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ACTIVATION OF BACTERIAL ENDOSPORES

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ABSTRACT

A. KEYNAN (Israel Institute of Biological Research, Ness Ziona, Israel), Z. EVENCHIK, H. O. HALVORSON, AND J. W. HASTINGS. Studies on the activation of bacterial endospores. J. Bacteriol. 88:313-318. 1964.—Heat activation of bacterial endospores was imitated by suspending spores in reducing agents (mercaptoethanol or thioglycolate) or in a pH less than 4.5. Urea (6 M) had no effect on spores. In addition to the well-known activation at 65 C for 45 min, spores were also activated by exposure to 34 C for 48 hr. The activation by heat and by reducing agents was reversible; the reverse reaction was temperature-dependent. No reversion occurred at −20 C, whereas at 28 C the spores reversed to their original dormant state within 72 hr.

It is suggested that the heat-activation phenomenon could be explained by assuming that heat or reducing agents change the tertiary structure of a protein responsible for the maintenance of the dormant state by reducing the disulfide linkages which stabilize the protein in a specific configuration. The partial denaturation of this protein is reversible by reoxidation of the reduced disulfide bonds.

It has been known for a long time (Evans and Curran, 1943) that fresh suspensions of some bacterial endospores will not (or only very slowly) germinate unless heated for some time. This process is known as heat activation. The temperature and duration of optimal heating for this effect vary widely among different species and even among different spore preparations of the same strain. Heat activation is lost during storage and is, therefore, a reversible process, but spores can be reactivated by a second treatment (Church and Halvorson, 1957; Powell and Hunter, 1955). In addition to the stimulation of germination, heat also activates certain enzymes which are not active in the dormant spore (Church and Halvorson, 1957; Krishna Murty, 1957). A reversal of calcium dipicolinate-activated spores by treatment with acid was reported by Lee and Ordal (1963).

Germination is the irreversible conversion of a dormant bacterial endospore into a metabolically active cell. Rapid and complete germination occurs only after activation, and is triggered by specific germination-inducing agents, such as L-alanine. The breaking of dormancy thus involves two superimposed mechanisms. The first consists of the reversible activation of the spore by heat or other agents, and the second consists of the irreversible germination process which can be induced only in the activated spore. Spores activated by storage for long periods of time will germinate upon addition of germination-inducing agents without heat treatment, but this is not reversible.

Although much work has been done describing different aspects of heat-activation (reviewed by Murrell, 1961), its mechanism is unknown and there is no theory which will account for all facts known about this phenomenon.

In the present paper, some experiments are described in which an attempt was made to imitate heat activation by chemicals, to investigate the influence of pH and temperature on activation, and to study the conditions under which heat activation is lost. From these observations, a working hypothesis is suggested to explain the molecular basis of these phenomena.

MATERIALS AND METHODS

Preparation of spore suspensions. Spores of *Bacillus cereus* strain T were grown in 30 liters of G medium (Church and Halvorson, 1957) in a 65-liter fermentor for 19 hr at 28 C. The conditions of aeration were: agitation at 550 rev/min and 2 liters of air per min (oxygen absorption rate, 0.6 mmoles of oxygen per liter per min). A detailed account of the method of growth will be published elsewhere (Miller, *personal communication*). The spores were washed several times in distilled water in a refrigerated centri-

fuge, lyophilized, and stored at 4 C. The spores used in the present investigation contained 5% dipicolinic acid, as determined by the method of Janssen, Lund, and Andersen (1958).

Heat activation of spores. Unless otherwise stated, a suspension of 1 mg of dry spores per ml of distilled water was heat-activated by exposure to a temperature of 65 C for 45 min. This was sufficient to give optimal germination rates under the conditions described below.

