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Initiation of *Bacillus* Spore Germination by Hydrostatic Pressure: Effect of Temperature

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Suspensions of *Bacillus cereus* T, *B. subtilis*, and *B. pumilus* spores in water or potassium phosphate buffer were germinated by hydrostatic pressures of between 325 and 975 atm. Kinetics of germination at temperatures within the range of 25 to 44°C were determined, and thermodynamic parameters were calculated. The optimum temperature for germination was dependent on pressure, species, suspending medium, and storage time after heat activation. Germination rates increased significantly with small increments of pressure, as indicated by high negative $\Delta V‡$ values of -230 ± 5 cm³/mol for buffered *B. subtilis* (500 to 700 atm) and *B. pumilus* (500 atm) spores and -254 ± 18 cm³/mol for aqueous *B. subtilis* (400 to 550 atm) spores at 40°C and -612 ± 41 cm³/mol for *B. cereus* (500 to 700 atm) spores at 25°C. The ranges of thermodynamic constants calculated at 40°C for buffered *B. pumilus* and *B. subtilis* spores at 500 and 600 atm and for aqueous *B. subtilis* spores at 500 atm were: Ea = 181,000 to 267,000 J/mol; $\Delta H‡$ = 178,000 to 264,000 J/mol; $\Delta G‡$ = 94,000 to 98,300 J/mol; $\Delta S‡$ = 264 to 544 J/mol per °K. These values are consistent with the concept that the transformation of a dormant to a germinating spore induced by hydrostatic pressure involves either hydration or a reduction in the viscosity of the spore core and a conformational change of an enzyme.

Germination of bacterial spores can be initiated by various chemicals or by physical methods (10). Clouston and Wills (6) have shown that hydrostatic pressures of a few hundred atmospheres suffice to initiate germination of *Bacillus pumilus* spores. The general application of this phenomenon to other bacterial spore species has been confirmed by Gould and Sale (11). The stimulation of germination by hydrostatic pressure contrasts with the inhibition that may be produced by comparatively low gas pressures (up to 100 atm) (8). Under appropriate conditions of time, temperature, and pH, spores germinated by hydrostatic pressure can be inactivated (6, 31). The kinetics of hydrostatic pressure-induced germination and inactivation can be interpreted in terms of consecutive first-order reactions (7).

The mechanism for initiation of germination of bacterial spores is unknown. Because hydrostatic compression provides a simple method, uncomplicated by the addition of chemicals, for studying initiation of bacterial spores, it is useful in investigating the breaking of dormancy. Using pressures of less than 1,000 atm, we have examined compression effects on the initiation of germination of spores of three *Bacillus* species. Other variables tested include the effects of temperature, compression time, and solvent. The kinetics have been analyzed, and thermodynamic constants have been calculated. Possible mechanisms of initiation of germination are discussed in terms of these data.

MATERIALS AND METHODS

Spore suspensions. *Bacillus cereus* T was grown and allowed to sporulate in modified G medium, further modified by reducing the yeast extract concentration to 0.1%, as described previously (28). For growth and sporulation of *Bacillus subtilis*, 1% tryptone was added to 15 liters of the same medium, which was vigorously aerated in 20-liter carboys incubated at 37°C for 36 h. Spores were harvested and washed six times with sterile distilled water, using a refrigerated MSE centrifuge at 7,000 × *g*. Working suspensions containing 10^7 to 3×10^7 spores per ml in 0.067 M potassium phosphate buffer, pH 6.8, or water were prepared from aqueous stock suspensions maintained at 1°C. *B. subtilis* spores, but not *B. cereus* T spores, were heat activated at 80°C for 10 or 15 min. More than 99% of the *B. subtilis* spores and 98% of the *B. cereus* T spores were fully refractile. *B. pumilus* spores were produced on soybean agar in Roux flasks and harvested with six washings of sterile distilled water using an International centrifuge (6). At least 95% of the

heat-activated working suspensions were fully refractile.

