

Effect of Temperature of Liquid Nitrogen on Radiation Resistance of Spores of *Clostridium botulinum*¹

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Received for publication 4 February 1965

ABSTRACT

GRECZ, NICHOLAS (U.S. Army Natick Laboratories, Natick, Mass.), O. P. SNYDER, A. A. WALKER, AND A. ANELLIS. Effect of temperature of liquid nitrogen on radiation resistance of spores of *Clostridium botulinum*. Appl. Microbiol. **13**:527-536. 1965.—An apparatus consisting of a Dewar flask and a relay system controlling the flow of liquid nitrogen permitted the irradiation of samples in tin cans or Pyrex tubes at temperatures ranging from 0 ± 1.5 C to -194 ± 2 C. An inoculated pack comprising 320 cans of ground beef containing 5×10^4 spores of *Clostridium botulinum* 33A per can (10 cans per radiation dose) was irradiated with Co^{60} at 0 and -196 C. Incubation was carried out at 30 C for 6 months. Approximately 0.9 Mrad more radiation was required to inactivate the spores at -196 C than at 0 C. Cans irradiated at -196 C showed partial spoilage at 3.6 Mrad and no spoilage at 3.9 Mrad; the corresponding spoilage-no spoilage doses at 0 C were 2.7 and 3.0, respectively. The majority of positive cans swelled in 2 to 14 days; occasional swelling occurred as late as 20 days. At progressively higher doses, swelling was delayed proportionally to the radiation dose received. The remaining nonswollen cans had no toxin after 6 months of storage, although occasional cans contained very low numbers of viable spores comprising on the average 0.1% of the original spore inoculum. The D_{10} values in phosphate buffer were 0.290 Mrad for 0 C and 0.396 Mrad for -196 C; in ground beef, the corresponding D_{10} values were 0.463 Mrad and 0.680 Mrad, respectively. These D_{10} values indicate that the lethal effect of γ rays decreased at -196 C as compared with 0 C by 13.5% in phosphate buffer, and by 47% in ground beef.

In radiation sterilization of foods, two opposing factors must be considered: (i) the destruction of microorganisms and (ii) the retention of the organoleptic qualities of the food.

The application of very low temperatures during irradiation appears to retard significantly the radiation-induced off-flavors and off-odors which are produced at ambient temperatures (Snyder, unpublished data; Coleby et al., 1961). Radiation-sensitive meats such as beef steaks retained excellent organoleptic quality when irradiated in liquid nitrogen (-196 C). It was, therefore, essential to investigate the effect of low temperatures on the radiation resistance of microorganisms which are of particular concern in radiation preservation of foods.

The present paper describes the apparatus for temperature control during irradiation, and presents data on the radiation resistance of spores of

Clostridium botulinum at 0 and -196 C in phosphate buffer and in ground beef.

MATERIALS AND METHODS

Microorganisms. Spores of a highly radiation-resistant strain, *C. botulinum* 33A, were produced by the method of Anellis and Koch (1960), and were irradiated in ground beef or in Sorensen's phosphate buffer (pH 7).

Buffer substrate. Approximately 10^4 spores in phosphate buffer were distributed into Pyrex tubes (10 by 150 mm) in 2-ml quantities and sealed in an oxygen flame. Ten replicate tubes were irradiated at each dose level. After irradiation, the survivors were determined by the end-point technique with the use of double-strength Wynne's broth (Wynne, Schmieding, and Daye, 1955) for recovery of viable organisms. Alternatively, the viable organisms were estimated by direct counts in oval culture tubes with Wynne's broth plus 1.5% agar.

These two techniques, i.e., the spoilage end-point, and the direct count technique, were also used for evaluation of spore survival in ground beef. With the end-point technique in glass tubes, the indications of viability of *C. botulinum* were turbidity, odor, and specific toxin; with canned beef, swelling of cans and specific toxin were the indicators.

¹ This paper has been assigned number 2273 in the series of papers approved for publication.

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Meat substrate. Ground beef of the following chemical composition was used: 19.68% protein, 20.81% fat, 57.32% moisture, 0.90% ash, 0.09% NaCl, 0.0% reducing sugar, and 1.46% free fatty acid; the pH was 5.9. No peroxide was found (potassium iodide method). The ground beef was heated to an internal temperature of 165 F (74 C), to inactivate the enzymes and to destroy the native vegetative flora of the product.

