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Thermal Injury and Recovery of *Salmonella typhimurium* and Its Effect on Enumeration Procedures

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Exposure of *Salmonella typhimurium* 7136 to sublethal heating produced a temporary change in the tolerance of the organism to a particular stress medium. After sublethal heat treatment at 48 C for 30 min, greater than 90% of the viable population was unable to reproduce on Levine Eosin Methylene Blue Agar containing 2% NaCl. This sensitivity was dependent on the *p*H of the heating menstruum. In addition, the heated cells displayed a sensitivity to Brilliant Green Agar, Levine Eosin Methylene Blue Agar, Salmonella-Shigella Agar, and Desoxycholate Citrate Agar. Unheated cells displayed a sensitivity to Brilliant Green Agar, Salmonella-Shigella Agar, and Desoxycholate Citrate Agar. When the injured cells were placed in a suitable medium (Trypticase Soy Broth), they recovered and grew at a rate equal to that of normal cells. Recovery was also possible in Nutrient Broth, Lactose Broth, and Lauryl Tryptose Broth. Although recovery of the injured cell occurred in Tetrathionate Broth and Selenite F Broth, they were less than ideal growth media for the organism.

The presence of *Salmonella* in food products has become a subject of great concern in recent years. The introduction of a greater variety and volume of partially heat-processed frozen convenience foods has given rise to a greater chance of the survival of these organisms in the prepared food. The effect of heat on the death of *Salmonella* has been a subject of frequent investigation (9, 10, 12), but the effect of sublethal heat or thermal injury has received scant attention. In this study, we attempted to relate this thermal injury effect on *Salmonella* to the problems that might occur during use of the more common methods for *Salmonella* detection (14).

MATERIALS AND METHODS

Injury procedure. Frozen stock cultures of *S. typhimurium* 7136 (obtained from the National Center for Urban and Industrial Health, Cincinnati, Ohio) were prepared by placing 0.1 ml of an actively growing culture into 10 ml of Trypticase Soy Broth (TSB) and freezing at −20 C. The frozen tube was thawed when needed, and the entire 10 ml was inoculated into 200 ml of TSB in a 500-ml flask. After 16 hr of incubation at 37 C on a rotary shaker, a 40-ml sample was centrifuged at 2,050 × *g* for 10 min at room temperature. The supernatant fluid was decanted, and the cells were washed once in 100 mM phosphate buffer (*p*H 6.0), centrifuged, and suspended in 5 ml of phosphate buffer. These cells were heat-treated at 48 C for 30 min by adding the 5 ml of suspension to 195 ml of preheated 100 mM phosphate buffer, *p*H 6.0, which was constantly agitated. The treated suspension contained a cell concentration of approximately 10^9 cells/ml.

Assay procedure. Samples were removed from the heating menstruum at various intervals 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) and surface-plated on Levine Eosin Methylene Blue (EMB) Agar containing 2% NaCl (EMB-NaCl). The plates were incubated at 37 C for 48 hr. The TSA counts represented the total number of viable cells present in the heating menstruum, and the EMB-NaCl count represented the number of uninjured cells present. The difference between the TSA count and the EMB-NaCl count represented the number of injured cells present in the heating menstruum. This TSA and EMB-NaCl system was the primary assay system used in this study to evaluate heat injury and recovery.

Other assay systems examined for measuring injury were chosen from selective media used during the normal detection procedures for *Salmonella*. The media tested consisted of Bismuth Sulfite Agar (Difco), Brilliant Green Agar (Difco and BBL), Desoxycholate Agar (BBL), Desoxycholate Citrate Agar (Difco), Endo Agar (Difco), and Salmonella-Shigella Agar (Difco). All media were prepared by

recommended procedures. Plates were poured immediately after the media were sterilized, dried overnight at 37 C, and used within 48 hr. Samples were surface-plated as previously described.

Studies of *p*H were done by heating the organism at 48 C for 30 min in 100-mM PO_4 buffer prepared at various *p*H values between the range of 5.7 to 8.0. The cells were washed in the same buffer as was used in the injury vessel.