Compression. Pressure apparatus and compression procedures were essentially the same as described previously (38). Triplicate samples of spore suspensions, generally contained in sealed 1-ml plastic syringes, were submitted to a constant pressure of between 300 and 1,000 atm. The hydraulic fluid was water. The temperature inside the stainless-steel pressure vessel was controlled to ±0.1°C. The effects of different pressures applied for various times and temperatures on spore germination were determined by measuring the loss of heat stability or refractility (determined from the fraction of spores that had become phase-dark after compression) or the increase in stainability (assessed as the fraction of the treated spores taking up dilute stain) (6).

Rate and thermodynamic constants. Least-squares linear regression analysis was used to determine specific reaction rate constants, k, at different pressures and temperatures for *B. pumilus* and *B. cereus* T spores according to the standard exponential inactivation equation: $S = e^{-kt}$, where S is the fraction of spores unchanged by compression at time t. For *B. subtilis* spores, k was determined using the following equation (4) based on probability arguments: $S = 1 - (1 - e^{-kt})^n$, where n is the "extrapolation number" (3). The best fit of the data was obtained by computer analysis to determine k and n.

According to the transition state theory, the effect of pressure on the rate constant k is:

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V‡}{RT}$$

where $\Delta V‡$ is the volume of activation for the reaction, R is the gas constant (8.314 J/mol per °K), and T is the absolute temperature. The slope of a semilogarithmic plot of log k against pressure (atmospheres) may be used to estimate $\Delta V‡$. With this method, straight lines were obtained between the pressures of 500 and 700 atm for *B. cereus* T and *B. subtilis* spores, and the slopes were determined by the least-squares method for linear regression analysis. However, for *B. pumilus* spores the plots, which were markedly curved, were fitted by multiple regression analysis according to the quadratic equation: $\ln k = a + bp + cp^2$.

Arrhenius plots of the logarithm of the reaction velocities against the reciprocal of the absolute temperature were constructed, the slopes of the resulting straight lines were calculated by the sum of the least squares method, and the apparent activation energy, μ or Ea, was determined from: slope = $-$ Ea/2.303R.

The thermodynamic constants of the activated state, i.e., the enthalpy $\Delta H‡$, the free energy $\Delta G‡$, and the entropy $\Delta S‡$ of activation, were calculated from equations of the theory of absolute reaction rates: $\Delta H‡ = \text{Ea} - RT$; $\Delta G‡ = RT \ln(\kappa T/hk)$; $T\Delta S‡ = \Delta H‡ - \Delta G‡$, where κ and h are Boltzmann and Planck constants, respectively.

RESULTS

Effect of pressure on the rate of initiation of germination of bacterial spores. Figure 1 shows the fraction of spores not stained by 0.1% crystal violet after different times at 555, 625, and 763 atm at 25°C for *B. cereus* T spores in phosphate buffer and at 481 and 595 atm at an average temperature of 37.3°C for aqueous suspensions of *B. subtilis* spores. These semilogarithmic plots show the exponential nature of germination initiated by pressure. *B. pumilus* spores (not shown) behaved similarly, with no lag period evident.

The absence of a marked lag period (shoulder) in pressure-initiated germination rate curves constructed by observing changes in stainability or refractility, as defined previously, enabled rate constants to be determined from simple equations for exponential relationships. By contrast, these equations are not suitable for determining germination rate constants based on optical density (absorbance) measurements of the suspension because, in general, optical density-time curves show a very marked lag period. Under certain conditions, slight shoulders occurred in plots of pressure-germinated *B. cereus* T spores. However, rate constants were determined using the simple expression $S = e^{-kt}$, because it provided a better fit to the experimental data than the expression $S = 1 - (1 - e^{-kt})^n$ used for *B. subtilis* spores.

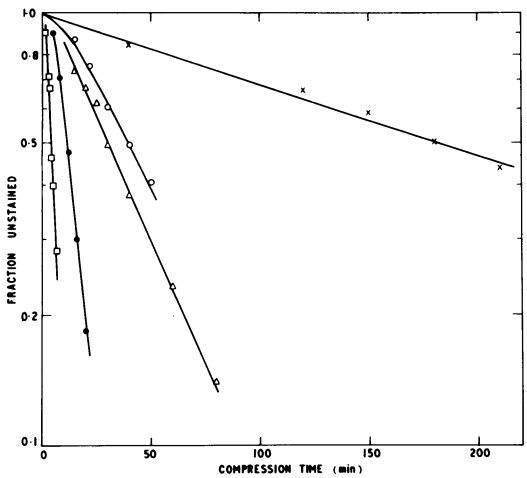

FIG. 1. *Effect of pressure applied at constant temperature for different times on initiation of germination of spores. Phosphate-buffered B. cereus T spores were compressed to 555* (×), *625* (△), *and 763* (□) *atm at 25°C. Aqueous B. subtilis spores were compressed to 481* (○) *and 595* (●) *atm at 37.3°C. Uptake of stain after compression was used as the criterion for germination. Curves fitted by regression analysis.*

