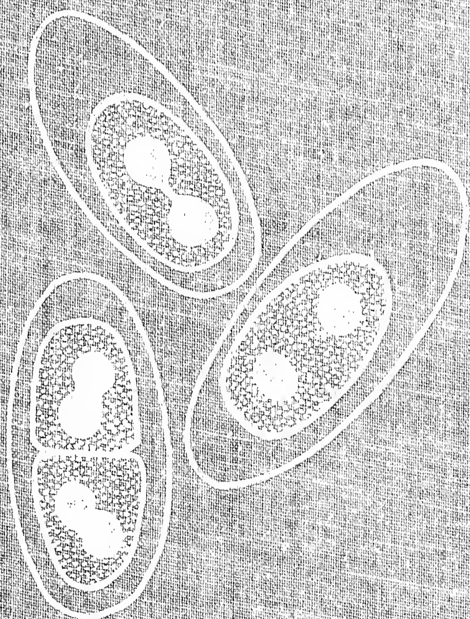

Spores

Edited by
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SPORES

Spores

A Symposium held at Allerton Park, Illinois October 11-12, 1956 under the auspices of the University of Illinois and with the support of the Office of Naval Research.

EDITED BY

H. ORIN HALVORSON

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Introduction

At the meeting of the Society of American Bacteriologists in Houston in 1956, a numer of papers dealing with bacterial spores were put together in one section and they engendered so much discussion that it could not be completed during the time allotted. As a result, a group of the interested persons got together for a "rump" session one evening and continued the discussion into the early hours of the morning. This assured us that a conference on spores would be worthwhile and as a result the members participating in this discussion suggested that I try to arrange such a conference at the University of Illinois some time during the fall of 1956. Following correspondence with Dr. Roger Reid of the Office of Naval Research, a spore conference was finally arranged to be held at Allerton Park on October 11-12, 1956, under the auspices of the University of Illinois and with the support from the Office of Naval Research. An attempt was made to invite to this conference all the persons in the United States who were actively engaged in research on sporulation, spore germination and the enzymes of spores. Also, persons were invited from Canada who were interested in spore cytology, as well as Mrs. Powell from Porton, England. The papers presented herein are those given at the spore conference together with the formal and informal discussions.

The people participating in this conference expressed their sincere appreciation to the Office of Naval Research for making this conference possible, and all of them felt that the discussions stimulated more widespread investigations into the properties of the bacterial spore.

H. Orin Halvorson
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Chairman, Committee on Arrangements

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The Mineral Requirements for Sporulation

Harold R. Curran

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ALTHOUGH scores of papers have been published on various aspects of spore formation, relatively few have been concerned directly with the role of minerals in sporogenesis. Salt effects on sporulation were reported by Behring as early as 1889, and by Schrieber in 1896. However, their real significance in the production of spores was not recognized until many years later. Thus, Cook, in his comprehensive review "Bacterial Spores" (1932), concluded "it does not appear that salts exert a direct influence on sporulation." The inability of early workers to devise chemically defined media that would produce spores in quantity contributed to the slow development of knowledge in this field. Cook (1931) and Tarr (1932) were the first to overcome this difficulty, the latter by showing that good sporulation by several aerobic species could be obtained by cultivation in a mineral salts medium containing low concentrations of secondary ammonium phosphate and sucrose. Roberts (1934) obtained 60-70% sporulation of *Bacillus subtilis* with suitable minerals supplemented with asparagine and levulose. Leifson (1931) studied the effects of inorganic salts on the sporulation of *Clostridium botulinum* in 1% peptone. The basal medium, which by itself produced no spores, was supplemented with a variety of mineral salts used alone or in combination. Sporulation occurred only when the supplement contained NH_4^+ and PO_4^{---} and to some extent SO_4^{--} ions. The Ca^{++} ion stimulated sporulation when added to NH_4^+ and PO_4^{---} ions.

Further evidence that peptone is deficient in minerals for sporulation was supplied by Fabian and Bryan (1933), who observed greatly increased sporulation of four mesophilic aerobes when the peptone solution was suitably fortified with cations of the univalent chloride salts; di- and tri-valent ions were without effect. Meat infusion tryptone glucose broth was shown by Knaysi (1945) to be deficient in Mg^{++} for the sporulation of *Bacillus mycoides*. The work of Foster and Heligman (1949) indicated that not only peptone but most complex organic media, when used in the fluid state, do not provide an adequate supply of minerals for the sporulation of *Bacillus cereus*; the chief limiting factor in asporogenic media was shown to be an

insufficiency of K^+. The specificity of Mn^{++} for the sporulation of *B. subtilis* was first shown by Charney and his associates (1951). In both chemically defined and in complex organic media, sporulation was negligible without added Mn^{++}, although this supplement was not required for full vegetative growth.

