Stimulation of Germination of Unactivated *Bacillus cereus* Spores by Ammonia

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Inclusion of ammonia in germinant mixtures containing L-alanine and inosine stimulated germination of unactivated *Bacillus cereus* spores at rates equal to those obtained using heat-activated spores without ammonia. D-Alanine had little effect on germination of heat-activated spores, but severely inhibited germination of unactivated spores in the presence of ammonia. Ammonia did not replace the requirement for either L-alanine or inosine: all three compounds were required for rapid germination. Kinetic analysis suggested that the functions of ammonia and L-alanine were more closely related than the functions of ammonia and inosine. With rate-saturating concentrations of L-alanine and inosine, germination rates showed saturation kinetics for ammonia with a K_m for NH_4Cl of 5 mM. Comparisons of the effects of salts, amines and pH on germination rates suggested that NH_4OH rather than NH_4^+ was the rate-limiting form of ammonia. In comparisons of various strains of *B. cereus*, stimulation of germination by ammonia occurred in all cases, although spores of most soil isolates germinated more rapidly than *B. cereus* T spores in the absence of ammonia.

INTRODUCTION

Germination of bacterial endospores involves a sequence of degradative reactions initiated by specific chemical agents (Gould & Dring, 1972; Setlow, 1981). Initiating agents (germinants) include amino acids, sugars, ribosides, or even simple inorganic salts, depending on the bacterial strain involved. Usually, stimulation of rapid germination by these substances requires prior activation of the spores by one of a variety of treatments. Most commonly, activation is obtained by heating aqueous suspensions of spores (at 60–115 °C, depending on the strain) for periods ranging from minutes to several hours (Keynan & Evenchik, 1969). Molecular mechanisms of activation and germination have not been established, despite extensive work in these areas.

The necessity for activation as a prerequisite for spore germination varies amongst species (Evans & Curran, 1943; Curran & Evans, 1945) and also depends on the composition of the medium in which spores were produced (Levinson, 1961) and the types and concentrations of germinating agents (Keynan & Evenchik, 1969). Nonetheless, and despite the uncertain ecological significance of its effects, activation of spores by sub-lethal heating is generally accepted as a convenient technique for increasing the germinability of spores (e.g., Foerster & Foster, 1966). The application of this technique poses few problems if its only purpose is to ensure rapid germination, but studies specifically concerned with the analysis of control mechanisms in spore germination are complicated by unknown variables associated with sub-lethal heat treatments (Powell & Hunter, 1955). The analysis of these control mechanisms would be simplified by avoiding the use of heat-activation when possible.

An alternative to heat-activation as a means for increasing spore germinability was encountered here during the course of an unrelated study of unactivated spores (Kieras *et al.*, 1978). We found that the requirement for activation for rapid germination of spores of *Bacillus cereus* T could be entirely eliminated by adding ammonium chloride to a commonly used

germinant mixture containing L-alanine and inosine. Stimulation of spore germination by ammonia was noted previously in several strains of *B. subtilis* (Wolf & Thorley, 1957) and a possible mechanism for this effect was discussed (Gould, 1978). The results reported here, describing the kinetics and specificity of ammonia-stimulated germination of spores of *B. cereus*, provide a foundation for further analysis of the mechanism of this phenomenon. In addition, however, our comparisons of the stimulatory effects produced by ammonia and heat-activation suggest that rapid germination of unactivated spores in the presence of ammonia involves regulatory mechanisms that appear to be bypassed during germination of heat-activated spores. The use of ammonia as a co-germinant for unactivated spores should facilitate the analysis of these regulatory mechanisms. (Preliminary results of this study were presented at the 80th Annual Meeting of the American Society for Microbiology, May 11–16, 1980, Miami, Fla., USA.)

METHODS

Bacterial strains. Unless otherwise indicated, experiments were performed using *B. cereus* T obtained from N. McCormick (University of Wisconsin) in 1967, maintained as spores in 50% (v/v) glycerol at -20°C . For strain comparisons as specified, additional stocks of *B. cereus* T were obtained from E. Juni (University of Michigan), L. Sacks (Western Regional Research Laboratories, USDA), J. C. Vary (University of Illinois), and R. S. Hanson (University of Wisconsin). Strains RP201–RP208 were independently isolated from local soils, identified as *B. cereus* by standard methods (Gordon *et al.*, 1973) and maintained on nutrient agar slants at 4°C . Of the soil isolates, strain RP201 had the rhizoid colony morphology typical of *B. cereus* var. *mycoides*, while the other strains had compact colony morphologies similar to that of *B. cereus* T.

