Repair of Thermal Injury of *Staphylococcus aureus*¹

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ABSTRACT

IANDOLO, JOHN J. (University of Illinois, Urbana), AND Z. JOHN ORDAL. Repair of thermal injury of *Staphylococcus aureus*. J. Bacteriol. 91:134-142. 1966.—Exposure of *Staphylococcus aureus* MF 31 to sublethal temperatures produced a temporary change in the salt tolerance and growth of the organism. After sublethal heat treatment at 55 C for 15 min, more than 99% of the viable population was unable to reproduce on media containing 7.5% NaCl. The data presented demonstrate that thermal injury, in part, occurred owing to changes in the cell membrane, which allowed soluble cellular components to leak into the heating menstruum. When the cells were placed in a limiting medium, complete recovery did not occur, regardless of the incubation time. The temperature and the pH which produced the optimal rate of recovery were similar to those described previously for the multiplication of uninjured cells. However, the rate of recovery as well as the unchanging total count during recovery indicated that cell multiplication was not a factor during the recovery process. The nutrient requirements for the complete recovery of injured cells consisted of a solution containing an energy source, such as glucose, a mixture of amino acids, and phosphate. The use of the metabolic inhibitors, penicillin, cycloserine, 2,4-dinitrophenol, and chloramphenicol, did not inhibit recovery. Actinomycin D, however, completely suppressed recovery. This result implied that ribonucleic acid synthesis was particularly involved; this inference was substantiated by radio tracer experiments. The rate at which label was incorporated in the nucleic acid fraction paralleled that of recovery and the return of salt tolerance.

Although an exaggerated lag phase is a cultural response to thermal injury (21, 22), it can more accurately be described as a recovery period. As defined by Harris (15), "it is the period of 'getting back' organisms from their (sublethal) environment." This is usually accomplished by incubating the treated organisms in a nutrient environment.

The use of metabolites or some type of nutrient medium for post-treatment incubation of damaged cells was first reported by Heinmetz, Taylor, and Lehman (17). They demonstrated that heat-treated organisms produced higher counts on minimal media if they were previously incubated in 0.2% solutions of various metabolites. Garvie (13), on the other hand, refuted these data by demonstrating that it was "virtually impossible" to purify media with respect to a nitrogen source.

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Consequently, she concluded that what Heinmetz et al. observed was the limited proliferation of the surviving cells. The results of Chambers, Tabak, and Kabler (6) and Hurwitz, Rosano, and Blattberg (19) concurred with those of Garvie. They also indicated that the apparent recovery of the injured organisms was consistent with the growth of the surviving organisms.

The controversy concerning recovery has not yet been resolved. Busta and Jezeski (4) reported that incubation of heat-induced salt-sensitive staphylococci in a nutrient medium resulted in the restoration of salt tolerance. Initially, they observed that a greater number of survivors appeared on Plate Count Agar than on *Staphylococcus* Medium No. 110. Subsequent incubation of the heat-shocked culture in skim milk resulted in equal numbers of organisms on both media. Later investigations by Stiles (Ph.D. Thesis, Univ. Illinois, Urbana, 1963) and Depperman (M.S. Thesis, Univ. Illinois, Urbana, 1965) indicated that the recovery of salt tolerance by heat-treated *Staphylococcus aureus* MF 31 oc-

curred under conditions which did not provide for the growth of the organisms.

The data presented in this communication demonstrate thermal injury, the environmental conditions affecting recovery, the minimal nutritional requirements needed for recovery, and information on the synthetic procedures involved in the recovery process.

MATERIALS AND METHODS

Injury procedure. Cultures of *S. aureus* MF 31 (20) were grown in Trypticase Soy Broth (TSB) for 18 to 24 hr on a rotary shaker at 37 C. Approximately 40 ml of the culture was removed and centrifuged at $2,050 \times g$ for 10 min. The spent medium was decanted and the cell pellet was suspended and washed in 100 mM phosphate buffer (pH 7.2). After centrifugation, the supernatant liquid was drawn off, and the cells were suspended in a minimal amount of phosphate buffer (5 ml of buffer per 40 ml of broth culture). The washed preparation was then added to 100 mM phosphate buffer which was equilibrated to 55 C (45 ml of heated buffer was used per 5 ml of buffered cell suspension). The mixture was heated for a minimum of 15 min. To minimize settling, the suspension was agitated for 10 sec at 5-min intervals.

