JOURNAL OF BACTERIOLOGY, Feb. 1969, p. 684-690

Vol. 97, No. 2
Printed in U.S.A.

Initiation of Germination and Inactivation of *Bacillus pumilus* Spores by Hydrostatic Pressure

J. G. CLOUSTON AND PAMELA A. WILLS
Australian Atomic Energy Commission, Lucas Heights, Sutherland, Australia

Received for publication 22 October 1968

The effect of hydrostatic pressures as high as 1,700 atm at 25 C on the heat and radiation resistance of *Bacillus pumilus* spores was studied. Phosphate-buffered spores were more sensitive to compression than spores suspended in distilled water. Measurements of the turbidity of suspensions, the viability, refractility, stainability, dry weight, and respiratory activity of spores, and calcium and dipicolinic acid release were made for different pressures and times. Initiation of germination occurred at pressures exceeding 500 atm and was the prerequisite for inactivation by compression. The rate of initiation increased with increasing pressure at constant temperature. This result is interpreted as a net decrease in the volume of the system during initiation as a result of increased solvation of the spore components.

Bacterial spores are less readily killed by hydrostatic pressure than are their vegetative forms or nonsporeforming bacteria (8). Similarly, spores are very resistant to heat, radiation, and chemical injury, but when induced to germinate their sensitivity to these treatments increases (12).

While studying factors influencing the radiation sensitivity of bacterial spores, enhanced inactivation was observed when the spores were hydrostatically compressed during gamma irradiation. In this paper, we show that the increased inactivation occurs because hydrostatic pressure initiates germination of *Bacillus pumilus* spores before inactivation. This conclusion is based on the effect of compression at as high as 1,700 atm, applied for various times at 25 C, on the heat and radiation sensitivity, turbidity, refractility, stainability, calcium and dipicolinic acid (DPA) release, respiratory activity, and dry weight of *B. pumilus* spores.

MATERIALS AND METHODS

Spore suspensions. The test organism was *B. pumilus* E601; originally from Ethicon, Inc., Somerville, N.J., it was obtained from Johnson and Johnson Pty. Ltd., Botany, New South Wales. After incubation in Nutrient Broth (Oxoid) at 37 C for 24 hr, three consecutive daily serial transfers were made. The final 24-hr culture was used to seed Roux bottles containing soybean agar (18). After incubation at 37 C for 48 hr and at room temperature in the dark for 14 days or until at least 90% sporulation had been achieved, the spores were harvested in 0.067 M phosphate buffer (*p*H 6.8, mono- and dipotassium). The suspension was centrifuged in a Wifug angle head centrifuge at 2,000 × *g* for 30 min, the supernatant fluid was discarded, and the spores were washed three times with chilled phosphate buffer.

After overnight refrigeration, the suspension was heated at 80 C for 15 min, rapidly cooled, and washed three more times with buffer. The spores were finally suspended in phosphate buffer to a concentration of about 5×10^9 to 10×10^9 spores/ml, and were stored at 4 C for a maximum period of 4 months before being discarded.

When aqueous suspensions were required, a portion of the buffered stock suspension was washed five times with sterile distilled water before resuspension in water.

Working suspensions containing 0.5×10^8 to 10×10^8 spores/ml were prepared from the stock suspensions. They were always mechanically shaken for 15 min before use.

Pressurization. Experiments were undertaken in (up to three) identical, stainless-steel pressure vessels connected in parallel to a hydrostatic air pressure pump. Hydrostatic pressures as high as 1,700 atm could be sustained for long periods. The hydraulic fluid was demineralized water. Compression and decompression produced a maximum rise and fall, respectively, of 2 C as measured by a thermistor probe. The temperature stabilized within 2 min of compression and, for these experiments, was thermostatically controlled at 25 ± 0.5 C.

Three different procedures were used for pressurizing the spore suspensions, depending on the volume subsequently required for analysis. Glass vials, 40 mm long and 9 mm in diameter, were used for the small volumes needed for estimating pressure,

heat, or radiation sensitivity, refractility, and stainability. Three vials, each containing about 0.8 ml of spore suspension and an equivalent amount of sterile silicone oil 550, could be treated simultaneously in a pressure vessel. The silicone oil, forming a layer on top of the spore suspension, transmitted the pressure but acted as a barrier against the hydraulic fluid. Direct turbidity measurements were possible with a 10-mm glass cuvette completely filled with suspension and sealed with a rubber stopper. To obtain the large volumes required for DPA and calcium analyses, 7-ml glass bottles sealed with washed sterile rubber serum caps were used. With the serum cap in place, the container was filled, taking care to exclude air bubbles, by use of a hypodermic syringe with a hypodermic needle acting as a vent. The response of the spores to pressure was independent of the type of container or the method used to transmit the pressure.

