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Sublethal Heat Stress of *Vibrio parahaemolyticus*

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When *Vibrio parahaemolyticus* ATCC 17802 was heated at 41°C for 30 min in 100 mM phosphate-3% NaCl buffer (pH 7.0), the plate counts obtained when using Trypticase soy agar containing 0.25% added NaCl (0.25 TSAS) were nearly 99.9% higher than plate counts using Trypticase soy agar containing 5.5% added NaCl (5.5 TSAS). A similar result was obtained when cells of *V. parahaemolyticus* were grown in a glucose salts medium (GSM) and heated at 45°C. The injured cells recovered salt tolerance within 3 h when placed in either 2.5 TSBS or GSM at 30°C. The addition of chloramphenicol, actinomycin D, or nalidixic acid to 2.5 TSBS during recovery of cells grown in 2.5 TSBS indicated that recovery was dependent upon protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) synthesis. Penicillin did not inhibit the recovery process. Heat-injured, GSM-grown cells required RNA synthesis but not DNA synthesis during recovery in GSM. Chemical analyses showed that total cellular RNA decreased and total cellular DNA remained constant during heat injury. The addition of [6-^{3}H]uracil, L-[U-^{14}C]leucine, and [*methyl*-^{3}H]thymidine to the recovery media confirmed the results of the antibiotic experiments.

Many of the processing conditions used in the manufacture and preparation of food products cause stress or injury to microorganisms that might be present. Sublethal stresses of microbial cells can result in impaired metabolic and reproductive functions, which materially hamper the ability of common analytical methods (e.g., selective media and restrictive growth temperatures) currently used to qualitatively and quantitatively detect their presence in foods. Since the demonstration that *Vibrio parahaemolyticus* causes food poisoning, various methods for detecting both normal and stressed cells of this microorganism in contaminated seafoods have been investigated (5, 26).

Considerable effort has been devoted to studying the effects of sublethal heat stress on microorganisms and to determining those biosynthetic processes involved in the recovery of injured cells. Researchers have found that thermal injury in *Staphylococcus aureus, Salmonella typhimurium, Streptococcus faecalis, Bacillus subtilis, Escherichia coli, Pseudomonas fluorescens, Vibrio marinus,* and *Clostridium perfringens* involved alterations in their structural, permeability, and biosynthetic characteristics. Cell membrane damage has been demonstrated by increased salt sensitivity of the organism (G. L. Ades, Ph.D. thesis, Virginia Polytechnic Institute and State Univ. [VPI&SU], Blacksburg, 1973; 8, 9, 15, 22), leakage of intracellular components such as proteins, amino acids, 260-nm-absorbing material, and metal ions (1, 13, 16, 21, 23), and an increased sensitivity to food preservatives (R. I. Tomlins, M.S. thesis, Univ. of Illinois, Urbana, 1969). An alteration of the metabolic mechanisms of injured cells has been evidenced by an increased lag time, an altered nutritional demand for recovery and growth, loss of enzyme activity, an increased rate of glucose uptake, degradation of ribosomal ribonucleic acid (RNA), and single- and double-strand breakage in deoxyribonucleic acid (DNA; 2, 6, 11, 12, 17-20, 24, 25). Repair of these thermally induced lesions occurs during the recovery of the heat-injured microorganisms.

It was the purpose of this investigation to demonstrate heat injury and recovery in *V. parahaemolyticus* and to investigate some of the biosynthetic processes vital in cellular repair.

MATERIALS AND METHODS

Organism. The organism used in this study was *V. parahaemolyticus* ATCC 17802 (obtained from Carl Vanderzant, Texas A&M University, College Station).

Media and diluent. The undefined medium used for growth and recovery was Trypticase soy broth (BBL) plus 2.5% NaCl (2.5 TSBS). The defined system used for growth and recovery was a glucose salts

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medium (GSM; 4). Trypticase soy agar (BBL) plus 0.25% NaCl and Trypticase soy agar plus 5.5% NaCl (0.25 TSAS and 5.5 TSAS) were utilized as the assay media. The diluent was 100 mM potassium phosphate-3% NaCl buffer (pH 7.0).

