Some Characteristics of Bacillus Cereus T Spores Sensitized to Muramidases

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SOME CHARACTERISTICS OF *Bacillus cereus* T SPORES
SENSITIZED TO MURAMIDASES.

by

PRAVIN C. PATEL

A THESIS SUBMITTED TO THE FACULTY OF THE
GRADUATE SCHOOL OF LOYOLA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE.

February
1974
DEDICATION

To my parents
and my wife, Madhu,
whose encouragement and love
have made this thesis possible.
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<tr>
<td>C</td>
<td>Centigrade</td>
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<td>cm</td>
<td>centimeter</td>
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<td>concn</td>
<td>concentration</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>CORLE</td>
<td>cortex lysing enzyme</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>g</td>
<td>gram</td>
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<td>hr</td>
<td>hour(s)</td>
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<td>KU</td>
<td>Klett unit</td>
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<td>l</td>
<td>liter</td>
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<td>MCE</td>
<td>mercaptoethanol</td>
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<td>nm</td>
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<td>OD</td>
<td>optical density</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TSA</td>
<td>Trypticase soy agar</td>
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<tr>
<td>TSB</td>
<td>Trypticase soy broth</td>
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<tr>
<td>sec</td>
<td>second(s)</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
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<td>w/v</td>
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INTRODUCTION

Bacterial spores are resistant not only to heat but also to radiation, deleterious chemicals and lytic enzymes to which their progenitor vegetative cells are susceptible. Theories dealing with the mechanisms of bacterial spore resistance have been reviewed by several authors (Sussman and Halvorson, 1966; Roberts and Hitchins, 1969; and Russell, 1971). Factors most frequently associated with the high degree of resistance of bacterial spores to deleterious agents include (i) presence of calcium and dipicolinic acid (DPA), (ii) low water content and (iii) the presence of the complex spore integument (exosporium, coat and cortex). However, the specific role(s) played by the coat in spores resistance to various physical and chemical agents is understood only to a limited extent.

A great deal of information regarding the ultrastructure (Fitz-James, 1960; Ohye and Murrell, 1962; Warth et al., 1963a; Gerhardt and Ribi, 1964; Holt and Leadbetter, 1969; Leadbetter and Holt, 1969; Hashimoto and Conti, 1971; and Short and Walker, 1971) and chemical composition (Murrell, 1967; 1969) of the spore coat is available in the literature. The coat of Bacillus cereus T spores consists of three recognizable layers (Hashimoto and Conti, 1971). An electron dense middle layer appears to be sandwiched by two thinner outer and inner layers. Spores of B. coagulans (Warth et al., 1963a) and B. megaterium (Fitz-James and Young, 1959) seem to have structurally more complex coats with multiple lamellae. In B. megaterium the spore coat consists of an inner fibrous coat and an outer dense thick coat (Freer and Levinson, 1967). The spore coat of B. subtilis Marburg is composed
of three layers: "a membranous outermost layer", "an electron opaque thick outer layer", and "an inner laminated layer" (Hiragi et al., 1967). Spores of *B. polymyxa* have thick, symmetrically ridged coats with few distinguishable layers (Holt and Leadbetter, 1969). Spores of *B. fastidiosus* possess asymmetrically ridged, lamellar spore coat layer (Holt and Leadbetter, 1969).

The freeze-etched surface of *B. cereus* spores is characterized by the presence of numerous small patches consisting of short fibers oriented in juxtaposition (Holt and Leadbetter, 1969; Hashimoto and Conti, 1971). In *B. licheniformis* and *B. subtilis*, the fibrils are regular and traverse most of the length of the spore. In *B. fastidiosus*, the fibrils are long, but are randomly arranged over the ridges (Holt and Leadbetter, 1969). The coat surface of *B. megaterium* and *B. polymyxa* is reported to have no rodlet crystalline structure (Holt and Leadbetter, 1969).

The spore coat of *Clostridium perfringens* can be differentiated into two distinct layers; an inner electron dense layer which surrounds the cortex and an outer one which is much thicker but less electron dense. (Hoeniger et al., 1968). The spore coats of *C1. botulinum* (Hodgkiss et al., 1967) and *C1. pectinovorum* (Fitz-James, 1962; Hoeniger and Headly, 1968) consists of a thin electron dense inner spore coat surrounded by a multilayered outer spore coat. In contrast, the outer coat of *C1. sporogenes* is an amorphous loose structure (Hoeniger and Headly, 1969). The unique feature of the spore coat of *C1. pasteurianum* is that it is made up of an anatomically homogenous multilayered (7 to 10 layers, each layer of the same thickness) structure (Mackey and Morris, 1971). The surface structure of the spore coats
as viewed by electron microscopy of freeze-etched preparations appears uniform. Thus crystalline rodlet structures as seen in many spores of bacilli are not present in clostridial spores (Hoeniger et al., 1968; Short and Walker, 1971).

The spore coat of bacilli accounts for 30 to 60% of the spore dry weight and contains up to 80% of the spore protein (Murrell, 1967; 1969). They are composed mostly of protein, the residue being lipid (0.9 - 3%), ash (1-3%), carbohydrates and sometimes considerable amount of phosphorus (Murrell, 1969). The protein of spore coats is high in glycine, lysine, aspartate, glutamate, and nonpolar amino acids. The cysteine content of spore coats is especially high, being four to five times that of vegetative cell protein (Vinter, 1959).

The spore coat protein has been solubilized by various chemicals including urea, mercaptoethanol (MCE), dithiothreitol (DTT), thioglycollate, sodium dodecyl sulfate (SDS) or alkali (Kondo and Foster, 1967a; Aronson and Fitz-James, 1968; Kawasaki et al., 1969; Sommerville et al., 1970). Repeated and prolonged extraction of B. cereus aesti (A-) spore coats with 1% thioglycollate or dithiothreitol at pH 10.5 resulted in solubilization of about 80% of the coat protein. The extracted protein had a nitrogen content of 16-17% (Aronson and Fitz-James, 1968). Aronson and Horn (1972 extracted the inner coat (corresponding to the middle layer as described by Hashimoto and Conti, 1971) and the outer coat of B. cereus T spores preferentially with dithioerythritol (DTE) at pH 10.3 and with DTE plus SDS, respectively. The proteins extracted from both coats migrated as a single
protein on polyacrylamide gels and had an average molecular weight of 12,000. The results of Aronson and Horn (1972) suggested that both coat layers contained predominantly one or two kinds of polypeptides.

Kondo and Foster (1967a) separated the lysozyme (muramidase) treated coats of *B. megaterium* spores into an alkali-soluble fraction, a paracrystal fraction (sonicate of the alkali-insoluble fraction) and a pronase-resistant residue. The paracrystal fraction appeared to represent the middle particulate layer of coat sandwiched or cemented between the alkali-soluble layer on one side and the resistant-residue on the other (Kondo and Foster, 1967b). The alkali-soluble layer contained one or more proteins, composed mostly of amino acids and 0.9% phosphorus. It was rich in glycine and exceptionally rich in tyrosine and contained very little cystine. The paracrystalline material resembled keratin in composition, being exceptionally rich in cysteine, glycine, dicarboxylic amino acids, and was solubilized by SDS. The residual insoluble fraction was low in cysteine but rich in lysine. It contained considerable amounts of muramic acid and only 30% amino acids and gave 30% ash. Sommerville et al. (1970) showed that the amino acid composition of the protein extracted from different species of bacilli (*B. thuringiensis*, *B. cereus*, *B. subtilis*, and *B. megaterium*) with urea and MCE, is similar. The urea-MCE extracts of all spores tested, including those of *Cj. roseum*, showed essentially identical behavior to crystal protein in disc electrophoresis and gave a single major band (Sommerville et al., 1970).
In spite of this wealth of literature on the morphology and chemical composition of the spore coats, little is known about the role they play in the physiology of the spore. The importance of the spore integument in the resistance and germination of bacterial spores may be inferred from the fact that the coat is directly exposed to exogenous environments. It has been suggested that it is unlikely that the exosporium affects heat resistance or that its possession confers any additional heat resistance (Murrell, 1969). Gerhardt et al. (1961) reported that removing the exosporium from *B. cereus* spores by passing through a pressure cell did not damage or cause germination of the residual spore structure. The work reported by Pierce and Fitz-James (1971) clearly suggests that the cortex is not necessary for producing the initial dehydration and heat resistance but its role appears to be to maintain and protect the dehydrated stable state of the resting spore protoplast.

The role(s) of spore coats in the physiology of bacterial spores can be best studied by examining the properties or behavior of spores in which the coat has been removed. Such spores may be obtained by either chemically solubilizing the coat (Gould *et al.*, 1970; Sommerville *et al.*, 1970; Aronson and Fitz-James, 1971; Cassier and Ryter, 1971; Wyatt and Waites, 1971; Duncan *et al.*, 1972; Waites *et al.*, 1972; Wood, 1972) or by isolating coatless mutants (Ryter *et al.*, 1961; Fitz-James, 1965; Ryter *et al.*, 1966; Cassier and Ryter, 1971).

