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Effect of Heat Treatment on Survival of, and Growth from, Spores of Nonproteolytic *Clostridium botulinum* at Refrigeration Temperatures

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Spores of five type B, five type E, and two type F strains of nonproteolytic *Clostridium botulinum* were inoculated into tubes of an anaerobic meat medium plus lysozyme to give approximately 10^6 spores per tube. Sets of tubes were then subjected to a heat treatment, cooled, and incubated at 6, 8, 10, 12, and 25°C for up to 60 days. Treatments equivalent to heating at 65°C for 364 min, 70°C for 8 min, and 75°C for 27 min had little effect on growth and toxin formation. After a treatment equivalent to heating at 80°C for 23 min, growth occurred at 6 and 8°C within 28 to 40 days. After a treatment equivalent to heating at 85°C for 19 min, growth occurred in some tubes at 6, 8, 10, or 12°C within 28 to 53 days and at 25°C in all tubes within 15 days. Following a treatment equivalent to heating at 95°C for 15 min, growth was detected in some tubes incubated at 25°C for fewer than 60 days but not in tubes incubated at 6 to 12°C. The results indicate that heat treatment of processed foods equivalent to maintenance at 85°C for 19 min combined with storage below 12°C and a shelf life of not more than 28 days would reduce the risk of growth from spores of nonproteolytic *C. botulinum* by a factor of 10^6. If foods intended for refrigerated distribution and storage are liable to be maintained at temperatures higher than 10°C, growth and toxin production by proteolytic strains of *C. botulinum* must also be prevented.

Clostridium botulinum produces an extremely potent neurotoxin, and growth of this bacterium in foods is liable to result in the production of sufficient toxin to cause severe illness or death. It is, therefore, imperative to ensure the safety of food with respect to *C. botulinum*. The safety and preservation of low-acid, refrigerated, processed foods with an extended shelf life, including sous-vide foods, rely mainly on heat treatment followed by refrigerated storage. Nonproteolytic strains of *C. botulinum* (types B, E, and F), unlike proteolytic strains, are capable of growth and toxin production at refrigeration temperatures and present a potential hazard in these foods (27). Growth and production of toxin by nonproteolytic strains of *C. botulinum* have been detected at temperatures as low as 3.3°C (7, 8, 41), and predictive mathematical models that show the effect of temperature and other factors on growth from vegetative cells and spores of nonproteolytic *C. botulinum* have been developed (2). There is little published information, however, that shows the combined effect of heat treatment and subsequent storage temperature on the survival of and growth from spores of nonproteolytic *C. botulinum*. Several groups of workers have reported that for spores of these bacteria heated in phosphate buffer at pH 7.0, the $D_{82.2°C}$ (time at 82.2°C required for a 10-fold reduction in viable numbers) was 2 min or less (26), but the apparent heat resistance of the spores can be increased considerably by the inclusion of lysozyme in the recovery medium (31–33). Lysozyme promotes growth from a small fraction of the heated spores, making this fraction apparently more heat resistant than the remainder of the spores and resulting in biphasic survival curves. This effect of lysozyme is significant because the enzyme, and others with a similar effect on spores of nonproteolytic *C. botulinum*, may be present in various types of food (27).

The aim of the work reported here was to determine the effect of heat treatment and subsequent incubation temperature on the ability of spores of nonproteolytic *C. botulinum* to survive and give growth in an anaerobic meat medium intended as a model food and, specifically, the combination of treatments required to reduce the risk of growth from spores of nonproteolytic *C. botulinum* by a factor of 10^6. In this work it was important to ensure that the meat medium represented a type of food that would provide the most favorable conditions for growth from heated spores, i.e., a "worst case" situation. For this reason, (i) during preparation the medium was adjusted from its initial pH of 5.8 (the natural pH of the meat) to pH 6.1 to 6.3, which is within the optimum range for growth of nonproteolytic *C. botulinum*; (ii) lysozyme, 10 μg/ml (625 U/ml), was added to the medium before heat treatment of the spores; and (iii) tubes of medium were prepared under strictly anaerobic conditions in order to ensure that traces of oxygen did not contribute to inhibition of *C. botulinum.*

MATERIALS AND METHODS

Bacteria. Cultures of nonproteolytic *C. botulinum* types B (Eklund 2B, Eklund 17B, Hobbs FT50, and Colworth 151), E (Beluga, Foster B96, and Sebald P34), and F (Eklund 202F and Craig 610) were originally obtained from J. S. Crowther, Unilever Research. Type B strain 2129B was from V. N. Scott (National Food Processors Association), type E strain VH was from C. L. Hatheway (Centers for Disease Control), and type E strain Hazen 36208 was from the National Collection of Industrial Bacteria (NCIB 10660). All were maintained as described previously (25). Tests were made to confirm that the concentration of toxin

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formed in peptone-yeast extract-glucose-starch (PYGS) medium (25) was higher than 1,000 mouse lethal doses per ml (9).

