APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1978, p. 403-407
0099-2240/78/0036-0403$02.00/0

Vol. 36, No. 3
Printed in U.S.A.

Sensitization of *Clostridium perfringens* Spores to Heat by Gamma Radiation†

DAVID E. GOMBAS AND REINALDO F. GOMEZ*

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received for publication 3 July 1978

Spores of *Clostridium perfringens*, type A, were given separate or sequential treatments of gamma radiation (0 to 0.7 Mrad) and/or high temperature (93 to 103°C). Prior heating, sufficient to inactivate 40 to 99% of the viable spores, had no effect on the subsequent radiation inactivation rate. Prior irradiation had a sensitizing effect on subsequently heated spores. The degree of sensitization to heat, as measured by thermal inactivation rate, increased with increased radiation pretreatment dose.

In 1938, Curran and Evans (4) reported that bacterial spores of several *Bacillus* species exposed to certain levels of ionizing radiation became sensitized to subsequent heating. Since that time, many other researchers have reported similar observations, i.e., a greater rate of spore inactivation by sequential treatments than by either treatment alone (6, 10, 11). The simultaneous treatments of heat and irradiation have also been shown to have a synergistic effect on spore inactivation in several cases (2, 7, 11, 13). The effects of these treatments in *Clostridium perfringens* spores have not been reported.

The following is a study of the effect of heat and ionizing radiation on the viability of *C. perfringens* spores, the inactivation rates manifested by each, separately and sequentially, and some of the factors possibly having a related effect.

MATERIALS AND METHODS

Organism. The organism used was *C. perfringens* type A (NCTC 8798), obtained from C. L. Duncan, Campbell Institute of Food Research, Camden, N.J.

Sporulation and cleaning of spores. Spores were grown in modified Duncan and Strong (5) sporulation medium, using 1.0% soluble starch and without activated charcoal. Spores were harvested by centrifugation (10,400 × *g*, 20 min). Spores were cleaned of cells and vegetative debris after harvest. A successful method for freeing spores from their sporangia and vegetative cells was suggested by a study by Chen and Duncan (3). After harvest, spores were left suspended in sodium phosphate buffer at 4°C for 1 week. Removal of vegetative debris was accomplished by a method suggested by Long and Williams (12). Soluble components were removed by two washings in distilled water (12,100 × *g*, 10 min). Spores and debris were subsequently pelleted in two layers by low-speed (1,475 × *g*), long-time (20 min) centrifugation. The bottom was composed of an adherent, tightly packed layer of clean spores, and the upper layer was gelatinous and composed of spores and vegetative debris. The upper layer could be removed by a gentle rocking motion in 5 to 10 ml of distilled water, as suggested by H. S. Levinson (personal communication). The gelatinous debris layer could then be homogenized by blending in a Vortex mixer and centrifuged again to remove more spores. This procedure was repeated until, by microscopic inspection, greater than 90% of all objects were refractile spores clean of vegetative debris.

Unless otherwise specified, the cleaned spores were suspended in reduced buffer (RB; containing 0.3 mg of cysteine and 1.0 μg of resazurin per ml of sodium phosphate buffer, 0.067 M, pH 7) and distributed in 2.5-ml samples into quick-break freeze-drying ampoules (Wheaton; A1910-2). The ampoules were flame-sealed under vacuum and stored at 4°C until use. The vials generally became anaerobic within 12 h, as indicated by clearing of resazurin.

Heat activation. Spores suspended in RB under anaerobic conditions were heat activated at 75°C for 10 min prior to all other treatments.

Determination of heat resistance. The vials containing the heat-activated spore suspensions were immersed in a thermostatically controlled oil bath (Neslab, Durham, N.H.). After heating, vials were cooled by immersion in ice water for 1 min, followed by immersion in a detergent-water solution to remove adherent oil.

Determination of radiation resistance. The vials were held in a beaker of ice water during gamma irradiation in a Gammacell-220 ^{60}Co source (Atomic Energy of Canada, Ltd.).

The dose rate was determined by Fricke dosimetry (1) to be 3.4 to 3.9 krad/min.

Viability determinations. Samples were serially diluted, 1 ml into 9 ml, with 0.1% peptone water. Spores were enumerated in the basal medium of Harmon et al. (8), containing 1.5% tryptose (Difco), 1.5% Soytone (Difco), 0.5% yeast extract (Difco), 0.1% sodium metabisulfite, 0.1% ferric ammonium citrate, and

† Publication no. 3424 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

2.0% agar, adjusted to pH 7.6 to 7.7 with sodium hydroxide. Henceforth, this medium will be referred to as Base. Samples of 5 ml were distributed into Hungate roll tubes (Bellco, Vineland, N.J.), autoclaved (121°C for 10 min), and stored at room temperature until use, but no more than 2 weeks.

