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Thermally Induced Ribonucleic Acid Degradation and Leakage of Substances from the Metabolic Pool in *Staphylococcus aureus*

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The effects of temperatures of 50 and 60 C on log-phase and stationary-phase cell suspensions of *Staphylococcus aureus* are described. There is a leakage of free amino acids, protein, and 260 mμ-absorbing material from both types of cell suspension, and membrane damage, as measured by the intracellular penetration of 8-anilino-1-naphthalene-sulfonic acid, may be partially related to this leakage. Ribonucleic acid (RNA) degradation at any one temperature is virtually the same for both types of cell suspension, proceeding initially at a more rapid rate at 60 C than at 50 C. However, at the lower temperature, there is a secondary breakdown of RNA, which may be the result of enzyme action on a particularly labile RNA fraction. With stationary-phase cell suspensions heated in 1 M sucrose, there is a more rapid degradation of RNA at 60 C than with cells in water. The results are discussed in relation to the biochemical effects of moist heat on the organism.

The exact mechanism whereby nonsporing bacteria suffer thermal damage is unknown; however, there are several changes observed in heated bacteria, and any one of these could theoretically be responsible for thermally induced death. Such effects include leakage of intracellular constituents (2, 3, 5, 7, 11), breakdown of ribonucleic acid (RNA) (7, 12), and protein coagulation (6). Previous experiments with *Staphylococcus aureus* showed a variation of leakage material with the temperature at which the cells were held (2, 3). We, therefore, studied the simultaneous rate of breakdown of RNA and the composition of the acid-soluble pool in, and the leakage of 260 mμ-absorbing substances, amino acids, and protein material from, heated bacteria. Because of differences observed previously (2, 3) between cells suspended in water and in 1 M sucrose, in some experiments cells were heated in both media.

MATERIALS AND METHODS

Suspensions of the organism. After growth in nutrient broth overnight for 18 hr at 37 C in a shaking incubator, the organism (*S. aureus* NCTC 3761) was centrifuged; the pellet was washed twice with sterile water and finally suspended in water to give ca. 10^{10} viable cells/ml (2, 3). In other experiments, portions of overnight cultures were inoculated into 80 ml of nutrient broth in 250-ml conical flasks, which were incubated at 37 C (0.1 C) in a thermostatically controlled water bath operating at 100 oscillations/min. When the organisms were in the logarithmic growth phase, they were centrifuged at 2,000 × *g*, washed twice with sterile water, and eventually resuspended in water to give ca. 10^{10} viable cells/ml. These two types of suspension are termed stationary-phase and log-phase cell suspensions, respectively.

Heating procedure. One part of a washed suspension was added to nine parts of suspending medium (water or sucrose) already at the desired temperature; when used, the sucrose had a final concentration of 1 M.

Analysis of cells. Samples (10 ml) were removed when required and were subjected to the following procedure. The samples were centrifuged at 20,000 × *g* in a MSE 18 (Measuring & Scientific Equipment, Ltd., London) refrigerated centrifuge at 2 C; the supernatant fluid (*a*) was carefully removed and examined for ultraviolet-absorbing material, protein, and amino acids, as described below. The centrifuge tubes containing the pellets were cooled to 2 C, 10 ml of 0.5 N perchloric acid (PCA) was added, and the tubes were maintained at 2 C for 30 min. The suspensions were centrifuged at 2 C and the supernatant fluids (*b*, comprising the acid-soluble pool) were carefully removed. The centrifuge tubes containing the pellets were placed in a water bath at 37 C, and 7.5 ml of 0.3 N potassium hydroxide was added. After 60 min at this temperature, the tubes were cooled to 2 C, 2.5 ml of 2 N PCA was added, and the tubes were centrifuged. The supernatant fluids (*c*) were carefully removed and retained. Volumes (10 ml) of 1 N PCA were added to the pellets, and the centrifuge tubes

were placed in a water bath at 70 C. After 20 min, the tubes were cooled to 2 C and centrifuged. The supernatant fluids (*d*) were carefully removed and retained for analysis.

