REVERSIBLE ACTIVATION FOR GERMINATION AND SUBSEQUENT CHANGES IN BACTERIAL SPORES[1]

W. H. LEE[2] AND Z. JOHN ORDAL

Department of Food Technology, University of Illinois, Urbana, Illinois

Received for publication 15 August 1962

ABSTRACT

LEE, W. H. (University of Illinois, Urbana) AND Z. JOHN ORDAL. Reversible activation for germination and subsequent changes in bacterial spores. J. Bacteriol. **85**:207–217. 1963.—It was possible to isolate refractile spores of *Bacillus megaterium*, from a calcium dipicolinate germination solution, that were activated and would germinate spontaneously in distilled water. Some of the characteristics of the initial phases of bacterial spore germination were determined by studying these unstable activated spores. Activated spores of *B. megaterium* were resistant to stains and possessed a heat resistance intermediate between that of dormant and of germinated spores. The spontaneous germination of activated spores was inhibited by copper, iron, silver, or mercury salts, saturated *o*-phenanthroline, or solutions having a low pH value, but not by many common inhibitors. These inhibitions could be partially or completely reversed by the addition of sodium dipicolinate. The activated spores could be deactivated and made similar to dormant spores by treatment with acid. Analyses of the exudates from the variously treated spore suspensions revealed that whatever inhibited the germination of activated spores also inhibited the release of spore material. The composition of the germination exudates was different than that of extracts of dormant spores. Although heavy suspensions of activated spores gradually became swollen and dark when suspended in solutions of *o*-phenanthroline or at pH 4, the materials released resembled those found in extracts of dormant spores rather than those of normal germination exudates.

Since the demonstration by Powell (1953) that bacterial spores contained dipicolinic acid (DPA), there has been much interest in its biological role. Several investigators have sought a correlation between the heat resistance of bacterial spores and their DPA content (Church and Halvorson, 1959; Black et al., 1960*a*; Pelcher, 1961). Doi and Halvorson (1961) presented evidence that DPA could be involved in an electron-transport system. Riemann and Ordal (1961) reported that calcium dipicolinate (CaDPA) caused the germination of spores of many species of the genera *Bacillus* and *Clostridium*. This investigation further characterizes the role of CaDPA in the germination of bacterial spores.

In many bacterial spore-germination systems, the changes occur so rapidly that they are considered simultaneous and irreversible. Little is known about the initial reaction or phases involved when a refractile dormant spore is changed to a germinated spore. One difficulty in studying the early phases of germination has been to find a suitable procedure to delay and to separate these changes. Riemann and Ordal (1961) demonstrated that the rate of germination was decreased by a reduction in temperature. They (Riemann and Ordal, *unpublished data*) also demonstrated that spores of Putrefactive Anaerobe 3679, exposed to CaDPA or ethylenediaminetetraacetic acid (EDTA) at low temperatures, were refractile but would germinate spontaneously when the spores were separated from the CaDPA or EDTA solutions and resuspended in distilled water.

This report describes the preparation of activated refractile spore suspensions of *Bacillus megaterium*. The suspensions were prepared by treating them in a solution of CaDPA at temperatures of 7 to 10 C. For convenience, these unstable spores, which will germinate in distilled water, are called activated spores. Likewise, CaDPA-activated spores stabilized by acid treatment are referred to as deactivated spores

[1] From a thesis submitted to the Department of Food Technology, in partial fulfillment of the requirements for the Ph.D. degree, University of Illinois, Urbana.

[2] Present address: Merck and Co., Danville, Pa.

to distinguish them from dormant spores. Some of the germinal changes which occurred in spore suspensions receiving various treatments are reported and discussed. In particular, the changes in thermal resistance and the release of DPA, amino acids, and peptides are correlated with other criteria of germination.

Materials and Methods

Spore preparation. B. megaterium (ATCC 13632) was cultured on nutrient agar that contained 0.05% $MnSO_4$ and 0.1% $CaCl_2$ and was made up with tap water. After 2 days of incubation at 30 C, the spore suspension was washed and freed of vegetative cells and debris by the method of Long and Williams (1958). The cleaned spores were stored in distilled water at 3 C. Spores stored in water at 3 C for 3 months or more remained refractile but responded more slowly to CaDPA activation than did fresh spores. Because of this change, new spore crops were grown at least every 2 months.

Criteria for germination. The germination of activated spores was indicated by an increase in the per cent transmission of the spore suspension at 650 mμ in a Coleman Junior spectrophotometer (model 6A). Spore refractility was determined microscopically by use of the agar slide method (Riemann, 1961).

Preparation of the spore exudates and extracts. Exudates for analysis were collected from treated spore suspensions (5 ml of 5×10^9 spores/ml or 67 mg of spores) after the spores had been exposed to the given conditions for 24 hr. The spore solids were separated from the exudate solution by centrifugation at 16,000 × g for 10 min. The spore solids were rinsed twice with 2 ml of water, and the rinsings were added to the supernatant solution. Extracts of dormant spores were obtained by boiling them in water for 90 min or by disruption in 50% ethanol with a Mickle disintegrator and Ballantoni glass beads in the room at 3 C. All samples were stored at −20 C until analyzed.

Paper chromatography of amino acids and DPA. Spore exudates were concentrated and applied to Whatman no. 1 paper for chromatography. The chromatograph was developed overnight in an ascending system by using *n*-butanol, ethanol, water, HCl (80:20:20:2; v/v) at 24 C. The chromatogram was air-dried and the residual HCl removed by steaming in an autoclave for 1 to 2 min. Amino acids were detected by spraying with 0.2% ninhydrin in *n*-butanol. After the amino acid color had formed at 25 C, the same chromatogram was sprayed with 0.5% thiourea, $Fe(NH_4)_2(SO_4)_2$, and acetic acid solution for the detection of DPA. The FeDPA complex color could be further intensified by spraying the chromatogram with 4% collidine in acetone.