Recovery procedure. TSB was used as the primary control medium for recovery. The heat-injured cells were inoculated directly into 100 ml of recovery medium in a 250-ml flask and were incubated at 37 C in still culture. The same assay system was used for recovery as was previously described for injury.

Pre-enrichment and enrichment media generally used for detection and identification of *Salmonella* were also tested for their ability to support recovery of heat-injured organisms. Pre-enrichment media tested were Lactose Broth, Nutrient Broth, and Lauryl Tryptose Broth, made up by using Difco formulations and procedures. Enrichment media tested were Selenite F Broth (Difco) and Tetrathionate Broth (Difco).

RESULTS AND DISCUSSION

Differences in survival of *S. typhimurium* 7193 were found when the heated cells were plated on TSA and EMB-NaCl (Fig. 1). After 30 min of heating, the total viable population remained constant, as represented by the counts on TSA, but greater than 90% of the cell population displayed a sensitivity to the EMB-NaCl medium, as represented by a decrease in counts. Similar results have been observed with *S. senftenberg* 775W (8), *Staphylococcus aureus* MF-31(3), and *Streptococcus faecalis* R57 (1), when heated and plated on a particular stress medium.

The degree of injury obtained during heating in PO_4 buffer at 48 C varied with a change in the *p*H of the buffer. Injury at *p*H 5.7 and 6.5 showed only a slight variation from that at *p*H 6.0. However, at *p*H 7.2 after 30 min of heating, approximately 30% of the cells died and 97% of the remaining cells were injured (Fig. 2). When the *p*H was raised to 8.0, 89% of the cells died after 30 min of heating and 96% of the remaining cells were injured. Therefore, the heat resistance of the organisms becomes greater as the *p*H of the heating menstruum is lowered from 8.0 to below the neutral range. Similar results were observed by Osborne et al. (9), who studied the effect of *p*H on the heat resistance of *Salmonella* in liquid whole egg and egg whites.

Injured cells also demonstrated a marked difference in their ability to grow on solid selective media used for the isolation and identification of *Salmonella* (Table 1). Most of the media supported good growth of the normal cells; however,

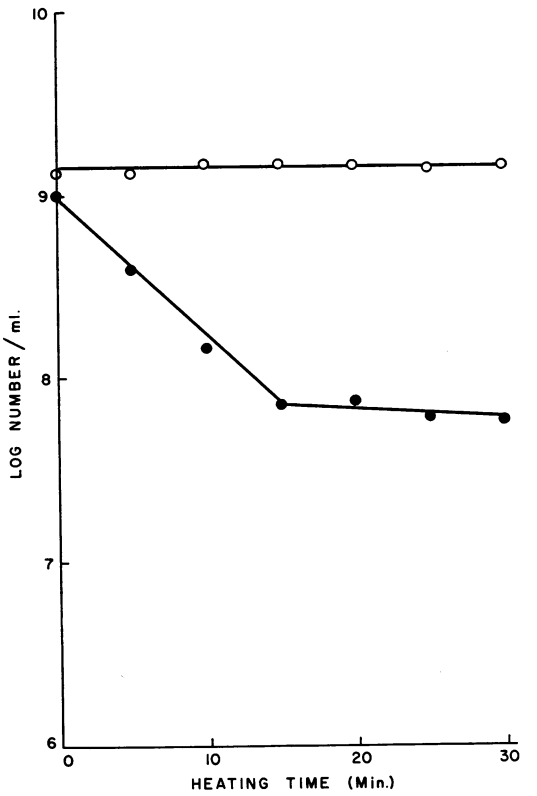

FIG. 1. *Survivor curves for S. typhimurium 7136 heated in 100 mM phosphate buffer, pH 6.0, at 48 C. TSA (○) was used as the control and EMB-NaCl (●) was used to show uninjured cells.*