Preparation of spores. One-litre cultures in modified G-medium (Vary & Halvorson, 1968) were grown in 6-litre flasks at 30°C on a rotary shaker at 200 r.p.m. These cultures were inoculated with exponential-phase cells (equivalent to 0.5 mg dry wt) from trypticase soy precultures. The cultures were harvested after 24 h, by which time release of free spores was usually complete. All harvesting and washing procedures were performed at $0-4^{\circ}\text{C}$. Spores were harvested and washed by centrifugation (5000 g, 5 min) and resuspended in washing media at 1–2 mg dry wt ml^{-1} . Three washes with distilled water were followed by three washes with 1 M-KCl, then four more washes with distilled water. Spores in distilled water (8 mg dry wt ml^{-1}) were stored on ice and used within 3 weeks of preparation.

The 1 M-KCl washes were originally included as a precaution for removing proteases and other sporangial contaminants (Tesone & Torriani, 1975). They were retained on principle, though they had no apparent effect on germination under the conditions used here. Spores could not be lyophilized or frozen without producing a temporary reduction in their germinability following rehydration or thawing. Problems in standardizing these effects were avoided by not using such storage techniques.

Germination assays. Germination was assayed as the decrease in absorbance of a suspension of spores, measured at 660 nm using a Gilford 240 spectrophotometer. Assays were done at 30°C in cuvettes of light path 1 cm, starting the reactions by adding a small volume of spore suspension ($<1\%$ of final vol.) to the germination reaction mixtures to give an initial absorbance of 0.6–0.8 (10^8 spores ml^{-1}). Except where specified, the basal germination reaction mixture (InAl) contained 1 mM-L-alanine, 1 mM-inosine, 20 mM-Tris and 100 mM-NaCl, adjusted to pH 8.4 using HCl. In some cases, adenosine was substituted for inosine in this mixture (AdAl). When NH_4Cl or other ionic substances were added to the mixture, the NaCl concentration was adjusted to maintain a total ionic strength equal to that of the basal mixture. Appropriate adjustments of ionic strength were also made in experiments in which pH variations altered the proportions of NH_4^+ and NH_4OH . Germination was not specifically affected by NaCl (Table 3).

Calculation of germination rates. Absorbance (660 nm) was recorded continuously on a strip chart recorder. After adding spores to germinants, absorbance typically remained constant for 1–5 min, then decreased in a sigmoidal manner as described by McCormick (1965). Germination rate was defined as the slope of the tangent to the most rapidly changing portion of the recording of A_{660} vs time, expressed as a percentage of the initial absorbance of the suspension ($\%A_{660} \text{ min}^{-1}$).

Heat-activation. Except where specified, spores for these experiments were not activated by heat or other means. In specific cases, spores were optimally heat-activated as follows. Spores (4–8 mg dry wt ml^{-1}) in 20 mM-sodium phosphate, pH 8, were heated at 65°C . Samples were taken periodically, cooled to 4°C on ice, washed twice with distilled water by centrifugation (5000 g, 5 min) and assayed for germinability in the standard InAl or AdAl reaction mixture. Depending on the particular spore preparation, activation at 65°C for 20–40 min was required to obtain maximal germination rates in these germinants. Maximally activated spores were stored at 0°C and used within 4 h of preparation.

Curve fitting. Saturation curves relating germination rates to concentrations of reactants were approximated using the Michaelis–Menten equation. Since the saturation curves frequently intersected the germination rate axis at non-zero values, conventional reciprocal plots were not suitable for estimating the parameters of the equation. Instead, apparent K_m and V_{max} values were obtained using a non-linear optimization program (Brent, 1971) that minimized the sum of squared differences between the observed rates and the rates predicted by the Michaelis–Menten equation.

