Antimetabolite Sensitivity and Magnesium Uptake by Thermally Stressed *Vibrio parahaemolyticus*

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Metabolic inhibitors were added to a culture medium inoculated with thermally stressed *Vibrio parahaemolyticus* to obtain information pertaining to biosynthetic processes required for recovery from heat damage. Ribonucleic acid and protein syntheses, in addition to membrane repair, were required during recovery of injured cells. Neither nucleic acid nor Mg^{2+} leakage was noted to occur during the time cells were subjected to heat stress. Studies revealed that Mg^{2+} was apparently taken up by cells of *V. parahaemolyticus* during the first 30 min after thermal treatment, indicating a possible increased requirement for Mg^{2+} for membrane and/or ribosome stability and repair.

Investigations involving the addition of metabolic inhibitors to growth media have revealed much about biosynthetic processes required for repair of heat-damaged bacteria. Processes observed to be necessary for recovery of heat-stressed gram-negative bacteria include syntheses of ribonucleic acid (RNA) (2, 11, 20), protein (17, 20), and, in some instances, adenosine 5'-triphosphate (17). In freeze-injured cells, damage to the cell membrane is accompanied by increased permeability to surface-active agents (15), whereas, for heat-injured cells, membrane damage is accompanied by leakage of intracellular components such as protein, amino acids, enzymes (9), and nucleic acids (17). The presence of Mg^{2+} in a heating medium serves to protect cells from injury (10), and its presence in media used to recover thermally injured cells is beneficial (5, 8).

The study reported here was designed to investigate some of the biosynthetic mechanisms required for repair to thermally injured cells of *Vibrio parahaemolyticus* and to determine whether there is Mg^{2+} leakage during heat treatment or uptake during the recovery process.

MATERIALS AND METHODS

Organism. *V. parahaemolyticus* 8700, isolated from stools of a patient suffering from gastroenteritis (13), was used in this study.

Procedure for determining injury site. Mid to late exponential growth phase cultures (20 ml) of *V. parahaemolyticus* grown at 30°C in tryptic soy broth (TSB, Difco Laboratories, Detroit, Mich.) (pH 7.2) containing 3% NaCl were transferred to a 500-ml Erlenmeyer flask containing 190 ml of a 3% NaCl (wt/vol) solution buffered at pH 7.3 with 0.1 M potassium phosphate (salt buffer). The buffer had been equili-

brated to 45°C by submerging flasks in a Thelco water bath (Precision Scientific, Chicago, Ill.) heated by a thermostatically controlled immersion heater (Haake, Saddle Brook, N.J.). Previous studies (5) showed that thermal injury resulted from heating at 45°C for 8 min. After continuous agitation for 8 min, aliquots of the suspension were cooled to 23 to 25°C and 1 ml was immediately added to the recovery medium (100 ml of 3% NaCl-TSB at 30°C) containing selected antimicrobial agents (see below). The flasks were shaken at 150 rpm on an Aquatherm waterbath shaker (New Brunswick Scientific, New Brunswick, N.J.) adjusted to 30°C. Nonheated (control) cells were diluted in salt buffer at 23°C to give approximately the same viable population (100 cells per ml) as the heated suspensions before inoculating 3% NaCl-TSB containing antimicrobial agents. At 30-min intervals, 1-ml samples were removed from the recovery medium, diluted in salt buffer, and surface plated on thiosulfate-citrate-bile salts-sucrose agar (TCBS, BBL). Colonies were counted after incubation at 37°C for 12 to 15 h.