These findings were confirmed and extended by Curran and Evans (1954), who also observed that iron, when used in relatively large amounts, replaced Mn^{++} in Mn-deficient media. Weinberg (1955) concurred in these findings but suggested that the sporogenic activity of the added $Fe^{++(+)}$ could be largely attributed to appreciable quantities of Mn^{++} contained as impurity in CP grade Fe compounds. In agreement with Weinberg, we have since found that a sample of $FeCl_3$ which in nutrient broth exhibited characteristic sporogenic activity for *B. subtilis* at 200 μg/ml, when purified by repeated extraction with HCl and with ether, became sporogenically inactive in the same medium at levels of iron in excess of 200 μg/ml. The spore-inducing property of the medium containing purified iron could be restored by small additions of Mn^{++}.

The careful studies of Grelet (1951, 1952a, 1952b) have contributed valuable information concerning the minerals required for sporulation by *Bacillus megatherium*. With shake cultures in a glucose-mineral-salts medium, the effect on sporulation was determined for a number of mineral constituents. Each constituent of the medium was decreased independently, and its approximately limiting concentration for sporulation determined. Depletion of glucose and nitrate in the control medium was followed by rapid and almost complete sporulation; similarly, sporulation remained at a high level with progressive reductions in the NO_3^-, SO_4^{--}, Cl^-, Na^+, $Fe^{++(+)}$, and Zn^{++}. In contrast, depletion of carbon and nitrogen, occurring in conjunction with a deficiency of K^+, Mg^{++}, or Mn^{++}, prevented most of the bacilli from sporulating, indicating the essentiality of these minerals for sporulation. A deficiency of Ca^{++} and PO_4^{---} reduced the number of spores; and omission of Ca^{++} from the formula, although without effect on growth, greatly hindered sporogenesis and yielded spores slightly refractile and weakly thermoresistant. Omission of Ca^{++}, but with Mn^{++} at a concentration equal to that of Ca^{++} in the control medium, yielded spores about equal in number to that of the control but with only 1/10 as many thermoresistant cells. When both Ca^{++} and Zn^{++} were omitted, sporulation did not occur; with omission of Zn^{++}, some Ca^{++} was necessary for sporulation.

Brewer *et al* (1946) described a chemically defined medium for *Bacillus anthracis* which produced yields in excess of one billion spores/ml. The medium contained a mixture of 18 amino acids, glucose, $NaHCO_3$, glutamine, nucleic acid components, mineral salts, and vitamins. Employing a

more dilute version of this medium, Brewer studied the effects of Ca^{++}, $Fe^{++(+)}$, Mg^{++}, and Mn^{++} ions on sporulation. Each ion was tested individually by varying its concentration, while the other ions were present in approximately optimal concentration, as determined by preliminary tests. In the absence of Mg^{++}, practically no growth occurred; 1.6 $\mu g/ml$ and higher of Mg^{++} induced good growth and sporulation. A good yield of spores was obtained in the absence of added calcium; however, addition of 20 $\mu g/ml$ of Ca^{++} increased the spore yield about 4.5 times. Inclusion of $Fe^{++(+)}$ in the formula was not critical for sporulation, although the addition of 0.7 $\mu g/ml$ increased the spore yield about 2.5 times. Added Mn^{++} was not essential to sporulation, but, as recognized by the authors, enough was probably present as an impurity to provide sporulation requirements of the organism. Replacement of the K phosphates with Na phosphates decreased spore yield about 1/3. The addition of Cu^{++}, Zn^{++}, Cd^{++}, and Co^{++} at levels of 0.1-1.0 $\mu g/ml$ of metallic ions was without effect upon sporulation.