Enumeration was done with the Virginia Polytechnic Institute anaerobic culture system (Bellco); molten and cooled (50 to 55°C) agar roll tubes were inoculated with 1 ml of appropriate dilutions under a constant stream of sterile nitrogen gas. The sealed roll tubes were then spun on a tube spinner (Bellco) until the agar was solidified. Roll tubes were incubated in a horizontal position to minimize "pooling" of the agar. Incubation was at 37°C in a thermostatically controlled Isotemp Incubator (Fisher).

RESULTS AND DISCUSSION

Thermal inactivation curves were determined for spores suspended in RB and are summarized in Fig. 1 (data have been corrected for thermal come-up time). For heating temperatures of 93, 98, and 103°C, decimal reduction times of 62, 11, and 1.1 min, respectively, were obtained by linear regression of the exponential portion of the curve. By plotting these and additional data (not shown) in an Arrhenius curve, an activation energy of 93 kcal (ca. 389.1 kJ) was obtained for thermal inactivation of nonirradiated spores in the range of 93 to 103°C.

Spores suspended in RB were irradiated at 0 to 4°C, and the decimal reduction dose was calculated by linear regression to be 0.28 to 0.34 Mrad. This inactivation rate is in general agreement with previous published results for this strain of *C. perfringens* (14). In RB, exponential inactivation was generally preceded by a shoulder of 0.25 to 0.3 Mrad. The shoulder was not included in the calculation of the radiation inactivation rate.

Figure 2 shows the results obtained for heat-activated spores heated at 103°C for 0, 0.5, and 1.5 min and then irradiated. The radiation inactivation rates were the same for control (heat-activated only) spores and for those heated at 103°C. Here again shoulders were not included in the calculation of radiation inactivation rates. Spores heated at 93 and 98°C, enough to reduce viability by 40 to 99%, prior to irradiation exhibited similar behavior to those heated at 103°C; no effect was seen on the radiation inactivation rate. However, preliminary heat treatments diminished the shoulder seen during ir-

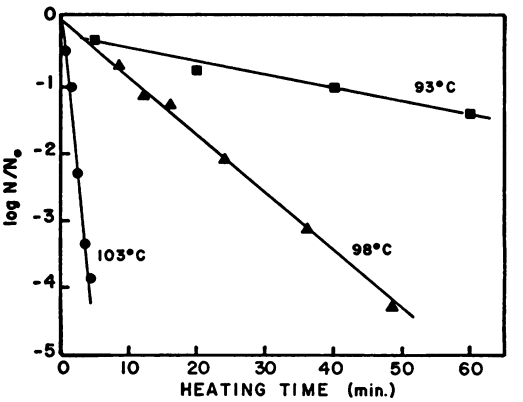

FIG. 1. *Thermal inactivation curves of C. perfringens spores heated at 93 (■), 98 (▲), and 103°C (●). Spores were suspended in RB and were heat activated prior to lethal heating. At various times, viable spores were enumerated in Base agar. A sample receiving only heat activation was taken as N_0.*

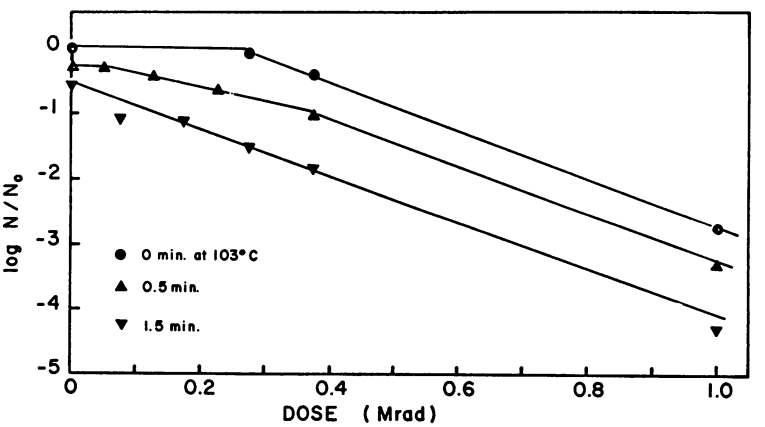

FIG. 2. *Radiation resistance of C. perfringens spores with and without prior heat treatments. (●) No lethal heat treatment prior to irradiation; (▲) spores heated for equivalent time of 0.5 min at 103°C; (▼) spores heated for equivalent time of 1.5 min at 103°C. All spores were suspended in RB and heat activated prior to other treatments. Spores were irradiated anaerobically at ice water temperature. Viable spores were enumerated in Base agar. A sample receiving only heat activation was taken as N_0.*

radiation of control spores. The reason for this effect is not known, although several explanations are possible. For example, preliminary heating may remove some radioprotective factors from the spore. Alternatively, if the presence of a shoulder is the result of sporal enzymes repairing initial radiation damage, the heat may act to inactivate these enzymes, thus diminishing the amount of radiation injury that the spore can successfully cope with.