Temperature control and culture agitation. Temperatures during growth, injury, and recovery were controlled within ±0.05°C by means of a Haake model E52 constant-temperature circulator (Haake Instruments, Inc., Rochelle Park, N.J.) in an insulated water bath. Continuous mixing of the cultures in a 250-ml, screw-capped Erlenmeyer flask was accomplished with a submersible magnetic stirrer (Cole-Parmer Instrument Co., Chicago, Ill.) placed in the water bath.

Growth and harvesting conditions. The cultures (125 ml) were grown at 35°C to the late logarithmic stage of growth. Cells grown in 2.5 TSBS were harvested at 6 h, and GSM-grown cells were harvested at 12 h. The cells were harvested by centrifugation in a Sorvall RC2-B refrigerated centrifuge (25°C) at 11,700 × *g* for 10 min. The supernatant was discarded, and the cell pellet was resuspended in 125 ml of 100 mM phosphate-3% NaCl buffer (pH 7.0) and centrifuged. The washed pellet was treated as described in the following sections.

Injury and recovery procedures. The washed cell pellet was resuspended in 5 ml of 100 mM phosphate-3% NaCl buffer (pH 7.0) and inoculated into 125 ml of the same buffer, pretempered at the desired injury temperature. Preliminary experiments showed that the injury temperature was 41°C for *V. parahaemolyticus* cells grown in 2.5 TSBS and 45°C for cells grown in GSM. The cells were heated for 30 min under constant agitation. At the termination of the heating period, the cells were harvested by centrifugation (25°C) at 11,700 × *g* for 10 min.

The pellet of injured cells was resuspended in 5 ml of 100 mM phosphate-3% NaCl buffer (pH 7.0) and added to 125 ml of the recovery medium pretempered at 30°C, to give 10^8 cells/ml. The recovery media were 2.5 TSBS and GSM. When metabolic inhibitors were used, the resuspended cells were diluted in buffer and added to 50 ml of the appropriate recovery medium containing the specific inhibitor, to give 10^6 cells/ml. When experiments involved a study of the incorporation of [6-^{3}H]uracil, [*methyl*-^{3}H]thymidine, and L-[U-^{14}C]leucine, the resuspended cells were diluted in buffer and added to 50 ml of the appropriate recovery medium containing the specific labeled compounds, to give 10^8 cells/ml.

Assay procedure. Assays for injury and recovery were done by a plate-counting technique. One-milliliter samples were removed from the heating menstruum or the recovery medium at various time intervals, serially diluted in 100 mM phosphate-3% NaCl buffer (pH 7.0), and pour-plated in duplicate with 0.25 TSAS and 5.5 TSAS. The plates were overlaid with the appropriate agar and incubated at 35°C for 24 to 48 h before counting. The 0.25 TSAS was used to estimate the total number of viable cells present in the heating menstruum or recovery medium, and the 5.5 TSAS was used to estimate the number of uninjured cells present. The difference between the 0.25 TSAS and the 5.5 TSAS counts was an estimate of the number of injured cells present.

Inhibitors. Metabolic inhibitors were added to the 2.5 TSBS recovery medium at the following concentrations: penicillin G (Calbiochem, Los Angeles, Calif.), 200 μg/ml; chloramphenicol (Calbiochem), 1.6 μg/ml; actinomycin D (Calbiochem), 5.0 μg/ml; and nalidixic acid (Sterling-Winthrop Research Institute, Rensselaer, N.Y.), 40.0 μg/ml. Actinomycin D (5.0 μg/ml) and nalidixic acid (80.0 μg/ml) were also used in the GSM recovery medium. The metabolic inhibitors were at a minimal inhibitory concentration for growth of normal cells, except for actinomycin D (B. S. Emswiler, Ph.D. thesis, VPI&SU, Blacksburg, 1974). Heat-injured *V. parahaemolyticus* cells were very sensitive to actinomycin D; therefore, 5 μg/ml rather than a minimal inhibitory concentration of 80 μg/ml was used.

