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Precursor Ribosomal Ribonucleic Acid and Ribosome Accumulation In Vivo During the Recovery of *Salmonella typhimurium* from Thermal Injury[1]

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When cells of *S. typhimurium* were heated at 48 C for 30 min in phosphate buffer (*p*H 6.0), they became sensitive to Levine Eosin Methylene Blue Agar containing 2% NaCl (EMB-NaCl). The inoculation of injured cells into fresh growth medium supported the return of their normal tolerance to EMB-NaCl within 6 hr. The fractionation of ribosomal ribonucleic acid (rRNA) from unheated and heat-injured cells by polyacrylamide gel electrophoresis demonstrated that after injury the 16*S* RNA species was totally degraded and the 23*S* RNA was partially degraded. Sucrose gradient analysis demonstrated that after injury the 30*S* ribosomal subunit was totally destroyed and the sedimentation coefficient of the 50*S* particle was decreased to 47*S*. During the recovery of cells from thermal injury, four species of rRNA accumulated which were demonstrated to have the following sedimentation coefficients: 16, 17, 23, and 24*S*. Under identical recovery conditions, 22, 26, and 28*S* precursors of the 30*S* ribosomal subunit and 31 and 48*S* precursors of the 50*S* ribosomal subunit accumulated along with both the 30 and 50*S* mature particles. The addition of chloramphenicol to the recovery medium inhibited both the maturation of 17*S* RNA and the production of mature 30*S* ribosomal subunits, but permitted the accumulation of a single 22*S* precursor particle. Chloramphenicol did not affect either the maturation of 24*S* RNA or the mechanism of formation of 50*S* ribosomal subunits during recovery. Very little old ribosomal protein was associated with the new rRNA synthesized during recovery. New ribosomal proteins were synthesized during recovery and they were found associated with the new rRNA in ribosomal particles. The rate-limiting step in the recovery of *S. typhimurium* from thermal injury was in the maturation of the newly synthesized rRNA.

When nonsporulating bacteria are subjected to a sublethal heat stress, the damaged cells lose many normal metabolic and biosynthetic characteristics (2, 4, 10, 24, 28). The inoculation of injured cells into a suitable medium directs the return of these characteristics during an extended lag time or recovery period, after which time the cells commence to grow at a rate equal to that of unheated cells (6, 10). The degradation of ribosomal ribonucleic acid of (rRNA) in vivo when cells of *Staphylococcus aureus* and *Escherichia coli* were exposed to elevated temperatures has been reported (2, 21, 24, 26), and a possible mechanism for rRNA degradation in *E. coli* under these conditions has been proposed (21).

Recently, we reported that the recovery of *Salmonella typhimurium* from thermal injury was dependent on RNA and protein synthesis (28). It was the purpose of this investigation to observe the stability of ribosomes and rRNA in *S. typhimurium* when cells were sublethally heat injured, and to determine the nature of the rRNA and ribosomal particles that were synthesized during the recovery process.

MATERIALS AND METHODS

The organism used in this study, *S. typhimurium* 7136, the citrate minimal (CM) growth medium, and the growth conditions have been previously reported (28).

[1] This report is from a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree in Food Science.

Injury procedure. Cells were harvested by centrifugation for 10 min at 8,000 × *g* at 0 to 2 C. The supernatent fluid was decanted, and the cells were washed once in 100 mM potassium phosphate buffer (*p*H 6.0). These cells were thermally injured at 48 C for 30 min by adding 10 ml of a cell suspension to 190 ml of 100 mM potassium phosphate buffer (*p*H 6.0), pretempered under constant agitation. The final cell concentration during injury was approximately 2×10^9 cells/ml.

Recovery medium. A modification of the CM growth medium was used for recovery. The citrate minimal phosphate (CMP) recovery medium had the following composition (g/liter): sodium citrate, 8.0; tris(hydroxymethyl) aminomethane (Tris), 12.0; hydrochloric acid (concentrated), 6.9 ml; ammonium chloride, 2.0; sodium phosphate, 0.2; sodium sulfate, 0.02; potassium chloride, 2.0, and trace metals: 0.5 mM Mg^{2+}, 0.3 mM Mn^{2+}, and 2×10^{-3} mM Fe^{2+}. The citrate plus salts were heat sterilized separately from the trace metal solutions which were filter sterilized. The desired concentrations of trace metals were added just before inoculation. Protein synthesis during recovery was inhibited by the addition of 100 μg of chloramphenicol per ml to the CMP medium.

Preparation of radioactive RNA and ribosomal particles. The preparation of standard ^{3}H-labeled RNA and protein was necessary as a control of several experiments. They were prepared by the addition of 2 μCi of uracil-*6*-^3H or DL-leucine-*4,5*-^3H per ml to a 15-hr culture of *S. typhimurium* in CM medium. After a further 5-hr incubation, the cells were removed from the spent medium, washed, and treated as required.

