in the temperature range 3 to 60 C. Another paper (*in preparation*) is concerned with changes in absorbancy and light-scattering properties of such suspensions.

Leakage of intracellular constituents from bacterial cells has been reported as a consequence of various physical or chemical treatments, e.g., rapid chilling (16, 17), freezing (18), storage (13), heating (5, 9, 14, 20), and exposure to various cationic substances and polypeptide antibiotics (7, 11, 15). It has been postulated that the presence of leakage material in the environment affects the subsequent viability of the remaining cells (13).

Studies of the effect of moist heat on bacteria have been developed in various ways, but mainly they have been designed to define nutritional changes induced by the heating process (6, 9, 10). Although such experiments undoubtedly provide valuable information, such changes do not explain the lethal damage induced in the bacterial cell as a result of its exposure to moist heat.

MATERIALS AND METHODS

Preparation of suspensions. S. aureus NCTC 3761 was grown for 18 hr in double strength nutrient broth (Oxoid Division of Oxo, Ltd., London, England) in Roux flasks at 37 C. The suspension was centrifuged, and the pellet was washed twice with sterile glass-distilled water. The suspension was adjusted to a turbidity corresponding to about 10^9 viable cells/ml, except in those experiments in which responses of cells suspended in water or in 1 M sucrose were to be compared. In such instances, cells were washed twice and resuspended in water to about 3×10^9 viable cells/ml; 1 part of this suspension was then added to 2 parts of water or of a 51.3% (w/v) solution of sucrose (analytical reagent grade) as necessary.

Storage procedure. Samples of 30 ml of the suspensions were placed in 250-ml conical flasks, previously equilibrated at the desired temperature, and were held in a thermostatically controlled water bath at the same temperature (±0.1 C). Such suspensions reached the desired temperature within 3 to 4 min.

Viability determination. Samples of 1 ml were removed at intervals and were serially diluted in sterile water. Viable-cell numbers were determined in duplicate by the pour-plate method.

Measurement of leakage material. Samples of 4 ml of the stored suspensions were removed at intervals and centrifuged at 3,000 × *g* for 30 min, and the absorbance at 260 mμ of the supernatant fluid was determined with the Unicam SP 800 spectrophotometer.

Ribonucleic acid (RNA)-like material (referred to as RNA in Fig. 2-4) was determined by the method of Ceriotti (4), with RNA (British Drug Houses, Ltd., London, England) as a reference standard. The test was carried out on the supernatant fluid after determining absorbance at 260 mμ.

Penetration of 8-anilino-1-naphthalene sulphonic acid into heated cells. Samples of 5 ml of heated suspensions were removed at intervals and centrifuged; the supernatant fluid was discarded, and the cells were dispersed again in 10 ml of water or 1 M sucrose. Of this suspension, 5 ml was added to 0.25 ml of a 5×10^{-3} M solution of 8-anilino-1-naphthalene sulfonic acid,

sodium salt (ANS; Eastman Chemical Products, Inc., Kingsport, Tenn.). The fluorescence was measured at 470 mμ with a spectrophotofluorometer (Aminco-Bowman; American Instrument Co., Inc., Silver Spring, Md.), by use of incident light of 400 mμ. The fluorometric blank was the remaining portion of heated suspension to which 0.25 ml of water or sucrose was added. A correction was made for fluorescence of a solution of the dye alone.

Total counts. These were carried out in duplicate with a Thoma counting chamber.

RESULTS

Suspensions of *S. aureus* stored in water at various temperatures show an increasing loss of viability as the temperature increases (1). A more complete analysis of the results is shown in Fig. 1a–f, the loss of viability of the cells being determined by inability to recover under optimal revival conditions (2). Suspensions lost viability at all temperatures studied (except at 3 C, at which point there was also negligible leakage), the rate of death increasing with temperature. Figure 1 also shows the leakage of 260 mμ-absorbing materials from the cells. There appeared to be a direct correlation between the rate of death and the rate and amount of leaked material over the temperature range

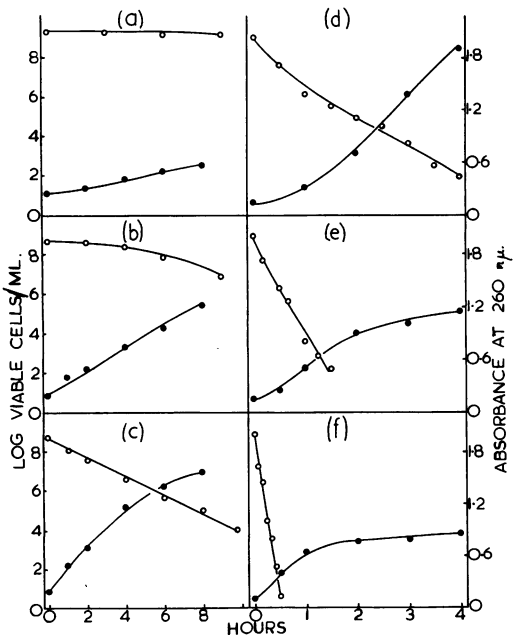

FIG. 1. *Effect of storage temperature on the viability of, and leakage of 260 mμ-absorbing materials from, washed suspensions of Staphylococcus aureus. Symbols: ○, viable cells/ml; ●, 260 mμ-absorbing materials. Temperatures: (a) 22 C; (b) 37 C; (c) 45 C; (d) 50 C; (e) 55 C; (f) 60 C.*