It is generally agreed that the spore coat functions as a protective layer against lytic enzymes but is not involved in resistance to heat and

The mechanism of spore resistance to heat, radiation and chemicals still remains unclear. Increased heat resistance has been attributed to increased calcium: DPA ratios (Levinson et al., 1961) and decreased magnesium: calcium ratios (Murrell and Warth, 1965). Spores of a DPA-less mutant of B. cereus T were found to be heat-sensitive (Halvorson and Swanson, 1969). However, recently Hanson and his associates (1972) have isolated mutant B. cereus T spores that produce low levels of calcium and magnesium and lacks DPA but remain thermoresistant. Spores in which the coat has been chemically solubilized are reported to be equally resistant to heat as compared to untreated spores (Gould et al., 1970; Sommerville et al., 1970; Aronson and Fitz-James, 1971; Cassier and Ryter, 1971; Wood, 1972). A mutant that form coatless spores have been reported (Ryter et al., 1961; 1966) but their heat resistance was not reported. However, spores have been produced without, or deficient in, coatlayers by growth in the presence of chloramphenicol added at stage V; these spores are refractile and resistant to mild heat treatments (10 min at 80 C), but are unstable and germinate within a few days at 0-1 C (Fitz-James, 1965).

Resistance to ionizing radiations has been claimed to be due to the disulfide rich coats (Vinter, 1960; 1961; 1962) whereas resistance to UV light has been suggested to be due to the presence of DPA (Grecz et al., 1973). Resistance to deleterious chemicals, desiccation and stains is still unknown. Resistance to stains has been associated with the cortex (Hashimoto and Naylor, 1958). Spores of a coat-less mutant (Cassier and Ryter, 1971)
of *Cl. perfringens* have been reported to be resistant to heat, ethanol and octanol. Spores of a *B. subtilis* mutant with partially impaired texture of the envelope have been reported to be heat sensitive but chloroform resistant (Balassa and Yamamoto, 1970). However, spores of another cortex-less mutant of *B. subtilis* reported by Millet and Ryter (1972) were found to have a normal spore coat and was resistant to chloroform. Therefore it is still not clear which component(s) (cortex, coat or DPA) is conferring resistance to various agents (heat, radiation, chemicals, etc.).

The biochemical mechanisms of spore germination has recently been reviewed by Gould and Dring (1972). There is an enormous amount of information available concerning the ways in which germination of different bacterial spores can be initiated. The chemical and physical reactions taking place during germination and outgrowth is also well documented (Strange and Hunter, 1969). However little is known about the specific reactions which occur in between, i.e., the connecting reactions that occur after the exposure of a spore to some germination inducers (Gould, 1969; Gould and Dring, 1972). The fact that diverse compounds such as L-alanine, inosine, glucose, calcium, dipicolinate and others can induce germination of various bacterial spores makes it difficult to speculate upon the existence of a common biochemical mechanism of initiation. Keynan and Evenchik (1969) have suggested that there is a different pathway or at least a different recognition site on the spore for the various germination stimulants. In fact the recent observations of Sogin et al. (1972) that different germination systems of *B. cereus* T spores had a different time-
temperature relationship of heat activation for obtaining optimal germinal response suggests multiple pathways for germination.

Spore lytic enzymes (muramidase-like enzymes) initially described by Strange and Dark (1957) and Gould and Hitchins (1965) have been shown to initiate germination of suitably sensitized spores (Gould and King, 1969; Frieben, 1971). Although the exact mechanism by which this enzyme is activated in spores by germination inducers is not known, it is hypothesized that the germination enzyme may be released from some binding site in the coats or other parts of the spore integument (Gould et al., 1966; Gould, 1969; Frieben, 1971; Gould and Dring, 1972). The triggering process in spore germination has been well distinguished from the lytic process (Hashimoto et al., 1969; Frieben, 1971).

It has been recently shown that the coat of spores may also play an important role in physiological germination (Cassier and Ryter, 1971; Frieben, 1971; Duncan et al., 1972). Spores of Cl. perfringens mutant devoid of spore coats (Cassier and Ryter, 1971) or normal clostridial spores treated with alkali or reducing agents (Cassier and Ryter, 1971, Duncan et al. 1972) were completely deprived of their ability to germinate in the presence of physiological germinants. Contrary to these results, Cl. bifermentans spores treated under similar conditions were reported to germinate at accelerated rates and to require lower concentrations of germinative substances for initiation (Wyatt and Waites, 1971; Waites et al., 1972).

Sommerville et al. (1970) reported similar findings that B. thuringiensis spores treated with urea-MCE or urea-MCE-SDS required a lower concentration
of nutrients to germinate than that required by untreated spores.

As mentioned earlier, the triggering process in spore germination has been distinguished from the lytic process (Hashimoto et al., 1969; Frieben, 1971). The spore lytic enzyme (muramidase-like enzyme) has been implicated to be involved in the second phase of the lytic process (microgermination; Frieben, 1971). In addition, chlorocresol, phenols, and other compounds are known to inhibit reversibly the initiation of germination of spores (Sierra, 1970; Frieben, 1971; Lewis and Jurd, 1972). Thus it appears feasible to isolate the dormant enzyme-integument complex in the presence of such an inhibitor. Once such a complex (cortex lysing enzyme: integument) is isolated it would be possible to carry out in vitro experiments to determine the role of germination inducers such as L-alanine and other low molecular weight compounds which are hypothesized to trigger the release of this enzyme (Gould et al., 1966; Frieben, 1971; Gould and Dring, 1972).

The purposes of this thesis research are: (i) to investigate the change of physical and physiological properties of B. cereus T spores, especially the alteration of resistance to heat, chemicals, enzymes, ultraviolet radiation, basic dyes and desiccation as the result of partial removal of the spore coat by chemical treatment; (ii) to examine germination characteristics of B. cereus T spores in which the spore coat has been partially removed by chemical treatment; and (iii) to elaborate a model for studying the mechanism of germination. Preliminary studies were carried out to test the hypothesis mentioned earlier namely that L-alanine and other low molecular weight compounds initiate germination by triggering the release of the
spore lytic enzyme bound to the spore integument. Optimum conditions for assaying the spore lytic enzyme have been determined.
11.

MATERIALS AND METHODS

Organism. The organism used throughout this study was *Bacillus cereus* strain T. Stock cultures were maintained on trypticase-soy agar (TSA), stored at 4°C. TSA was prepared by adding 1.5% agar agar (Baltimore Biological Laboratory) to trypticase soy broth (TSB, Baltimore Biological Laboratory, Baltimore, Maryland).

Production of spores. Large quantities of mature, heat-resistant *B. cereus* T spores were produced in the "modified G-medium" of Hashimoto et al. (1960). TSA plates were streaked from a stock TSA slant culture of *B. cereus* T. After 18 hour (hr) of incubation at 30°C pure colonies were subcultured in TSA slants. The slants were incubated at 30°C for 18 hr. The slant cultures were then washed with sterile TSB (1 ml) with a sterile pasteur pipette. Washings from 2-3 slants were inoculated in a 250 ml Erlenmeyer flask containing 20 ml TSB and incubated at 30°C for 2-4 hr on a Gyratory shaker (Model G 25, New Brunswick Scientific Co., New Brunswick, New Jersey). To obtain a seed inoculum for the production of spores, the 20 ml culture was inoculated into 200 ml TSB (10% inoculum) and incubated for 4-6 hr on the Gyratory shaker at 30°C. This seed inoculum, comprised of filamentous vegetative cells in the exponential phase of growth was then aseptically inoculated in 40 ml amounts each, into 400 ml sterile "modified G medium" contained in 2 l Erlenmeyer flasks fitted with stainless steel baffles to increase aeration. The freshly inoculated medium was incubated at 30°C for 24-30 hr under maximum aeration on the Gyratory shaker. Samples were removed periodically to follow the progress of sporulation under the phase contrast microscope. Under these
conditions of sporulation, greater than 99% of the cells formed free spores after 28 hr of incubation.

Harvesting and storage of spores. Free spores were collected by centrifugation in plastic bottles. The spores were pooled and subsequently washed more than 10 times with cold distilled water and were determined to be free of vegetative cells and debris by phase contrast microscopy. Dense, aqueous spore suspensions were dispensed in small glass vials and stored at -20°C until use.

Sensitization of spores to muramidase (lysozyme). Various methods for solubilizing spore coat protein have been described previously by many investigators (Kondo and Foster, 1967a; Gould and King, 1969; Kawasaki et al., 1969; Sommerville et al., 1970; Aronson and Fitz-James, 1971; Cassier and Ryter, 1971). A modification of these methods has been used in this investigation. Dormant B. cereus T spores (2 × 10⁹ spores/ml) were extracted in 4 M urea (certified A.C.S. obtained from Fisher Sci. Co., New Jersey or Schwarz/Mann, Orangeburg, New York.) prepared in 0.1 M sodium phosphate buffer pH 6.5, containing 1% MCE (Sigma Chemical Co., St. Louis, Mo.) and 1% SDS (Fisher Sci. Co.). Extraction was carried out at room temperature (25°C) with constant agitation for different periods of time. The extracted spores were washed several times by means of centrifugation with distilled water. Muramidase (3.2.1.17) sensitivity of extracted spores was tested by mixing either 1 ml sample of spore suspension with 1 ml of lysozyme (muramidase; Sigma Chemical Co., St. Louis, Mo.) solution (200 µg
per ml in 0.1 M carbonate-bicarbonate buffer pH 9.2) or 1 ml of cortex lysing enzyme (muramidase-like enzyme; see below). The mixture was incubated at room temperature when lysozyme was used or at 35°C when cortex lysing enzyme was used, and aliquots were removed after appropriate intervals and were observed for muramidase sensitivity (phase darkening) under a phase contrast microscope.