RNA extraction. RNA was extracted from normal heat-injured and recovered cells by a modification of the phenol method originally described by Kirby (11). The cell pellet was washed once in and then suspended in 8 ml of cold extracting medium containing, 0.01 M Tris-hydrochloride buffer (*p*H 7.6), 0.06 M potassium chloride, 0.006 M β-mercaptoethanol, and 0.01 M magnesium acetate. The suspension was passed through the French pressure cell at 5,000 psi and collected directly into an equal volume of cold (0 C) water-saturated phenol containing 0.5% sodium dodecyl sulfate. The mixture was held cold for 20 min with occasional vigorous shaking and then centrifuged for 10 min at 14,000 × *g*. The aqueous phase was removed and reextracted twice more with half volumes of water-saturated phenol. Finally, the RNA was precipitated by adding two volumes of cold 100% ethyl alcohol to the aqueous phase. This mixture was held at −20 C for 3 hr with occasional shaking, and the RNA was pelleted by centrifugation at 5,000 × *g* for 10 min. The RNA pellet was gently resuspended in a minimal volume of 100 mM Tris-hydrochloride buffer (*p*H 5.0). To remove contaminating deoxyribonucleic acid (DNA), 5 μg of ribonuclease-free deoxyribonuclease (Worthington Biochemical Corp., N.J.) per ml, was added to the RNA solution and held for 30 min at 0 C. The RNA was then reprecipitated, suspended in a minimal volume of Tris-hydrochloride buffer (*p*H 5.0), and held frozen at −20 C.

RNA fractionation by polyacrylamide gel electrophoresis. The separation of rRNA species by polyacrylamide gel electrophoresis is well documented (3, 9, 16). Polyacrylamide gels (3%) were prepared in Tris-acetate buffer (*p*H 7.0 to 7.2), by the method originally described by Bishop et al. (3). All gels were preelectrophoresed at 5 ma/gel for 30 min before the RNA sample was applied. The RNA samples were layered directly on top of the gel after mixing the RNA sample with one drop of glycerol, and electrophoresis was continued. The electrophoresis was done at a constant current of 5 ma/gel for 6 hr to obtain the desired separation of the rRNA species.

For assay, the gels were immediately frozen in crushed solid CO_2 and sliced into 1.0-mm slices by using a gel slicer (The Mickle Laboratory Engineering Co., Surrey, England). The gel slices were placed directly into scintillation vials, 0.5 ml of 30% hydrogen peroxide was added, and the gels were digested for 12 hr at 70 C. The scintillation vials were allowed to cool, 15 ml of scintillation fluid was added, and the samples were counted.

Crude extract preparation and sucrose gradient separation. The cell pellet of either normal, injured, or recovered cells, after one washing, was resuspended in 6 ml of 0.01 M Tris-hydrochloride buffer (*p*H 7.2) containing 5×10^{-4} M Mg^{2+}. The cells were disrupted in the French pressure cell at 5,000 psi at 0 C. Whole cells and cell debris were removed by centrifugation at 27,000 × *g* for 30 min at 0 to 2 C.

A 10-ml linear sucrose gradient (20 to 5%) was prepared by using sucrose solutions made up in Tris-hydrochloride buffer (*p*H 7.2) containing 5×10^{-4} M Mg^{2+}. Approximately 0.25 ml of the fresh crude extract sample was placed on the top of the gradient and then centrifuged, by using an SB-283 rotor in an International preparative ultracentrifuge model B-60 (International Equipment Co., Needham Heights, Mass.) for 6.5 hr at 26,500 rev/min. After centrifugation, the bottoms of the tubes were punctured, and six-drop fractions were collected directly into scintillation vials; scintillation fluid was added, and the samples were counted.

Measurements of radioactivity. All radioactive samples were counted on a Tri-Carb liquid scintillation spectrometer (model 3320; Packard Instrument Co., Downers Grove, Ill.). Most of the experiments involved the counting of $^3H/^{32}P$ or $^3H/^{14}C$ doubly labeled samples. In such cases, the spectrometer was set at an optimum counting efficiency that gave a low degree of channel cross-over. In the $^3H/^{14}C$-labeled samples, the counting efficiencies were 40 and 69%, respectively, and a spillover of 14% of the ^{14}C count in the ^3H channel was corrected for. In the $^3H/^{32}P$-labeled samples, the counting efficiencies were 51.5 and 98.6%, respectively. The ^3H cross-over (0.5%) and ^{32}P cross-over (1.7%) were ignored since the values were low. All radioactive samples were counted after adding 15 ml of scintillation fluid containing 0.7% 2,5-diphenyloxazole, 0.03% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, and 10% naphthalene in dioxane.

RESULTS

Injury and recovery. The heating of *S. typhimurium* at 48 C produced a population of cells that became increasingly sensitive to Levine

Eosin Methylene Blue Agar containing 2% NaCl (EMB-NaCl). After 30 min of heating, 99.9% of the total viable population were incompetent at growth on EMB-NaCl (Fig. 1A). The total viable count on Trypticase Soy Agar (TSA)-citrate medium (28) remained quite constant throughout this heating procedure, although some loss in viability was evident (Fig. 1A). The inoculation of injured cells into fresh medium supported the rapid return of the cells' normal tolerance to the EMB-NaCl medium; within 6 hr, the injured cells were fully recovered (Fig. 1B). During the recovery period, the total viable count on TSA-citrate remained quite constant.