Antimicrobial agents studied. Inhibitors of specific biosynthetic processes were added to 3% NaCl-TSB recovery medium to detect heat-damaged sites. Cycloserine, 2,4-dinitrophenol, kanamycin sulfate, rifampin, actinomycin D, and nalidixic acid were prepared in distilled water at concentrations 100-fold greater than that required for the test and filter sterilized through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.). Sodium deoxycholate and sodium lauryl sulfate were prepared in distilled water and autoclaved for 15 min at 121°C. Concentrations found to have minimal or no effect on control cells were selected to test the response of heated cells. Shortly before initiating tests to determine the site of cell injury, 1 ml of the appropriate metabolic inhibitor was added to a 250-ml Erlenmeyer flask containing 100 ml of the 3% NaCl-TSB recovery medium (30°C). This procedure was followed to avoid potential time-dependent reactions between the inhibitors and constituents in the recovery medium.

A scheme was arbitrarily established to interpret results from inhibitor studies. The difference (percentage) in viable cell population between cultures with and without a test inhibitor was determined. This evaluation was made for both control and heated suspensions after various incubation periods. Ideally, after any given growth period there should be no difference in viable counts in control cultures with and without a test inhibitor. However, in some instances, the inhibitor had a slight detrimental effect on growth. Corrections were made for the effect of the inhibitor on control cells, and the net percentage decrease in viable population of the heated cells was obtained.

Based on the net magnitude of the percentage difference between control and heated cell populations, an arbitrary scale was established: none (−, no effect); slight (+, 0–20); moderate (++, 20–40); and marked (+++, greater than 40). A sample calculation follows.

Kanamycin sulfate (10 μg/ml) after a 60-min recovery time: control (no agent), 178% of initial count; (with agent), 169% of initial count—correction term = 178 − 169 = 9; heated (no agent), 124% of initial count; (with agent), 96% of initial count—heated cell percentage decrease due to inhibitor = 124 − 96 = 28. Thus, the net percentage decrease in viable population for heated cells due to kanamycin sulfate = (heated cell percentage decrease due to inhibitor) − (correction term) = 28 − 9 = 19 = +, or slight effect.

Magnesium and nucleic acid leakage studies. A mid to late exponential growth phase culture of *V. parahaemolyticus* grown in TSB containing 0.5, 3.0, or 7.5% NaCl was centrifuged at 3,000 × *g* at 25°C for 20 min using a Sorvall RC2-B centrifuge (DuPont Instruments, Newtown, Conn.). The pellet was resuspended in 10 ml of 3% NaCl (pH 7.2). Centrifugation and resuspension of cells gave sufficiently high populations to permit the detection of both nucleic acid and Mg^{2+} leakage, if either were to occur. An 8-ml sample of cell suspension was inoculated into 72 ml of 3% NaCl solution tempered at 45°C. Aliquots (10 ml) were taken at various heating intervals and either diluted in salt buffer and plated on TCBS (to determine viable population) or centrifuged at 25,000 × *g* for 8 min. The supernatant was examined for nucleic acid and Mg^{2+} content. Leakage of 260-nm-absorbing materials was determined using a Beckman DB-G grating spectrophotometer with a hydrogen lamp source (Beckman Instruments, Inc., Fullerton, Calif.). Magnesium concentration in the supernatant was determined using a Perkin-Elmer 404 Atomic Absorption Spectrometer (Perkin-Elmer, Norwalk, Conn.). Blanks consisted of the noninoculated 3% NaCl solution. Nonheated cells were examined similarly for leakage of Mg^{2+} and 260-nm-absorbing materials.

Magnesium uptake by injured cells. The same general procedure outlined in the previous section was followed to test for magnesium uptake by injured cells. After cells were heated for 8 min at 45°C, 8-ml samples were immediately placed in 72 ml of 3% NaCl (30°C) containing one of three Mg^{2+} levels (0, 12.7, and 25.4 μg of Mg^{2+} per ml as $MgCl_2 \cdot 6H_2O$). At 30-min intervals, 10-ml samples were removed and either diluted in salt buffer and plated on TCBS to determine viable population or centrifuged at 25,000 × *g* for 8 min. The supernatant was analyzed for Mg^{2+} concentration. As

in the leakage study, blanks consisted of the uninoculated 3% NaCl diluent. Control cells were examined similarly after diluting to approximately the same population per milliliter as the heated cells.