Preparation of cortex lysing enzyme (CORLE). CORLE from B. cereus T spores was prepared as described by Frieben (1971). Spores [15 to 25 mg (dry weight) per ml] were suspended in distilled water containing 5 X 10^-4 M disodium ethylenediamine tetraacetate (EDTA) and 25 µmole/ml DTT (Sigma Chemical Co.), mixed with acid-washed glass beads (type 1, Sigma Chemical Co.). The spores were disintegrated in 5 ml portions for 30-45 min at 4°C in a Mickle tissue disintegrator (Brinkmann Instruments, Westburg, N.Y.) with 4.5 g of glass beads. The suspension of disrupted spores was centrifuged at 10,000 x g for 30 min at 4°C, and the supernatant fraction was centrifuged at 25,000 x g for 30 min at 4°C. The supernatant fraction contained the lytic enzyme activity and was used directly without any dilution. In some cases, spores were disrupted in 0.1% (V/V) phenol prepared in 0.1 M sodium phosphate buffer (pH 7.0). The supernatant fluid obtained after centrifugation at 25,000 x g for 30 min was assayed for CORLE activity. The pellet containing the spore debris (integument) was processed in the following different ways: (a) treated with 0.05 M Tris-HCl [tris (hydroxymethyl)aminomethane-hydrochloride] buffer (pH 8.3), or (b) treated with the buffer mentioned in (a) containing L-alanine (5 mg/ml;
Sigma Chemical Co.) and adenosine (2 mg/ml; Sigma Chemical Co.). The mixtures were incubated for 1 hr and then centrifuged as mentioned above. The supernatant fluids obtained were assayed for CORLE activity as described below.

**Phase-contrast photomicroscopy.** Refractile (ungerminated) and phase-dark (germinated) spores were examined with a phase contrast microscope by using an oil immersion objective (dark medium, x 100, numerical aperture 1.25; Nikon). Photomicroscopy was made on panchromatic film (Kodak Plus-X) with a Nikon camera equipped with an automatic exposure system attached to a phase-contrast microscope.

**Viability of spores.** Viability of spores was tested by conventional plating techniques and by a microscopic method. In the first method spore suspensions were serially diluted in sterile distilled water and 0.1 ml samples were plated in triplicate on TSA by using standard procedures. Plates were incubated for 18-30 hr at room temperature, and colonies were counted on a New Brunswick colony counter (New Brunswick Scientific Co., New Brunswick, N.J.). In the second method an aliquot of spores was suspended in 2 ml of TSB supplemented with L-alanine (5 mg/ml) and adenosine (2 mg/ml) and incubated at 30°C on a rotary shaker. After 1, 1 1/2, 2, 2 1/2 and 3 hr samples were removed and examined under the phase-contrast microscope for outgrowth and division.

**Dipicolinic acid (DPA) and calcium determination.** DPA (Aldrich Chemical Co., Milwaukee, Wis.) was determined colorimetrically by the method described by Janssen et al. (1958). Calcium was determined by atomic absorption
spectrophotometry. Spore suspensions were autoclaved at 121°C for 20 min. The supernatant fluid obtained after centrifugation of the autoclaved spore suspension was used for DPA and Ca analysis.

**Dry weight determination.** Samples of spore suspension (1.00 × 10⁹ spores/ml; 10 ml) were filtered through pre-dried, pre-weighed filters (0.45 µm pore size, 47 mm diameter, Millipore Corporation, Bedford, Massachusetts.), washed 3 times with 5 ml distilled water and dried in an oven at 95°C for 48 hr.

**Separation of untreated and extracted spores by using gradient centrifugation.** The specific gravity of spores was determined by means of gradient centrifugation using Renografin (Reno M-60, E.R. Squibb & Sons, Inc., New York.). This method is based on the fact that Renografin absorbs strongly in the ultraviolet region with a maximum absorption at 260 nm and that a linear correlation exists between the absorbance at 260 nm and the density for Renografin solutions (Tamir and Gilvarg, 1966). This makes it possible to evaluate the density of any particular fraction from a gradient. The method used in preparing gradients was essentially the same as that described by Tamir and Gilvarg (1966). The tubes were centrifuged in an International Equipment Co. centrifuge (model B35, Needham Hts., Massachusetts.) equipped with a swinging bucket rotor (No. SB269) at 17,000 rev/min for either 30 min or 180 min, at 4°C. Fractions were collected by puncturing the bottom of the tube and their absorbance at 260 nm was measured after dilution to 10⁻⁴.
Polyacrylamide gel electrophoresis. The sample gel contained about 100 µg/ml of the protein extracted by 4 M urea -1% MCE and 1% SDS. Polyacrylamide (Eastman Kodak Co., Rochester, New York.) (7.5%) gels were prepared, and electrophoresis was carried out, as described by Davis (1964) for 45 min at 3 miliamperes per running tube at 4 C. Bromophenol blue (0.001%) was used as a marker and amido black (Merck and Co., Rahway, N.J.) (1.0%) in acetic acid (7%) as a stain. Destaining was done electrophoretically using 7% acetic acid.

Protein determination. Protein was estimated by the method of Lowry et al. (1951) using crystalline bovine serum albumin (Sigma Chemical Co.) as standard.

Resistance to heat. A 1 ml sample of an aqueous spore suspension (2 x 10^7/ml) was dispensed into several sterile screw cap pyrex glass tubes (15 x 125 mm). All tubes were exposed to heat at the same time in a thermostatically controlled water bath (Model 81, Precision Scientific Co., Chicago, Ill.), held at either 80, 85 or 90 C sequentially for specified periods. At intervals a tube was withdrawn and immediately chilled in ice. Viable counts were determined in triplicate by standard plate count method described previously.

Resistance to ultraviolet irradiation. A 2 ml sample of a spore suspension containing about 2 x 10^7 spores/ml in distilled water were placed into petri dishes (35 x 10 mm, Bio-Quest, Falcon Plastics, 1950 Williams Drive, Oxnard, Ca.) and exposed to various doses of UV light peak emission 2537° A, 233 µ
watts/sq cm at a distance of 45 cm from the lamp source (a mercury vapor lamp, Mineralight, Model RS1, Ultra-violet Prod. Inc., San Gabriel, California) with the cover removed. Viable spores after exposure were determined in triplicate by the plate count method described above.

**Resistance to glutaraldehyde.** Spore suspensions (about 2 x 10^7/ml) were mixed with 0.25% glutaraldehyde (Sigma Chemical Co.) in 0.3% NaHCO_3 [pH 8.0, Pepper and Chandler (1963)] and were allowed to stand at room temperature for varying periods. Treated spores were immediately diluted in distilled water and viable spores were determined by the standard viable plate count technique described above. Our preliminary tests showed that 0.0001% glutaraldehyde had no lethal effect on the spores.

**Resistance to desiccation.** Tubes containing 1 ml of an aqueous suspension of spores were lyophilized and kept in vacuo or dry air for different periods of time. At intervals samples were removed and the viability of the spores was determined by the standard plate count technique.

**Resistance to basic dyes.** Spores smeared on clean cover glasses were mixed with a drop of 1% methylene blue. The spores which failed to stain within 10 min at room temperature were considered as resistant to the dye.

**Heat activation of spores.** Spores in aqueous suspension (1 mg of spores per ml) were heated for 30 min in a thermostatically-controlled water bath maintained at a temperature of 65 C. Activation was stopped by chilling in an ice bath.
Germination induced by low molecular weight compounds. The following organic compounds were tested for their ability to initiate germination of normal and sensitized (spores extracted with 4 M urea, 1% MCE, 1% SDS for 3 hr at 25°C) spores: L-alanine and adenosine, L-alanine, adenosine, inosine, L-cysteine, L-serine, L-threonine, L-glycine, L-proline and D-glucose (Fisher Sci. Co.). The reaction mixture consisted of spores (initial OD at 450 nm = ca. 0.3), 15 or 50 mM prospective germination compound, 50 mM Tris-maleate (Fisher Chem. Co.) buffer pH 7.0 and 100 mM NaCl in a total value of 3 ml. Germination was measured in two ways. In one method, germination was followed spectrophotometrically by recording the decrease in OD at 450 nm using a Bausch and Lomb, Spectronic-70 spectrophotometer. In the second method, the percentage of spores which had lost their refractility was determined under a phase contrast microscope; a total of 200 spores were counted for each sample. The amino acids and ribosides were obtained from Sigma Chemical Company.

Different concentrations of phenol (Mallinckrodt Chemical Works, St. Louis, Mo.), 5% ethanol and 5% methanol were tested to see if they had any effect on L-alanine and adenosine induced germination of B. cereus T spores. The effect of these inhibitors on CORLE induced germination of sensitized spores was also investigated.

Germination induced by cortex-lysing enzyme. To test the germination induced by CORLE, spores were suspended in a solution containing 50 mM Tris-HCl buffer (pH 7.5,) 100 mM NaCl, and 0.5 ml of crude enzyme (25,000 x g
supernatant) in a total volume of 3.0 ml and maintained at 35 C. The reaction mixture was incubated at 35 C (optimum temperature for lytic activity of CORLE; Frieben, 1971) and the OD was monitored with a spectrophotometer at 450 nm. Lytic activity of the enzyme was also tested on heated (90 C for 30 min) or autoclaved (121 C for 20 min) sensitized spores.