Table 1 shows rate constants for germination of spores of *B. cereus* T (phosphate buffer, varying pressures, constant temperature), *B. pumilus* (phosphate buffer, constant pressure, varying temperatures), and *B. subtilis* (phosphate buffer, constant pressure, varying temperatures; aqueous, varying pressures and temperature). To enable more meaningful comparisons to be made between rates obtained under different conditions, particularly where germination is delayed, the times required to initiate germination in 50% of the spore population (t_{50}) were also determined.

The difference among species is readily seen. When the pressure was increased by 139 atm from 555 to 694 atm at 25°C, the rate of initiation of *B. cereus* spores increased 30-fold and t_{50} was reduced by 177 to 4 min. For aqueous *B. subtilis* spores, when the pressure was increased by 114 atm from 481 atm at 37.0°C to 595 atm at 37.6°C, the germination rate increased fivefold and t_{50} was reduced by 28 to 11.5 min.

Effect of temperature on the initiation of germination by pressure. As with alternative methods for initiating spore germination, there is an optimum temperature for germination initiated by pressure. The optimum temperature for germination of *B. subtilis* or *B. pumilus* spores was determined by estimating the fraction of spores remaining fully phase-bright or heat-stable after compression was applied at different temperatures for a constant time.

For both buffered *B. subtilis* at 631 atm and *B. pumilus* spores at 654 atm the optimum temperature was 49.5°C (Fig. 2). For aqueous *B. subtilis* spores compressed to 400 atm, the optimum temperature for initiation of germination was 46.5°C. This was reduced to 43.5°C when the pressure was increased by 150 to 550 atm (37).

Figure 2 shows also that the physiological state of the spore influences the optimum temperature and the degree of germination. When buffered *B. subtilis* spores were freshly heat-activated at 80°C for 10 min, compression caused 85% of the spores to germinate at the optimum temperature of 49.5°C. After 5 months of storage of the suspension at 4°C, the optimum temperature had shifted either to 54°C with only 45% of the spores germinating, if measured by refractility, or to 58°C with 63% germination, if measured by loss of heat resistance. Initial germination levels were restored after a second heat activation (unpublished data).

To determine accurately the effects of temperature at constant pressure on the rate of germination, spores were examined at varying intervals for their ability to take up stain. Figure 3 is a semilogarithmic plot of the fractions of buffered *B. subtilis* spores remaining unstained after different times of compression

TABLE 1. *Effect of pressure and temperature on rate of initiation of germination of Bacillus spores*

Species	Suspending medium	Pressure (atm)	Temp (°C)	Rate constant, k, ± SE[a] (per min)	n[b]	t_{50}[c] (min)
B. cereus T	Phosphate buffer	555	25.0	$3.77 \pm 0.31 \times 10^{-3}$ (15)[d]		181.0
		625	25.0	$2.62 \pm 0.14 \times 10^{-2}$ (23)		40.0
		694	25.0	$1.22 \pm 0.07 \times 10^{-1}$ (17)		4.3
		763	25.0	$2.63 \pm 0.26 \times 10^{-1}$ (10)		2.1
B. pumilus	Phosphate buffer	653	32.6	$1.03 \pm 0.05 \times 10^{-2}$ (15)		66.4
		653	33.7	$1.74 \pm 0.09 \times 10^{-2}$ (18)		38.5
		653	35.5	$2.54 \pm 0.15 \times 10^{-2}$ (17)		23.4
		653	36.6	$3.07 \pm 0.12 \times 10^{-2}$ (14)		17.7
B. subtilis	Phosphate buffer	631	35.9	$2.31 \pm 0.17 \times 10^{-2}$ (21)	1.7	46.8
		631	38.7	$4.89 \pm 0.20 \times 10^{-2}$ (20)	1.9	24.1
		631	40.8	$6.49 \pm 0.31 \times 10^{-2}$ (16)	2.0	18.5
		631	42.9	$1.08 \pm 0.24 \times 10^{-1}$ (13)	2.4	13.0
B. subtilis	Water	481	35.5	$5.52 \pm 0.90 \times 10^{-3}$ (18)	1.05	182.0
		481	37.0	$2.76 \pm 0.22 \times 10^{-2}$ (18)	1.7	39.5
		481	39.8	$6.98 \pm 0.36 \times 10^{-2}$ (11)	2.7	20.7
		481	42.7	$1.78 \pm 0.17 \times 10^{-1}$ (11)	2.5	8.1
		595	37.6	$1.37 \pm 0.05 \times 10^{-1}$ (16)	3.0	11.5