considerable depression in counts of uninjured cells was observed on Brilliant Green Agar, Salmonella-Shigella Agar, and Desoxycholate Citrate Agar. After the 30-min heating period, the heat-injured cells were also sensitive to EMB Agar and EMB-NaCl as well as the three previously mentioned media. Both normal and thermally injured cells displayed no sensitivity to MacConkey Agar, Endo Agar, Bismuth Sulfite Agar, and Desoxycholate Agar. The productivity of injured cells on Brilliant Green Agar was greater than that of normal cells. This difference was greater when BBL agar was used than when Difco agar was used. Read and Reyes (11) observed that different species of *Salmonella* varied greatly in their productivity on various lots of Brilliant Green Agar, ranging all the way from 100% of a TSA count to what they described as generally unproductive or less than 0.02% of the TSA count. These results indicate that Brilliant Green Agar is less than an ideal medium for indicating numbers of both normal and stressed *Salmonella*. These data demonstrate some of the

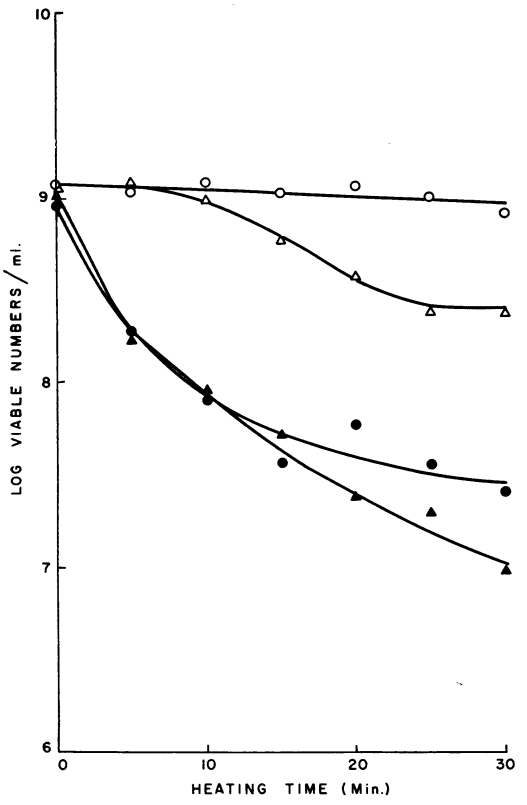

FIG. 2. *Survivor curves for S. typhimurium 7136 heated in 100 mM phosphate buffer at various pH values at 48 C. Symbols: ○, cells heated in pH 7.2 buffer and plated on TSA; ●, cells heated in pH 7.2 buffer and plated on EMB-NaCl; △, cells heated in pH 8.0 buffer and plated on TSA; ▲, cells heated in pH 8.0 buffer and plated on EMB-NaCl.*

problems involved in obtaining accurate counts of *Salmonella* present in a test product when plated on various selective media.

In addition to sensitivity to various selective media, the heated cells also displayed an increased lag when placed in TSB (Fig. 3). This extended lag with heated cells is a frequently observed phenomenon (1, 3–5, 8). During this extended lag, the cells completely recovered their tolerance to the EMB-NaCl medium and commenced to grow at a rate equal to that of normal cells and reached the same total viable population. Both the shape of the recovery curve and the absence of an increase in the TSA count indicate that this is a recovery rather than a growth process.

Injured cells were also able to recover in media used for pre-enrichment of *Salmonella*, namely Lactose Broth, Nutrient Broth, and Lauryl Tryptose Broth (Fig. 4). The recovery rate in all

TABLE 1. *Comparison of the productivity of various selective media for uninjured and thermally[a] injured cells of S. typhimurium 7136*

Selective agar	Per cent of initial TSA count	
	Normal cells	Thermally injured cells
Trypticase Soy	100	76
MacConkey	100	79
Endo	100	77
Bismuth Sulfite	100	76
Desoxycholate	95	73
Brilliant Green	39	66
EMB	94	37
Salmonella-Shigella	82	35
EMB-NaCl	85	2.3
Desoxycholate citrate	25	2.0

[a] Cells were thermally injured by heating in 100 mM PO_4 buffer, *p*H 6.0, at 48 C for 30 min.