Estimation of free amino acids by paper electrophoresis and scanning. Free amino acids were separated by high-voltage paper electrophoresis by using the method of Michl (1959), modified in two respects. First, instead of using cellophane membrane tabs to prevent oversaturation, two rows of 16 holes (6-mm diam) were punched in the paper 3 in. from each end. Second, the paper was cooled in a chlorobenzene bath in a room at 10 C, instead of with cooled plates. Samples were applied in 1-in. bands, 6 in. from the cathode, on Whatman no. 1 paper (5.5 by 22 in.) The solvent used was 12% acetic and 2.65% formic acid (v/v) in water. Separation was complete after 90 min at 0.5 ma per cm width and 30 v per cm, supplied by a Beckman Spinco Constat power unit. The amino acids were developed by dipping the strips in 0.2% ninhydrin in *n*-butanol. The density of both standard and unknown amino acids was determined by using a Beckman Spinco Analytrol equipped with a B-5 cam at 500 mμ.

Paper electrophoresis of spore protein and peptide and their dinitrophenyl derivatives. The protein and peptide components of spore germination exudates from 1 to 5 mg of spores were separated and characterized by the paper electrophoresis procedure of Fynn and De Mayo (1951) which was used by Strange and Powell (1954) for spore-peptide identification. A better resolution of the peptide was obtained when the paper electrophoresis was performed at 20 ma and 500 to 600 v per cell for 1 hr at 25 C instead of 120 v for 20 hr. The equipment used consisted of a Beckman Spinco Constat power unit, a Durrum Cell, B-2 buffer (pH 8.6 barbital, 0.075 ionic strength), and Schleicher & Schuell 2043 A paper strips (Block, Durrum, and Zweig, 1958). After development, the paper strips were dried in an oven (100 C) for 10 min and then stained overnight in a saturated solution (1:200 dilution) of Buffalo Black NBR (National Aniline) or Amidoschwartz 10 B in 10% acetic acid methanolic solution. The strips were then rinsed in fresh 10% acetic acid metha-

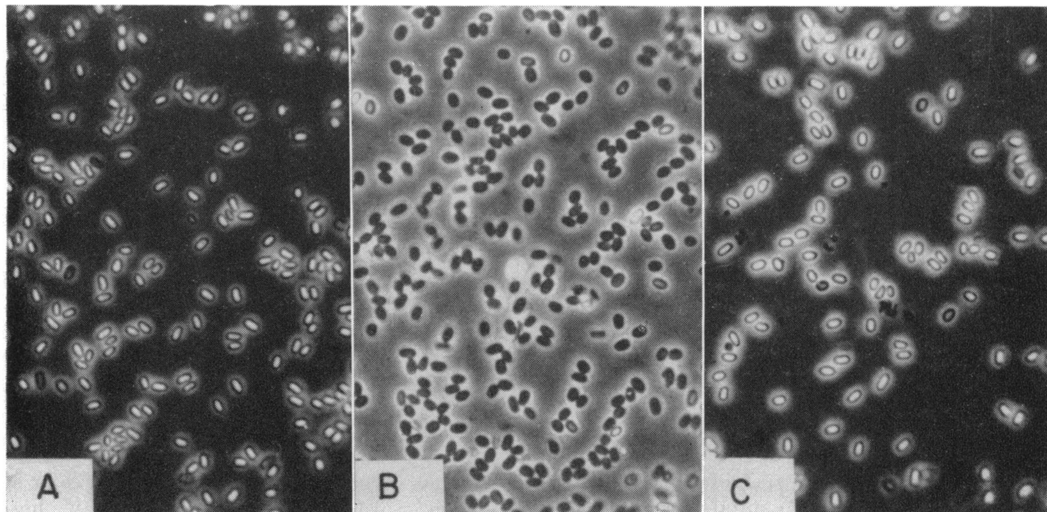

FIG. 1. *Dark-phase contrast photomicrographs of activated, germinated, and deactivated spores of Bacillus megaterium.* A = *spores immediately after activation and washing.* B = *germinated spores. Spores were held in distilled water at 24 C for 1 day following activation and washing.* C = *deactivated spores. Washed activated spores were suspended in 12 mM H_3PO_4 for 1 hr at 3 C, washed in 10 mM phosphate buffer (pH 7), and resuspended in 10 mM $MgSO_4$. Photo taken after 20 days at 3 C.*

nolic solution for 0.5 hr and air-dried. In some experiments, the exudates were also reacted with fluorodinitrobenzene to make the yellow dinitrophenyl derivatives by the method of Levy (1954), and the samples were dialyzed with four to five changes of distilled water in a 24-hr period. The dialyzed dinitrophenyl derivatives of protein and peptide were separated by the electrophoresis method described above.

Analytical methods. Ammonia was determined by the microdiffusion method of Conway (1950). Reducing sugars were determined by the method of Somogyi (1945) before and after hydrolysis with 6 N HCl at 106 C for 3 hr. The ninhydrin-positive materials were determined by the colorimetric method of Moore and Stein (1948). The free amino acids of two samples were quantitatively determined in a Spinco Model 120 amino acid analyzer patterned after the method of Spackman, Stein, and Moore (1958).