Extraction and colorimetric determination of RNA and DNA. Samples (30-ml) were removed from the 41°C injury vessel and centrifuged at 20,000 × *g* for 10 min at 25°C. The resulting supernatants were discarded, and the RNA content of the cell pellets was determined according to the procedure of Herbert et al. (14). DNA was determined by Burton's (7) modification of the diphenylamine reaction after hot perchloric acid extraction. Yeast RNA (Sigma Chemical Co., St. Louis, Mo.) and calf thymus DNA type I (Sigma Chemical Co.) were used as standards.

Radiotracer experiments. Labeled uracil, thymidine, and leucine were added to the 2.5 TSBS recovery medium at the following final concentrations: [6-^{3}H]uracil (New England Nuclear Corp., Boston, Mass.; specific activity, 27.5 Ci/mmol), 5 μCi/ml, plus 10^{-1} mM carrier uracil; [*methyl*-^{3}H]thymidine (New England Nuclear Corp.; specific activity, 6.7 Ci/mmol), 5 μCi/ml; and L-[U-^{14}C]leucine (New England Nuclear Corp.; specific activity, 281 mCi/mmol), 0.2 μCi/ml. Concentrations of the same compounds in GSM during recovery were as follows: [6-^{3}H]uracil, 20 μCi/ml, plus 10^{-1} mM carrier uracil; [*methyl*-^{3}H]thymidine, 10 μCi/ml, plus 10^{-3} mM carrier thymidine; and L-[U-^{14}C]leucine, 0.2 μCi/ml, plus 2×10^{-3} mM non-radioactive leucine.

One-milliliter samples were taken from the recovery flasks at 30-min intervals and mixed with an equal volume of cold (0°C) 10% trichloroacetic acid. The samples were held at 0°C for 30 min, and the acid-insoluble fractions were collected by vacuum filtering on 0.45-μm pore size membrane filters of 47 mm in diameter (Millipore Corp., Bedford, Mass.). The precipitates were washed with three 3-ml volumes of cold (0°C) 5% trichloroacetic acid, and the filters were dried under a heat lamp and placed in scintillation vials containing 15 ml of scintillation fluid [5 g of 2,5-diphenyloxazole and 0.3 g of 2,2'-*p*-phenylene-bis-(5-phenyloxazole) per liter of liquid scintillation counting-grade toluene]. All radioactive samples were counted in a Beckman LS-100 liquid scintillation system for 10 min or until a 1.5% error had been attained, whichever occurred first.

RESULTS AND DISCUSSION

Injury and recovery curves. When cells of *V. parahaemolyticus* ATCC 17802 were grown in 2.5 TSBS and then placed in 100 mM potassium phosphate-3% NaCl buffer (pH 7.0) at 41°C,

there was an increase in sensitivity to 5.5 TSAS as heating time increased (Fig. 1A). In comparison to 5.5 TSAS, there was only a slight increase in sensitivity to 0.25 TSAS with increasing heating time. The difference between the 0.25 TSAS and 5.5 TSAS counts was an estimate of the number of injured cells. At the end of 30 min of heating, about 99.9% of the total viable cell population were unable to grow in 5.5 TSAS. The subsequent inoculation of the heat-injured cells into the recovery medium (2.5 TSBS) at 30°C supported the return of salt tolerance within 3 h, as evidenced by the increase in counts on 5.5 TSAS (Fig. 1B). The increase in salt sensitivity during heating was a result of loss of selective permeability of the cell membrane. The return of salt tolerance was most likely due to repair of heat-induced membrane damage. Beuchat (5) and Vanderzant et al. (26) have demonstrated that the increased sensitivity of stressed *V. parahaemolyticus* cells to various selective isolation media adversely affects detection and enumeration of the stressed cells.