Inoculation and canning. The ground beef was chilled to 2 to 4 C, inoculated with heat-shocked (80 C for 10 min) spores of *C. botulinum* 33A (2.5×10^8 spores per gram), and thoroughly mixed to

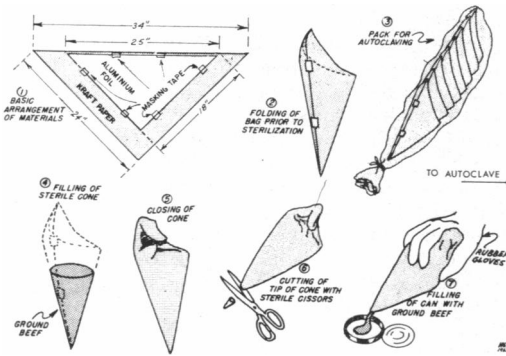


FIG. 1. Use of the baker's cone for rapid filling of cans with ground beef.

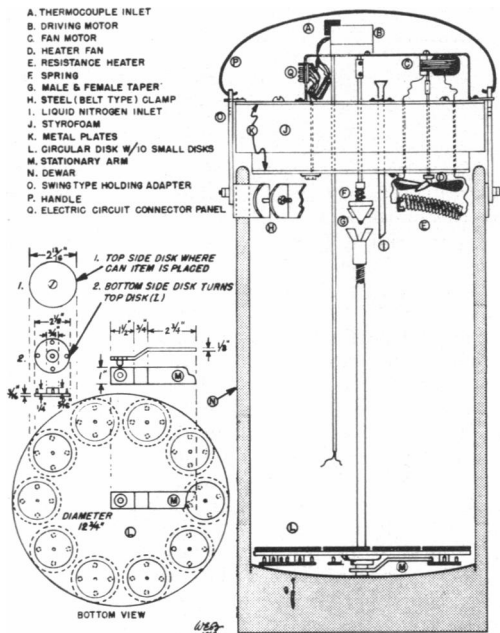


FIG. 2. Schematic drawing of Dewar flask with a platform for irradiation of TDT cans at controlled temperatures, including a section through the interior of the flask.

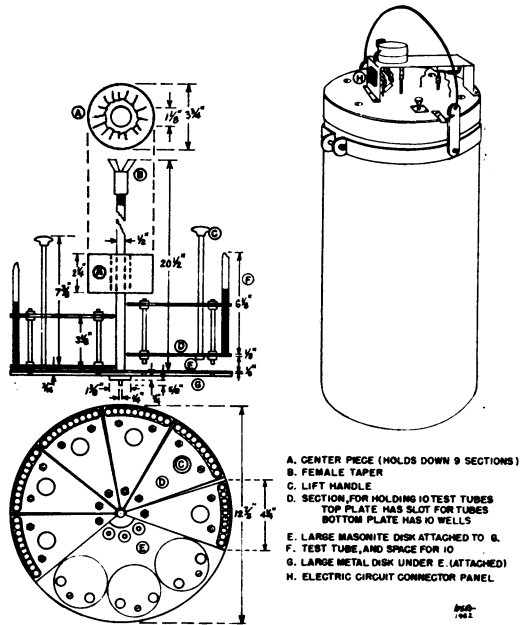


FIG. 3. Schematic drawing of Dewar flask with a platform for irradiation of sealed Pyrex tubes (10×150 mm) at controlled temperatures, including an overall view of the flask.

insure uniform distribution of the inoculum. The spore inoculum in Sorensen's phosphate buffer (pH 7.0) comprised 2% of the total weight of ground beef. The uninoculated meat was compensated with an equivalent amount of sterile buffer. Approximately 20-g portions of ground beef were placed into each 208×006 thermal-death-time (TDT) can (American Can Co., Chicago, Ill.). A baker's cone was used for rapid distribution of the ground meat into TDT cans (Fig. 1).

The filled cans were sealed in a stream of sterile nitrogen and placed into a freezer at -24 C. All cans used in each of the two experiments were prepared at the same time. Appropriate lots of samples were withdrawn randomly from the freezer for each radiation experiment. During the entire handling procedure prior to irradiation, the cans were always kept at a temperature sufficiently low to prevent germination of the spores.

Temperature-control equipment. The temperature-control equipment used for irradiation of the cans of inoculated ground beef and other bacteriological samples was designed and constructed by O. P. Snyder (*unpublished data*). The apparatus consisted of a large Dewar flask equipped with temperature-regulating devices (Fig. 2 and 3). During irradiation of the samples, the Dewar flask was placed on the platform in the center of the Co^{60} source at Cook Electric Co., Morton Grove, Ill. Temperature control was achieved by a thermocouple-solenoid circuit (Fig. 4), activating

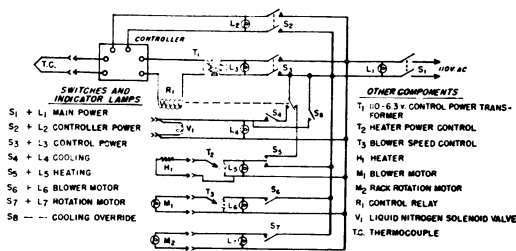


FIG. 4. Simplified schematic wiring diagram for Dewar temperature control thermocouple-solenoid circuit.