The influence of mineral salts on sporulation is reflected in the results of experiments involving the use of a nutrient medium both in fluid form and solidified with agar. Thus Roberts and Baldwin (1937) observed that agar in amounts as small as 0.06%, when added to 1% peptone solution, definitely increased spore production of *B. subtilis*, and subsequently (1942) that the percentage sporulation in vigorously aerated peptone broth was considerably less than in unaerated medium made slightly viscous with agar. Since sporulation of *B. subtilis* is more rapid and attains materially higher levels in shake than in stationary cultures, these results must be attributed in part at least to minerals supplied by the agar — the latter contributes relatively large amounts of Mg^{++}, K^{+}, and Ca^{++} and biologically significant amounts of Mn^{++} and $Fe^{++(+)}$.

The mineral requirements of thermophiles in relation to sporulation have received very little attention. Ward (1947) observed that sporulation of *Bacillus thermoacidurans* in proteose-peptone agar was increased by the addition of Li^{++}, Mg^{++}, Ca^{++}, $Fe^{++(+)}$, Zn^{++}, or Mn^{++}.

The addition of NO_3^- or NO_2^- to nutrient agar has been reported to increase the sporulation of *Bacillus stearothermophilus* (Dahl, 1955). Schmidt's (1950) studies on the same organism, limited to NaCl and $CaCl_2$, indicated no clear-cut effect. Subsequently the same investigator observed that the addition of Mn^{++} to nutrient agar greatly increased sporulation of *B. stearothermophilus* (unpublished data). Sporulation by *Bacillus coagulans* var. *thermoacidurans* in three peptone-containing agar media was shown to be greatly enhanced by the addition of Mn^{++}, Co^{++}, or Ni^{++} (Amaha *et al*, 1956).

Apart from their utility in regulation of pH, bicarbonates may promote the formation of spores by increasing CO_2 concentration, as shown by Powell and Hunter (1955).

Proliferating vegetative cells, when centrifuged, washed, and shaken in distilled water, form spores only if the prior growth medium is conducive to sporulation. However, *B. subtilis* derived from an asporogenous medium sporulates in distilled water when supplied with both yeast extract and salts, but not if one of these supplements is omitted (Murrell, 1955), indicating that the spore-generating mechanism is not seriously impaired by limited cultivation in asporogenous media.

The known facts, briefly surveyed, indicate that mineral salts are essential for the formation of bacterial spores. The fact that synthetic media without added salts do not produce spores, and the fact that the inadequacy for spore formation of many complex organic media can be corrected by the addition of suitable minerals support this contention. The level of minerals required for sporulation varies with the organism and with cultural conditions such as kind and concentration of nutrients, oxygen supply, temperature, and pH. The importance of manganese in the sporulating process is conspicuously evident. It is required by a wide variety of mesophilic and thermophilic aerobes, at concentrations above those needed for active vegetative growth, and, with one apparent exception, cannot be replaced by any other mineral element. Manganese has been found to broaden the temperature and pH range over which sporulation occurs (Amaha *et al.* 1956).

The specific function of the minerals in the spore-forming process is unknown. It may be presumed that they exercise a catalytic role in the activation of many enzyme systems. They may thus expedite the complex enzymatic processes involving intracellular protein degradation and resynthesis postulated by Hardwick and Foster as the basic mechanism in sporogenesis. There is evidence that organic or inorganic phosphorus may supply some of the energy used for the synthesis of spore constituents. The presence of calcium above certain minimum levels seems to be a factor in the production of thermostable spores; it may well be that calcium contributes to the stability of spores by forming internal bonds with the low molecular weight peptides which comprise much of the spore substance.

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Discussion

E. D. Weinberg

Dr. Curran has given us an excellent review of the literature in this area of spore research and his paper should be of great value to future investigators in this field.

In any study of mineral nutrition, the *usual* obstacle is the presence of unsuspected or undetectable trace amounts of metallic ions in the environment. Even in our present-day synthetic media there may exist unknown metal contaminants that are required in higher concentrations for sporulation than for vegetative growth. Or perhaps there exist in some media in use today trace metals that inhibit sporulation. To illustrate such a possibility we can recall the extreme variations in amount of diphtheria toxin obtained in different culture media before 1936. At that time it was finally realized that although one part of iron in ten million parts of medium is required for maximum toxin production, as little as five times that quantity of iron permits perfectly good cellular multiplication of diphtheria bacilli but completely suppresses toxin production.

Fortunately, however, the development of methods for the preparation of synthetic demetalled media and for the detection of trace metal contaminants has progressed greatly during the past decade. And recently the Society of General Microbiology has made a start towards our understanding of the mineral composition of *complex* media of plant and animal orgin.