[a] SE, Standard error.
[b] n estimated by computer to fit $S = 1 - (1 - e^{-kt})^n$.
[c] Time required to initiate germination in 50% of spore population.
[d] Numbers in parentheses indicate number of observations.

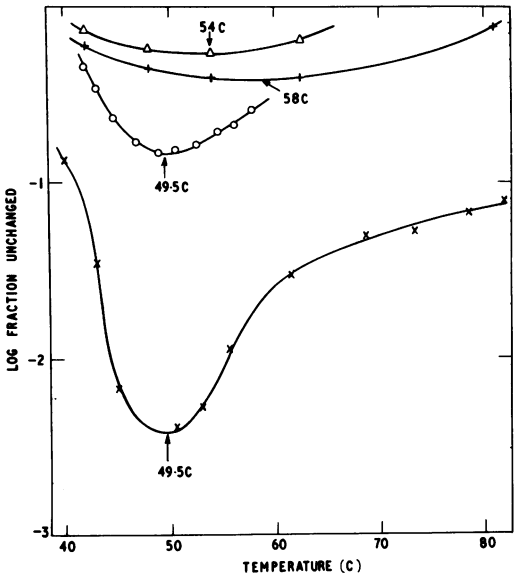

FIG. 2. *Optimum temperature for pressure-initiated germination of heat-activated B. pumilus and B. subtilis spores suspended in 0.067 M potassium phosphate buffer, pH 6.8. Compression was at 645 atm for 15 min on B. pumilus spores and at 631 atm for 10 min on B. subtilis spores (new) or on the same suspension of spores stored for 5 months at 4°C (aged). Degree of germination is expressed as the fraction of spores initially present remaining fully phase-bright (B. subtilis, new [○], aged [△]) or heat-stable (B. subtilis, aged [+]; B. pumilus [×]) after compression. Heat activation, compression, refractility, and heat stability determinations were carried out as described in Materials and Methods. Curves fitted by hand.*

to 631 atm at temperatures of 35.9, 38.7, 40.8, and 42.9°C. Similar plots (not shown) were obtained for aqueous *B. subtilis* spores compressed to 481 atm at 35.5, 37, 39.8, and 42.7°C, and to 595 atm at 37.6°C. Plots of the fraction of buffered *B. pumilus* spores remaining fully phase-bright after varying periods at 653 atm and 32.6, 33.7, 35.5, or 36.6°C were analogous.

Rate constants determined by regression analysis and t_{50} values (Table 1) show that within this temperature range the rates of initiation of germination by pressure increased markedly with temperature. At 481 atm aqueous *B. subtilis* spores germinated 6.5 times faster at 42.7 than at 37°C; at 631 atm the spores germinated 4.7 times faster at 42.9 than at 35.9°C. Comparable results were obtained from phosphate-buffered *B. pumilus* spores. A rise in temperature of 4°C was sufficient to reduce the time required for 50% of the spores to germinate from 66.4 to 17.7 min.

