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Effect of Various Gas Atmospheres on Destruction of Microorganisms in Dry Heat[1]

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ABSTRACT

The heat resistance of dry bacterial spores was tested in various gases at temperatures ranging from 121.1 to 160 C (250 to 320 F). Spores of *Clostridium sporogenes* (PA 3679) were heated in air, carbon dioxide, and helium; spores of *Bacillus subtilis* 5230 were heated in these gases and also in oxygen and in nitrogen. The surrounding gas influenced the heat resistance, but the differences among gases were small. *D* values were about 7 min at 148.9 C (300 F); *z* values were about 18.3 C (33 F) for *B. subtilis*, and about 21.7 C (39 F) for *C. sporogenes*. The resistance of *B. subtilis* in carbon dioxide was about the same as in air, but lower than in all other gases; resistance in helium and nitrogen was about the same, and was higher than in all other gases. *C. sporogenes* had the least resistance in air; the resistance was about the same in carbon dioxide and helium. For *B. subtilis*, the gases in order of increasing heat re sistance were carbon dioxide, air, oxygen, helium, and nitrogen, and for *C. sporogenes*, air, carbon dioxide, and helium. Neither oxygen content nor molecular weight of the gas appeared to have a marked influence on dry-heat resistance of the spores, whereas the more inert gases seemed to yield larger *D* values.

The resistance of bacterial spores to dry heat is substantially greater than that to wet heat. Since the temperature coefficients, or *z* values, of resistance to wet and dry heat are different, a different mechanism of dry-heat destruction has been postulated; oxidation has been suggested as a possible destructive mechanism. The effect of oxygen may be verified by determining the resistance to dry heat in gases other than air or oxygen.

This paper presents the results of heat resistance tests of *Bacillus subtilis* and *Clostridium sporogenes* spores exposed to air and a number of other gases. The basic procedure was to seal the dried spores in small cans filled with the test gas, and then heat the sealed cans in miniature steam retorts.

MATERIALS AND METHODS

Preparation of spores. The organisms used in this study were *B. subtilis* 5230 (15u), grown as described by Pflug (3), and *C. sporogenes* PA 3679-45, grown in pork infusion broth as described by Townsend et al. (7). (Cultures of *C. sporogenes* PA 3679-45 were obtained from C. F. Schmidt, Continental Can Co., Chicago, Ill.) A master suspension, from which subsamples were drawn, was prepared for each organism and stored in a refrigerator throughout the test period; spore concentration was about 10^8 per milliliter in 0.066 M phosphate buffer, *p*H 7.0. The resistance to wet heat, evaluated by fraction negative methods, was: for *B. subtilis* spores, $D_{121.1C}(D_{250\ F})$ was 0.48 min, *z* was 9.4 C (17 F); for *C. sporogenes* spores, $D_{121.1C}(D_{250\ F})$ was 0.84 min, *z* was 10.6 C (19 F). These cultures were considered to have high wet-heat resistance.

Spores for test were dispensed as needed from a micropipette into small (outer diameter, 11 mm; depth, 8 mm), tin plate cups, in amounts of 0.01 ml of suspension per cup. Prior to filling, the cups were washed, dried, put in petri dishes, and sterilized in a hot-air oven. The filled cups were dried at 25 C for 24 hr in a vacuum (725 mm of mercury), and were stored in a desiccator at room temperature until used, usually within 2 weeks after filling.

The initial number dispensed was determined by plate counts each time a set of cups was prepared for test. The number of viable spores per milliliter of master suspension remained constant throughout the tests, which extended over 18 months. The thermal resistance of "dry" spores is a function of the moisture content, as shown by Murrell and Scott (2). The moisture content of dried spores was estimated by measuring the weight of water lost on drying by the

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above procedure and of water lost on drying for 24 hr at 110 C under 725 mm of mercury (five 0.3-ml samples of *B. subtilis* spores, 8.0×10^6 spores per milliliter in 0.066 M phosphate buffer, were dried by each procedure). If the weight at the end of the higher-temperature drying period represents "bone-dry" spores, then the moisture content of spores dried at the lower temperature would be about 9% dry basis. This estimate is crude because the spore weight is a very small fraction of sample weight—most of the dry weight is buffer. The additional weight loss on drying at the higher temperature amounted to about 0.03 mg per original 30 mg of wet sample, whereas the weight of the buffer was about 0.3 mg.