Germination rate normalizations. In experiments testing the effects of pH on reactions containing NH_4Cl , germination rates were normalized in the following way. It was assumed that processes rate-limiting for germination would have an intrinsic pH-dependence not directly related to the ionization of ammonia. The intrinsic pH-dependence was approximated as the boundary of all germination rate vs pH profiles at all NH_4Cl concentrations (Fig. 2, dashed line). Observed germination rates at a given pH were normalized using this estimated intrinsic pH-dependence curve and the relation $V_n(pH) = V_o(pH) \times [V_i(8.5)/V_i(pH)]$, where: $V_n(pH)$ = normalized rate at given pH; $V_o(pH)$ = observed rate at given pH; $V_i(8.5)$ = rate at optimum of intrinsic pH-dependence curve; and $V_i(pH)$ = rate at given pH of intrinsic pH-dependence curve.

Materials. Inorganic reagents were analytical reagent grade from various commercial sources. Yeast extract and nutrient agar were from Difco, trypticase soy from Baltimore Biological Laboratories (Cockeysville, MD 21030, USA), and other organic reagents from Sigma.

RESULTS

Elimination of activation requirement for rapid germination

Freshly harvested spores of *B. cereus* T germinated slowly or not at all in InAl or AdAl unless the spores were first activated by heating at 65 °C (see Methods). Unactivated spores germinated rapidly, however, when NH_4Cl was included in germination reactions with InAl or AdAl (Table 1). As reported by others, inosine was more effective than adenosine as a co-germinant with L-alanine (Powell & Hunter, 1955; Gould, 1969), but germination rates with both InAl and AdAl varied considerably in successive spore preparations, particularly in the presence of NH_4Cl (see below). The stimulatory effects of heat-activation and NH_4Cl were partially additive in the AdAl germinant, but not in InAl, where maximal rates were observed using NH_4Cl with spores that had not been activated by heat (Table 1). NH_4Cl did not initiate germination in the absence of InAl or AdAl, whether the spores were heat-activated or not (data not shown).

Effect of D-alanine on germination of activated and unactivated spores

D-Alanine inhibits germination induced by L-alanine in *B. cereus* and other species (Hills, 1949; Fey *et al.*, 1964; Stewart *et al.*, 1981). In *B. cereus* T, the inhibitory effect of D-alanine is diminished in the presence of adenosine or inosine when using heat-activated spores (Yousten, 1975). We compared the effects of D-alanine on germination stimulated by heat-activation or

Table 1. Comparisons of germination rates stimulated by heat-activation or by supplementing germinants with NH_4Cl , or both, with different spore crops

Germinants*	Spore preparation no.	Germination rate (% A_{660} min ⁻¹)			
		Unactivated spores		Activated spores†	
		Control	+ NH_4Cl	Control	+ NH_4Cl
L-Alanine + inosine	9	2.8	42	30	37
	17	2.7	46	37	44
	56	2.6	62	43	53
L-Alanine + adenosine	17	0‡	26	31	41
	56	0‡	44	24	51

* Reaction mixture contained 1 mM indicated germinants, 20 mM-Tris/HCl, and either 100 mM-NaCl (controls) or 100 mM- NH_4Cl , pH 8.4.

† Spore activation pretreatment: 65 °C, 20–40 min (see Methods).

‡ No change in absorbance during experimental period (30 min).

Fig. 1. Inhibition of germination by D-alanine. Germinant mixtures contained inosine and L-alanine (1 mM each), 20 mM-Tris, and the indicated concentrations of D-alanine; the pH was adjusted to 8.4 with HCl in all cases. ●, Germination rates of unactivated spores, stimulated by supplementing the reaction mixture with 0.1 M-NH₄Cl; ■, germination rates of heat-activated spores, with NaCl to maintain an ionic strength of 0.1 M.

Table 2. *Apparent Michaelis-Menten parameters for individual germinants*

Parameters were determined by non-linear optimization (see Methods). All reactions were buffered at pH 8.4 with 20 mM-Tris/HCl. Parameters for L-alanine were calculated in the presence of constant 1 mM-inosine; parameters for inosine were calculated in the presence of constant 1 mM-L-alanine. The reaction mixture for 0.5 mM-NH₄Cl also contained NaCl to maintain an ionic strength of 0.1 M.

NH ₄ Cl concentration	L-Alanine		Inosine	
	<i>K_m</i> (μM)	<i>V_{max}</i> (% <i>A</i> ₆₆₀ min ⁻¹)	<i>K_m</i> (μM)	<i>V_{max}</i> (% <i>A</i> ₆₆₀ min ⁻¹)
0.5 mM (limiting)	8	8.3	170	11
100 mM (saturating)	100	41	73	38

NH₄Cl to assess the functional similarity of these two stimulatory treatments. As shown in Fig. 1, germination of unactivated spores in the presence of ammonia was severely inhibited by D-alanine, while heat-activated spores were relatively insensitive to D-alanine.