Irradiation. Gamma radiation was accomplished by means of a ^{60}Co source with cylindrical geometry. Dose rate was measured by ferrous ammonium sulfate chemical dosimeters (19) with $G = 15.5$. Three samples could be irradiated simultaneously, the vials fitting into a metal jig which could be accurately located inside the pressure vessel. Variation in dose between the three positions of the vials was less than 4%. The pressure vessel in the radiation field was connected in parallel with two other pressure vessels. With this arrangement, simultaneous, comparative experiments with or without radiation could be performed.

Viable counts. The number of spores surviving compression, irradiation, or heat, or a combination of these treatments, was estimated from colony counts. Immediately after the treatment ended, serial dilutions in 0.067 M phosphate buffer were prepared from measured portions (usually 0.1 ml) taken from two or three vials. The "drop plate" counting method was employed (14), using plates prepared from Nutrient Broth (Oxoid) and 1% agar (Oxoid no. 3). After 24 hr of incubation at 37 C, the colonies were counted with a New Brunswick colony counter. Further incubation did not increase the number of colonies present. The viable count was calculated from at least two different dilutions, with weighting for dilution (3).

Heat sensitivity. The serial dilutions used in the total viable count estimations were heated with shaking at 80 C for 15 min. After rapid cooling, drop plate counts were made as before, and the number of heat-sensitive forms was calculated by difference.

Inactivation and survival after compression. From viable counts performed before and after compression, the number of inactivated spores was calculated by difference. Because spores surviving compression were either resistant or sensitive to heat, the surviving fraction (V) has been defined as $V = N_s + N_g = 1 - N_k$, where N_s = number of survivors which are heat resistant/initial number of spores; N_g = number of survivors which are heat sensitive/initial number of spores; N_k = number of spores which have been inactivated/initial number of spores.

Turbidity. Changes in the turbidity of spore suspensions were followed by measuring optical density (OD) at 625 nm with a Unicam SP700 recording spectrophotometer. Working suspensions were adjusted to give an initial OD of about 0.8 (4×10^8 spores per ml), with phosphate buffer as the blank. By pressurizing the suspension in a 10-mm cuvette, OD could be measured directly, immediately after decompression. The turbidity changes were normalized to OD_0 and were plotted as $T = OD_t/OD_0$, where OD_t = optical density after t min and OD_0 = initial OD of the suspension.

Refractility and stainability. Refractility was measured on samples in a Thoma counter with a phase microscope. The numbers of bright and dark spores present before and after compression were counted, and the fraction of bright spores surviving compression was calculated. Similarly, heat-fixed smears stained with Loeffler's alkaline methylene blue for 7 min were used to estimate the fraction of unstained spores present in the spore suspensions after compression.

Detection and measurement of soluble constituents present in the supernatant fluids of pressurized spore suspensions. After centrifuging the spore suspensions at $2,000 \times g$ for 30 min, the supernatant fluids were used to estimate DPA and calcium.

With a Unicam SP700 recording spectrophotometer, absorbance of the supernatant fluid was measured at 264.8, 269.8, 274.8, and 277.8 nm. DPA concentration was calculated with the quaternary function M as described by Lewis (10). (Function N was used in DPA analyses of dried spores.) The results for DPA released are recorded as a fraction of the initial DPA content.

Calcium analyses were performed by flame photometry, with slight modification of the normal procedure because of the accompanying high concentrations of potassium and phosphorus. The results for release of Ca^{++} are recorded as a fraction of initial Ca^{++} present in the spore.

Dry weight. Portions of stock spore suspensions were dried at 60 C in a vacuum oven to a constant weight.

Respiratory activity. The oxygen consumption of phosphate-buffered spore suspensions was measured with a Radiometer oxygen-carbon dioxide analyzer (4). A spore suspension (1.5×10^9 per ml) was compressed for 60 min at 820 atm, causing about 60% of the spores to lose their heat resistance. After decompression, 7.9 ml was transferred to an oxygen measuring flask. The suspension was then diluted with 2.0 ml of sterile phosphate buffer and 0.1 ml of a 3.6% glucose solution, giving a final concentration of 0.002 M glucose. Measurements of oxygen uptake were made at 37 C and compared with the oxygen uptake of unpressurized spores under the same conditions.