Germination conditions and measurements. After heat activation, the spores were cooled to room temperature, centrifuged, and resuspended in 0.01 M phosphate buffer (pH 7.8) to a final spore concentration of 2 mg/ml. Germination was initiated by addition of L-alanine and adenosine to give final concentrations of 10 and 5 μg/ml, respectively. The course of germination at 30 C was measured spectrophotometrically as described previously (Keynan, Murrell, and Halvorson, 1962). The rate of germination was calculated as the per cent decrease in optical density per minute during the first 20 or 30 min after addition of the germination-inducing agent. The "germination lag" (time interval between addition of L-alanine and beginning of decrease of optical density) in all these experiments was constant, and of the order of 2 to 3 min.

Reagents. Dipicolinic acid was obtained from Aldrich Chemical Corp., Milwaukee, Wis. Fresh solutions of mercaptoethanol and thioglycolic acid were prepared prior to each experiment. Other reagents were of analytical grade.

RESULTS

Activation of spores by reducing agents. If the changes that occur in spores during heat activation involve splitting of covalent or ionic bonds, then one might expect that certain chemical agents could mimic or spare heat treatment. Several compounds, which are not themselves germinating agents, were found to serve as activation agents, thus imitating the effect of heat.

An experiment was conducted to illustrate this effect (Fig. 1). Freshly prepared spores remained dormant for 22 hr or more upon incubation in succinate buffer at pH 5.6 at 28 C. After a longer incubation in this buffer and under the same conditions (36 hr), there occurred some spontaneous activation. Activation by heat treatment at all times was fully effective. Incubation in the presence of either mercapoethanol or thioglycolic acid resulted in a partial activation of the spores. A minimal time of preincubation with the reducing agent was required, since no effect was observed in less than 12 hr.

Effect of pH on activation. The activation of spores in the presence or absence of reducing agents is influenced by pH. Below pH 4.5 (Fig. 2), the pH dependence of spontaneous activation parallels that of activation induced by reducing agents, whereas between pH 4.5 and 7.2 the activation was more pronounced in the presence of reducing agents. If we assume that these two effects are additive, then the pH optimum for activation by reducing agents is about 6.0 (curve E).

The marked activation which occurred in spores incubated at a low pH is particularly interesting, and may in fact be a significant method of breaking dormancy in nature. To exclude a specific role of succinate in this phenomenon, the experiments were repeated with citrate and phosphate buffers over the same pH range.

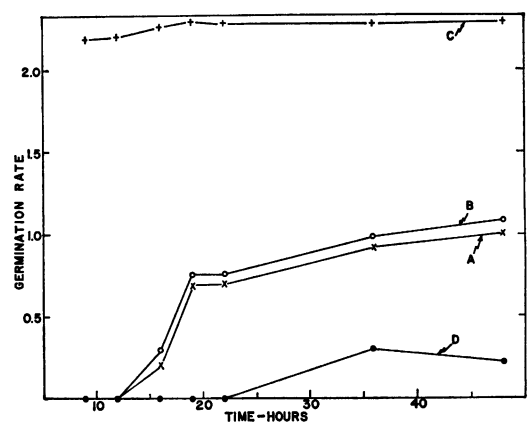

FIG. 1. *Effect of preincubation with mercaptoethanol and thioglycolic acid on germination rates of a spore suspension of Bacillus cereus T. Non-heat activated spore suspensions (1 mg of dry spores per ml) in 0.2 M succinate buffer (pH 5.6) were incubated at 28 C for varying times in the presence of (A) 0.2 M mercaptoethanol, (B) 0.02 M thioglycolic acid, and (C and D) 0.2 M succinate buffer. Samples were taken at times indicated, washed twice in distilled water, and resuspended in 0.1 M phosphate buffer (pH 7.8); only C was heat-activated before the final resuspension in phosphate buffer. Germination was induced and rate of germination was measured over the first 30 min as described in Materials and Methods.*

In all cases, the results were the same as those described in Fig. 2.

