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Requirements of *Salmonella typhimurium* for Recovery from Thermal Injury

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The heating of *Salmonella typhimurium* 7136 at 48 C for 30 min produces a population of cells that are incompetent at division on Levine Eosin Methylene Blue Agar containing 2.0% NaCl (EMB-NaCl). When these injured cells were placed in fresh citrate salts medium they recovered, and regained their tolerance to the EMB-NaCl medium and grew out. The addition of the selective inhibitors rifamycin, 5-fluorouracil, 2,4-dinitrophenol, chlorotetracycline, chloramphenicol, and 5-methyltryptophan to the recovery medium showed that the recovery process was dependent on ribosomal ribonucleic acid (RNA) synthesis, adenosine triphosphate synthesis, and the synthesis of new protein. These results were substantiated by incorporation experiments, which demonstrated that during recovery no deoxyribonucleic acid synthesis, and hence no cell division, occurred. Ribosomal RNA was synthesized during recovery, but its synthesis was not the rate-limiting step. A small but significant amount of protein was also formed during the latter part of the recovery period.

The complex phenomenon of cellular injury and recovery in nonsporulating bacteria has been under investigation for several years. It is well documented that many different sublethal stresses will produce physiological damage in microorganisms, heat (1, 9, 11, 21), freezing or chilling (19, 26), ultraviolet irradiations (2), freeze-drying (23), and various chemicals (12,13).

The characteristic lesions caused by sublethal heating have been shown to be: (i) the loss of cellular membrane integrity, as shown by the leakage of amino acids, potassium ions, and 260-nm absorbing material (9); (ii) the degradation of ribosomal ribonucleic acid (rRNA; references 21, 25); and (iii) some, although not extensive, metabolic damage (3; M. D. Pierson, Ph.D. thesis, Univ. of Illinois, Urbana, 1970).

Most of the investigations into the nature of thermal injury and recovery have been pursued by using *Staphylococcus aureus* and other gram-positive microorganisms (1, 3, 4, 9, 21, 25). Although our knowledge, as yet, is incomplete in terms of understanding the complex changes occurring in a gram-positive organism such as *S. aureus* during thermal injury and recovery, there is even less information available on gram-negative organisms. This study was undertaken to investigate thermal injury in a gram-negative organism, *Salmonella typhimurium*.

MATERIALS AND METHODS

Injury procedure. Cultures of *S. typhimurium* 7136 (obtained from the National Center for Urban and Industrial Health, Cincinnati, Ohio) were grown in a citrate minimal salts (CM) medium. Frozen stock cultures were prepared by inoculating 0.1 ml of an actively growing culture into 10 ml of sterile CM medium without the trace metals and freezing at −20 C. The frozen tube was thawed when needed and the entire 10 ml was inoculated into 190 ml of CM medium. The culture was incubated on a rotary shaker at 37 C for 20 hr. Cells were harvested by centrifugation for 10 min at 8,000 × *g* at 0 to 2 C. The supernatant fluid was decanted and the cells were washed once in 100 mM potassium phosphate buffer. These cells were heat-injured at 48 C for 30 min by adding the 10 ml of cell suspension to 190 ml of 100 mM potassium phosphate buffer (*p*H 6.0), pretempered under constant agitation.

Medium. The medium used for growth and recovery, and in the incorporation experiments, was a CM medium of the following composition (grams/liter): sodium citrate, 8.0; potassium phosphate (monobasic), 3.0; potassium phosphate (dibasic), 7.0; ammonium sulfate, 1.0; and trace metals Mg^{2+}, 5×10^{-1} mM, Mn^{2+}, 3×10^{-1} mM, and Fe^{2+}, 2×10^{-3} mM. 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.