Media. Several recovery media were used to collect the data. TSB was employed as the control recovery medium and as the vehicle for inhibitor and C^{14} studies. The medium proposed by Fildes et al. (10) was also tested, and led to a mixture which consisted of 0.25% glucose; 30 mM phosphate buffer (pH 7.2); 0.4 mM L-alanine, L-valine, L-leucine, L-glycine, L-proline, L-hydroxyproline, L-aspartic acid, and L-glutamic acid; 0.15 mM L-methionine, L-phenylalanine, L-tyrosine, L-arginine hydrochloride, L-histidine hydrochloride, and L-lysine hydrochloride. To test various energy sources in this medium, stock solutions of filter-sterilized mannitol, ribose, sodium citrate, galactose, sodium pyruvate, and glycerol were prepared and used at the same concentration as glucose.

The effect of temperature on the recovery process was determined by transferring the thermally injured cells to TSB and incubating at selected temperatures in the range of 5 to 45 C. Samples were removed for plating at 2-hr intervals during incubation. In the pH studies, TSB was adjusted to the desired pH and recovery was similarly followed. In this case, the inoculated recovery media were incubated at 37 C.

Assay procedure. Samples (1 ml) were removed from the recovery medium at intervals and diluted in sterile distilled water. One set of dilution bottles was prepared for a given treatment at each sampling time, and triplicate pour plates made with Trypticase Soy Agar (TSA) and Trypticase Soy Agar containing 7.5% NaCl (TSAS) were inoculated with these preparations. The plates were incubated at 37 C for 72 hr and then were counted. The total count was given on TSA and the count of recovered cells was made on TSAS. The difference in the initial counts on TSA and TSAS was equal to the number of injured cells in the suspension.

Inhibitors. Inhibitors were added to the TSB re-

covery medium to obtain information on synthetic processes involved in recovery. Chloramphenicol (100 $\mu\text{g/ml}$) was used to inhibit protein synthesis (3, 12). Cycloserine (100 $\mu\text{g/ml}$) and penicillin (100 $\mu\text{g/ml}$) were used to evaluate the role of cell-wall synthesis (18). Actinomycin D (5 $\mu\text{g/ml}$) was used to inhibit ribonucleic acid (RNA) synthesis (23), and 2,4-dinitrophenol (100 $\mu\text{g/ml}$) was used to inhibit adenosine triphosphate (ATP) formation and the normal functioning of the citric acid cycle (11).

Chemical tests. The suspending menstruum, containing the materials leached from the cells during heating, was concentrated to one-tenth of its original volume by lyophilization. Ninhydrin-positive material was analyzed as glutamic acid equivalents (8). Potassium was assayed by flame photometry (8). RNA was measured by the orcinol test and deoxyribonucleic acid (DNA), by the diphenylamine test (1).

The presence of glucosamine and glycerol was determined by paper chromatography on Whatman no. 1 paper. Isopropanol-acetic acid-water (3:1:1) was used as the solvent system (2). For detection, the chromatograms were sprayed either with the aniline-oxalate or the periodate-benzidine sprays (2). Paper chromatographic methods were also used for the detection of nucleic acid derivatives. Whatman no. 1 paper was used with a 5% (w/v) aqueous Na_2HPO_4 solution saturated with isoamyl alcohol as the solvent system (2). The spots were located by scanning the strip with ultraviolet light at 260 μm .

Spectrophotometric measurements were made with a Cary model 11M recording spectrophotometer, with the use of a 1 M phosphate buffer reference solution to compensate for the concentration of the heating menstruum.