C. perfringens spores were then tested to determine what effect the reverse treatment, i.e., radiation followed by heat, had on spore inactivation. Spores were again heat activated, then given a lethal dose of gamma radiation (0.3 to 0.7 Mrad), yielding a 40 to 99% reduction in colony-forming units. These spores were then tested for their thermal resistance at three temperatures (93, 98, and 103°C). At all three temperatures, spores which had received a preliminary dose of radiation were more sensitive to subsequent heating than spores which received no irradiation (Fig. 3, 4, 5; Table 1). For example, at 98°C, decimal reduction times of 4.8, 3.7, and 1.9 min were obtained after preirradiation treatments of 0.3, 0.5, and 0.7 Mrad, respectively, as compared with 11 min for nonirradiated spores. This compares well with results obtained with *Clostridium botulinum* spores (9), where preirradiation to a dose of 0.37 Mrad had the effect of doubling the thermal inactivation rate at 99°C. The thermal inactivation rate of *Clostrid-*

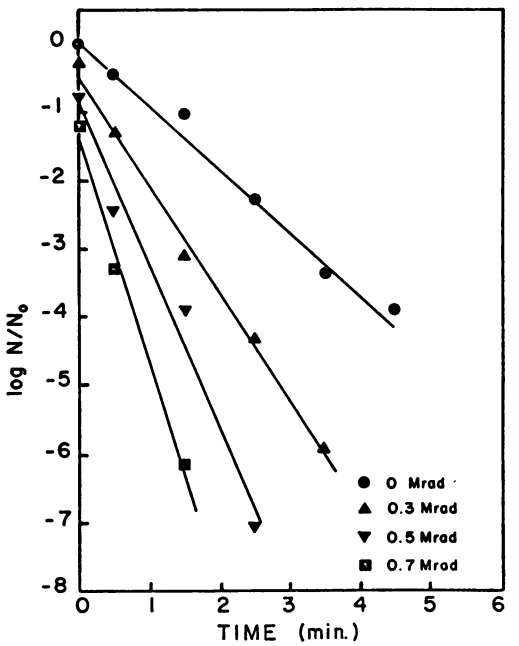

FIG. 3. *Effect of preliminary doses of gamma radiation on thermal resistance of C. perfringens spores at 103°C. (●) 0 Mrad; (▲) 0.3 Mrad; (▼) 0.5 Mrad; (■) 0.7 Mrad. Spores were suspended in RB and heat activated prior to other treatments. Irradiation was at ice water temperature under anaerobic conditions. Samples were taken at various times during lethal heating, and viable spores were enumerated in Base agar. A sample receiving only heat activation was taken as N_0.*

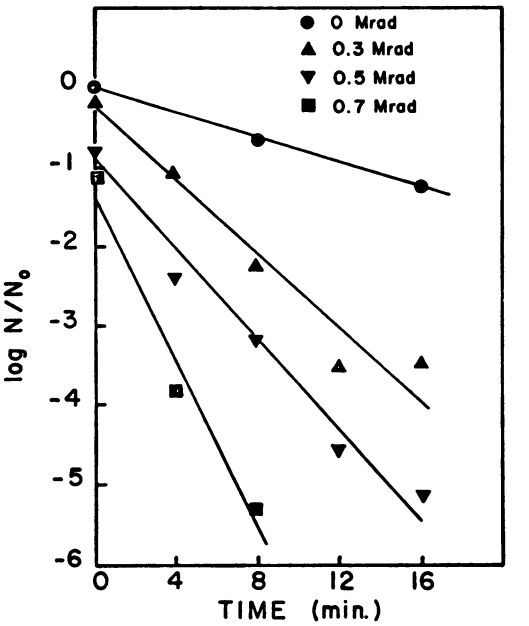

FIG. 4. *Effect of preliminary doses of gamma radiation on thermal resistance of C. perfringens spores at 98°C. (●) 0 Mrad; (▲) 0.3 Mrad; (▼) 0.5 Mrad; (■) 0.7 Mrad. Same conditions as Fig. 3.*

