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Stability of Ribosomes of *Staphylococcus aureus* S6 Sublethally Heated in Different Buffers

ANDRE HURST* AND ASHTON HUGHES

Microbiology Research Division, Health Protection Branch, Health and Welfare Canada, Ottawa, Canada K1A OL2

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Cells of *Staphylococcus aureus* heated at 52°C in magnesium-chelating buffers [pH 7.2, 50 mM potassium phosphate or 50 mM tris(hydroxymethyl)-aminomethane containing 1 mM ethylenediaminetetraacetic acid] leaked 260-nm absorbing material, shown to be RNA, and suffered destruction of their ribosomes. These cells did not regain their salt tolerance when repair was carried out in the presence of actinomycin D (5 μg/ml). Cells similarly heated in magnesium-conserving buffers [pH 7.2, 50 mM tris(hydroxymethyl)aminomethane containing 10 mM $MgCl_2$ or piperazine buffer] did not leak RNA, suffered no ribosomal damage when heated for 15 min, and recovered, at least partially, in the presence of actinomycin D. Ribosomal damage is, therefore, a consequence of Mg^{2+} loss and is not an effect of heat per se. Cells suspended in either Mg^{2+}-chelating or Mg^{2+}-conserving buffers lost salt tolerance to about the same extent during heating at 52°C. Therefore, sublethal heat injury can not be attributed to ribosomal damage.

Sublethal heating causes pleiotrophic cellular damage (for review see 4). The pioneering work of Iandolo and Ordal (7) established the importance of ribosomal damage. They showed that sublethally damaged *Staphylococcus aureus* could not repair heat-induced injury when incubated with actinomycin D. Sogin and Ordal (15) demonstrated ribosomal destruction after heating. Haight and Ordal (3) suggested that the ribosomal damage observed could be due to enzyme activity. Rosenthal et al. (14) and Rosenthal and Iandolo (13) observed the preferential destruction of the 30S particle and the destruction of its constituent 16S RNA. They could not account for this observation in terms of damage by enzyme(s).

Strange and Shon (16) working with mildly heated *Aerobacter aerogenes* showed that the organism was killed and injured because of ribosomal damage. The organism also suffered Mg^{2+} loss, and Strange and Shon suggested that Mg^{2+} loss triggered ribosomal destruction. Hurst et al. showed that *S. aureus* also lost Mg^{2+} during sublethal heating (6).

Since Mg^{2+} is classically concerned with ribosome integrity, we reexamined this question with *S. aureus*. In this paper we show that heating in buffers that release cellular Mg [potassium phosphate or tris(hydroxymethyl)aminomethane (Tris) plus ethylenediaminetetraacetic acid (EDTA)] causes ribosomal destruction. Heating in buffers that do not release Mg (piperazine or Tris plus Mg) does not cause ribosomal destruction. Therefore, the effect on ribosomes is not due to heat per se, but is a consequence of Mg loss.

MATERIALS AND METHODS

Organism, culturing conditions, media, and plate counting. The food poisoning strain of *S. aureus* S6 was grown and cultured essentially as described earlier (5).

Sublethal heat injury. Stationary-phase cultures in HK medium (5) having an optical density at 600 nm (OD_{600}) of about 1.3 (when diluted 1:9 with distilled water), a pH of 8.2, and a population of 1.3×10^{10} colony-forming units per ml were centrifuged at 6,000 × *g* for 10 min. The pellet from 30 ml was washed in 30 ml of buffer (room temperature), centrifuged again, and resuspended in 3 ml of buffer. This concentrated cell suspension (3 ml) was added to 200 ml of preheated and vigorously stirred buffer at 52°C. All buffers used for heating were 50 mM (pH 7.2) and were as follows: potassium phosphate (KP), Tris (Sigma Chemical Co., St. Louis, Mo.) containing either 10^{-2} M $MgCl_2$ (TM) or 10^{-3} M EDTA (TE) and piperazine-*N*,*N*^{1}-bis(2-ethanesulfonic acid) (PIPES, Calbiochem, La Jolla, Calif.). Injury was assayed by plate count in tryptone soy agar (TSA, Difco Laboratories, Detriot, Mich.) and TSA containing 7.5% NaCl (TSAS), to estimate the numbers of salt-sensitive survivors. The techniques used were as previously described (5).

