Functional Relationships between L- and D-Alanine, Inosine and NH$_4$Cl during Germination of Spores of *Bacillus cereus* T

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(Received 1 March 1988; revised 27 June 1988)

The results of a physiological study of the interaction between NH$_4$Cl, inosine, and the stereoisomers of alanine during germination of spores of *Bacillus cereus* T are presented. Detailed kinetics for the germination of unheated spores in moderate concentrations of L-alanine (in the absence of auto-inhibition due to alanine racemase) are established, as is the specificity of the stimulatory effect of NH$_4$Cl in relation to other salts, amines, and germinants. The results suggest that NH$_4$Cl and inosine affect an early step in germination closely related to the function of an L-alanine receptor.

**INTRODUCTION**

L-Alanine has long been known to be a particularly potent germinant for spores of many species of *Bacillus* (Hills, 1949, 1950; Gould, 1969). In a previous report we established that NH$_4$Cl causes a 10-fold stimulation of the germination rate of unactivated spores (i.e. spores not subjected to the traditional heat-shock at sublethal temperatures prior to germination assays) of *B. cereus* T in a mixture containing L-alanine and either inosine (InA1) or adenosine (AdA1) (Preston & Douthit, 1984a). This stimulation is comparable in magnitude to that obtained with conventional heat-shock pretreatments. It differed from that of heat-shocked spores in being considerably more sensitive to inhibition by D-alanine. Consequently, it appeared that regulatory mechanisms not present in heat-shocked spores might be accessible for analysis if NH$_4$Cl were included as a co-germinant (Preston & Douthit, 1984a).

Germination kinetics in varying concentrations of L-alanine, inosine and NH$_4$Cl suggested that the function of NH$_4$Cl during germination might be more closely related to L-alanine than to inosine (Preston & Douthit, 1984a). However, since both inosine and L-alanine appeared to be essential for an appreciable germination rate, it was difficult to analyse functional relationships between these co-germinants. Subsequently, we discovered that inosine is not essential for germination of unactivated spores: such spores germinate readily in L-alanine alone, if precautions are taken to eliminate endogenous and exogenous sources of the germination inhibitor D-alanine (Preston & Douthit, 1984b). That finding greatly simplified the analysis of co-germinant functions. We now present the results of a physiological study of the interaction between NH$_4$Cl, inosine and the stereoisomers of alanine during germination of spores of *B. cereus* T. We establish detailed kinetics for the germination of unheated spores in moderate concentrations of L-alanine (in the absence of auto-inhibition due to alanine racemase), and we compare the effect of NH$_4$Cl with the effects of other salts, amines and germinants.

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Abbreviations: InAl, 1 mM-inosine, 1 mM-L-alanine, 100 mM-NaCl, 20 mM-Tris/HCl, pH 8.4; AdAl, as InAl but with inosine replaced by adenosine; DCS, d-cycloserine; MeNH$_2$, methylamine; HOEtNH$_2$, ethanolamine.

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METHODS

Strains, media and germination assays. Spores of B. cereus T were prepared and germination assays were done as previously described, with germination rate defined as the rate of loss of optical density (450 nm) of a suspension of spores (Preston & Douthit, 1984b). Where specified, alanine racemase in the spores was inhibited by either of two methods. Racemase was inhibited by including 5 mM-hydroxylamine in the germination assay medium or spores were treated with D-cycloserine (DCS) prior to germination assays to inactivate the racemase irreversibly. Treatment with DCS was for 15–20 min (Preston & Douthit, 1984b).

Triggering assay. The extent and rate of triggering by L-alanine were assayed in the following manner. Parallel germination reactions were initiated with 1 mM-L-alanine (see above), then the triggering reaction was quenched by adding 1 mM-D-alanine at specific times. The reactions were allowed to continue until all triggered spores had germinated, at which point the optical density of the spore suspension stabilized. The extent of triggering, or commitment, was then calculated as the fractional change in optical density of the quenched spore suspension relative to the fractional change in optical density typical of complete germination (0:52), expressed as a percentage. That is, the percentage triggered = [(initial OD450 − final OD450)/initial OD450 × 52] × 100. The rate of triggering was taken as the slope of the curve relating the extent of triggering to the times at which parallel reactions were quenched with D-alanine (see Fig. 1).

