JOURNAL OF BACTERIOLOGY, Oct. 1967, p. 1082-1087

Vol. 94, No. 4
Printed in U.S.A.

# Regeneration of Ribosomes and Ribosomal Ribonucleic Acid During Repair of Thermal Injury to *Staphylococcus aureus*

STEPHEN J. SOGIN AND Z. JOHN ORDAL
*Department of Food Science, University of Illinois, Urbana, Illinois 61801*

Received for publication 19 July 1967

Heating *Staphylococcus aureus* MF31 at 55 C for 15 min renders the organisms unable to reproduce on agar containing 7.5% NaCl (1). The heated organisms exhibited an extended lag period during which the organisms regained their ability to grow on the 7.5% NaCl-agar. Inhibitor and antibiotic data indicated that protein synthesis is not involved in this recovery process but nucleic acid synthesis is suggested (3). The data presented here further substantiate the noninvolvement of protein synthesis during recovery and further demonstrate the site of the thermally induced nucleic acid lesion. Methylated albumin kieselguhr column analysis showed the lesion site to be the ribosomal ribonucleic acid (rRNA). The rRNA is resynthesized during the extended lag period. Sucrose gradient analysis demonstrated that a ribosomal peak was undetectable subsequent to the thermal treatment, but this peak was regenerated during the recovery period.

An extended lag phase is a culture response to thermal injury (4, 5). This extended lag phase can perhaps be more accurately described as a recovery period. Harris (2) explains this as "the period of 'getting back' organisms from their sublethal environment." This is commonly accomplished by incubating the organisms in a nutrient broth. Busta and Jezeski (1) reported that, when staphylococci were subjected to a sublethal heat treatment, the survivor count on plate-count agar was greater than in Staphylococcus Medium No. 110. They demonstrated that the heat treatment rendered the cells salt sensitive, but that their salt tolerance returned if the treated cells were incubated in skim milk. This observation was confirmed by Iandolo and Ordal (3), who further demonstrated that salt tolerance could be regained in a medium which would not support growth and multiplication of the organism. They also provided data that indicated that nucleic acid resynthesis was an important aspect during recovery.

The data presented in this communication characterize in greater detail the nucleic acid changes during thermal injury and recovery from such injury. The data show that during thermal injury there is a marked degradation of the ribosomal ribonucleic acid (rRNA) and that resynthesis occurs during the recovery period.

## MATERIALS AND METHODS

*Injury procedures.* Cultures of *Staphylococcus aureus* MF31 were grown in Trypticase Soy Broth for 16 to 18 hr on a rotary shaker at 37 C. The cultures were harvested by centrifugation at 2,050 × *g* for 10 min. The spent medium was decanted and the cell pellet was suspended and washed in phosphate buffer (0.1 M, *p*H 7.2). After centrifugation, the cell pellet was suspended in a minimal amount of phosphate buffer and added to a pretempered phosphate buffer (55 C) such that the final volume of the heating menstruum was 1.2 times the culture volume. The mixture was then heated for a minimum of 15 min.

*Media.* Two media were employed to follow the recovery of injured cells; Trypticase Soy Broth was used as the recovery medium except in the experiment in which 5-methyltryptophan (5MT) was employed as an inhibitor of protein synthesis. In this case, the recovery medium consisted of (per liter): Casamino Acids, 17 g; glucose, 2.5 g; $K_2HPO_4$, 2.5 g; NaCl, 5 g; sucrose, 0.7 g; and raffinose, 0.3 g; with 1.0 mg of either tryptophan or 5MT per ml added. Incubation was at 37 C.

*Assay procedures.* Assay of injury and recovery was performed by using the plate-count technique. Samples (1 ml) were removed from the recovery medium at intervals and diluted in sterile water. Trypticase Soy

Agar (TSA) and Trypticase Soy Agar containing 7.5% NaCl (TSAS) were employed to determine the total cell count (TSA counts) and the number of injured or recovered cells (TSAS counts) (1). The difference in the initial counts on TSA and TSAS was equal to the number of injured cells in the suspension.