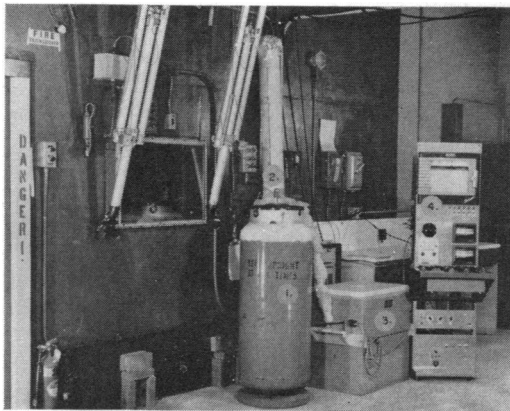


FIG. 5. Wide temperature control system. Arrangement of instruments outside the cobalt-60 source room at Cook Electric Co., Morton Grove, Ill. 1. Liquid nitrogen tank and heavily insulated copper tubing; 2. connecting the tank to the Dewar flask in the cobalt-60 source room; 3. tempering box; 4. control and recording panel for the temperature-control assembly.

either injection of liquid nitrogen (for low temperatures) or the heating element (for elevated temperatures). Temperature levels of 0 ± 1.5 C and -194 ± 2 C maintained in the Dewar flask were recorded continuously during irradiation of the respective samples.

As indicated in Fig. 2 and 3, two exchangeable platforms could be used in the Dewar flask—one for irradiation of TDT cans (Fig. 2) and another for irradiation of 10 by 150 mm sealed Pyrex tubes (Fig. 3). The platform rotated around its central axis at 1 rev/min, thus providing a uniform integrated radiation dose to each individual sample. In addition to the rotation around the central axis of the platform, the TDT cans were also rotated around their own axis at the rate of 0.25 rev/min.

Nine stacks of TDT cans, five cans in each stack, were irradiated by use of the appropriate platform; thus, a total of 40 to 45 TDT cans were irradiated simultaneously. The platform for irradiation of sealed Pyrex tubes consisted of nine seg-

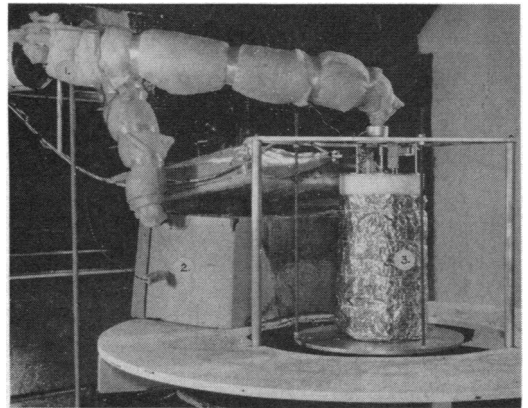


FIG. 6. Overall view of the temperature-control Dewar flask holding the samples during irradiation. The photograph shows the Dewar flask placed at the center platform of Cook Electric Co.'s cobalt-60 source. Heavy insulated copper tubing serves for injection of liquid nitrogen. 1. Solenoid valves; 2. insulated irradiation box for irradiation of food products; 3. Dewar for irradiation of microbiological samples.



FIG. 7. Control panel. 1. West strip chart recorder; 2. master control panel; 3. temperature control panel; 4. K3 potentiometer; 5. power supply; 6. master power control panel.

ments, each of which could be removed individually after the desired time of irradiation. Each segment provided spaces for 10 tubes (10 by 150 mm), thus yielding a maximal capacity of 90 tubes. Prior to irradiation, the samples were brought to the desired temperature in a special tempering box (Fig. 5). The tempering box was operated similarly to the Dewar flask. It was constructed of styrofoam insulating plates. The equipment installed at the Co⁶⁰ source of Cook Electric Co. is shown in Fig. 5, 6, and 7.

Dosimetry for inoculated ground beef in TDT cans. Values for γ -ray dose rate were determined by irradiating polystyrene cells (5/8 by 2 inches; 1.6 by 5 cm) filled with ferrous sulfate in the horizontal position in a no. 2 can filled with polystyrene. The dose was measured at 1-inch (2.5-cm) intervals along the entire vertical height of the no. 2 can. The values from top to bottom showed a variation of $\pm 1\%$.

Each TDT can was related to the corresponding dose value by marking the position of the individual cans in the can holder during irradiation.

For irradiation of spores in phosphate buffer in sealed test tubes (10 by 150 mm), the dose rate was determined in similar tubes (10 by 150 mm) containing ferrous sulfate. The tubes were placed for dosimetry into the test-tube holder illustrated in Fig. 3 F.

