APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1983, p. 360–365
0099-2240/83/020360-06$02.00/0

Vol. 45, No. 2

Enhanced Recovery of Injured *Escherichia coli* by Compounds That Degrade Hydrogen Peroxide or Block Its Formation

LINDA C. McDONALD,[1] CAMERON R. HACKNEY,[1]* AND BIBEK RAY[2]

Department of Food Science, Louisiana Agricultural Experiment Station, Center for Agricultural Sciences and Rural Development, Louisiana State University, Baton Rouge, Louisiana 70803,[1] and Department of Animal Science, University of Wyoming, Laramie, Wyoming 82071[2]

Received 18 June 1982/Accepted 27 October 1982

Escherichia coli LSUFS was injured either by freezing at −10°C or by heating at 57°C for 12 min. Surviving cells were recovered on nonselective tryptone-glucose extract agar and selective violet red bile agar supplemented with compounds that degrade hydrogen peroxide or block its formation. Various concentrations of the following compounds were tested: sodium pyruvate, 3,3′-thiodipropionic acid, catalase, ascorbic acid, potassium permanganate, sodium thioglycolate, dimethylsulfoxide, ethoxyquin, *n*-propyl gallate, α-tocopherol sodium metabisulfite, and ferrous sulfate. Sodium pyruvate and 3,3′-thiodipropionic acid, when added to either medium, significantly ($P > 0.01$) increased recovery of injured cells. More than 90% of the heat-injured cells and 40 to 90% of the freeze-injured cells failed to grow on unsupplemented tryptone-glucose extract agar. Supplementation of violet red bile agar increased recovery, but the counts remained considerably lower than the tryptone-glucose extract agar counts. The repair detection procedure of Speck et al. (M. Speck, B. Ray, R. Read, Jr., Appl. Microbiol. **29**:549–550, 1975) was greatly improved by the addition of pyruvate or 3,3′-thiodipropionic acid. However, when this improved repair detection procedure was applied to foods, pyruvate-supplemented media showed some false-positives. We therefore recommend that 3,3′-thiodipropionic acid be used to supplement media in the repair detection procedure.

Microorganisms may become sublethally injured after exposure to many chemical and physical stresses (6, 10, 14, 18, 21). Injury may be measured by the difference in counts when stressed cells are simultaneously enumerated on selective and nonselective media. Only uninjured cells are recovered on selective medium, whereas the nonselective medium is assumed to recover both injured and uninjured cells (6, 11, 14). However, agents lethal to injured cells may be formed spontaneously in either selective or nonselective medium (2, 4, 7). Media containing manganese, citrate, or both will autooxidize and form peroxides in concentrations lethal to bacterial cells stressed by ionization (4). Carlsson et al. (7) reported the formation of superoxide radicals and hydrogen peroxide in anaerobic broth media exposed to oxygen. The amount of phosphate and reducing sugars influences H_2O_2 formation. When thioglycolate is present in the media, H_2O_2 formation is inhibited but superoxide radicals are still formed.

Supplementation of media with compounds which degrade H_2O_2 has been studied (2, 16, 20). Baird-Parker and Davenport (2) reported that incorporation of pyruvate into selective media enhances the recovery of *Staphylococcus aureus*. Martin et al. (16) noted improved recovery of injured *S. aureus*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, and *Escherichia coli* when media selective for these microorganisms are supplemented with either catalase or sodium pyruvate (hereafter referred to as pyruvate). Rayman et al. (20) reported that the addition of pyruvate to nonselective tryptic soy agar greatly increases recovery of heat-injured *Salmonella senftenberg*.

