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Factors Affecting Inactivation of *Moraxella-Acinetobacter* Cells in an Irradiation Process

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The effect of various stages of the irradiation processing of beef on the injury and inactivation of radiation-resistant *Moraxella-Acinetobactor* cells was studied. *Moraxella-Acinetobacter* cells were more resistant to heat inactivation and injury when heated in meat with salts (0.75% NaCl and 0.375% sodium tripolyphosphate) than in meat without salts. These salts had no effect on radiation resistance. Both radiation- and heat-injured cells were unable to form colonies at 30°C in plate count agar containing 0.8% NaCl. Neither unstressed nor heat-stressed cells were able to multiply in minced beef incubated at 30°C for 12 h. Only after the beef was diluted 1:10 with peptone water were the heat-injured cells able to repair and eventually multiply. Heated cells were more sensitive to radiation inactivation and injury than unheated cells. After repair, the cells regained their resistance to both NaCl and irradiation. Freezing and storage at −40°C for 14 days had only a slight effect on either unstressed or heat-stressed cells.

Members of the *Moraxella-Acinetobacter* (M-A) group of bacteria have been found in unirradiated (11, 13, 18, 23) and irradiated meats (22). In the processing of kosher poultry (carcasses covered with dry salt), the two major genera found on carcasses at the time of spoilage at 4.5°C were *Moraxella* and *Pseudomonas*, and members of the *Moraxella* genus had the higher salt tolerance (11). Kraft et al. (13) showed that the predominant flora in beef patties after frozen storage consisted of species of M-A (42%) and *Pseudomonas* (32%). Seideman et al. (18) found that the initial microbial flora of wholesale cuts of beef consisted primarily of M-A species and coryneform bacteria. Several cultures of M-A cells, extremely radiation resistant at −30°C, were isolated from raw beef (24).

In the irradiation process, meat is usually formulated to contain 0.5 to 0.8% (wt/wt) NaCl and 0.3 to 0.4% (wt/wt) sodium tripolyphosphate to improve the juiciness of the final product (19). The formulated meat is heated to inactivate autolytic enzymes, chilled, vacuum packed in cans or pouches, frozen, and subjected to high-dose (>1 Mrad) gamma radiation. The usual thermal treatment (internal temperature of about 70°C for up to 9 h) before irradiation would eliminate radiation-resistant M-A cells (8). However, in other products to be preserved by irradiation, the heat treatment may be less severe and M-A cells may not be thermally destroyed. Other factors which may affect the survival of M-A cells are (i) salts, (ii) freezing, (iii) enhanced sensitivity of heat-stressed cells to subsequent treatments, and (iv) repair of heat-injured cells.

The purpose of this study was to determine: (i) the effect of NaCl and sodium tripolyphosphate in beef on thermal inactivation of M-A cells in beef, inactivation or repair of thermally stressed cells during subsequent incubation, and radiation resistance of M-A cells; (ii) the sensitivity of unstressed and thermally stressed cells to freezing; and (iii) the radiation resistance of thermally stressed cells before and after resuscitation.

MATERIALS AND METHODS

Test organism. A radiation-resistant culture, isolated from beef and designated as M-A number 7 by Welch and Maxcy (24), was kindly provided by R. B. Maxcy, University of Nebraska, Lincoln. The culture was maintained at 5°C on plate count agar (PCA). Cultures for experiments were obtained by inoculating cells from a slant culture into plate count broth (Difco Laboratories, Detroit, Mich.) and incubating at 32°C on rotary shaker (450 rpm) for 22 h. The cultures were harvested by centrifugation (2,000 × *g*) and resuspended in 20 ml of sterile plate count broth to give a final concentration of about 7×10^9 cells per ml.

Inoculating the beef. Radiation-sterilized (4.1 Mrads) minced beef, unsupplemented or supplemented with 0.75% NaCl and 0.375% (wt/wt) sodium tripolyphosphate, was inoculated with a 22-h M-A culture to give a final concentration of about 10^8 cells per g of beef. After grinding five times to assure the even distribution of M-A cells, the inoculated ground beef was placed in a polyethylene pouch and pressed to a uniform thickness of 3 mm. Each pouch was heat sealed under vacuum.

Thermal treatment and recovery. The heat treatment and recovery of cells was as described previously (8). Unless otherwise specified, beef samples without and with salts were heated at 65°C for 40 and 45 min, respectively.