Oddly enough, the chief difficulty in mineral nutrition that confronted workers in sporulation for a half-century was not the presence of an undetected metallic ion in their media but rather the opposite situation: that is, the lack of sufficient potassium and manganese, and sometimes magnesium, in complex media derived from animal products. How could anyone have predicted that peptone and meat extract and skim milk contain sufficient available manganese for the growth of most microbial cells including those of the genus *Bacillus* but contain insufficient available manganese to enable cells of aerobic bacilli to efficiently sporulate? And think for a moment of

the very small amount of manganese that must be added to nutrient broth to obtain spore formation: one part in twenty million. It can certainly be stated without exaggeration that if such a small quantity of available manganese had been naturally contained in nutrient and other complex broths, the literature on sporulation research during the first half of this century would be considerably different.

The discovery of the manganese requirement for sporulation of aerobic bacilli is a good example of a contribution made by applied research to basic microbiology. As you may recall, Dr. Charney and his associates in 1951 were engaged in an antibiotic screening program and by chance discovered that mycelia of a *Streptomyces* culture stimulate growth and sporulation of bacilli on trypticase soy agar. Their curiosity was aroused by this observation; they proceeded to find that the active principle in the mycelium is manganese and that in trypticase soy and synthetic broths sporulation but not growth is dependent on an external supply of this inorganic ion. In the complex solid medium it is likely that constituents of the agar bind the low concentration of native manganese so that only a suboptimal quantity is available for growth.

Further studies of the mineral requirements for sporulation can contribute information that could be of immediate use in applied work. Such studies might include the development of effective methods of binding or of releasing essential minerals in the environment, which would result in either suppression or stimulation of sporulation. Another example of an applied use of inorganic nutrilite research is in analytical microbiology: at least two useful methods of assay of manganese based on Charney's discovery have already been published.

Future work in this general area of basic sporulation research should certainly include studies on the functions of the essential minerals. Dr. Curran has pointed out two types of possible functions: a structural function as demonstrated by the moderate calcium requirement and a catalytic function as demonstrated perhaps by the small manganese requirement. We are all familiar with the fact that some metals can substitute for others in the activation of enzyme systems; and apparently magnesium, cobalt, or nickel can, in a few cases, substitute for manganese. It would be of interest to learn if such alkaline earth metals as strontium and barium can substitute for calcium in its structural function.

With respect to the catalytic function of manganese, the remote possibility that the metal is involved in suppressing spore-inhibiting substances should be kept in mind. Such inhibiting substances could be other metals that might be antagonized by manganese or they could be organic molecules that might be dissimilated by enzymes that require manganese.

Other future studies should include a reexamination of the mineral requirements for sporulation of cells of the genus *Clostridium* using basal media rendered as metal-free as possible. Synthetic media are usually more complicated for species of *Clostridium* than for species of *Bacillus* and therefore might be considered more difficult to demineralize. However, it may be recalled that Drs. Shankar and Bard at Indiana University in 1952 grew cells of *C. perfringens* in demetalled media plus known concentrations of certain essential ions. A sequel to their study might consist of the testing of various combinations of metallic ions with this basal medium to learn if sporulation, in addition to vegetative growth, could be obtained.

A different type of unsolved problem in this general area of sporulation research concerns the possible occurrence of metals that are required for vegetative growth but not for spore formation. The solution of this problem would be facilitated, of course, by the development of techniques for demineralizing washed vegetative cells without impairing their viability (or should I say without injuring their ability to sporulate).

Other problems in sporulation research that would be aided by a complete knowledge of the mineral requirements for spore formation include those associated with the age and previous history of the cells. For example: during which phase of vegetative growth are the various essential metals required?

I believe that as the conference progresses, additional problems associated with mineral requirements may occur to us—and, perhaps some answers to the problems Dr. Curran and I have outlined will also be forthcoming.

Informal Discussion

CURRAN: I would like to comment on Dr. Weinberg's mention of an assay procedure for manganese. According to my information, the chemists lack a delicate test for detecting manganese in the presence of considerable amounts of iron. I think about 0.1% is the least they can detect. In connection with our studies on the need of manganese for sporulation, we find, by plotting the logarithms of spores against the concentration of manganese, that the sporulation curve rises abruptly around .01 to 03 μg of manganese, so this may provide us with a relatively delicate test for manganese in the presence of large quantities of iron.