Calculation of D_{10} values. The average D_{10} value, i.e., dose necessary for destruction of 90% of the initial spore population, was calculated by the formula of Schmidt and Nank (1960):

$$D_{10} = \frac{\text{Mrad}}{\log M - \log S}$$

where M is the total number of spores per radiation dose level and S is the number of surviving spores, assuming at least one survivor per positive tube. The term D_{10} was used in preference to the conventional D without subscript, in conformity with

Matsuyama, Thornley, and Ingram (1964a), to designate 10% survival.

RESULTS

Effect of freezing on viability of spores. Information on the effect of freezing *per se* on the viability of spores is important in connection with irradiation of spores at low temperatures.

Repeated experiments showed that rapid freezing in liquid nitrogen (-196°C), taking approximately 30 sec for solidification, injured, to some degree, the viability of *C. botulinum* 33A spores. A reduction in numbers was recorded in two experiments in which spores in polystyrene tubes were frozen by rapid immersion into liquid nitrogen and held in the frozen state for 5 min and 2 hr.

The injury appeared to occur during the initial freezing at -196°C ; the length of the subsequent holding period at this temperature seemed not to be of importance. Retardation of growth in subcultures was most pronounced during the initial stages of growth in the recovery medium (Fig. 8), thus suggesting that spore injury by low temperature may be partially repairable by incubation of the spore in the recovery medium for 7 to 8 days.

Apparently, neither slow freezing of spores at -18°C , which required approximately 1 hr for solidification, nor further chilling of the slowly (-18°C) frozen spores to a temperature of -196°C had an effect on spore viability. Rather, it appeared that the injury was caused by violent physical changes occurring during rapid freezing at -196°C (e.g., expansion, disorderly displacement, pressure within the liquid, rapid crystallization, etc.).

Radiation resistance of spores. One unexpected finding was that results obtained by end-point

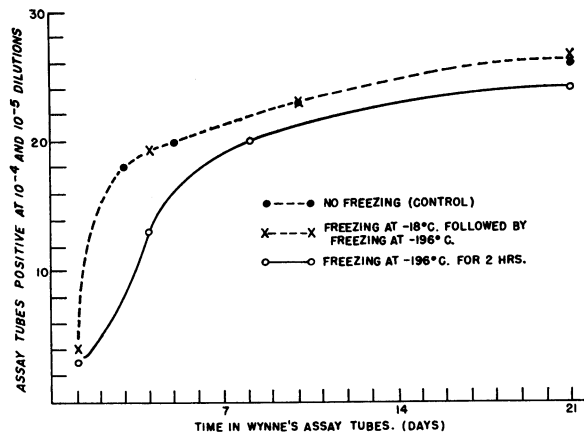


FIG. 8. Effect of slow freezing at -18°C and of rapid freezing at -196°C on viability of spores of *Clostridium botulinum* 33A.

spoilage techniques and those obtained by direct counts were substantially different at both temperatures and in both substrates, i.e., at 0 and -196 C, in phosphate buffer, and in ground beef.

Phosphate buffer (pH 7). Table 1 summarizes the data obtained by end-point techniques with 10 tubes with 10^4 spores of *C. botulinum* 33A per tube at each radiation dose level. At 0 C, 1.6 Mrad inactivated all spores, whereas 1.5 Mrad did not; at -196 C, the corresponding inactivation-non-inactivation doses were 1.9 and 1.6 Mrad. D_{10} values calculated from these data were $D_{10} = 0.306$ Mrad for 0 C and $D_{10} = 0.321$ Mrad for -196 C.

A second experiment in buffer done with colony count techniques is summarized in Fig. 9. The D_{10} values were $D_{10} = 0.275$ Mrad for 0 C and $D_{10} = 0.462$ for -196 C. Thus, the average D_{10} values from the two experiments in buffer were $D_{10} = 0.290$ for 0 C and $D_{10} = 0.396$ Mrad for -196 C; i.e., the lethal effect of radiation at -196 C decreased by approximately 13.5%.

Ground beef. The results of two experiments

TABLE 1. Radiation resistance of spores of *Clostridium botulinum* 33A in phosphate buffer (pH 7) at 0 and -196 C*

Dose Mrad	0 C		-196 C	
	No. of tubes showing survivors	D_{10} value†	No. of tubes showing survivors	D_{10} value†
1.0	—‡		10	
1.1	10		—	
1.2	8	.293	—	
1.3	5	.302	5	.302
1.4	3	.310	—	
1.5	2	.319	—	
1.6	0		2	.341
1.7	0		—	
1.8	0		—	
1.9	—		0	
Avg		.306		.321

* Ten Pyrex glass tubes were irradiated at each dose level. Each tube contained 10^4 spores in phosphate buffer. The experiment was evaluated by end-point techniques.