Assay procedures. Assays of injury and recovery were done by using a plate counting technique. Samples (1 ml) were withdrawn at various intervals from the injury

vessel, or from the recovery flask, and diluted in 0.1% peptone-distilled water blanks. The samples were taken from common dilution bottles and pour plated on Trypticase Soy Agar (TSA; from BBL) containing 0.25% sodium citrate (TSA-citrate) and surface-plated on Levine Eosin Methylene Blue Agar (Difco) containing 2.0% NaCl (EMB-NaCl). The plates were incubated at 37 C for 48 hr. The TSA-citrate counts gave a measure of all viable cells both injured and uninjured. The EMB-NaCl count gave an estimation of the uninjured population.

Inhibitors. Metabolic inhibitors were added to the CM recovery medium during recovery of heat injury in the following concentrations. Chloramphenicol (Calbiochem, Los Angeles, Calif.), 100 μg/ml; chlorotetracycline (American Cynamid Co., Pearl River, N.Y.), 30 μg/ml; 5-fluorouracil (Calbiochem), 100 μg/ml; 2,4-dinitrophenol (Eastman Organic Chemicals, Rochester, N.Y.), 75 μg/ml; 5-methyltryptophan (Calbiochem), 0.5 mg/ml; and rifamycin (Calbiochem), 5 μg/ml.

Radiotracer technique. To measure the synthesis of steady state ribonucleic acid (RNA) fractions, a mixture of uracil-*6-*^{3}*H* (New England Nuclear Corp., Boston, Mass., specific activity 12.2 Ci/mmole), at a final concentration of 20 μCi/ml, plus 10^{-1} mM carrier uracil was added to the CM recovery medium. Likewise, protein synthesis during recovery and subsequent growth was measured by the incorporation of L-leucine-*U-*^{14}*C* (New England Corp., specific activity 260 mCi/mmole), at a final concentration of 0.2 μCi/ml, plus 2×10^{-4} mM nonradioactive L-leucine. The synthesis of deoxyribonucleic acid (DNA) during recovery and subsequent growth was measured by the incorporation of thymidine-*methyl-*^{3}*H* (New England Nuclear Corp., specific activity 6.7 Ci/mmole), at a final concentration of 10 μCi/ml plus 1×10^{-3} mM nonradioactive thymidine. Samples (1 ml) were taken at 15-min intervals and mixed with an equal volume of cold (0 C), 10% trichloroacetic acid. The samples were allowed to stand at 0 C for 30 min, and the acid-insoluble fraction was collected on 0.22-μm membrane filters (Millipore Corp., Bedford, Mass.).

The precipitates were washed with two volumes of cold (0 C), 5% trichloroacetic acid, and the filters were dried and placed in scintillation vials containing 15 ml of scintillation fluid (5 g of 2,5 diphenyloxazole and 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene per liter of toluene).

All radioactive samples were counted in a Tri-Carb liquid scintillation spectrometer, model 3320 (Packard Instrument Co., Inc., Downers Grove, Ill.). For the doubly labeled samples, the efficiencies of counting were 40% and 69% for ^{3}H and ^{14}C, respectively. A spillover of 14% of the ^{14}C count in the ^{3}H channel was corrected for. The effect of quenching from the filter discs was ignored since all samples were quenched to the same extent.

RESULTS AND DISCUSSION

Plate counting procedure to estimate injury and recovery. When cells of *S. typhimurium* 7136 were heated at 48 C, they showed an increasing sensitivity to EMB-NaCl (Fig. 1). The total viable count on TSA-citrate remained fairly constant during this heating procedure, although some death was evident. The counts on the EMB-NaCl medium which were obtained from the same dilution bottles of heat-treated organisms reflected the number of uninjured cells. Hence, the difference in the two viable cell counts gave the extent of the injury at any time during the heating procedure. The cells rapidly lost their ability to grow on EMB-NaCl within 15 min of heating, and continued heating resulted in further injury, although at a much slower rate (Fig. 1). At the end of 30-min heating at 48 C, 99.9% of the total viable cell population was incompetent at growth on EMB-NaCl. These data were consistent with the observations of Clark and Ordal (5).