RESULTS

Although the *B. pumilus* spores were heated at 80 C for 15 min during preparation of the spore crop, no further heat treatment was given before compression. No significant increases in the colony counts of the unpressurized spores were

ever noted after the second heating at 80 C for 15 min, showing that the initial heat activation was retained during storage.

Inactivation of spores by hydrostatic pressure. When compression was maintained for 270 min, viability was not affected until a threshold pressure of about 600 atm was attained (Fig. 1). As the pressure increased, the number of survivors decreased, the decrease being greater for spores suspended in buffer. With buffered spores, the response altered at about 1,350 atm, and further increases in pressure had little effect on the degree of inactivation. This residual effect was not studied further. Because the apparatus was limited to a maximum pressure of 1,700 atm, it was not possible to note whether similar tailing occurred at higher pressures for the more pressure-resistant spores suspended in water.

Heat sensitivity of compressed spores. Compression of buffered spores at 475 atm for 240 min did not affect their resistance to heat (Table 1). When the pressure was increased by 70 atm (545 atm), the concentration of heat-resistant spores decreased by 17%; with an additional 65 atm (610 atm), it decreased by 31%. It was not possible to decrease the pressure at which a change in heat resistance first became apparent by increasing the time of compression. Spores remained unaffected by 375 atm applied for 1,440 min.

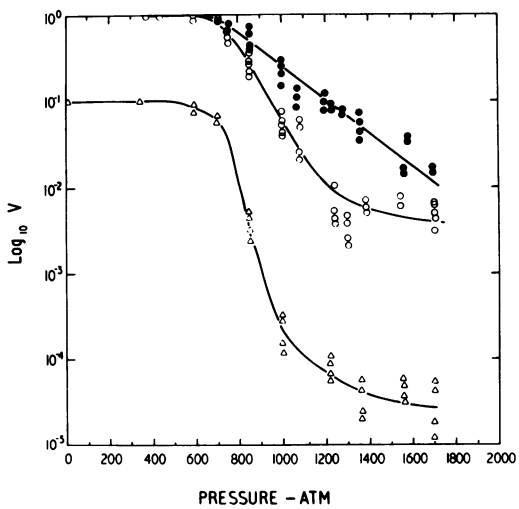

FIG. 1. *Lethal effect at 25 C of hydrostatic compression maintained for 270 min in the presence and absence of* ^{60}*Co gamma radiation on B. pumilus spores. The radiation dose was 0.310 MR. The curves were fitted by hand. Symbols:* ●, *spores suspended in distilled water, compressed but not irradiated;* ○, *spores suspended in 0.067 M potassium phosphate buffer (pH 6.8), compressed but not irradiated;* △, *spores suspended in 0.067 M potassium phosphate buffer (pH 6.8), compressed and irradiated simultaneously.*

TABLE 1. *Fraction of heat-stable (N_s), heat-labile (N_g), and inactivated (N_k) spores after compression of B. pumilus heat-stable spores suspended in 0.067 M potassium phosphate buffer at 25 C*

Pressure (atm)	Time (min)	Fraction		
		N_s	N_g	N_k
1	240	1.00	0.00	0.00
475	240	1.00	0.00	0.00
545	240	0.83	0.17	0.00
610	240	0.69	0.26	0.05
665	240	0.24	0.36	0.40
820	10	0.84	0.15	0.01
	20	0.55	0.38	0.07
	40	0.23	0.65	0.12
	240	0.009	0.54	0.451
950	10	0.65	0.34	0.01
	20	0.20	0.73	0.07
	40	0.11	0.72	0.17
	240	0.007	0.068	0.925
1,085	10	0.16	0.84	0.00
	20	0.049	0.731	0.220
	40	0.007	0.493	0.500
	240	0.0002	0.004	0.996

The relationship between the concentrations of heat-resistant, heat-sensitive, and inactivated spores and the time of compression in a buffered suspension at 820 atm is shown in Fig. 2. At pressures below the threshold of about 500 atm, no changes developed (Table 1). Other studies showed that, when spores were compressed in water for 270 min at 1,220 atm, 84% of the surviving fraction was heat-stable; at 1,500 atm, 69% was heat-stable.