In each of the stages described above, the walls of the centrifuge tubes were dried with cellulose tissues to remove traces of liquid.

RNA in supernatant fluids *a*, *b*, and *c* was determined by absorbance at 260 mμ, with yeast RNA (British Drug Houses, B.D.H., London) used as standard. Deoxyribonucleic acid (DNA) in supernatant fluid *d* was determined by absorbance at 260 mμ, with thymus gland DNA (B.D.H.) used as standard, and by the method of Burton (4). The presence of 0.5 N PCA did not affect the absorbances of these solutions. Amino acids and proteins in supernatant fluids *a* and *b* were determined, respectively, by the ninhydrin method, with leucine used as standard, and by the method of Lowry et al. (8), with bovine albumin used as standard. Before their determination in supernatant liquid *b*, the PCA was neutralized with sodium hydroxide.

RNA is expressed throughout this paper as a percentage of the total amount in the cells; the total amount of RNA (i.e., in the cells, including the pool plus that leaked) was constant over the periods tested.

Penetration of 8-anilino-1-naphthalene-sulfonic acid (ANS) into heated cells. This was carried out as previously described (2), with a 5×10^{-3} M solution of ANS, incident light of 400 mμ, and measuring fluorescence at 470 mμ.

Viable-counting technique. Viable counts were made as described previously (1, 2), by using the pour-plate method, a plating medium consisting of nutrient agar containing 1% (w/v) yeast extract (Difco), and an incubation period of 48 hr.

RESULTS

The amount of 260 mμ-absorbing material released from log-phase cell suspensions was considerably higher at a particular temperature (50 or 60 C) than that from stationary-phase cell suspensions at the same temperature (Fig. 1). However, this may be accounted for by the fact that cells in the former suspension contained about 50% more RNA than those in the latter. Consequently, as stated in Materials and Methods, to obtain a more accurate comparison of thermally induced death in both types of suspension, it was better to express RNA leaked or present in the acid-soluble nucleotide pool as a percentage of the total amount of RNA present in the cell.

The death rate of log-phase cell suspensions at 50 C was only marginally greater than that of stationary-phase cell suspensions at this temperature; whereas, at 60 C, the former were killed considerably more rapidly than the latter (Table 1).

The penetration of ANS into log-phase and stationary-phase suspensions previously held for various periods at 50 and 60 C is shown in Fig. 2. The rate of penetration was greater at 60 than at 50 C, in both cases.

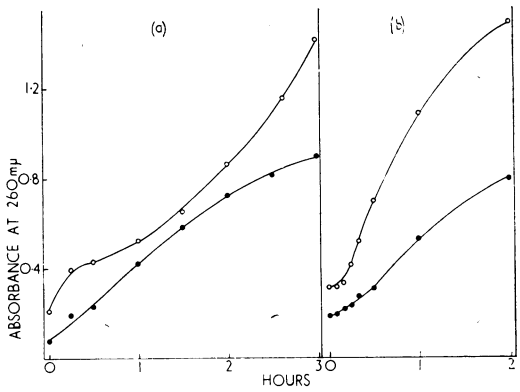

FIG. 1. *Leakage from log-phase (○) and stationary-phase (●) cell suspensions of Staphylococcus aureus at (a) 50 C and (b) 60 C.*

TABLE 1. *Loss of viability of log- and stationary-phase cell suspensions of Staphylococcus aureus at 50 and 60 C*

Holding temperature	Time	Viability[a]	
		Log-phase cell suspension	Stationary-phase cell suspension
C	*min*		
50	0	1×10^9	1×10^9
	15	2.35×10^8	—
	30	1.4×10^7	8.9×10^7
	60	—	6.4×10^6
	90	2.6×10^5	6.2×10^5
	120	1.7×10^4	8.5×10^4
	150	1.3×10^3	6.7×10^4
	180	1.4×10^2	1.7×10^4
60	0	1×10^9	1×10^9
	1.25	2.1×10^7	—
	2.5	9.6×10^5	3.9×10^7
	5	8×10^4	7.4×10^6
	7.5	1.3×10^4	2×10^6
	10	5.7×10^3	3.6×10^5

[a] Number of viable cells per milliliter.