Effects of ammonia with individual germinants

Both L-alanine and inosine (or adenosine) were essential components of reaction mixtures supporting rapid germination of unactivated spores in the presence of NH₄Cl. With some spore preparations, slow germination (<1%*A*₆₆₀ min⁻¹) did occur in reaction mixtures containing only NH₄Cl and L-alanine (or only NH₄Cl and inosine), but these results could not be reliably reproduced in successive spore crops. As noted elsewhere (Gould, 1969), endogenous sources of omitted co-germinants have been implicated in some germinations occurring in 'incomplete' mixtures of germinants, and this could account for our variable results with NH₄Cl and either L-alanine or inosine as germinants. In any case, NH₄Cl was clearly not a substitute for either L-alanine or inosine, and it appeared that the functions of all three of these substances were mutually interdependent.

We attempted a partial kinetic analysis of this interdependence by measuring the effect of NH₄Cl on the quantitative requirements for L-alanine and inosine. Germination rates were assayed using either rate-limiting or saturating (see below) NH₄Cl concentrations in reaction mixtures having either L-alanine or inosine present at a rate-saturating concentration while the other co-germinant was variable. The resulting rate-saturation curves were approximated by Michaelis-Menten functions (see Methods), with the optimal parameters as shown (Table 2). The apparent *K_m* values for L-alanine and inosine were both altered by 100 mM-NH₄Cl, but the magnitude of the change was considerably higher for L-alanine. While suggesting a closer

Table 3. *Effects of univalent ions on germination rates*

Salt added to reaction*	Germination rate (% A_{660} min ⁻¹)
None	2.5
NaCl	4.4
KCl	0.0
LiCl	0.8
CsCl	1.2
Tris/HCl	3.7
NH ₄ Cl	38.0

* The reaction mixtures contained 20 mM indicated salt, 1 mM-L-alanine, 1 mM-inosine, 2 mM-Tris/HCl, pH 8.4.

biochemical relationship between the functions of NH₄Cl and L-alanine rather than NH₄Cl and inosine, these results remain open to a variety of interpretations. Nonetheless, they confirmed that neither L-alanine nor inosine was rate-limiting for germination when present in a concentration of 1 mM (1 mM $\gg$ K_m), allowing the following determination of the sensitivity of InAl-induced germination for NH₄Cl.

Kinetics of response to ammonia

Germination rates in InAl showed saturation kinetics with respect to NH₄Cl concentration (see Fig. 4; note the logarithmic abscissa scale, and ammonia expressed as NH₄OH: see below). As shown (see also Table 1), germination in InAl occurred slowly in the absence of NH₄Cl, implicating either an endogenous source of NH₄Cl (or its equivalent) or a germination mechanism not strictly dependent on NH₄Cl. Using a Michaelis-Menten approximation, the response of the germination rate to NH₄Cl had an apparent K_m of 5 mM NH₄Cl and an apparent V_{max} of 35 % A_{660} min⁻¹ (K_m = 0.7 mM NH₄OH, from pK_a for NH₄⁺: see below). These parameters and the germination rate in the absence of NH₄Cl varied considerably in successive spore preparations (K_m for NH₄Cl varied from 2 to 6 mM; V_{max} varied from 20–60 % A_{660} min⁻¹; rates of controls lacking NH₄Cl varied from 2 to 6 % A_{660} min⁻¹). Systematic alterations in details of spore preparation protocols failed to reveal the source of this variability (see Discussion).

Absence of general ionic stimulation

Germination of spores of various *Bacillus* species is reportedly influenced by the ionic composition of the germination reaction mixture (Foerster & Foster, 1966; Gould, 1969), and we initially supposed that the stimulatory effect of NH₄Cl involved similar ionic effects. However, univalent cation chlorides other than NH₄Cl were ineffective in stimulating germination under our conditions (Table 3). As reported previously K⁺ ions were inhibitory to germination (Krisk, 1961).