Plots of the fraction of spores remaining fully phase-bright or unstained after 30 min at constant temperature and different pressures flatten out at the lower pressures. This suggests a limiting pressure, which decreases with increasing temperature and below which no measurable change can be detected in the compressed spores. The limiting pressures for *B. subtilis* spores in water were 440 and 360 atm at 35 and 39.9°C, respectively (Fig. 4). An increase in temperature of less than 5°C is sufficient to reduce by 125 atm the pressure required to initiate germination in 50% of the spores. *B. cereus* T (aqueous), *B. subtilis* (phosphate buffer), and *B. pumilus* (phosphate buffer) spores behaved similarly. Rate constants at different pressures, required for estimating volumes of activation, were calculated as described in Materials and Methods.

Thermodynamic constants. At constant temperature, semilogarithmic plots of rate constants against pressure were linear for *B. cereus* T and *B. subtilis* spores over a narrow pressure range (Fig. 5a) and curved for *B. pumilus* spores (Fig. 5b). The volumes of activation, $\Delta V\ddagger$, of the various reactions were calculated from the slopes of the curves (Table 2).

The mean $\Delta V\ddagger$ value for *B. subtilis* spores was -231 cm^3/mol within the ranges 500 to 750 atm and 35 to 43°C. Over the same pressure

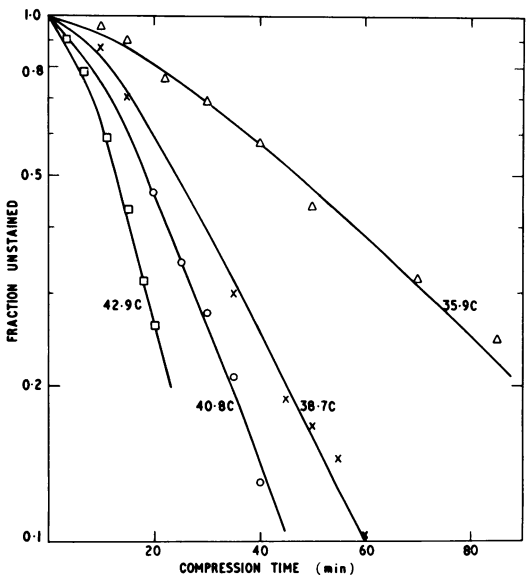

FIG. 3. *Effect of temperature applied at constant pressure (631 atm) for different times on germination of phosphate-buffered B. subtilis spores. Uptake of stain after compression was used as the criterion for germination. Curves fitted by regression analysis.*

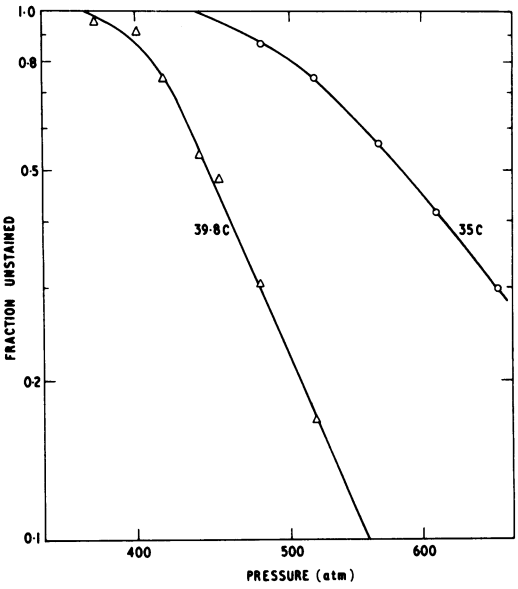

FIG. 4. *Effect of pressure applied for 30 min on germination of aqueous B. subtilis spores at 35°C* (○) *and 39.8°C* (△). *Uptake of stain after compression was used as the criterion for germination. Curves fitted by hand.*

range, temperature markedly influenced $\Delta V\ddagger$ for *B. pumilus* spores. At 500 atm, $\Delta V\ddagger$ altered from −332 to −95 cm³/mol when the temperature rose from 36 to 44°C. Similarly, at 700 atm

TABLE 2. *Volume change of activation of spores germinated by hydrostatic pressure*

Species	Suspending medium	Pressure (atm)	Temp (°C)	$\Delta V\ddagger \pm$ SE[a] (cm³/mol)
B. subtilis	Buffer	550–570	38.7	−223 ± 16
		500–700	40.5	−231 ± 4
		500–650	42.9	−225 ± 9
	Water	550–700	35.0	−221 ± 16
		400–550	39.8	−254 ± 18
B. cereus T	Buffer	500–700	25.0	−612 ± 41
B. pumilus	Buffer	400	40.2	−351 ± 49
			44.2	−350 ± 8
		500	36.0	−332 ± 37
			40.2	−229 ± 5
			44.2	−95 ± 17
		600	33.7	−212 ± 12
			36.0	−204 ± 6
			40.2	−107 ± 59
		700	31.0	−248 ± 12
			33.7	−152 ± 2
			36.0	−76 ± 26

[a] SE, Standard error.