Preparation of spores and assessment of spore heat resistance. Spores were produced on a two-phase medium and washed as described by Peck et al. (31). In order to confirm that the spores had the expected level of heat resistance, the effect of heating at 85°C in phosphate buffer (pH 7.0; 0.067 M) was determined by the submerged tube procedure of Kooiman and Geers (21), as detailed previously (31).

Numbers of spores that survived the heat treatments were determined by preparation of serial dilutions in PYGS medium, using strict anaerobic technique (18, 25), followed by inoculation of samples onto PYGS medium plus lysozyme, 10 μg/ml (hen egg white lysozyme, 62,500 U/mg; L6876; Sigma, Poole, United Kingdom [U.K.]). All plates were incubated anaerobically at 30°C under a headspace of H_2-CO_2 (90:10, vol/vol), and the number of colonies was counted after 5 days.

Preparation of meat medium. Raw, minced meat with a fat content of approximately 18.6% (wt/wt) (Brooks, Norwich, U.K.) was ground in a domestic food mixer (Magimix 5000) for approximately 30 s. An equivalent volume of water was added, and the mixture was steamed in a boiling-water bath for 10 min to cook the meat. The meat slurry was then ground in the food mixer a second time for approximately 60 s to produce very small particles. Additions were made so that the final medium was composed of the following: minced beef, 500 g; glucose, 10 g; NaCl, 10 g; soluble starch, 10 g; glass-distilled water, to 1,000 g. The final medium was prepared by strict anaerobic technique (18) and was boiled under oxygen-free H_2-CO_2-N_2 (10:5:85, vol/vol/vol) for 15 min with constant stirring. Evaporation was minimized by the use of a trap, the flask was weighed at the end of boiling to estimate any loss of water, and deoxygenated glass-distilled water (prepared by strict anaerobic technique) was added when necessary to give the required weight. The medium was cooled to 50°C in a stream of oxygen-free H_2-CO_2-N_2, and the pH was adjusted from approximately 5.8 to 6.4 with 5 M KOH. After the medium was held at 50°C in a stream of oxygen-free H_2-CO_2-N_2 for 1 h, the pH was again adjusted to 6.4 (if necessary). A total of approximately 5 ml of 5 M KOH was added per liter of medium. The medium was continuously stirred and maintained at 50°C to keep the fat molten, while 20-ml volumes were dispensed by strict anaerobic technique into anaerobic culture tubes (18 by 150 mm; Bellco, Vineland, N.J.), which were then capped with grey butyl rubber septa (Jencons Scientific, Leighton Buzzard, U.K.) and sealed. This procedure ensured an even distribution of fat throughout the tubes. The medium was sterilized by autoclaving at 121°C for 15 min, stored at 1°C, and used within 2 weeks of preparation. The pH of the medium after autoclaving was 6.1 to 6.3, and the final fat content was 9.3% (wt/wt).

On the day prior to inoculation, sterile lysozyme was added to all tubes. A solution of 1 mg of lysozyme per ml was prepared in deoxygenated glass-distilled water (prepared by strict anaerobic technique) and sterilized by filtration (0.22-μm pore size, Millex-GV; Millipore, Watford, U.K.), and 0.2 ml was added to each tube to give a final concentration of 10 μg/ml (625 U/ml). All of these manipulations were carried out in an anaerobic cabinet filled with oxygen-free H_2-CO_2-N_2 (10:5:85, vol/vol/vol).