Although we are not able to offer a simple explanation for the activation which occurred at a low pH, it appears not to be related simply to the breaking of hydrogen bonds, since high concentrations of urea (6 M) had no effect on either the pH activation or the temperature activation described below.

Effect of temperature. In spores of *B. cereus* T, the rate of germination increases almost linearly with the time of heat-activation up to a maximum (O'Connor and Halvorson, 1961). The effect of the temperature of heat activation upon the germination rate is illustrated in Fig. 3. The critical temperature for activation is dependent upon the duration of the heat treatment. The maximal germination rate (curve D) is achieved with a 45-min incubation time at 60 C or with a 48-hr incubation time at 34 C. However, for a given incubation time, as the temperature of incubation is decreased, the rate of germination rapidly declines from the maximal germination rate, and approaches that of the dormant spore. A characteristic feature of this relationship is the existence of a critical temperature range, indicating that the apparent energy of activation for the process is very high. This suggests that the phenomenon may involve the disruption of tertiary structures in some molecules; the curves in Fig. 3 resemble the so-called melting curves for double-stranded nucleic acids—or the curves for temperature-denaturation of proteins.

Attempts were made to shift the temperature response by incubation in the presence of reducing agents. As shown in Fig. 3, no great sparing effect was measured; the criticial temperature in the presence of these agents was no more than a degree or two lower than the controls.

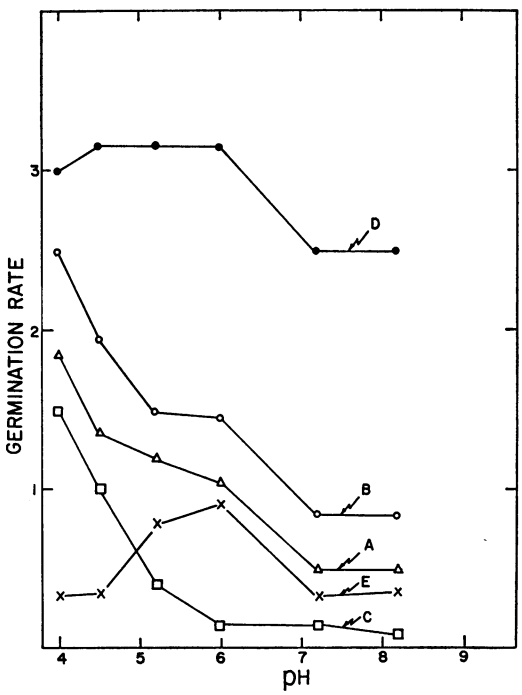

FIG. 2. *Effect of the pH of preincubation on germination rates of spore suspensions of Bacillus cereus T. Non-heat activated spore suspensions (1 mg of dry spores per ml) were incubated at 28 C for 48 hr at the pH indicated with 0.2 M succinate buffer between pH 4 and 6, and with 0.2 M phosphate buffer between pH 7.2 and 8.2. (A) 0.02 M mercaptoethanol, (B) 0.02 M thioglycolic acid, (C) without heat shock, (D) with heat shock. Curve E was obtained by subtracting curve C from curve A. See text for additional details.*