RESULTS

Activation of spores. Spore activation was accomplished by placing dormant spores in a solution of 40 mM CaDPA (pH 7.0). The necessary conditions (time and temperature) had to be established for each spore crop. The first three spore crops were activated after 70 min at 10 C, whereas two later crops were activated after 70 min at 7 C. The activation solution was prepared immediately before use by mixing solutions of $CaCl_2$ and NaDPA to yield the desired concentration in 1.6 mM tris(hydroxymethyl)aminomethane (tris) buffer (Riemann and Ordal, 1961). Activation was stopped by adding 2 to 3 volumes of crushed ice and water, and the preparation was immediately centrifuged. The spore pellet was resuspended in crushed ice and water, recentrifuged, and finally resuspended in cold distilled water. Following activation, the spores were refractile (Fig. 1A) and were resistant to staining by crystal violet. The activated spores would germinate (Fig. 1B) rapidly in water at ambient temperature but more slowly if the spore suspension was kept in an ice bath. For this reason, the spores were studied immediately after activation. Figure 2 is a schematic outline of the activation procedure and subsequent treatments used to characterize the activated spores. Included also is the percentage of refractile spores in the variously treated preparations.

The properties of an activated spore suspension depended on the extent of activation. Spores activated at a higher temperature or for a longer period of time germinated more rapidly than

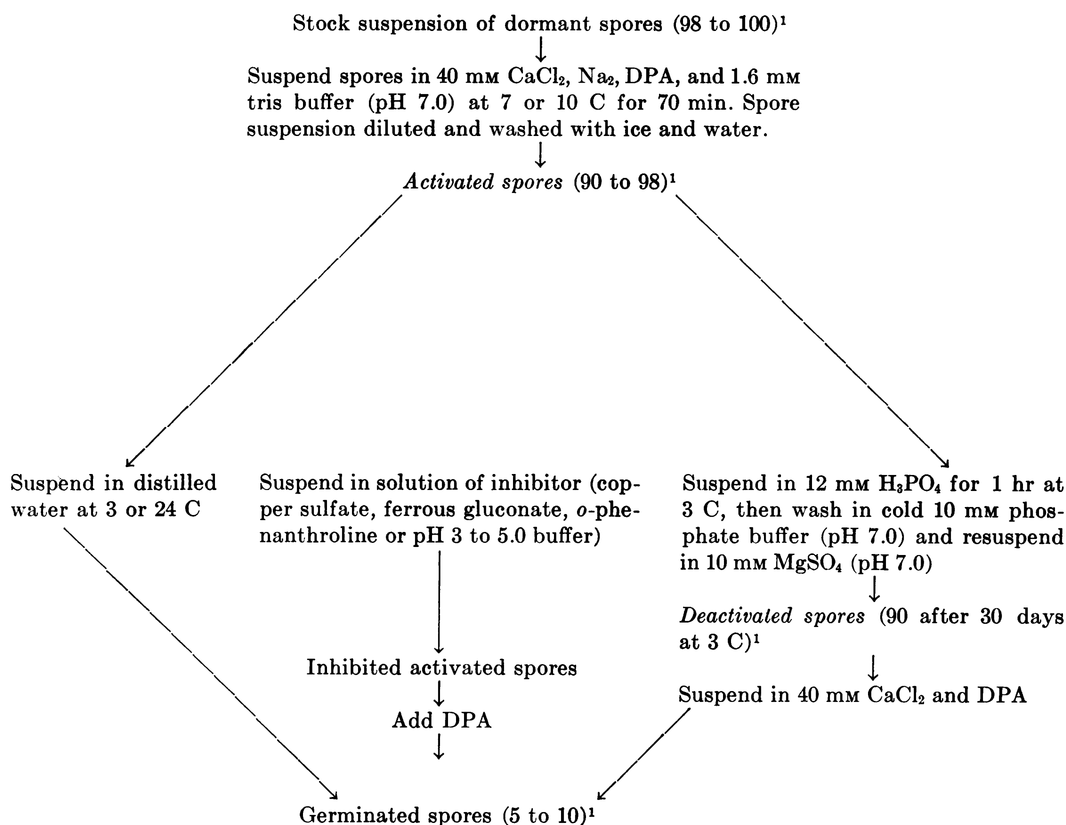

FIG. 2. *General schema for the production and characterization of activated and deactivated spores.*
[1] Percent refractile spores in suspension after indicated treatment.

those receiving a reduced activation treatment. It was also noted that the increase in the percentage of light transmission of some partially inhibited activated-spore suspensions was due to a uniform graying or reduction in refractility rather than to more complete germination of some spores in the suspension.

Cation inhibition of the germination of activated spores. Murty and Halvorson (1957) reported that various cations inhibited the germination of dormant spores of *B. cereus*, and that the inhibitions could be reversed by chelating agents. Germinal changes of activated spores were consistently inhibited by the addition of cation salts of Cu^{++}, Fe^{++}, Ag^{+}, and Hg^{++} in concentrations of 1 to 5 mM at neutral pH (pH 6.8 to 7.0). As Ag^{+} and Hg^{++} were toxic and did not permit subsequent outgrowth, they were not extensively investigated. Figure 3 demonstrates the effect of Cu^{++} and Fe^{++} on the germination rate of activated spores. The activated spore control was over 90% germinated after 90 min, whereas 1 mM $CuSO_4$ completely inhibited the germination of all preparations tested for at least 24 hr. Results with ferrous gluconate were variable. It only partially inhibited the activated spore preparation used to provide the data for Fig. 3, but completely inhibited germination of other activated spore preparations. When these cation-inhibited spore suspensions were examined after several days at 24 C, it was noted that the spore coats of such spores began to show signs of disintegration. The inhibition by copper was partially counteracted by the addition of 50 mM DPA but not by 50 mM EDTA. The inhibition by ferrous ion was counteracted by DPA or EDTA. The addition of 10 mM L-alanine had no demonstrable effect in counteracting these inhibitions. Cation salts which were tested but did not inhibit the germination of activated spores at pH 7 were 5 mM $CaCl_2$, $MnCl_2$, $NiSO_4$, and $CoCl_2$, and 10 mM $MgSO_4$.