When the cells were grown in GSM and then heated at 45°C, a similar pattern of injury was obtained. The cells rapidly lost their ability to grow on 5.5 TSAS, and after 30 min of heating at 45°C greater than 99% of the cell population were unable to grow in 5.5 TSAS. Inoculation of the injured cells into GSM at 30°C supported recovery of the injured cells within 3 h (data not presented).

Effect of metabolic inhibitors on recovery. Metabolic inhibitors were added to the recovery media to elucidate some of the biosynthetic processes involved during recovery. Penicillin G, chloramphenicol, actinomycin D, and nalidixic acid were added to separate recovery flasks simultaneously with the introduction of the thermally stressed *V. parahaemolyticus* cells to the recovery media. Penicillin G inhibits the synthesis of bacterial cell walls by blocking the cross-linking of linear peptido-polysaccharide chains into the complex peptidoglycan (10). Chloramphenicol inhibits protein synthesis without affecting RNA synthesis, and actinomycin D blocks the synthesis of RNA on a DNA template (10). The mode of action of nalidixic acid is the specific inhibition of DNA synthesis (3).

Heat-injured cells placed in 2.5 TSBS containing penicillin G recovered at the same rate and developed complete tolerance to 5.5 TSAS within 3 h, as did injured cells in the absence of penicillin G (Fig. 2). Penicillin G did not affect recovery but did inhibit subsequent multiplication and caused death of some cells after 3 h. It was therefore assumed that cell wall synthesis was not necessary for recovery. Chloramphenicol permitted a significant amount of recovery;

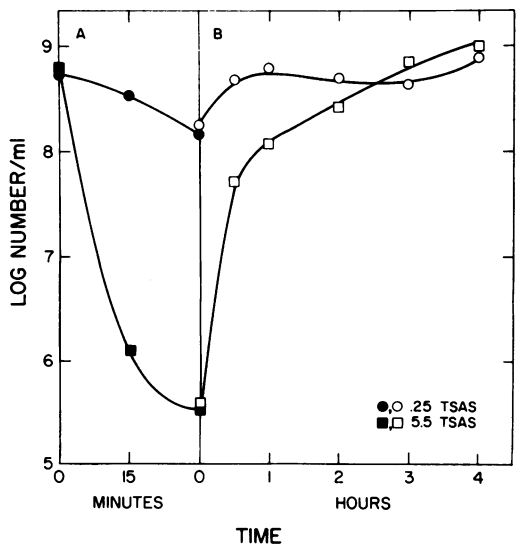

FIG. 1. *(A) Survival curves for 2.5 TSBS-grown V. parahaemolyticus ATCC 17802 heated in 100 mM phosphate-3% NaCl buffer (pH 7.0) at 41°C. (B) Recovery curve for 2.5 TSBS-grown V. parahaemolyticus ATCC 17802 heated for 30 min at 41°C and then inoculated into 2.5 TSBS and recovered at 30°C. Samples were plated on 0.25 TSAS and 5.5 TSAS.*

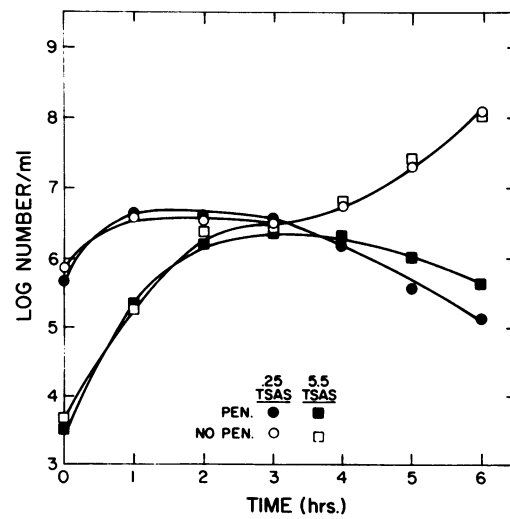

FIG. 2. *Effect of penicillin G (PEN) on the recovery of 2.5 TSBS-grown, heat-injured V. parahaemolyticus ATCC 17802. The cells were heated at 41°C in 100 mM phosphate-3% NaCl buffer (pH 7.0) for 30 min and recovered at 30°C in 2.5 TSBS and 2.5 TSBS containing 200 μg of PEN per ml. Samples were plated on 0.25 TSAS and 5.5 TSAS.*