Effects of pH and NH₄Cl concentration on germination rate

The results shown in Table 3 did not support a role for NH₄Cl as a general ionic germination stimulant, but were consistent with a specific requirement for the NH₄⁺ cation. The results were also consistent with a requirement for NH₄OH as a specific hydrolysis product of NH₄⁺, in which case ionic considerations would be secondary in establishing the specificity of the stimulatory effects of ammonia. We tested for the involvement of NH₄OH by measuring the relative effects of pH and total NH₄Cl concentration on the rate of germination in InAl (Fig. 2). The pH optimum for germination was more alkaline in the presence of decreased amounts of total NH₄Cl, suggesting that NH₄OH might be the relevant rate-limiting substance on the acidic sides of the pH profiles. However, in the format shown (Fig. 2), the data were difficult to interpret quantitatively in terms of the titration of NH₄Cl.

The relationship between the data shown (Fig. 2) and the titration of NH₄⁺ was quantified in the following manner. We calculated the amounts of NH₄⁺ and NH₄OH in the various reactions under the specified conditions of pH and total NH₄Cl concentration, using pK_a of 9.2 for NH₄⁺.

Fig. 2. Effect of pH on germination rates in InAl containing different NH_4Cl concentrations. Reaction mixtures contained inosine and L-alanine (1 mM each), 20 mM-Tris and NH_4Cl at 50 mM ($\bullet$), 10 mM ($\blacksquare$), or 2 mM ($\blacktriangle$). The pH was adjusted with NaOH or HCl, and ionic strength maintained constant at 0.1 M with NaCl. The dashed line shows the estimated intrinsic pH-dependence (see text).

Fig. 3. Replots of germination rates of Fig. 2 as functions of concentrations of NH_4^+ and NH_4OH calculated from reaction mixture pH values (using $\text{pK}_a = 9.2$ for NH_4^+ ionization). Rates were normalized as described in the text. Reaction mixtures are described in the legend to Fig. 2. Solid line, filled symbols: rates as a function of NH_4OH concentration. Dashed line, open symbols: rates as a function of NH_4^+ concentration. The amounts of NH_4Cl initially added were: 50 mM ($\circ$, $\bullet$), 10 mM ($\square$, $\blacksquare$) and 2 mM ($\triangle$, $\blacktriangle$).

The observed germination rates were then normalized, based on an assumed intrinsic pH-dependence not related to the ionization of ammonia (dashed line, Fig. 2, and see Methods). These normalized rates were then plotted against the calculated concentrations of the two forms of ammonia, with the result shown in Fig. 3. Only the NH_4OH plot had a reasonably continuous curve, with rates generally increasing with concentration rather than decreasing as in the NH_4^+ plot. A Michaelis-Menten approximation to the initial portion of the NH_4OH plot (Fig. 3, data points below 10 mM- NH_4OH) provided an apparent K_m for NH_4OH of 0.8 mM. The apparent K_m for NH_4OH calculated from experiments performed at constant pH (see above) was 0.3–0.8 mM, in good agreement with the result obtained here. The decrease in germination rates at concentrations of NH_4OH exceeding 10 mM remains to be explained, but could be a toxic effect unrelated to the mechanism of the stimulatory function of ammonia.

Stimulation by other weak bases

If NH_4OH rather than NH_4^+ were the active form of ammonia in stimulating spore germination, as suggested above, other weak bases might have similar effects. To test this possibility, a number of structurally diverse amines and amino acids with widely varying ionization constants were assayed for their ability to stimulate germination in InAl. Glutamine, glutamic acid, γ -aminobutyrate, asparagine, lysine and urea had no effect on germination in InAl when added at concentrations of 1 mM, while glycine and putrescine were inhibitory (data

Fig. 4. Stimulation of germination by weak bases. Reaction mixtures contained inosine and L-alanine (1 mM each), 20 mM-Tris, NaCl to maintain an ionic strength of 0.1 M and the indicated concentrations of the non-ionic forms of: NH_4OH , $\text{pK}_a = 9.2$ (●); methylamine, $\text{pK}_a = 10.6$ (▼); ethanolamine, $\text{pK}_a = 9.5$ (■); or hydroxylamine, $\text{pK}_a = 6.0$ (▲). The reaction mixtures were adjusted to pH 8.4 with HCl or NaOH. A logarithmic abscissa is used for convenience of comparisons in a single graph. The dashed line indicates the germination rates of controls lacking ammonia or amines.

not shown). However, methylamine, ethanolamine and hydroxylamine were stimulatory to varying extents (Fig. 4). Comparing the non-ionized forms of these substances, methylamine and ammonia appeared to be equally stimulatory at low concentrations, but higher concentrations of methylamine inhibited germination. Both ethanolamine and hydroxylamine were considerably less effective than ammonia at low concentrations, although hydroxylamine became quite stimulatory at high concentrations (Fig. 4).