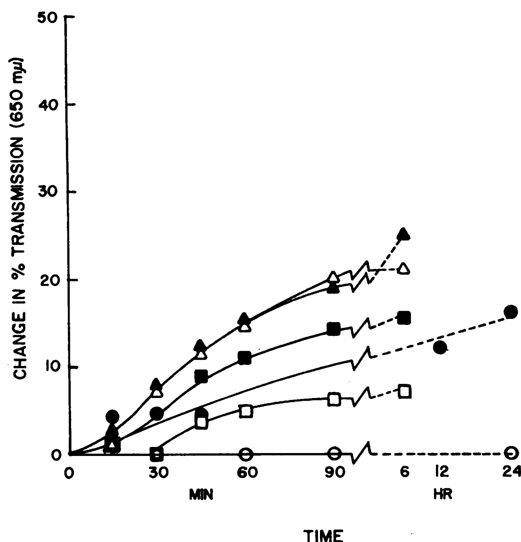

FIG. 3. *Inhibitory effect of copper and ferrous ion solutions on the germination of activated spores of Bacillus megaterium and its reversal by DPA or EDTA. All solutions in 50 mM acetate buffer (pH 6.8) at 24 C. Ordinate: scale units increase in % transmission. All suspensions adjusted to 50% transmission at zero time.* △, *control*; ○, *1 mM* $CuSO_4$; ●, *1 mM* $CuSO_4$ *plus 10 mM DPA;* □, *1 mM ferrous gluconate;* ■, *1 mM ferrous gluconate plus 10 mM DPA;* ▲, *1 mM ferrous gluconate plus 10 mM EDTA.*

Inhibition of the germination of activated spores by o-phenanthroline. Although numerous compounds have been reported to inhibit the germination of dormant spores, of the many compounds tested only a saturated solution of *o*-phenanthroline (300 ppm) inhibited the germination of activated spores (Fig. 4). *o*-Phenanthroline is a strong chelating agent and has been used to bind cations in the study of the relationship of metals to enzymatic activity (Valle, 1960). Surprisingly, the *o*-phenanthroline inhibition could be counteracted by the addition of 10 mM DPA or 25 mM phosphate buffer at pH 7. The inhibitory effect of *o*-phenanthroline was variable for different spore harvests and occasionally only lasted for about 6 to 8 hr. Heavy spore suspensions of 5×10^9 spores/ml also counteracted the inhibitory effect of *o*-phenanthroline. Inhibitors that failed to inhibit the germination of activated spores at pH 7 were: 5 mM D-alanine, NaCN, NaF, $NaAsO_3$, $NaNO_2$, atebrine, dipyridyl, 2,4-dinitrophenol, and pyridine, and 10 mM NaN_3, NH_2OH, iodoacetate, and ethyloxamate.

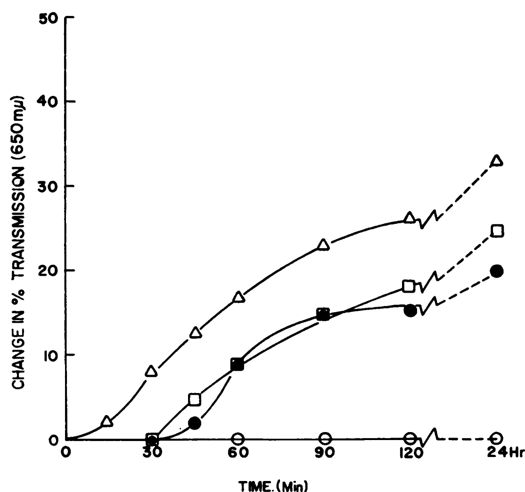

FIG. 4. *Inhibitory effect of o-phenanthroline on the germination of activated spores of Bacillus megaterium and its reversal by DPA or phosphate. Temperature, 24 C. Ordinate same as in Fig. 3.* △, *control activated spores in distilled water;* ○, *saturated solution of o-phenanthroline;* ●, *saturated solution of o-phenanthroline in 50 mM phosphate buffer (pH 7);* □, *saturated solution of o-phenanthroline plus 10 mM DPA.*

Inhibition of the germination of activated spores by low pH. With many dormant spores, germination does not occur at pH 4 or below (Vas and Proszt, 1957). Figure 5 demonstrates that germination of an activated spore suspension was related to pH. At pH 7.0, over 90% of the activated spores were germinated after 120 min, at pH 5.0 there was some darkening, and at pH 3.5 the spores were still refractile. Inhibition due to low pH was partially counteracted by the addition of 10 mM DPA. The response of activated spores to inhibition by low pH was dependent on the degree of the CaDPA activation treatment. For example, when a sample of the same crop of dormant spores was activated at 7.6 C for 70 min instead of at 7 C as in Fig. 5, the spores activated at the higher temperature germinated at pH 5 but not at pH 4. Heavy spore suspensions (5×10^9 spores/ml) were less affected at pH 4 and gradually darkened (germinated).