*Biochemical techniques.* Bacterial extracts were prepared by adding an equal volume of 0.11- to 0.12-mm glass beads to a 10 to 20% cell suspension in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.4, containing 10 $\mu$g of deoxyribonuclease per ml. The cell-bead suspension was agitated in a Braun cell homogenizer for 6 min at 2,000 cycles/min. This process was carried out in the cold, with cooling provided by the adiabatic expansion of $CO_2$ gas through a capillary leading to the homogenizing chamber. The cell-bead suspension was then centrifuged at 10,000 $\times$ *g* for 10 min to remove cell debris and beads. RNA was extracted by the phenol-Duponol method of Kirby (6). The purity was assayed by determining the ratios of absorbance at 260 and 280 m$\mu$. All optical densities were read on a Beckman DU spectrophotometer. The RNA was chromatographed on a methylated albumin kieselguhr column by using 500 ml of a 0.1 to 1.2 M NaCl gradient in 50 mM phosphate buffer (*p*H 6.8) for elution, and by collecting 4-ml fractions (7). RNA (2 mg) was charged by using 100 ml of 50 mM phosphate buffer containing 0.1 M NaCl. The charging buffer was also collected in 4-ml fractions.

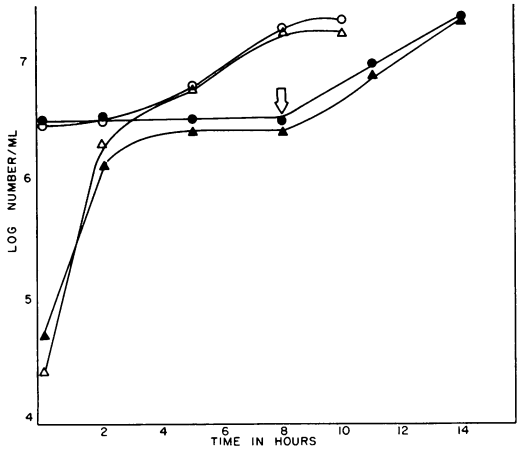


FIG. 1. *Effect of the competitive inhibitor 5 methyl tryptophan (5MT) on the recovery of salt tolerance of heat-injured cells of Staphylococcus aureus MF41. The cells were thermally injured by heating at 55 C in 100 mM phosphate buffer, pH 7.2. The cells were then recovered in media (see Materials and Methods) with and without the presence of 1 mg/ml of 5MT. Symbols: (○) counts from medium without 5MT on Trypticase Soy Agar (TSA); (△) counts from medium without 5MT on Trypticase Soy Agar containing 7.5% NaCl (TSAS); (●) counts from media with 5MT on TSA; (▲) counts from media with 5MT on TSAS; (↓) addition of a fourfold excess of tryptophan to relieve the inhibitory effects of 5MT.*

Crude extracts were prepared for zone centrifugation by using the supernatant fluid from the procedure for the preparation of bacterial extracts. Zone centrifugation of crude extracts was performed in 20 to 5% linear gradients of sucrose in 0.01 M Tris buffer (*p*H 7.2) containing $10^{-2}$ M magnesium acetate. The gradients were centrifuged in the 25 SW rotor in a Spinco model L-2-50 at 25,000 rev/min for 6 hr at 5 C.

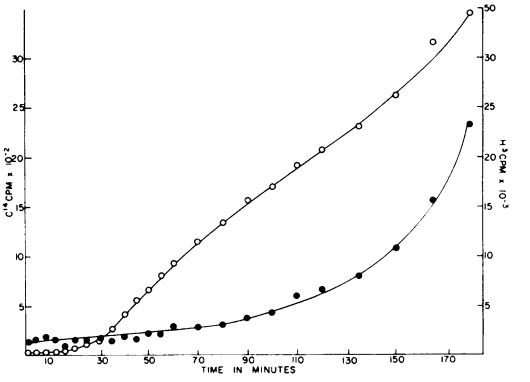


FIG. 2. *Incorporation of uniformly labeled $^{14}C$-glutamic acid and uracil-6-$^{3}H$ into trichloroacetic acid-precipitable fraction of recovering heat-injured cells of Staphylococcus aureus MF31 in Trypticase Soy Broth (TSB). Cells were heated for 15 min at 55 C in 100 mM phosphate buffer, pH 7.2. The injured cells were then transferred to the recovery medium (TSB). One-ml samples were removed, made up to a final concentration of 5% trichloroacetic acid, and were chilled. The precipitate was collected on filters (0.22 $\mu$; Millipore Corp., Bedford, Mass.), see Materials and Methods. Symbols (○) uracil-6-$H^3$ counts; (●) uniformly labeled $^{14}C$-glutamic acid counts.*