**Calculation of the germination rate.** Germination rate was calculated as described previously by Frieben (1971). The percent decrease in OD of a germinating spore suspension was plotted against time, and the germination rate (per cent OD loss per minute) was calculated from the linear portion of the curve i.e., that portion where there was the greatest linear decrease in OD.

**Effect of pH on CORLE activity.** The method described before for determining CORLE induced germination was followed using the following different buffer systems: 0.05 M sodium phosphate buffer, 0.05 M citrate phosphate buffer, 0.05 M Tris-maleate buffer, and Tris-HCl buffer with and without 100 mM NaCl.

**Effect of different ions on CORLE activity.** In view of our preliminary finding that a cation such as Na⁺ was absolutely necessary for CORLE activity, the effects of different ions on the activity of CORLE were examined. The test system consisted of autoclaved sensitized spores (OD at 450 nm = ca. 0.3-0.4) 50 mM Tris-HCl buffer (pH 7.5), crude enzyme (25,000 x g supernatant) and a known concentration of the following different salts: NaCl, KCl, CaCl₂, MgSO₄, MnSO₄, and spermine diphosphate (Sigma Chemical Co.).
Germination was followed spectrophotometrically as described before.

**Preparation of ultrathin sections.** Samples were harvested and suspended in 4% glutaraldehyde (Polyscience Inc., Rydal, Pa.) in a modified Veronal acetate buffer (Kellenberger *et al.*, 1958) for 2 hr at 4 °C. This was followed by 2% osmium tetroxide (in the same buffer) for 18 hr at 4 °C. The fixed cells were subsequently treated with 0.5% uranyl acetate (J.T. Baker Chemical Co., Phillipsburg, N.J.) for 2 hr at room temperature and dehydrated by passage through a graded acetone (Scientific Products, Evanston, Ill.) series embedded in Epon (Miller Stephenson Chemical Co., Danbury, Conn.), and polymerized at 60 °C for 20 to 24 hr. The sections were cut with glass knives on an LKB Ultratome and mounted on Formvar-covered 300-mesh copper grids. Sections were stained with lead citrate [K & K Laboratories, Plainview, New York; Reynolds, (1963)] before observation with a Hitachi HU-11 A electron microscope.

**Freeze-etched preparation.** Freeze-etched preparations were made in a Balzars (Santa Ana, Calif.) apparatus model BA 360 M by the method of Friedman *et al.* (1968), except that 4 to 6% sodium hypochlorite was used in place of Eau de Javelle to clean the replica.
RESULTS

1. Alteration of spore properties due to partial solubilization of spore coat protein(s).

The treatment with 4 M urea-1% MCE-1% SDS for 3 hr progressively removed protein(s) from *B. cereus* T spores without affecting viability (Fig. 1) or their appearance under the phase contrast microscope (Fig. 2b). As shown in Fig. 1 and Table 1, all the spores extracted with 4 M urea-1% MCE-1% SDS at room temperature for up to 21 hr were fully viable. Increasing the concentration of urea to 8 M, MCE to 10% and SDS to 2% or increasing the time (up to 21 hr) and temperature (up to 80 °C) of extraction did not affect the viability of extracted spores (Table 1).

It is also apparent from Fig. 1 that after 2 hr of extraction spores became completely sensitive to lysozyme. These sensitized spores, upon exposure to lysozyme, underwent germination-like changes (Fig. 2d). Spores rendered sensitive to lysozyme by chemical treatment will be referred to as sensitized spores.

Protein solubilized and dry weight lost. Approximately 62 µg of protein was solubilized from 1 mg of spores after extraction in 4 M urea-1% MCE-1% SDS for 3 hr (Fig. 1). This protein was shown to migrate with the tracker dye as a single major band in 7.5% polyacrylamide gels (Fig. 3) which is in agreement with the results of other workers (Sommerville *et al.*, 1970; Wyatt and Waites, 1971; Aronson and Horn, 1972; Wood, 1972). The solubilized protein can only partially account for the dry weight loss of sensitized spores. Since a treatment of *B. cereus* T spores with 4 M urea-1% MCE-1% SDS
Fig. 2 Phase contrast photomicrographs of *B. cereus* T spores. Note that the refractility of normal spores (2a) and spores sensitized by 4 M urea-1% MCE-1% SDS extraction for 3 hr (2b) is similar. However, when the spores are incubated in lysozyme (100 µg/ml) the sensitized spores rapidly lose their refractility and undergo germination-like changes (2d) whereas the normal spores remain completely unaffected (2c).
Table 1

Effect of different conditions of culture on germinability of lysozyme-sensitive and lysozyme-resistant spores.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Germinability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50%</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0%</td>
</tr>
<tr>
<td>UV</td>
<td>25%</td>
</tr>
</tbody>
</table>

In this particular assay, 30% of the spores became lysozyme (muramidase) sensitive (phase shift) after approximately 1 h of incubation in lysozyme (muramidase).
### TABLE 1

**Effect of different conditions of urea-MCE-SDS extraction on germinability, lysozyme sensitivity and viability of *B. cereus* T spores.**

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Germinability&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Lysozyme (muramidase)&lt;sup&gt;c&lt;/sup&gt; sensitivity</th>
<th>Viability&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>4 M urea-1% MCE - for 3 hr at 25 C</td>
<td>+</td>
<td>S&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>4 M urea-1% MCE-1% SDS for 3 hr at 25 C</td>
<td>+</td>
<td>S</td>
<td>+</td>
</tr>
<tr>
<td>8 M urea-5% MCE-2% SDS for 20 hr at 25 C</td>
<td>+</td>
<td>S</td>
<td>+</td>
</tr>
<tr>
<td>8 M urea-10% MCE-2% SDS for 3 hr at 50 C</td>
<td>+</td>
<td>S</td>
<td>+</td>
</tr>
<tr>
<td>8 M urea-10% MCE-2% SDS for 1 hr at 70 C</td>
<td>+</td>
<td>S</td>
<td>+</td>
</tr>
<tr>
<td>8 M urea-10% MCE-2% SDS for 1 hr at 80 C</td>
<td>+</td>
<td>S</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Spores were treated with different concentrations of urea-MCE-SDS at the indicated temperatures and times. After treatment, spores were washed as described in Materials and Methods.

<sup>b</sup> Germinability was tested in L-alanine and adenosine prepared in Tris-HCl buffer (0.05 M, pH 8.3) after a minimum incubation for 2 hr at 30 C. +: > 99% spores germinated.

<sup>c</sup> Lysozyme sensitivity was determined by incubating spores for 30 min in 50 mM phosphate buffer (pH 6.5) containing 100 µg lysozyme/ml. R: Resistant to lysozyme (muramidase) S: Sensitive to lysozyme (muramidase)

<sup>d</sup> Viability was determined microscopically as described in Materials and Methods. Germinability, lysozyme (muramidase) sensitivity and viability was confirmed by phase microscopy as described in Materials and Methods. +: > 99% spores are viable.

<sup>e</sup> In this particular case ca. 50% of the spores became lysozyme (muramidase) sensitive (phase dark) after approximately 5 hr incubation in lysozyme (muramidase).
Fig. 3 Polyacrylamide gel electrophoresis of protein solubilized from *B. cereus* T spores with 4 M urea-1% MCE-1% SDS for 3 hr. About 100 µg protein was mixed in the sample gel; only one major band was detected.
for 3 or more temperaturc treatments, the protein without affecting their permeability as well as viability, 3 in extracts, spores were sequentially used in all experiments as the sensitized spores unless otherwise stated.

Ultrastructure. The effect of sensitization on the ultrastructure of heat-rectum T spores was investigated by the electron microscopy. The electron microscopic appearance of a normal spore is shown above (Shearn and Conti, 1971), the spore rodlet crystalline structure is shown in Fig. 4b. The rodlet structure seems to be preserved by the treatment and at pericoat. Fig. 4c shows the electron microscope observation of a completely naked spore. Almost all of the pericoat of the surface of sensitized spores (also see Fig. 5b).

Fig. 5a illustrates a portion of a thin-sectioned normal spore. There is a fairly thick middle layer sandwiched by the outer and inner layers. The middle layer has been correlated to the layer containing the rodlet crystalline structure in freeze-etching preparations (Mandel and Conti, 1972).

By the sensitization treatment, the middle electron dense layer of the coat is primarily solubilized (Fig. 5b). The outer and inner layers of
for 3 hr at room temperature maximally removed the protein without affecting their germinability as well as viability, 3 hr extracted spores were subsequently used in all experiments as the sensitized spores unless otherwise stated.

Ultrastructure. The effect of sensitization on the ultrastructure of _B. cereus_ T spores was investigated. Fig. 4a shows the electron microscopic appearance of a normal freeze etched spore. As shown before (Hashimoto and Conti, 1971), the spore coat of _B. cereus_ T contains a patchwork layer of rodlet crystalline structure. A spore undergoing sensitization treatment is shown in Fig. 4b. The rodlet crystalline structure seems to be removed by the treatment and at places the rodlet structure underwent disorientation. Fig. 4c shows the electron microscopic appearance of a completely sensitized spore. Almost all of the crystalline structure was removed. The micrograph also shows bleb-formation at certain places on the surface of sensitized spores (also see Fig. 5b).

Fig. 5a illustrates an electron micrograph of a portion of a thin sectioned normal spore. As shown, the spore coat consists of 3 layers. There is a fairly thick middle layer sandwiched by the outer and inner layers. The middle layer has been correlated to the layer containing the rodlet crystalline structure in freeze etched preparations (Hashimoto and Conti, 1971).