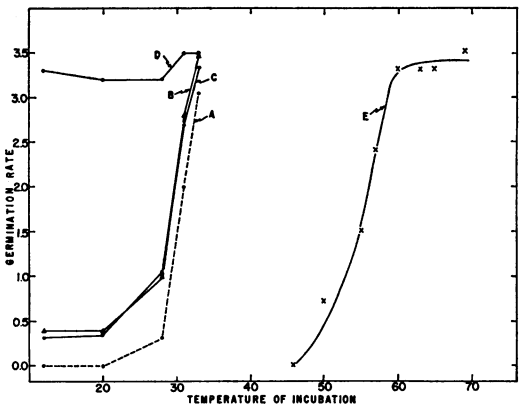

FIG. 3. *Critical temperatures for heat activation. The experiments illustrated at the left of the figure (A, B, C, and D) were carried out by incubating spore suspensions for 48 hr, whereas in curve E a 45-min incubation time was used. The spores were suspended during incubation in 0.2 M succinate buffer (pH 5.6). They were then washed twice in distilled water, resuspended in phosphate buffer at pH 7.8, and rates of germination over the first 20 min were determined. (A) Incubation for 48 hr followed by germination assay. (B) As in A but with 0.02 M mercaptoethanol during incubation. (C) As in A but with 0.02 M thioglycolic acid during incubation. (D) As in A but with heat activation (65 C for 45 min) after incubation; this illustrates potentiality of spores. (E) Incubation for 45 min followed by germination assay.*

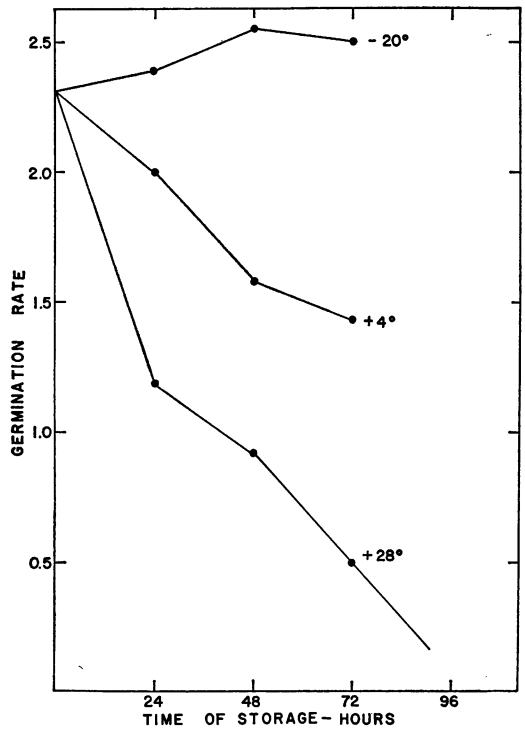

FIG. 4. *Influence of temperature on the rate of deactivation of heat-activated spores of Bacillus cereus T. Suspensions of freshly heat-activated spores were suspended in distilled water and stored at the temperature indicated. After 24 and 48 hr, the spores were centrifuged and resuspended in phosphate buffer (pH 7.8), and the rates of germination were estimated over the first 20 min as described in Materials and Methods. The rate at zero time was measured immediately after the heat shock.*

Reversal of heat activation of spores. The conversion of a dormant spore to an activated spore is a reversible process. Two properties of activated spores, rapid germination and the newly acquired metabolic activity (Church and Halvorson, 1957; Powell and Hunter, 1955), are lost when activated spores are stored for some period of time.

The reversal of heat activation was temperature-dependent (Fig. 4). After 72 hr of storage at 28 C, the rate of germination decreased by 80%, to nearly that of a dormant spore. At 4 C, however, the rate of germination decreased only 40% during the same period of time. At −20 C, no deactivation was observed. When the spores stored at 28 or 4 C were re-exposed to a second heat treatment, they germinated normally in the presence of L-alanine and adenosine.

Activation caused by reducing agents was also reversible. Spores activated by mercaptoethanol, washed, and resuspended in distilled water lost their ability to germinate after 48 hr. After a heat treatment or re-exposure to reducing agents, they were fully reactivated.

DISCUSSION

The activation of dormant biological systems by temperature is not restricted to bacterial spores. For example, temperature is a factor involved in breaking dormancy in such diverse systems as fungal spores, plant seeds, tree buds, and the diapause in insects. Although the effect of "heat activation" on bacterial spores is well documented (Murrell, 1961), there exists no hypothesis consistent with all known facts which could explain this phenomenon at the molecular level.