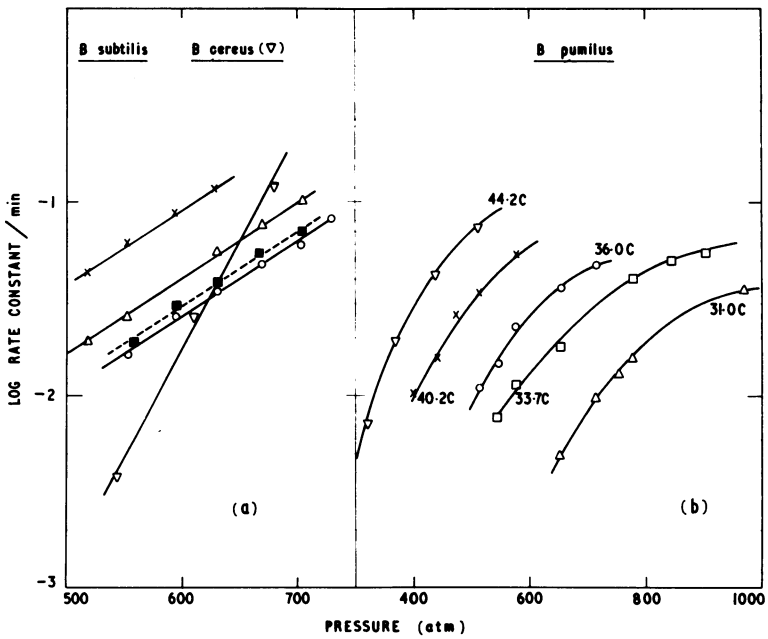

FIG. 5. *Effect of pressure at constant temperature on rate of initiation of germination of B. subtilis and B. cereus (a) and B. pumilus (b) spores. Rate constants were determined from the fraction of spores remaining phase-bright or unstained after 30 min of compression. Curves fitted by linear (a) or multiple regression (b) analysis. Symbols: (a) Phosphate-buffered B. subtilis spores at 38.7°C* (○), *40.5°C* (△), *42.9°C* (×); *aqueous B. subtilis spores, 35°C* (■); *phosphate-buffered B. cereus spores, 25°* (⁻). *(b) Phosphate-buffered B. pumilus spores at 31.0°C* (△), *33.7°C* (□), *36.0°C* (○), *40.2°C* (×); *44.2°C* (⁻).

an increase of 5°C in temperature from 31 to 36°C sufficed to reduce $\Delta V\ddagger$ from −248 to −76 cm^3/mol.

From regression equations calculated to find the line of best fit for the experimental data used to construct Fig. 5a and b, specific reaction rates were estimated at 500 atm for *B. pumilus* and phosphate-buffered *B. subtilis* spores. A straight line is obtained when the logarithm of the reaction rates is plotted against the reciprocal of the absolute temperature in an Arrhenius-type plot (Fig. 6). For aqueous *B. subtilis* spores rate constants were experimentally determined at 481 atm. Apparent activation energy (Ea), free energy ($\Delta G\ddagger$), enthalpy ($\Delta H\ddagger$), and the entropy of activation ($\Delta S\ddagger$) were calculated at 40°C and pressures of 500 and 600 atm (Table 3).