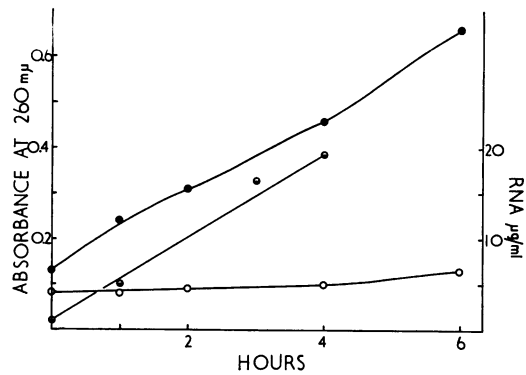

FIG. 2. *Release of intracellular constituents from suspensions of Staphylococcus aureus at 37 C. Release of 260 mμ-absorbing materials from suspensions in water (●) and in 1 M sucrose (○), and of RNA from suspensions in water (◒).*

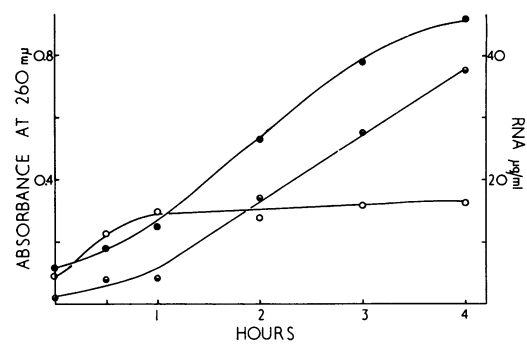

FIG. 3. *Release of intracellular constituents from suspensions of Staphylococcus aureus at 50 C. Release of 260 mμ-absorbing materials from suspensions in water (●) and in 1 M sucrose (○), and of RNA from suspensions in water (◒).*

22 to 45 C (Fig. 1a, b, c). A similar relationship occurred at 50 C (Fig. 1d), whereas, at 55 and 60 C (Fig. 1e and f, respectively), there was a different pattern of response: a comparatively rapid initial rate of leakage was followed by the attainment of what was virtually the maximal level; in addition, the death rate was linear.

It was considered that leakage could be occurring as a result of primary damage to the cytoplasmic membrane. Thus, the amount of leakage was compared from cells stored at 37, 50, and 60 C in water and in the osmotic stabilizer, 1 M sucrose (Fig. 2–4). In addition, the RNA content (see Materials and Methods) from the supernatant fluids of suspensions of *S. aureus* stored in water at these temperatures is shown in Fig. 2–4. At 37 C, sucrose almost completely

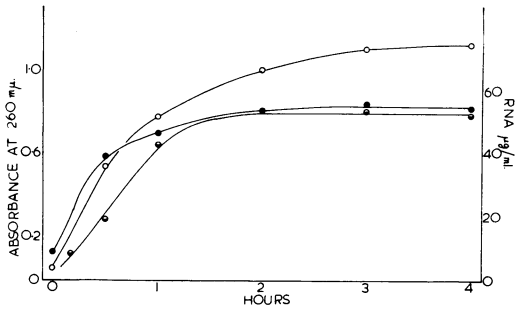

FIG. 4. *Release of intracellular constituents from suspensions of Staphylococcus aureus at 60 C. Release of 260 mμ-absorbing materials from suspension in water (●) and in 1 M sucrose (○), and of RNA from suspensions in water (◒).*

TABLE 1. *Percentage of survival of suspensions of Staphylococcus aureus stored at various temperatures in water or 1 M sucrose*[a]

Time (min)	Storage temperature and suspending medium					
	37 C		50 C		60 C	
	Water	Sucrose	Water	Sucrose	Water	Sucrose
0	100	100	100	100	100	100
5					0.31	42
10						0.1
20					0.0001	0.0005
30			3.3	48	<0.0001	0.0003
40						<0.0001
60			0.83	14		
120			0.045	0.14		
240	86	72	0.00014	0.00012		
480	78	44				

[a] Original numbers of viable cells (per ml) at 37, 50, and 60 C were 6.4 × 10^8, 7.1 × 10^8 and 6.0 × 10^8, respectively.

prevented leakage (Fig. 2), and at 50 C leakage was greatly reduced (Fig. 3). However, at 60 C leakage was greater in sucrose than in water (Fig. 4). Table 1 indicates, however, that sucrose has some protective effect on the viability of cells stored at both 50 and 60 C.