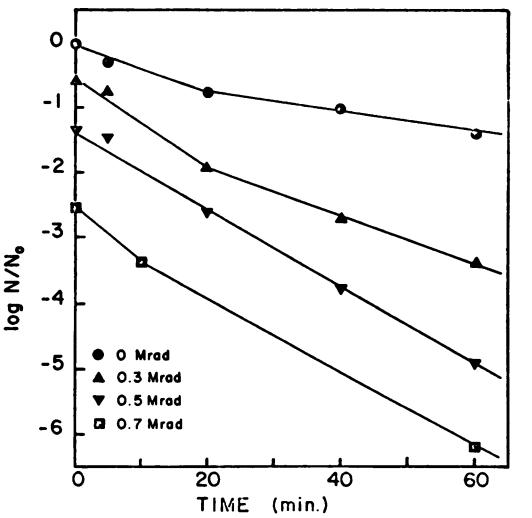

FIG. 5. *Effect of preliminary doses of gamma radiation on thermal resistance of C. perfringens spores at 93°C. (●) 0 Mrad; (▲) 0.3 Mrad; (▼) 0.5 Mrad; (■) 0.7 Mrad. Same conditions as Fig. 3.*

ium sporogenes spores was reportedly increased 10-fold at 100°C by a preliminary irradiation dose of 0.71 Mrad (11).

It is possible that the radiation sensitizing effect on spores is actually a secondary effect of radiation and in fact is caused by radiolytic products formed in the suspending buffer. Two experiments were carried out to test this possibility.

Several vials, containing spores suspended in RB, were heat activated, and half were subsequently irradiated. After irradiation, the spores were separated from the suspending buffer by centrifugation. The buffer was sterilized through a 0.45-μm filter (Millipore) to remove any remaining spores. The same procedure was followed for unirradiated spores. The spores were then remixed with the appropriate buffer, such that irradiated spores were suspended in either irradiated buffer or nonirradiated buffer, and the same for unirradiated spores. These mixtures were then resealed in sterile vials and tested for heat resistance by determining the degree of inactivation after 12 min at 97°C. The results are summarized in Table 2. The sensitization effect is always associated with irradiated spores, as opposed to the suspending buffer. This eliminated the possibility of a radiolytic product in the irradiated buffer being responsible for the sensitization. However, this did not remove the possibility of a radiation-catalyzed reaction between a buffer component and the spore itself. This possibility was eliminated by testing for thermal resistance in spores which were suspended only in distilled water during irradiation. It was found that these spores showed the same degree of sensitization to 98°C as spores irradiated in RB.

C. sporogenes spores are reported to be equally sensitized to heating at 100°C by preliminary irradiation under an air atmosphere at room temperature or at −78°C (11). This argues against the possibility that the causative agent(s) of radiation sensitization are radiolytic products which decompose when exposed to air, or radiolytic products with very short half-lives unless stabilized by freezing. Also, it has been reported that *Bacillus megaterium* spores become sensitized to heating after being irradiated in the dry state (10). Although this result could conceivably be caused by factors other than the primary effects of irradiation, e.g., exposure of spore components to radiolytic products formed in the atmosphere surrounding and permeating the lyophilized spore, this is further supportive evidence that sensitization is not a result of radiation-catalyzed reactions between the suspending menstruum and the spore.

Although radiation sensitization to heat has been demonstrated in several species of bacterial spores, no results have been reported as to the duration of the effect, i.e., whether or not the spore is capable of repairing the sensitization.

Suspended in RB, spores were irradiated, then held at 4°C for various lengths of time before being tested for heat resistance by heating at 98°C for 12 min. As seen in Fig. 6, the spores remained sensitized to heat for up to 28 h, indicating that the sensitizing effect is not repaired by the spores, at least not under these experi-

TABLE 1. *Radiation sensitization of spores to heat*

Radiation dose (Mrad)	*D* value (min)[a] at		
	93°C	98°C	103°C
0	62	11	1.1
0.3	27	4.8	0.64
0.5	17	3.7	0.42
0.7	15	1.9	0.31

[a] Decimal reduction times.

TABLE 2. *Effect of irradiation of spores and/or suspending medium on heat resistance*

Radiation treatment[a]		Viable counts[b]	
Spores	Buffer	After heat activation	After 12 min at 97°C
nr	nr	2.8×10^7	1.7×10^6
nr	r	7.5×10^7	1.5×10^6
r	nr	8.1×10^6	1.0×10^2
r	r	9.5×10^6	1.0×10^2
u	u	1.7×10^7	3.5×10^1

[a] r, Irradiated with 0.5 Mrad; nr, not irradiated; u, irradiated with 0.5 Mrad, but spores were not separated from buffer (unopened vial).