Radiation sensitivity of compressed spores. Figure 1 shows that the radiation sensitivity of the spore was not altered by compression until a pressure of about 500 atm was reached. Above this pressure, the degree of inactivation for a constant period of compression and a constant radiation dose increased with increasing pressure. The shapes of the survival curves for the compressed buffered spores in the presence or absence of radiation are similar, both tending to tail at about the same pressure (1,350 atm). The cumulative effect of simultaneous treatment by compression and irradiation on the surviving fraction (V) was greater than expected when each treatment acted independently. The increased radiation sensitivity of the compressed spores was due, at least in part, to the decreased radiation resistance of the heat-labile cells as compared with the heat-stable spores (Fig. 2).

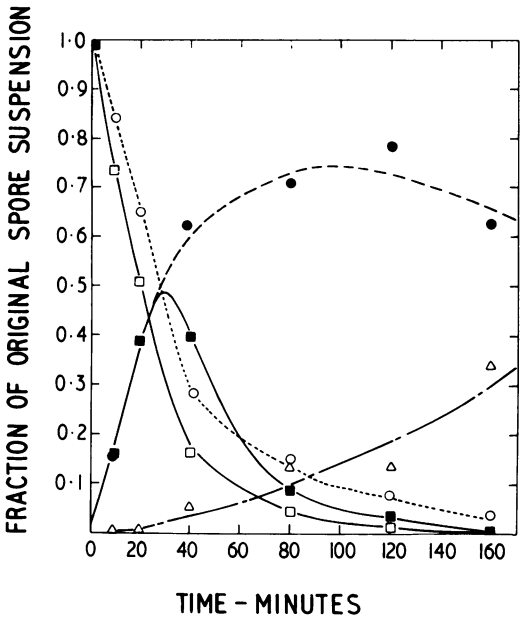

FIG. 2. *Heat-stable and heat-labile B. pumilus spores surviving 820 atm of compression for different periods in the presence and absence of gamma radiation. Spores were suspended at 25 C in 0.067 M potassium phosphate buffer (pH 6.8). Radiation dose rate was 858 R/min. The curves were fitted by hand. Symbols: ○, heat-stable fraction in the absence of radiation; ●, heat-stable fraction in the presence of radiation; □, heat-labile fraction in the absence of radiation; ■, heat-labile fraction in the presence of radiation; △, fraction inactivated by compression in the absence of radiation.*

Turbidity changes with compression. The turbidity of a compressed, buffered spore suspension decreased with increasing time of compression (Fig. 3). The sigmoid shape of the experimental curve is characteristic of turbidity changes when germination of bacterial spores is initiated by exogenous chemicals (11).

Loss of refractility and increase in stainability with compression. After compression, the number of phase-bright spores decreased and the number of cells which could be stained increased. These changes in refractility and stainability corresponded with the changed heat resistance of compressed spores (Table 2 and Fig. 3).

Presence of soluble constituents in the supernatant fluid from compressed spore suspensions. Compression above the threshold released DPA and calcium from the spores. As with heat and radiation resistance, this threshold pressure could not be lowered by increasing the time of compression. Thus, the ultraviolet visible spectrum of the supernatant fluid was unchanged after compression of spores at 400 atm for 950 min.

With higher pressures, examination of the supernatant fluid revealed the characteristic absorption spectrum of calcium dipicolinate with maxima at 269.8 and 277.8 nm and minima at 264.8 and 274.5 nm. When buffered spores were pressurized for 40 min at 1,085 atm, corresponding to a 99.0% loss of heat resistance, the amount of DPA released was 5.6% of the spore (dry weight). This figure is in good agreement with the 5.9% DPA found in dried unpressurized spores, as spores contained 0.35% DPA after the application of sufficient compression to cause 99.9% of the spores to lose their heat resistance.

At constant pressure, the concentration of DPA in the supernatant fluid increased with increasing periods of compression (Table 2). The release of DPA appears to lag behind the loss of heat resistance. Inaccuracies in the spectrophotometric method at low DPA concentrations (under our experimental conditions, less than 2 μg/ml when compression was maintained for 80 min or less) may possibly contribute to this apparent lag. However, it should be noted that Woese and Morowitz (20), when studying the kinetics of alanine-induced germination of *B. subtilis*, showed the existence of a similar lag in DPA release.