Preparation of spore suspension and inoculation of meat medium. A suspension of spores (approximately 5×10^6/ml) was prepared in deoxygenated saline (0.85%, wt/vol). This suspension contained an equal number of spores of each of 12 strains of nonproteolytic *C. botulinum* (five type B strains, five type E strains, and two type F strains). From this suspension, 0.2-ml volumes were added by syringe to sealed tubes of medium that had been prewarmed at 45°C for 10 min, to melt and mix the fat. The tubes were then shaken to disperse the added spores. Ten replicate tubes were used for each heat treatment-incubation temperature. The inoculated tubes were heat treated within 30 min of the addition of spores. At the end of the experiment, the number of viable spores present in the unheated spore suspension was determined by preparation of duplicate dilution series and inoculation of appropriate dilutions onto four replicate plates of PYGS medium. After anaerobic incubation at 30°C for 4 days, the numbers of colonies were counted.

Measurement of heat treatments applied. In order to monitor the temperature at the center of each tube, between four and eight micro-thermistor probes (Grant Instruments, Cambridge, U.K.) were sealed singly into tubes of uninoculated meat medium. Each probe had been calibrated previously against certified precision mercury-in-glass thermometers in a range of 55 to 95°C. The temperature was recorded to the nearest 0.05°C. The heat treatment applied was assessed by placing the temperature-monitoring tubes (i.e., those with thermistor probes) towards the center of the rack containing tubes to be heat treated; this ensured that they received the lowest heat treatment of all tubes. An appropriate heat treatment was applied by immersing the rack of tubes rapidly in a large water bath (W38 bath; Grant Instruments) set at the desired temperature, and the treatment was monitored by a precision mercury-in-glass thermometer. When the core temperature of the meat medium was within 0.1°C of that required, timing was started. After an appropriate period of time at the desired temperature, the rack of tubes was removed and plunged into a deep ice bath. The tubes were then shaken vigorously to effect a rapid cooling. When the core temperature of the meat medium had fallen below 10°C in all monitored tubes, the tubes were dried and transferred to an appropriate incubator. Tubes incubated at 6 and 8°C were maintained in anaerobic jars (H_2-CO_2, 90:10 [vol/vol]) to ensure strict anaerobic conditions during the long incubation period at low temperatures. Each heat treatment, from the time of addition of the spore suspension through the period of heating, maintenance at high temperature, and cooling, was carried out in approximately 60 to 80 min, with the exception of the heat treatment at 65°C. Tubes for each heating temperature were all heated at the same time.

Temperatures were recorded by a 12-bit Squirrel data logger (1200 series; Grant Instruments), with the response of the probes monitored every 5 or 6 s. The data were analyzed on a microcomputer. For each measured temperature (T_m), the lethal rate (L) with reference to the target temperature (T_t) was calculated from the formula, $L = 10^{[(T_m - T_t)/z]}$, where z is the change in temperature, in °C, resulting in a 10-fold change in decimal reduction time. A z value of 8°C was used (26). The lethality of the heat treatment (K), in terms of the equivalent time (minutes) at the target temperature (T_t), was calculated from the equation, $K = \Sigma(L \cdot \Delta t)$, where Δt is the time (minutes) between temperature measurements. This procedure is similar to the one described by Pflug (35).

Incubation of tubes after heat treatment. After heat treatment and cooling, tubes of meat medium previously inoculated with spores of *C. botulinum* were incubated in low-temperature incubators (Astell-Hearson, model MK III), the temperatures of which were monitored with platinum resistance thermometers connected to a data logger and recorded at intervals of 30 min. The platinum resistance thermometers (British Standard Grade II) were calibrated to an accuracy of ±0.1°C over the range of use and were positioned in air close to the incubated tubes. At the end of each experiment, the data were analyzed to determine the mean temperature and the variation in temperature.

Determination of growth and toxin production. Tubes were examined at least every 2 to 3 days for signs of growth. Growth of *C. botulinum* in tubes of the meat medium was indicated by obvious formation of gas. In some circumstances a few gas bubbles or minor cracks in the meat medium occurred as a result of the manipulations; this was not taken to indicate growth.