Radiotracer procedures. Amounts of 10 μc each of uniformly labeled C^{14} -aspartic acid (39 mc/mole) and uniformly labeled C^{14} -glycine (21 mc/mole) were added to 50 ml of TSB. The final concentration of radioactive material used was 0.4 $\mu\text{c/ml}$ of recovery medium. The dry weights of the cell suspensions to be added were determined from a previously constructed standard curve. After inoculation of the recovery medium, 5-ml samples were removed at intervals and Millipore-filtered. The cells retained on the filter were washed three times with cold distilled water. The washed cells were transferred from the filter membrane to test tubes and were recovered by centrifugation. The amino acid pool and other cold acid-soluble molecules were extracted at 3 C for 30 min with 5 ml of 0.25 N HClO_4 (28). The cells were recovered by centrifugation and the supernatant liquid was decanted and saved for analysis. After two washes in cold distilled water, the nucleic acids were removed by two consecutive extractions with 2.5-ml portions of 0.5 N HClO_4 for 15 min at 100 C (28). The remaining cell residue was washed twice with cold distilled water, harvested, and suspended in 5 ml of distilled water. The acid extracts from both procedures (cold and hot extraction) were neutralized with 3 N KOH, and the resultant KClO_4 was precipitated at 3 C. The mixture was filtered, and the filtrates were saved for analysis.

The scintillator solution consisted of 5 g per liter

FIG. 1. Effect of 7.5% NaCl on the survival of *Staphylococcus aureus* MF 31 heated for varying lengths of time. The cells were heated in 100 mM phosphate buffer at pH 7.2 at 55 C. Trypticase Soy Agar (●) was used as the control and Trypticase Soy Agar containing 7.5% NaCl (○) was used to determine the effect of NaCl.

of 2,5-diphenyloxazole (PPO) and 0.3 g per liter of 1,4-bis-2(4-methyl-5-phenoxazolyl)-benzene (dimethyl POPOP) (16) dissolved in a 50:50 mixture of toluene-absolute ethyl alcohol. Dispersion of the neutralized acid extracts in the scintillator was aided by the addition of 1 M hydroxide of Hyamine 10-X [*p* - (diisobutylcresoxethoxyethyl) - dimethylbenzyl - ammonium chloride] (26). The cell residue was suspended in the scintillator with the addition of Cab-O-Sil (Packard Instrument Co., La Grange, Ill.) thixotropic gel powder. Portions (1 ml) of the various fractions were added to 15 ml of scintillator solution containing Hyamine 10-X or Cab-O-Sil gel. The samples were counted for 10 min in a Packard Tri-Carb liquid scintillation spectrometer. The final data were interpreted on the basis of counts per minute per milligram (dry weight).

RESULTS

Differences in the survival of *S. aureus* MF 31 were found when the heated cells were plated on TSA and TSAS (Fig. 1). The total viable population, indicated by the TSA counts, was essentially unchanged after the first 15 min of heating. Thereafter, additional heating produced a slow rate of decline in the number of organisms detected. When the same suspensions of heat-treated organisms were plated on TSAS (Fig. 1), differing results were obtained. After a short exposure to heat (5 min), 90% of the viable population were salt-sensitive and unable to grow on TSAS. Additional heat treatment increased the

FIG. 2. Growth of *Staphylococcus aureus* MF 31 in Trypticase Soy Broth before and after sublethal heat treatment. The cells were heated in 100 mM phosphate buffer at pH 7.2 for 15 min at 55 C. The unheated control (▲) represents average counts from Trypticase Soy Agar (TSA) and TSA containing 7.5% NaCl (TSAS). The heated cells plated on TSA are represented by the closed circles (●) and the heated cells plated on TSAS are represented by the open circles (○).

number of salt-sensitive cells in the suspension. After 15-min exposure, the TSAS counts decreased to about 0.01% of the total or TSA count. These data are consistent with the observations of Busta and Jezeski (4) that suspensions of *S. aureus* 196-E lost their salt tolerance after heat shock. Further exposure to heat (longer than 15 min) indicated that little additional salt sensitivity was induced.

In addition to salt sensitivity, discussed above, the treated cells (heated for 15 min at 55 C) demonstrated an increased lag phase when inoculated into TSB (Fig. 2). During the extended lag phase, readjustment in the form of repair to the cell occurred. This was confirmed by the gradual return of salt tolerance. A detailed characterization of this recovery process will be presented in subsequent figures. The thermally induced lag substantiates the reports of Kaufman et al. (22) and Jackson and Woodbine (21), who found that

FIG. 3. Recovery of salt tolerance by thermally injured cells of *Staphylococcus aureus* MF 31. The cells were injured by heating in 100 mM phosphate buffer at pH 7.2 for 15 min at 55 C. Symbols: ●, total counts on Trypticase Soy Agar (TSA) of both recovery media employed; ○, counts on TSA containing 7.5% NaCl (TSAS) of injured cells incubated at 37 C in Trypticase Soy Broth; △, counts on TSAS of injured cells incubated at 37 C in the concentrated heating medium of Stiles (Ph.D. Thesis, Univ. Illinois, Urbana, 1963).

increased lag phases were a manifestation of thermal injury to the cell. Further, the recovery of salt tolerance which occurs during the extended lag phase confirms these observations. Upon emergence from the lag phase, the treated cells grew at the normal rate and achieved a total viable population that was comparable to the unheated control. These data demonstrated the complete repair of the heated cells.