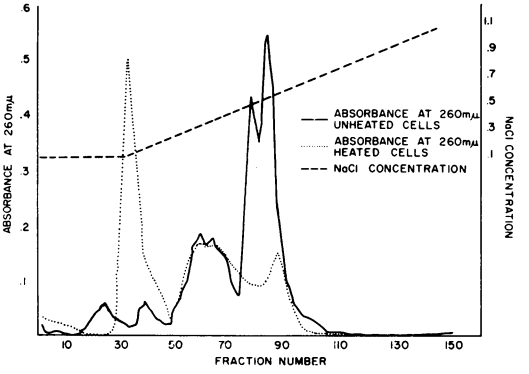


FIG. 3. *Comparison of elution patterns from methylated albumin kieselguhr (MAK) columns of RNA from heated and unheated cells of Staphylococcus aureus MF31. The treated cells were heated for 15 min at 55 C in 100 mM phosphate buffer, pH 7.2. Solid line: absorbance at 260 m$\mu$ of RNA from unheated cells of S. aureus MF 31; Dotted line: absorbance at 260 m$\mu$ of RNA from heated cells of S. aureus MF31. dashed line: NaCl concentration.*

After centrifugation, 0.8-ml fractions were read at 260 mμ. Zone centrifugation of extracted RNA was performed in 20 to 5% linear gradients of sucrose in acetate buffer 0.01 M (*p*H 5.0) with 0.1 M NaCl. The gradients were centrifuged as above for 16 hr. Fractions were collected and read at 260 mμ.

*Radiotracer procedures.* Uracil-6-$^{3}H$ (1 μc/ml) and uracil-2-$^{14}C$ (0.1 μc/ml) were used to label the steady-state RNA fractions during growth or recovery. To test for protein synthesis, $^{14}$C-glutamic acid (0.1 μc/ml) was added to the recovery medium, and the uptake was followed. All samples were precipitated with

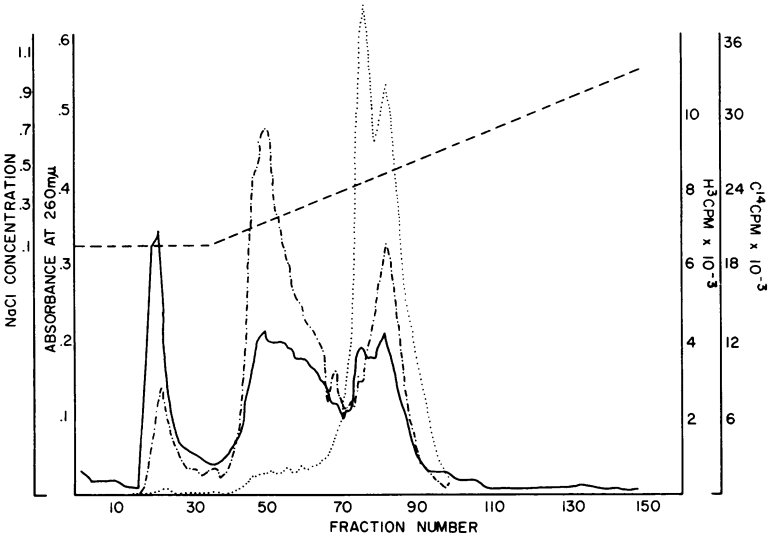


FIG. 4. *Elution pattern from a methylated albumin kieselguhr column of RNA extracted from heated cells of Staphylococcus aureus MF31 and allowed to recover for 90 min in Trypticase Soy Broth (TSB) containing 0.1 μc/ml of uracil-2-$^{14}C$. The cells were grown in TSB containing 1 μc/ml of uracil-6-$^{3}H$ previous to heat treatment and were heated for 15 min in 100 mM phosphate buffer, pH 7.2. Solid line: absorbance at 260 mμ; dotted line: $^{14}C$ counts; dot-dash line: $^{3}H$ counts; dash line: NaCl concentration.*

