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Release of dipicolinic acid and calcium and activation of *Bacillus stearothermophilus* spores as a function of time, temperature and pH

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The kinetics of release of dipicolinic acid and calcium from *Bacillus stearothermophilus* spores and the rate of increase of colony count have been shown to be determined by conditions of time, temperature and pH. The apparent activation energies for release of dipicolinic acid, calcium and colony count increase were similar. The results support the hypothesis that breaking of dormancy involves a rupture of dipicolinic acid bonds and that the nature of these bonds rather than the dipicolinic acid content determines dormancy and resistance.

Spores of *Bacillus stearothermophilus* are specially dormant in the sense that less than 10% germinate and produce colonies on conventional media without prior heat treatment (Cook & Brown, 1964), but about 50% germinate and form colonies after optimum heat activation. Brown, Brown & Porter (1968) have shown that in the presence of 0·5 N HCl at 25°, the colony count of *B. stearothermophilus* spores increased to the value of the total (chamber) count and that dipicolinic acid (DPA) was progressively released. DPA is a characteristic component of bacterial spores (Powell, 1953) and spores with reduced contents of DPA germinate spontaneously after heat activation (Keynan, Murrell & Halvorson, 1962). The length of heat activation necessary for maximum germination was related to the DPA content (Keynan, Murrell & Halvorson, 1961). Keynan, Evenchik, & others (1964) and Keynan, Issahary-Brand & Evenchik (1965) found that low pH had effects on germination rates similar to those of heat activation, but did not attempt to correlate this with release of DPA. Lewis, Snell & Alderton (1965), using colony counts, reported activation of *B. stearothermophilus* at a pH of 1·5, but they found that this treatment (pH 1·5 for 80 min at 25°) did not remove DPA. The present work shows that the kinetics of DPA release and dormancy loss were determined by the conditions of time, temperature and pH. A brief report of part of this work has appeared previously (Brown & Melling, 1967).

MATERIALS AND METHODS

Preparation and counting of spore suspensions. The method of preparation and counting of aqueous spore suspensions of *Bacillus stearothermophilus* NCIB 8919 was as described by Cook & Brown (1964). To attain appropriate pH values for kinetic studies the following were used: Sorensen's citrate, Clark and Lubs KCl-HCl or HCl.

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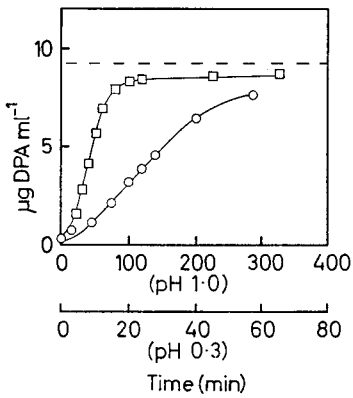

FIG. 1. Release of dipicolinic acid at 25° from *B. stearothermophilus* spores at pH 1·0 (○) and pH 0·3 (□). Results at pH 1·0 and pH 0·3 are on different time scales. Broken line = total DPA.

Assay of dipicolinic acid. DPA released from spores was measured polarographically using method I of Porter, Brown & Brown (1967).

Assay of calcium and magnesium. Calcium and magnesium were assayed using a Unicam SP 90 atomic absorption spectrophotometer.

RESULTS

Effect of pH and temperature on DPA release

Spores were suspended at various pH values at 25° and samples assayed at intervals for DPA. After an initial lag the release of DPA was linear with time, it then slowed down before maximum release (Fig. 1). Total DPA content was determined by assay of autoclaved spore aliquots (Porter & others 1967). The rate of release of DPA from spores at 25° decreased with increase in pH and the logarithm of the linear part of the curve (log k) was linear with pH (Fig. 2A). At pH 2 it was necessary to make measurements over about 4 days to determine the rate of DPA release at 25°. It can be

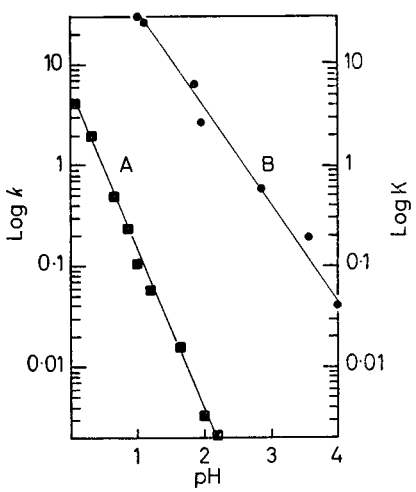

FIG. 2. Effect of pH on rates of release of dipicolinic acid (curve A, ■) and increase in colony count (curve B, ●) in *B. stearothermophilus* spores at 25°.

predicted from Fig. 2A that a comparable release of DPA at pH 4 would take about 12 years at 25°, the predicted k being $2{\cdot}5 \times 10^{-6}$ μg DPA ml^{-1} min^{-1}. For this reason rates of release of DPA from spores at pH 4 were measured using temperatures of 100, 95, 85 and 80° in a further attempt to predict rates of release at temperatures around 25° (Fig. 3). An Arrhenius plot of log k versus 1/T°K was linear and corresponded to an apparent activation energy of about 46 kcal (190 kJ) (Fig. 4A). Extrapolation of this line gave a value of k, corresponding to 25°, of $2{\cdot}5 \times 10^{-6}$ μg DPA ml^{-1} min^{-1}. This coincided with the value predicted by the plot of log k versus pH.