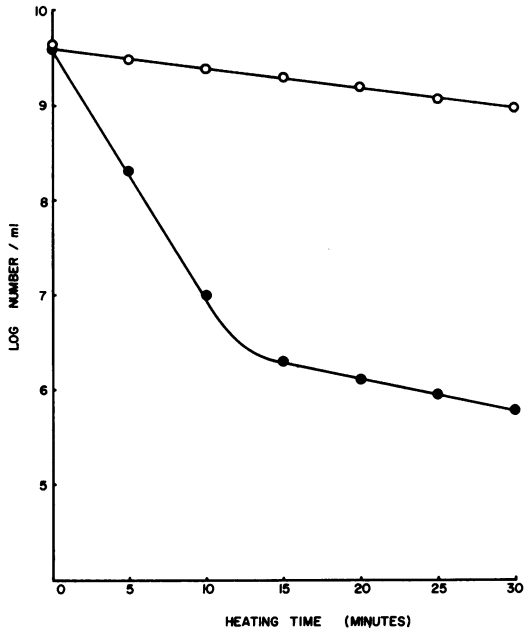

FIG. 1. *Survival curves for Salmonella typhimurium 7136 heated in 100 mM potassium phosphate buffer, pH 6.0, at 48 C. Symbols:* ○, *cells plated on TSA-citrate to give a total viable count;* ●, *cells plated on EMB-NaCl to give the uninjured population.*

The differential plating procedure was a slight modification of that proposed by Clark and Ordal (5) for Trypticase Soy Broth-grown *S. typhimurium*. Injured or recovering cells of *S. typhimurium* originally grown on CM medium were not quantiatively enumerated on TSA. The addition of 0.25% sodium citrate to the TSA medium overcame this problem.

During recovery from thermal stress, a frequently observed phenomenon is an extended lag time (9, 11). During this recovery period, the cells rapidly regain their competence on the EMB-

NaCl medium and later commence to grow at a rate equal to that of an unheated control, eventually reaching the same total viable population (5).

Effect of RNA synthesis inhibitors on recovering cells. To understand the mechanisms involved during the recovery process, various metabolic inhibitors were added to the recovery medium. It has been shown that, during the heat injury of *S. aureus*, ribosomal RNA (rRNA) was degraded, and resynthesis of the degraded rRNA occurred during the recovery process (25; L. J. Rosenthal, Ph.D. thesis, Kansas State Univ., Lawrence, 1969). To determine whether rRNA synthesis was required during the recovery of *S. typhimurium* from heat injury, heat-injured cells were allowed to recover in the presence of 5 μg of rifamycin or 100 μg of 5-fluorouracil per ml (Fig. 2). Rifamycin has been shown to be a potent inhibitor of RNA synthesis, irreversibly binding to DNA-dependent RNA polymerase (7, 24, 28). Figure 2 shows that no recovery occurred, also that the heat-injured population was sensitive to rifamycin, since the TSA-citrate count fell to approximately the zero time level of EMB-NaCl count. The counts on the EMB-NaCl medium slowly declined from their original value, indicating that uninjured cells that were originally competent on EMB-NaCl became sensitive to EMB-NaCl in the presence of rifamycin. The control experiments demonstrated that the injured population recovered their tolerance to EMB-NaCl in 3 hr and cell division occurred after 5 hr of recovery.

The dependence of the recovery system on rRNA synthesis was similarly demonstrated by using 5-fluorouracil (Fig. 2). The TSA-citrate and EMB-NaCl curves were similar to those produced by rifamycin inhibition. The data also demonstrated that the injured cell population was more sensitive to 5-fluorouracil than the uninjured population. There was an apparent tendency for recovery during the first 2 hr, after which time the EMB-NaCl counts slowly decreased, only just falling below the initial zero time count after 23 hr.