Freezing. Beef samples, heated (for 40 min at 65°C or 0.3 min at 80°C) and unheated, were frozen rapidly (25 s) by dipping them in liquid nitrogen or frozen slowly (50 to 60 min) in a freezer at −40 or −10°C. The pouches were held frozen at the temperature of freezing indicated for 1 to 14 days and subsequently thawed. Beef was rapidly thawed (60 s) by dipping the pouches in a water bath at 30°C. Other samples of beef were slowly thawed (120 min) in a refrigerator at 5°C.

Irradiation of beef. Inoculated beef samples in pouches, heated (65°C) and unheated, were frozen and irradiated at −30 ± 5°C. The samples were irradiated with a ^{137}Cs gamma source with a dose rate of 13,000 rads/min.

Irradiation of diluted beef with heat-stressed cells before and after repair and with unstressed cells. Inoculated beef samples (20 g) in pouches were heated (65°C), blended with 180 ml of 0.1% peptone, and then divided into two samples. One sample was incubated at 30°C for 8 h to allow the heat-stressed cells to repair, and then 6 ml of the incubated suspension was transferred to sterile Pyrex tubes (16 by 150 mm), vacuum sealed (125 mm), frozen, and irradiated at −30 ± 5°C. The second sample was not incubated at 30°C but otherwise was treated in the same manner. Unheated beef samples, with unstressed M-A cells, were handled in the same manner as the second heated sample. After irradiation the beef suspension was thawed at 30°C, appropriately diluted, and pour plated.

Determination of cell injury and repair. Uninjured cells were able to form colonies on both PCA and PCA plus 0.8% NaCl. However, some of the stressed cells produced colonies on PCA but not on PCA plus 0.8 NaCl. The difference in colony counts with and without 0.8% NaCl was a measure of injury. The gain of ability to form colonies in the presence of 0.8% NaCl was a measure of repair.

Each experiment described in this paper was repeated at least three times, and the results shown represent the average of all experiments.

RESULTS

Effect of salts in beef on heat inactivation of M-A cells. A comparison of survival curves of M-A cells at 70°C in beef with 0.75% NaCl and 0.375% sodium tripolyphosphate and in beef without salts is shown in Fig. 1. M-A cells were more resistant to heat in the presence of the salts; i.e., the *D* value increased from 4.7 to 6.6 min. Furthermore, in the absence of salts in the beef there were fewer uninjured M-A cells (able to form colonies on PCA + 0.8% NaCl) as a result of heat treatment than in beef that contained salts (Table 1). Salts (0.75% NaCl and 0.375% sodium tripolyphosphate) in beef did not have any effect on the radiation resistance of M-A cells in beef at −30 ± 5°C (data not shown).

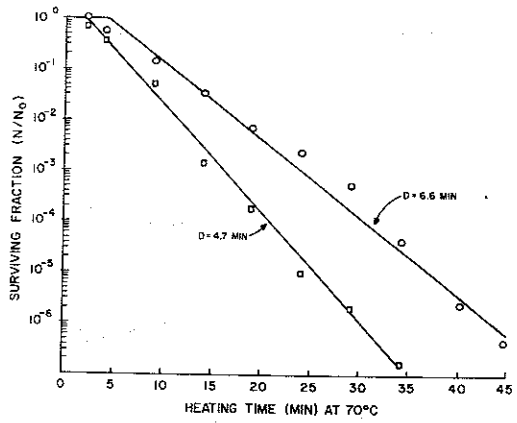

FIG. 1. *Effect of salts in beef on the thermal inactivation of M-A cells at 70°C. Symbols: ○, beef with salts; □, beef without salts.*

TABLE 1. *Effect of salts in beef on the uninjured fraction of M-A cells after heat treatment*

Heating time (min)	Heating temp (°C)	Log of uninjured fraction[a]	
		Meat with salts	Meat without salts
45	65	−2.982	−5.210
60	65	−4.145	−7.507
14	70	−4.523	−5.401
19	70	−5.728	−6.723

[a] Uninjured fraction = numbers of colonies formed on PCA plus 0.8% NaCl after the heat treatment divided by the initial number of unheated M-A cells.

Effect of incubation at 30°C on heat-stressed cells in beef or diluted beef. Inoculated beef (with and without salts) was heated at 65°C to reduce the total M-A population by approximately one log cycle. The samples were then incubated at 30°C for up to 8 h. There was no apparent change in the number of either total cells or uninjured cells in minced beef with salts (Fig. 2A). However, in beef without salts there was a continual reduction in the total number of M-A cells (Fig. 2B). The reduction in numbers was due to injured cells. When the 20-g meat sample, with or without salts, was blended with 180 ml of peptone water (diluted beef) after the heat treatment, the M-A cells repaired without multiplication during incubation at 30°C (Fig. 3). After 8 h the numbers of total cells (PCA) and uninjured cells (PCA plus 0.8% NaCl) were almost equal. In these experiments cells started to multiply by about 9 h, as evidenced by increased counts on PCA.