Effect of pH and temperature on increase in colony count

Colony counts were made of spore suspensions at 25° in buffers at various pH values. The spore suspensions were replicates of those used for DPA studies except that nutrient broth (Oxoid, half strength) was present. Preliminary experiments had shown that the release of DPA was unaffected by the presence of nutrient broth in the buffer, whereas colony counts were drastically reduced in the absence of broth from the system (Fig. 5A). Without this protective effect of broth we were not able to obtain quantitative results for colony counts at low pH. Suspensions at the various pH values were instantly quenched before plating by using nutrient broth as a counting diluent. Colony counts increased linearly with time in the presence of broth at each of several pH values at 25° before tailing off to a maximum (Fig. 5). No lag was observed. The rate of increase of count increased with decreased pH, although the maximum count was reduced at the lower extremes of pH due to a killing effect superimposed on activation. The presence of broth always protected against this killing effect. The logarithm of the initial rate of increase in count (log K) was linear with pH (Fig. 2B). Change of pH had a greater effect on rate of loss of DPA than upon rate of loss of dormancy (Fig. 2). The slope of unity for Fig. 2B indicated that the rate-limiting step for dormancy loss was hydrogen ion catalysed.

As a comparison with the DPA study, the initial rate of increase of count (K) was determined at pH 4 using several elevated temperatures and was found to increase with rise in temperature (Fig. 5B). An Arrhenius plot of log K versus I/T°K was linear above a value of I/T°K corresponding to about 50° (Figure 4B). Below this temperature log K was constant. Clearly the contribution of the test conditions to activation at this temperature was not significantly greater than the activation due to the time, temperature and pH conditions at which the spores were subsequently incubated on agar plates. The slope of the Arrhenius plot above 50° corresponded to the same apparent activation energy of 46 kcal (190 kJ) as for release of DPA (Fig. 4A).

Kinetics of cation release

The kinetics of Ca^{2+} and Mg^{2+} release, in addition to DPA and dormancy loss, were studied using a spore suspension prepared as described except that growth temperature was 53°. The release of Ca^{2+} at different temperatures followed kinetics identical to those for DPA (Fig. 6). The apparent activation energy of 32 kcal (134 kJ) for Ca^{2+} release was identical to that for DPA release and close to the 35 kcal for dormancy loss measured for this second spore suspension. The kinetics of Mg^{2+} loss was different with a rapid initial release which was temperature dependent. This

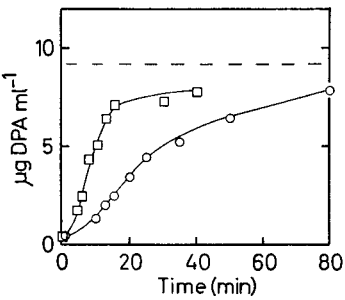

FIG. 3. Release of dipicolinic acid from *B. stearothermophilus* spores at pH 4·0 at 90° (○) and 95° (□). Broken line = total DPA.

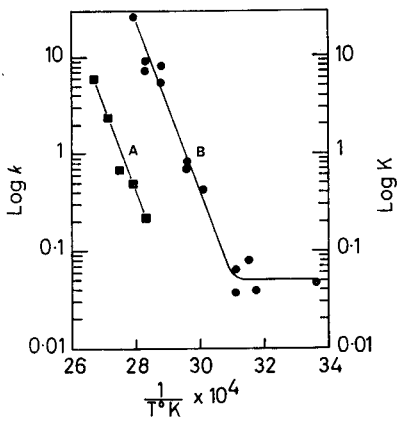

FIG. 4. Arrhenius plot of rates of release of dipicolinic acid (curve A, ■) and rates of increase of colony count (curve B, ●) in *B. stearothermophilus* spores at pH 4·0.

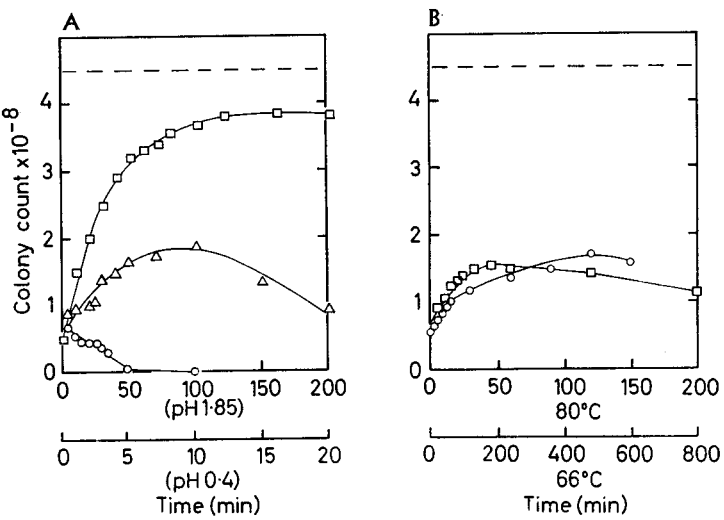

FIG. 5. A. Colony count of *B. stearothermophilus* spores suspended at 25° for various times in nutrient broth at pH 1·85 (□), and pH 0·4 (△) and at pH 0·4 without nutrient broth (○). Results at pH 1·85 and pH 0·4 are plotted on different time scales.