ORIN HALVORSON: Did the spores produced in low concentrations of manganese show any differences other than the difference in heat resistance?

CURRAN: This again is Grelet's work. He found that in the absence of manganese the spores were smaller when he started below certain levels of manganese and their refractivity was lower. In one of his tests using an

excess of manganese he got about as many spores as he did using both manganese and calcium, but they were smaller, less refractile and considerably less heat resistant.

POWELL: We have made some studies in our laboratories on the sporulation of laboratory strains of *Bacillus cereus*, *B. subtilis* and *B. megatherium*.[1] The effect of glucose (10^{-2}M) and manganese (10^{-4}M) addition on sporulation in shaken tryptic meat digest medium (100 mg. nitrogen/100 ml.) was tested. All the above organisms grew well but showed no signs of sporulation in the test medium with no additions or with added glucose.

Manganese addition stimulated almost complete sporulation of *B. cereus* in the presence but not in the absence of glucose, of *B. subtilis* in the absence but not in the presence of glucose, and of *B. megatherium* both in the presence and absence of glucose. These results were not always repeatable with different batches of meat digest medium prepared in an identical manner and of equivalent nitrogen content. For example, in four other batches of medium, only in one batch was sporulation of *B. cereus* stimulated by addition of manganese and glucose. In all the batches of medium tested, *B. sphaericus* sporulated well in the absence of any additions.

ROTH: We have studied the sporulation of *Bacillus anthracis* in a casein hydrolysate medium to which we added calcium, magnesium and manganese. When we used tap water with these minerals, we obtained very good sporulation, 5 x 10^9 spores per ml. but when we used deionized water with the same minerals, we obtained very few spores. This indicates that there are other trace mineral requirements for sporulation besides calcium, magnesium and manganese.

ORDAL: I want to comment on the requirement of manganese for proper cell division. We found that *B. coagulans*, in a manganese deficient medium, at 55° grows reasonably well, but produces only filaments, whereas in a culture containing small quantities of manganese, it produces typical short rods.

SUSSMAN: I was struck by the fact that two effects of manganese are mentioned in the literature and I am wondering if these have been noted on the same organism? I refer to the effect that Dr. Curran has mentioned, namely, its ability to induce sporulation, and secondly to bring about germination. Have any experiments been done to determine the amount of manganese needed to induce sporulation as compared to the amount required for subsequent activation of the spores?

CURRAN: As far as I know, there are no quantitative data available on this point.

[1] Powell, J. F., Unpublished results.

An Approach to Synchronous Growth for Spore Production in *Clostridium roseum*

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THE production of large amounts of anaerobic spores free from vegetative cells is not possible with conventional methods, but means to do this must be found if an adequate study of the physiology of these spores is to be made. Culture methods used heretofore result in a mixture of all possible cell forms: young vegetative cells, cells in various stages of sporulation, free spores, and spores in various stages of germination. It is virtually impossible to harvest clean spores from such a mixture.

We have been able to solve this problem by using methods that give a pseudosynchronous growth of the vegetative cells. By transferring a very heavy inoculum of organisms in the log phase of growth into a suitable sporulation medium, a culture is produced in which sporulation is initiated in every cell, after only a few generations, and every cell produces a spore at about the same time. If the growth medium is properly balanced with nutrients, those required for germination are practically exhausted during growth, and the spores which are formed cannot germinate in the original medium.

The culture may need to stand for some time before the spores are set free, but as long as the spores do not germinate in the growth medium, the final result is a suspension of relatively clean spores that can be harvested with little difficulty.

Materials and Methods

Preparation of inoculum for pseudosynchronous growth. A stock spore suspension of *Clostridium roseum* was prepared by growing the organism in 1.5 percent trypticase medium for 48 hours at 30°C. Six-ounce screw-cap bottles each contained 100 cc. of this medium; and a volume of the spore suspension equal to 10 percent of the medium to be inoculated was designated as the 10 percent inoculum. The spore suspension was subjected to heat shock at 65°C. for 30 minutes, after which a 10 percent inoculum was transferred to fresh trypticase medium contained in the bottles every three hours. This was repeated three times. On the fourth transfer the inoculation was made into the test flask in which the spores were to be produced.