Data presented in this paper are mean values of a minimum of four trials run in duplicate.

RESULTS

Determination of injury site. Neither 2,4-dinitrophenol (7.35 μg/ml) nor nalidixic acid (0.025 μg/ml) had inhibitory effects on the recovery of heat-injured *V. parahaemolyticus* (Table 1). Higher levels of both antimetabolites inhibited growth of both control and heated cells.

Delayed effects on recovery were observed for cycloserine and kanamycin sulfate (both at 10 μg/ml), with a marked to moderate effect commencing 30 and 90 min after heat treatment, respectively. Inhibitory effects of kanamycin sulfate were not observed after 90 min of incubation, whereas inhibition continued in 3% NaCl-TSB containing cycloserine.

Rifampin (0.025 μg/ml) and actinomycin D (0.7 μg/ml) markedly inhibited the repair of heated cells immediately after transferring to the recovery medium. This effect continued throughout the incubation period. Sodium lauryl sulfate and sodium deoxycholate (100 μg/ml) resulted in a moderate to marked inhibitory effect on the recovery of heated cells after 60 min which continued throughout the recovery period.

Magnesium and nucleic acid leakage studies. No increase in absorbance at 260 nm or in Mg^{2+} concentration was observed in supernatants collected from suspensions of cells heated at 45°C or maintained at 22°C for periods up to 30 min. Plating of heated and control cells on TCBS at 10-min intervals over a 30-min period revealed that substantial death occurred

TABLE 1. *Action of antimicrobial agents on recovery of heat-injured V. parahaemolyticus in TSB containing 3% NaCl*

Agent (μg/ml)	Effect (at min after heating)[a]			
	30	60	90	120
2,4-Dinitrophenol (7.35)	−	−	−	−
Cycloserine (10.0)	+	+++	+++	+++
Kanamycin sulfate (10.0)	+	+	++	−
Rifampin (0.025)	+++	+++	+++	+++
Actinomycin D (0.7)	+++	+++	+++	+++
Nalidixic acid (0.025)	−	−	−	−
Sodium lauryl sulfate (100.0)	+	++	+++	+++
Sodium deoxycholate (100.0)	+	+++	+++	+++

[a] Inhibition: none (−); slight (+); moderate (++); marked (+++).

at 45°C (Fig. 1).

Magnesium uptake by heated cells. Magnesium uptake was evident for both heated and nonheated cells originally grown in TSB containing each of the three NaCl levels (Fig. 2). Consistently lower Mg^{2+} concentrations were noted in supernatants from suspensions inoculated with heated cells, as compared with those from control cells. Uptake was most pronounced for heated cells during the first 30 min after transferring to holding media. This observation was consistent for heated cells which were grown in TSB at all three NaCl levels; however, uptake was most marked for cells grown in 7.5% NaCl-TSB. For cells grown in this medium, there appeared to be a release of Mg^{2+} from heated and control cells between 30 and 90 min when holding in 3% NaCl solution initially containing 25.4 µg of Mg^{2+} per ml. This may be due in part to autolysis. At 12.7 µg of Mg^{2+} per ml, this effect was not as pronounced for heated or nonheated cells.

Plate counts made at 30-min intervals re-

vealed that the number of cells capable of forming colonies on TCBS increased during the 90-min holding period, regardless of whether cells had been heat stressed and regardless of the presence of added Mg^{2+} in the 3% NaCl holding medium (Fig. 3).