The recovery of salt tolerance by sublethally heated cells of *S. aureus* MF 31 is presented in Fig. 3. After a 15-min treatment at 55 C, 99.5% of the total population were initially unable to grow on TSAS, but were still viable, as shown by the count on TSA. Incubation of the suspension in TSB and the concentrated heating menstruum of Stiles (Ph.D. Thesis, Univ. Illinois, Urbana, 1963) allowed the population to regain tolerance to NaCl. In TSB this process was complete in 4 hr. In the medium proposed by Stiles, only 44% regained the ability to grow in the presence of 7.5% NaCl, regardless of the length of time the cells were incubated in the recovery medium. The shape of this curve suggested that essential nutrients were depleted. Similar results were obtained when other limiting media were used. The constancy of the TSA counts indicated that re-

TABLE 1. Effect of incubation temperature and pH on the recovery of thermally injured cells of *Staphylococcus aureus* MF 31 in Trypticase Soy Broth

Temp	Per cent recovery at 2 hr*	pH	Per cent recovery at 2 hr*
C			
3	0	4	0
7	10	5	2
20	34	5.8	25
30	75	6.3	35
37	80	7.2	72
45	9	8	27
		9	9

* Cells were thermally injured by heating for 15 min at 55 C in 100 mM phosphate buffer (pH 7.2). Recovery was followed by incubating the injured cells in Trypticase Soy Broth at the indicated temperature or pH (at 37 C).

covery was not dependent upon multiplication of the organism.

Recovery in TSB was assayed at several incubation temperatures and pH values (Table 1). The minimal recovery temperature appeared to fall between 3 and 7 C. As the incubation temperature of the recovery mixture was increased to 30 C, a rapid rise in the recovery rate was observed. The maximal recovery rate occurred at 37 C, but little difference was actually found for the growth of the test organism (20) and appeared to reflect the functioning of one or more synthetic pathways.

The composition of the heating menstruum was studied to determine whether heating caused the loss of vital cellular components. The 280:260 ratio of this material was 0.4, which implied that the solution was 100% nucleic acid. The diphenylamine test for DNA was negative; however, the orcinol test indicated that 40 μ moles of orcinol-positive compounds were released per 100 mg of cell dry weight. This assay is positive in the presence of ribose, but, since the free sugar could not be detected chromatographically, ribose was assumed to be bound in nucleotides. This was confirmed by the appearance of 260 m μ -absorbing spots on chromatograms of the heating menstruum.

Although the above data indicate that the substances released were primarily nucleic acid-like in nature, assays for ninhydrin-positive material revealed that 23.5 μ moles of glutamic acid equivalents per 100 mg of cell dry weight were also released. Hydrolysis of the concentrated heating menstruum in 6 N HCl and subsequent analysis for amino acids gave similar results, confirming the release of free amino acids.

FIG. 4. Release of intracellular substances from *Staphylococcus aureus* MF 31 after sublethal heating. The cells were heated in 100 mM sodium phosphate buffer at pH 7.2 at 55 C. Symbols: $\circ$, microequivalents of K^+ per 100 mg of cell dry weight heated; $\triangle$, micro-moles of glutamic acid equivalents per 100 mg of cell dry weight heated; $\blacksquare$, appearance of absorbance at 260 mμ.

Chromatographic analyses for glucosamine and glycerol were negative, indicating that the cell wall remained stable during heating.

The loss of RNA-like material and free amino acids from the cell during sublethal heating also suggested that modification of the permeability barrier occurred. To test this hypothesis, the release of intracellular potassium during heating was assayed. This method of assay for membrane integrity was chosen, since Mitchell and Moyle (24) and Christian (7) concluded that potassium was actively concentrated within cells of *S. aureus*. Further, Christian (7) demonstrated that washing of the cells in aqueous solutions did not mediate the release of potassium whereas butanol caused the immediate loss of potassium plus intracellular ultraviolet-absorbing and ninhydrin-positive materials.