The results for calcium (Table 3) showed that, for the initial 60 min, the rate of calcium release and the rate of uptake of stain were the same. Spores containing 22.2 μg of Ca^{++} per mg (dry weight) released 98% of their calcium content in 950 min at 820 atm. Because Tables 2 and 3 refer to different spore batches, neither the relative rates of Ca^{++} and DPA release nor their absolute values can be compared.

Decrease in dry weight. The dry weight of compressed spores was lower than that of uncompressed spores. After compression at 1,085 atm for 80 min, only 0.001% of buffered *B. pumilus* spores remained heat-stable. The dry weight of these cells (7.1×10^{10} spores) was 13.3 mg, compared with an initial dry weight of 20.7 mg (7.1×10^{10} spores). The loss is equivalent to a 36% decrease in dry weight.

Respiratory activity. Metabolic activity is apparent in spores which have been compressed. In 0.002 M glucose, oxygen was consumed by *B. pumilus* spores after compression for 60 min at 820 atm (Fig. 4). The oxygen uptake of unpressurized spores was negligible. In a similar experiment, when 0.008 M glucose was added to spores after they had been compressed at 850 atm for 80 min, the rate of oxygen uptake of the compressed spores was 10 times that of the control spores.

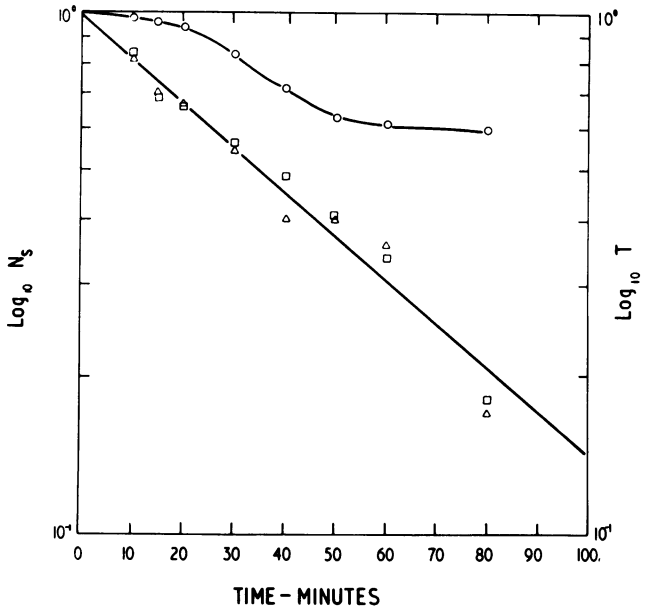

FIG. 3. *Effect of compression at 820 atm on the turbidity (T) and fractions of heat-resistant and unstained B. pumilus spores suspended in 0.067 M potassium phosphate buffer (pH 6.8) at 25 C. The least squares line for retention of heat and stain resistance is shown. The sigmoid curve is drawn by hand. Symbols: △, heat-resistant fraction; □, unstained fraction; ○, turbidity.*

TABLE 2. *Effect of compression of B. pumilus spores at 25 C on their refractility, stainability, heat stability, and DPA release*[a]

Pressure (atm)	Time (min)	Phase bright	Unstained	Heat stable	DPA released
820	10	0.75	0.83	0.81	0.08
	15	0.60	0.69	0.70	0.16
	20	0.66	0.67	0.66	0.14
	30		0.56	0.54	0.22
	40		0.49	0.40	0.42
	50		0.42	0.41	0.52
	60	0.14	0.34	0.36	0.63
	80	0.22	0.18	0.17	0.76
1,085	40	0.07	0.07	0.01	0.95

[a] Spores were suspended in 0.067 M potassium phosphate buffer (*p*H 6.8). The results are expressed as a ratio of the initial concentration.

DISCUSSION

Before *B. pumilus* spores are inactivated at 25 C by hydrostatic pressure, they undergo a physiological change. This only occurs when the pressure rises above a certain critical or "threshold" level, and then the rate of change is influenced by the presence or absence of ions in the suspending fluid.