Reproducibility of germination results presented in this paper is not significantly different from that described by Preston & Douthit (1984a, b).

Materials. Reagents were as previously described (Preston & Douthit, 1984b). Stock solutions of L- and D-alanine were made in chromic-acid-washed glassware, stored on ice, and replaced after 1 week to avoid adventitious racemization (Preston & Douthit, 1984c).

RESULTS

Stimulation of the trigger function of L-alanine by NH4Cl

L-Alanine-induced germination is dependent on L-alanine only during the initial 'triggering' or 'commitment' phase of germination. Post-commitment reactions, such as loss of spore calcium and the decrease in optical density of spore suspensions, occur even in spores washed free of L-alanine and in the presence of the inhibitor D-alanine (Halmann & Keynan, 1962; Stewart et al., 1981). To determine whether the stimulatory effect of NH4Cl on L-alanine-induced germination is due to pre- or post-commitment reactions, we tested the ability of NH4Cl to stimulate the rate of triggering as defined by the rate of escape from sensitivity to inhibition by D-alanine (see Methods). The rate of triggering increased 3.6-fold when 40 mM-NH4Cl was included in the germination reaction medium (Fig. 1). Thus, the stimulatory function of NH4Cl is related to the triggering function of L-alanine.

Specificity of NH4Cl as co-germinant with L-alanine

Several experiments were done in an attempt to determine whether other compounds would be more effective germinants in the presence of NH4Cl. Since it appears that some amino acids are effective germinants only in the presence of inosine or other ribosides (Gould, 1969), we tested some of them for germinative activity per se and also in the presence of NH4Cl and/or inosine. Possible auto-inhibitory metabolism (akin to racemization of L-alanine) was minimized by doing these assays with very low spore concentrations. Racemase inhibitors were excluded from the assays to avoid non-specific inhibition of the germinative functions of the amino acids (Table 1). Of the amino acids tested, only L-alanine was germinative by itself or in the presence of NH4Cl without inosine. Discounting the relatively slight stimulations obtained by adding NH4Cl to most of the amino acid + inosine mixtures, it appeared that the stimulatory properties of NH4Cl were restricted to germination occurring in the presence of L-alanine, L-α-aminobutyrate, and apparently L-serine also. The inhibition of germination in inosine by NH4Cl (Table 1) was not reproducible from one spore preparation to the next. In fact, many preparations failed to germinate at all in inosine by itself (within a 30 min observation period), as was previously reported for this strain (Warren & Gould, 1968). In some, but not all, of the spore preparations that failed to germinate in inosine alone, the addition of NH4Cl actually stimulated germination at low rates (<1%/OD450 min⁻¹). We could not identify factors responsible for the variable response to inosine, and we did not establish the ionic specificity of
Germination of spores of Bacillus cereus T

Fig. 1. Effect of NH₄Cl on triggering during germination in L-alanine. Germination reaction mixtures (30°C, pH 8.4) contained 20 μg spores ml⁻¹, 20 mM-Tris, 1 mM-L-alanine, and 5 mM-hydroxylamine with (▲, △) or without (■, □) 40 mM-NH₄Cl. Reactions (2.5 ml) were started by adding 25 μl of spore suspension to the reaction mixture. With time, the germination was quenched with 25 μl 0.1 M-D-alanine. The fraction triggered (▲, ■) was calculated (see Methods) when the optical density of the suspension stabilized (20 min after starting the reaction). For comparison with triggering, the change in optical density of parallel reactions not quenched with D-alanine is also shown (△, □). Complete germination corresponded to a 52% decrease in the optical density of the suspension.

Table 1. Germinative activities of amino acids alone and in the presence of NH₄Cl and/or inosine

Reaction mixtures (30°C) contained 20 mM-Tris and 1.5 μg spores ml⁻¹ with the indicated additional components. Concentrations of amino acids and inosine were 1 mM; NH₄Cl was 40 mM. All mixtures were adjusted to pH 8.4 with NaOH or HCl. Germination rates reported in this Table for mixtures containing L-alanine are lower than those reported in Table 2 and Fig. 3 for two reasons. In part, the lower rates here reflect auto-inhibition due to L-alanine racemization, which is appreciable even at very low spore concentrations (Preston & Douthit, 1984b). In addition, the spore preparation used for the experiments of this Table responded to L-alanine somewhat more slowly than other preparations, for unknown reasons. In buffered, 1 mM-L-alanine, the germination rate of DCS-treated spores from this batch was 1.2%OD₄₅₀ min⁻¹, compared to the more typical 2.2%OD₄₅₀ min⁻¹ reported in Table 2.