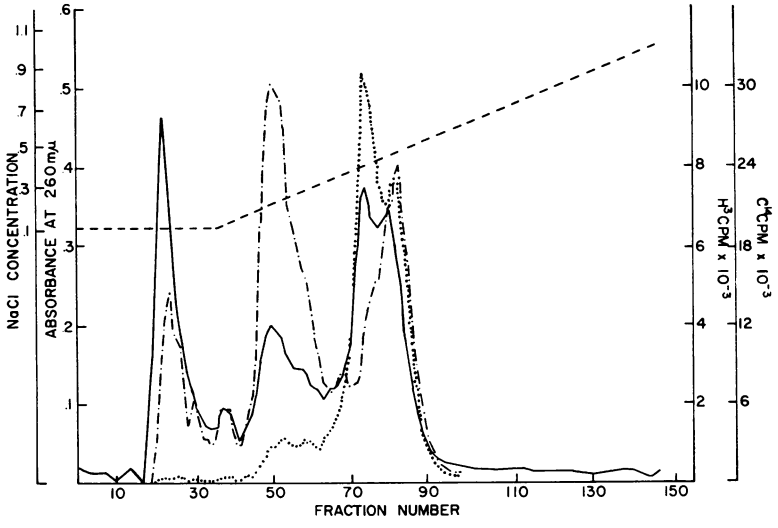


FIG. 5. *Elution pattern from a methylated albumin kieselguhr column of RNA extracted from Staphylococcus aureus MF31 and allowed to recover for 150 min in Trypticase Soy Broth (TSB) containing 0.1 μc/ml of uracil-2-$^{14}C$. The cells were grown in TSB containing 1 μc/ml of uracil-6-$^{3}H$ before heat treatment, and were heated for 15 min at 55 C in 100 mM phosphate buffer, pH 7.2. Solid line: absorbance at 260 mμ; dotted line: $^{14}C$ counts; dash-dot line: $^{3}H$ counts; dash line: NaCl concentration.*

cold (5 C) trichloroacetic acid and were collected on membrane filters (22-$\mu$ pore size; Millipore Corp., Bedford, Mass.). The precipitates were washed with 2 volumes of cold (5C) trichloroacetic acid, dried, and placed in scintillation-counting vials with toluene containing 5 g/liter of 2,5-diphenyloxazole (PPO) and 3 g/liter of 1,4-bis-2-(4-methyl-5-phenoxazoyl)-benzene (dimethyl POPOP) as scintillation fluid.

The RNA samples that had been subjected to zone centrifugation were increased to 2.8 ml with distilled water, and were added directly to 15 ml of dioxane scintillation fluid containing 7 g of PPO, 0.3 g of POPOP, and 100 g of naphthalene per liter.

Samples were counted in a Packard model 314 E liquid scintillation counter. The isotopes were counted with an efficiency of 18 and 65% for the $^{3}H$ and $^{14}C$ samples, respectively.

## Results and Discussion

By using the plate-count procedure (TSA counts versus TSAS counts) to assay for thermal injury and recovery from injury, Iandolo and Ordal (3) demonstrated that recovery was not affected by the addition of 100 $\mu$g of chloramphenicol per ml to the recovery medium. This suggested that protein synthesis per se was not involved in recovery (the regaining of salt tolerance). We have confirmed this, and have, in addition (Fig. 1), employed the competitive inhibitor of protein synthesis, 5MT. When 5MT was added to the recovery medium, the return to salt tolerance by the thermally injured cells was normal, but the cells were unable to undergo cell division (multiplication). When the competitive effect of 5MT was relieved by the addition of a fourfold excess of tryptophan, cell division followed.

To further characterize the relationship between protein synthesi., if any, and RNA synthesis during recovery and cell multiplication, the uptake of uniformly labeled $^{14}C$-glutamic acid and uracil-6-$^{3}H$ was followed during recovery and subsequent multiplication of thermally injured cells (Fig. 2). As expected, the uracil isotope uptake began after a short lag,