The degradation of RNA and the composition of the acid-soluble nucleotide pool in, and analyses of leakage material from, log- and stationary-phase suspensions at 50 and 60 C are shown in Fig. 3 and 4, respectively. These analyses were made over long periods to determine whether (i) changes could be correlated with viability loss, (ii) changes continued after the majority of the cells had been killed. At 50 C, with log-phase cell suspensions (Fig. 3a), there was an initial rapid breakdown of RNA concurrent with a rapid loss from the cells of leakage substances, thus reducing

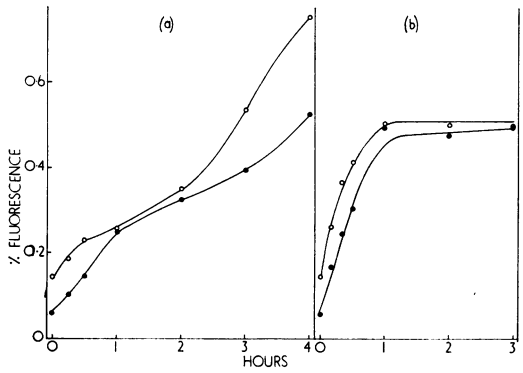

FIG. 2. *Penetration of 8-anilino-1-naphthalene-sulfonic acid into cells of log-phase* (○) *and stationary-phase* (●) *suspensions of Staphylococcus aureus at* (*a*) *50 C and* (*b*) *60 C.*

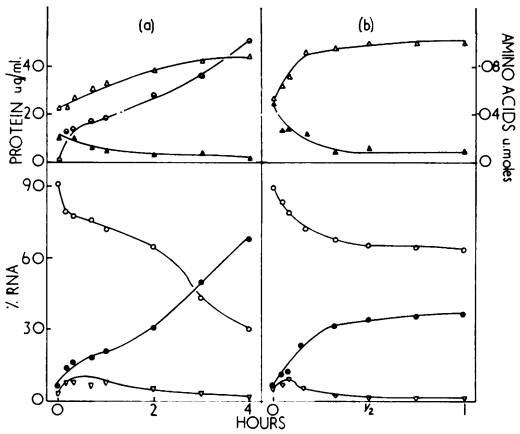

FIG. 3. *Analysis of cells of log-phase suspensions of Staphylococcus aureus at* (*a*) *50 C and* (*b*) *60 C. Upper portion: protein leaked* (◒)*; amino acids leaked* (△)*; amino acids in pool* (▲)*. Lower portion: total RNA in the cells* (○)*; RNA leaked from the cells* (●)*; RNA content of the pool* (▽).

the pool material; with stationary-phase suspensions at this temperature (Fig. 4a), there was likewise a marked increase in pool RNA, and this preceded leakage of RNA-like material. The total RNA in the cells decreased at a linear rate. The amount of protein in the acid-soluble pool (not shown in Fig. 3a and 4a) was very small, but did not appear to decrease; thus, the protein that leaked from the cell was not degraded. This statement is substantiated by the finding that the total amount (i.e., the sum of leaked plus pool) of amino acids remained approximately constant.

Figures 3b and 4b have different time scales along the abscissa from those used in Fig. 3a and 4a. At 60 C, with log-phase cell suspensions (Fig. 3b), RNA breakdown and leakage were more rapid, with no lag, than with stationary-phase cell suspensions at this temperature (Fig. 4b); however, the degree of RNA degradation after 1 hr at 60 C was approximately the same with both types of cell suspension. The leakage of amino acids at 60 C from the two suspensions appeared to be similar.

Also of interest was the fact that the total amount of leakage of 260 mμ-absorbing material at 50 C was always greater than that at 60 C, irrespective of which cell suspension was being used; this confirmed earlier findings (2, 3).

The amount of DNA in the cells remained constant at both temperatures and therefore is not included in the figures. This result indicates that no degradation and no leakage of DNA occurred on exposure of the cells to temperatures of 50 and 60 C.