† The average D_{10} value, i.e., dose allowing survival of 10% of the initial spore population, (Schmidt and Nank, 1960). $D_{10} = \text{Mrad}/\log M - \log S$, where M = the total number of spores per radiation dose level (in this case, $10^4 \times 10 = 10^5$ spores per 10 tubes) and S = the number of surviving spores assuming at least one survivor per positive tube.

‡ Not tested.

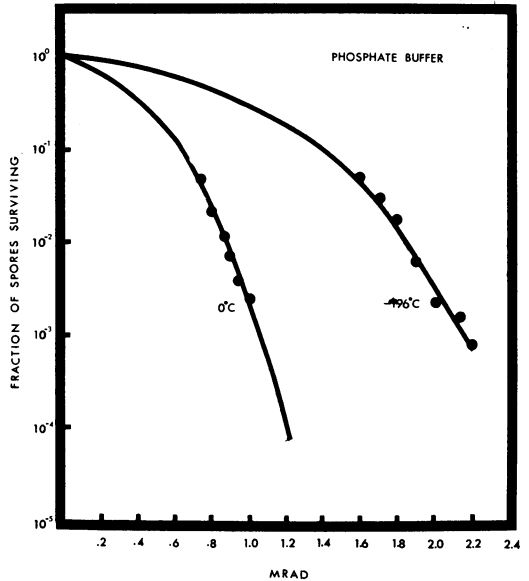


FIG. 9. Radiation resistance of spores of *Clostridium botulinum* 33A in phosphate buffer (pH 7) at 0 and -196 C. The initial inoculum was 1.3×10^4 spores per ml, with 10 tubes irradiated at each radiation dose level.

with 2.5×10^3 spores of *C. botulinum* 33A per gram of ground beef irradiated at 0 and -196 C and evaluated by end-point techniques were in reasonable agreement (Table 2). In experiment I, the temperature of -196 C was achieved by direct immersion of the cans into liquid nitrogen during irradiation. In experiment II, the cans were cooled in the vapor phase above liquid nitrogen. Immersion into liquid nitrogen was avoided in all recent experiments for two reasons: (i) the radiation dosimetry in the liquid substrate was not directly comparable with the dosimetry in the gaseous environment at 0 C, and (ii) liquid nitrogen occasionally penetrated into the cans and, on subsequent warming, produced a nitrogen swell due to expansion of the trapped gas.

Irradiation at -196 C to 2.5 to 3.3 Mrad gave 100% spoilage; 3.0 to 3.6 Mrad yielded partial spoilage, whereas 3.9 Mrad eliminated all swelling. The corresponding doses at 0 C were 2.0 Mrad (no effect), 2.5 to 2.75 Mrad (partial swelling), and 3.0 Mrad (no swelling). Thus, in the temperature range between 0 and -196 C, the increase in the radiation dose required to effect the same degree of destruction of spores of *C. botulinum* 33A in beef was 0.85 to 0.90 Mrad. The initial spore inoculum was 2.5×10^3 spores per gram $\times 20$ g of beef per can $\times 10$ cans per radiation dose = 5×10^5 spores per radiation dose.

The D_{10} values calculated from data in Table 2

TABLE 2. Swelling of cans of ground beef inoculated with spores of *Clostridium botulinum* 33A

Expt*	Dose	0 C		-196 C	
		No. of swollen cans	D_{10} value†	No. of swollen cans	D_{10} value†
	<i>Mrad</i>		<i>Mrad</i>		<i>Mrad</i>
I	0.0	10		10	
	1.0	10		10	
	1.5	10		10	
	2.0	10		10	
	2.5	1	.439	10	
	3.0	0		9	.632
	3.5	0		4	.687
	4.0	0		0	
	4.5	0		0	
II	2.25	8	.469	—	
	2.50	0		—	
	2.75	1	.483	—	
	3.0	—		10	
	3.3	—		10	
	3.6	—		5	.720
	3.9	—		0	
	4.2	—		0	
	4.5	—		0	
	Avg		.463		.680

* In experiment I the cans irradiated at -196 C were immersed into liquid nitrogen during irradiation, whereas in Experiment II the cans were kept in the vapor over liquid nitrogen. The necessary corrections for dosimetry were made to compensate for the changed environment. Ten cans were irradiated at each dose level. Each can was inoculated with 5×10^4 spores of *C. botulinum* 33A. The irradiated cans were incubated at 30 C for 6 months.

† The average D_{10} value, i.e., the dose necessary for destruction of 90% of the initial spore population (Schmidt and Nank, 1960). $D_{10} = \text{Mrad}/(\log M - \log S)$, where M = the total number of spores per radiation dose level (in this case, 2.5×10^8 spores per g \times 20 g per can \times 10 cans per radiation dose = 5×10^8 spores per radiation dose) and S = the number of surviving spores, assuming at least one survivor per swollen can.

were $D_{10} = 0.463$ Mrad for 0 C, and $D_{10} = 0.680$ Mrad for -196 C, and increase of approximately 47%.