The appearance of potassium in the clarified heating menstuum was linear with respect to time (Fig. 4). After 15-min treatment, approximately 20 to 30% (16.4 $\mu\text{eq}/100 \text{ mg}$ cell dry weight) of the total cellular potassium was leached from the cell, and that remaining was released by additional heating. Further evidence of membrane

damage was indicated by considerable loss of the soluble pool components. This was demonstrated by following the appearance of 260 mμ-absorbing and ninhydrin-positive materials in the heating menstuum (Fig. 4). A similar linear release of material was observed. The overall rates were slower than that observed for potassium, although substantial amounts of materials were found. After 15-min heating, 23.5 μmoles of glutamic acid equivalents per 100 mg of cell dry weight (orcinol test) were found in the heating menstuum (14).

A synthetic medium was sought which would support recovery as efficiently as TSB. The medium proposed by Fildes et al. (10) met this requirement, even though our strain was unable to multiply in it. Additional experimentation indicated that the $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, thiamine,

FIG. 5. Effect of defined media on the recovery of salt tolerance by thermally injured cells of *Staphylococcus aureus* MF 31. The cells were injured by heating in 100 mM phosphate buffer at pH 7.2 for 15 min at 55 C. Symbols: $\bullet$, average counts from Trypticase Soy Agar (TSA) of all recovery media; $\circ$, count on TSA containing 7.5% NaCl (TSAS) of injured cells incubated in 0.25% glucose, 30 mM (pH 7.2) phosphate-buffered amino acids (see Materials and Methods); $\bullet$, TSAS counts in 30 mM phosphate buffer at pH 7.2; $\blacktriangle$, TSAS counts in 30 mM phosphate-buffered amino acids (pH 7.2); $\triangle$, TSAS counts in 30 mM phosphate buffer (pH 7.2) and 0.25% glucose. Recovery was followed at 37 C.

TABLE 2. Effect of constituent changes in the recovery medium on the recovery of salt tolerance by thermally injured *Staphylococcus aureus* MF 31

Medium*	Recovery after 4 hr
	%
Control†	100
30 mM Tris-succinate buffer, 0.25% glucose, amino acids†	None
30 mM Tris-maleate buffer, 0.25% glucose, amino acids†	None
30 mM Veronal buffer, 0.25% glucose, amino acids†	None
30 mM phosphate buffer, 0.25% glucose, 50 mM NH ₄ Cl	None
30 mM Veronal buffer, 0.25% glucose, amino acids, 30 mM KH ₂ PO ₄	100
30 mM phosphate buffer, 0.25% glucose, 1.5 mM aspartic acid, 48.5 mM NH ₄ Cl	10
30 mM phosphate buffer, 0.25% glucose, 1.5 mM glutamic acid, alanine, glycine, lysine, and aspartic acid, 42.5 mM NH ₄ Cl	60
30 mM phosphate buffer, 0.25% glucose, 1.5 mM leucine, valine, proline, glycine, aspartic acid, phenylalanine, arginine, and cysteine, 38 mM NH ₄ Cl	63

* The pH of all media was 7.2.

† The control medium consisted of 0.25% glucose, 30 mM phosphate buffer (pH 7.2), and the amino acids listed in Materials and Methods.

nicotinamide, and dithiodiglycolic acid of the above medium were not needed for the cells to recover salt tolerance. The necessary constituents were a phosphate-buffered amino acid mixture (pH 7.2) and 0.25% glucose. When a medium containing these components was used for recovery (Fig. 5), the results were comparable to those observed in TSB. Recovery was complete in 4 hr, and, although not presented as such, assumed linear proportions as a function of time. When the injured cells were incubated in the buffered amino acids alone, no recovery could be detected. Likewise, no recovery was observed in phosphate buffer alone. In the presence of 0.25% glucose and phosphate buffer, a substantial decrease in the salt-insensitive population was observed. This effect, although difficult to explain adequately, may be due to degradative metabolism in the absence of a compatible nitrogen source.