Test gas environment. The spores were sealed in small thermal death time (TDT) cans 208 × 006 [2.5 inches (6.4 cm) in diameter and 3/16 inch (0.8 cm) deep, as described by Townsend et al. (6)] filled with the test gas. Figure 1 shows the cups in a TDT can. The cans were sealed in an isolator box from which air was exhausted and which was refilled with the test gas (Fig. 2). The isolator (25-ft³ capacity) was made of polyvinyl chloride; it was fitted with rubber gloves, a transfer lock, and the various valves necessary to evacuate and refill it. Standard procedure was to evacuate the isolator and refill with test gas three times, giving a residual air content (assuming perfect mixing) of 0.003%. A positive test gas pressure of 10 mm of mercury and a flow rate of 0.3 ft³/min were maintained in the isolator during transfer and sealing. Cans and can covers were sterilized separately in hot air and kept in petri dishes. All the materials used inside the isolator, i.e., cans, covers, filled cups, and forceps, were first put in a vacuum oven which was then pumped out once and refilled with the test gas. These materials were transferred from the petri dishes to the cans, and the cans were sealed. All the cans for a particular temperature-gas combination were filled and sealed on the same day.

Purified-grade test gases, namely, helium, nitrogen, oxygen, and carbon dioxide, were obtained locally.

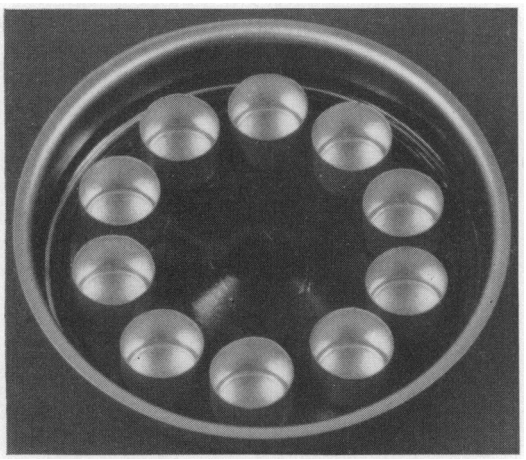

FIG. 1. *Cups in a thermal death time can.*

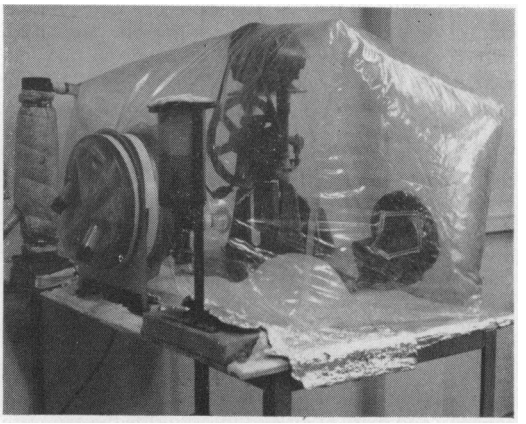

FIG. 2. *Isolator box with closing machine inside.*

Carbon dioxide, the principal impurity in "oxygen," was removed by passing the gas through a carbon dioxide absorber of Ascarite. Oxygen was removed from "nitrogen" by passing the gas through hot (300 C) reduced copper. Water was removed from "carbon dioxide" by passing the gas through two columns of desiccant (1 lb of CaO and 5 lb of $CaSO_4$). These treatments reduced the impurities to less than 20 ppm (chromatographic analysis); helium contained impurities of 5.81 ppm of CO_2 and 17.6 ppm of N_2 and was not treated. Air for the tests was the surrounding air in the laboratory.

Heating the TDT cans. The TDT cans, once sealed with spores, cups, and test gas inside, were heated in saturated steam in miniature retorts similar to those described by Schmidt et al. (5). Each can was filled with 10 cups. The miniature retorts held 12 cans, and two retorts were available.