Description of the test flask. A two-liter Erlenmeyer flask was fitted with a side arm which was covered with a rubber cap before autoclaving. Samples could be removed from the flask with a hypodermic syringe. City gas was passed aseptically through the flask immediately after inoculation until all oxygen was removed both from the medium and from the space above the medium. A rubber stopper with an inlet and outlet tube was used in the mouth of the flask with a suitable water trap to insure anaerobic conditions. A magnetic stirring rod placed in the flask before sterilization provided a means for constant stirring of the medium in the flask during growth of the anaerobe. All media were used immediately after preparation.

Test media. The original test medium [the medium used in the test flask] was composed of the following:

[1]	Trypticase	1.5%
	Sodium Chloride	0.5%
	K_2HPO_4	M/90
	Dextrose	0.2%
	Initial pH	7.0 to 7.2

The modified tris test medium was composed of the following:

[2]	Trypticase	1.5%
	Sodium Chloride	0.5%
	K_2HPO_4	M/30
	Tris buffer	M/80
	Dextrose	0.2%
	Initial pH	7.5 to 8.0

Counting of the spores. Percentage of spores was determined by staining with crystal violet; the spores remain unstained while the sporangia, the germinated spores, and the vegetative cells absorb the dye.

Results

When the original test medium was used, sporulation occurred usually within five and one-half to six and one-half hours. Table I summarizes the results obtained.

It is obvious that not all cells were producing spores at the same time. This was thought to be due to one of two things: possibly the culture was not in synchronous cell division or the medium itself was not satisfactory for complete simultaneous sporulation. Many investigators have obtained synchronous growth of bacteria by lowering the temperature of a culture for a period of time and then allowing the culture to incubate at the normal growth temperature. We used this technique for *Clostridium roseum*. Just prior to the inoculation of the test flask with the active culture, the inoc-

Table I

Degree of sporulation obtained with medium 1

Time (hours)	*Percent spores*
2	0
3	0
4	0
5	0
5½	45
6	45
7	64
8½	75
21	96 (lysed sporangia)

ulum was chilled to 10°C. for thirty minutes. Then the inoculum was placed in the test flask, which was held at 30°C., whereupon sporulation time and percentage sporulation were followed. This approach did not give complete and simultaneous sporulation, and furthermore the beginning of sporulation was delayed for thirty minutes.

Clostridium roseum, like other members of the butyric acid group of anaerobes, produces a large amount of acid. It occurred to us that the actively growing culture might be lowering the pH sufficiently to interfere with the production of spores. Accordingly, the pH of the original test medium was determined at different intervals prior to and including the period of actual sporulation. The drop in pH during the eight-hour period was from 7.2 to 6.0, which has been shown to be inhibitory to total sporulation. After testing various combinations of buffer, the above medium was modified to maintain the pH as close to neutrality as possible. The new medium is referred to as the modified tris test medium. It was our hope that by modifying the medium to maintain a constant pH, it would be possible to obtain 100 percent simultaneous sporulation in the five and one-half hour period. Table II shows the results which were obtained using the modified tris test medium.

It can be seen from this table that, although the pH was maintained around neutrality, not all cells produced spores at the same time. Further incubation nevertheless did give almost 100 percent sporulation in a seven-hour period. Table II also shows that after seven hours the percentage of spores dropped. This is presumably due to germination of some of the spores in the growth medium.

During the preparation of the inoculum for use in the test flask, the culture should not be allowed to overincubate. That is, the culture must not be

TABLE II

Degree of sporulation obtained with medium 2

Time (hours)	*pH*	*Percent spores*
2	7.5	0
3	7.5	0
4½	7.3	0
5	7.3	20
5½	7.3	50
6	7.3	77
7	7.3	96
20	7.0	57

in the process of sporulation prior to transfer to the test flask. We have found that if the above happens, it delays rapid growth of the culture in the test flask by five or more hours, and interferes with the subsequent process of sporulation. It became apparent that, in order to avoid the above complications, it was necessary to find out the approximate time at which the inoculum was committed to sporulation. This problem was first approached by assuming that growth of a vegetative cell and sporulation were separate processes. While it is known that this organism requires anaerobic conditions for growth, it is *not* known whether the sporulation process is so restricted. We tested this by aeration with penicillin after the vegetative cells were presumed to have been committed to sporulation. If spores could be formed from such cells under aerobic conditions, we could thus separate the process of growth from sporulation.