Several hypotheses concerning the nature of heat activation have been published. They are mainly based on the assumption either that heat releases a substance necessary for germination or that heat inactivates an inhibitor of germination. The first hypothesis was postulated by Goddart (1939), who assumed that in *Neurospora* the germination rate is determined by the internal concentration of a stimulatory compound which is limiting during dormancy but produced by a heat-activated reaction. Levinson (1961) noted that heat reduces the glucose or L-alanine requirements for germination of spores of *Bacillus megaterium*. He proposed that heat causes the release of a germination stimulant within the spore, which does not support germination alone, but can react with exogenously added compounds to increase the germination rate. The release of dipicolinic acid and L-alanine during heat activation lends support to this hypothesis. According to this hypothesis, the reversibility of heat activation could be explained by assuming that the substances released during heat activation diffuse out during storage. This explanation, however, is not consistent with the facts described in the present system. First, diffusion is relatively temperature-independent, whereas uptake is strongly temperature-dependent. Second, washing of heat-activated spores, which would be expected to remove the endogenous stimulant, has no effect on the reversibility of heat activation.

To test the specific proposal that dipicolinic

acid, a compound known to be released during heat activation (Levinson and Hyatt, 1960), might be one of the factors responsible for the increase in rate of germination after heating, several of the experiments described in Fig. 1 to 3 were repeated in the presence of dipicolinic acid. We also investigated the effect of exogenously added dipicolinic acid on heat activation itself. In all these cases, dipicolinic acid had no effect.

The second hypothesis that heat inactivates substances which prevent germination of spores is based on the fact that such germination-inhibiting substances are known to exist in plant seeds or mold spores (Juhren, Went, and Phillips, 1956). The reversibility of heat activation also makes this explanation unlikely.

Spores contain five times more sulfur than is present in vegetative cells (Vinter, 1960, 1961, 1962). This additional sulfur is concentrated in spore coats as cystine. It is, therefore, likely that the macromolecule responsible for maintaining the dormant state is a coat protein rich in cystine, stabilized in a specific configuration by S—S linkages. Reduction of these linkages would change the tertiary structure, resulting in the partial unfolding of the protein. This change in the structure of the protein could be responsible either for the uncovering of the active enzymatic sites necessary for germination or for the increase of the accessibility of the substrate to them.

This notion that heat activation is a reversible denaturation-like phenomenon of a spore protein would be consistent with the known facts of heat activation, as well as with those concerning the activation by reducing agents or by low pH. According to this theory, the reverse reaction would consist of the reoxidation of SH groups, reformation of the appropriate S—S bridges, and therefore of the original tertiary structure of the protein. A direct proof for the assumption that protein containing S—S bonds protects certain sites in the spore was given by Gould and Hitchins (1963). They demonstrated that spores are sensitized to lysozyme when treated with reagents which rupture disulfide bonds. The reducing agents used in our experiments did not replace heat activation completely. This might be explained by assuming that in spores the S—S bonds are nonrandomly distributed, and only some of them are accessible to added reducing agents.

Heat activation of spores has generally been studied by exposing spores for a short duration to comparatively high temperatures (65 to 70 C), far above the physiological temperature optimal for these organisms. The ecological significance of this fact was rather obscure, and most workers assumed that "heat activation" was a convenient but artificial way of initiating the physiological "aging" process in the laboratory. The findings described in this paper indicate that when longer exposure times are involved, considerably lower temperatures are effective in activating the bacterial spore. In fact, temperatures such as 30 C are ones which one could well expect to occur in soil or in decomposing organic material, indicating conditions favorable to germination. It can also be assumed that "aging" might simply be a "temperature activation" at a lower temperature for longer periods of time, as suggested by the relationship illustrated in Fig. 3. The one difference between these two phenomena is that "aging" is nonreversible. In this connection, it should be noted that in several of our experiments spores stored for 3 years, although still responsive to heat activation, had lost the ability to reverse this reaction. If we assume that some kind of equilibrium exists between activation and its reversal in spores, then the "aging" phenomenon could also be viewed as loss of the ability to reverse activation.

ACKNOWLEDGMENTS

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