Effect of freezing on inactivation and injury of M-A cells in beef. The effect of freezing was studied with unstressed and heat-stressed

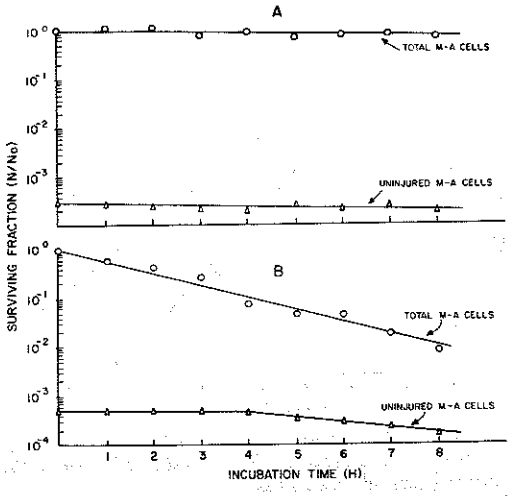

FIG. 2. *Effect of incubation at 30°C on the inactivation of M-A cells heat stressed and incubated in beef with (A) and without (B) salts. N_o is the number of colonies formed on PCA after heat treatment. Symbols: ○, cells plated with PCA; △, cells plated with PCA plus 0.8% NaCl.*

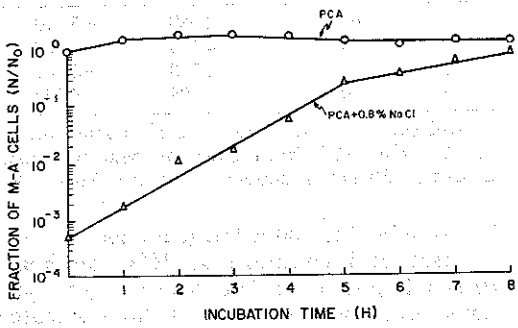

FIG. 3. *Repair of heat-stressed M-A cells in beef blended with peptone water (1:10) and incubated at 30°C. N_o is the number of colonies formed on PCA after heat treatment. Symbols: ○, cells plated with PCA; △, cells plated with PCA plus 0.8% NaCl.*

cells (40 min at 65°C or 0.3 min at 80°C). After either heat treatment, the number of colonies on PCA was reduced by approximately one log cycle, and more cells were heat injured at 80°C than at 65°C. The number of uninjured cells capable of forming colonies on PCA plus 0.8% NaCl was reduced by 2 to 2.5 log cycles at 65°C, whereas at 80°C this number was reduced by 3 to 3.5 log cycles (8). No more than 10 to 15% of the unheated M-A cells were inactivated or injured during frozen storage (−40°C) for 14 days (Fig. 4A and B). Heat-stressed cells were more sensitive to freezing. However, less than one log cycle of the heat-stressed cells was inactivated

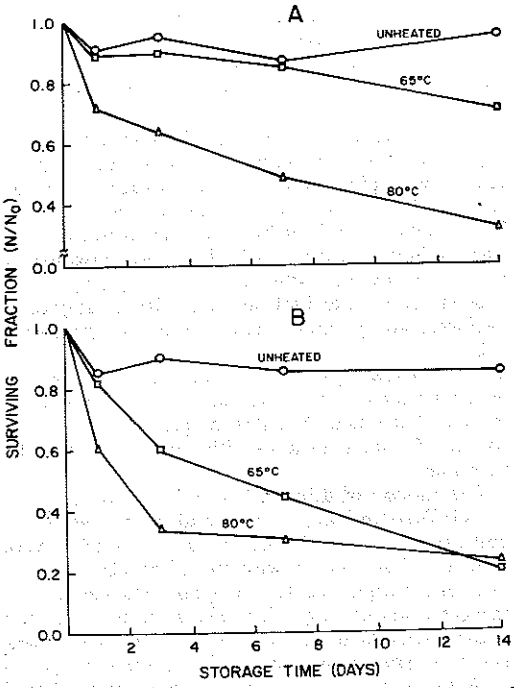

FIG. 4. *Effect of storage at −40°C on unstressed and heat-stressed M-A cells in beef with salts. The meat was rapidly frozen and thawed as described in the text. (A) Recovery on PCA; N_o is the number of colonies formed on PCA before freezing. (B) Recovery on PCA plus 0.8% NaCl; N_o is the number of colonies formed on PCA plus 0.8% NaCl before freezing. Symbols: ○, unheated cells; □, cells heated at 65°C for 40 min; △, cells heated at 80°C for 0.3 min.*