To a limited extent, these results supported the hypothesis that the non-ionic forms of ammonia and the amines were the stimulatory forms of these substances. However, this support rested on the assumption that similar mechanisms were involved in the observed stimulatory effects, and this appeared not to be true, at least in the case of hydroxylamine. It was reported elsewhere that, by inhibiting alanine racemase in *B. cereus* spores, hydroxylamine could relieve an auto-inhibition of germination arising from isomerization of L-alanine (Jones & Gould, 1968). Experiments subsequent to the above comparisons of amines (to be published elsewhere) revealed that inhibition of alanine racemase could account for almost all of the stimulatory effect of hydroxylamine (Fig. 4) but did not account for any of the stimulatory effect of NH_4Cl . Apparently, then, the mechanism responsible for the effects of ammonia has a pronounced specificity for ammonia rather than the amines tested here, with the pH-dependence (Fig. 3) most strongly supporting the significance of the uncharged form, NH_4OH .

Stimulation of other Bacillus cereus strains by ammonia

Germination requirements of sporeformers are both species- and strain-dependent (Foerster & Foster, 1966; Thorley & Wolf, 1961). To determine whether or not stimulation of germination by ammonia was peculiar to our laboratory strain of *B. cereus* T, we compared the effects of ammonia on the germination of spores of various strains of *B. cereus* (Table 4). All strains of *B. cereus* T tested (obtained from other laboratories, see Methods) responded to germinants and ammonia in a manner similar to our laboratory strain. However, spores of *B. cereus* strains recently isolated from soil responded in two different ways. In the case of an isolate having the colony morphology typical of *B. cereus* var. *mycoides* (RP201), although ammonia appeared to be stimulatory to some extent, germination rates were unusually low in InAl , whether or not ammonia was present. Germination rates of the other soil isolates were significantly stimulated by a moderate concentration of ammonia, but their relatively rapid germination in the absence of ammonia led to a decrease in the percentage stimulation by ammonia, compared to the *B. cereus* T strains (Table 4). Stimulation of germination by ammonia was observed to be strain-dependent in *B. subtilis* also, with ammonia used as the sole (exogenous) germinant or in combination with glucose or L-alanine as co-germinants (Wolf & Thorley, 1957).

Table 4. *Stimulatory effect of ammonia on germination of spores of different strains of B. cereus*

Strain	Source	Germination rate* (% A_{660} min ⁻¹)		Stimulation† (%)
		Control	+ NH ₄ Cl	
<i>B. cereus</i> T (five strains)	Various laboratories‡	4.0–5.8	14–22	150–240
RP201§	Soil	1.2	3.4	180
RP203	Soil	14	18	29
RP207	Soil	12	19	58
RP208	Soil	15	21	40

* The germination reaction mixture contained 1 mM-L-alanine, 1 mM-inosine, 20 mM-Tris/HCl and either 10 mM-NaCl (controls) or 10 mM-NH₄Cl, pH 8.4.

† Stimulation = [(NH₄Cl rate – control rate)/control rate] × 100.

‡ Strains obtained from five laboratory sources, see Methods.

§ *B. cereus* var. *mycoides*.

DISCUSSION

In this report, we have shown that spores of *B. cereus* T will germinate rapidly without prior activation when incubated in a germinant mixture containing L-alanine and inosine, supplemented with ammonium chloride. The results reported here are the initial stages of a continuing investigation of the functional relationships between these co-germinants and unactivated spores. While the identification of ammonia as a relevant co-germinant for spores of *B. cereus* seems a promising finding for the analysis of mechanisms regulating spore germination, a discussion of the possible biochemical basis for the stimulatory effects of ammonia would be premature at this time. Instead, the results reported here are more appropriately discussed in connection with possible relationships between heat-activation and germination and techniques for analysing these processes.