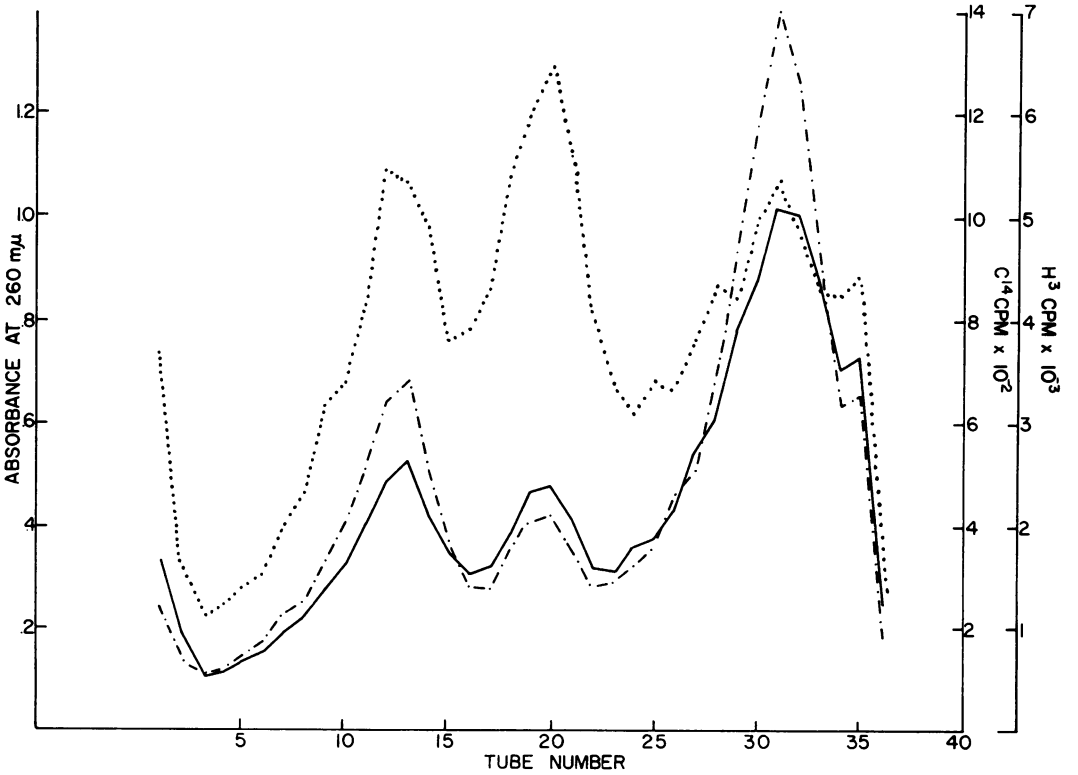


FIG. 6. *Zone centrifugation pattern of RNA extracted from cells of Staphylococcus aureus MF31 grown in Trypticase Soy Broth (TSB) containing 1 $\mu$c/ml of uracil-6-$^{3}H$, sublethally heated, and allowed to recover for 150 min in TSB containing 0.1 $\mu$c/ml of uracil-2-$^{14}C$. The cells were heated for 15 min at 55 C in 100 mM phosphate buffer, pH 7.2. RNA preparations were spun for 16 hr in a Spinco model L2-50 ultracentrifuge at 25,000 rev/min using a SW-25 rotor, through a 5 to 20% linear gradient of sucrose on 0.01 M acetate buffer with 0.1 M NaCl, pH 5. Solid line: absorbance at 260 m$\mu$; dotted line: $^{14}C$ counts; dash-dot line: $^{3}H$ counts.*

but there was a marked delay in the uptake of the labeled glutamic acid. This delay for the uptake of the amino acid was approximately equal to the recovery time (Fig. 1) and further substantiated the finding that recovery preceded protein synthesis.

The inhibition of recovery by actinomycin D (3) and the results of the preceding experiments pointed to the desirability of characterizing the RNA changes in injured and recovering cells. This was first achieved by the use of MAK profiles. The profile of RNA extracted from normal cells was compared with that extracted from heat-injured cells (Fig. 3). The ribosomal RNA of *S. aureus* MF31 is eluted from the column by 0.5 to 0.6 M NaCl, whereas, with *Escherichia coli* (7) the rRNA elutes with the 0.7 to 0.8 M NaCl portion of the gradient. When the profile of RNA material from the heat-injured cells is compared to that of the unheated cells, it is apparent that a striking change has taken place. The ribosomal peak of the heated cells is markedly reduced in size and elutes where one would normally expect to find the 23*S* material. The peak for the soluble RNA is enlarged and a considerable amount of material appears in the void region, material that does not bind in the column. This material consisted of degradation products of RNA, as will be substantiated later in this paper.