Ribosomal RNA degradation during thermal injury. To assess the nature and extent of the rRNA degradation produced by heat injury, the rRNA in control and test cells was steady-state labeled with ^{3}H and ^{14}C, respectively. After the ^{14}C-labeled cells were heat injured, the extracted rRNA species were co-electrophoresed on polyacrylamide gels (Fig. 2). This method gave an excellent separation of the control 23 and 16*S* rRNA (closed circles), due to their difference in molecular size (16). The data demonstrated that, after heat injury at 48 C, cells of *S. typhimurium* had completely lost their 16*S* RNA species. Only minor degradation of the 23*S* RNA species was incurred during heat injury. The injured cell rRNA profile (Fig. 2) was completely random at sedimentation coefficients smaller than 23*S* due to the presence of high-molecular-weight RNA degradation products. The material that migrated to a position intermediate between 23 and 16*S* region of the gel was the ribonuclease degradation products from the 23*S* rRNA species. This was evidence that 23*S* RNA had suffered some degradation during the injury procedure. The total loss of 16*S* RNA has been previously reported in *S. aureus* after a sublethal heat injury (24).

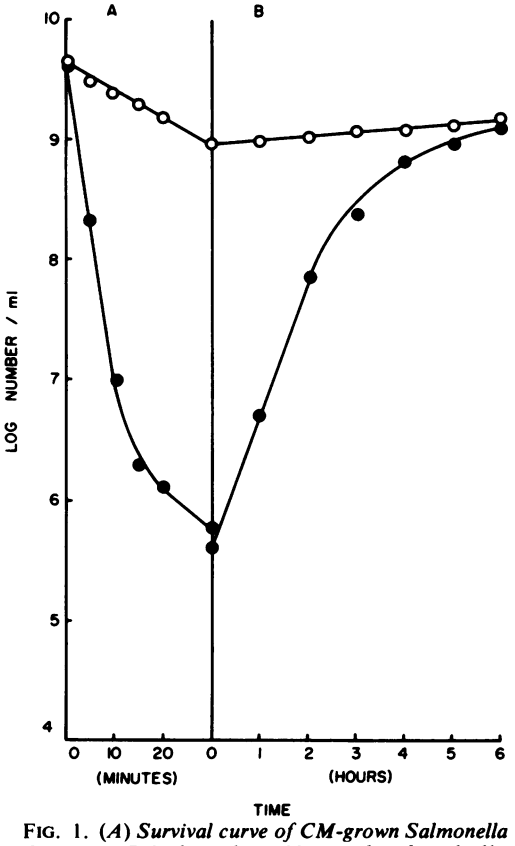

FIG. 1. *(A) Survival curve of CM-grown Salmonella typhimurium 7136 heated in 100 mM phosphate buffer (pH 6.0) at 48 C. Symbols: O, samples plated on TSA-citrate; ●, samples plated on EMB-NaCl. (B) Recovery curve of CM-grown S. typhimurium 7136 heat injured at 48 C for 30 min, inoculated into fresh CM medium, and recovered at 37 C. Symbols: O, samples plated on TSA-citrate; ●, samples plated on EMB-NaCl.*

Ribosome degradation during injury. Since considerable rRNA degradation occurred during thermal injury, further studies were initiated to observe the status of ribosomal particles after thermal injury. Crude lysates of ^{3}H-uracil-labeled control cells and ^{14}C-uracil-labeled heat-injured cells were cosedimented through a 20 to 5% linear sucrose gradient (Fig. 3). The control cells (closed circles) contained both 50 and 30*S* ribosomal subunits. The heat-treated preparation

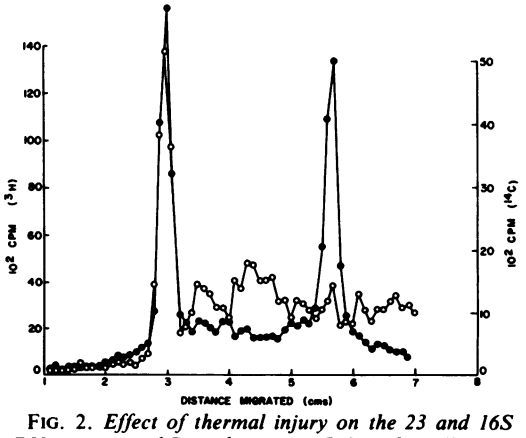

FIG. 2. *Effect of thermal injury on the 23 and 16S rRNA species of S. typhimurium 7136. The cells were steady-state-labeled with uracil-6-^{3}H and uracil-2-^{14}C for 5 hr before harvesting. The ^{14}C-labeled cells were heat injured at 48 C for 30 min. The RNA was extracted from both control and heat-injured cells and co-electrophoresed on a 3.0% polyacrylamide gel for 6 hr at 5 ma/gel. The gel was frozen, sliced into 1 mm slices, digested, and counted. Symbols: ●, uracil-6-^{3}H counts; O, uracil-2-^{14}C counts.*

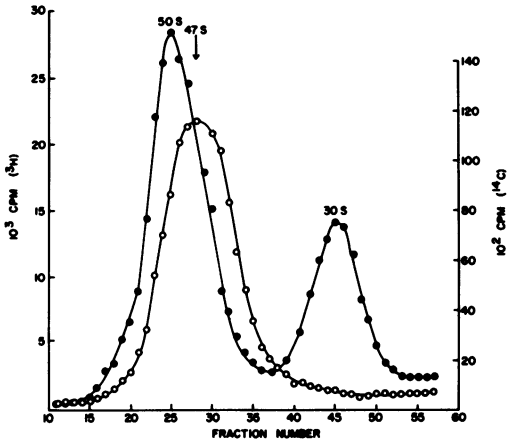

FIG. 3. *Effect of thermal injury on the 50 and 30S ribosomal particles of S. typhimurium 7136. The cells were steady-state-labeled with uracil-6-^3H and uracil-2-^{14}C for 5 hr before harvesting. The ^{14}C-labeled cells were heat injured at 48 C for 30 min. Both control and injured cells were lysed into 0.01 M Tris-hydrochloride buffer (pH 7.2) containing 5 × 10^{-4} M Mg^{2+}. Sedimentation analysis of the crude extract was carried out on a 20 to 5% linear sucrose gradient prepared in the same buffer for 6.5 hr at 26,500 rev/min. Six-drop fractions were collected directly into scintillation vials and counted. Symbols: ●, uracil-6-^3H counts; ○, uracil-2-^{14}C counts.*