Detailed comparisons of the germinability of activated and unactivated spores were not attempted in this study, for reasons to be discussed below. Nonetheless, our results establish that the use of ammonia as a stimulatory co-germinant is not functionally equivalent to heat-activation. Evidence supporting this view includes: (1) the requirement for a combination of both optimal heat-activation and rate-saturating concentrations of ammonia for maximal germination rates in AdAl (Table 1); (2) the fact that germination stimulated by heat-activation and ammonia differed in sensitivity to the inhibitor, D-alanine (Fig. 1); (3) the inability of ammonia to establish a stable activated state like that produced by heat-activation, based on observations of low germination rates after preincubation of spores in ammonia, removal of ammonia by washing and germination in InAl lacking ammonia (results not shown). On the basis of these functional differences, it can be concluded that rapid germination in the presence of ammonia involves regulatory mechanisms not evident in the germination of heat-activated spores.

The increased germinability of bacterial spores following heat-activation can be viewed as a relaxation of various requirements for germination, such that lower concentrations of specific germinants are required, fewer co-germinants are necessary for maximal germination rates and/or a greater variety of chemical agents acquire germinative activity (Lewis, 1969). In the present case, heat-activation appeared to 'relax' the requirement for ammonia for rapid germination of *B. cereus* T spores (Table 1). This 'relaxation' of germination requirements by heat-activation can be interpreted as a simplification assisting the analysis of underlying germination mechanisms, but there are difficulties with this view. The biochemical basis for the effect of heat-activation on spore germinability has not been determined in any instance (Keynan & Evenchik, 1969; Yousten, 1975), and it has been suggested that heat-activation could mobilize unknown co-germinants (Levinson, 1961). These co-germinants could easily escape detection by functioning only at the elevated temperature of activation treatments (Lawrence, 1955). Difficulties attending the use of heat-activation as a poorly understood germination

stimulant were clearly exemplified in a study in which spores apparently germinated in 'distilled water' following heat-activation (Powell & Hunter, 1955). Rather than indicating extreme 'relaxation' of germination requirements, this apparently spontaneous germination appeared to involve unidentified endogenous germinants that were particularly effective during centrifugal packing of activated spores (Powell & Hunter, 1955).

In view of such difficulties in accurately defining relevant changes in spore properties following heat-activation, it seems questionable that its use in 'relaxing' germination requirements actually simplifies the analysis of germination mechanisms. As an alternative view, we suggest that, in general, the use of heat-activation for increasing spore germinability may hinder the analysis of germination mechanisms by concealing requirements for co-germinants relevant to the operation of these mechanisms. As an effective and specific chemical substitute for heat-activation, ammonia may be one such co-germinant, at least for spores of *B. cereus*. In the present study, the tight functional interdependence of ammonia, L-alanine, and inosine allowed no clear demonstration of the biochemical significance of the stimulatory effect of ammonia. However, our results have established the feasibility of analysing mechanisms of rapid germination of *B. cereus* spores without routinely relying on the poorly defined stimulatory effect of heat-activation.

At the start of this study, it seemed curious that stimulation of germination by ammonia had not been investigated in detail following the observations of Wolf & Thorley (1957), particularly in view of the possible role of L-alanine dehydrogenase in spore germination (O'Connor & Halvorson, 1961; Freese *et al.*, 1964). For that reason, numerous experiments were performed to ensure that the apparent novelty of this phenomenon in *B. cereus* was not due to peculiarities of technique in this laboratory. Attempts to identify influential contaminants in reagents used for spore production or germination assays gave negative results. Spores produced in a modification of Nakata's (1964) synthetic medium for *B. cereus* were indistinguishable from spores produced in G-medium in their sensitivity to stimulation of germination by ammonia. Details of spore production and washing protocols were varied either purposely or incidentally during several years of experimentation with ammonia-stimulated germination, again with no systematic influence on the ability of ammonia to stimulate germination in InAl. Our laboratory strain of *B. cereus* T was not at all unique in its response to ammonia, although strains of *B. cereus* isolated from soil did show a considerably smaller dependence on ammonia for rapid germination (Table 4). Taking the reproducibility of the stimulatory effect of ammonia to be reasonably well established by these results, it appears that the delayed recognition of this phenomenon in *B. cereus* might be attributed to a combination of simple oversight, strain variability and the absence of the requirement for ammonia for the germination of heat-activated spores.

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