Colorimetric tests. Cell pellets were washed with cold perchloric acid (PCA) and extracted three times with 0.5 N PCA at 70°C for 30 min, and the combined extracts were used for estimating DNA by the diphenylamine amino reaction (1) and RNA by the orcinol

test (11). The residue of the extracted pellet was dissolved in 1 N NaOH, and protein was determined by the Lowry et al. test (10).

The same colorimetric tests were used on filtrates of treated cells. The filtrates were divided into two portions. One half was diluted 1:1 with 1N PCA and used for estimating DNA or RNA. The other half was diluted 1:1 with 2 N NaOH and used for protein estimation.

The following highly purified standards were used: DNA from *B. subtilis* (Calbiochem); RNA from yeast (Calbiochem); protein from bovine serum albumin (Sigma).

Leakage of cells during heat treatment. Samples of about 1 ml were withdrawn with a Pasteur pipette during the heat treatment and delivered into a pistonless syringe, which had a Sweeney filter attachment containing a membrane filter (Millipore Corp., Bedford, Mass., HAW PO 1300). This was injected into Vacutainer tubes (Becton, Dickinson & Co., Rutherford, N.J.). The whole process from sampling to completion of filtration lasted about 30 s. The OD of the filtrate was measured at 260 nm in a Gilford model 240 spectrophotometer.

Sucrose gradient centrifuging of ribosomes. Marker ribosomes were prepared after lysostaphin treatment of cells essentially as described by Rosenthal and Iandolo (13). The markers were centrifuged for 2.75 h in a Beckman SW56 rotor at 350,000 × *g* in 5 to 20% sucrose gradients made up in 10^{-2} M Tris plus Mg^{2+} at concentrations of 10^{-2}, 10^{-3}, and 10^{-4} M.

Lysostaphin treatment was not suitable for preparing ribosomes from injured cells presumably because, owing to leakage, these cells had lost turgidity. The ribosomal profiles shown in this paper were prepared as follows. Concentrated cell suspension (6 ml), prepared as described above for sublethal injury, was added to 400 ml of buffer either at room temperature (control) or at 52°C (injured). Cells from both treatments were cooled with stirring in ice water and then centrifuged at 6,000 × *g* for 15 min in a Sorval RC2B centrifuge. All subsequent operations were done at 5°C. Pellets were washed once in the same buffer, resuspended in 30 ml of TMK (Tris, 0.01 M; magnesium acetate, 0.01 M; KCl, 0.1 M; pH 7.0 at 0°C) plus 10 g of microbeads and treated for 5 min with a Braun disintegrator. Beads and broken cells were removed by centrifuging at 1,000 × *g* for 15 min followed by centrifuging at 6,000 × *g* for 15 min. Ribosomes were obtained by centrifuging the clear supernatant in an SW27.1 head at 100,000 × *g* for 9 h; the pellet was resuspended in 1 ml of buffer (10 mM Tris containing 10^{-4} M Mg^{2+}) and vigorously stirred for 9 h at 5°C. Undissolved aggregates were discarded after centrifuging at 12,000 × *g* for 20 min. These preparations (0.2 ml) were then layered on the gradients and centrifuged as described above for the marker ribosomes. Tubes were punctured from the bottom, fractions of 5 drops were collected and diluted with 0.5 ml of distilled water, and the absorbance at 260 nm was determined.

RESULTS

Leakage during heating at 52°C. The release of 260-nm absorbing material was dependent on the heating menstrum. Heating in Tris plus EDTA (TE) released more material than heating in Tris plus magnesium (TM) (Fig. 1). Heating in PIPES was about the same as heating in TM, and heating in potassium phosphate was about the same as heating in TE. Colorimetric estimations showed that the 260-nm substances released in the filtrate were RNA. Filtrates contained no DNA, and the protein concentration did not follow the increase in OD_{260}. Analysis of cell pellets during heating showed no loss of DNA and protein, but about ⅔ of the RNA was lost (data not shown).

Ribosomes were prepared from cells sampled at intervals during heating in KP at 52°C. A change in the ribosomal profile could be observed after as short a time as 30 s (not shown). In comparison to the unheated samples, the peak corresponding to the 30S particle had disappeared after 1 min, and the 50S peak became about 45 to 40S (Fig. 2). No ribosomal particles were detected after heating for 8 min (Fig. 2).

In contrast, the ribosomes appeared quite stable at 52°C when the heating menstrum was TM. Figure 3 shows virtually no difference between the ribosomes obtained from cells in TM after 15 min at room temperature or at 52°C.