however, after 1 h the lethal effect of the antibiotic became evident (Fig. 3). The fact that there was partial but incomplete recovery in the presence of chloramphenicol indicates that only part of the injured cell population suffered loss of protein, which was necessary for recovery. The addition of actinomycin D to the 2.5 TSBS recovery medium not only arrested recovery but also initiated a definite loss in viability of cells (Fig. 4). Similar results were obtained during the recovery of GSM-grown, heat-injured cells in GSM containing actinomycin D. These results indicated that degradation and resynthesis of RNA may have been involved in sublethal thermal injury and recovery of *V. parahaemolyticus*. Due to the loss of cell viability in the presence of actinomycin D, the role of RNA synthesis in recovery is not entirely clear.

The addition of nalidixic acid to the 2.5 TSBS recovery medium inhibited recovery of the thermally stressed cells and initiated a definite loss in viability (Fig. 5). The injured cell population was more sensitive to nalidixic acid than the uninjured cells, since the 0.25 TSAS counts decreased at a much faster rate than the 5.5 TSAS counts. The opposite effect was observed when nalidixic acid was added to GSM (Fig. 6). The GSM-grown, heat-injured cells in the presence of nalidixic acid recovered at the same time as did those in the absence of nalidixic acid. These results indicated that DNA damage occurred in

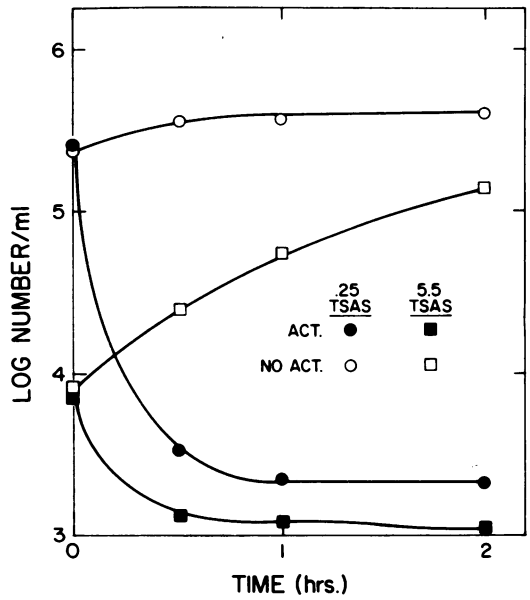

FIG. 4. *Effect of actinomycin D (ACT) on the recovery of 2.5 TSBS-grown, heat-injured V. parahaemolyticus ATCC 17802. The cells were heated at 41°C in 100 mM phosphate-3% NaCl buffer (pH 7.0) for 30 min and recovered at 30°C in 2.5 TSBS and 2.5 TSBS containing 5 μg of ACT per ml. Samples were plated on 0.25 TSAS and 5.5 TSAS.*