In a second experiment in ground beef (Fig. 10), colony-count techniques revealed that 2.21×10^8 spores per gram of ground beef were reduced at about the same rate at 0 and -196 C up to 1.5 Mrad. However, at doses above 1.5 Mrad the survival curves separated. The radiation doses necessary for destruction of the entire

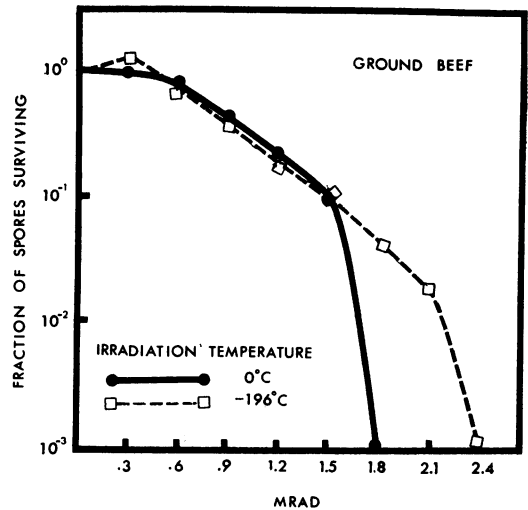


FIG. 10. Radiation survival curves of *Clostridium botulinum* 33A in canned ground beef at 0 and -196 C. Initial spore inoculum was 2.21×10^8 spores per gram of beef.

TABLE 3. Swelling of inoculated and uninoculated cans of ground beef irradiated in liquid nitrogen to 3.5 Mrad*

Dose	Spores of <i>Clostridium botulinum</i> 33A added per can	No. of swollen cans of 10 tested	Days required for swelling
<i>Mrad</i>	0.0	2	1
		8	2
0.0	None	2	3
		3	6
		2	13
		1	23
		1	27
		1	38
3.5	None	0	6 months

* The ground beef was heated to an internal temperature of 74 C to inactivate the enzymes and the vegetative microbial flora prior to canning and irradiation.

inoculum were 1.8 Mrad at 0 C, and 2.4 Mrad at -196 C.

These results indicate that in ground beef at low temperatures radiation survival of spores was complicated by what appears to be an extensive shoulder in the survival curve. This phenomenon deserves additional detailed study, so no D_{10} values have been calculated from these data at this time.

Uninoculated cans. A dose of 3.5 Mrad at 0 and

TABLE 4. Rate of swelling during incubation at 30 C of cans of ground beef inoculated with *Clostridium botulinum** and irradiated at -196 and 0 C

Radiation temp	Dose	No. of cans swollen during incubation at 30 C for													Total swollen (of 10 cans)
		1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days	9 days	10 days	11 days	12 days	13 days	
-196	0.0	2	8												10
	1.0		10												10
	1.5		10												10
	2.0					10									10
	2.5					2									10
	3.0							1	8						9
	3.5									8	1				4
	4.0 and 4.5†													3	0
0	0.0			1				9							10
	1.0			10											10
	1.5						9		1						10
	2.0								3		5				10
	2.5												2		1†
	3.0, 3.5, 4.0, and 4.5‡														0

* The inoculum was 5×10^4 spores of *C. botulinum* 33A per can. The data are for experiment I in which the cans irradiated at -196 C were immersed into liquid nitrogen during irradiation to achieve this low temperature.

† At these doses, no cans were swollen after 6 months.

‡ One can swollen after 20 days at 30 C.

-196 C was sufficient to arrest swelling of cans of ground pasteurized beef which was not experimentally infected with spores of *C. botulinum* (Table 3), whereas the nonirradiated cans spoiled all within 3 to 38 days.

After 6 months of storage, uninoculated meat irradiated to 3.5 Mrad had a pH of 6.1 to 6.4. No toxin and no viable organisms could be detected either in heat-shocked (10 min at 80 C) or in nonheated samples.

Rate of swelling of cans. As indicated in Table 4 and Fig. 11, 69.23% of the cans inoculated with *C. botulinum* spores swelled during the first week of incubation at 30 C, 29.80% swelled during the second week, and 0.96% during the third week. The time required for swelling was progressively longer with increasing doses of irradiation. Thus, all cans irradiated to 0.0, 1.0, and 1.5 Mrad swelled during the first week, whereas cans irradiated to 2.0 to 3.5 Mrad swelled during the second week.

The time required for swelling of uninoculated unirradiated cans (Table 3) was more unpredictable, as would be expected from the variability of the natural flora in ground beef.