Samples of the inoculated test medium were removed at different time intervals and placed in aeration tubes with penicillin to give a final concentration of 100 units of penicillin per milliliter. The tubes were then aerated until the test flask showed 96 percent sporulation, as determined by staining. The results are shown in Table III.

It appears from these results that there is a gradual production of spores. Aeration with penicillin completely arrests further sporulation, but it has no effect on the spores already formed; thus penicillin and aeration together block spore formation even after cells are committed.

The effect of aeration and penicillin has been somewhat clarified from the following experiment. Samples of the inoculated test medium were removed from the test flask at times when the culture was undergoing sporulation. The cells were washed free from all nutrients under anaerobic conditions, and allowed to incubate at 30°C. in distilled water also under anaerobic conditions. The culture sporulated completely although there were

TABLE III

Degree of sporulation in aerated samples containing penicillin

Hour of sampling	*Percent sporulation of control*	*Percent sporulation in test sample*
2	0	0
3	0	0
4½	0	0
5	20	18
5½	50	57
6	77	79
7	96	—

no nutrients available for further growth of the cells. If one assumes that this method stops growth of the vegetative cells, then it is reasonable to assume that at the time of withdrawal of the sample the biochemical changes culminating in the production of a spore have already started in every cell in the sample. Such a suspension of cells can successfully complete the process of sporulation in the absence of external nutrients. Presumably the aeration-penicillin treatment of such samples is interferring with the successful completion of the biochemical changes finally resulting in the formation of a spore.

If, at the end of seven hours of incubation, 96 percent sporulation has occurred, samples are removed and are aerated with or without penicillin at 30°C. Lysis of the sporangia then occurs within 24 hours with no germination of the freed spores.

We have found that when a culture of *Clostridium roseum* actively growing in the vegetative state is placed at 4°C. for one to two weeks, total lysis of the vegetative cell occurs. This phenomenon also has been found to occur when a culture with 96 percent spores in the sporangia and 4 percent vegetative cells is held at 4°C. for one to two weeks. In the latter case, not only do the vegetative cells lyse, but also do the sporangia.

In conclusion, from this work we have developed a better method of producing spores from *Clostridium roseum*. Also, by using methods given here, more information can be obtained about the sporulation process of this organism.

Discussion

William L. Brown

My task is an easy one since no data have been published on this specific subject. Mr. Collier's paper is timely as his work fits well into the general

problem of spore production. I am sure you will want to question him about his procedures and results, so my discussion of his paper will be short.

In order to study spore germination it is necessary to produce free ungerminated spores and separate them from the vegetative cells, germinated spores, and the sporangia that contain spores. Mr. Collier has presented an approach to the problem that has several merits. With some anaerobes an incubation period of 2 to 3 weeks is recommended for optimum sporulation. He has been able to produce spores from *Cl. roseum* in a matter of 5 to 7 hours. It is also interesting to note that the percentage sporulation is quite high if the culture is examined prior to the so-called "recycling" process.

He was able to lyse the vegetative cells by storage at 4°C. for 1 to 2 weeks. With another anaerobe, PA 3679, we were not successful in lysing the cells at 4°C., but we did use lysozyme to remove the vegetative cells from the harvested spore crop. This procedure was very successful.

From the information presented, it seems that there was a gradual production of spores, and the combination of aeration with penicillin blocked or arrested further sporulation of the culture. Aeration alone or penicillin alone will also block sporulation. This suggests a similarity between these results and the endotrophic sporulation of aerobic bacilli.

Hardwick and Foster (1953) studied the enzymatic changes during sporogenesis in some aerobic bacteria and advanced the hpyothesis that sporogenesis occurs at the expense of proteins (or enzymes) pre-existing in the vegetative cell. The procedure was similar to that of Halvorson and Spiegelman (1952) who established, by means of amino acid analogues, amino acid involvement in adaptive enzyme formation in yeast. Additional work may show a similiar type of mechanism in the sporulation of anaerobic bacteria.

The Michigan State workers also report that a synchronized technique will work for the production of PA 3679 spores. This is a new procedure and I feel that is a fruitful field of endeavor.

Informal Discussion

RAYMAN: In the commercial butyl fermentation, the culture must be transferred five to six times to get good production of solvents. The yield of butyl alcohol and acetone is poor for the first and second transfers, but improves up to the fifth. After that, the yield drops so that by the eighth or ninth transfer it is practically nothing. Mr. Collier, I am wondering if you are getting a sporulation-germination cycle that is equating itself about the fifth transfer. This is my explanation of why the yield of solvents is

improved after a few transfers in the butyl alcohol fermentation and why you get more spores.