The physiological changes we observed have features identical with those associated with initiation of germination. Germination has often been defined simply in terms of decreased heat resistance and refractility, increased stainability, and altered turbidity (1, 11). A more extensive definition is given by Rode and Foster (15), who list the following criteria as evidence of spore germination: ability to form colonies on a suitable growth medium, increased heat and radiation sensitivity, reduction in turbidity of a spore suspension, loss of refractility and increased uptake of dilute basic stains which may be associated with swelling of the spores, a decrease of about

TABLE 3. *Effect of compression (820 atm) at 25 C on the stainability and calcium release by B. pumilus spores*[a]

Time (min)	Stained	Ca^{++} released
10	0.10	0.12
20	0.21	0.17
30	0.36	0.30
40	0.50	0.43
50	0.53	0.47
60	0.64	0.56
80	0.67	0.56
100	0.75	0.64
950	0.98	0.98

[a] Spores were suspended in 0.067 M potassium phosphate buffer (*p*H 6.8). The results are expressed as ratios of the total number of cells and initial Ca^{++}.

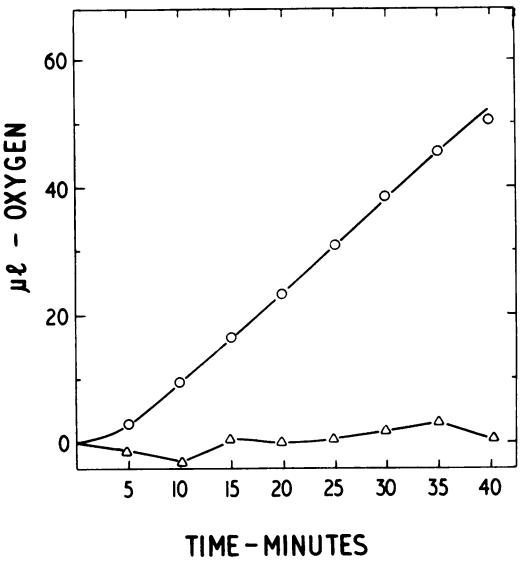

FIG. 4. *Oxygen uptake in 0.002 M glucose at 37 C by 1.16 × 10^{10} B. pumilus spores after compression at 820 atm for 60 min at 25 C. The spores were suspended in 0.067 M potassium phosphate buffer (pH 6.8). Symbols:* ○, *compressed spores;* △, *unpressurized spores.*

one-third in the dry weight of the spores, the presence of DPA, calcium, and a nondialyzable peptide in the spore exudate, and increased respiratory activity at the expense of glucose. With the exception of cell size and peptide release which were not studied, all of the above criteria were met by the compressed *B. pumilus* spores. Therefore, it is concluded that at 25 C hydrostatic compression initiates germination of *B. pumilus* spores. The physiological change is essentially identical with that induced by many exogenous chemicals at atmospheric pressure and physiological temperatures (5, 7, 16, 17) or by mechanical abrasion (15).

The conclusion drawn from the existence of a threshold pressure is that compression does not simply accelerate a process occurring slowly at 25 C. In the absence of compression, spores in distilled water or buffer were stable for long periods. For *B. pumilus*, the transformation seems to occur at about 500 atm. Metabolic activity begins and the process is irreversible. Although the presence of potassium and phosphate ions enhances the rate of initiation at the same pressure relative to distilled water, the critical pressure for initiation remains unchanged. Within the pressure range and at the temperature investigated, initiation is the necessary requirement for inactivation by hydrostatic pressure. In view of the contention that ions are important initiators of germination (16), the nature of the solute may well influence the rate of germination and inactivation.

The difference between the rates of pressure inactivation of spores suspended in phosphate buffer (*p*H 6.8) and those suspended in distilled water (*p*H 6.0) cannot simply be attributed to a *p*H effect. At 1,700 atm, the difference in *p*H between the two suspensions would be slight, the *p*H of the phosphate buffer being reduced by about 0.8 units (2) and the *p*H of the water being reduced by only 0.3 units (6). The release of calcium and DPA implies dissociation of a complex between these substances and some spore material, perhaps protein as proposed by Young (21), and suggests that charged groups which were previously stabilized by the complex are now present in the spore. Increased solvation of the spore components, in the heat-labile state of the spore relative to the resistant state, would contribute to a change in the free energy of the spore, the electrostrictive forces accompanying solvation causing a decrease in the specific volume of the system. The acceleration of the rate of initiation with increasing pressure suggests that the reactions associated with loss of heat and radiation resistance involve a net decrease in the molecular volume of the system. The release of peptides (13, 14) and amino acids (9) also suggests a molecular volume decrease. Thus, hydrostatic pressure could initiate germination by favoring a solvated state.