Methods have been developed which allow injured microorganisms to repair before being exposed to a selective medium (12, 18, 22). The repair detection procedure of Speck et al. (22) allows injured microorganisms to repair in a small volume of nonselective medium before being overlaid with the desired selective medium. However, this method does not take into consideration the possible inhibitory effects of peroxides spontaneously formed in the nonselective medium. The purpose of this study was to determine the effect on injured-cell recovery of medium supplementation with compounds

TABLE 1. Source, type, concentration and function of supplements

Supplement	Concn	Function	Time added relative to sterilization	Source
Campylobacter growth supplement[a]	0.75%	Degrades H_2O_2, scavenges O_2^- radical (13)	After	Oxoid Ltd.
Catalase	75 U/ml	Degrades H_2O_2 (16)	After	Sigma Chemical Co.
Dimethyl sulfoxide	0.5 and 1.0%	Scavenges O_2^- (17)	After	Sigma Chemical Co.
Ethoxyquin	0.1 and 0.05%	Antioxidizes (15)	After	Pfaltz & Bauer
Potassium permanganate	0.15 and 0.25%	Degrades H_2O_2 (20)	After	J. T. Baker Chemical Co.
n-Propyl gallate	0.125 and 0.25%	Antioxidizes (15)	Before	Sigma Chemical Co.
Sodium ascorbate	0.5 and 1.0%	Antioxidizes (15)	After	Sigma Chemical Co.
Sodium metabisulfite + ferrous sulfate	0.25% each	Scavenges O_2^- (13)	After	Mallinckrodt Chemical Co.
Pyruvate	0.33, 0.66, and 1.2%	Degrades H_2O_2 (16)	Before	Sigma Chemical Co.
Thioglycolate	0.02 and 0.05%	Blocks formation of H_2O_2 (7)	Before	Difco Laboratories
TDPA	0.25, 0.05, and 1.0%	Antioxidizes (15)	Before	Aldrich Chemical Co.
Vitamin E	50 U/ml	Antioxidizes (15)	After	Sigma Chemical Co.

[a] Contains pyruvate, sodium metabisulfite, and ferrous sulfate (0.25% each).

that either degrade H_2O_2 or block its formation. We then applied these results to established repair detection procedures (18, 22).

MATERIALS AND METHODS

Test organisms and method of injury. *E. coli* LSUFS (type 1) was used throughout. It was maintained on tryptone-glucose extract agar (Difco Laboratories, Detroit, Mich. [TGE]) slants and transferred weekly.

For injury studies, the cells from the slants were inoculated into 10 ml of tryptic soy broth (Difco) and incubated overnight at 35°C. The contents of the tube were then transferred to 90 ml of tryptic soy broth and further incubated. Growth was followed spectrophotometrically (Spectronic 70; Bausch & Lomb, Inc., Rochester, N.Y.) at 650 nm to an absorbance of 1.0. This procedure yielded cells in the late exponential growth phase. The cells were harvested by centrifugation at 7,000 × *g* for 12 min and suspended in sterile distilled water to an optical density at 650 nm of 1.0.

Portions of the cell suspensions (5 ml) were subjected to stress by freezing at −10°C for 48 h or by heating at 57°C for 12 min.

Enumeration of cells. Cells were enumerated before and after being stressed by pour plating with nonselective TGE and selective violet red bile agar (Difco [VRBA]) supplemented with various concentrations of compounds that either have an antioxidant function or degrade hydrogen peroxide (Table 1). Pyruvate, sodium ascorbate, 3,3'-thiodipropionic acid (TDPA), and sodium thioglycolate were added before autoclaving (TGE) or boiling (VRBA). These compounds had been dissolved in distilled water and, with the exception of TDPA, the pH had been adjusted to 7.3 with 1.0 N sodium hydroxide. The pH of TDPA had been adjusted to 6.5 with 10 N NaOH and then to 7.3 with 1.0 N NaOH. Heating to approximately 60°C dissolved TDPA more quickly. The desired medium was then added and prepared according to the directions of the manufacturer.

Solutions of catalase, *n*-propyl gallate, ethoxyquin, sodium metabisulfite, ferrous sulfate, and dimethyl sulfoxide were filter sterilized and added to the media after cooling to 45°C. Vitamin E was dissolved in a small volume of ethanol before addition to the tempered media.