DISCUSSION

Determination of injury site. 2,4-Dinitrophenol acts to uncouple oxidative phosphorylation (12). Data presented here showing that 2,4-dinitrophenol was ineffective against heat-stressed *V. parahaemolyticus* agree with observations reported by Pierson (Ph.D. Thesis, University of Illinois, Urbana, 1970) indicating that 2,4-dinitrophenol inhibited recovery of heat-injured *Salmonella typhimurium* in a citrate medium but not in media containing glucose. The recovery medium (3% NaCl-TSB) used in the present study contains glucose as a carbon and energy source and thus, although oxidative phosphorylation is blocked, sufficient adenosine 5'-triphosphate could be formed through sub-
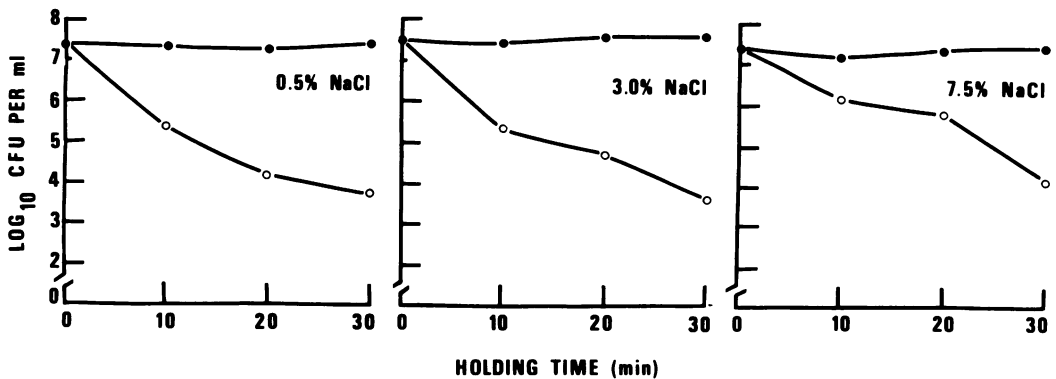

FIG. 1. *Effect of holding time in 3% NaCl on viability (colony-forming units, CFU) of control (22°C, ●) and heated (45°C, ○) cells of V. parahaemolyticus. Cells were originally grown in TSB containing 0.5, 3.0, and 7.5% NaCl.*
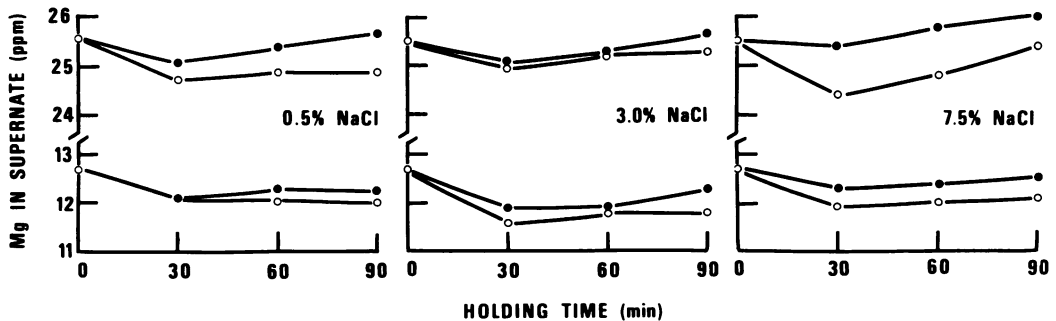