Inhibitors like 5-fluoruracil, which are analogues of natural bases, are incorporated into all molecular species of RNA, but the incorporation into rRNA and eventually ribosomes has the most damaging effect on the cell. Different species of microorganisms vary in their susceptibility to 5-fluoruracil. For example, in *Bacillus subtilis* 55 to 65% of the RNA-uracil was substituted by the fluorine derivative with only minor alterations to its physiology (22), whereas *Escherichia coli* suffered a loss in viability when 5-fluorouracil was incorporated into the cell, and especially into the ribosomal particles (10). Clearly, rRNA synthesis was required for recovery since two rRNA synthesis inhibitors having very different modes of action both inhibited recovery. These results concurred with the data of other workers (9, 25; L. J. Rosenthal, Ph.D. thesis, Kansas State Univ., 1969) who showed the requirement of rRNA synthesis during the recovery of *S. aureus* from heat injury.

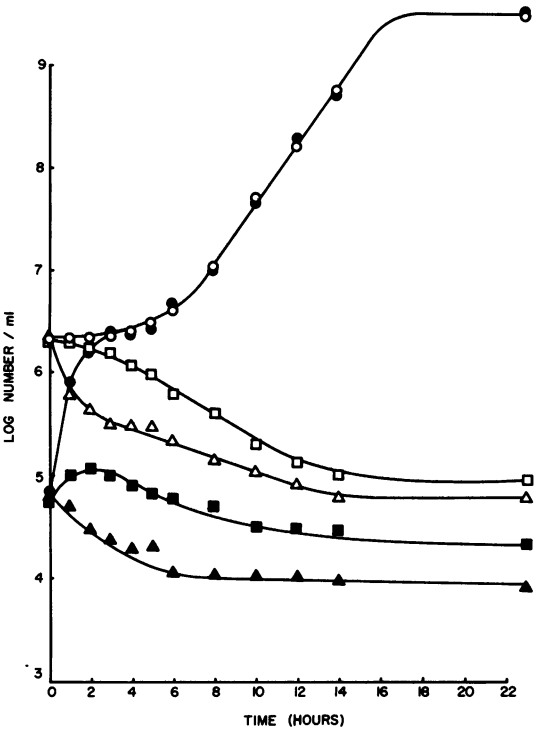

FIG. 2. *Recovery and growth of heat-injured Salmonella typhimurium 7136 incubated in CM medium plus 5 μg of rifamycin or 100 μg of 5-fluorouracil per ml. The cells were heat-injured in 100 mM potassium phosphate buffer, pH 6.0, at 48 C for 30 min. Cells incubated in CM medium: ○, plated on TSA-citrate; ●, plated on EMB-NaCl. Cells incubated in CM plus 5 μg of rifamycin per ml: △, plated on TSA-citrate; ▲, plated on EMB-NaCl. Cells incubated in CM plus 100 μg of 5-fluorouracil per ml: □, plated on TSA-citrate; ■, plated on EMB-NaCl.*

Effect of 2,4-dinitrophenol on recovering cells. The importance of an energy requirement for a cell to recover was amply demonstrated when injured cells were inoculated into the recovery medium containing 75 μg of 2,4-dinitrophenol per ml (Fig. 3). The TSA-citrate and the EMB-NaCl counts both decreased with first order kinetics. The injured population of cells was more sensitive to the 2,4-dinitrophenol than the uninjured population, since the TSA-citrate count