Stabilization of activated spores, deactivation. Activation was reversible in the sense that both spontaneous germination and further germinal changes could be arrested and the activated spores stabilized. Deactivation was discovered

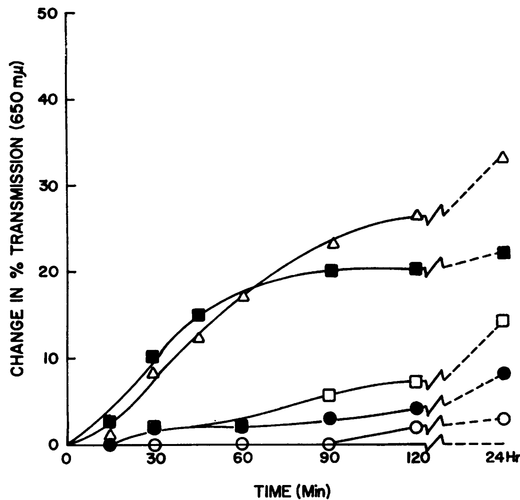

FIG. 5. *Inhibitory effects of acetate buffers (H+) at low pH on the germination of activated spores of Bacillus megaterium and its partial reversal by DPA. Ordinate same as in Fig. 3.* △, *control spores in water or acetate buffer (pH 6.8 to 7.0);* ○, *50 mM acetate buffer (pH 3.5);* ●, *50 mM acetate buffer (pH 3.5) plus 10 mM DPA;* □, *50 mM acetate buffer (pH 5.0);* ■, *50 mM acetate buffer (pH 5.0) plus 10 mM DPA.*

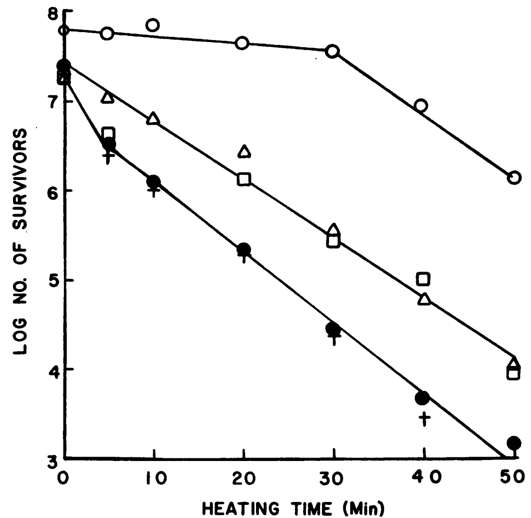

FIG. 6. *Survival at 84 C of dormant, activated, and deactivated spores of Bacillus megaterium in 25 mM barbital buffer (pH 7).* ○, *dormant spores;* △, *activated spores;* □, *deactivated spores;* ●, *deactivated spores stored in 50 mM $MgSO_4$;* +, *deactivated spores stored in 10 mM $CuSO_4$.*

when an activated spore suspension was suspended in 8 mM acetic acid for 1 day. These spores retained their refractility even after the acetic acid was removed and they were resuspended in neutral buffer. Additional study demonstrated that various acid treatments served to deactivate an activated spore suspension. The following procedure was adopted to produce deactivated spores for further study. Freshly activated spores were immediately suspended in 0.1% H_3PO_4 for 1 hr at 3 C. The acid was removed by centrifugation, and the deactivated spores were washed with 10 mM phosphate buffer (pH 7) at 3 C. The deactivated spores were then resuspended in 10 mM $MgSO_4$ (pH 7), and stored at 3 C. Deactivated spores were stored in $MgSO_4$ solution because a comparative study indicated that the spores retained their refractility better in 10 mM $CaCl_2$, $MgSO_4$, or $MnCl_2$ than in 10 mM $CuSO_4$, $CoCl_2$, $ZnCl_2$, ferrous gluconate, or water at pH 7. Figure 1C is a photomicrograph of a deactivated spore suspension that had been stored for 20 days at 3 C. The control activated spore suspension was 87% germinated after a storage period of 1 day at 3 C. Some DPA was lost during activation and deactivation. In one test, the control (dormant) spores contained 9.0% DPA, whereas after deactivation comparable spores contained 8.1% DPA.

Heat resistance of treated spores. The effect of the several treatments on the thermal resistance of the treated spores is presented in Fig. 6. These survivor curves indicate that the various treatments only slightly affected the thermal resistance of the treated spores. Although there was a small loss in thermal resistance during activation and some additional loss during storage, all preparations had thermal-resistance properties characteristic of dormant spores rather than of germinated spores or vegetative cells. The survivor curves for deactivated spores treated in 10 mM $CaCl_2$, $CoCl_2$, $MnSO_4$, or $ZnCl_2$ were similar to those of the deactivated spores treated with $CuSO_4$ or $MgSO_4$. The various treatments had no effect on the viability of the spores, as the zero-heating-time counts on comparable samples were almost the same.

Germination requirements of deactivated spores. The germination requirements for deactivated and dormant spores were similar. Both types germinated in a 40 mM solution of $CaCl_2$ and DPA, but did not when the concentration was reduced to 20 mM. It was, therefore, possible to start the germination process by the CaDPA activation at low temperature, stop it by the

TABLE 1. *Estimation* of free amino acids released by activated† and dormant spores*

Condition	Glutamate group‡	Leucine group	Alanine	Glycine	Arginine, histidine	Lysine
Activated spores suspended in						
Water at 24 C	10	10	5	4	5	6
Acetate, 50 mM (pH 3)	1	0	0.1	0	1	0
Acetate, 50 mM (pH 4)	1	0	0	0.5	2	0
$CuSO_4$, 9 mM (pH 6.8)	2	0	0	0	0	0
Saturated *o*-phenanthroline	7	0.1	0	0	1.5	0.5
Water at 3 C	5	1.5	1.5	2	3	0.5
Deactivated spores (supernatant solution)	0	0	±	0	±	±
Activation solution (40 mM CaDPA)	±	0	0	0	0	0
Deactivation solution (12 mM H_3PO_4)	0	0	0	0	0	0
Dormant spores (extract)						
Boiled in water for 90 min	7	0	0	0	3	0
Broken in 50% ethanol	5	0	0	0	3	0.5
Stored in water at 3 C for 40 days, then boiled in water	5	3	1.5	1.5	5	1.5

* Based on paper electrophoresis of solutions; see text.