The overall height of the cups was about 0.5 mm greater than the inside height of the TDT cans, so the can bottoms were recessed, by use of a jig and hydraulic press, to provide additional depth. Even with the extended depth of can, the cups were in pressure contact with the bottom of the TDT cans, thus insuring rapid heat transfer to the bottom of the cups.

During heating, the pressure in the retort exceeded that inside the cans, helping to make good contact between can and cup, but introducing the problem of steam leakage into the cans. Leakage was tested by filling some cans with weighed desiccant, processing for 5 hr at the highest test temperature, and reweighing. The sealing machine was adjusted until it produced seams which did not leak. This can-sealer was then used exclusively for these studies.

Heating and cooling lag corrections. Heating and cooling rates were measured to correct process time, because the cups did not reach process temperature the instant steam was turned on, and they did not cool below lethal temperatures the instant the cooling water was introduced into the retort. Several cup-can systems were specially prepared by soldering one thermocouple (30-gauge copper-constantan) to the bottom of a cup and suspending another in the air

near the center of the cup; the lead wires were brought out of the can through a hole in the side which was then sealed with epoxy resin. Time-temperature data were measured during heating and cooling of several cans with cups containing thermocouples. The data were plotted, and the f and j values were determined by the method of Ball and Olson (1). The lag correction factor, t_c, was calculated, by use of the general method of Ball and Olson (1), for z = 19.4 C (35 F). At the bottom of the cup, f_h = 0.5 min, j = 0.2 to 0.4 min, and t_c = 0.3 min; for the thermocouple in the center of the cup, f_h = 0.5 min, j = 1.5 min, and t_c = 0.7 min. The bottom-of-the-cup correction was used because the spores were on the bottom of the cup. The heating rates at the cup bottom and at the center of the cup were very high because the rate of heat transfer from steam to the can was high, the heat capacity and mass of air and metal inside the can were small, and the ratio of surface to mass heated was high.

Subculture and incubation. The cans were opened with a flame-sterilized, aseptic can opener, and the cups were removed with sterile forceps and transferred to test tubes of growth medium. The recovery medium for *B. subtilis* was dextrose-tryptone-starch broth with 0.04% bromocresol purple indicator, and for *C. sporogenes* was beef liver infusion with 0.2% sodium thioglycolate, 0.2 % K_2HPO_4, and 0.1% $NaHCO_3$. The tubes of liver broth were stratified with a paraffin-mineral oil mixture. All subcultures were incubated at 37 C. Tubes of *B. subtilis* were called positive if acid production, evidenced by the color change, and the characteristic pellicle appeared within 2 weeks; tubes of *C. sporogenes* were called positive if they showed gas production and had a characteristic putrefactive odor within 30 days.

Analysis of data. The data were analyzed by Schmidt's (4) method, in which all the data at a given temperature are plotted as fraction-of-units-negative (FN) versus time on arithmetic probability coordinates, and a straight line drawn. The heating time, corrected for lag, when the fraction of replicate cups is 50% (U_{FN50}) was read directly from the graph. The Schmidt method makes use of all FN values except 0 and 100%; however, in the present analysis, only FN values between 16 and 84% were used.

D values were calculated from the equation $D = U/(\log N_o - \log N_U)$, where N_o is the initial number of organisms per replicate unit (cups in the present study), and N_U is the number of organisms surviving after heating time U. Log N_U is -0.16 when the fraction negative is 50%; the final simplified equation is $D = U_{FN50}/\log N_U + 0.16$.

Prior to the final tests reported herein, preliminary tests were run with both spore species to find the approximate process times required. Three TDT cans, each containing 10 cups, were heated for five or six widely spaced process times at each temperature of interest. Once the time for U_{FN50} was approximately located, a final set of six to eight process times more closely spaced about the expected U_{FN50} were run; 30 cups were used, with 10 cups per can at each time. This procedure yielded at least three times at each temperature and gas combination at which the fraction negative was between 16 and 84%. Each D value, then, was based on the results of from 90 to 120 cups.