(open circles) contained a single peak, with a sedimentation coefficient of 47*S*. There was no 30*S* peak present in the injured extract. The total loss of the 30*S* ribosomal particle has been previously reported after *S. aureus* was subjected to a sublethal thermal injury (24, 26).

Characterization of the rRNA synthesized during recovery. The facts that a low concentration of rifamycin inhibited recovery and ^3H-uracil was incorporated into recovering cells of *S. typhimurium* (28) demonstrated that rRNA regeneration was required for recovery of thermally injured cells of *S. typhimurium*. It was necessary to determine the nature of the rRNA synthesized during the recovery period. Tritium-labeled control rRNA was co-electrophoresed with ^{32}P labeled rRNA which was synthesized during the recovery period (Fig. 4). The control, ^3H-labeled rRNA (closed circles), showed two distinct, clearly separated peaks corresponding to 23 and 16*S* rRNA. The ^{32}P-labeled rRNA (open circles) was fractionated into four peaks under the same condition. A small peak which migrated coincident with the 16*S* RNA control and a larger peak which migrated more slowly than the control 16*S* RNA were produced. The larger peak was shown by extrapolation on the gels to have a sedimentation value of 17*S* (15). In this system, complete separation of the 16 and 17*S* RNA species was achieved. Two other peaks, both smaller than the peaks observed in the 16 to 17*S* region were found associated with the 23 to 24*S* region of the gel. The larger of these two peaks migrated coincident with the control 23*S* RNA; the small peak which migrated more slowly had a sedimentation coefficient of 24*S* (15). The 17 and 24*S* RNA species represented an accumulation of precursor 16*S* and precursor 23*S* rRNA.

Characterization of the ribosomal particles regenerated during recovery. During recovery from thermal injury, large amounts of rRNA were synthesized, with a significant proportion of this new rRNA present in the higher molecular weight precursor form (Fig. 4). It was therefore necessary to determine the sedimentation coefficients of the ribosomal particles which contained these precursor rRNA species. Unlabeled cells of *S. typhimurium* were thermally injured and then recovered for 4 hr in the CMP medium containing ^{32}P. A crude lysate from these partially recovered cells was cosedimented along with ^3H-labeled control ribosomes through a 20 to 5% linear sucrose gradient (Fig. 5). The control, ^3H-labeled profile (closed circles), gave the distinctly separated peaks corresponding to the 50 and 30*S* ribosomal subunits. The ^{32}P-labeled ribosomal particles (open circles) produced during recovery

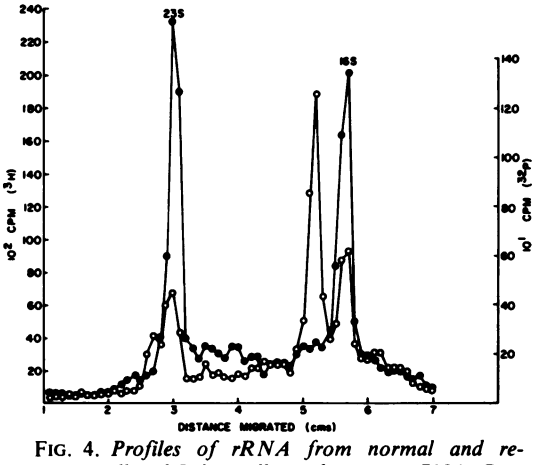

FIG. 4. *Profiles of rRNA from normal and recovering cells of Salmonella typhimurium 7136. Control cells were steady-state-labeled with uracil-6-^3H for 5 hr before harvesting, and the RNA was extracted. Test cells (unlabeled) were heat injured and recovered for 4 hr in CMP containing 2 μCi of $Na_2H^{32}PO_4$ per ml, and the RNA was extracted. The two samples of RNA were mixed and co-electrophoresed on a 3.0% polyacrylamide gel for 6 hr at 5 ma/gel. The gel was then frozen, sliced into 1 mm slices, digested, and counted. Symbols: ●, uracil-6-^3H counts; ○, ^{32}P counts.*