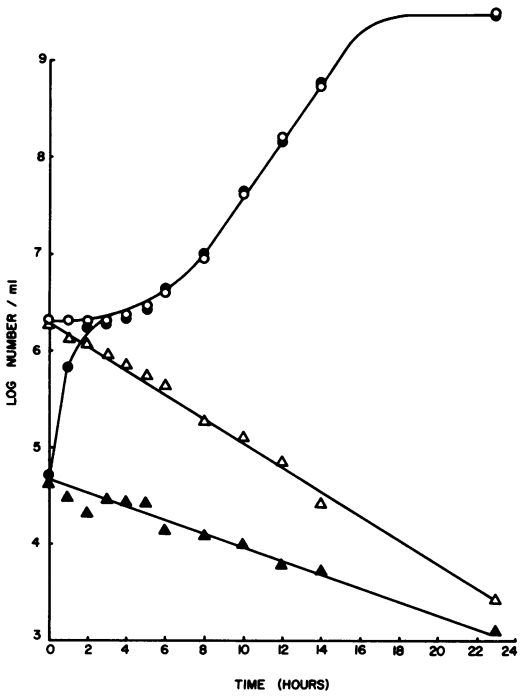

FIG. 3. *Recovery and growth of heat-injured Salmonella typhimurium 7136 incubated in CM plus 75 μg of 2,4-dinitrophenol per ml. The cells were heat-injured in 100 mM potassium phosphate buffer, pH 6.0, for 30 min at 48 C. Cells incubated in CM: ○, plated on TSA-citrate; ●, plated on EMB-NaCl. Cells incubated in CM plus 75 μg of 2,4-dinitrophenol per ml: △, plated on TSA-citrate; ▲, plated on EMB-NaCl.*

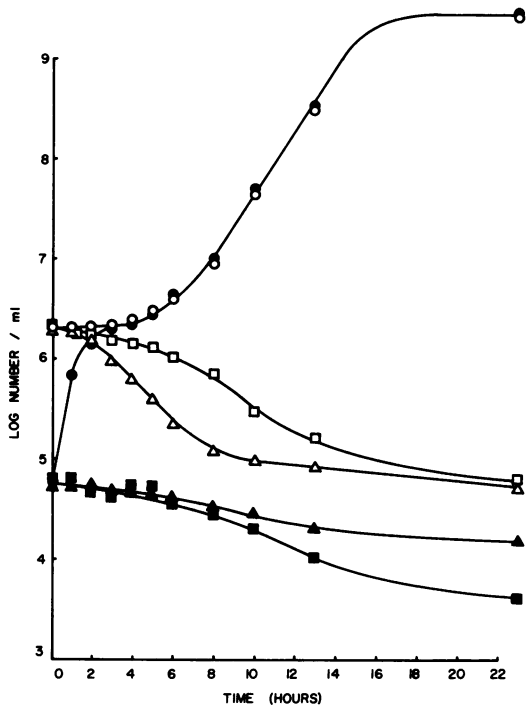

FIG. 4. *Recovery and growth of heat-injured Salmonella typhimurium 7136 incubated in CM plus 100 μg of chloramphenicol or 30 μg of chlorotetracycline per ml. The cells were heat-injured in 100 mM potassium phosphate buffer, pH 6.0, for 30 min at 48 C. Cells incubated in CM: ○, plated on TSA-citrate; ●, plated on EMB-NaCl. Cells incubated in CM plus 100 μg of chloramphenicol per ml; □, plated on TSA-citrate; ■, plated on EMB-NaCl. Cells incubated in CM plus 30 μg of chlorotetracycline per ml: △, plated on TSA-citrate; ▲, plated on EMB-NaCl.*

decreased at a faster rate than the EMB-NaCl counts.

As citrate was the only carbon source available to the cell, the major part of the adenosine triphosphate formed was from oxidative phosphorylation along the electron transport chain. It is well documented that 2,4-dinitrophenol is able to uncouple oxidative phosphorylation by an unknown mechanism (20). However, it has been demonstrated in *S. aureus* (9) and *S. typhimurium* (M. D. Pierson, Ph.D. thesis, Univ. of Illinois, 1970) that, if the recovery medium contains a compound from which substantial quantities of adenosine triphosphate are synthesized by substrate level phosphorylation (e.g., glucose), this effect of 2,4-dinitrophenol is not observed.