FIG. 2. *Magnesium uptake by heated and nonheated (control) cells of V. parahaemolyticus. Cells were grown in TSB containing 0.5, 3.0, and 7.5% NaCl before heating at 45°C for 8 min (○) or holding at 22°C for 8 min (●) and then transferring to 3% NaCl initially containing two levels of Mg^{2+} (12.7 and 25.4 µg/ml). Supernatants were analyzed for Mg^{2+} after holding periods of up to 90 min at 30°C.*
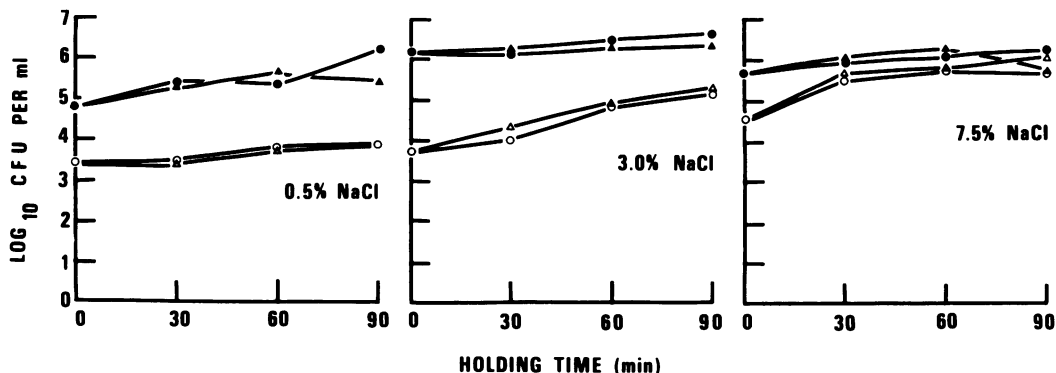
FIG. 3. *Viable counts (colony-forming units, CFU) for heated and nonheated cells of V. parahaemolyticus held for up to 90 min at 30°C after treatment. Cells were grown in TSB containing 0.5, 3.0, and 7.5% NaCl. Symbols:* ○, *cells heated in 3% NaCl for 8 min at 45°C (recovery in 3% NaCl);* ●, *cells held in 3% NaCl for 8 min at 22°C (recovery in 3% NaCl);* △, *cells heated in 3% NaCl for 8 min at 45°C (recovery in 3% NaCl plus 25.4 μg of Mg^{2+} per ml);* ▲, *cells held in 3% NaCl for 8 min at 22°C (recovery in 3% NaCl plus 25.4 μg of Mg^{2+} per ml).*

strate-level phosphorylation to aid in recovery.

Alanine racemase and D-alanine synthetase activities are inhibited by cycloserine, thereby blocking the selective incorporation of D-alanine from L-alanine into the cell wall peptidoglycan (14). The marked inhibitory effect of this antimetabolite on repair processes of heated *V. parahaemolyticus* cells indicates that cell wall synthesis is a critical step in recovery.

Protein synthesis is inhibited as a result of kanamycin sulfate acting on the 30S ribosomal subunit sometime during initiation, elongation, or termination (19). Protein synthesis was apparently involved during the first 90 min of recovery; this effect was also noted for heat-injured *S. typhimurium* (20).

Messenger RNA chain initiation (21) or chain propagation of messenger RNA beyond the first four nucleotides on a double-helical deoxyribonucleic acid (DNA) template (16) are inhibited by rifampin and actinomycin D. The continued inhibitory effect of these agents throughout the recovery period indicated that messenger RNA synthesis was necessary for recovery of heat-stressed cells of *V. parahaemolyticus* and was required within 30 min after injury.

Nalidixic acid selectively inhibits the synthesis of new DNA and possibly DNA repair by interfering with the attachment of DNA to the cell membrane (4). This inhibitor had no effect on the recovery of heated cells of *V. parahaemolyticus*, indicating that DNA polymerase activity may not be required for recovery from thermal injury. Gomez and Sinskey (3) reported that metabolic injury resulting from heat stress depended upon pretreatment growth conditions, metabolic injury occurring when cells were placed in a complex recovery medium. Emswiler

et al. (2) showed that DNA damage was detected in heat-injured *V. parahaemolyticus* when cells were recovered in complex media. These observations differ from those presented here and may be due to several factors, including the age of cells (6 h), heating time (30 min), and the additional stresses of washing and centrifugation induced during sample preparation.

The extent of injury to the cell lipopolysaccharide layer which normally protects the membrane lipoprotein layer against the lytic action of sodium lauryl sulfate and sodium deoxycholate (15) was determined by adding these surface-active agents to the recovery medium. Results indicate that lipopolysaccharide damage was present in heat-injured *V. parahaemolyticus*.