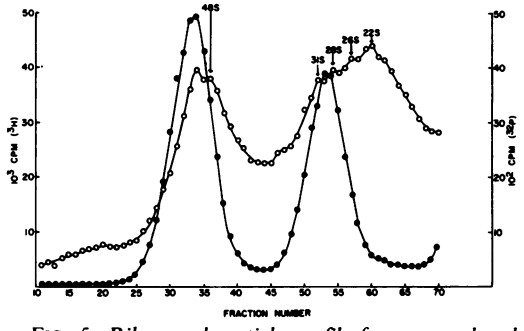

FIG. 5. *Ribosomal particle profile from normal and recovering cells of S. typhimurium 7136. Control cells were steady-state-labeled with uracil-6-^3H for 5 hr before harvesting. Injured cells (unlabeled) were recovered for 4 hr in CMP containing 2 μCi of $Na_2H^{32}PO_4$ per ml. Both control and recovering cells were lysed into 0.01 M Tris-hydrochloride buffer (pH 7.2) containing 5 × 10^{-4} M Mg^{2+}. Sedimentation analysis of the crude extracts was carried out on a 20 to 5% linear sucrose gradient, prepared in the same buffer, for 6.5 hr at 26,500 rev/min. Six-drop fractions were collected directly into scintillation vials and counted. Symbols: ●, uracil-6-^3H counts; ○, ^{32}P counts.*

were also fractionated into two broad peaks under the same conditions. However, each of these two ^{32}P-labeled peaks could be identified as a composite of several partially separated peaks of distinctly different sedimentation coefficients. In the 50*S* region of the gradient, there was a substantial peak coincident with the control 50*S* particle and another slightly lighter peak that had a sedimentation coefficient of 48*S*. The broad peak associated with the 30*S* area of the gradient was fractionated into four subpeaks with the following sedimentation values: 31, 28, 26, and 22*S*. The majority of the rRNA in this peak sedimented with a value of 22*S*.

The ribosomal particle profile produced after 5 hr of recovery, although similar to that in Fig. 5, did show a shift in the relative amounts of the 30*S* precursor particles with time. The major peak in the 22 to 30*S* region, after 5 hr of recovery, was coincident with the 30*S* peak of the control ribosomes.

Characterization of the rRNA synthesized during chloramphenicol-inhibited recovery. We have previously reported (28) that 100 μg of chloramphenicol per ml inhibited the recovery of thermally injured cells of *S. typhimurium*, as measured by an increased viability on EMB-NaCl medium. However, during the chloramphenicol-inhibited recovery, extensive RNA synthesis was demonstrated by ^3H-uracil incorporation (R. I. Tomlins, Ph.D. Thesis, University of Illinois, 1971). Therefore, to determine the effect of chloramphenicol on the synthesis and maturation of the rRNA formed during recovery, control ^3H-labeled rRNA was co-electrophoresed with ^{32}P-labeled rRNA which was synthesized during the chloramphenicol-inhibited recovery of *S. typhimurium*. The results are presented in Fig. 6. The control, ^3H-labeled rRNA (closed circles), was separated into two peaks: 23 and 16*S* RNA. The ^{32}P-labeled rRNA was separated into three peaks under the same conditions. No 16*S* RNA was detected in the chloramphenicol-recovered RNA, but there was a large peak which migrated slightly slower than the 16*S* RNA. This peak had a sedimentation coefficient of 17*S*. The larger peak in the 23 to 24*S* region of the gel migrated identically with the 23*S* RNA of the control. The smaller, slower moving peak had a sedimentation coefficient of 24*S*. It was clear that the presence of chloramphenicol had not affected the synthesis of either 24 or 17*S* RNA nor the maturation of 24*S* RNA; however, the maturation of 17*S* RNA was completely abolished in the presence of chloramphenicol.

Characterization of the ribosomal particles regenerated during the chloramphenicol-inhibited recovery. The addition of chloramphenicol to the recovery medium inhibited the maturation of 17*S* RNA but did not affect the maturation of 24*S* RNA (Fig. 6). When a crude lysate from ^{32}P-labeled chloramphenicol recovered cells was co-sedimented with ^3H-labeled control ribosomes,

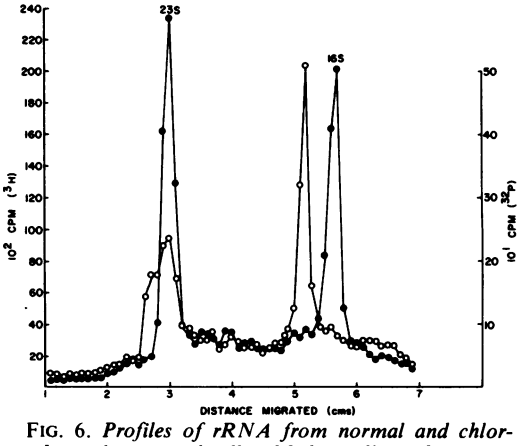

FIG. 6. *Profiles of rRNA from normal and chloramphenicol-recovered cells of Salmonella typhimurium 7136. Control cells were steady-state-labeled with uracil-6-^3H for 5 hr before harvesting, and the RNA was extracted. Test cells (unlabeled) were heat injured and recovered for 4 hr in CMP plus 2 μCi of $Na_2H^{32}PO_4$ containing 100 μg of chloramphenicol per ml, and the RNA was extracted. The two samples of RNA were mixed and co-electrophoresed on a 3.0% polyacrylamide gel for 6 hr at 5 ma/gel. The gel was then frozen, sliced into 1-mm slices, digested, and counted. Symbols: ●, uracil-6-^3H counts; ○, ^{32}P counts.*

the profiles in Fig. 7 were obtained. The control profile (closed circles) showed two well separated peaks corresponding to the 50 and 30*S* ribosomal subunits. The ribonucleoprotein particles synthesized during the chloramphenicol-inhibited recovery produced two major peaks under the same sedimentation conditions (open circles). In the 50*S* region, there was a substantial peak which sedimented identically with the 50*S* control particle. However, there was a shoulder on the peak which suggested the accumulation of a slightly lighter ribonucleoprotein particle which had a sedimentation coefficient of 48*S*. The second and major ribonucleoprotein peak produced was lighter than the control 30*S* particle, having a sedimentation coefficient of 22*S*. This peak also contained a shoulder which indicated the accumulation of a 32*S* precursor particle.