By the sensitization treatment the middle electron dense layer of the coat is primarily solubilized (Fig. 5b). The outer and inner layers of the
Fig. 4. Freeze-etched surface of the spore coat of *B. cereus* T spores during various stages of sensitization. Normal spore (Fig. 4a) undergoing sensitization (Fig. 4b) and a completely sensitized spore (Fig. 4c). Note that the rodlet crystalline structure of the normal spore coat (Fig. 4a) is progressively removed by urea-MCE-SDS treatment (Fig. 4b and 4c).

[Electromicrographs taken by Dr. T. Hashimoto]
Fig. 5. Electron micrographs of a normal (a) and sensitized (b) *B. cereus* T spores. The normal spore coat contains an electron dense middle layer (M) sandwiched between the outer (O) and inner less dense layers. As seen in the sensitized spores, most of the middle layer is solubilized and the outer and inner layer show bleb (B) formation. 

CX: Cortex; EX: Exosporium.

[Electronmicrographs taken by Dr. T. Hashimoto]
A significant amount of DPA or Ca solubilized by the sensitization treatment at a heat reference. Contrary to other reports (Souvenelle et al., 1970; Apoama and Fitz-James, 1971; Casale and Sykes, 1974; Wood, 1972) fully sensitized spores were slightly more sensitive to heat than...
spore coat tended to be removed as blebs (Fig. 5b) as often observed in the surface of gram negative bacteria exposed to EDTA or a detergent (Schnaitman, 1971).

Specific gravity. Fig. 6 illustrates the separation of sensitized spores and normal spores by Renografin gradient centrifugation. Centrifuging the tubes at 17,000 x g for 30 min through 180 min did not change the position of the two bands (Fig. 6) indicating that the spores in the two bands were at equilibrium with the corresponding Renografin density. Upon fractionation of the gradient, samples were washed with distilled water to remove the Renografin. The fractions obtained from band 1 corresponded to normal spores, whereas the fractions obtained from band 2 corresponded to sensitized spores. The parallel determination of specific gravity revealed that normal spores had a density of 1.256 g/cm³, whereas sensitized spores were slightly denser and had a density of 1.266 g/cm³. Normal but germinated spores had an average density of 1.140 g/cm³.

DPA and calcium. Table 2 summarizes the DPA and calcium content of normal and sensitized spores. Normal spores contained 118 µg of DPA and 33 µg of Ca²⁺ per 1.85 x 10⁹ spores whereas the same number of sensitized spores contained the same amount of DPA and 29 µg of Ca²⁺. Thus there was no significant amount of DPA or Ca solubilized by the sensitization procedure.

Heat resistance. Contrary to other reports (Sommerville et al., 1970; Aronson and Fitz-James, 1971; Cassier and Ryter, 1971; Wood, 1972) fully sensitized spores seem to be slightly more sensitive to moist heat than
Fig. 6. The Renografin density gradient tubes showing the separation bands of normal (arrow 1) and sensitized (arrow 2) B. cereus T spores. Tube A was layered with normal spores while tube B was layered with a mixture of normal and sensitized spores before centrifugation at 17,000 x g for 30 min. The gradient range of Renografin is 48% at the top and 60% at the bottom.
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TABLE 2

Dipicolinic acid and calcium contents of normal and sensitized *B. cereus* T spores.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DPA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Calcium&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CA:DPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>118</td>
<td>33</td>
<td>1.17</td>
</tr>
<tr>
<td>Sensitized spores</td>
<td>118</td>
<td>29</td>
<td>1.02</td>
</tr>
</tbody>
</table>

<sup>a</sup> Normal spores were sensitized by extracting in 4 M urea-1% MCE-1% SDS for 3 hr as described in Materials and Methods.

<sup>b</sup> Expressed as µg of dipicolinic acid or calcium extractable from $1.85 \times 10^9$ spores by autoclaving in distilled water.
Fig. 7. Effect of moist heat on normal and sensitized *B. cereus* spores. Spores were heated at either 80°C, 85°C or 90°C for specific periods of time and survivors were enumerated as described in Materials and Methods.
percent survival vs. exposure time (min)

- normal (80 & 85°C)
- 80°C sensitized
- 85°C
- normal (90°C)
- sensitized (90°C)
normal spores, if a proper temperature of heat inactivation was employed (Fig. 7). The D90 value (time required to reduce spore population by one log value at 90°C) for normal and sensitized spores was found to be 24 min and 13 min respectively. It should be noted that there was no significant difference in the heat sensitivity between the two types of spores when 80°C or 85°C was employed for testing heat resistance.

**Chemical resistance.** The difference in resistance of normal and sensitized spores to 0.125% glutaraldehyde (pH 8.0) is illustrated in Fig. 8. Sensitized spores were killed by glutaraldehyde much faster than normal spores but slower than germinated spores.

**UV resistance.** The survival curves of normal and sensitized *B. cereus* T. spores after exposing to UV irradiation is illustrated in Fig. 9. Both sensitized and normal spores were equally resistant to UV light.

**Resistance to dyes.** Sensitized spores resisted staining by 1% methylene blue even though they were devoid of an intact spore coat. Fig. 10 illustrates the appearance of spores stained with 1% methylene blue. It is evident that the normal and sensitized spores remain unstained.

**Resistance to desiccation.** The effect of desiccation on the viability of spores is summarized in Table 3. As compared to normal spores, the extracted spores were equally resistant to desiccation. Desiccating both normal and sensitized spores for as long as two weeks did not decrease their viability.
Fig. 8. Survival of *B. cereus* T spores in 0.125% glutaraldehyde - 0.3% NaHCO₃ solution (pH 8.0 at room temperature).
Germinated and sensitized survival curves as a function of exposure time (min).
Fig. 9. Resistance of normal and sensitized spores to ultraviolet radiation. Note that there is no significant difference in resistance between normal and sensitized spores. Conditions of the experiment were as described in Materials and Methods.
Fig. 10. Light micrographs of *B. cereus* T spores stained with 1% methylene blue. Note that there is no difference between stainability of normal (10a) and sensitized (10b) spores.
10a

10b

10 \mu m
TABLE 3

Effect of desiccation\(^a\) on the viability of normal and sensitized spores.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viable spores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>None</td>
<td>$3.20 \times 10^7$</td>
</tr>
<tr>
<td>Lyophilization</td>
<td>$2.74 \times 10^7$</td>
</tr>
<tr>
<td>Lyophilization followed by 1 day desiccation</td>
<td>$3.08 \times 10^7$</td>
</tr>
<tr>
<td>Lyophilization followed by 2 weeks desiccation</td>
<td>$3.49 \times 10^7$</td>
</tr>
</tbody>
</table>

\(^a\) Desiccation was carried out in dry air as described in Materials and Methods.
2. Effects of sensitization treatment on germination of *B. cereus* T spores.

The changes in germination requirements of *B. cereus* T spores as the result of the sensitization treatment and its effects on the rate of physiological germination are summarized in Table 4. While the normal spores could be germinated effectively by L-alanine alone, inosine alone, or by L-alanine and adenosine, and less effectively by L-cysteine or adenosine, the sensitized spores could be germinated only by L-alanine or L-alanine and adenosine and somewhat less effectively by L-cysteine, but almost not at all by inosine or adenosine alone (Table 4). At a given concentration of a germination inducer the sensitized spores in general germinated much more slowly than the normal spores (Table 4).

In contrast to the observation reported for certain clostridial spores (Waites et al., 1972) the sensitization treatment did not relax the germination requirements of *B. cereus* T spores. Compounds other than those effective for normal spore germination were totally ineffective for inducing germination of sensitized spores under the condition employed (Table 4).

Even after the most extensive sensitization treatment (increasing the time of extraction up to 20 hr or extracting in 8 M urea-10% MCE-2% SDS at 80 °C for 1 hr) the spores were fully capable of germinating and subsequent outgrowth (Table 1). By means of sensitization treatment it was not possible to obtain spores which were totally lysozyme dependent for their germination as reported in certain clostridial spores (Cassier and Ryter, 1971; Duncan et al., 1972).
### TABLE 4
Germination of normal and sensitized spores of *B. cereus* T.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Normal spores</th>
<th>Sensitized spores&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Germination</td>
<td>Percent phase dark spores&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>rate&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>L-alanine (15 mM)</td>
<td>+</td>
<td>5.40</td>
</tr>
<tr>
<td>L-alanine (15 mM)</td>
<td></td>
<td>1.50</td>
</tr>
<tr>
<td>Adenosine (15 mM)</td>
<td></td>
<td>0.19</td>
</tr>
<tr>
<td>Inosine (15 mM)</td>
<td></td>
<td>3.25</td>
</tr>
<tr>
<td>D-Glucose (50 mM)</td>
<td></td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>L-Cysteine (50 mM)</td>
<td></td>
<td>&lt; 0.63</td>
</tr>
<tr>
<td>L-Proline (50 mM)</td>
<td></td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>L-Serine (50 mM)</td>
<td></td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>L-Threonine (50 mM)</td>
<td></td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>L-Glycine (50 mM)</td>
<td></td>
<td>&lt; 0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Spores were treated with 4 M urea-1% MCE-1% SDS for 3 hr and washed thoroughly with distilled water before use as described in Materials and Methods.