Leakage at 37°C. The leakage of the 260-nm absorbing material was tested by using cells first heated at 52°C for 4 min, cooled, and then incubated at 37°C. It is evident that leakage at 37°C was similar to leakage at 52°C (Fig. 4). Far more leakage occurred under the Mg^{2+} re-

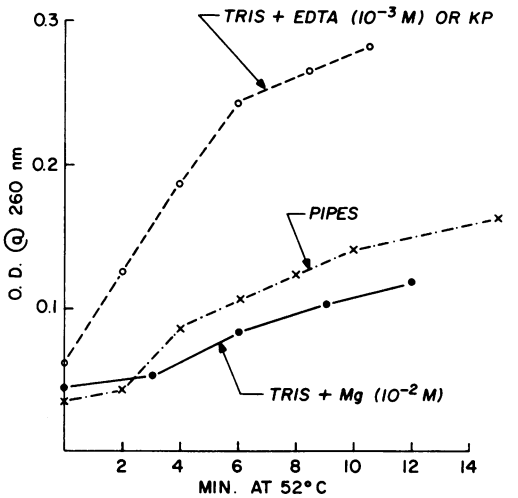

FIG. 1. *Release of 260-nm absorbing material from cells of S. aureus heated at 52°C and suspended in 50 mM (pH 7.2) buffers (TE, KP, TM, and PIPES). Details of the filtration procedure are described in the text.*

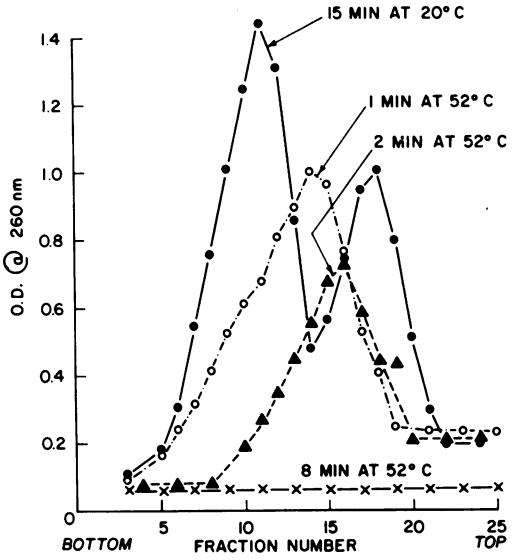

FIG. 2. *Sucrose gradient (5 to 20%) centrifuging of ribosomes of S. aureus. Unheated control and samples during heating at 52°C in 50 mM (pH 7.2) KP.*

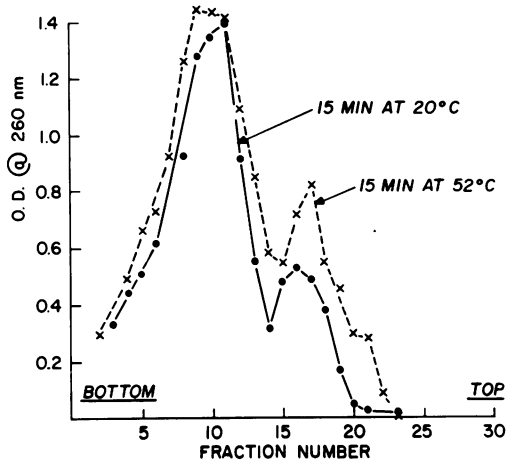

FIG. 3. *Sucrose gradient (5 to 20%) centrifuging of ribosomes of S. aureus unheated or heated for 15 min in 50 mM TM (pH 7.2).*

leasing conditions of TE (or KP, data not shown) than if the Mg^{2+} nonreleasing conditions of PIPES (or TM, data not shown) were used. Sucrose gradient centrifugation showed that in such TE-treated cells the 30S ribosomes were missing, and during incubation the 50S particle was gradually destroyed (data not shown).