The initial *p*H of staphylococcal suspensions was 7.0, and at the end of the heating treatments (1 hr at 60 C or 4 hr at 50 C), the maximal *p*H fall was to 6.6. Thus, it is unlikely that *p*H changes in the external environment play any significant part in the lethal processes.

Sucrose, at a concentration of 1 M, has been shown (2) to greatly reduce leakage from stationary-phase cell suspensions held at 50 C; whereas, at 60 C, there was an increased leakage in both instances as compared with cells held in water at the corresponding temperature. An attempt to explain these phenomena was made by heating such suspensions in water and in sucrose at 50 and 60 C and comparing RNA degradation, etc. The results obtained (Fig. 5a) show that the rate and extent of RNA degradation of cells in sucrose at

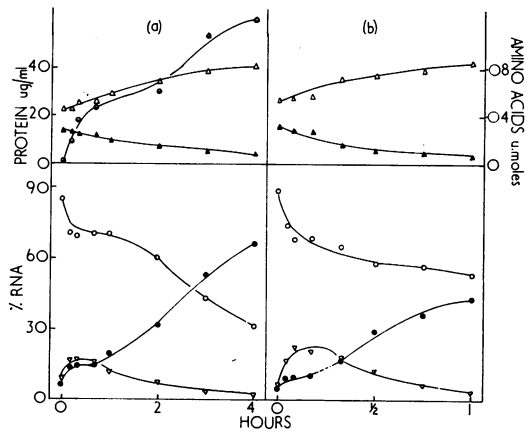

FIG. 4. *Analysis of cells of stationary-phase suspensions of Staphylococcus aureus at* (*a*) *50 C and* (*b*) *60 C. Symbols as in Fig. 3.*

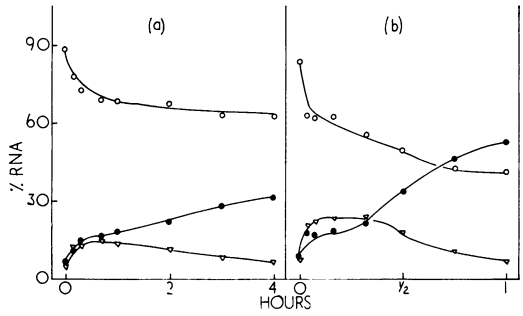

FIG. 5. *Effect of the presence of 1 M sucrose during the heating process on the analysis of cells of stationary-phase suspensions of Staphylococcus aureus at (a) 50 C and (b) 60 C. Total RNA in the cells (○); RNA leaked from the cells (●); RNA content of the pool (▽).*

50 C decreased (cf. with Fig. 4a) with a consequent small increase only in pool RNA. At 60 C, there was an increased amount of leakage with cells in sucrose (Fig. 5b) as compared to those in water (Fig. 4b), and this increased leakage was the result of a greater degradation of RNA, and thence of an increased content in the pool in cells heated in sucrose at this temperature.

DISCUSSION

The results presented in this paper show that several changes occur in *S. aureus* cells exposed to high temperatures. Certain differences and similarities may be observed between stationary- and log-phase cell suspensions of this strain. The pattern of amino acid release from cells of log-phase suspensions at 50 C is similar to that from cells of stationary-phase suspensions at this temperature, and there is virtually no increase in the total amounts of free amino acids during the heating period; this is also true at 60 C. However, at the higher temperature, amino acids are released more rapidly from log- than from stationary-phase cell suspensions, and the former suspensions are considerably more sensitive to 60 C (Table 1). However, at this temperature, the rate of penetration of ANS into the organisms is virtually the same with both types of cell suspension, the initial higher reading with log-phase cell suspensions denoting a naturally greater permeability. This "natural permeability" might, therefore, be responsible for the more rapid leakage of amino acids from cells of log-phase suspensions.

The leakage of protein from log- and stationary-phase cell suspensions at 50 C shows a fairly close relationship. At 60 C, protein leakage occurred to a very small degree, and thus has not been included in the figures. This considerable reduction in leakage presumably occurs as a result of protein coagulation either intracellularly or intercellularly.