To follow the RNA changes during recovery, the culture was grown in TSB containing 1.0 μc/ml of uracil-6-$^3H$. The cells were harvested and subjected to the normal heat treatment. The treated cells were transferred to Trypticase Soy Broth containing 0.1 μc/ml of uracil-2-$^{14}C$ and incubated for 90 min. The RNA was extracted from these recovering cells and was chromatographed on a methylated albumin kieselguhr column (Fig. 4). The RNA synthesized prior to

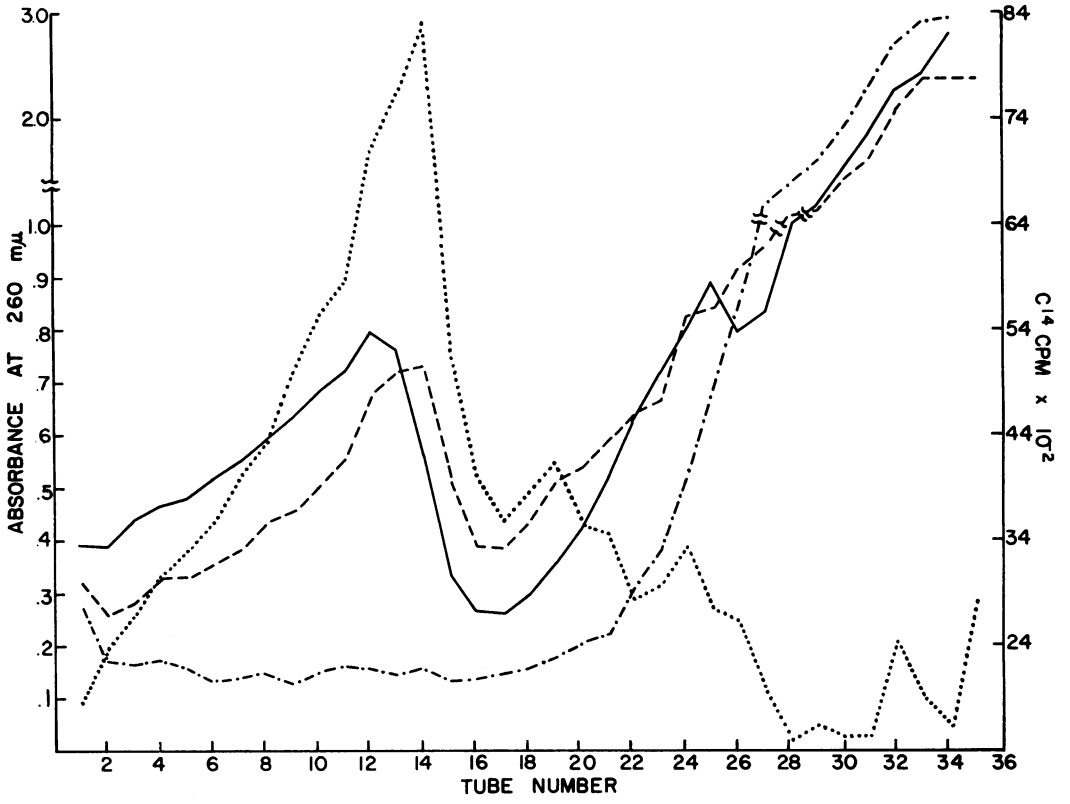


FIG. 7. *Zone centrifugation analysis of crude extracts of heated, unheated, and 120-min recovered cells of Staphylococcus aureus MF31. Cells were heated at 55 C for 15 min. The recovered cells were incubated in Trypticase Soy Broth containing 0.1 μc/ml of uracil-2-$^{14}C$. Preparations (see Materials and Methods) were spun in a Spinco L2-50 ultracentrifuge at 25,000 rev/min for 6 hr, through a linear gradient of 5 to 20% sucrose in tris-(hydroxymethyl) aminomethane buffer containing $10^{-2}$ M magnesium acetate, pH 7.2. Solid line: absorbance 250 mμ unheated cells; dash-dot line: absorbance 260 mμ heated cells; dash line: absorbance 260 mμ 120 min recovered cells; dotted line: $^{14}C$ counts.*

the heat treatment ($^3H$ count) shows a profile similar to that of the heated-cell RNA (Fig. 3). The newly synthesized RNA is incorporated into the 16*S* region. Also to be noted is the increase in the total ribosomal RNA as evidenced by the increase in the material absorbing at 260 m$\mu$ in the ribosomal region (compare with Fig. 3, profile for RNA from heated cells).