RESULTS AND DISCUSSION

D values from the dry-heat resistance studies of *B. subtilis* spores heated in carbon dioxide, air, oxygen, helium, and nitrogen are shown in Table 1; thermal-resistance curves are shown in Fig. 3. The initial number of spores per cup was 8.0×10^4. The results for *B. subtilis* fell into three groups: resistance was about the same in helium and nitrogen, but greater than in the other gases; resistance in air and oxygen was about the same; and resistance was lowest in carbon dioxide, but probably not different from the resistance in air.

TABLE 1. *D values of Bacillus subtilis 5230 spores heated in various dry gases*[a]

Temp		Gas				
C	F	Carbon dioxide	Air	Oxygen	Helium	Nitrogen
121.1	250	170	190	210	240	250
132.2	270	40	48	48	60	60
143.3	290	11.0	11.5	11.5	13.5	11.5
148.9	300	5.8	5.2	5.8	6.6	7.4
154.5	310	2.8	2.4	3.2	3.4	
160.0	320	1.40	1.46	1.47	1.63	1.69

[a] Values given in minutes.

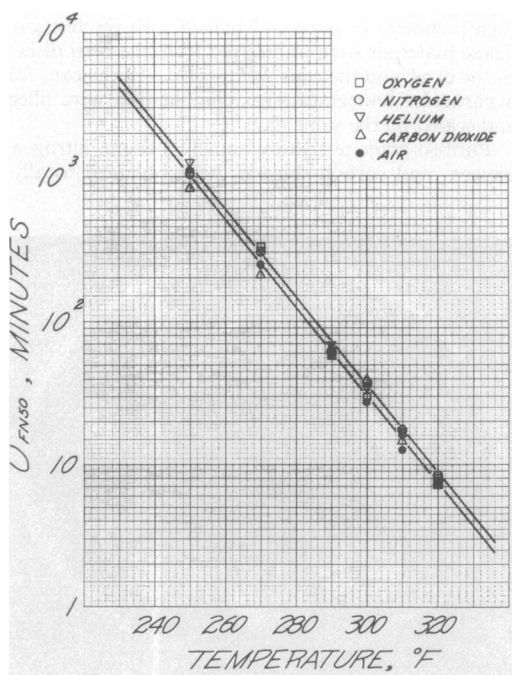

FIG. 3. *Temperature coefficient curve for Bacillus subtilis 5230; in this graph, the heating time to produce negative growth in 50% of the samples, U_{FN50}, is plotted versus temperature.*

The temperature coefficients (z values) for all five gases were within ±0.5 C of 18.3 C (33 F).

A measure of experimental reproducibility, including such variations as temperature, time of test, initial number, gas container, and degree of spore clumping, was provided by a number of replications done at 160 C (320 F). These D values (Table 2) illustrate the reproducibility of results with the five gases (180 cups for each D value determination). The coefficient of variations of D, all gases considered to have the same variation, was about 2.5%.

D values of *C. sporogenes* spores heated in air, carbon dioxide, and helium are shown in Table 3; thermal-resistance curves are shown in Fig. 4. The initial number of spores per cup was different for each gas: 6.5×10^4 in air, 4.3×10^4 in carbon dioxide, and 6.6×10^4 in helium. The results for *C. sporogenes* fell into two groups: the resistances in carbon dioxide and helium were about the same; resistance was lowest in air. The temperature coefficients for all three gases were within ±0.5 C of 21.7 C (39 F).

For a given spore species, there were differences in resistance to dry heat among the several gases tested, but these differences were small (maximal differences were by a factor of 1.7). The dry-heat resistances (both D and z values) of the two species were clearly different, all gases considered together. The temperature region in which the D values for the two species were about the same was from 143.3 to 148.9 C (290 to 300 F). Because *C. sporogenes* had a larger z value, its D values were larger than those for *B. subtilis* at temperatures above 143.3 to 148.9 C (290 to 300 F) and smaller below these temperatures. In wet heat at 121.1 C (250 F), the D value of *C. sporogenes* was greater than that of *B. subtilis*.