To determine whether phosphate buffer satisfied a substrate requirement in addition to controlling pH, tris(hydroxymethyl)aminomethane (Tris)-succinate, Tris-maleate, and Veronal

buffer at the same concentration and pH as the phosphate buffer were substituted for phosphate in the amino acid and glucose recovery medium (Table 2). With these buffer systems, no recovery was observed even on prolonged incubation. However, when 30 mM KH₂PO₄ was added to the Veronal-buffered medium, 100% recovery was observed in 4 hr. This demonstrated that phosphate was a necessary component of the recovery medium, and indicated that recovery of salt tolerance required a medium consisting of a relatively complex nitrogen source, an energy source, and phosphate.

Further attempts to simplify the recovery medium involved replacement of the amino acid mixture with NH₄Cl (Table 2). With NH₄Cl as the sole nitrogen source, no recovery was observed, but as increasing numbers of amino acids were added to the medium containing NH₄Cl recovery was substantially increased.

When the energy source was replaced with other carbohydrates and metabolites, varying results were obtained. Mannitol, ribose, tri-

FIG. 6. Effect of antibiotics on the recovery of salt tolerance by thermally injured cells of *Staphylococcus aureus* incubated at 37 C in Trypticase Soy Broth (TSB). The cells were injured by heating in 100 mM phosphate buffer at pH 7.2 for 15 min at 55 C. Symbols: ●, average count on Trypticase Soy Agar (TSA) of all recovery conditions; ○, TSA containing 7.5% NaCl (TSAS) count of TSB with 100 µg/ml of chloramphenicol; □, TSAS count of TSB with 100 µg/ml of penicillin; ▲, TSAS count of TSB with 100 µg/ml of cycloserine; △, TSAS count of TSB with 5 µg/ml of actinomycin D.

FIG. 7. Incorporation of uniformly labeled C^{14} -glycine and uniformly labeled C^{14} aspartic acid into the soluble pools and nucleic acid of recovering cells of *Staphylococcus aureus* MF 31 incubated at 37 C. The cells were injured by heating in 100 mM phosphate buffer at pH 7.2 for 15 min at 55 C. The recovery medium, Trypticase Soy Broth, contained 100 μ g/ml of chloramphenicol, 100 μ g/ml of penicillin, and 0.2 μ g/ml each of uniformly labeled C^{14} glycine and aspartic acid. Symbols: O, label uptake into the soluble pool; ●, label uptake into the nucleic acid in the absence of actinomycin D; Δ, label uptake into the soluble pool; ▲, label uptake into the nucleic acid in the presence of 5 μ g/ml of actinomycin D.

sodium citrate, and galactose were unable to support recovery. Strasters and Winkler (29) pointed out that glucose-grown staphylococci were unable to utilize ribose as an energy source, and the same may be true for mannitol, tri-sodium citrate, and galactose. However, this was not evaluated. When injured cells were incubated in sodium pyruvate, glycerol, and glucose, the amount of recovery observed after 2 hr of incubation was 10, 45, and 75%, respectively. This suggested that glycolysis intermediates may be able to serve as energy sources, but the rate and degree of recovery attained appeared to be a function of the energy available to the cell from these compounds. This contention was supported by the fact that the uncoupling agent, 1,4-dinitrophenol (100 μ g/ml), did not inhibit recovery.

Antibiotics, to which this organism was sensitive at concentrations below those used in the recovery experiments, were used to characterize further the synthetic mechanisms involved (Fig.

6). Chloramphenicol (3), penicillin, and cycloserine, at a concentration of 100 μ g/ml (18), did not affect normal recovery, but actinomycin D at 5 μ g/ml (23) brought about inhibition. These results suggest that neither protein synthesis nor cell-wall synthesis was intimately involved, but rather that RNA synthesis was necessary for the injured cells to recover salt tolerance. As further evidence of this, the incorporation of uniformly labeled C^{14} -aspartic acid and glycine was followed into the nucleic acid and soluble pools of recovering cells (Fig. 7). In the absence of actinomycin, the soluble pool material was concentrated before nucleic acid synthesis began. After this event, RNA was labeled at a rapid rate. Plate counts of the suspension indicated that the return of salt tolerance paralleled the synthesis of RNA. When actinomycin D was added to the recovery medium, the concentration of the soluble pool material was delayed, and the rate of synthesis and the total amount of RNA found were substantially decreased. As expected, assay of the recovery by plate count demonstrated that the recovery of salt tolerance was inhibited regardless of the length of time the cells were incubated in the recovery medium. These data demonstrated that RNA synthesis controlled the recovery of salt tolerance in thermally injured cells of *S. aureus* MF 31.