Samples of medium to be tested for toxin were frozen on the day of sampling and later centrifuged (12,000 × *g*, 10°C, 30 min), and the supernatant was stored in a frozen state until it was tested for toxin. Tests for the presence of toxin were made by intraperitoneal injection into mice, using a method essentially the same as that described in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (9), with the following modifications: four mice were always used instead of two, the mice were observed for 4 days rather than 3 days, and the presence of toxin was confirmed by neutralization with specific antisera (detailed below). All samples were treated with trypsin prior to injection. The limit of sensitivity of the test was 2 mouse lethal doses per ml of culture supernatant. Samples were tested for toxin at days 0 (to confirm absence of toxin), 14 (treatments incubated at 25°C), 28 (treatments incubated at 25°C and negative for toxin at day 14 and treatments incubated at 6 to 12°C), and 60 (all treatments previously negative for toxin). If all tubes in a set showed growth, or all showed no growth, three replicate tubes from each treatment were tested for the presence of toxin; if some but not all of the tubes in a set showed growth, five of the replicate tubes were tested. For each treatment in which toxin was detected, the contents of all replicate tubes that contained toxin were pooled and the presence of type B, E, or F toxin was confirmed by neutralization with a mixture of antisera to these toxins, or the type of toxin was determined by neutralization with the individual, specific antiserum.

RESULTS

Heat resistance of spores. Heat treatment of spores at 85°C resulted in biphasic survival curves. Approximately 0.1 to 1.0% of the heated spores were permeable to lysozyme in the plating medium in the case of all strains except Foster B96, in which nearly 20% of spores were permeable to lysozyme. Heat treatment at 85°C for 60 min resulted in a reduction by a factor of $10^{1.9}$ in the number of viable spores of strain Foster B96 and by a factor of 10^5 in the number of spores of strain Colworth 151. Results for the other strains (four type B, four type E, and two type F) were between these values. These results were similar to those for other batches of spores in previous work (33).

Numbers of spores added to tubes of meat medium before heat treatment. The mean numbers, and 95% confidence intervals, of spores in the mixture of strains that were added to each tube containing 20 ml of medium prior to heat treatment were $(7.4 \pm 0.8) \times 10^5$ (experiment 1) and $(7.8 \pm 0.9) \times 10^5$ (experiment 2).

Heat treatments applied. Thermistors sealed into tubes of uninoculated meat medium recorded the heat treatments applied. When the temperature in the tubes approached the target value, the slow rate of increase in temperature caused some difficulty in deciding when the temperature at the core of the tubes of meat medium was within 0.1°C of the target

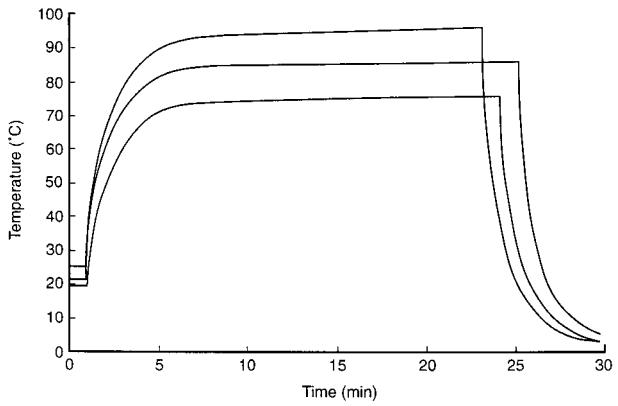

FIG. 1. Changes in temperature of meat medium, inoculated with spores of nonproteolytic *C. botulinum*, during heat treatment. The temperature in reference tubes of uninoculated medium during heating to 75, 85, and 95°C in experiment 1 was measured at intervals of 5 s; mean temperatures are shown.

temperature and, consequently, the point at which the come-up period ended and the time when heat treatment at the required temperature started (Fig. 1). The come-up period contributed significantly to the lethality of all heat treatments because for a large part of the come-up time the core temperature in the meat medium was close to the maximum temperature (Fig. 1; Table 1). The cooldown period was only 2 to 3 min in all treatments (Fig. 1; Table 1). The lethal effect of the total heat treatment is expressed as the equivalent time at the target temperature (Table 1), and this is the best definition of the heat treatments given.

Incubation temperature after heat treatment. The actual mean incubation temperatures were 5.7, 8.0, 10.0, 11.8, and 25.1°C in experiment 1 and 6.1, 8.1, 9.8, 11.8, and 25.0°C in experiment 2. For more than 92% of the time of incubation (60 days) in experiment 1 and 97% of the time in experiment 2, the temperatures were within ±0.5°C of the mean, and in no case was the measurement more than ±1.0°C of the mean. The measured temperature was that of air in the incubator and not that of the meat medium. It would be expected that the temperature of the meat medium would vary less than that of the air in the incubators.