Effect of actinomycin D on repair. Cultures were sublethally heated in either KP or PIPES for 15 min at 52°C. They were then washed and suspended in HK medium and incubated at 37°C with aeration (5). The repair part of the experiment was done either with actinomycin D (5 μg/ml) or without (control). The results (Fig. 5) show that 95% of the cells repaired their salt tolerance in 3 h. In the same time, 65% of the cells heated in PIPES recovered

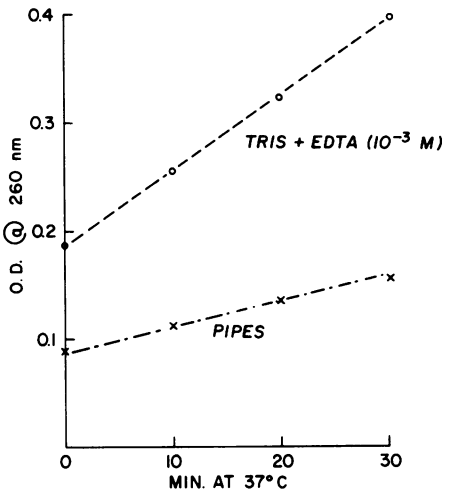

FIG. 4. *Release of 260-nm absorbing material from cells of S. aureus suspended in either 50 mM TE or in 50 mM PIPES. The suspensions were first heated for 4 min at 52°C, cooled, and incubated at 37°C. Time zero is the start of the incubation at 37°C.*

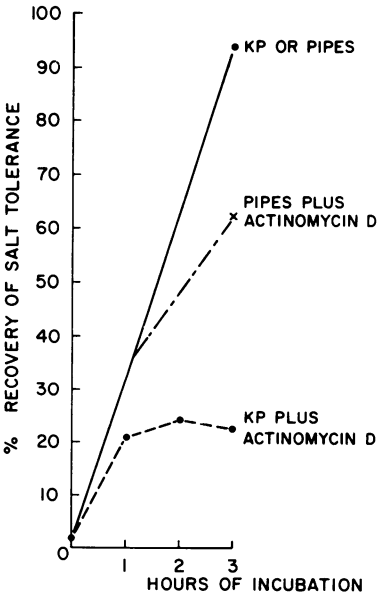

FIG. 5. *Effect of actinomycin D (5 μg/ml) on repair of salt tolerance in S. aureus heated for 15 min at 52°C in either 50 mM KP or 50 mM PIPES. Recovery in HK medium (5) at 37°C expressed as counts per milliliter in TSAS × 100/counts per milliliter in TSA.*

in the presence of actinomycin D, but recovery was only 18% when cells were heated in KP.

Correlation between sublethal heat injury and ribosomal damage. The nature of the heating medium affected ribosomal damage, but not the loss of salt tolerance. Heating in Mg^{2+}-chelating buffers (TE or KP), which destroys ribosomes, caused about the same degree of injury as heating in Mg^{2+}-conserving buffers (TM or PIPES) in which no ribosomal damage was detected. The result of one such experiment is shown in Fig. 6, when loss of salt tolerance was about the same after heating in TM or TE.

DISCUSSION

The purpose of this paper is to demonstrate that ribosomal damage is not the primary effect of sublethal heating. The data in Fig. 6 clearly show that there is no correlation between loss of salt tolerance and ribosomal damage. Salt tolerance was lost to about the same degree whether or not cells sustained ribosomal damage. This agrees with the results of Lee and Goepfert (9), who showed that in *Salmonella typhimurium*, the primary effect of sublethal heat injury was not damage to ribosomes.

The destruction of the 30S ribosomal particle appears to be a direct consequence of Mg loss. In sucrose gradients, its peak diminished in about the minimum time required to carry out the manipulations (data not shown) and was totally missing at 1 min (Fig. 2). In leakage experiments, the zero-time OD_{260} was always higher in Mg-chelating than in Mg-conserving buffers (Fig. 1). The speed of these reactions argues against enzyme involvement in the destruction of the 30S particle. However, the 50S particle was more resistant. The leakage experiment (Fig. 4) at 37°C involved only this particle, the sample having received prior heating at 52°C for 4 min, a treatment shown to destroy the 30S particle in less than 1 min. After the loss of Mg, nucleases may have become activated. Such divalent cation-inhibited nucleases are known in *Escherichia coli* (2) and in the pancreas (12), but have not been described in *S. aureus*.