<sup>b</sup> Spores (OD at 450 nm = ca. 0.3-0.4) were incubated with specified concentrations of germinants in 50 mM Tris-maleate buffer (pH 7.0) supplemented with 100 mM NaCl. Germination was followed spectrophotometrically and the germination rate was determined as described in Materials and Methods.

<sup>c</sup> The percent of spores that germinated after 2 hr in each germinant. The percentage of germinated spores was determined by counting a total of 200 spores in a wet mount preparation under a phase contrast microscope.
Kinetics of L-alanine and adenosine induced germination of sensitized spores.

Fig. 11 illustrates the kinetics of L-alanine and adenosine induced germination of normal and sensitized B. cereus T spores. It is clear from this figure that the extraction for up to 3 hr progressively decreased the rate of physiological germination but further extraction under this condition did not affect their germination rate further (Fig. 12). The sensitization treatment also prolonged the lag time.

The effect of extraction on the extent of L-alanine and adenosine induced germination of B. cereus T spores is also depicted in Fig. 11. It is evident that the extraction process rendered the spores to germinate to a greater extent than normal spores. It appears that the extraction process increased the lag time for initiation of germination, decreased the rate of germination and increased the extent of germination of B. cereus T spores.

The lag time for germination of B. cereus T spores is known to be shortened when spores are preheated for 30 min at 65 C prior to exposing them to germination inducers (Vary and McCormick, 1965; Hashimoto et al., 1969). Similar findings were obtained in the present investigation (Fig. 13). As seen in Fig. 13, heat-activation did not increase the subsequent germination rate of normal spores but it did shorten the lag time for germination. Sensitized spores, in which the spore coat is partially solubilized, failed to respond to heat activation, i.e., there was no significant difference between the lag times of heat activated (65 C for 30 min) and unheated sensitized spores.
Fig. 11. Effect of extended time of extraction on the germination of sensitized spores. Spores were incubated in 50 mM Tris-HCl buffer (pH 8.3) containing L-alanine (5 mg/ml) and adenosine (2 mg/ml). Sensitized spores were obtained as described in Materials and Methods.

Symbols: ○—○, normal spores; ▲—▲, 3 hr sensitized spores; □—□, 9 hr sensitized spores; ◊—◊, 18 hr sensitized spores.
Fig. 12. Plot of $\ln \ln 1/Y$ vs $\ln t$ from the germination curves described in Fig. 11. Symbols: □—□, normal spores; ○—○, 3 hr sensitized spores; ★—★, 9 hr sensitized spores; ○—○, 18 hr sensitized spores. The above plot was drawn using the formula described by Vary and McCormick (1965).

\[ a = \ln \text{this formula } Y = e^{-kt^c} \]

when $Y = \text{ODi-ODt}/\text{ODi-ODf}$, represents the fraction germinated at time $t$ (where OD$_i$ is the initial OD, OD$_f$ is the limiting OD as the germination process approaches completion, and, OD$_t$ is the OD at time $t$). A plot of $\ln \ln 1/Y$ versus $\ln t$ gave a straight line with a negative slope and led to the above formula:

\[ K = \ln 1/Y_0 \]

\[ Y_0 = Y \text{ at time } t, \text{ and} \]

\[ c = \text{constant} \]
Fig. 13. Effect of heat activation on the germination of normal and sensitized \textit{B. cereus T.} spores. Experimental conditions were same as described in Fig. 11. Symbols: \(\textcircled{O}\), dormant normal spores; \(\textcircled{Q}\), heat activated normal spores; \(\square\), dormant sensitized (3 hr) spores; \(\blacktriangle\), heat activated sensitized (3 hr) spores.

The experiments reported in this section were done primarily to elaborate a suitable model system for studying the mechanism of germination. It is well established that the major changes occurring during germination of bacterial spores include rapid depolymerization of the cortex, and release from the spore of some of the oligosaccharide along with calcium and dipicolinic acid (Sussman and Halvorson, 1966; Gould, 1969). Spore lytic enzyme(s) initially described by Strange and Dark (1957) and Gould and Hitchins (1965) has been shown to initiate germination-like changes in sensitized spores (Gould and King, 1969; Frieben, 1971). The spore lytic enzyme has been implicated in germination (Frieben, 1971). It has also been suggested that the germination enzyme is bound to the spore integument (Gould, 1969; Frieben, 1971; Duncan et al., 1972; Gould and Dring, 1972). What is not certain is whether depolymerization of the cortex by the lytic enzyme is the prime event in germination or some other biochemical reaction(s) involving germination inducers precedes the lytic action of the enzyme(s) (Gould, 1969). It is well known that certain sporostats reversibly inhibit germination by preventing the triggering or initiating reactions (Sierra, 1970; Frieben, 1971; Lewis and Jurd, 1972). Whether such inhibitors interfere with the lytic activity of cortex lysing enzyme itself or not remained unclear.

In order to study the relation between triggering agents and the lytic process in vitro we attempted to isolate an enzyme integument complex by disruption of intact B. cereus T spores in the presence of initiation
inhibitors. Once such an enzyme-integument complex could be isolated, it would be useful for studying the mechanism of physiological or L-alanine induced germination in vitro. It would then be possible to elucidate the role of low molecular weight compounds (L-alanine, adenosine, etc.) in the initiation of bacterial spore germination.

In order to establish such an in vitro germination system, it is necessary to: (i) Investigate an assay system for cortex lysing enzyme; determine the optimum pH, temperature, cation requirement and suitable substrate for assaying the enzyme(s), (ii) Elaborate a method for isolating enzyme-integument complex; this may be achieved by using low temperature and suitable reversible inhibitors during disruption of spores or by use of inhibitors for the initiation of germination of spores.

Factors affecting the activity of CORLE: Selection of proper substrates for detecting activity of enzyme. Fig. 14 depicts the action of the lytic enzyme on variously treated spores. The enzyme was active on both unheated and heated (at 90°C for 30 min or at 121°C for 20 min) sensitized spores, but not on normal spores. Heated sensitized spores did not lose their optical density as much as unheated sensitized spores. Heated (121°C for 20 min) sensitized spores were used in the rest of the experiments, unless otherwise mentioned, for assaying and standardizing the lytic activity of the enzyme.

Optimum pH and cation requirements of the spore lytic enzyme(s). The enzyme seemed to be most active on sensitized spores at pH 7.5 (Fig. 15). It should be noted, however, that the lytic activity of the enzyme was
Fig. 14. Action of spore lytic enzyme on sensitized B. cereus T spores.

Spores were incubated in 50 mM sodium phosphate buffer (pH 7.5) containing 0.5 ml of the crude enzyme (25,000 x g supernatant). The mixture was incubated at 35°C. The enzyme preparation contained 840 µg protein/ml.

Symbols: ○—○, boiled (10 min) enzyme + sensitized spores; ▲—▲, normal spores; □—□, heated (121°C for 20 min) sensitized spores; ▲—▲, heated (90°C for 30 min) sensitized spores; ◦—◦, unheated sensitized spores.

Although the data are not shown here or elsewhere, no spontaneous germination of sensitized spores in water or buffers took place, under all conditions tested.
Fig. 15. Effect of pH on the lytic activity of CORLE. The test system consisted of heated (121°C for 20 min) sensitized spores (OD at 450 nm = 0.4), 0.5 ml crude enzyme (840 µg protein/ml, 25,000 x g supernatant) and the appropriate buffer (50 mM) in a total volume of 3.0 ml. The mixture was incubated at 35°C. O—O, phosphate buffer; ▲—▲, citrate phosphate buffer; □—□, Tris-maleate buffer; ✫—✫, Tris-HCl buffer; ●—●, Tris-HCl + 50 mM NaCl.
completely undetectable when Tris-HCl buffer was used (Fig. 15). The apparent inhibitory effect of Tris-HCl buffer was subsequently attributed to the absence of cation in the buffer. As shown in Fig. 15 addition of sodium chloride (50 mM) to Tris-HCl buffer, resulted in rapid restoration of the lytic activity on heated (121 C for 20 min) sensitized spores.

The effects of various cations on the lytic activity of the enzyme are shown in Fig. 16. It was found that in addition to sodium and potassium, magnesium and calcium ions had stimulatory effects on the activity of the enzyme (Fig. 16). Although the data are not shown, spermine diphosphate and manganese also stimulated the activity of CORLE. Sodium and potassium ions showed the maximum stimulatory effect at 100 and 200 mM cation concentration respectively, while the divalent ions calcium, magnesium, and manganese showed an optimum at 10 mM cation concentration (Fig. 16). The fact that EDTA progressively decreased the activity of CORLE when a given concentration of calcium ions was present, clearly indicated that cations are required for the lytic activity of the enzyme.

Effect of inhibitors on germination of B. cereus T. spores. Phenol or ethanol are known to inhibit the initiation of the physiological germination of normal B. cereus T spores induced by L-alanine and adenosine (Fig. 17). Although the data are not shown, both normal and sensitized spores failed to initiate germination in the presence of inducers (L-alanine and adenosine) when incubated with either 0.1% phenol or 5% ethanol for as long as 5 hr. However 33% of the spores initiated germination in L-alanine and adenosine when 5% methanol was used as the inhibitor. Removal of the
Fig. 16. Cation dependence of the lytic activity of CORLE. The test system consisted of 2 ml of sensitized spores (OD at 450 nm = ca. 0.3 - 0.4) in 50 mM Tris-HCl buffer (pH 7.5), 0.5 ml of the crude enzyme (25,000 x g supernatant), and 0.5 ml of a known concentration of the cation. The mixture was incubated at 35°C. Symbols indicate final concentration of cation used.