Effect of protein synthesis inhibitors on recovering cells. It has been reported that recovery from heat injury in *S. aureus* and *Streptococcus faecalis* is independent of protein synthesis (3, 4, 9, 25). In *S. typhimurium* recovery from heat injury is dependent on the formation of new protein (Fig. 4). When protein synthesis in recovering cells is stopped by the addition of 30 μg of chlorotetracycline per ml to the recovery medium, viable counts on both the TSA-citrate and EMB-NaCl media decreased with time. The injured population showed a greater susceptibility since there was a greater loss in counts on the TSA-citrate than on the EMB-NaCl medium. This result was substantiated by the addition of 100 μg of chloramphenicol per ml to the recovery medium (Fig. 4). Both chlorotetracycline (8, 15) and chloramphenicol (6, 29) are very potent inhibitors of protein synthesis.

Sogin and Ordal (25) used 5-methyltryptophan as an inhibitor of protein synthesis (14). They showed that, under such an inhibition, injured cells of *S. aureus* recovered, and growth ensued after the inhibition was relieved by the addition of a fourfold excess of L-tryptophan. When this experiment was performed with heat-injured *S. typhimurium*, a similar result was obtained (Fig.

5). The injured cells recovered their tolerance to EMB-NaCl in the presence of 5-methyltryptophan and grew out upon the addition of excess L-tryptophan. Moyed (18) demonstrated that 5-methyltryptophan blocks the biosynthesis of tryptophan by the allosteric inhibition of anthranilate synthetase. It has also been reported that 5-methyltryptophan is a leaky inhibitor since small amounts of protein are synthesized in its presence (17). The recovery of injured cells in the presence of 5-methyltryptophan could be due to either the synthesis of a small quantity of tryptophan, by incorporation of 5-methyltryptophan into the newly formed protein (17), or the synthesis of protein that was extremely low in or completely devoid of tryptophan.

The fact that *S. typhimurium* recovered but did not grow in the presence of 5-methyltryptophan indicated that large amounts of other enzymes and structural proteins were not required for recovery from heat injury. This has been supported by Pierson (Ph.D. thesis, Univ. of Illinois, 1970), who showed that the activities of glycolytic and tricarboxylic acid cycle enzymes in heat-injured *S. typhimurium* were similar to those of the unheated control. The results from the protein synthesis inhibitors (Fig. 4 and Fig. 5) demonstrated that some new protein was required for an injured cell to recover, and that the enzyme formed contained either negligible quantities of tryptophan or incorporated 5-methyltryptophan into the molecule without any loss in function.

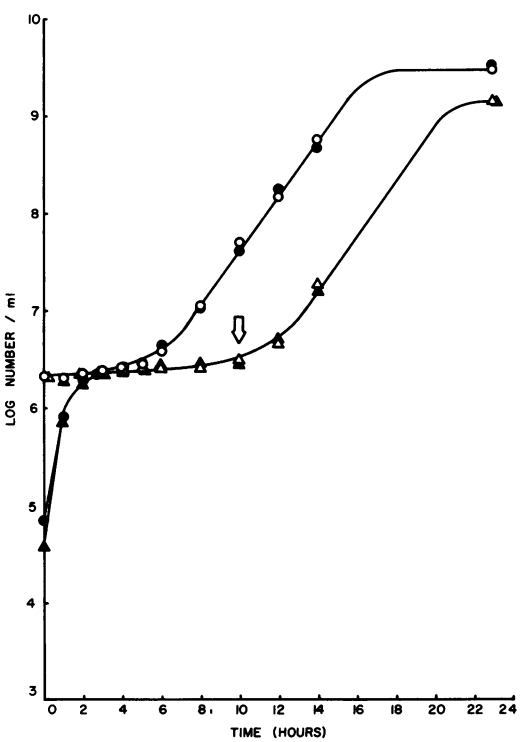

FIG. 5. *Recovery and growth of heat-injured Salmonella typhimurium 7136 incubated in CM plus 0.5 mg of 5-methyltryptophan per ml. The cells were heat-injured in 100 mM potassium phosphate buffer, pH 6.0, for 30 min at 48 C. Cells incubated in CM:* ○, *plated on TSA-citrate;* ●, *plated on EMB-NaCl. Cells incubated in CM plus 0.5 mg/ml 5-methyltryptophan:* △, *plated on TSA-citrate;* ▲, *plated on EMB-NaCl.* ⇩, *addition of a 4 fold excess of L-tryptophan.*