Growth of nonproteolytic *C. botulinum* in meat medium after heat treatment and incubation at chill temperature. Growth of *C. botulinum* was recorded when formation of gas was evident. For each treatment, 10 replicate tubes were used. During incubation some tubes were removed before 60 days to test for the presence of toxin (see Materials and Methods); thus, the number of tubes per treatment incubated throughout the full 60 days was fewer than 10. After heat treatments at the target temperatures of 80°C or lower, growth occurred in all tubes at all incubation temperatures within 60 days (Table 2; Fig. 2). Heat treatments at 65 and 70°C had a negligible effect in reducing the period before growth, and a heat treatment at 75°C had a very small effect at low incubation temperatures (Table 2; Fig. 2). Heat treatment at 80°C more than doubled the period required before growth occurred, with growth in at least one tube detected after 40 days or less at all incubation temperatures. After the heat treatments at 85°C, growth was detected in only a few of the tubes incubated for the full 60 days at 6°C (3 of 14) or 8°C (2 of 14), in most tubes incubated at 10°C (8 of 14), and in all tubes incubated at 12 and 25°C. The shortest incubation times before growth was detected were after 6 days at 25°C, 42 days at 12 and 10°C, and 53 days at 6 and 8°C (Table 2; Fig. 2). After the heat treatment at 95°C, no growth was detected in tubes incubated at 6 to 12°C. Of the 10 tubes heated at 95°C and incubated at 25°C, 4 were incubated for 60 days (the remaining six tubes were used to test for toxin). One of these four tubes showed growth after 32 days, but no other tubes showed growth after 60 days (Table 2). Of the tubes heated at 95°C and incubated at 6, 8, 10, and 12°C for 60 days, four from each temperature were transferred to 25°C and incubated for a further 60 days. Growth occurred in 2 of these 16 tubes in 14 days; thus, survivors were detected in 3 of 20 of the tubes heated at 95°C. The heat treatment at 95°C was not sufficient, therefore, to give reliable inactivation of 10^6 spores of nonproteolytic *C. botulinum.*

Toxin formation by nonproteolytic *C. botulinum.* Three or five tubes from each treatment were tested for toxin after 14, 28, and 60 days, and the type of toxin formed was determined. No toxin was detected in heated or unheated tubes at day 0. Toxin was detected in the great majority of tubes in which growth was recorded (Table 3); the only exceptions were one tube heated at 85°C and incubated at 10°C for 60 days (replicates of this tube were positive for toxin) and one tube heated at 95°C and incubated at 25°C for 60 days. Toxin was detected in only one tube (heated at 85°C and incubated for 28 days at 12°C) in which growth had not been detected from gas production, and the level of toxin was too low to be typed. Growth was observed later in other tubes subjected to this treatment. In every case in which the type of toxin was determined, type

TABLE 1. Summary of heat treatments applied to spores of nonproteolytic *C. botulinum* in a meat medium

Expt	Maximum temp of heat treatment (°C)		Time (min)			Target time (min) at target temp	Heat lethality (*K*) expressed as equivalent time (min) at target temp[a]
	Target	Actual	Come-up[b]	At target temp[c]	Cooldown[d]		
1	65	65.0	9.0	359.9	3.0	360	364.0
	70	70.0	11.3	2.4	3.0	2	8.2
	75	75.0	13.7	9.2	3.0	10	16.9
	85	85.0	14.8	9.3	2.8	10	17.3
	95	95.3	12.3	9.8	3.2	10	15.2
2	75	75.0	23.4	10.1	2.0	10	27.0
	80	80.0	19.3	10.3	2.1	10	23.3
	85	85.0	14.2	11.0	2.1	10	19.2

[a] Calculated by the procedure described in the text.
[b] Time required for temperature at the center of meat medium to reach within 0.1°C of the target maximum temperature.
[c] Time that temperature at the center of meat medium was within 0.1°C of the target maximum temperature or higher.
[d] Time required for temperature at the center of meat medium to fall from within 0.1°C of the target maximum temperature to less than 10°C.