Figures 2–4 also show that, as would be expected, the appearance of 260 mμ-absorbing substances in supernatant fluids of heated aqueous suspensions is paralleled by the release of RNA-like material from the cells.

DISCUSSION

Iandolo and Ordal (10) reported that heating at 55 C caused the release of intracellular constituents from cells of *S. aureus*; the present study demonstrates that there are certain parallels between the rate and amount of leakage and the loss of viability of the bacteria stored in water.

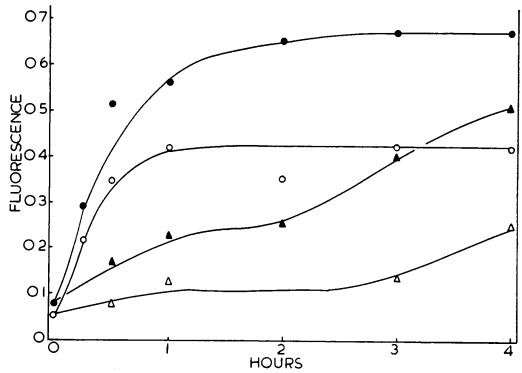

FIG. 5. *Penetration of ANS into heated Staphylococcus aureus cells. Suspensions at 50 C in water (▲) and in 1 M sucrose (△). Suspensions at 60 C in water (●) and in 1 M sucrose (○).*

A distinction may be drawn between two ranges of temperature. Up to 45 C, the organism is capable of growth on or in a suitable nutrient medium. In this range, it is likely that the death of cells in a non-nutrient medium is not caused by physical or chemical effects of heat, but by a starvation process accelerated by temperature. It may be inferred that a temperature above this could induce thermal damage in the bacterial cell, as manifested by the fact that there is an altered pattern of leakage above 50 C, and by the change in response to the presence of sucrose. The leakage of intracellular material from cells held at temperatures above 50 C could conceivably be reduced by a sealing-off of the membrane, as has been reported with high concentrations of chlorhexidine acting on *Escherichia coli* (4, 9). This hypothesis for heat-treated cells of *S. aureus* was tested by studying the penetration of ANS into cells heated in water and sucrose at 50 and 60 C. The results (Fig. 5) show that membrane damage in cells held in water is significantly greater at 60 C than at 50 C. Thus, sealing-off of the membrane does not occur as a result of heat treatment of this organism. With cells heated in sucrose, damage to the membrane is again greater at 60 C than at 50 C; however, it must be noted that the mere presence of sucrose may reduce the rate of penetration into the cells of ANS. It is considered unlikely that sucrose has any effect on the actual fluorescence: when cells were heated in water, centrifuged, resuspended, and treated with ANS, almost identical readings were obtained with water or sucrose as the resuspending medium. Thus, the results shown in Fig. 5 suggest that sucrose decreases membrane damage. We therefore conclude that leakage is not solely effected by membrane damage, but must be the

result of some intracellular changes in the stability of RNA. Release of intracellular constituents could also occur as a result of cell lysis (12). However, no significant decrease in total counts has been observed over a 4.5-hr period. Thus, in general, the integrity of the outer layers of the cell is not affected by storage at high temperatures, and lysis does not account for the leakage of intracellular substances.

Postgate and Hunter (13) reported that during starvation RNA degradation occurs and that heat destroys the osmotic integrity of cells of *Aerobacter aerogenes*. The results of the present study show the expected similarity between leakage of 260 mμ-absorbing substances and RNA-like material. This leakage may be due to the breakdown of RNA (19) with passage of the resultant smaller molecular weight substances out of the cell. Hansen and Riemann (5) have also shown that the loss of stainability of gram-negative cells exposed to heat results from a loss of nucleic acids. The loss of endocellular material from chilled cells is a cause and not an effect of death in *A. aerogenes* (17). However, with *S. aureus*, the reverse is true for cells held at 50 to 60 C.

A comparison of the leakage pattern from bacteria heated in water and sucrose again demonstrates the differences between 50 and 60 C. Sucrose failed to prevent leakage at 60 C, although membrane damage appeared to be reduced (Fig. 5). This has been consistently observed in our experiments. The presence of sucrose gave some protection against thermally induced death. This indicates that leakage at 60 C is not a primary effect of moist heat on *S. aureus*. A possible explanation of these findings is that at 60 C, with suspensions in water, intracellular protein coagulation enmeshes RNA-like material and therefore prevents further leakage. At this temperature, also, sucrose, owing to its water activity, could reduce protein coagulation, so that increased leakage could result. The partial protection afforded by sucrose against leakage at 50 C cannot, as yet, be explained in these terms.

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