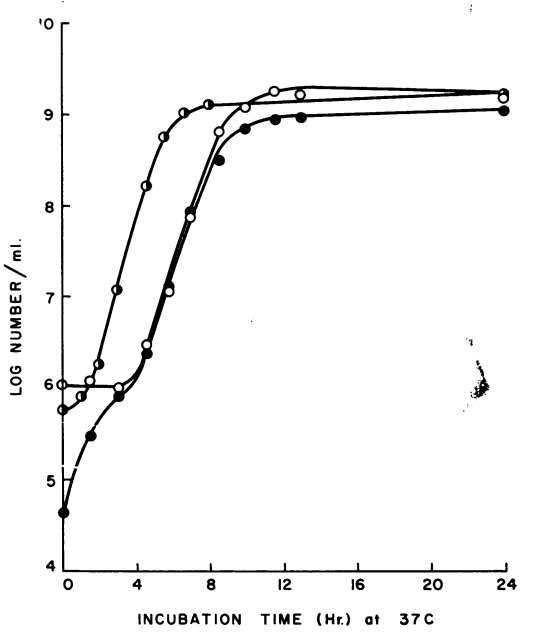

FIG. 3. *Recovery and growth of heat-injured S. typhimurium 7136, in TSB. The cells were heated in 100 mM phosphate buffer, pH 6.0, at 48 C for 30 min. Symbols: ○, heated cells plated on TSA; ●, heated cells plated on EMB-NaCl; ◑, unheated cells plated on TSA.*

of these media was slightly slower than in TSB, growth rates after completion of recovery were approximately the same, and total population reached after 24 hr of incubation was between 40 and 70% lower than that reached in TSB. Similar

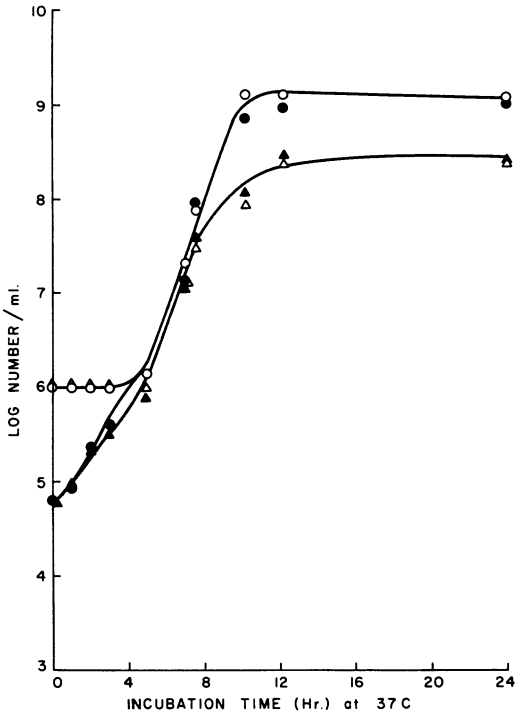

FIG. 4. *Recovery and growth of heat-injured S. typhimurium 7136 in TSB and pre-enrichment media. The cells were heated in 100 mM phosphate buffer, pH 6.0, at 48 C for 30 min. Symbols: ○, incubated in TSB and plated on TSA; ●, incubated in TSB and plated on EMB-NaCl; △, incubated in Nutrient Broth, Lactose Broth, or Lauryl Tryptose Broth and plated on TSA; ▲, incubated in Nutrient Broth, Lactose Broth, or Lauryl Tryptose Broth and plated on EMB-NaCl.*