Magnesium and nucleic acid leakage studies. Since lipopolysaccharide damage was shown to occur during thermal treatment of *V. parahaemolyticus*, an additional study was conducted to determine whether damage was accompanied by leakage of intracellular nucleic acids or Mg^{2+}. The lack of nucleic acid leakage observed for heated *V. parahaemolyticus* was consistent with findings by Lee and Goepfert (10) for *S. typhimurium*. They reported that phosphate buffer was more deleterious to cells during heating than was a medium containing NaCl but no phosphate. Phosphate buffer was noted to activate ribonuclease (18); this would result in degradation of RNA and subsequent release through the damaged membrane into the suspending medium.

The lack of Mg^{2+} leakage may be due to an increased active involvement of the ion in repair mechanisms and/or active transport back into the cell if it is released. In the presence of

phosphate buffer, sufficient stress would be placed upon the organism so that active Mg^{2+} transport might be unable to occur, giving results similar to those reported by Hughes and Hurst (7) for *Staphylococcus aureus*. Plating of heated and control cells of *V. parahaemolyticus* on TCBS at 10-min intervals demonstrated that substantial death occurred at 45°C as evidenced by decreased colony counts for heated cells after extended heating times.

Magnesium uptake by heated cells. Previous studies showed that the addition of Mg^{2+} to liquid media generally led to a reduced lag time for injured *V. parahaemolyticus* and to increased counts on TCBS recovery agar (5). When it was observed that apparently no 260-nm-absorbing materials or Mg^{2+} leaked from injured cells and that both membrane and RNA damage occurred during heating, an additional study was undertaken to determine whether injured cells took up Mg^{2+} at a faster rate than did control cells when held in 3% NaCl immediately after treatment.

Increased Mg^{2+} uptake was observed for heated cells which were originally grown in TSB containing all three levels of NaCl. However, uptake was most marked for cells grown at the nonoptimal salt levels (0.5 and 7.5%). It is concluded that, although Mg^{2+} does not leak from cells of *V. parahaemolyticus* during heating, heat-injured cells require increased levels of Mg^{2+} during the repair process. Furthermore, the requirement for Mg^{2+} is immediate (within 30 min) and not dependent upon external nutrient sources. These observations lend support to a previous study indicating that Mg^{2+} enhances recovery of heated cells of *V. parahaemolyticus* (5).

Viable plate counts made at 30-min intervals during the holding (recovery) period revealed that there was an increased colony count with increased holding time for both heated and control cells after respective treatments. This increase in count confirms that repair and perhaps cell division may be taking place even in the absence of added nutrients. Within 90 min, repair progressed to an extent that recoverable population levels were observed that were near those occurring before heat treatment.

Observations presented here for *V. parahaemolyticus* are in accord with those reported for *S. aureus* (7, 8) and *Escherichia coli* (6). Injured *S. aureus* had an extremely high affinity for Mg^{2+}, even in the presence of ethylenediaminetetraacetic acid. Repair of these injured cells was associated with Mg^{2+} uptake; repaired cells bound Mg^{2+} less tightly than did injured cells. The addition of 5 mM Mg^{2+} to the recovery medium protected sublethally heated cells of *E.*

coli from death and structural injury. In the present study, Mg^{2+} is probably required for membrane stability (1) and ribosome stability (12) and repair.

The experiments described in this and previous studies (5) demonstrate the possible utility of adding Mg^{2+} to media used for the enumeration of *V. parahaemolyticus* from processed foods. Magnesium was especially beneficial to cells which had undergone heat treatment and then were immediately placed in media containing adequate nutrients for growth (5). Omission of Mg^{2+} from recovery media may lead to an underestimation of the extent of contamination in seafoods. It may be advisable, therefore, to supplement with Mg^{2+} media routinely used in procedures for detecting *V. parahaemolyticus*. Further studies are necessary to determine the effect of the presence and concentration of Mg^{2+} on growth of competitive microflora naturally occurring in seafoods before decisive recommendations can be made.

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