All plates were incubated for 24 h at 35°C. Relative recovery efficiency was calculated from the colony counts obtained with the supplemented and unsupplemented media.

Repair detection procedure. Stressed cells were serially diluted and plated with 5 ml of TGE. The plates were incubated at 25°C (room temperature) for up to 2 h to facilitate repair. At intervals, duplicate plates were overlaid with 10 to 15 ml of VRBA to provide selective environments. In the modified procedures, TGE, VRBA, or both were supplemented with either pyruvate (0.66%) or TDPA (1.0%) before plating. The plates were incubated at 35°C for 24 h and colonies were counted. Coliform colonies in the repair detection procedure appear identical to coliform colonies in VRBA (19, 22).

Enumeration of coliforms from foods. Food samples were obtained from retail stores in the Baton Rouge area, transported to the Food Science Department, Louisiana State University, and stored frozen or refrigerated until tested. For analysis, we blended 25 g of each sample with 225 ml of sterile peptone-salinewater (0.1% peptone [Difco Laboratories, Detroit, Mich.], 0.85% NaCl, 0.01% Antifoam B [Sigma Chemical Co., St. Louis, Mo.]). Serial dilutions were made in peptone-saline-water as needed. Coliforms were enumerated directly with VRBA by the repair detec-

TABLE 2. Percent increase in colony counts due to supplementation after freezing cells for 48 h at −10°C

Supplement and concn	% Increase after supplementation[a]	
	TGE	VRBA
Pyruvate		
1.2%	1,633[b]	1,512[b]
0.66%	2,100[b]	1,129[b]
0.33%	1,350[b]	443[b]
TDPA		
1%	2,566[b]	857[b]
0.5%	867[c]	479[b]
0.25%	213	200[c]
Sodium ascorbate		
1.0%	503[c]	71
0.5%	227	14
Catalase	106	100[c]
Thioglycolate		
0.05%	59	−29
0.02%	29	−43
Dimethyl sulfoxide		
0.5%	43	71
Pyruvate + TDPA		
0.25% + 1.0%[d]	2,133[b]	829[b]
0.25% + 0.5%[d]	1,027[c]	629[b]
Campylobacter growth supplement	89	20
Vitamin E	−22	15

[a] Percent increase = [(counts in supplemented medium − counts in unsupplemented medium)/counts in unsupplemented medium] × 100. Ethoxyquin, potassium permanganate, *n*-propyl gallate, and sodium metabisulfite plus ferrous sulfate were lethal to *E. coli* cells, and no cells were recovered on either VRBA or TGE supplemented with these compounds. Experiments were repeated three times.

[b] Counts were significantly ($P > 0.01$) higher than those in unsupplemented medium.

[c] Counts were significantly ($P > 0.05$) higher than those in unsupplemented medium.

[d] Respectively.

tion procedure of Speck et al. (22) and the modified repair detection procedure (2-h repair time at 25°C) described above. All plates were incubated for 24 h at 35°C, and characteristic colonies were counted as coliforms. Representative colonies were confirmed by establishing the ability to form gas in brilliant green lactose-bile broth (Difco) in 48 h at 35°C.

Ability of microorganisms to utilize pyruvate and TDPA as substrates for acid production. Purple broth base (Difco) was supplemented with either 0.66% pyruvate or 1% TDPA, and the final pH was adjusted to 6.8. Tubes containing 10 ml of purple broth base were inoculated with *E. coli*, soil homogenate, ground beef homogenate, or oyster homogenate. The tubes were incubated at 35°C for 48 h.

Statistical analyses. Statistical analyses were done with logarithms of the counts in an analysis of variance (3). Logarithmic means were separated by the Fisher protected least-significant-difference method (23).