<table>
<thead>
<tr>
<th>Basal germinant</th>
<th>None</th>
<th>NH₄Cl</th>
<th>Inosine</th>
<th>NH₄Cl + inosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>0.3</td>
<td>7.0</td>
<td>12</td>
<td>54</td>
</tr>
<tr>
<td>L-α-Aminobutyrate</td>
<td>0</td>
<td>0</td>
<td>5.5</td>
<td>26</td>
</tr>
<tr>
<td>L-Serine</td>
<td>0</td>
<td>0</td>
<td>1.7</td>
<td>16</td>
</tr>
<tr>
<td>Glycine</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>5.6</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
<td>4.1</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>6.6</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0</td>
<td>0</td>
<td>2.6</td>
<td>1.8</td>
</tr>
<tr>
<td>L-Valine</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* A value of zero indicates that no detectable germination occurred during the period of observation (at least 30 min).

the minor effects of NH₄Cl on inosine-induced germination. Warren & Gould (1968) suggested that slow germination in inosine might depend on an endogenous source of an amino acid. Alternatively, a reaction that normally requires an exogenous amino acid might be ‘leaky’ in certain spore preparations, such that the spores could germinate slowly in inosine without either endogenous or exogenous amino acids. Until the phenomenon can be studied reproducibly, it seems reasonable to consider the slow germination sometimes obtained in inosine as a pathology peculiar to specific spore preparations.
Table 2. Stimulatory effects of NH₄Cl and inosine on germination induced by L-alanine

Spores were pretreated with DCS to inactivate alanine racemase. Reaction mixtures (30 °C, pH 8.4) contained 1 μg spores ml⁻¹, 1 mM-L-alanine, 20 mM-Tris, and, where specified, 1 mM-inosine and/or 40 mM-NH₄Cl. These concentrations of co-germinants were rate-saturating for the high sensitivity response to L-alanine (see text).

<table>
<thead>
<tr>
<th>Germination rate in L-alanine (%OD₄₅₀ min⁻¹)*</th>
<th>Increase (fold) due to NH₄Cl*</th>
</tr>
</thead>
<tbody>
<tr>
<td>- NH₄Cl</td>
<td>+ NH₄Cl</td>
</tr>
<tr>
<td>- Inosine 2-2 (9%)</td>
<td>10 (20%)</td>
</tr>
<tr>
<td>+ Inosine 18 (11%)</td>
<td>68 (15%)</td>
</tr>
<tr>
<td>Increase due to inosine 8-2 (7%)</td>
<td>6-3 (8%)</td>
</tr>
<tr>
<td></td>
<td>7-5 (Average increase)</td>
</tr>
</tbody>
</table>

Observed and predicted rates with both NH₄Cl and inosine present:

Observed rate ................................................. 68%OD₄₅₀ min⁻¹

Additive calculated rate ........................................... 26%OD₄₅₀ min⁻¹
Rate calculated by additive combination of individual stimulations due to NH₄Cl and inosine. The two stimulatory factors for each co-germinant (in the presence of and absence of the other co-germinant) were not considered significantly different and were averaged for this calculation. Rate = 7.5 × (2-2%OD₄₅₀ min⁻¹) + 4-2 × (2-2%OD₄₅₀ min⁻¹).†

Multiplicative calculated rate ................................. 69
Rate calculated by multiplicative combination of individual stimulations due to both co-germinants. As in the additive case, an averaged stimulatory factor was used for each co-germinant. Rate = 2-2%OD₄₅₀ min⁻¹ × 7-5 × 4-2.

* Data are the means (with coefficients of variation in parentheses) for results obtained in four independent experiments involving two spore preparations grown and assayed at different times.
† An equivalent result is obtained by simple addition of the mean germination rates in L-alanine + inosine and L-alanine + NH₄Cl (10% OD₄₅₀ min⁻¹ + 18% OD₄₅₀ min⁻¹ = 28% OD₄₅₀ min⁻¹).