or injured during subsequent storage at −40°C. Since the population heated at 65°C underwent less injury than the population heated at 80°C, the latter was more susceptible to inactivation during subsequent storage at −40°C (Fig. 4A). About 35% of the population surviving the heat treatment at 80°C remained viable after 14 days at −40°C (Fig. 4A), and about 42% of this population was injured. Although salts in the meat did play a protective role during heating, they had no apparent effect during frozen storage (data not shown). Freezing and holding the inoculated beef at −10°C gave essentially the same results as the treatment at −40°C.

There was no significant difference in the inactivation or injury of either unstressed or stressed M-A cells between either rapid (liquid nitrogen) and slow (freezer) freezing or rapid versus slow thawing.

Radiation resistance of unstressed and heat-stressed M-A cells in beef. Meat samples containing salts and inoculated with M-A cells were heated at 65°C for 45 min to inactivate

about one log cycle of the cells. Cells treated in this manner were more sensitive to radiation inactivation (Fig. 5) when the heated beef was subsequently irradiated at −30 ± 5°C than were unheated cells. The major effect of the heat treatment was to eliminate the shoulder in the subsequent radiation survival curve. After about two decimal reductions the inactivation rates were similar for both heated and control cells.

Radiation resistance of heat-stressed M-A cells before and after repair in diluted beef. Heat-stressed M-A cells repaired in diluted beef (Fig. 3) but not in beef (Fig. 2A) when incubated at 30°C for 8 h. Thus, to determine the effect of repair of heat-stressed cells on subsequent irradiation, it was necessary to work with diluted beef (as described in Materials and Methods) after the initial heat treatment. As in beef, heated cells were more sensitive to radiation inactivation (Fig. 6A) and injury (Fig. 6B) than were unheated cells. However, if the heated cells were allowed to repair before irradiation, they regained their radiation resistance. In addition, the radiation resistance of repaired cells slightly exceeded that of unheated cells. This increase was observed in each of three separate experiments and was statistically significant.

DISCUSSION

It was previously shown that a thermal treatment used to inactivate autolytic enzymes before irradiation would injure, if not eliminate, radiation-resistant M-A cells (8). Salts (0.75% NaCl and 0.375% sodium tripolyphosphate) in the meat played both a protective and an inhibitory role. These salts were protective during thermal treatment, yet had no effect on the radiation resistance of M-A cells. However, both radiation- and heat-injured M-A cells were intolerant of 0.8% NaCl in PCA.

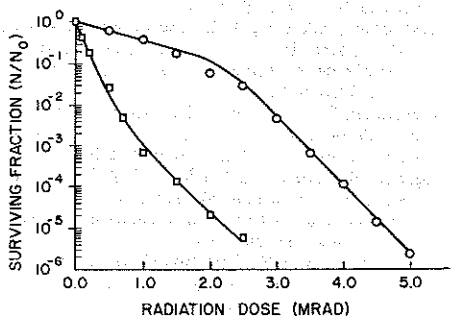

FIG. 5. *Effect of heat stress on the radiation inactivation at −30°C of M-A cells in beef with salts. Recovery on PCA. N_0 is the number of colonies formed on PCA before irradiation. Symbols: ○, unheated cells; □, cells heated in beef at 65°C for 45 min.*

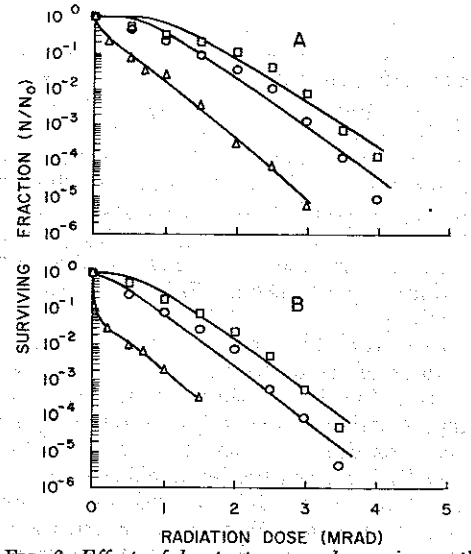

FIG. 6. *Effect of heat stress and repair on the radiation inactivation and injury of M-A cells in diluted beef at −30°C. (A) Recovery on PCA. N_0 is the number of unirradiated cells forming colonies on PCA. (B) Recovery on PCA plus 0.8% NaCl. N_0 is the number of unirradiated cells forming colonies on PCA plus 0.8% NaCl. Symbols: ○, unheated cells; cells heated at 65°C for 45 min in beef before, △, and after, □, 8 h of repair at 30°C.*