The elution pattern of RNA prepared from cells treated in an identical manner, except that they were allowed to recover for 150 min in Trypticase Soy Broth, shows a similar result to that of cells allowed to recover for 90 min (Fig. 5). A further increase in material absorbing at 260 m$\mu$ in the ribosomal region was noted. The observation that an increased amount of label incorporated into the 16*S* ribosomal material correlated with the heat-treated RNA eluting from the column with the 23*S* material, implied that 16*S* ribosomal material is more heat labile than is 23*S* RNA. Sucrose gradient analysis of the RNA isolated from the 150-min-recovered cells failed to confirm this result (Fig. 6). The RNA synthesized before heat treatment was separable into 16*S* and 23*S* components, and was detected in an amount approximately proportional to that of the RNA unheated cells ($^3H$ counts, Fig. 6). The previous determination that a greater amount of label was incorporated into the 16*S* material was confirmed ($^{14}C$ counts, Fig. 6).

To determine the effect of heat on ribosomes, the ribosomes of the heat-treated, nontreated, and 120-min recovered cells were subjected to analysis on sucrose gradients. The ribosomes of unheated *S. aureus* showed a peak in the portion of the gradient which corresponds approximately to the area of *E. coli* ribosomes having a 70*S* sedimentation coefficient (solid line, Fig. 7). After heat treatment, this peak could not be detected by using this technique (dash-dot line, Fig. 7). The result indicated that the ribosomes were broken down as a result of the heat treatment. The RNA detected by methylated albumin kieselguhr column analysis subsequent to heat treatment may thus be detached from the protein moiety of the ribosome, and therefore inactive.

When the cells were allowed to recover for 120 min in Trypticase Soy Broth containing 0.1 $\mu$c of uracil-2-$^{14}C$, the 70*S* peak regenerated and sedimented at approximately the same portion of the gradient as the unheated 70*S* ribosomal material. The majority of the radioactive material was incorporated into this peak, with the two subsidiary peaks corresponding to the 50*S* and 30*S* peaks as found in *E. coli* (dotted line, Fig. 7).

The extended lag period in heat-injured *S. aureus* is in part due to the breakdown of the ribosomes as a result of the heat treatment. The cells are unable to reproduce until the ribosomes are regenerated. The effect of the high-salt agar may restrict the cell from accumulating the precursor material for ribosome synthesis, and the cell eventually becomes senescent and subsequently expires. We feel that the results of this work in part clarify one of the phenomena responsible for an extended lag period as a result of thermal injury to *S. aureus* MF31.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Research Grants EF-00126 and EF-00840 of the Division of Environmental Engineering and Food Protection.

The technical assistance of Carol W. Clark is appreciated.

LITERATURE CITED

1. BUSTA, F. F., AND J. J. JEZESKI. 1963. Effect of sodium chloride concentration in an agar medium on growth of heat-shocked *Staphylococcus aureus*. Appl. Microbiol. **11**:404–407.
2. HARRIS, N. D. 1963. The influence of the recovery medium and the incubation temperature on the survival of damaged bacteria. J. Appl. Bacteriol. **26**:387–397.
3. IANDOLO, J. J., AND Z. J. ORDAL. 1966. Repair of thermal injury of *Staphylococcus aureus*. J. Bacteriol. **91**:134–142.
4. JACKSON, H., AND M. WOODBINE. 1963. The effect of sublethal heat treatment on the growth of *Staphylococcus aureus*. J. Appl. Bacteriol. **26**:152–158.
5. KAUFMANN, O. W., L. G. HARMON, O. C. PAILTHORP, AND I. J. PFLUG. 1959. Effect of heat treatment on the growth of surviving cells. J. Bacteriol. **78**:834–838.
6. KIRBY, K. S. 1956. A new method for the isolation of ribonucleic acids from mammalian tissue. Biochem. J. **64**:405–408.
7. MANDELL, J. D., AND A. D. HERSHEY. 1960. A fractionation column for analysis of nucleic acids. Anal. Biochem. **1**:66–67.