The RNA content of heated cells shows some interesting findings. At 50 C, the initial leakage of RNA-like material occurs more rapidly with log-phase than with stationary-phase cell suspensions. However, this is not the result of an increased degradation of RNA, as RNA breakdown is closely related in both types of cell suspension. The total RNA content of the cells of both suspensions shows a diphasic type of reduction, whereas at 60 C no such diphasic pattern is presented. This could suggest that, at 50 C, the "secondary" breakdown which takes place may be the result of enzyme action; this enzyme would be destroyed at 60 C, so that no secondary degradation would occur at this temperature. The initial rapid degree of RNA degradation observed could be owing to a particularly labile RNA fraction. At 60 C, also, RNA breakdown occurs at roughly the same rate in the cells of both log-phase and stationary-phase suspensions, despite the increased sensitivity of the former at this temperature. It would, however, be premature at this stage to consider that RNA degradation is not related to thermally induced bacterial death. It is of interest to note here that 5% (v/v) *n*-butyl alcohol treatment of *S. aureus*, causing maximal permeability damage, released approximately 10% of the total cell RNA. Any additional leakage from heat-treated bacteria would thus be presumed to be the result of RNA degradation.

A consideration of the degradation of RNA in log-phase cell suspensions leads to the following conclusions: (i) the rate is more rapid at 60 than at 50 C; (ii) the overall amount is greater at 50 than at 60 C, which, in turn, is responsible for (iii) the increased overall leakage at the lower temperature observed here and in previous papers (2, 3). Exactly the same conclusions apply with RNA breakdown in stationary-phase cell suspensions.

Stationary-phase cell suspensions in 1 M sucrose exposed to 50 and 60 C show certain differences from suspensions in water. Thus, there is a greater degree of RNA degradation at 60 C in cells in sucrose than in those in water. Why this is so is not immediately apparent, but this result does partially explain the repeatedly observed finding that there is greater leakage of RNA-like material at 60 C from cells in sucrose than from those in water (2, 3). There appears to be a greater amount of pool RNA at 60 C (Fig. 5,b) than at 50 C (Fig. 5,a), and the overall amount of leakage is higher at 60 than at 50 C. It is considered that these facts, taken as a whole, represent a decreased protein coagulation in cells in sucrose, compared to cells in water at 60 C, so that no

"RNA protection" (i.e., enmeshing of RNA by protein material) ensues.

It is difficult to fit these and previous (2, 3) findings into a pattern which adequately and unequivocally explains thermal damage in the cell. To the best of our knowledge, only Califano (5) has previously commented upon the fact that, with *S. aureus*, the amount of RNA leakage does not necessarily parallel temperature increase. Our results here and elsewhere (2, 3) have expanded considerably on this finding and serve as a cautionary note to those who assume that high temperatures (about 100 C) necessarily release the maximal amount of intracellular material from the organism.

Iandolo and Ordal (7) suggested that a series of changes attributable to heat occurred in *S. aureus*, in which a weakening of the cell membrane, a coincident loss of the soluble pools, and a simultaneous degradation of RNA were involved. Strange and Shon (12) considered that depletion of RNA was not the primary cause of death in *Aerobacter aerogenes* at 47 C, but that RNA degradation, with a resultant rapid increase in endogenous pool materials, contributed to the lethal effect. In each paper, however, only one temperature was studied, and our results show that seemingly different events may be involved at different temperatures. In a temperature-sensitive strain of *Escherichia coli*, which could grow normally at 28 C but not at 40 C, degradation of ribosomal (r-) RNA occurred at the higher temperature (9). Moreover, ribosomes of the thermophilic organism *Bacillus stearothermophilus* undergo denaturation at a much higher temperature than do those of a mesophilic organism, *E. coli* (10).

There is, therefore, a considerable weight of evidence to suggest that RNA degradation is linked with thermally induced death in nonsporing bacteria. It must also be observed in the present work, however, that some RNA breakdown occurs after a loss of cell viability has taken place.

Further studies, involving the use of cell-free constituents and of recovery [along the lines described by Iandolo and Ordal (7)], should lead to a better understanding of the role of RNA degradation in the lethal effect of moist heat on *S. aureus*.

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