Examination of cans after 6 months of incubation at 30 C. Swollen cans contained 1,000 to 10,000

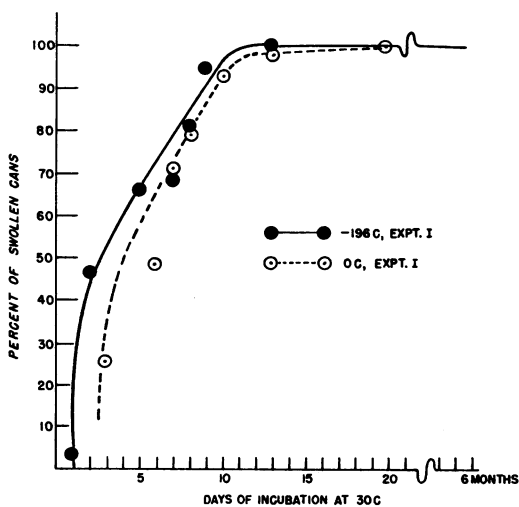


FIG. 11. Rate of swelling of cans during 6 months of incubation at 30 C.

MLD of type A botulinum toxin per gram of ground beef. Cans containing 1,000 MLD of toxin had approximately 10^4 organisms per gram, and those with 10,000 MLD of toxin had approximately 10^5 *C. botulinum* organisms per gram.

TABLE 5. Viable spores in nonswollen cans of irradiated ground beef after 6 months of incubation at 30 C

Irradiation temp	Expt*	Dose	No. of cans of a total of 10 containing spores	Minimal probable no. of spores†
-196	I	<i>Mrad</i>		
		3.0		
		3.5	6	1
		4.0	10	6
0	I	4.5	10	0
		3.6	5	0
		3.9, 4.2, 4.5	10	0
-196	II	2.5	9	0
		3.0, 3.5, 4.0, 4.5	10	0
		2.25	2	0
		2.50	10	0
-196	I (uninoculated control)	2.75	10	3
		3.5	10	0
Total			150	10

* Initial inoculum prior to irradiation was 2.5×10^3 spores of *Clostridium botulinum* 33A per gram of ground beef.

† The number of spores recovered ranged <1 to 7.36 organisms per gram, except for one can at 4.0 Mrad (experiment I, -196 C) which contained 1.73×10^2 spores per gram.

Nonswollen cans. None of the nonswollen cans contained detectable amounts of *C. botulinum* toxin. However, approximately 7% of the nonswollen cans contained very low numbers of viable spores which were able to survive 80 C for 10 min. These spores germinated, grew, and produced type-specific toxin in Wynne's broth (Wynne et al., 1955) whether heat shocked or not. The number of spores ranged from <1 to 7.36 organisms per gram (Table 5) and, thus, comprised approximately 0.04 to 0.4% of the original inoculum. In one seemingly exceptional case, 1.73×10^2 spores, i.e., approximately 7% of the original inoculum, could be recovered from a can irradiated to 4.0 Mrad at -196 C. It is not clear whether these spores were unable to germinate because of natural dormancy, radiation-induced dormancy, or because of a more exacting nutrient requirement.

Quality of beef irradiated at -196 and 0 C. By visual examination, the beef irradiated at -196 C had a considerably better appearance and odor than corresponding samples irradiated at 0 C. However, no attempt was made to evaluate quantitatively the improvement in quality of beef by low temperature irradiation.

DISCUSSION

The recent advance of liquid nitrogen into commercial food processing (Mischon, 1961; Samuel, 1962) suggests the feasibility of the economical use of this refrigerant in low-temperature radiation preservation of food. The advantage of low temperatures in reducing the radiation-induced off-flavors and off-odors would be particularly beneficial in sterilization of radiation-sensitive foods, such as beef, which become unacceptable when irradiated at ambient temperatures.

Early investigators (Brasch and Huber, 1948; Huber, Brasch, and Waly, 1953) observed that freezing was very effective in preventing undesirable organoleptic changes in irradiated foods. Later reports appeared to indicate that freezing had no significant benefit (Pratt and Ecklund, 1956; Cain, Bubl, and Anderon, 1956; Schultz et al., 1956). However, more recent investigations showed again that freezing at -75 C provided an improvement in the flavor of raw meat by a factor of at least 2.5; furthermore, it has been shown that the degree of freezing (i.e., the actual temperature) influenced the amount of radiation-induced flavor changes. Irradiation in liquid nitrogen resulted in the least quality change, but a large proportion of protection was already achieved at temperatures as high as -20 C (Coleby et al., 1961).

Protection against radiation-induced deterioration of foods afforded by low temperatures may be explained by the fact that low temperatures preclude mass transfer of radicals in frozen aqueous systems but allow electron excitation and migration (Huber, 1961). Trapped immobile radicals are formed in the frozen aqueous system which disappear on warming but long before melting (Matheson and Smaller, 1958).