RESULTS

E. coli cells stressed by freezing at −10°C for 48 h were enumerated by pour plating simultaneously with unsupplemented and supplemented TGE or VRBA. The percent increase in colony counts in a supplemented medium over the counts in a corresponding unsupplemented medium was determined (Table 2). In VRBA, the largest percent increases were obtained when the medium was supplemented with pyruvate, whereas supplementation of TGE with TDPA (1.0%) and pyruvate (0.66%) gave comparable results. No significant ($P > 0.05$) difference was noted between the supplements, nor were additive effects observed when they were used together. Catalase (75 U/ml) was not as effective as either pyruvate or TDPA, at least by the pour plating method. However, catalase at 750 U per plate was as effective as pyruvate when injured cells were enumerated by spread plating (data not shown). Most of the other compounds tested did not significantly ($P > 0.05$) increase the recovery of injured cells. Antioxidants containing phenolic groups, i.e., *n*-propyl gallate and ethoxyquin, were lethal to cells (data not shown).

Heat-injured cells also were enumerated with supplemented and unsupplemented media, and the percent increase in colony counts was calculated (Table 3). Supplementation with pyruvate

TABLE 3. Percent increase in colony counts due to supplementation after heating for 12 min at 57°C

Supplement and concn	% Increase after supplementation[a]	
	TGE	VRBA
Pyruvate		
1.2%	2,384[b]	223
0.66%	3,205[b]	513[b]
0.33%	2,553[b]	575[b]
TDPA		
1%	2,521[b]	351
0.5%	1,194[b]	448[c]
0.25%	668[c]	545[b]
Catalase		
75 U/ml	25	50
Thioglycolate		
0.05%	395	109
0.02%	500[c]	174
Dimethyl sulfoxide		
1.0%	−56	287
0.5%	−36	287
Sodium ascorbate		
1%	−77	129
0.5%	−63	57

[a] See Table 2, footnote *a*.

[b] Counts were significantly ($P > 0.01$) higher than those in unsupplemented medium.

[c] Counts were significantly ($P > 0.05$) higher than those in unsupplemented medium.

enhanced recovery of heat-stressed cells in both TGE and VRBA. Supplementation with TDPA gave relatively lower but not significantly ($P > 0.01$) different recoveries. These results are generally similar to those observed for freeze-stressed cells. Some compounds, such as dimethyl sulfoxide, decreased recovery in TGE but enhanced recovery in VRBA.

Figure 1 shows the recovery of freezed-stressed cells in TGE and VRBA with and without pyruvate or TDPA. Supplementation of VRBA increased the recovery of injured cells to levels that were not significantly ($P > 0.05$) different from counts on unsupplemented TGE. However, significantly ($P > 0.01$) more cells were recovered when TGE was supplemented with either TDPA or pyruvate.

Injured cells will repair if placed in the proper environment (10, 12, 14, 19). A 2-h incubation in TGE at 25°C, before overlaying with VRBA, was necessary for the repair of injured cells by the previously described repair detection procedure (22) (Fig. 2). TGE supplemented with TDPA recovered 90% more cells than unsupplemented TGE. The shortest repair time (90 min) was noted when both TGE and VRBA overlay were supplemented. After approximately 90 min at 25°C, these counts were equal to those in TGE supplemented with TDPA (Fig. 2). Similar results were observed when pyruvate was used as a supplement.