† Spores activated at 7.6 C for 70 min and the exudates collected after 24 hr at 24 C, unless otherwise indicated.

‡ Values in table expressed as mmoles of amino acid per 100 g of dry spores.

acid deactivation treatment, and start germination again by a second exposure to CaDPA. The deactivated spores were like dormant spores in that 10 mM L-alanine, glucose, or adenosine, 20 mM CaDPA, or 40 mM NaDPA or EDTA did not stimulate germination even after 24 hr at 24 C. The similarity in germination requirements for the deactivated and dormant spores indicated that the activation effect was removed by the acid treatment and that the deactivated spores were physiologically similar to dormant spores.

Exudate analyses. To further characterize biochemical changes during activation, germination, and inhibition, exudates were collected and analyzed. As very little material was released from inhibited or deactivated spores, supernatants were collected after the activated spores (7.6 C for 70 min) had been subjected to a particular condition for 24 hr. The long treatment period and the heavy (5×10^9 spores/ml) suspension used caused some preparations to lose their refractility; spores in water at either 3 or 24 C, in acetate at pH 4, or in *o*-phenanthroline lost their refractility by the time the supernatant was removed, whereas spores suspended in 5 mM $CuSO_4$ or acetic acid were still refractile. The deactivated spore suspension which provided the supernatant for analysis contained 17% partially refractile spores, but the germination changes had been arrested as the refractility did not change on continued storage.

The estimations of free amino acids found in the samples analyzed are presented in Table 1. The data show that glutamate is the major component of extracts of fresh dormant spores, but that extracts of refractile dormant spores aged at 3 C demonstrated an accumulation of additional free amino acids. To substantiate the findings obtained by paper chromatography and electrophoresis, the extract from fresh dormant spores and the exudate from activated spores allowed to germinate were quantitatively analyzed (Table 2). In contrast to dormant spores, the germination exudate of activated spores contained 14 free amino acids and 5 unknown ninhydrin-positive materials. Powell and Strange (1953) identified the same amino acids except phenylalanine in the germination exudate of the *B. megaterium* spores they studied.

The quantitative analysis of DPA and peptides, and the electrophoretic identification of peptide are presented in Table 3. As Powell and Strange (1953) demonstrated the hexosamine and reducing-sugar values of various germination exudates to be within a few per cent of each other, the reducing-sugar value of the hydrolyzed sam-

TABLE 2. *Free amino acid* composition of ethanolic extract of broken dormant spores and of the germination exudate of activated spores germinated in water*

Amino acids	Dormant spores†	Activated germinated spores‡
Aspartic acid	0	1.58
Glutamic acid	7	7.76
Methionine	0	2.02
Threonine	0.192	2.54
Serine	±	1.11
Leucine	0	2.68
Isoleucine	0	2.01
Valine	0	2.88
Tyrosine	0	1.01
Phenylalanine	0	1.31
Alanine	0	5.32
Glycine	0	3.86
Histidine	0	0.84
Arginine	0.253	0
Lysine	0	6.65
Ammonia	±	9.1

* Determined by Spinco model 120 amino acid analyzer. Results expressed as mmoles of amino acid per 100 g of spores.

† Dormant spores broken in and extracted with 50% ethanol by using Mickle disintegrator.

‡ Activated spores suspended in water. Supernatant collected after 24 hr at 24 C. No apparent spore lysis as indicated by phase-contrast microscopic examination, or change in per cent transmission of the suspension.

ple was used as a measure of the amount of peptide in the sample. The peptide material extracted from dormant spores under conditions which excluded enzymatic activity did not have an electrophoretic mobility similar to that of peptides obtained from germination exudates. The exudates of the activated spores that germinated at 3 C contained only 20% of the peptide and 10 to 50% of the free amino acids found in the normal germination exudate collected at 24 C. The results show that the breakdown and release of materials were retarded at 3 C. Exudates from inhibited activated spores and deactivated spores were different in composition than the normal germination exudate. The exudates collected from activated spores inhibited by $CuSO_4$ and acetic acid contained only small amounts of glutamate and DPA. When dormant spores were incubated in 50 mM acetic acid for 24 hr at 24 C, only a small amount of DPA was detected. The peptide and free amino acid composition of exudates, collected from activated spores suspended in acetate solution at pH 4 or in *o*-phenanthroline, was more like that of an extract of dormant spores than a normal germination exudate. These two solutions did not stop the release of DPA and peptide material, but the production or release of NH_3 and the other 12 amino acids was retarded. Under certain adverse conditions, such as low temperature or pH, activated spores appeared to be germinated and swollen when examined microscopically, but their germination exudates were not normal.

Very small amounts of material were released from the spores by the activation and deactivation treatments. This may be the reason why the activation and deactivation treatments were successful. The small amounts of materials released from the deactivated spores and the fact that they remained refractile for long periods indicated that the release of material started by activation had been arrested. In these analyses, it was not possible to determine whether the small amount of material found originated from the activated spores or primarily from the 5 to 10% partially germinated spores present in these preparations. Generally, whatever inhibited the germination of activated spores also inhibited the release of exudate material.