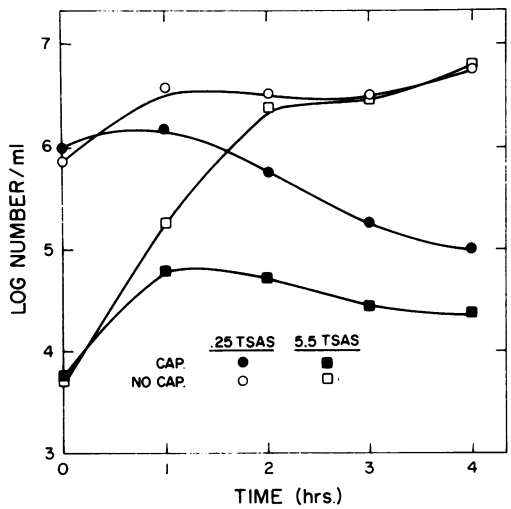

FIG. 3. *Effect of chloramphenicol (CAP) on the recovery of 2.5 TSBS-grown, heat-injured V. parahaemolyticus ATCC 17802. The cells were heated at 41°C in 100 mM phosphate-3% NaCl buffer (pH 7.0) for 30 min and recovered at 30°C in 2.5 TSBS and 2.5 TSBS containing 1.6 μg of CAP per ml. Samples were plated on 0.25 TSAS and 5.5 TSAS.*

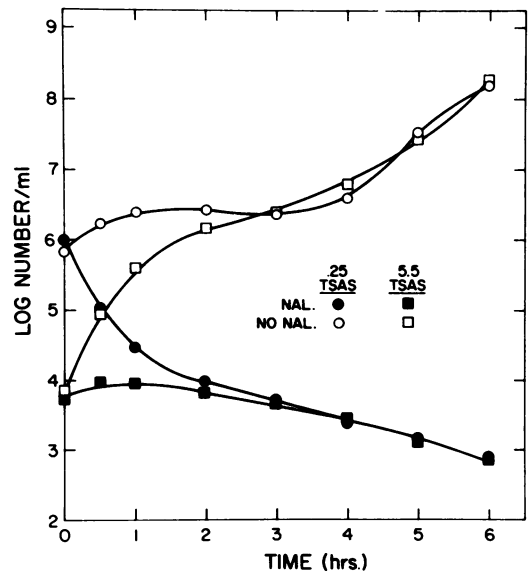

FIG. 5. *Effect of nalidixic acid (NAL) on the recovery of 2.5 TSBS-grown, heat-injured V. parahaemolyticus ATCC 17802. The cells were heated at 41°C in 100 mM phosphate-3% NaCl buffer (pH 7.0) for 30 min and recovered at 30°C in 2.5 TSBS and 2.5 TSBS containing 40 μg of NAL per ml. Samples were plated on 0.25 TSAS and 5.5 TSAS.*

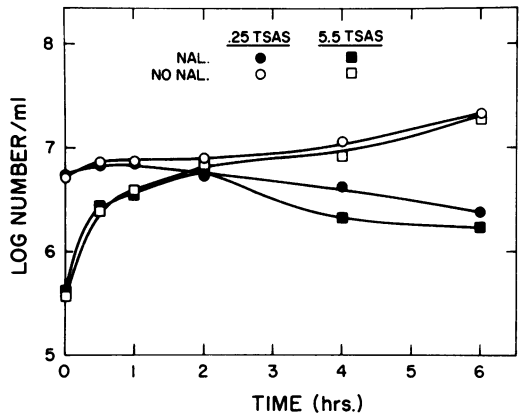

FIG. 6. *Effect of nalidixic acid (NAL) on the recovery of GSM-grown, heat-injured V. parahaemolyticus ATCC 17802. The cells were heated at 45°C in 100 mM phosphate-3% NaCl buffer (pH 7.0) for 30 min and recovered at 30°C in GSM and GSM containing 80 μg of NAL per ml. Samples were plated on 0.25 TSAS and 5.5 TSAS.*

heat-injured, 2.5 TSBS-grown *V. parahaemolyticus* but not in heat-injured, GSM-grown cells. Gomez and Sinskey (11) found evidence of DNA single-strand breakage with concomitant losses in viability when *S. typhimurium* LT-2 cells grown in a glucose salts broth were heated and subsequently placed in more complex media, such as Trypticase soy broth enriched with yeast extract or glucose salts broth supplemented with amino acids. They did not observe DNA breakage when the heat-injured cells were placed in the minimal medium, glucose salts broth. The possibility exists that DNA damage in 2.5 TSBS-grown cells of *V. parahaemolyticus* occurred when the heat-injured cells were placed in the complex recovery medium. Since there was no apparent DNA damage to GSM-grown cells during heat treatment and recovery in GSM, it is possible that the DNA damage incurred by the 2.5 TSBS-grown cells was the result of a combination of heat treatment and subsequent placement in a complex recovery medium.