Independence of recovery on DNA synthesis. To prove to what extent DNA synthesis and hence cell division was involved in the recovery process, heat-injured cells were recovered in the presence of thymidine-*methyl*-^{3}H and L-leucine-*U*-^{14}C (Fig. 6). The ^{14}C-L-leucine incorporation into protein was used as a control. The ^{3}H-thymidine counts were very low throughout the first 5 hr of recovery but started to increase at a rapid rate thereafter. This coincided with the incubation time, after which multiplication was demonstrated (Fig. 2). These data are conclusive evidence that the biosynthetic processes observed after heat injury were due to recovery of the cells and not to a certain population of the cells undergoing division.

Dependence of recovery on RNA and protein synthesis. The incorporation of uracil-*6*-^{3}H and L-leucine-*U*-^{14}C into recovering cells of heat-injured *S. typhimurium* is shown in Fig. 7. After a slight lag, the ^{3}H-uracil counts increased linearly until they reached a plateau after 4 hr of recovery. The ^{3}H-uracil counts remained constant for 1 hr and increased again after 5 hr of recovery. The initial lag in the ^{3}H-uracil counts could be explained by the formation of new pools of intermediates required in RNA synthesis which were lost in the heating menstruum during injury (*unpublished data*) and from turnover of RNA molecules containing unlabeled uracil. The plateau reached after 4 hr indicated that the recovering cells had reached the required ribosome population for the particular growth rate of the cells in the citrate salts medium (16).

If the recovery process were dependent only on rRNA synthesis, the kinetics of synthesis during recovery and growth would have appeared similar to those reported in shift-up experiments (16). In such experiments no plateau in RNA synthesis is observed. The appearance of a plateau in rRNA synthesis during the recovery of heat-injured *S. typhimurium* indicated that, although rRNA synthesis was a requirement for recovery, its synthesis was not the rate-limiting step.

It is not surprising that very little protein synthesis took place during the early stages of recov-

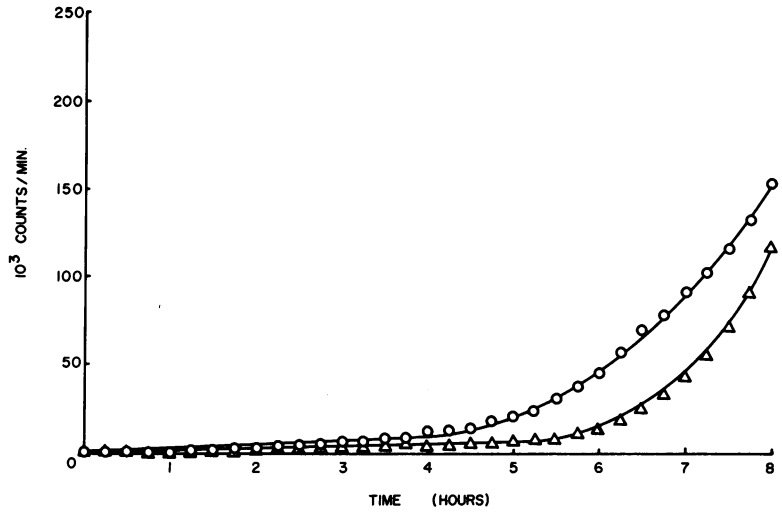

FIG. 6. *Incorporation of uniformly labeled ^{14}C-L-leucine and thymidine-methyl-^{3}H into the trichloroacetic acid-precipitable fraction of heat-injured Salmonella typhimurium 7136 during recovery and subsequent growth. The cells were heat-injured in 100 mM potassium phosphate buffer, pH 6.0, at 48 C for 30 min. The injured cells were then transferred to the CM recovery medium. One-milliliter samples were taken and mixed with an equal volume of cold 10% trichloroacetic acid. The precipitate was collected on 0.22-μm membrane filters, washed, dried, and counted. Symbols: O, uniformly labeled ^{14}C-L-leucine counts; Δ, thymidine-methyl-^{3}H counts.*