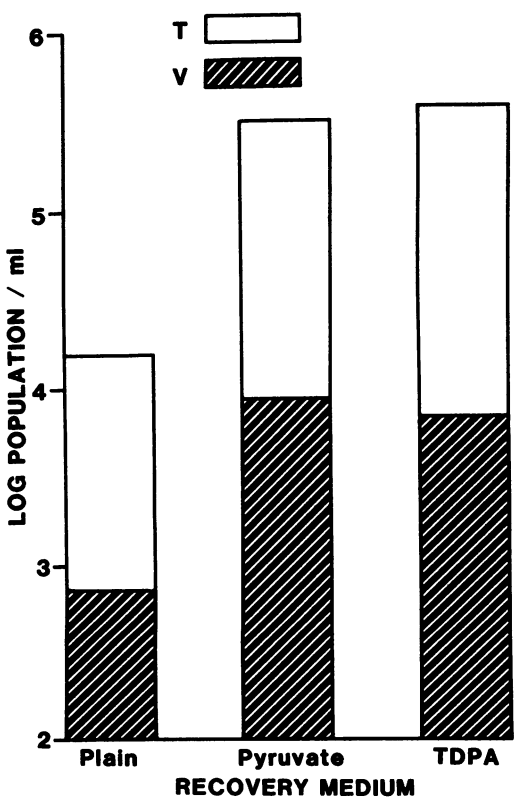

FIG. 1. Recovery of freezed-stressed *E. coli* cells in supplemented and unsupplemented media. The cells were frozen at −10°C for 48 h and plated in TGE (T) and VRBA (V) with and without pyruvate or TDPA. Data are means of three trials.

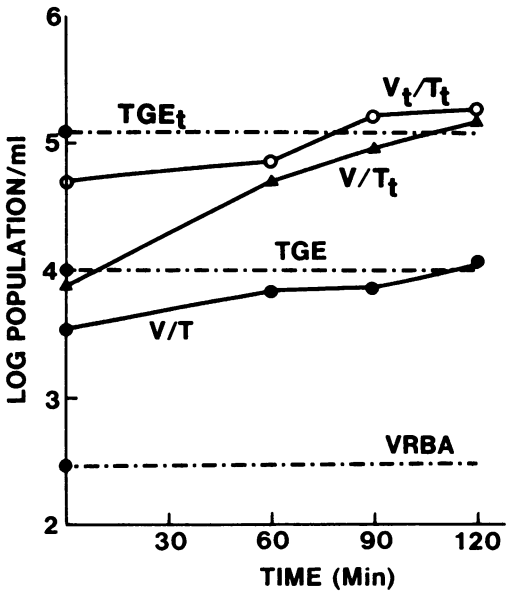

FIG. 2. Recovery of stressed *E. coli* cells by various plating procedures. The cells were frozen at −10°C for 48 h, thawed, and plated with VRBA (V), TGE, and TGE supplemented with TDPA. Repair time was determined by plating the cells in 5 ml of TGE (T) or TGE supplemented with TDPA (T_t) followed by overlaying the medium at 30-min intervals with VRBA or VRBA supplemented with TDPA (V_t).

The improved repair detection procedure was compared with VRBA and the previously described repair detection procedure for the detection of coliforms from foods (Table 4). Significantly ($P > 0.01$) higher counts were noted with the improved repair detection procedure when either pyruvate or TDPA was used as a supplement. An increase in the number of false-positives was observed in pyruvate-supplemented plates for some foods. This was not noted when TDPA was used. An organism was considered to be falsely positive if it failed to produce gas in brilliant green lactose-bile broth within 48 h at 35°C. In VRBA, lactose is used by coliforms to produce acid. Since pyruvate can also be used as a substrate for energy production, it is possible that noncoliform cells were using pyruvate to produce acid. *E. coli* and organisms from a number of sources, including oyster homogenate, hamburger homogenate, and soil homogenate, were able to produce acid in purple broth base containing pyruvate (data not shown). No

TABLE 4. Logarithmic mean of coliform-positive food samples tested by different plating procedures

Food	No. positive/ no. tested	Log means of positive samples[a]			
		VRBA	Unsupplemented media[b]	Media + TDPA[b,c]	Media + pyruvate[b,c]
Ice cream	9/15	3.6×10^2	4.9×10^2	6.8×10^2	6.9×10^2
Cheese	3/15	3.7×10^2	5.2×10^2	6.7×10^2	6.1×10^2
Oysters	4/5	7.8×10^4	3.3×10^5	3.9×10^5	3.8×10^5
Hamburger (frozen)	7/9	1.3×10^4	1.2×10^4	1.9×10^4	2.0×10^{4d}
Sausage	3/3	6.3×10^5	8.5×10^5	2.6×10^6	2.2×10^{6d}
Chicken salad	0/3				
Pot pies	0/3				

[a] Colonies were confirmed by establishing gas production in brilliant green lactose-bile broth. Counts were adjusted when false-positives were found.