Previous studies established that, in the absence of inosine, heat-shocked spores of B. cereus T would germinate in a variety of amino acids other than L-alanine (Gould, 1969), and Krask (1961) described this strain’s response to L-cysteine in some detail. In our hands, heat-shocked spores (65 °C, 2 h) germinated in 10 mM-L-cysteine at a rate of 2-6%OD₄₅₀ min⁻¹, compared to a rate of 6-6%OD₄₅₀ min⁻¹ for the same spores in 1 mM-L-alanine. In L-alanine, the germination rate of heat-shocked spores was increased 140% by including 40 mM-NH₄Cl in the reaction mixture, but in L-cysteine, the rate of germination was inhibited 40% by either NH₄Cl or NaCl. Thus, NH₄Cl had no specific effect on germination initiated by L-cysteine, despite the fact that heat-shocked spores were capable of responding to L-cysteine, unlike unheated spores (Table 1).

Independence of the stimulatory effects of NH₄Cl and inosine

Since slow germination in inosine (observed with some spore preparations) was considered insignificant (see above), it appeared that inosine and NH₄Cl essentially were not germinative by themselves or together (Table 1). Inosine stimulated germination in L-alanine by the same amount in the presence or absence of NH₄Cl. Similarly, NH₄Cl caused a comparable relative rate increase in the presence or absence of inosine (Table 2). The stimulatory effect of a combination of inosine and NH₄Cl was equal to the product, rather than the sum, of the individual effects of these co-germinants (Table 2). Thus inosine and NH₄Cl were apparently functioning independently of each other as effectors of L-alanine-induced germination.

We emphasize that the results shown in Table 2 required the use of suitable inhibitors of alanine racemase. The production of traces of D-alanine by an active racemase would disproportionately inhibit slow germination reactions in L-alanine (in the absence of NH₄Cl or inosine) thereby obscuring the independence of the stimulatory effects of NH₄Cl and inosine. For example, with an active racemase the addition of NH₄Cl to L-alanine would provide a much greater stimulatory factor than obtained by adding NH₄Cl to a mixture of L-alanine and inosine. Similarly, inosine would provide very different stimulatory factors when added to L-alanine or L-alanine + NH₄Cl, if racemization were occurring. For this reason, the type of analysis...
Germination of spores of Bacillus cereus T

Fig. 2. Effects of salts and weak bases on germination rates in L-alanine. Spores were preincubated in DCS to inactivate alanine racemase. Germination reaction mixtures (30 °C, pH 8.4) contained 20 μg spores ml⁻¹, 2 mM-Tris, 1 mM-L-alanine, and the indicated concentrations of NH₄Cl (●), MeNH₂ (▲), HGeVNH₂ (▲), KCl (▲) or NaCl (▲). For the weak bases, the indicated concentration is the total concentration of both the ionized and non-ionized chemical species.

Table 3. Effect of monovalent cations on germination rates in L-alanine and L-alanine + inosine, with spores containing either active or inactive alanine racemase

<table>
<thead>
<tr>
<th>Salt added</th>
<th>L-Alanine</th>
<th>L-Alanine + inosine</th>
<th>L-Alanine + inosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inactive racemase*</td>
<td>Normal racemase†</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.62 (0)</td>
<td>19 (0)</td>
<td>13 (0)</td>
</tr>
<tr>
<td>Tris/HCl</td>
<td>2.3 (14)</td>
<td>23 (7)</td>
<td>12 (−2)</td>
</tr>
<tr>
<td>LiCl</td>
<td>7.7 (60)</td>
<td>20 (2)</td>
<td>8.1 (−8)</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.8 (10)</td>
<td>27 (14)</td>
<td>14 (2)</td>
</tr>
<tr>
<td>KCl</td>
<td>2.8 (19)</td>
<td>29 (18)</td>
<td>3.9 (−15)</td>
</tr>
<tr>
<td>RbCl</td>
<td>6.0 (46)</td>
<td>33 (25)</td>
<td>7.4 (−9)</td>
</tr>
<tr>
<td>CsCl</td>
<td>7.8 (61)</td>
<td>21 (4)</td>
<td>4.8 (−13)</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>12.4 (100)</td>
<td>76 (100)</td>
<td>74 (100)</td>
</tr>
</tbody>
</table>

* Alanine racemase was inactivated by pretreating spores with DCS as described in Methods.
† Normal racemase activity would severely inhibit germination in L-alanine in the absence of inosine, with the concentrations of spores used in these assays. Therefore, comparisons of the effects of monovalent cations were not attempted using L-alanine by itself as a germinant.

presented in Table 2 fails when applied to similar data in Table 1, where the germination rate in L-alanine is disproportionately inhibited by racemization.