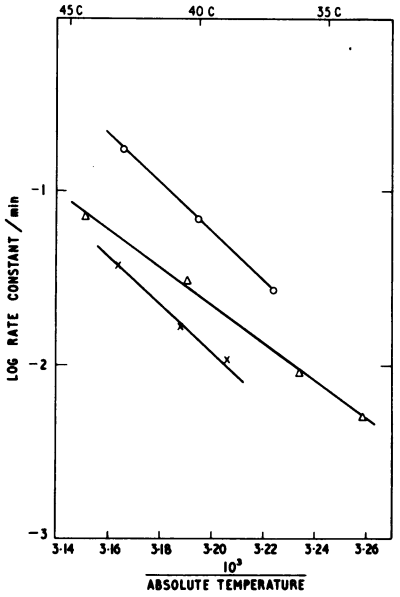

FIG. 6. *Arrhenius plot of logarithm of the rate constant for pressure germination of Bacillus spores and reciprocal of the absolute temperature. Curves fitted by linear regression analysis. Symbols: phosphate-buffered B. pumilus (△) and B. subtilis (×) spores at 500 atm; aqueous B. subtilis spores at 481 atm (○).*

The Ea, $\Delta H\ddagger$, and $\Delta S\ddagger$ values are significantly different between species but are apparently not influenced within species by alterations in the suspending medium.

DISCUSSION

Germination of bacterial spores is most frequently followed by observing changes in the absorbance of the spore suspension with time. Although the methodology is simple, the technique has inherent disadvantages that make interspecies (or intraspecies) and strain comparisons difficult. For example, initial optical densities will vary according to the chemical composition of the medium and the concentration of spores; final optical densities may depend on the degree of germination; and different conditions will influence the duration of the lag period for quantitative estimation of germination rates. Complex expressions that take some of these factors into account have been developed (25, 35).

Although refractility, stainability, and heat stability measurements of germinating spores are more time consuming than optical density determinations, the resulting curves of percentage or fraction of unchanged spores, when plotted against time, are directly comparable and can be fitted by relatively simple equations for rate constant determinations. Application of this technique to calculate rate constants for pressure-initiated germination of *Bacillus* spores allowed the effects of temperature, solvent, and pressure within and between different species to be compared.

The optimum temperature and the degree of spore germination initiated by "physiological"

TABLE 3. *Kinetics and thermodynamic constants for germination of spores initiated by hydrostatic pressure at 40°C*

Constant	Pressure (atm)	*B. pumilus* (buffer)	*B. subtilis*	
			Buffer	Water
k (per min)	500	2.59×10^{-2}	1.48×10^{-2}	8.05×10^{-2}
	600	5.70×10^{-2}	3.72×10^{-2}	
Ea (J/mol)	500	2.09×10^{5}	2.64×10^{5}	2.67×10^{5}
	600	1.81×10^{5}	2.48×10^{5}	
$\Delta H\ddagger$ (J/mol)	500	2.06×10^{5}	2.62×10^{5}	2.65×10^{5}
	600	1.78×10^{5}	2.45×10^{5}	
$\Delta G\ddagger$ (J/mol)	500	9.71×10^{4}	9.83×10^{4}	9.42×10^{4}
	600	9.50×10^{4}	9.58×10^{4}	
$\Delta S\ddagger$ (J/mol per °K)	500	3.47×10^{2}	5.19×10^{2}	5.44×10^{2}
	600	2.62×10^{2}	4.77×10^{2}	

germinants may be influenced by species or strain (24, 33), previous heat activation treatment (2), germinant (33) and its concentration (1), pH (1, 34), and by the presence or absence of specific cations in the germination medium (18). It has been reported previously that the optimum temperature for germination initiated by pressure is similarly influenced by spore species, germinant, pH, and cation content of the suspending fluid (11, 36–38). The effect of loss of heat activation on the ability of spores to germinate under pressure (Fig. 2), a characteristic of physiological germination (17), provides additional evidence that pressure does not induce germination by non-physiological "chemical" or "mechanical" means (30).

Observations of apparently higher than "normal" optimum temperatures for pressure-initiated germination of *B. subtilis* and *B. pumilus* spores do not invalidate this concept of physiological germination. Different strains of *B. subtilis* spores germinate in physiological germinants with optimum temperature of 30 to 45°C (2, 33; H. P. Fleming, Ph.D. thesis, University of Illinois, Urbana, 1963). Although *B. pumilus* spores have not been studied in such detail, optimum temperature for their germination could be similar to that of *B. subtilis* spores because of the close relationship between these species (5, 9, 39). Higher optimum temperatures for germination induced by pressure would be expected, as it is known that pressure can retard thermal inactivation of enzymes (14, 27) and bacterial spores (13) and also heat denaturation of proteins (32).