Effect of heat injury on cellular RNA and DNA content. Heat treatment at 41°C caused a decrease in cellular RNA content. There was an immediate effect on the RNA component, with a net loss of approximately 48% during the 30-min heating period (B. S. Emswiler, Ph.D. thesis). The DNA content of the cells remained unchanged. The metabolic inhibitor studies using nalidixic acid indicated that DNA repair was involved during recovery of 2.5 TSBS-grown, heat-injured cells. The fact that cellular DNA content remained unchanged during heat injury suggested that DNA degradation did not involve leakage of DNA components from the injured cells. Strange and Shon (23) reported no change in DNA content of *Aerobacter aerogenes* during heat treatment at 47°C. Gomez and Sinskey (11) reported that DNA damage in heat-injured *S. typhimurium* LT-2 was in the form of single-strand breakage. It may be that DNA damage in 2.5 TSBS-grown, heat-injured *V. parahaemolyticus* also involved limited DNA strand breakage, in which case leakage from the cells would not occur.

Incorporation of [6-^{3}H]uracil and L-[^{14}C]-leucine. The incorporation of [6-^{3}H]uracil and L-[^{14}C]leucine during recovery of the 2.5 TSBS-grown, heat-injured cells in the presence and in the absence of the RNA inhibitor actinomycin D is shown in Fig. 7. The [6-^{3}H]uracil counts increased rapidly during the first 2 h of recovery in the absence of the inhibitor. This was followed by a plateau in label incorporation at 2-h recovery, which preceded recovery to salt tolerance by about 1 h. The plateau reached at 2 h indicated that the recovering cells had synthesized their full complement of RNA and that there was some other event that was rate limiting for complete recovery. After a 30-min lag period, the L-[^{14}C]leucine counts increased, indicating that protein synthesis was occurring during recovery of the injured cells. Very little protein synthesis occurred during the early stages of recovery, since heat injury had possi-

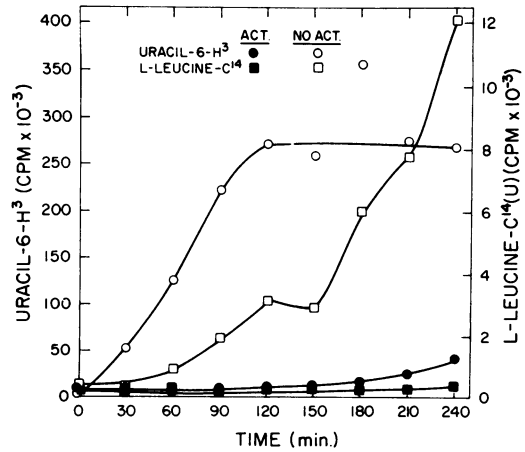

FIG. 7. *Incorporation of [6-^{3}H]uracil and L-[U-^{14}C]leucine into the cold trichloroacetic acid-insoluble fraction of 2.5 TSBS-grown, heat-injured V. parahaemolyticus ATCC 17802 during recovery. The cells were heated at 41°C in 100 mM phosphate-3% NaCl buffer (pH 7.0) for 30 min and recovered at 30°C in 2.5 TSBS and 2.5 TSBS containing 5 μg of actinomycin D (ACT) per ml.*

bly degraded the ribosomal RNA and ribosomal particles required in protein synthesis. There was no incorporation of either [6-^{3}H]uracil or L-[^{14}C]leucine in the presence of actinomycin D. The presence of the inhibitor prevented the synthesis of RNA and the recovery of the injured cells.