TABLE 2. Combined effect of heat treatment in a meat medium and subsequent incubation temperature on growth from spores of nonproteolytic *C. botulinum*[a]

Heating temp (°C)	Incubation time (days) to first observation of growth at specified incubation temp/incubation time (days) to growth in all tubes									
	6°C		8°C		10°C		12°C		25°C	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
None	8/11	7/9	4/6	5/7	4/4	5/5	2/4	3/3	1/1	1/1
65	11/15	NT[b]	4/7	NT	4/5	NT	2/4	NT	1/1	NT
70	8/13	NT	6/6	NT	4/7	NT	4/4	NT	1/1	NT
75	13/18	16/16	11/11	9/12	6/7	7/9	5/5	5/5	1/1	1/2
80	NT	40/47	NT	23/40	NT	19/23	NT	12/19	NT	3/5
85	NG[c]	53/(3/7)[d]	NG	53/(2/7)	42/(1/7)	49/60	47[e]/60	42/49	6/15	9/14
95	NG	NT	NG	NT	NG	NT	NG	NT	32/(1/4)	NT

[a] Tubes were inoculated with spores from 12 strains of nonproteolytic *C. botulinum* to give 10^6 spores per tube. Full details of the heat treatments applied are given in Table 1. For each experiment there were initially 10 tubes per treatment (heating and incubation). Sample tubes were removed to be tested for toxin on days 14 (tubes incubated at 25°C only), 28 (tubes incubated at 25°C previously negative for toxin and tubes incubated at 6 to 12°C), and 60 (tubes previously negative for toxin). Three tubes were tested if all tubes either showed growth or no growth, and five tubes were tested if some, but not all, showed growth.

[b] NT, treatment not tested.

[c] NG, no growth observed at day 60.

[d] Value in parentheses is number of tubes positive/total at day 60.

[e] Toxin detected at day 28 at a very low concentration.

B was present, while toxins of types E and F were detected less frequently (Table 3).

DISCUSSION

In the experiments reported here, we investigated the combination of heat treatment and temperature and time of incubation required to reduce the risk of growth from spores of nonproteolytic *C. botulinum* by a factor of approximately 10^6. A reasoned proposal regarding the reduction in risk of growth of nonproteolytic *C. botulinum* that should be provided in the production of refrigerated, processed foods with an extended shelf life does not appear to have been published. Regulations and guidelines for the production of refrigerated, processed foods with an extended shelf life have been produced in several countries (26, 40); in some cases they have failed to comment in quantitative terms on the degree of safety with respect to *C. botulinum*. In the United Kingdom, the Richmond Committee stated, without a supporting argument, that for sous-vide meals with an extended shelf life the thermal process "should reduce

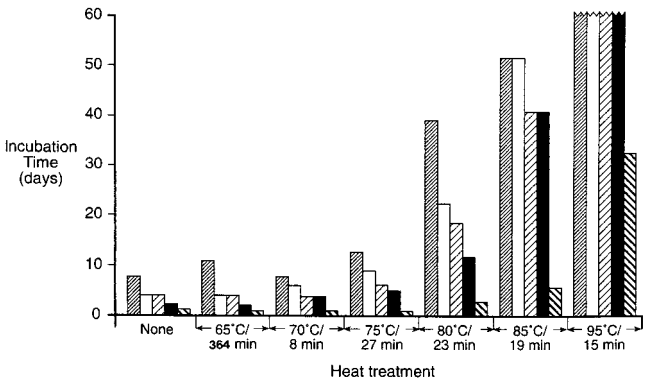

FIG. 2. Effect of heat treatment and incubation temperature on number of days required for growth from spores of nonproteolytic *C. botulinum* in a meat medium. The histogram shows the incubation time required before the first tube from each treatment (heat treatment-incubation temperature) showed growth. Before being heated, tubes were inoculated at 10^6 spores per tube with spores from 12 strains of nonproteolytic *C. botulinum*. Full details of the heat treatments applied are given in Table 1. Target incubation temperatures: ▨, 6°C; □, 8°C; ▨ 10°C; ■, 12°C; ▧, 25°C.

non-proteolytic strains of *C. botulinum* by a factor of 10^6 (6D process)" (4). This criterion was adopted by the Advisory Committee on the Microbiological Safety of Food in its report, "Vacuum Packaging and Associated Processes" (1).