[b] Counts enumerated in Base (see the text).

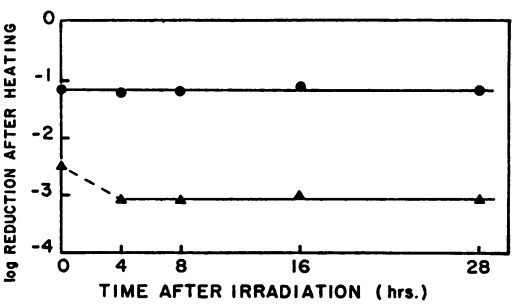

FIG. 6. *Duration of sensitization to heat of C. perfringens spores after gamma irradiation of 0.3 Mrad. Both nonirradiated (●) and irradiated (▲) spores were held anaerobically in RB at 4°C until heating. Spores were heated at 98°C for 12 min before enumerating in Base agar. All spores were heat activated prior to other treatments. A sample receiving only heat activation was taken as N_0.*

mental conditions. It is not yet known whether the sensitization effect can, in fact, be reversed.

Gamma radiation was found to have a sensitization effect on the subsequent heat resistance of *C. perfringens* spores, as has been observed with other bacterial spores. The reverse treatment, high heat followed by gamma irradiation, was found to have only an additive effect on the rate of *C. perfringens* spore inactivation, although heat may play a role in reducing the radiation shoulder. Furthermore, the effect of radiation on heat resistance was found to be independent of the suspending menstruum and of time between radiation and heating when the spores are held in RB. Although further work is required to elucidate the mechanisms of heat and irradiation synergism, we suggest that the effect may be of a structural nature. Current work in our laboratories is aimed at determining the effect of ionizing radiation on spore structure and its relationship to heat resistance.

ACKNOWLEDGMENTS

This work was supported by grant DAAG 29-76-C-0034 from the U.S. Army Research Office, Department of the Army. R.F.G. was supported by a grant from Lilly Research Laboratories.

LITERATURE CITED

1. **Anonymous.** 1972. Standard method of test for adsorbed gamma radiation dose in the Fricke dosimeter. American Society for Testing and Materials, D 1671-72.
2. **Briggs, A., and S. Yazdany.** 1974. Resistance of Bacillus spores to combined sporicidal treatments. J. Appl. Bacteriol. **37**:623-631.
3. **Chen, L. K., and C. L. Duncan.** 1974. Sporangial autolysis in *Clostridium perfringens* type A. Biochim. Biophys. Acta **338**:605-618.
4. **Curran, H. R., and F. R. Evans.** 1938. Sensitizing bacterial spores to heat by exposing them to ultraviolet light. J. Bacteriol. **36**:455-465.
5. **Duncan, C. L., and D. H. Strong.** 1968. Improved medium for sporulation of *Clostridium perfringens*. Appl. Microbiol. **16**:82-89.
6. **Farkas, J., and T. A. Roberts.** 1976. The effect of sodium chloride, gamma irradiation and/or heating on germination and development of spores of *Bacillus cereus* T in single germinants and complex media. Acta Aliment. **5**:289-302.
7. **Fisher, D. A., and I. J. Pflug.** 1977. Effect of combined heat and radiation on microbial destruction. Appl. Environ. Microbiol. **33**:1170-1176.
8. **Harmon, S. M., D. A. Kautter, and J. T. Peeler.** 1971. Improved medium for enumeration of *Clostridium perfringens*. Appl. Microbiol. **22**:688-692.
9. **Kempe, L. L.** 1955. Combined effects of heat and radiation in food sterilization. Appl. Microbiol. **3**:346-352.
10. **Levinson, H. S., and M. T. Hyatt.** 1966. Sequence of events during *Bacillus megaterium* spore germination. J. Bacteriol. **91**:1811-1818.
11. **Licciardello, J. J., and J. T. R. Nickerson.** 1963. Effect of radiation environment on the thermal resistance of irradiated spores of *Clostridium sporogenes* P. A. 3679. J. Food Sci. **27**:211-218.
12. **Long, S. K., and O. B. Williams.** 1958. Method for removal of vegetative cells from bacterial spore preparations. J. Bacteriol. **76**:332.
13. **Pallas, J. E., III, and M. K. Hamdy.** 1976. Effects of thermoradiation on bacteria. Appl. Environ. Microbiol. **32**:250-256.
14. **Roberts, T. A.** 1968. Heat and irradiation resistance and activation of spores of *Clostridium welchii*. J. Appl. Bacteriol. **31**:133-144.