B. Colony counts of *B. stearothermophilus* spores in nutrient broth at pH 4·0 after heating at 80° (□) and 66° (○) for various times. Results at 80 and 66° are plotted on different time scales. Broken line = total (chamber) count.

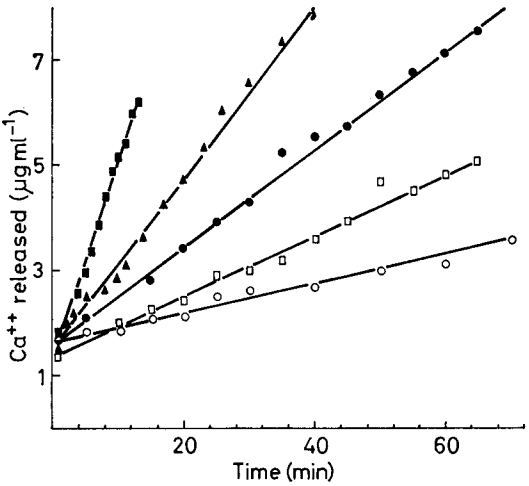

FIG. 6. Effect of temperature on release of calcium from *B. stearothermophilus* spores at pH 2:70° (■), 65° (▲), 60° (●), 55° (□), 50° (○).

rapidity together with the limitations of sensitivity of the Mg^{2+} assay precluded a quantitative kinetic study for Mg^{2+} loss. The difference in activation energies (32/35 kcal compared to 46 kcal) between the two suspensions may possibly be attributed to the difference in growth temperature together with the inherent variability of spore batches grown on agar. Nevertheless it is significant that for each batch the energetics for loss of dormancy mirrored that for DPA release.

DISCUSSION

Our results show that the kinetics of release of DPA and calcium and of loss of dormancy are predictable from the conditions of time, temperature and pH (Figs 2, 4, and 6). The similarity between the apparent activation energies for dormancy loss and release of DPA and Ca^{2+} indicates that these processes may be closely related. Lewis & others (1965) reported that treatment of *B. stearothermophilus* spores at pH 1·5 for 80 min at 25° did not remove DPA. Our data, for suspensions used in this work, would predict a release at 25° of 0·024 μg DPA ml^{-1} min^{-1} and detection would depend on the actual concentration. The kinetics of increase of colony count are no less predictable, but the interpretation is complicated by the contribution to the overall conditions of the time, temperature and pH of incubation (Figs 2B and 4B). This may explain the different pH relation for DPA release and colony count increase Fig. 2). Since it is not possible in aqueous systems to avoid conditions of time, temperature and pH, the terms "heat shock" and "heat activation" are misleading. The colony count data support the results of Busta & Ordal (1964) who reported that activation for colony formation was occurring throughout the range of test temperatures.

A DPA-protein complex has been suggested as a possible protective mechanism in spore resistance (Powell, 1957; Sussman & Halvorson, 1966). Mishiro & Ochi (1966) have shown protein stabilization *in vitro* by DPA. Sadoff (1969) has reported reversible *in vitro* heat stabilization of spore proteins by modification of the ionic environment.

Much published work has attempted to relate spore DPA content and resistance. Murrell (1969) concluded that the spore DPA content was not the determining factor in the degree of heat resistance. Our proposal (Brown & Melling, 1967) that the nature of the DPA bonds rather than the amount of DPA was the determining factor for dormancy and resistance has received further support by Grecz & Tang (1970) and Grecz, Tang & Rajan (1972). These workers studied the stability of Ca^{2+}-DPA complexes in relation to pH and temperature. They obtained evidence supporting the idea that spore polymers are stabilized by the binding of Ca^{2+}-DPA chelates. It is significant that mutants of *Bacillus cereus* T produce spores devoid of DPA and that these germinated spontaneously in cultures if not harvested (Halvorson & Swanson, 1969). Washed DPA—less spores on the other hand are more dormant than the wild type, although less heat resistant. The mechanism of dormancy with washed spores of DPA-less mutants is unknown.

The identical apparent activation energies for DPA release and dormancy loss are consistent with our original hypothesis that breaking of dormancy involves essentially a rupture of DPA bonds as a function of time, temperature and pH (Brown & Melling, 1967).

In conclusion we quote the 'working hypothesis' of Powell (1957) that the resting spore is a highly condensed waterproofed structure stabilized by the incorporation of calcium dipicolinate.

Acknowledgements

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