Although values for the volume change of activation (Table 2) appear to be high, values within this range have been reported for protein denaturation and for gel-sol transitions (15). Pressure accelerates reactions with negative volume changes of activation. The very high negative value of 612 cm^3/mol for *B. cereus* T spores correlates with its ease of germination at low temperatures compared with those of *B. pumilus* and *B. subtilis* spores. At constant pressures of 500 atm and above, the decrease in $\Delta V‡$ values for *B. pumilus* spores with increasing temperatures demonstrates that there is an upper temperature at which the germination rate will not be increased. Similarly, for constant temperature, for example, 36°C, the decrease in $\Delta V‡$ with increasing pressure indicates that there is an optimum pressure for maximum rate of initiation of germination. Further increases in pressure will result in a lower rate of initiation.

Activation energy, Ea, can be considered as the energy threshold that must be exceeded before a reaction can occur. Values of Ea of between 41,860 and 83,720 J (10 and 20 kcal; 18, 20, 26) for germination initiated by conventional techniques fall within the range for enzymatic reactions. For *Clostridium sporogenes* PA3679 spores initiated by 20 mM ethylenediaminetetraacetic acid (29), an Ea value of about 188,000 J can be calculated. The Ea values in Table 3 for pressure-initiated germination are higher and similar to those reported for the effect of heat activation on germination rates (19). However, it can be predicted that with higher pressures, activation energies would decline to those reported for other germination systems.

Low et al. (21) have pointed out that it is better to use $\Delta G‡$, the free energy of activation, than the activation energy for interspecies comparisons of the efficiency of an enzyme to reduce the energy barrier and allow a reaction to proceed. The use of Ea as an index of catalytic efficiency would be valid only if different species had equivalent entropies of activation, $\Delta S‡$. From Table 3 it can be seen at 500 atm that irrespective of species or suspending medium, there is an inverse relationship between $\Delta G‡$ and rate constant values. Thus, *B. subtilis* spores in buffer have the highest $\Delta G‡$ value (98,300 J/mol) and the lowest k value (1.48×10^{-2}/min). When the spores are suspended in water, the values are reversed; $\Delta G‡$ is lower (94,200 J/mol), and k is higher (8.05×10^{-2}/min). The corresponding constants for *B. pumilus* spores are intermediate in value. No such relationship exists between the rate constants and the activation energies. The correlation between $\Delta G‡$ and reaction rate provides indirect evidence, supported by the high enthalpies of activation, that initiation of germination involves a temperature-dependent, enzyme-controlled reaction. Low $\Delta H‡$ values are indicative of temperature-independent chemical reactions (12).

Low and Somero have suggested (23) that if one or more water-constricting groups on a protein surface become increasingly exposed to water during the formation of the activated enzyme-substrate complex, $\Delta V‡$ and $\Delta G‡$ will be reduced. The addition of salts to the medium will generally hinder the hydration of these exposed groups, $\Delta V‡$ and $\Delta G‡$ will increase, and the reaction rate will be inhibited. The decreased rate of germination of *B. subtilis* spores at 500 atm and 40°C in phosphate buffer compared with water follows this pattern.

Enzymes exist in the protoplasts of dormant

spores in an inactive or inhibited state. Keynan (16) has suggested four alternative explanations for their inactivity: cross-linking between enzymes or other spore proteins, unfavorable ionic environment, dehydration, and reversible enzyme inhibition. Hydrostatic pressure could influence any of these proposed mechanisms (15) and thereby break dormancy.

Interpretation of thermodynamic constants calculated for biological systems is difficult because they represent the net effect from a possible multiplicity of reactions. Nevertheless, the values obtained for pressure-initiated germination are consistent with the proposition that the transformation from a dormant to a metabolizing spore involves hydration or a reduction in the viscosity of the core with a subsequent conformational change of an enzyme. Changes in enzyme conformation are often associated with large activation volume changes (22). The effect of pressure on solvation of intracellular gels is well known (15). Solvation of the core cytoplasm could enable mobilization of ions, which could either enhance catalytic enzyme action or free ions previously acting as enzyme inhibitors (23).

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