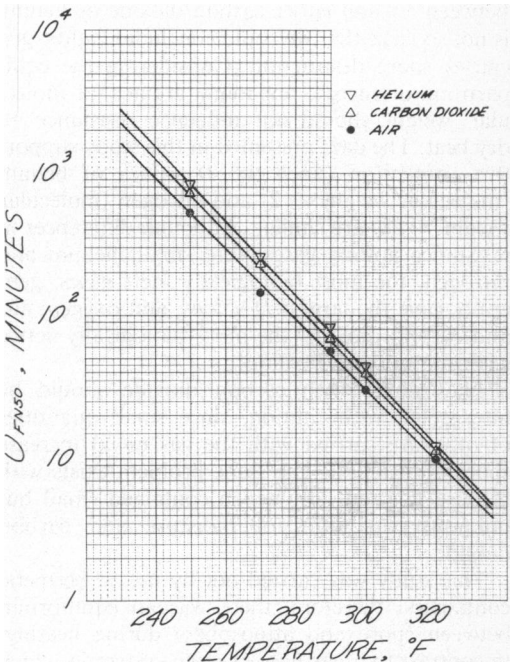

FIG. 4. *Temperature coefficient curve for Clostridium sporogenes PA 3679; in this graph, the heating time to produce negative growth in 50% of the samples, U_{FN50}, is plotted versus temperature.*

TABLE 2. *D values of Bacillus subtilis 5230 spores heated in various dry gases at 160 C (320 F)*

Carbon dioxide	Air	Oxygen	Helium	Nitrogen
1.38	1.46	1.46	1.70	1.66
1.38	1.46	1.46	1.62	1.70
1.46	1.46	1.50	1.58	1.66
1.38	1.46			1.74
1.40[a]	1.46[a]	1.47[a]	1.63[a]	1.69[a]

[a] Average value for each gas.

TABLE 3. *D values of Clostridium sporogenes PA 3679 spores heated in various dry gases*[a]

Temp		Gas		
C	F	Air	Carbon dioxide	Helium
121.1	250	100	150	170
132.2	270	34	48	52
143.3	290	11	14	17
148.9	300	6.0	8.0	8.8
160.0	320	1.95	2.40	2.40

[a] Values given in minutes.

Both spore species were tested in three of the gases, namely, air, carbon dioxide, and helium. Resistance of both species in helium was the highest. However, with *B. subtilis*, the resistance in carbon dioxide was a little lower than that in air, whereas, with *C. sporogenes*, the resistance in carbon dioxide was greater than the resistance in air. Whether this fact is related to the different oxygen requirements of the vegetative forms is a matter for speculation.

If oxygen content of the gas is responsible for the major destructive effect, resistance to dry heat should be least in 100% oxygen, followed by air, and then the other gases. The data do not support this opinion in the case of *B. subtilis*; in fact, the resistance in air was not measurably different from that in oxygen, and the resistance was even lower in carbon dioxide. Resistance of *C. sporogenes* was least in air, but the difference

between air and either carbon dioxide or helium is not so large that we could conclude that oxygen causes spore destruction. Considering the equipartition of energy, one might argue that molecular weight should not influence resistance to dry heat. The data presented in this study support this contention, since the *D* values of helium (molecular weight = 2) and nitrogen (molecular weight = 28) are similar. Although differences in resistance among gases were small, helium and nitrogen, the most biologically inert gases, generally gave the largest *D* values, and oxygen, air, and carbon dioxide, the more biologically active gases, gave lower *D* values.

The results with carbon dioxide should be interpreted with caution, since small quantities of moisture carried with the gas could increase spore destruction. The same problem exists with the use of room air, which contained small but unknown quantities of moisture and carbon dioxide.

This study was carried out by use of hermetic containers; therefore, there was an equilibrium between spore and atmosphere during heating, in contrast to a gas-flow or open system in which there could be continual molecular diffusion under a constant driving force during the heating period. The spores in this study were in phosphate buffer, which may in itself enhance resistance to dry heat.

ACKNOWLEDGMENT

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