Further experimentation is needed to explain which of the two salts caused the increased heat resistance. Polyphosphates have been shown to decrease the thermal resistance of bacteria when present in the heating menstruum (23). The effect of NaCl on the resistance of vegetative cells and spores varies with the genus. Sodium chloride in the heating menstruum protected *Escherichia coli*, *Pseudomonas fluorescens* (4), *Salmonella* (1), and putrefactive anaerobes (6), but hastened the thermal inactivation of *Staphylococcus aureus* (4) and spores of *Bacillus stearothermophilus* (2). Okazawa et al. (17) showed that NaCl sensitized *E. coli* K-12 to irradiation. However, up to 2.5% NaCl had no effect on the radiation resistance of *Yersinia enterocolitica* (7). When M-A cells were heated in minced beef with salts and subsequently stored for 8 h at 30°C, the total viable cells and uninjured cells remained constant during storage (Fig. 2A). There was a decrease in the total viable count when the cells were heated and stored in the absence of salts (Fig. 2B). Most of this decrease was due to the inactivation of heat-injured cells (Fig. 2B). The apparent stabilizing effect of salts is probably due to the fact that fewer cells were injured when heated in beef containing salt (Table 1). Those M-A cells surviving the thermal treatment, in either the presence or absence of

salts, were unable to multiply or repair in minced beef incubated at 30°C for 8 h. Snyder and Maxcy (20) previously showed that unstressed M-A cells were unable to multiply in ground beef. They presented evidence indicating that the cells were unable to multiply due to a limiting water activity. In our experiments, when the water activity of beef was increased by the addition of peptone water, most of the heat-injured M-A cells were able to repair in the absence of multiplication within 8 h (Fig. 3) and eventually to multiply.

Others have shown that: (i) freezing and thawing rates affected the percentage of bacteria inactivated (3, 15, 21); (ii) the lower the storage temperature, the lower the rate of inactivation of bacteria during storage (10); and (iii) NaCl markedly increased the lethal effect of freezing (10). However, we did not find any of these factors to have an effect on the inactivation or injury of M-A cells. Unstressed M-A cells were relatively insensitive to freezing. Heat-stressed cells showed only a slightly enhanced sensitivity to freezing (Fig. 4).

Maxcy and Rowley (14) showed that a heat treatment sufficient to inactivate about 90% of a given M-A population, previous or subsequent to irradiation at −30°C, eliminated the shoulder in the radiation survival curve and increased the number of cells inactivated by a given dose of irradiation. They suggested that this phenomenon may be related to the cell's repair mechanism. Others have suggested that the extreme radiation resistance of asporogenous cells may be due to an efficient repair mechanism (9, 12). We showed a similar sensitization to irradiation by a previous thermal treatment of either inoculated or diluted beef. Furthermore, we demonstrated that, if conditions (e.g., time, temperature, available water) were right for the repair of the heat-stressed cells, the cells were able to regain their resistance to 0.8% NaCl and irradiation. The repair population appeared to be a little more radiation resistant than the unheated population. Further studies are required to help explain why the repaired heat-stressed M-A cells are more radiation resistant than unheated cells. Such an increase in radiation resistance may be related to membrane changes during repair. Duitschaever and Jordan (5) found that heat-injured cells that were allowed to repair were more resistant to salt and heat than cells not previously heated. They speculated that the changes in fatty acid reflected a change in the physical state and functional properties of the membrane of the heat-injured but repaired cells. Other factors which should be considered include changes in the microecosystem and possible differences in the physiological state between a stationary-phase culture (unheated cells) and a culture that has just undergone repair. Notermans and Kampelmacher (16) showed that cells attached to broiler skins were more heat resistant that unattached cells.

Factors affecting the elimination of radiation-resistant M-A cells during irradiation processing include thermal inactivation and injury, sensitivity of injured cells to NaCl and irradiation, and inability of cells to multiply and injured cells to repair in undiluted beef.

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