Uptake of labeled uracil by injured cells recovering in GSM in the absence of actinomycin D (data not presented) occurred at a much slower rate than in the nondefined 2.5 TSBS system. As was expected, actinomycin D inhibited incorporation of labeled uracil. Incorporation of labeled leucine occurred during recovery in the presence and in the absence of actinomycin D. These data suggest that there was no direct correlation between RNA and protein synthesis during recovery of GSM-grown, heat-injured cells and that heat-induced RNA damage was not as extensive in injured GSM cells as in injured 2.5 TSBS cells.

Incorporation of [*methyl*-^{3}H]thymidine and L-[^{14}C]leucine. Figure 8 shows the incorporation of tritiated thymidine and L-[U-^{14}C]leucine during recovery of 2.5 TSBS-grown, heat-injured *V. parahaemolyticus* in the presence and in the absence of the DNA inhibitor nalidixic acid. There was very rapid incorporation of tritiated thymidine during the first 30 min of recovery in the absence of nalidixic acid, which was followed by a plateau that lasted throughout the remainder of the recovery period. The appearance of a plateau in labeled thymidine incorporation indicated that, although DNA synthesis was a requirement for recovery, its synthesis was not the rate-limiting step. A direct count of the total ^{14}C and ^{3}H radioactivity in the recovery flask indicated that the plateau was not due to insufficient labeled thymidine. After a 1-h lag period, the labeled leucine counts increased, again indicating that protein synthesis was involved in recovery. There was no incorporation of labeled leucine in the presence of nalidixic acid. However, there was a small amount of tritiated thymidine incorporation in the presence of nalidixic acid, which could have been the result of a leaky type of inhibition mechanism or the incorporation of thymidine into compounds other than DNA.

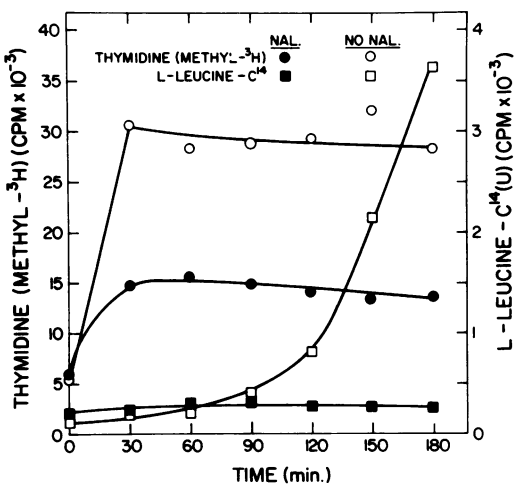

FIG. 8. *Incorporation of [methyl-^{3}H]thymidine and L-[U-^{14}C]leucine into the cold trichloroacetic acid-insoluble fraction of 2.5 TSBS-grown, heat-injured V. parahaemolyticus ATCC 17802 during recovery. The cells were heated at 41°C in 100 mM phosphate-3% NaCl buffer (pH 7.0) for 30 min and recovered at 30°C in 2.5 TSBS and 2.5 TSBS containing 40 μg of nalidixic acid (NAL) per ml.*

There was little or no incorporation of tritiated thymidine into GSM-grown, heat-injured cells during recovery in GSM in either the presence or absence of nalidixic acid (data not presented). These results substantiated previous conclusions that DNA synthesis was not involved during recovery of GSM cells. Labeled leucine counts increased during recovery in the presence and absence of nalidixic acid and reached a plateau at 2 h.

The nature of the DNA damage in 2.5 TSBS-grown, heat-injured *V. parahaemolyticus* ATCC 17802 is not known. Also, it cannot be explained why DNA damage occurred in heat-injured 2.5 TSBS cells but not in heat-injured GSM cells. Since there was no DNA damage in the GSM minimal medium system, it is possible that the DNA damage incurred by the 2.5 TSBS cells was the result of a combination of heat treatment and subsequent placement of the injured cells in a complex recovery medium (11). These are interesting findings in the field of thermal injury, but the significance and frequency with which these results occur in thermally stressed microorganisms has not yet been determined.

ACKNOWLEDGMENTS

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