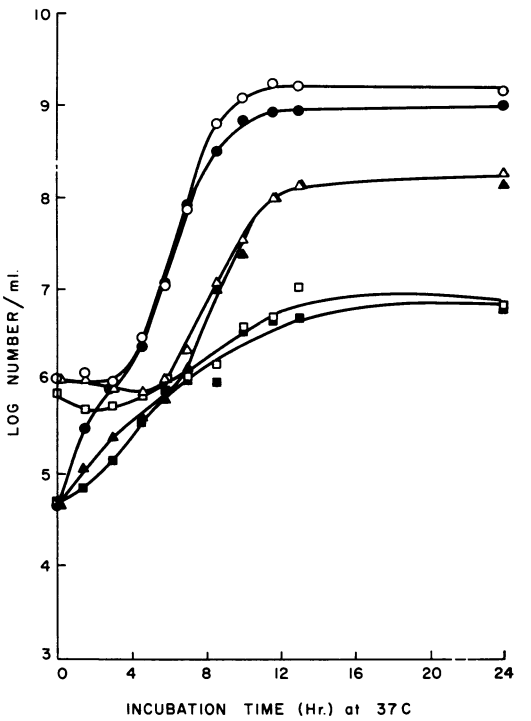

FIG. 5. *Recovery and growth of heat-injured S. typhimurium 7136 in TSB and enrichment media. The cells were heated in 100 mM phosphate buffer, pH 6.0, at 48 C for 30 min. Symbols: ○, incubated in TSB and plated on TSA; ●, incubated in TSB and plated on EMB-NaCl; △, incubated in Tetrathionate Broth and plated on TSA; ▲, incubated in Tetrathionate Broth and plated on EMB-NaCl; □, incubated in Selenite F Broth and plated on TSA; ■, incubated in Selenite F Broth and plated on EMB-NaCl.*

experiments with heat-injured *S. senftenberg* 775W showed that Lauryl Tryptose Broth is superior to either Lactose Broth or Nutrient Broth for recovery (8). Thus, it appears that the recovery requirements for *S. senftenberg* 775W are more exacting than for *S. typhimurium* 7136.

There are many variations of enrichment media used for *Salmonella* which have been developed for use for specific food products (2, 7, 13). However, for this study we used only Tetrathionate Broth and Selenite F Broth without any additives. The recovery and growth of injured cells in these media are shown in Fig. 5 The TSA count decreased during the recovery period in both of these media, this decrease being much more pronounced in the Selenite F Broth than in the Tetrathionate Broth. The rates of recovery of the injured cells in the two media did not differ greatly; however, after recovery the cells grew at a much faster rate and reached a much higher total population in Tetrathionate Broth than in Selenite F Broth. TSB was far superior to both Tetrathionate Broth and Selenite F Broth for recovery of injured cells as well as for measuring total population reached after 24 hr. The fact that up to 30% of the injured cells die when placed in enrichment media emphasizes the need for use of a less toxic pre-enrichment medium before inoculation into an enrichment medium. If the initial number of *Salmonella* present in a particular product is low and these cells have been exposed to some degree of heat stress, the death of injured cells in the enrichment media could cause significant error in the determination of the number of *Salmonella* present in the product. As previously described, this death does not occur in the less selective pre-enrichment media. North (6) substantiated these findings by use of Lactose Broth pre-enrichment for detecting *Salmonella* when present in small

numbers in dried egg products and found fewer skips by this method than by direct enrichment.

The experimental results have shown that cells of *S. typhimurium* 7136 can be heat-injured, as expressed by the assay systems of sensitivity to an EMB-NaCl medium, an extended lag phase of growth, and sensitivity to various selective media used for identification of *Salmonella* organisms. The overall effect of the heating process was enhanced by raising the *p*H of the heating menstruum from 6.0 to 8.0. However, for the purpose of this study, *p*H 6.0 provided the most desirable conditions. The heat-injured cells were able to recover in a rich medium such as TSB and also in preenrichment and enrichment media used for the determination of the presence of *Salmonella*. The data suggest that, when a procedure for determination of *Salmonella* is used and the suspect organisms have been exposed to some degree of thermal stress, it is important to include each step in the isolation procedure. Elimination of any step would probably yield erroneous results, since the test organism shows sensitivity to both enrichment media and selective media.

ACKNOWLEDGMENT

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