[b] Cells were allowed to repair for 2 h at 25°C in the nonselective media before overlaying with the selective media.

[c] Counts obtained by the modified repair detection procedure were significantly ($P > 0.01$) greater than counts obtained by the unmodified repair detection procedure and VRBA.

[d] Oyster, hamburger, and sausage samples contained organisms which mimicked coliforms when plated on VRBA plus pyruvate overlaid with TGE plus pyruvate.

acid production was noted when purple broth base containing TDPA was similarly inoculated.

DISCUSSION

When enumerating a particular group of microorganisms from foods, a selective medium must be used. Therefore, only uninjured cells are detected. This creates a discrepancy between the counts obtained and the actual number of organisms present. When the repair detection procedure (22) was developed, it was felt that all coliforms, injured as well as uninjured, could be recovered. However, certain discrepancies have been noted. Some nonselective media recover more injured cells than others. For example, Draughon and Nelson (8) reported that TGE recover more injured cells than Trypticase soy agar. In addition, Rayman et al. (20) and Bissonnette et al. (G. K. Bissonnette and C. B. Law, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N99, p. 180) reported that the recovery of heat-injured *S. senftenberg* and acid-injured *E. coli* was improved in nonselective media supplemented with catalase. This indicated that lethal peroxide concentrations may have been present in nonselective media.

Injured cells may have reduced catalase and superoxide dismutase activity, which may explain the toxic effects of relatively low peroxide levels in media (1, 5, 16). The death of injured cells may also be caused by the formation of hydroxyl radicals. If H_2O_2 accumulates in the media in the presence of superoxide radicals, hydroxyl radicals will be formed (9). The latter are the most potent oxidants known and may be especially lethal to injured cells (9).

The susceptibility of injured cells to peroxides may be a general phenomenon. In this study, several compounds, notably pyruvate and TDPA, improved the recovery of injured cells in both selective and nonselective media. Catalase did not appreciably improve recovery of stressed *E. coli* by the pour plate method. This was probably due to an insufficient concentration of the enzyme, since catalase concentrated on the medium surface was as effective as pyruvate.

The type of injury influenced the effectiveness of the various supplements. For example, sodium ascorbate improved recovery of freeze-injured *E. coli* but was detrimental to heat-injured cells. On the other hand, thioglycolate had the opposite effect.

When the repair detection procedure was modified by supplementing the media with either pyruvate or TDPA, recovery of injured cells was enhanced. These two supplements were equally effective in aiding the recovery of *E. coli* cells. No significant ($P > 0.01$) differences were noted between the number of cells recovered by the modified repair detection procedure and the number recovered on supplemented TGE.

Significantly ($P > 0.01$) higher coliform counts were obtained from foods by the modified repair detection procedure. However, the percentage of false-positives was higher in pyruvate-supplemented media than in unsupplemented VRBA. No increase in the percentage of false-positives was noted in TDPA-supplemented media, compared with unsupplemented VRBA. Because of its effectiveness and low cost, we think TDPA should be used as a supplement for the modified repair detection procedure.

LITERATURE CITED

1. **Andrews, G. P., and S. E. Martin.** 1979. Catalase activity during the recovery of heat-stressed *Staphylococcus aureus* MF-31. Appl. Environ. Microbiol. **38**:390–394.
2. **Baird-Parker, A., and E. Davenport.** 1965. The effect of