It is relevant to consider the safety factor that applies to refrigerated, processed foods with an extended shelf life in relation to the safety factors that apply in the production of other foods. It is generally accepted that the thermal process for canned, low-acid foods should be at least sufficient to reduce the number of viable spores of proteolytic *C. botulinum* by a factor of 10^{12} (12D process) (16, 17, 36). In shelf-stable, canned, cured meats preserved by a combination of thermoprocessing, salt, and nitrite, the effect of the preservation has been estimated as equivalent to a reduction in the risk of growth of *C. botulinum* by a factor of not more than 10^8 (16). Reduction in the concentration of salt and nitrite in these products would be liable to reduce this degree of protection. In the case of perishable cured meats, the preservation of which depends on a combination of salt, nitrite, and refrigeration, Hauschild (15) concluded that vacuum-packed bacon was one of the best protected of the meats considered, the then current manufacturing process resulting in reduction by a factor of 10^5 to 10^7 in the risk of growth of *C. botulinum* in conditions of temperature abuse. The degree of safety of other perishable cured meats with respect to *C. botulinum* differed by several log units. These considerations led us to decide that we should aim for a combination of heat treatment and incubation conditions that would reduce the risk of growth of nonproteolytic *C. botulinum* by a factor of the order of 10^6.

Guidelines and Codes of Practice often stipulate that storage of these foods should be at very low refrigeration temperatures. During storage in retail and domestic refrigerators, however, such foods may be liable to maintenance at temperatures up to 8 or 10°C. At 6, 8, and 10°C, doubling times of 23, 9.0, and 5.7 h have been reported for nonproteolytic *C. botulinum* type B (14); thus, in 7 days at these temperatures the theoretical increases in numbers would be 150-, 4×10^5-, and 7.4×10^8-fold, respectively. In the present work, with an inoculum of about 3.8×10^4 spores per ml, growth in tubes of unheated medium was observed after 4 days at 8 and 10°C. Other workers, using inoculated-sample studies at these temperatures (usually with lower inocula), have reported toxin detection after 5 to 9 days in fish and crabmeat (3, 10, 11, 19,

TABLE 3. Combined effect of heat treatment in a meat medium and subsequent incubation temperature on the toxin formed from spores of nonproteolytic *C. botulinum*[a]

Heating temp (°C)	Type of toxin detected at specified incubation temp									
	6°C		8°C		10°C		12°C		25°C	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
None	B (28)[b]	B (28)	B, E (28)	B (28)	B, E, F (28)	B (28)	B, E, F (28)	+[c] (28)	B, E, F (14)	B, E (14)
65	B (28)	NT[d]	B (28)	NT	B, F (28)	NT	B, F (28)	NT	B, E, F (14)	NT
70	B (28)	NT	B (28)	NT	B, E, F (28)	NT	B, E, F (28)	NT	B, E, F (14)	NT
75	B (28)	+ (28)	B (28)	B (28)	B, F (28)	B (28)	B, F (28)	B (28)	B, E, F (14)	B, E (14)
80	NT	+ (60)	NT	+ (28)	NT	+ (28)	NT	B (28)	NT	B (14)
85	No toxin (60)[e]	+ (60)	No toxin (60)[e]	+ (60)	No toxin (60)[e]	+ (60)	B[f] (60)	+ (60)	B, E (14)	B (14)
95	No toxin (60)[e]	NT	No toxin (60)[e]	NT	No toxin (60)[e]	NT	No toxin (60)[e]	NT	No toxin (60)[g]	NT

[a] Tubes were inoculated with spores from 12 strains of nonproteolytic *C. botulinum* to give 10^6 spores per tube. Full details of the heat treatments applied are given in Table 1. Time to visible growth is given in Table 2. For each experiment there were initially 10 tubes per treatment (heating and incubation). Sample tubes were removed to be tested for toxin on days 14 (tubes incubated at 25°C only), 28 (tubes incubated at 25°C previously negative for toxin and tubes incubated at 6 to 12°C), and 60 (tubes previously negative for toxin). Three tubes were tested if all tubes either showed growth or no growth, and five tubes were tested if some, but not all, showed growth. The contents of tubes positive for toxin were pooled, and inactivation by type B, E, and F antitoxin was confirmed; in some cases, the toxin was also typed.

[b] Time (days of incubation) sample was removed for toxin test.

[c] Positive (+) toxin neutralized with antiserum to types B, E, and F but not with type B antiserum alone.

[d] NT, treatment not tested.

[e] No growth observed by day 60 (see Table 2).

[f] Toxin detected at day 28, but concentration was too low to type.

[g] Growth observed (See Table 2).

20, 22–24, 29, 38, 42), 7 to 8 days in poultry meat (12, 28), and 8 to 9 days in sous-vide-type products (2, 29).