Microbial flora. The literature indicates that at very low temperatures microorganisms become increasingly resistant to ionizing radiations; however, the increase in resistance may not be commensurate with the protection afforded to foods by these temperatures. In this sense, low temperatures may be of value in radiation sterilization of those meats which develop unacceptable flavor changes at ambient temperatures.

The natural flora of foods is generally comprised of vegetative cells and spores. Both types exhibit distinct sensitivities to radiation and temperature changes.

Vegetative cells comprise the major portion of the flora of food stuffs. Freezing protects vegetative cells of *Escherichia coli* against radiation by a factor of two to five times. (Hollaender and Stapleton, 1953; Houtermans, 1954; Bellamy and Lawton, 1955; Stapleton and Edington, 1956).

Similar protection was reported for *Pseudomonas aeruginosa* (Moos, 1952), *Staphylococcus aureus* (Bellamy and Lawton, 1955), and slowly frozen yeasts (Wood and Taylor, 1957). For several vegetative organisms, the sensitivity in the frozen state was found to be comparable to that of the dry organisms (Bellamy and Lawton, 1954).

Coleby et al. (1961) reported that the mixed flora of minced beef, with an initial load of approximately 0.5×10^6 organisms, was reduced after irradiation with 0.2 Mrad to fewer than 10^4 survivors at -80 to -20 C and to 10^3 at 0 to 18 C. The radiation sensitivity of the microorganisms decreased in a rather sudden manner between 0 and -20 C. This may correlate with the solidification of water in the cell within this critical temperature range. A radiation-resistant vegetative organism *Streptococcus faecium* R53 yielded a D_{10} of 0.300 Mrad when irradiated at -79 C (Matsuyama et al., 1964a). This resistance was closely approaching that of some of the highly radioresistant spores of *C. botulinum* irradiated at 0 C (Anellis and Koch, 1962).

The main objective in radiation sterilization of foods is the destruction of bacterial spores, particularly *C. botulinum*. In contrast to vegetative cells, the spores appear to be affected by low temperatures to a much smaller degree, or not at all. Similar results were reported for aerobic spore-formers (Houtermans, 1956; Proctor et al., 1955; Fuld, Proctor, and Goldblith, 1957; Powers, Webb, and Ehret, 1960) of an organism related to *C. sporogenes* (Fuld et al., 1957), for *C. perfringens* (Matsuyama et al., 1964b), and for *C. botulinum* (Denny et al., 1959; Ingram and Thornley, 1961).

Some investigators have reported a reduction in radiation resistance of spores in the frozen state (Edwards, Peterson, and Cummings, 1954; Pepper, Buffa, and Chandler, 1956), whereas others have recorded an increase in resistance at low temperatures (Denny et al., 1959; Ingram et al., 1959). Kempe and Graikoski (*unpublished data*) found that the radiation resistance of spores of *C. botulinum* 62A at -75 C was approximately the same as at 20 to 30 C.

A recent systematic study by Powers et al. (1960) indicated that the X-ray resistance of dry spores of *Bacillus megaterium* was temperature-independent below -148 C, whereas from -148 to 37 C there was a steady increase in sensitivity, amounting to about 45%.

C. botulinum spores were irradiated in frozen phosphate-buffer by Denny et al. (1959) and in raw minced pork and beef at 0 and -78 C by Ingram et al. (1959) and Ingram and Thornley (1961). In both cases, a relatively small reduction

in radiation sensitivity of spores at the lower temperature was noted.

An approximate 0.9 Mrad increase in dose requirement was established for comparable destruction of spores of *C. botulinum* 33A in ground beef between temperatures of 0 and -196 C. If 4.5 Mrad is accepted as the safe sterilization dose at 0 C (Schmidt, 1961), then by simple addition one would estimate that 5.4 Mrad would be needed at -196 C for comparable sterility.

If the 12 D concept is applied, then $D_{10} = 0.680$ Mrad for -196 C in beef yields a dose requirement of approximately 8.16 Mrad for commercially safe radiation sterilization of beef at -196 C. However, the application of the 12 D concept to radiation sterilization at -196 C is rather problematic. Work in progress suggests that low spore inocula (10^2 to 10^3 spores per gram), such as used in the present experiment, yield especially high D_{10} values. In this sense, the above calculations should be viewed as preliminary approximations. Actual doses for safe commercial sterilization of foods at -196 C should be established by detailed studies, with special consideration of each particular food product to be irradiated.

ACKNOWLEDGMENT

Our thanks are due to M. D. Schneider for participation in the initial experiments of this project, to D. Berkowitz for competent and enthusiastic technical assistance, to O. Wolford for preparing Fig. 4, and to T. C. Tang for preparing Fig. 9 and 10.

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