DISCUSSION

Calcium dipicolinate-activated bacterial spores represent an early phase of germination, and some of their properties are quite different from those of germinated or dormant spores. The significance of the findings presented above will be discussed.

The most distinctive characteristic of activated spores was that their rate of germination could be controlled or stopped altogether. The rate of germination could be regulated by adjusting the pH or temperature as well as the concentration of activated spores. A stronger acid treatment deactivated the activated spores and made them stable. The exact role of the H^+ cannot be evaluated at this time, but it is known that acid extracts DPA from dormant spores (Foster, 1960). The acid treatment may have caused the removal of DPA that was attached to particular sites of the activated spore, with the result that stability was regained and such spores were made similar to dormant spores.

TABLE 3. *Composition of exudates and extracts from activated and dormant spores*

Condition	Ninhydrin*† value	NH_3†‡	Reducing sugar†		Paper electrophoresis		DPA†
			Unhydrolyzed	Hydrolyzed	Protein	Peptide	
Activated spores suspended in:							
Water at 24 C	59	26.4	0.75	16.2	+	+	47.5
Acetate, 50 mM (pH 3)	5.9	0	0	1.5		±	13.8
Acetate, 50 mM (pH 4)	15.8	0	0	7.7	+	+	35.4
$CuSO_4$, 9 mM (pH 6.8)	3.4	0	±	±	0	0	2.8
Saturated *o*-phenanthroline	16.9	0	0	8.1	0	+	39.6
Water at 3 C	28.6	14.7	0	3	0	±	44.4
Deactivated spores (supernatant solution)	5.6	0	0	1.5	0	0	5.1
Activation solution (40 mM CaDPA)	2.8	1.9	0	±	0	0	—
Deactivation solution (12 mM H_3PO_4)	1.8	0	0.3	1.8	0	0	0.6
Dormant spores (extract)							
Boiled in water for 90 min	12.9	0	0	9.2	++	±	42.5
Broken in 50% ethanol	15.4	0	±	±	+	0	45

* Monosodium glutamate used as reference standard.
† Expressed as mmoles per 100 g of spores.
‡ Spores were activated at 7.6 C for 70 min and the exudates were collected after 24 hr at 24 C.

Germination of activated spores was inhibited by various cations (H^+, Ag^+, Cu^{++}, Fe^{++}, and Hg^+) and by the chelating agent *o*-phenanthroline. Although the nature and action of these inhibitors are divergent, their inhibitions were partially reversed by NaDPA. This suggested that these inhibitors were competitive with NaDPA. Curran, Brunstetter, and Myers (1943) and others have demonstrated that spores contain more cations than do vegetative cells. Riemann (1961) proposed that exogenous CaDPA could be chelated to spore material and could disrupt a normal stereostructure. Vinter (1961) reported that bacterial spore coats were rich in cysteine. Perhaps, like ion-exchange resin, dormant spores could provide an insoluble matrix for the chelation of CaDPA and other soluble materials which would make them "insoluble" in water.

Other studies have indicated that the dormant spore is anhydrous and yet permeable to water. Ross and Billing (1957) concluded that dormant spores were anhydrous, on the basis of their refractive index measurements. Murrell and Scott (1958) demonstrated that D_2O exchanged freely with 99% of the spore water, and Black et al. (1960) demonstrated that small molecules penetrated 40% of the spore volume. Powell (1957) as well as Rode and Foster (1960) stated that there is a marked permeability change during germination. The data presented here emphasize that dormant spores are impermeable to the extent that they release only a limited amount of soluble constituents. The analysis of the exudates of the various activated spore suspensions indicated that the permeability (as reflected by the release of spore material) was altered during activation. The increased permeability of activated spores and the effect of the several inhibitors in reducing this increase indicated that exogenous DPA affected the permeability of such spores. When Cu^{++}, Fe^{++}, or H^+ was added to the activated spore suspensions, the release of spore material was decreased but could be partially counteracted by the addition of NaDPA. Riemann (1961) postulated that the DPA of a normal dormant spore, if released from the normal internal complex, was enough to germinate bacterial spores. It is possible that heat shock, mechanical abrasion, and other germination stimuli may directly or indirectly upset the DPA stability of the dormant spore and initiate a permeability change that would result in germination. The relationship of the release of DPA to changes in spore permeability as a result of the action and metabolism of other germination agents has yet to be studied.

The exudate analyses demonstrated that the

free amino acid composition and the electrophoretic mobility of the peptide material extracted from newly harvested dormant spores was different from that present in a normal germination exudate. In contrast to dormant spores, the germination exudate of activated spores contained 14 amino acids and additional unknown ninhydrin-positive material. Under adverse germination conditions, such as low temperature or pH, dense suspensions of activated spores appeared to be germinated (dark) or swollen when examined microscopically, but the exudates contained a reduced amount of spore material. Likewise, although glutamate was the major free amino acid found in the extracts of newly harvested dormant spores, extracts made from refractile dormant spores aged for 40 days or more at 3 C contained significant amounts of additional amino acids. During aging, free amino acids accumulated in dormant spores, without any apparent changes in refractility. Conversely, activated spores germinated at 3 C released less than the normal amount of spore material, and those germinated in the presence of *o*-phenanthroline had an exudate with a reduced amino acid content. These observations imply that biochemical changes gradually occur in refractile dormant spores kept in the cold, and that physical changes (loss of re-refractility) can occur to activated spores in an inhibitory solution without normal biochemical changes. Changes in the spore coat undoubtedly occur during germination; Strange and Dark (1957) described a lytic enzyme which solubilizes a peptide from isolated spore coats, and Levinson and Wrigley (1960) recorded, by means of electron micrographs, the disintegration of the outer coat of *B. megaterium*. The exact role of lytic and proteolytic enzymes during spore germination in relation to physical changes is not yet known. Further studies on the breakdown of spore materials during germination as related to physical changes induced by different germinating systems are needed to clarify this point.