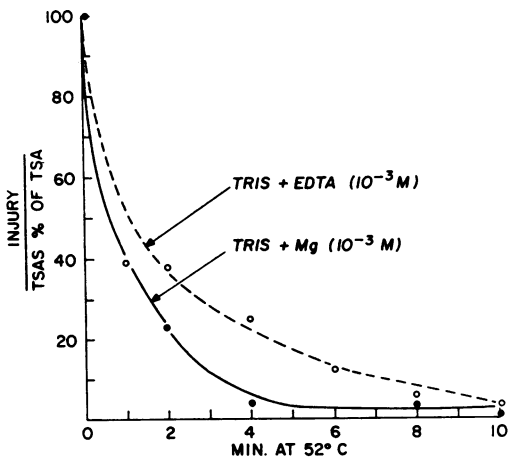

FIG. 6. *Loss of salt tolerance of S. aureus heated at 52°C in either 50 mM TE or TM. Injury is expressed as counts per milliliter in TSAS × 100/counts per milliliter in TSA.*

Under experimental conditions similar to our own, treatment of 50S ribosomes of *E. coli* at a low Mg concentration caused irreversible unfolding. This was measured by the diminishing S value of the particle (8). Results presented in Fig. 2 also show loss in the S value of the 50S particle so that it is likely that we were observing the unfolding of this particle, which may have initiated its destruction.

When Mg^{2+} was chelated by heating in KP or TE, cells leaked 260-nm material (shown to be RNA), ribosomes were rapidly destroyed, and such damaged cells were unable to repair their salt resistance in the presence of actinomycin D (Fig. 5). However, if the suspending medium conserved cellular Mg^{2+} (TM or PIPES), heating caused little cellular leakage (Fig. 1), no ribosomal destruction occurred (Fig. 3), and the injured cells effected repair in the presence of actinomycin D. These results clearly show that ribosomal damage is the consequence of Mg^{2+} loss and not of heating.

LITERATURE CITED

1. **Burton, K.** 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
2. **Datta, A. K., and S. K. Niyogi.** 1976. Biochemistry and physiology of bacterial nucleases. Prog. Nucleic Acid Res. Mol. Biol. **17**:271–308.
3. **Haight, R. D., and Z. J. Ordal.** 1969. Thermally induced degradation of staphylococcal ribosomes. Can. J. Microbiol. **15**:15–19.
4. **Hurst, A.** 1977. Bacterial injury: a review. Can. J. Microbiol. **23**:934–946.
5. **Hurst, A., A. Hughes, J. L. Beare-Rogers, and D. L. Collins-Thompson.** 1973. Physiological studies on the recovery of salt tolerance by *Staphylococcus aureus* after sublethal heating. J. Bacteriol. **116**:901–907.
6. **Hurst, A., A. Hughes, D. L. Collins-Thompson, and B. G. Shah.** 1974. Relationship between loss of magnesium and loss of salt-tolerance after sublethal heating of *Staphylococcus aureus*. Can. J. Microbiol. **20**:1153–1158.
7. **Iandolo, J. J., and Z. J. Ordal.** 1966. Repair of thermal injury of *Staphylococcus aureus*. J. Bacteriol. **91**:134–142.
8. **Ivanov, D. A., and M. I. Lerman.** 1976. Loosening and unfolding of *E. coli* 50S ribosomal subunits: dependence on magnesium content and temperature. Mol. Biol. Rep. **3**:39–46.
9. **Lee, A. C., and J. M. Goepfert.** 1975. Influence of selected solutes on thermally induced death and injury in *Salmonella typhimurium*. J. Milk Food Technol. **38**:195–200.

10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
11. **Mejbaum, W.** 1939. Uber die Bestimmung kleiner Pentosemengen insbesondere die merivaten der Adenylesaure. Hoppe-Seyler's Z. Physiol. Chem. **258**:117–120.
12. **Morrill, G. A., and M. M. Reiss.** 1969. Inhibition of enzymatic degradation of RNA by bound calcium and magnesium. Biochim. Biophys. Acta **179**:43–49.
13. **Rosenthal, L. J., and J. J. Iandolo.** 1970. Thermally induced intracellular alteration of ribosomal ribonucleic acid. J. Bacteriol. **103**:833–835.
14. **Rosenthal, L. J., S. E. Martin, M. W. Pariza, and J. J. Iandolo.** 1972. Ribosome synthesis in thermally shocked cells of *Staphylococcus aureus*. J. Bacteriol. **109**:243–249.
15. **Sogin, S. J., and Z. J. Ordal.** 1967. Regeneration of ribosomes and ribosomal ribonucleic acid during repair of thermal injury to *Staphylococcus aureus*. J. Bacteriol. **94**:1082–1087.
16. **Strange, R. E., and M. Shon.** 1964. Effects of thermal stress on viability and ribonucleic acid of *Aerobacter aerogenes* in aqueous suspension. J. Gen. Microbiol. **34**:99–114.