- - , 0 mM; -X-, 50 mM; - , 100 mM; - - , 200 mM; - , 500 mM; - , 10 mM Ca in presence of 5 mM EDTA; - - , 10 mM Ca in presence of 2.5 mM EDTA, O-O, 10 mM.
Fig. 17. Effect of 0.1% phenol, 5% ethanol or 5% methanol on L-alanine and adenosine induced germination of B. cereus T spores.

Normal and sensitized spores were incubated in the presence of 50 mM Tris-HCl buffer (pH 8.3) containing L-alanine (5 mg/ml) and adenosine (2 mg/ml) with or without the indicated inhibitor. The mixture was incubated at room temperature. •—•, Normal spores without any inhibitors; •—•, sensitized spores without any inhibitors; □—□, normal spores with 0.1% phenol; ○—○, normal spores with 5% ethanol; ▲—▲, normal spores with 5% methanol.
% REDUCTION IN OD_{450} nm

TIME (MIN)
inhibitors by centrifugation and subsequent resuspension of the pellet in
L-alanine and adenosine solution resulted in rapid and complete germination
of the spore suspension. These results suggest that alcohols and phenols
inhibit germination of B. cereus T spores by interfering with the triggering
mechanism(s). These findings are in agreement with the results of other
workers (Slepecky, 1963; Sierra, 1970; Trujillo and Laible, 1970; Lewis and

The above suggestion was further substantiated by an additional experi­
ment in which the inhibitor was added to the spore suspension at different
times after the initiation of germination (Fig. 18). It is evident from
Fig. 18 that only those spores which had not initiated germination were
inhibited by 5% alcohol. As shown in Fig. 18, the extent of completion of
germination of the spore suspension depended on the time of addition of the
inhibitor. Addition of the inhibitor after all the spores had initiated
resulted in complete germination of the whole population of spores.

The effect of 0.1% phenol and 5% ethanol on the activity of CORLE it­
self is shown in Fig. 19. Neither phenol nor alcohol inhibited the
activity of CORLE.

The above studies with sporostats support the idea that reaction(s)
triggered by L-alanine or adenosine is required before CORLE is activated
or released from a bound state during the early stages of germination. As
mentioned by others (Frieben, 1971; Lewis and Jurd, 1972), the initial
triggering event is susceptible to sporostats but the cortex lytic process
is not. So far, it has not been possible to obtain an enzyme-integument
Fig. 18. Effect of ethanol on the germination of B. cereus T spores.

Ethanol (5% final concentration) was added at specified time interval after heat activated normal spores (65°C for 30 min) were mixed with 50 mM Tris-HCl buffer (pH 8.3) containing L-alanine (5 mg/ml), and adenosine (2 mg/ml). The mixture was incubated at room temperature as described in Materials and Methods.
TIME (MIN) OF ADDITION OF ETHANOL (5%)
Fig. 19. Effect of 0.1% phenol and 5% ethanol on the activity of CORLE. Sensitized spores (OD at 450 nm = ca. 0.3-0.4) in 50 mM sodium phosphate buffer (pH 7.5), 0.5 ml CORLE (25,000 x g supernatant), and with or without the indicated inhibitors in a final volume of 3.0 ml. The mixture was incubated at 35 C.

▲—▲, sensitized spores without any inhibitors;
O—O, sensitized spores with 0.1% phenol;
■—■, sensitized spores with 5% ethanol.
complex. In a few exploratory experiments it was found that the bulk portion of CORLE was mostly released into the supernatant fluid upon the breakage of spores even in the presence of 0.1% phenol. (Fig. 20).
Fig. 20. Activity on sensitized spores of CORLE prepared under different conditions of disruption. The methods are elaborated in Materials and Methods. The figure indicates the activity of CORLE prepared (i) under normal conditions of disruption (---), i.e. in the absence of any inhibitors) and (ii) in the presence of 0.1% phenol (□□□). No enzyme was recovered when the integument obtained in (ii) was pelleted and was resuspended either in (iii) 50 mM Tris-HCl (pH 8.3, ▲▲▲) buffer only or in (iv) 50 mM Tris-HCl buffer (pH 8.3, ●●●) containing L-alanine (5 mg/ml) and adenosine (2 mg/ml).
DISCUSSION

The present study has shown that the integrity of the coat is important for maintaining the maximum resistance of bacterial spores not only to heat but also to some chemicals and to muramidases. The spore coat also seems to play an important role in physiological germination.

The fact that extraction of *B. cereus* T spores with 4 M urea-1% MCE-1% SDS did not affect their viability (Fig. 1, Table 1) or refractility (Fig. 2) seems to indicate that the integrity of the spore coat is not essential for the viability or refractility of spores.

The urea-MCE-SDS extract of *B. cereus* T spores appears to contain predominantly protein which migrates as a single band during polyacrylamide gel electrophoresis (Fig. 3). Similar results were obtained by Sommerville *et al.* (1970) with spores of *B. thuringiensis* and other bacilli; by Wyatt and Waites (1972) with spores of *Cl. bifermentans*, by Wood (1972) with *B. subtilis* spores; and, by Aronson and Horn (1972) with *B. cereus* T spores.

The increase of specific gravity of spores after 4 M urea-1% MCE-1% SDS extraction (Fig. 6) or similar treatments was also noted by Aronson and Fitz-James (1971) and by Wood (1972). No explanation for the change in density of sensitized spores was given by these workers. This increase in density may be best explained by assuming the spore to be made up of an inner dense core region (nucleic acid, calcium and dipicolinate) and an outer less dense integument (mostly polysaccharide and protein). The loss of relatively lighter components (protein) due to sensitization treatment results in the relative gain in specific density.
The spore coat seems to be involved in resistance of spores to lytic enzymes (Fig. 1, 2). This is probably due to the alteration and subsequent solubilization of the spore coat (Fig. 4, 5) by the extraction procedure allowing the direct access of lytic enzymes to the susceptible underlying cortex (Warth et al., 1963b).

Heat resistance of spores is generally a function of time with respect to temperature. Sussman and Halvorson (1966), Roberts and Hitchins (1969) and Russell (1971) have reviewed some theories of bacterial spore heat resistance. Various workers have related the heat resistance of bacterial spores to their cation and DPA content (Levinson et al., 1961; Murrell and Warth, 1965). Increased heat resistance has been attributed to increased calcium: dipicolinate ratios (Levinson et al., 1961) and decreased magnesium: calcium ratios (Murrell and Warth, 1965). In this investigation it was found that the sensitized spores did not differ significantly from normal spores with respect to their DPA and calcium content (Table 2). However, it is evident from Fig. 7 that sensitized spores are less heat resistant than normal spores if appropriate temperatures are used. The data presented seem to indicate that an intact coat may be required for attaining the maximal degree of heat resistance in bacterial spores.

Halvorson and Swanson (1969) from their studies with DPA-less mutants suggested that DPA is the primary factor in conferring heat resistance on spores. However it was observed (Halvorson and Swanson, 1969) that the "spent medium" from the DPA-less mutant contained more methionine and cysteic acid than was found in the "spent medium" in which the wild type
spores were produced. They could not interpret the significance of this latter finding. It is well established that one of the major biochemical events associated with spore coat formation is the incorporation of cystine into the spore coat at the time of spore coat formation. (Aronson and Fitz-James, 1968). It is probable that the DPA-less mutant (Halvorson and Swanson, 1969) may have an incomplete immature spore coat. It is likely therefore that the decreased heat resistance of the DPA-less mutant spores may be due to the dual effect of an abnormal spore coat and lack of DPA.

In contrast to our observation, other workers (Gould et al., 1970; Sommerville et al., 1970; Aronson and Fitz-James, 1971; Cassier and Ryter, 1971; Wood, 1972) found no alteration in the resistance to heat in sensitized spores. This discrepancy may be due to (i) The difference in temperature selected for determining heat resistance; Aronson and Fitz-James (1971) did not mention their condition of testing heat resistance, whereas Sommerville et al (1970) used a suboptimum temperature of heat inactivation. Our data (Fig. 7) indicate that heat sensitivity of sensitized spores can be demonstrated only at 85 °C, and more pronouncedly at 90 °C, but not at 80 °C. (ii) The different condition used for sensitizing spores; Gould and his coworkers (1970) sensitized spores by treatment with 7 M urea containing 10% MCE, followed by 0.1 M NaOH to sensitize B. subtilis spores. However when we used alkali under similar conditions of treatment (4 M urea, 1% MCE, followed by 0.1 M NaOH treatment), sensitized spores tended to aggregate rendering the quantitative viable count difficult. (iii) Difference in the nature of spore coats of different species; Holt and Leadbetter (1969) and
Short and Walker (1971) reported that the fine morphology of the coat of spores varied significantly from species to species. Hence, difference in resistance to heat may be due to a difference in the morphology and integrity of the spore coats of different spores.