Specificity of the stimulatory effect of NH₄Cl in relation to other monovalent cations

Previous work had indicated that the requirement for NH₄Cl is specific, compared to the effects of the alkali-metal cations, for stimulating germination of untreated spores in a mixture of 1 mM-L-alanine and 1 mM-inosine (InAl) (Preston & Douthit, 1984a). We re-evaluated the effects of the alkali metals on germination in InAl and in 1 mM-L-alanine to determine the extent to which non-specific ionic effects influenced the germination rates of spores in which alanine racemase had been inactivated. Contrary to the results obtained earlier with spores that had an
active racemase, both NaCl and KCl were moderately stimulatory to germination initiated by both L-alanine (Fig. 2, Table 3) and InAl. Neither salt was nearly as effective as NH₄Cl under these germination conditions, nor was Tris/HCl, used as standard assay buffer. Several other alkali metals were quite stimulatory; others were less so, or were inhibitory, depending on the nature of the germinants used and the activity of the alanine racemase in the spores (Table 3). All alkali cations except Na⁺ were inhibitory to germination in InAl using spores with an active racemase, as previously shown (Preston & Douthit, 1984a), while all cations were stimulatory for germination in L-alanine or InAl when spores with an inactive racemase were used. NH₄Cl was unique in stimulating InAl-induced germination of spores that had an active racemase.

Specificity of the stimulatory effect of NH₄Cl in relation to other weak bases

Previous comparison of NH₄Cl, methylamine (MeNH₂) and ethanolamine (HOEtNH₂) as stimulants for germination in InAl (Preston & Douthit, 1984a) revealed effects that included, in the case of MeNH₂, an optimum concentration for stimulation (16 mM). We re-evaluated MeNH₂ and HOEtNH₂ as potentially stimulatory analogues of NH₄Cl, using spores lacking racemase activity and therefore not subject to the non-specific ionic inhibitory effects observed above. As shown (Fig. 2), both MeNH₂ and HOEtNH₂ stimulated germination under these conditions, when present at concentrations up to 100 mM. Analysis of the MeNH₂ and HOEtNH₂ by HPLC ruled out the possibility that contamination by NH₃ was the cause of the stimulatory effect of these amines. These results suggest that apparent specificity of the stimulatory effect for NH₄Cl reported earlier (Preston & Douthit, 1984a) was an artifact caused by non-specific ionic inhibition at high concentrations of amines.

Kinetic analysis of NH₄Cl-stimulated germination

Preliminary experiments had shown that the kinetics of unactivated spore germination in L-alanine were complex, suggestive of high and low sensitivity germination systems. After eliminating endogenous production of the germination inhibitor D-alanine (Preston & Douthit, 1984b), we examined the kinetics of the response to L- and D-alanine in the absence of NH₄Cl, and in the presence of approximately Kₛ⁻ (5 mM) and saturating- (40 mM) concentrations of NH₄Cl.

High sensitivity system. Examination of the kinetics of germination rates at low concentrations of L-alanine revealed an apparent positive cooperativity in the response to L-alanine, as evident in the sigmoid shape of the plots of germination rates as a function of L-alanine concentration (Fig. 3). The apparent Kₛ for L-alanine in the absence of D-alanine was 50–100 μM, depending on the NH₄Cl concentration, and it was extremely sensitive to D-alanine, being roughly doubled by 5 μM D-alanine, regardless of the NH₄Cl concentration.