Participation of old ribosomal proteins in the formation of new ribonucleoprotein particles. It has been reported that under conditions of physiological stress which produces ribosome degradation (e.g., Mg^{2+} ion starvation), ribosomal proteins are conserved; during the recovery period, the new ribonucleoprotein particles formed contained new rRNA and old ribosomal proteins (14, 18). It has also been reported that, during the recovery of *S. aureus* from heat injury, the new ribosomal particles contained only old ribosomal proteins (L. J. Rosenthal, Ph.D. Thesis, Kansas State University, Manhattan, Kansas, 1969).

To evaluate the participation of old ribosomal proteins in the formation of new ribonucleoprotein particles produced during recovery, the ribosomal proteins in cells of *S. typhimurium* were steady-state-labeled with ^{3}H-leucine before injury. After heat injury, the cells were recovered for 5 hr in CMP medium containing ^{32}P. A crude extract of these cells was fractionated on a 20 to 5% linear sucrose gradient (Fig. 8). The profile representing old ribosomal proteins (open circles) produced two peaks. The larger peak associated with the 47*S* region of the gradient was expected since the 50*S* ribosomal particle was not totally degraded during heat injury. The smaller peak which sedimented in the 22 to 30*S* region of the gradient showed only a minor association of the prelabeled ribosomal proteins in the newly synthesized ribonucleoprotein particles.

Ribosomal protein synthesis during recovery. The results presented in Fig. 8 demonstrated that there was limited conservation and later incorporation of old ribosomal proteins into the ribonucleoprotein particles formed during recovery. The fact that new particles were formed meant

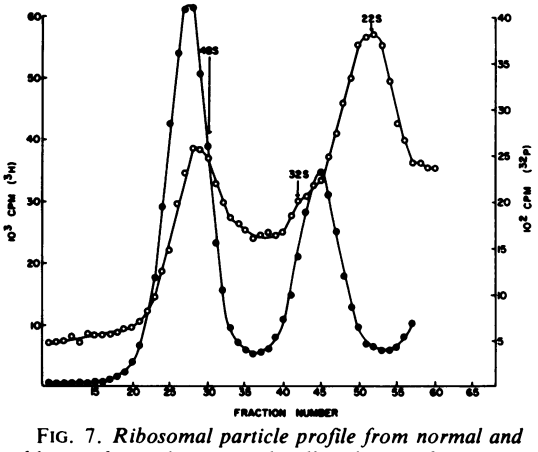

FIG. 7. *Ribosomal particle profile from normal and chloramphenicol-recovered cells of S. typhimurium 7136. Control cells were steady-state-labeled with uracil-6-^{3}H for 5 hr before harvesting. Injured cells (unlabeled) were recovered for 4 hr in CMP containing 100 μg of chloramphenicol per ml plus 2 μCi of $Na_2H^{32}PO_4$ per ml. Both control and recovered cells were lysed into 0.01 M Tris-hydrochloride buffer (pH 7.2) containing 5 × 10^{-4} M Mg^{2+}. Sedimentation analysis of the crude extracts was carried out on a 20 to 5% linear sucrose gradient, prepared in the same buffer for 6.5 hr at 26,500 rev/min. Six-drop fractions were collected directly into scintillation vials and counted. Symbols: ●, uracil-6-^{3}H counts; ○, ^{32}P counts.*

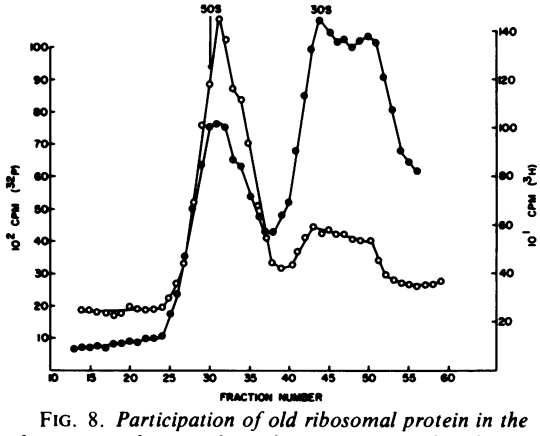

FIG. 8. *Participation of old ribosomal protein in the formation of new ribonucleoprotein particles during recovery. Cells of S. typhimurium 7136 were steady-state labeled with DL-leucine-4,5-^{3}H, injured, and then recovered for 5 hr in CMP containing 2 μCi of $Na_2H^{32}PO_4$ per ml. The cells were lysed into 0.01 M Tris-hydrochloride buffer (pH 7.2) containing 5 × 10^{-4} M Mg^{2+}. Sedimentation analysis of the crude extract was carried out on a 20 to 5% linear sucrose gradient, prepared with the same buffer for 6.5 hr at 26,500 rev/min. Six-drop fractions were collected directly into scintillation vials and counted. The positions of the 50 and 30S markers were determined from a control experiment. Symbols: ●, ^{32}P counts; ○, DL-leucine-4,5-^{3}H counts.*

that recovering cells must have synthesized new ribosomal proteins. To test this hypothesis, unlabeled, heat-injured cells were recovered for 5 hr in CMP medium containing both ^{32}P- and ^{3}H-leucine. After recovery, a crude extract of the cells was subjected to fractionation on a 20 to 5% linear sucrose gradient (Fig. 9). The synthesis of new ribosomal protein associated with the ribonucleoprotein particles formed during recovery was significant (open circles). The new ribosomal protein profile followed the RNA profile quite closely. However, the RNA to protein ratio was higher in the 30*S* region of the gradient than at any other position. This was possibly due to the presence of a protein-deficient ribonucleoprotein precursor of the 50*S* ribosomal subunit which had a sedimentation value of about 30*S*.