The heat treatments used in the present work were chosen to be relevant to heat treatments that have been considered for use in the production of processed foods, depending on the required shelf life. The target time of 2 min at 70°C was included because this is the heat treatment specified to ensure the destruction of *Listeria monocytogenes* in pasteurized products, as stipulated, for instance, in the United Kingdom for precooked, refrigerated meals produced in catering operations and designed for storage at 0 to 3°C for up to 5 days (6).

Heat treatment of the meat medium containing spores of nonproteolytic *C. botulinum* at 65°C for 364 min, 70°C for 8 min, and 75°C for 27 min caused little delay before growth of nonproteolytic *C. botulinum*. Heat treatment equivalent to exposure to 80°C for 23 min increased the delay, but growth was detected within 40 days in some tubes at all incubation temperatures. After heat treatments equivalent to maintenance at 85°C for 19 min, growth was detected in some tubes at all incubation temperatures and was accompanied by formation of toxin. In tubes given this heat treatment and then incubated at 12°C, a low concentration of toxin was detected after 28 days, although visible growth was only observed after 42 days. This indicates that heat treatment of processed foods equivalent to maintenance at 85°C for 19 min combined with storage below 12°C and a shelf life of not more than 28 days would reduce the risk of growth from spores of nonproteolytic *C. botulinum* by a factor of 10^6. To provide the same safety factor for a longer shelf life, heat treatment at 85°C for a longer period of time or at a higher temperature would be necessary.

Following heat treatment equivalent to maintenance at 95°C for 15 min, and then incubation at 25°C, growth was observed in 3 of 20 tubes; only 1 of these tubes was tested for toxin, which was not detected. This may have been due to a low incidence of non-toxin-forming cells of *C. botulinum*. In a similar subsequent experiment, growth and formation of toxin occurred in 4 of 30 tubes exposed to the this treatment (37), giving a total of 7 of 50 tubes that showed growth after heat treatment equivalent to 15 min at 95°C, followed by incubation at 25°C for up to 60 days. A heat treatment equivalent to maintenance at 95°C for 15 min, therefore, failed to prevent growth at 25°C within 60 days, i.e., failed to reduce the risk of growth of spores of nonproteolytic *C. botulinum* by a factor of 10^6, but the combination of this heat treatment and incubation of tubes at 12°C or lower prevented growth and toxin formation within 60 days. If processed foods intended for storage at refrigeration temperatures are liable to be maintained at temperatures higher than 10°C, growth and toxin production by proteolytic strains of *C. botulinum* must also be prevented (16).

Previous work in this laboratory has shown that the inclusion of lysozyme in the plating medium greatly increases the number of spores of nonproteolytic *C. botulinum* that give growth after heat treatment at 75 to 95°C in phosphate buffer (31–33). Lysozyme is likely to be present in certain foods (27, 39) and was added, therefore, to the meat medium used in the present work. Lysozyme is relatively heat stable, particularly in acidic conditions (27, 39). The heat resistance of lysozyme appears to be strongly dependent on the composition of the heating medium; thus, the extent to which it would be inactivated by the heat treatments in the present experiments could not be predicted. Subsequent experiments in this laboratory have shown that when lysozyme was included in this meat medium at a concentration of 10 μg/ml, its activity was not inactivated completely by the heat treatments given in this study (34).

The type of toxin formed in a representative number of tubes in these experiments was determined. Type B toxin was detected in all of these tubes, while type E and F toxins were detected less frequently. This may have been due to one or more of the following factors: (i) greater numbers of type B spores than of types E and F may have survived the heat treatment; (ii) type B spores may have germinated more rapidly; (iii) type B vegetative cells may have multiplied more rapidly, and associated production of organic acids and other metabolites and bacteriocins may have inhibited other strains; (iv) greater amounts of toxin may have been formed by type B strains. Studies in which a mixture of spores of nonproteolytic strains of types B, E, and F have been inoculated into fish that was then stored at 4 to 30°C have also shown that type B was the main toxin formed (3, 10, 11, 19).

Further work is required to investigate the effect of heat

treatment and subsequent storage temperature on spores of nonproteolytic *C. botulinum* in other types of food. Other foods may have a greater protective effect on spores and on lysozyme and may contain a higher concentration of lysozyme or of other lytic enzymes with similar effects on spores (27). The addition to refrigerated, processed foods of lysozymes, including a genetically modified lysozyme with additional disulfide bonds to increase heat resistance, and of other lytic enzymes as preservative factors, as proposed in several publications (5, 13, 30, 39), might increase the risk of growth of *C. botulinum.*

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