The sigmoid plots of Fig. 3 are suggestive of allosteric enzymology, as though D-alanine were a heterotropic inhibitor that shifts the curves toward higher L-alanine concentrations. Hill plots (data not shown) were linear at the lower concentrations of L-alanine, with Hill coefficients nearly independent of D-alanine concentration, being roughly 2 in the absence of NH₄Cl, and 2:3–3:6 in the presence of 5 or 40 mM NH₄Cl. Although D-alanine is a competitive inhibitor of L-alanine-induced germination in the sense that its inhibition was overcome by raising the concentration of L-alanine, it is not adequately described as a simple competitive inhibitor, given the complexity of the germinative response to both L- and D-alanine. When plotted in Lineweaver–Burk form (Fig. 4), the kinetics are approximately linear only in a very restricted range of L- and D-alanine concentrations. Over a wider range of concentrations, the curves display a concave-up curvature typical of positive co-operativity in response to L-alanine.

The curves from experiments done in the absence of D-alanine appear to be hyperbolic rather than sigmoidal. However, Hill plots of the data obtained in the absence of D-alanine (not shown) revealed Hill coefficients of 1·6 (0·99), 2·4 (0·98) and 3·2 (0·94) for reactions containing zero, 5 mM and 40 mM NH₄Cl, respectively (correlation coefficients). The curves obtained in the absence of D-alanine appear to be hyperbolic (despite the co-operativity implied by their Hill coefficients), probably for reasons of kinetics discussed elsewhere (Segel, 1975; cf. p. 365).
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Fig. 3. Effects of NH₄Cl and D-alanine on germination rates in low concentrations of L-alanine. Spores were preincubated in DCS to inactivate alanine racemase. Germination reaction mixtures (30 °C, pH 8.4) contained 20 μg spores ml⁻¹, 20 mM-Tris, the indicated concentration of L-alanine, without NH₄Cl (a), or supplemented with 5 mM-NH₄Cl (b) or 40 mM-NH₄Cl (c). The reaction mixtures also contained D-alanine as follows: none (○); 5 μM (▲); 10 μM (■); 20 μM (▼).

Fig. 4. Effect of D-alanine on germination rates in L-alanine. The data from Fig. 3(c) are shown here as a Lineweaver–Burk plot. The germination reaction mixtures contained D-alanine as follows: none (○); 5 μM (▲); 10 μM (■); 20 μM (▼). Inset: a similar plot, with expanded scale, showing the low affinity response at very high L-alanine concentrations (data from Fig. 3 and Fig. 5).

Low sensitivity system. Lineweaver–Burk plots of germination rates at various concentrations of L-alanine revealed a sharp break at high concentrations, indicative of a distinct low-sensitivity response to the germinant (Fig. 4, insert). As visualized in direct plots, the response of spores to high concentrations of L-alanine (Fig. 5, curves A, B and C) is characterized by a Kᵣ for L-alanine of about 50 mM (based on a visual estimate of the alanine concentration required for half-maximal germination rates), with both the Vₘₐₓ and Kᵣ being relatively insensitive to NH₄Cl. (Note that the change in the maximum germination rates evident in Fig. 5 at different NH₄Cl concentrations was due mainly to changes in rates near the ordinate that reflect an increasing high-sensitivity response; see above, Fig. 3.) In the presence of fixed low
Fig. 5. Effect of NH₄Cl and D-alanine on germination rates in high concentrations of L-alanine. Spores were preincubated in DCS to inactivate alanine racemase. Germination reaction mixtures (30 °C, pH 8.4) contained 20 μg spores ml⁻¹; 20 mM-Tris, the indicated concentration of L-alanine (A), and either 5 mM NH₄Cl (B), 40 mM-NH₄Cl (C), 40 mM-NH₄Cl + 1 mM-D-alanine (D) or 40 mM-NH₄Cl + 5 mM-D-alanine (E).

concentrations of D-alanine (Fig. 5, curves D and E) germination rates in the low-sensitivity region were severely inhibited, and there was a threshold concentration for L-alanine below which no detectable germination occurred within the period of observation (30 min).

DISCUSSION

Physiological analysis of bacterial spore germination has been hampered by uncritical use of heat-shocked spores and by uncontrolled racemization of L-alanine during germination assays. The results presented here reveal several important characteristics of germination observed using unheated spores, with alanine racemase activity eliminated by active site-directed, irreversible inhibition (Preston & Douthit, 1984b). Germination rates have a sigmoid dependence on L-alanine concentration (Fig. 3), consistent with long-standing hypotheses concerning the role of allosteric control during germination (Woese et al., 1968; Lewis, 1969). Demonstration of the extreme sensitivity of unheated spores to D-alanine (Figs 3–5) emphasizes the central roles of the two alanine isomers and alanine racemase as regulatory agents during germination. Our previous finding that heat-shock desensitizes spores to D-alanine (Preston & Douthit, 1984b), when combined with the co-operative kinetics of germination in response to L-alanine shown here (Figs 3 and 4) supports the view that heat-shock probably perturbs the normal functioning of germination mechanisms and should be used with circumspection.