DISCUSSION

The thermal injury conditions which rendered cells of *S. typhimurium* sensitive to EMB-NaCl medium also produced extensive degradation of 16*S* RNA with minor degradation of the 23*S* RNA species. The same injury conditions completely destroyed the 30*S* ribosomal subunit and produced an alteration in the 50*S* ribosomal subunit causing it to sediment at 47*S*. The production of a 47*S* particle during thermal injury could have been a result of either the heat treatment alone or to the imbalance in the ribosomal particle population due to the depletion of the 30*S* ribosomal subunits after heat injury (7).

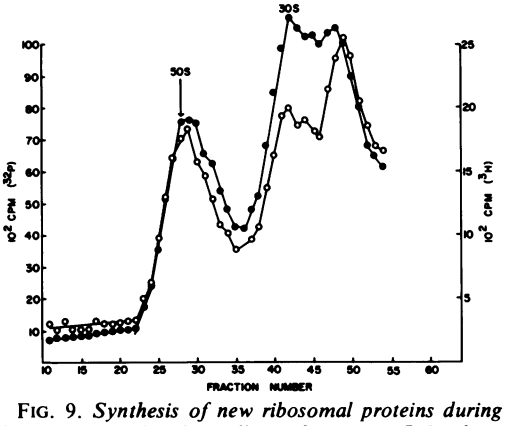

FIG. 9. *Synthesis of new ribosomal proteins during the recovery of Salmonella typhimurium 7136 from thermal injury. Unlabeled cells were injured and then recovered for 5 hr in CMP containing 2 μCi of $Na_2H^{32}PO_4$ per ml and 2 μCi of* DL-*leucine-4,5-^{3}H per ml. The cells were lysed into 0.01* M *Tris-hydrochloride buffer (pH 7.2) containing 5×10^{-4}* M *Mg^{2+}. Sedimentation analysis of the crude extracts was carried out on a 20 to 5% linear sucrose gradient, prepared in the same buffer for 6.5 hr at 26,500 rev/min. Six-drop fractions were collected directly into scintillation vials and counted. The position of the 50 and 30S markers were determined from a control experiment. Symbols: ●, ^{32}P counts; ○,* DL-*leucine-4,5-^{3}H counts.*

The inoculation of thermally injured cells into fresh growth medium supported a return of the cells' normal tolerance to EMB-NaCl. During the recovery period, large quantities of RNA were synthesized with a substantial accumulation of 17 and 24*S* precursor RNA species.

Recently, experiments have been performed which demonstrated that both 16 and 23*S* RNA were not primary transcription products. By using the kinetic analysis developed by Britten and McCarthy (5), Hecht and his co-workers (8, 9) demonstrated that the formation of 5*S* and mature 16*S* RNA in *Bacillus subtilis* was third order; thus, they concluded that there must be at least one and possibly two precursors to these species. The synthesis of precursor 16*S* RNA, 17*S* RNA was first order, and hence this molecule was a primary transcription product.

The differences in electrophoretic mobility between precursor and mature rRNA forms cannot be explained by a conformational difference alone and must be due also to a molecular-weight difference. By using the method of Bishop et al. (3), the molecular-weight differences of the precursor and mature rRNA species accumulated during recovery (Fig. 4) were calculated assuming the molecular weights of mature 16 and 23*S* RNA to be 0.55×10^6 and 1.1×10^6 daltons, respectively. This calculation showed that precursor and mature 16*S* RNA had a molecular-weight difference of 60,000 daltons, and precursor and mature 23*S* RNA had a molecular-weight difference of 80,000 daltons. These values corresponded closely to those obtained from pulse-labeling experiments using *B. subtilis* (9) and *E. coli* (1), and from oligonucleotide fingerprinting (25).

During recovery, 22, 26, and 28*S* precursors of the 30*S* ribosomal subunit and 31 and 48*S* precursors of the 50*S* ribosomal subunit accumulated along with both the 30 and 50*S* mature particles (Fig. 5). Pulse labeling of actively growing bacterial cultures has been used to observe the natural precursors of mature 30 and 50*S* ribosomal subunits. Mangiarotti et al. (17) using this technique identified two successive precursors of the 50*S* subunit, which sedimented at 32 and 43*S*, respectively. They also identified a single 30*S* precursor which sedimented at 26*S*. The later work of Osawa et al. (23) was in basic agreement with this data with the exception that they proposed another 30*S* precursor with a sedimentation coefficient of 22*S*. The 32 and 43*S* precursor particles were shown to contain precursor 23*S* RNA (17, 23). Both the 22 and 26*S*