LITERATURE CITED

BLACK, S. H., T. HASHIMOTO, AND P. GERHARDT. 1960. Calcium reversal of the heat susceptibility and dipicolinate deficiency of spores formed "endotropically" in water. Can. J. Microbiol. **6**:213–224.

BLACK, S. H., R. E. MACDONALD, T. HASHIMOTO, AND P. GERHARDT. 1960. Permeability of dormant bacterial spores. Nature **185**:782–783.

BLOCK, R. J., E. L. DURRUM, AND G. ZWEIG. 1958. A manual of paper chromatography and paper electrophoresis, 2nd ed. Academic Press, Inc., New York.

CHURCH, B. O., AND H. HALVORSON. 1959. Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. Nature **183**:124–125.

CONWAY, E. J. 1950. Microdiffusion analysis and volumetric error. Crosley Lockwood & Son, Ltd., London.

CURRAN, H. R., B. C. BRUNSTETTER, AND A. T. MYERS. 1943. Spectrochemical analysis of vegetative cells and spores of bacteria. J. Bacteriol. **45**:485–494.

DOI, R. H., AND H. HALVORSON. 1961. Comparison of electron transport systems in vegetative cells and spores of *Bacillus cereus*. J. Bacteriol. **81**:51–58.

FLYNN, F. V., AND O. P. DEMAYO. 1951. Microelectrophoresis of protein on filter paper. Lancet **261**:235–239.

FOSTER, J. W. 1960. Dipicolinic acid and bacterial spores, p. 1–38. Lectures on the theoretical and applied aspects of modern microbiology. University of Maryland, College Park.

LEVINSON, H. S., AND A. S. WRIGLEY. 1960. Spore germination and emergence of *Bacillus megaterium*. Science **131**:1382.

LEVY, A. L. 1954. A paper chromatographic method for the quantitative estimation of amino acids. Nature **174**:126–127.

LONG, S. K., AND O. B. WILLIAMS. 1958. Method for removal of vegetative cells from bacterial spore preparations. J. Bacteriol. **76**:332.

MICHL, H. 1959. High voltage electrophoresis. Chromatog. Rev. **1**:9–38.

MOORE, S., AND W. H. STEIN. 1948. Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem. **176**:367–388.

MURRELL, W. G., AND W. J. SCOTT. 1958. The permeability of bacterial spores to water. Intern. Congr. Microbiol., 7th, Stockholm, p. 26.

MURTY, G. G. K., AND H. O. HALVORSON. 1957. Effect of enzyme inhibitors on the germination and respiration of and growth from *Bacillus cereus* var. *terminalis* spores. J. Bacteriol. **73**:230–234.

PELCHER, E. A. 1961. Some characteristics of spores of *Bacillus cereus* produced by a replacement technique. M.S. thesis. University of Illinois, Urbana.

POWELL, J. F. 1953. Isolation of dipicolinic acid (pyridine-2,6-dicarboxylic acid) from spores

of *Bacillus megaterium*. Biochem. J. **54**:210–211.

POWELL, J. F. 1957. Biochemical changes occurring during spore germination in *Bacillus* species. J. Appl. Bacteriol. **20**:349–358.

POWELL, J. F., AND R. E. STRANGE. 1953. Biochemical changes occurring in the germination of bacterial spores. Biochem. J. **54**:205–209.

RIEMANN, H., AND Z. J. ORDAL. 1961. Germination of bacterial endospores with calcium and dipicolinic acid. Science **133**:1703–1704.

RIEMANN, H. 1961. Germination of bacteria by chelating agents, p. 24–48. *In* H. O. Halvorson [ed.], Spores II. Burgess Publishing Co., Minneapolis.

RODE, L. J., AND J. W. FOSTER. 1960. Mechanical germination of bacterial spores. Proc. Natl. Acad. Sci. U.S. **46**:118–128.

ROSS, K. F. A., AND E. BILLING. 1957. The water and solid content of living bacterial spores and vegetative cells as indicated by refractive index measurements. J. Gen. Microbiol. **16**:418–425.

SOMOGYI, M. 1945. The determination of blood sugar. J. Biol. Chem. **160**:61–73.

SPACKMAN, D. H., W. H. STEIN, AND S. MOORE. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. **30**:1190–1206.

STRANGE, R. E., AND J. F. POWELL. 1954. Hexosamine-containing peptides in spores of *Bacillus subtilis*, *B. megaterium* and *B. cereus*. Biochem. J. **58**:80–85.

STRANGE, R. E., AND F. A. DARK. 1957. A cell-wall lytic enzyme associated with *Bacillus* species. J. Gen. Microbiol. **16**:236–249.

VALLE, B. L. 1960. Metal and enzyme interaction: correlation of composition, function, and structures, p. 225–276. *In* P. D. Boyer, H. Lardy, and K. Myrback [ed.], The enzymes, vol. 3, 2nd ed. Academic Press, Inc., New York.

VAS, K., AND G. PROSZT. 1957. Effect of temperature and hydrogen ion concentration in the germination of spores of *Bacillus cereus*. Nature **179**:1301–1302.

VINTNER, V. 1961. The formation of cysteine-rich structure in sporulating cells and its possible role in the resistance of spores, p. 127–141. *In* H. O. Halvorson [ed.], Spores II. Burgess Publishing Co., Minneapolis.