Although quantitative differences depending on the environment are to be expected, there is no reason to suppose that the results with *B. pumilus* are a special case. It is likely that any member of the genus *Bacillus* will respond in the same way, and that compression will accelerate any process stimulating germination.

Hydrostatic compression of bacterial spores may provide a method for the quantitative study of initiation of germination in the presence or absence of exogenous compounds.

ACKNOWLEDGMENTS

The authors appreciate the discussions on high pressure technique with A. H. Ewald and R. Truer and the experimental assistance of W. Moyle, D. O'Regan, and K. Fenn.

LITERATURE CITED

1. Campbell, L. L. 1957. Bacterial spore germination—definitions and methods of study, p. 33–38. *In* H. O. Halvorson (ed.), Spores. Am. Inst. Biol. Sci. Publ. no. 5, Washington, D.C.
2. Distèche, A. 1959. *p*H measurements with a glass electrode withstanding 1500 kg/cm² hydrostatic pressure. Rev. Sci. Instr. 30:474–478.
3. Farmiloe, F. J., S. J. Cornford, J. B. M. Coppock, and M. Ingram. 1954. Survival of *Bacillus subtilis* spores in the baking of bread. J. Sci. Food Agr. 5:292–304.

4. Flenley, D. C., J. S. Miller, and H. A. Rees. 1967. Accuracy of oxygen and carbon dioxide electrodes. Brit. Med. J. 2:349–352.
5. Foerster, H. F., and J. W. Foster, 1966. Response of *Bacillus* spores to combinations of germinative compounds. J. Bacteriol. 91:1168–1177.
6. Hamann, S. D. 1963. The ionization of water at high pressures. J. Phys. Chem. 67:2233–2235.
7. Hills, G. M. 1949. Chemical factors in the germination of spore-bearing aerobes. The effect of yeast extract on the germination of *Bacillus anthracis* and its replacement by adenosine. Biochem. J. 45:353–362.
8. Larson, W. P., T. B. Hartzell, and H. S. Diehl. 1918. The effects of high pressure on bacteria. J. Infect. Diseases 22:271–279.
9. Lee, W. H., and Z. J. Ordal. 1963. Reversible activation for germination and subsequent changes in bacterial spores. J. Bacteriol. 85:207–217.
10. Lewis, J. C. 1967. Determination of dipicolinic acid in bacterial spores by ultraviolet spectrometry of the calcium chelate. Anal. Biochem. 19:327–337.
11. McCormick, N. G. 1965. Kinetics of spore germination. J. Bacteriol. 89:1180–1185.
12. Murrell, W. G. 1961. Spore formation and germination as a microbial reaction to the environment. Symp. Soc. Gen. Microbiol. 11:100–150.
13. Powell, J. F., and R. E. Strange. 1953. Biochemical changes occurring during the germination of bacterial spores. Biochem. J. 54:205–209.
14. Reed, R. W., and G. B. Reed. 1948. "Drop plate" method of counting viable bacteria. Can. J. Res. 26:317–326.
15. Rode, L. J., and J. W. Foster. 1960. Mechanical germination of bacterial spores. Proc. Natl. Acad. Sci. U.S. 46:118–128.
16. Rode, L. J., and J. W. Foster. 1962. Ions and germination of spores of *Bacillus cereus T*. Nature 194:1300–1301.
17. Rode, L. J., and J. W. Foster. 1962. Ionic germination of spores of *Bacillus megaterium* QM B 1551. Arch. Mikrobiol. 43:183–200.
18. Smith, N. R., R. E. Gordon, and F. E. Clark. 1952. Aerobic sporeforming bacteria. U.S. Dept. Agr. Monograph no. 16.
19. Weiss, J., A. O. Allen, and H. A. Schwarz. 1956. Use of Fricke ferrous sulphate dosimeter for gamma ray doses in the range 4 to 40 KV, p. 179–181. Proc. Intern. Conf. Peaceful Uses Atomic Energy, vol. 14.
20. Woese, C., and H. J. Morowitz. 1958. Kinetics of the release of dipicolinic acid from spores of *Bacillus subtilis*. J. Bacteriol. 76:81–83.
21. Young, I. E. 1958. A relationship between the free amino acid pool, dipicolinic acid and calcium from resting spores of *Bacillus megaterium*. Can. J. Microbiol. 5:197–202.