Although spores are more resistant than vegetative cells to most chemicals, very little is known about the mechanism by which spores acquire this resistance. The possible mechanisms by which spores may become resistant to chemicals was recently discussed by Roberts and Hitchins (1969). The results obtained in the present investigation (Fig. 8) strongly suggest an important role for the spore coat of *B. cereus* T spores in resistance towards glutaraldehyde. Balassa and Yamamoto (1970) isolated a *B. subtilis* mutant which produced heat sensitive but chloroform and octanol resistant spores, whereas the wild type spores were resistant to all these agents. This was attributed by these workers (Balassa and Yamamoto, 1970) to a morphological difference of the envelopes between the mutant and wild type spores. The mutant spores had a better resolved lamellar structure of the coats and the cortex was more transparent than the wild type spores. The importance of spore coat in chemical resistance was further shown by Millet and Ryter (1972) who reported that cortex-less mutant spores of *B. subtilis*, which possessed spore coats, was resistant to chloroform. Cassier and Ryter (1971), however, reported that spores of *Clostridium perfringens* treated with reducing agents and alkali were still resistant to ethanol and octanol.

Romig and Wyss (1957) and Vinter (1960, 1961, 1962) have shown that resistance of spores to radiation is acquired during sporulation at the
time of coat formation. The addition of cysteine or thioproline to sporulating cultures of B. cereus slightly increased radiation resistance (Vinter and Vechet, 1964). Spore resistance was also correlated to an increased ratio of disulfide bonds to thiol groups (Bott and Lundgren, 1964) in two strains of B. cereus. However, when spores were treated with thioglycollic acid, which ruptured at least 10-30% of the spore disulfide bonds, they did not lose resistance to gamma radiation. It is doubtful that these disulfide bonds play a significant role in radioprotection of spores (Hitchins et al., 1966).

The results obtained in the present investigation (Fig. 9) are compatible with the idea that the spore coat is not involved in resistance to UV light (Sommerville et al., 1970). Resistance to UV light is more likely due to a modified state of spore cytoplasm and DNA (Donnellan and Setlow, 1965; Donnellan and Stafford, 1968; Stafford and Donnellan, 1968; Varghese, 1970) or the presence of DPA (Woese, 1959; Leif and Herbert, 1960; Berg and Grecz, 1970, Grecz et al., 1973). Recently Grecz et al. (1973) have shown that resistance to UV is related to the amount of DPA in the spores and that inactivation by UV is reduced if DPA-less spores are irradiated through calcium dipicolinate filters.

The inability of normal and sensitized spores to be stained by 1% methylene blue suggests that the coat may not be the sole permeability barrier for bacterial spores. It is likely that the cortex may be involved in blocking the permeation of the dye into the spore protoplast. It was shown earlier that stain resistance develops during formation of the cortex
in sporulating cells (Hashimoto and Naylor, 1958; Hashimoto et al., 1960).

It is generally recognized that different organisms show different survival rates when desiccated or lyophilized (freeze-dried; Fry, 1966). In the present investigation it was shown that both sensitized and normal spores resisted desiccation or lyophilization to the same extent, suggesting that the integrity of the spore coat does not play a significant role in the acquisition of resistance of bacterial spores to desiccation. Gould and his associates (1966) have shown that the germination enzyme involved in germination could be inactivated by oxidation or by other reagents such as p-chloromercuribenzoate, iodine or CuSO$_4$. Recently it was shown by Stewart and Lee (1972) that desiccation of mosses affected the activity of a photosynthetic enzyme, NADP$^+$ glyceraldehyde-3-phosphate dehydrogenase, by oxidizing its sulfhydryl residues. Its activity, however, was restored by the addition of reduced glutathione. The fact that sensitized spores resisted desiccation in vacuo or in air (oxygen) suggests that the enzymes essential for germination and subsequent outgrowth appear to be protected even after the extensive modification of coat layers.

Other workers have demonstrated that treatment of spores with protein denaturing and protein solubilizing agents affect their germination requirements (Sommerville et al., 1970; Cassier and Ryter, 1971; Wyatt and Waites, 1971; Duncan et al., 1972; Waites et al., 1972). Treatment of Cl. perfringens spores with alkali rendered them totally dependant on lysozyme or an initiation protein for germination (Cassier and Ryter; 1971; Duncan et al., 1972). On the other hand, some investigators reported relaxation of
germination requirements as a result of sensitization treatment of spores. Treatment of *B. thuringiensis* spores (Sommerville *et al.*, 1970) with urea-MCE, increased their subsequent germination rate and lowered the concentration of germinants required for rapid germination. Similarly, alkali treatment of *C. bifermentans* spores relaxed their germination requirements and increased their subsequent germination rate (Wyatt and Waites, 1971; Waites *et al.*, 1972). These workers (Wyatt and Waites, 1971; Waites *et al.*, 1972) attributed the increased germination rate to the increased permeability of the spore to germinants.

In contrast to these observations, we observed consistently that sensitized spores of *B. cereus* T obtained by urea-MCE-SDS extraction exhibited a remarkably slow germination in all nutrients tested (Table 4). This may be due to the partial solubilization of enzymes involved in germination (Frieben, 1971).

Keynan and Evenchik (1969) and Sogin *et al.*, (1972), suggested that there may be different recognition sites on the spore for the various germination inducers. Our findings (Table 4) that the sensitized *B. cereus* T spores retained their capability of being germinated by some nutrients (L-alanine or L-cysteine) but not by ribosides (inosine or adenosine) supports their theory. Our results also imply that enzymes involved in inosine or adenosine induced germination may be located on the spore surface and are modified, inactivated or solubilized during the sensitization treatment.
Although Gould et al. (1966) considered that the spore lytic enzyme of B. cereus was contained within the central core of the spore, the recent observations of Frieben (1971) that the urea-MCE-SDS treatment could solubilize the lytic enzyme without affecting viability makes the localization of the lytic enzyme in the core very unlikely in B. cereus.

Excellent reviews (Lewis, 1969; Slepecky, 1969; Gould, 1969; 1970; Vinter, 1970; Gould and Dring, 1972) on germination are available in the literature. However, the exact biochemical mechanism of germination is at the present time only poorly understood. Various mechanism(s) of germination suggested are (i) connecting reactions in which the initiators are thought to undergo metabolic degradation, (ii) participation of DPA (iii) involvement of spore lytic enzymes and (iv) role of free ion levels within spores. These hypothetical mechanism(s) of germination have been reviewed by Gould and Dring (1972).

Although we were not able to establish a workable model for studying germination in vitro by isolating an enzyme-integument complex in our limited trials, the facts that various compounds such as phenols and alcohols reversibly inhibit the initiation but not the progress of physiological germination still strongly suggest the feasibility of such attempts in the future. However, our preliminary investigation along this line of research has added several new pieces of information which might be useful in the assay of CORLE of B. cereus T spores.

The optimum pH for CORLE activity was found to be 7.5 (Fig. 15) and it was subsequently found that the enzyme required a cation for its lytic
activity (Fig. 16). Furthermore, the enzyme could act either on unheated or heated sensitized spores (Fig. 14).

The finding that sensitized spores were susceptible to the lytic action of CORLE in various buffers tested except Tris-HCl buffer suggested that either certain cations were absolutely required for the lytic activity of the enzyme, or that Tris-HCl contained inhibitors for the enzyme. The data presented in Fig. 16 favors the former possibility. It is interesting to note that Addington (1969) observed that sodium ions were essential for spontaneous germination of extensively heat activated spores. The efficient germination of spores in Tris-HCl buffer in the absence of exogenous cations (Boylen and Ensign, 1968; Hashimoto et al., 1969) may be due to the presence of sufficient endogenous ions in spores. It is unlikely that the endogenous calcium dipicolinate may provide the essential ions, as viable (unheated) sensitized spores containing the normal amounts of calcium and DPA, failed to undergo germination-like changes when CORLE was used as the inducer (unpublished data).
SUMMARY

Some properties of *B. cereus* T spores sensitized to muramidases by chemical treatment were investigated in order to elucidate the role(s) of the spore coat in resistance to different physical and chemical agents and in physiological germination. Treatment of spores with 4M urea-1% mercaptoethanol-1% sodium dodecyl sulfate for 2-3 hr at room temperature rendered the spores completely susceptible to muramidase (lysosozyme and cortex lysing enzyme), without loss of viability. Electron microscopy of spores undergoing sensitization revealed that the main change occurred in the spore coat; an electron dense middle layer apparently associated with the rodlet structure of the coat was gradually removed while the outer and inner coats were removed as blebs during the sensitization treatment. The fully sensitized spores lost 15-25% of their initial dry weight. This loss in dry weight was partially accounted for by the protein solubilized. The protein solubilized migrated as a single major band on polyacrylamide gel electrophoresis. The sensitized spores still appeared refractile under a phase contrast microscope and retained most of their calcium and dipicolinic acid but showed a reduction in dry weight (15-25%) and a slight increase in specific gravity (1.256 to 1.266).

The sensitized spores displayed an almost equal degree of resistance to ultraviolet radiation, desiccation and stain as that of the normal spores but became more susceptible to glutaraldehyde and heat.

The sensitized spores germinated at a slower rate than the normal spores in the presence of some physiological germinants. In addition, the
sensitized spores lost their ability to be germinated by ribosides, especially by inosine.

It was concluded that the coat of *B. cereus* T spores plays a significant role in the resistance of spores to moist heat, lytic enzymes and certain chemicals, and in physiological germination.

The autoclaved, sensitized spores were shown to be a suitable substrate for demonstrating and quantitating the lytic activity of the cortex lysing enzyme (CORLE). Using such spores, it was shown that CORLE required cations for its full activity.

Preliminary attempts to establish a model system to study the mechanism of germination at subcellular level were not successful.
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APPROVAL SHEET

The thesis submitted by Pravin C. Patel has been read and approved by the members of the Advisory Committee listed below.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

July 16th, 1973

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