recovery medium on the isolation of *Staphylococcus aureus* after heat-treatment and after storage of frozen or dried cells. J. Appl. Bacteriol. **28**:390–420.
3. **Bar, A., and J. Goodnight.** 1972. Statistical analysis systems. North Carolina State University, Raleigh, N.C.
4. **Barry, V. C., M. L. Conalty, J. M. Denneny, and F. Winder.** 1956. Peroxide formation in bacteriological media. Nature (London) **178**:596–597.
5. **Bucker, E. R., and S. E. Martin.** 1982. Effect of free-radical scavengers on enumeration of thermally stressed cells of *Staphylococcus aureus* MF-31. Appl. Environ. Microbiol. **43**:1020–1025.
6. **Busta, F.** 1976. Practical implication of injured microorganisms in food. J. Milk Food Technol. **39**:138–145.
7. **Carlsson, J., G. Nyberg, and J. Wrethén.** 1978. Hydrogen peroxide and superoxide radical formation in anaerobic broth media exposed to atmospheric oxygen. Appl. Environ. Microbiol. **36**:223–229.
8. **Draughon, F. A., and P. J. Nelson.** 1981. Comparison of modified direct-plating procedures for recovery of injured *Escherichia coli*. J. Food Sci. **46**:1188–1191.
9. **Fridovich, I.** 1976. Oxygen radicals, hydrogen peroxide, and oxygen toxicity, p. 239–277. *In* W. A. Pryor (ed.), Free radicals in biology. Academic Press, Inc., New York.
10. **Hackney, C. R., and G. Bissonnette.** 1978. Recovery of indicator bacteria in acid mine streams. J. Water Pollut. Control. Fed. **50**:775–780.
11. **Hackney, C. R., B. Ray, and M. L. Speck.** 1979. Repair detection procedure for enumeration of fecal coliforms and enterococci from seafoods and marine environments. Appl. Environ. Microbiol. **37**:947–953.
12. **Hartman, P. A., P. S. Hartman, and W. W. Lanz.** 1975. Violet red bile 2 agar for stressed coliforms. Appl. Microbiol. **29**:537–539.
13. **Hoffman, P. S., H. A. George, N. R. Krieg, and R. M. Smibert.** 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. jejuni. II. Role of exogenous superoxide anions and hydrogen peroxide. Can. J. Microbiol. **25**:8–16.
14. **Hurst, A.** 1980. Injury and its effect on survival and recovery, p. 205–214. *In* International Commission on Microbiological Specifications for Foods (ed.), Microbial ecology of foods. Academic Press, Inc., New York.
15. **Lindsay, R. C.** 1976. Other desirable constituents of food, p. 465–513. *In* O. R. Fennema (ed.), Principles of food science, part 1. Food chemistry. Marcel Dekker, Inc., New York.
16. **Martin, S. E., R. S. Flowers, and Z. J. Ordal.** 1976. Catalase: its effect on microbial enumeration. Appl. Environ. Microbiol. **32**:731–734.
17. **McCord, J. M., and I. Fridovich.** 1969. The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. J. Biol. Chem. **244**:6056–6063.
18. **Ray, B., and M. L. Speck.** 1973. Enumeration of *Escherichia coli* in frozen samples after recovery from injury. Appl. Microbiol. **25**:499–503.
19. **Ray, B., and M. L. Speck.** 1978. Plating procedure for the enumeration of coliforms from dairy products. Appl. Environ. Microbiol. **35**:820–822.
20. **Rayman, M. K., B. Aris, and H. B. El Derea.** 1978. The effect of compounds which degrade hydrogen peroxide on the enumeration of heat stressed cells of *Salmonella senftenberg*. Can. J. Microbiol. **24**:883–885.
21. **Scheusner, D. L., F. F. Busta, and M. L. Speck.** 1971. Injury of bacteria by sanitizers. Appl. Microbiol. **21**:41–45.
22. **Speck, M. L., B. Ray, and R. B. Read, Jr.** 1975. Repair and enumeration of injured coliforms by a plating procedure. Appl. Microbiol. **29**:549–550.
23. **Steel, R. G., and J. H. Torrie.** 1960. Principles and procedures of statistics. McGraw-Hill Book Co., Inc. New York.