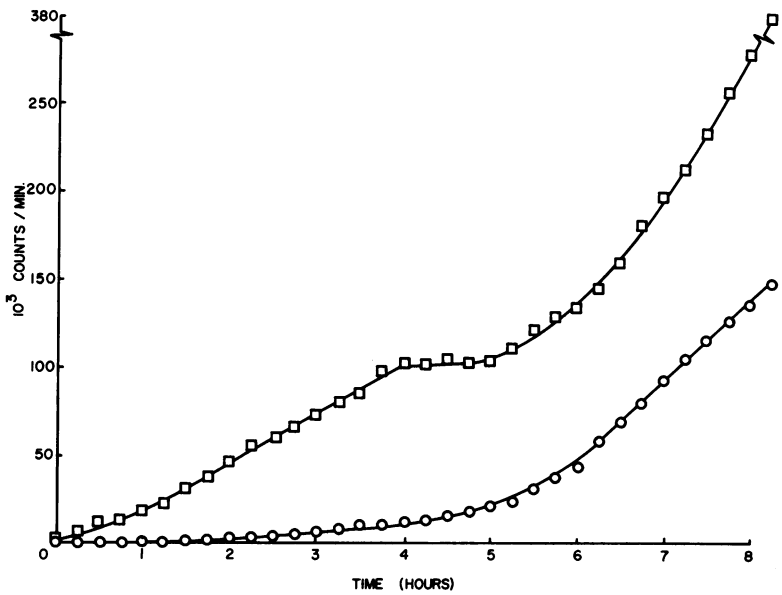

FIG. 7. *Incorporation of uniformly labeled ^{14}C-L-leucine and uracil-6-^{3}H into the trichloroacetic acid-precipitable fraction of heat-injured Salmonella typhimurium 7136 during recovery and subsequent growth. The cells were heat-injured in 100 mM potassium phosphate buffer, pH 6.0, at 48 C for 30 min. The injured cells were then transferred to the CM recovery medium. One-milliliter samples were taken and mixed with an equal volume of cold 10% trichloroacetic acid. The precipitate was collected on 0.22-μm membrane filters, washed, dried, and counted. Symbols: O, uniformly labeled ^{14}C-L-leucine counts; □, uracil-6-^{3}H counts.*

ery, since heat injury had degraded the rRNA and ribosomal particles which are required in protein synthesis. However, during the 3rd through 5th hr of recovery, the ^{14}C-leucine counts incorporated into protein started to increase (Fig. 7). There were probably sufficient ribosomes present after 3 hr of recovery to support this small amount of protein synthesis.

After 5 hr of recovery, the ^{3}H and ^{14}C counts increased very rapidly owing to growth. This can be verified by comparing the time course of the radioactive counts (Fig. 7) with the control cell counts (Fig. 2) and the DNA synthesis curve (Fig. 6).

Since RNA synthesis accounts for the bulk of the biosynthesis occurring during recovery, it is most likely that the functional protein formed is in some way involved in ribosome formation. One possibility is a nuclease. Tanichi et al. (27) reported on the amino acid composition and sequence of an extracellular staphylococcal nuclease which contained only one tryptophan residue. It is, therefore, not unlikely that a nuclease of *S. typhimurium* could also contain this negligible level of tryptophan. This would explain recovery in the presence of 5-methyltryptophan (Fig. 5) on the basis that tryptophan was not necessary for the formation of the enzyme. However, other possibilites exist: a methylase or a ribosomal protein, either of which could be thermolabile and consequently inactivated during heat injury. The rate-limiting step during recovery of *S. typhimurium* could then be attributed to the synthesis and activity of this functional protein.

ACKNOWLEDGMENTS

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