precursors of the 30*S* particle contained precursor 16*S* RNA. During the recovery of thermally injured cells of *S. typhimurium*, a steady-state accumulation of ribosomal precursor particles was observed (Fig. 5). Recovering cells were initially unable to synthesize protein due to the depletion of 30*S* ribosomes during thermal injury (Fig. 3). Consequently, such cells were in a similar physiological state to normal cells after treatment with a low concentration of chloramphenicol (23). The precursor particles formed during recovery were probably similar to those identified from normal cells during protein synthesis inhibition (23). The 22 and 26*S* precursor particles have been previously observed by pulse-labeling (12) and by inhibition of growing *E. coli* with low concentrations of chloramphenicol (23). A 28*S* ribonucleoprotein particle which contained 16*S* rRNA has only been identified in extracts of *E. coli* treated with 5-fluorouracil (12). During recovery, two precursors of the 50*S* particle, with sedimentation coefficients of 31 and 48*S*, accumulated (Fig. 5). The 31*S* particle observed was possibly the same as the 32*S* precursor particle that was previously described (12). The presence of a 48*S* precursor of the 50*S* particle has not been previously reported. It was possible that the 48*S* particle which accumulated during recovery (Fig. 5 and 7) was similar to the 47*S* particle produced by heat injury (Fig. 3), with the exception that the 48*S* particle contained 24*S* RNA, and the 47*S* particle contained 23*S* RNA. There are many references to a 43*S* precursor of the 50*S* subunit, but the accumulation of this species was not observed during recovery.

The addition of chloramphenicol (100 μg/ml) to the recovery medium completely abolished the maturation of precursor 16*S* RNA. However, the ratio of precursor to mature 23*S* RNA was similar, irrespective of the presence of chloramphenicol. These data indicated that the mechanisms of maturation of precursor 16 and 23*S* rRNA species were different. The maturation of precursor 23*S* RNA during chloramphenicol inhibition of growing *E. coli* was previously observed (B. H. Browstein and P. S. Sypherd, Bacteriol. Proc., p. 49, 1970). Under the same recovery conditions, the formation of mature 30*S* particles was inhibited, and a 22*S* precursor particle accumulated. The normal flow of precursors into mature 50*S* ribosomal subunits during recovery was unaffected by chloramphenicol.

The cessation of protein synthesis by any one of a number of suitable inhibitors has caused the accumulation of precursor ribosomal particles. High concentrations of chloramphenicol (200 μg/ml) produced an accumulation of 18 and 25*S* particles which contained 16 and 23*S* RNA, respectively (13). In contrast, low concentrations of chloramphenicol (0.6 to 1.5 μg/ml) caused the accumulation of 42, 32, 26, and 22*S* ribonucleoprotein particles in growing *E. coli* in addition to both mature 50*S* and 30*S* ribosomes (12). The accumulation of a 22*S* precursor particle has been reported in some of the *sad* mutants of *E. coli* (7) and in a cold-sensitive mutant of *S. typhimurium* isolated by Tai et al. (27). However, the accumulation of a 22*S* ribonucleoprotein precursor particle with the exclusion of all other 30*S* precursors, on the addition of chloramphenicol to a bacterial culture, has not been previously reported. Why a 22*S* precursor of the 30*S* particle accumulated during the chloramphenicol-inhibited recovery of *S. typhimurium* merits further discussion. The original work described by Nomura and Watson (19) demonstrated that a high concentration of chloramphenicol added to growing *E. coli* produced only an 18*S* precursor of the 30*S* particle. The so called RI particle observed by Traub and Nomura (20, 29), during the in vitro reconstitution of functional 30*S* ribosomes at 0 C from 16*S* RNA and 30*S* ribosomal proteins, had a sedimentation coefficient of 21*S*. The *sad-38* and *sad-410* cold-sensitive mutants of *E. coli* (20) were shown to accumulate both 21 and 32*S* particles at 20 C. The 21*S* particle contained precursor 16*S* RNA and nine ribosomal proteins. All of these ribosomal proteins were involved in the early steps of the ribosome assembly process according to Nomura's in vitro scheme (20). Consequently, the 21*S* particles accumulated at 20 C by the *sad* mutants were considered to be the in vivo equivalent of the RI particle formed in vitro. The data presented in Fig. 7 demonstrated the accumulation of 22*S* particle when heat-injured cells were incubated in the presence of chloramphenicol. It would then appear that the thermal injury process caused some irreversible damage to the S proteins without altering the binding properties of the RI proteins. The synthesis of new S proteins during recovery would therefore be required for the formation of the other precursors and, eventually, mature 30*S* ribosomal particles.

Recent experiments by Lefkovits and DiGirolamo (14) showed that complete mature 30 and 50*S* ribosomal particles were formed during the recovery of cells from Mg^{2+} ion starvation in the presence of chloramphenicol. The data reported here demonstrated that there was a different mechanism for ribosome formation during the recovery of cells from thermal injury (Fig. 8 and 9). The 50*S* ribosomal particle formation during recovery was not affected by the addition of chloramphenicol, and its formation was as efficient with either old or newly synthesized ribo-

somal proteins. When the synthesis of new ribosomal proteins was inhibited, preexisting RI proteins reassociated with new 17*S* RNA with the formation of a 22*S* particle. However, in the absence of chloramphenicol, recovering cells preferentially synthesized new ribosomal proteins rather than reusing preexisting ribosomal proteins in the synthesis of new ribosomal particles. If this interpretation is correct, we have to consider the possibility that there exists a coordination between rRNA and ribosomal protein synthesis during the recovery of *S. typhimurium* from thermal injury.

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