The existence of two ranges of L-alanine concentration that give different kinetic responses (high and low sensitivity: Figs 3–5) has not been previously reported, most likely because the high sensitivity, sigmoid response normally would be masked by racemase activity in a typical spectrophotometric germination assay. The significance of the low sensitivity response is not clear. It may reflect an inherent kinetic property of the same L-alanine receptor that mediates the high sensitivity response, although other interpretations are possible. In view of the fact that this low sensitivity response also is susceptible to inhibition by palanine (and since 50–100 mM-L-alanine has been routinely used in many germination studies) the low sensitivity response should not be discounted without further study.

L-Alanine was the only amino acid (among ten tested) that caused germination of unheated spores and supported the co-germinative activity of NH₄Cl (Table 1). In the presence of inosine, the apparent $K_s$ for L-alanine is sufficiently low (3 μM: Warren & Gould, 1968; 9 μM: Preston & Douthit, 1984a) that cross-contamination of commercial amino acids becomes problematic for interpreting small stimulatory effects (e.g. see Hedblom & Adler, 1983). The rates observed using the ternary mixtures do set an upper limit for the effectiveness of the various amino acids in these mixtures, and in most cases (except with L-serine) this upper limit is considerably lower than the high rates observed with L-alanine and its analogue L-α-aminobutyrate.
Analysis of the stimulatory effect of NH₄Cl in relation to co-germinants revealed that it affects the early 'triggering' activity of L-alanine (Fig. 1), but not germination initiated by inosine or L-cysteine. Those results argue against a role for NH₄Cl during the later stages of germination that would be independent of specific initiating agents. Without knowledge of the identity or biochemical function of the L-alanine receptor, it is not yet possible to directly test the effect of NH₄Cl on that receptor.

The stimulatory effect of NH₄Cl is essentially independent of the absolute germination rate. Although addition of 1 mM-inosine to a reaction mixture containing 1 mM-L-alanine increased the germination rate by nearly an order of magnitude, NH₄Cl provided a similar stimulation in the presence and absence of inosine (Table 2). This relationship between the effects of NH₄Cl and inosine, both being essentially independent of each other (and so independent of the absolute germination rate), can be most simply interpreted as the result of separate functional modifications of a common rate-limiting process during germination. If so, then these results suggest that inosine stimulates the same L-alanine-dependent triggering reaction that has been shown to be stimulated by NH₄Cl (Fig. 1).

Salts and amines had an unexpected variety of effects on germination rates, depending on the level of activity of the endogenous alanine racemase (Table 3). It seems likely that the overall effects of amines and NH₄Cl are due to a combination of non-specific ionic and non-specific weak base effects. Beyond that, the magnitudes of the various effects are difficult to interpret with the current data. The unexpected relationship between alanine racemase activity and the effects of certain salts is an intriguing finding for which we have no explanation.

We emphasize, as has been pointed out by others (Stewart et al., 1981), that the change in optical density that accompanies bacterial spore germination is not the earliest observable event during germination, and that germination rates based on changes in optical density are not necessarily proportional to simple enzymic reaction rates. For these reasons, conclusions concerning early 'triggering' events based on the kinetics of absorbance changes are necessarily tentative. NH₄Cl stimulated the triggering function of L-alanine, one of the earliest detectable events during germination, and we assume that the increased germination rate in the presence of NH₄Cl is mainly a consequence of the increased triggering rate. However, NH₄Cl increased the triggering rate only 3.6-fold while providing a 5-fold increase in germination rate (Figs 1 and 2). At present, we cannot account for the slight discrepancy between these two effects.

This project was supported in part by BRSG S07-RR07050-18, awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health and by an Edwin H. Edwards Scholarship (R. A. P.). We thank A. S. Sussman, H. Ikuma and C. S. Yocum for continuing interest and support.

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