DISCUSSION

The data presented demonstrated that a repairable cellular injury was induced in *S. aureus* MF 31 by sublethal heating. The temporary damage to findings originally reported by Busta and Jezeski (4). In the absence of inhibitory concentrations of NaCl, an increase in the lag phase of growth consistent with the data of Kaufman et al. (22) and Jackson and Woodbine (21) was observed. During this time, salt tolerance returned, and appeared to be coincident with the end of the extended lag. Upon emergence from the lag phase, the cells showed complete repair of the injury. This was indicated by the similarity of the generation time and total population of injured and uninjured cells.

It is significant to note that recovery occurred in the absence of growth. The constant TSA count, which was more rapid than the normal growth rate (20), tended to refute the contention of Garvie (13), Chambers et al. (6), and Hurwitz et al. (19) that recovery is a result of multiplication. Rather, it suggested that recovery involved enzymatic activity directed towards repair. The responses encountered at the incubation temperatures and pH values tested also reinforced this contention.

The data obtained concerning the minimal

requirements of recovery are more appropriately interpreted in light of the specific inhibitions induced. As mentioned above, cell-wall synthesis and protein synthesis were not required for the recovery of salt tolerance, but the inhibition of RNA synthesis halted the process. Therefore, synthesis of this compound appeared to control the system.

Gale and Folkes (12) showed that normal RNA synthesis in *S. aureus* requires a complex mixture of amino acids composed mainly of those used in this study. They also indicated that simple mixtures of four or five amino acids stimulated the synthesis of some RNA but when they were added alone only a small effect was observed. This is substantiated by the data in Table 2. In the absence of amino acids no recovery was found, but as increasing numbers were added the degree of recovery increased. The formation of RNA was dependent upon the organic nitrogen and carbon skeletons supplied by the amino acids. This was also demonstrated by the tracer experiments. Once the amino acid pool reached maximal concentration, the amino acids were used to synthesize RNA. It should be noted that the control recovery medium used in the C^{14} studies contained 100 $\mu\text{g}/\text{ml}$ of penicillin and chloramphenicol to inhibit protein and cell-wall synthesis, whereas the other contained these antibiotics and actinomycin D.

Reconcentration of the soluble pools was mediated by energy supplied through glucose oxidation. The use of glucose alone or amino acids alone resulted in no recovery of the injured population. Stiles and Witter (27), on the other hand, reported that the limited recovery of salt tolerance by *S. aureus* MF 31 occurred in the presence of 5% glucose alone. However, glucose was added to the heating menstruum which contained considerable amounts of the amino acid pool and other soluble molecules of the cell. It would, therefore, appear that recovery in glucose alone, as reported by these investigators, was related to the presence of cellular components extracted by heat. The data presented substantiate this contention and, in fact, demonstrate that, in addition to glucose, organic nitrogen and phosphate are required.

Although the involvement of RNA in the recovery process appears to be primary, we also observed a transient loss of the concentrating mechanisms of the cell. Intracellular compounds not normally found in the suspending menstruum could be demonstrated after heating. Potassium, the free amino acid pool, and RNA derivatives were able to leak out. Damage to the membrane may be slight since reformation of the pools is

rapid and occurs before salt sensitivity returns. This also occurred in the cultures inhibited by actinomycin D, and suggests that, in addition to the loss of the nucleotide pool, heat may also mediate the degradation of RNA, as Califano (5), Eigner, Boedtker, and Michaels (9), Moats (25), and Strange and Shon (28) indicated.

The results discussed are indicative of a series of events attributable to heat. The first seems to be a weakening of the cell membrane with a coincident loss of the soluble pools. Simultaneously, RNA which was degraded by the heat treatment is also able to escape through the weakened membrane. For recovery to take place, these materials must be resynthesized and re-concentrated, and the membrane must be repaired. Concentration of amino acids is necessary, since in their absence RNA cannot be synthesized. Once this is accomplished, the return of salt tolerance parallels the synthesis of RNA.

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