ORIN HALVORSON: We have assumed that, if we continue to grow these cultures in the vegetative state. we favor the growth of non-sporeforming mutants, and therefore we want to avoid too many transfers in the vegetative state.

SUSSMAN: The observation by Mr. Collier that cells only partially sporulated will complete the process in distilled water needs. I think, a little clarification. Is it possible that in distilled water you are getting lysis which would provide nutrients for this process?

COLLIER: I made total counts in all of these studies and found that the lysis of vegetative cells without spores was negligible, but nutrients could have come from the sporangia that lysed.

ROBINOW: Faced with the problem of having cultures in the early stages of spore formation for classwork at 3:15 in the afternoon. I found quite some time ago the following procedure useful. When *Bacillus megaterium* is grown overnight at 16°C. on potato extract agar, confluent growths are produced. When these cultures, 18 hours later, are transferred to 37°, rapid and simultaneous spore formation occurs three hours later. Five hours later approximately two-thirds of the cells are in the advanced stages of spore formation which is useful for cytological and teaching purposes.

CAMPBELL: Dr. Brown, what concentration of lysozyme did you use in cleaning your spores?

BROWN: We used a concentration of about 0.5 mg per ml. We realize this is high, but we had a very heavy suspension and we used this routinely to separate the spores and it worked well with PA 3679. Mr. Titus has used it successfully with *B. sterothermophilus*.

LUND: In regard to using lysozyme with PA 3679, we have also done that and we used about the same concentration, but we adjusted the pH to 11 where there seems to be an automatic, almost immediate dissolution of all vegetative cells. We thought at first that this gave us clean suspensions; however, upon examing the cells under the phase microscope, we thought we saw shadows around those spores that were the shadows of the original sporangia. We tried staining these by various methods and finally found that hematoxylin gave a stainable outline of the original cell.

POWELL: We've recently had a great gleam of hope about the preparation of clean spore suspensions and we find that when a laboratory strain of *Cereus* sporulates, it builds up a very strong lytic system which will attack vegetative cell walls. Now, my colleague, Mr. Strange, has done more work on this enzyme than I have, so perhaps it is a little premature.

but I think that this does offer some hope of cleaning up spore suspensions. The enzyme is very like lysozyme in its activity. This is a study we do intend to get on with. We are now making the enzyme in a concentrated form and are studying its properties. The next thing we mean to do is to see if it will help in this dreadful problem of getting rid of these horrible vegetative cells.

KRASK: I would like to mention a technique we have developed with lysozyme that may or may not be applicable to some of this work. We find that some organisms not susceptible to lysozyme at neutral pH become susceptible in the presence of versine and tris buffer.

HARRELL: I wonder if Mr. Collier would like to comment on the problem of getting spores in large volumes of media. When we get the volume of medium over two liters, we get poor results. We get good vegetative growth but few spores. If we keep the volume of the medium less than 1500 ml. we get good sporulation.

COLLIER: We have had the same experience. We believe that the pH of the medium may be a factor and therefore we want to repeat some of these experiments with tris buffer. The medium appears to become more acid in large than in small volumes.

The Effect of Nutritional and Environmental Conditions of Sporulation

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Knaysi (1948) in his review entitled "The Endospore of Bacteria" gave us a rather complete account of the information available up to that time on the effects of nutritional and environmental factors on sporulation, especially in regard to members of the genus *Bacillus.*

It is my purpose today to bring to your attention some of the pertinent data that have appeared since then, and to present, rather briefly, several examples from unpublished data obtained in our laboratory. It is also my concern to stimulate discussion on some of the important issues which are still ambiguous and controversial.

First of all, I must emphasize the fact that our knowledge about "why and how" a vegetative cell sporulates is still very limited. Four main hypotheses have been suggested as explanations. The first and oldest is that of Behring (1889): "Sporulation is an intermediate stage in the normal development of the bacterial cell, which may be partially or completely inhibited by some partial physiological damage short of total prevention of growth." The second is Knaysi's (1948): "Endospores are formed by healthy cells facing starvation." The third, which is a more specific version of Behring's is that of Foster and Heiligman (1949b): "Sporulation is a sequence of integrated biochemical reactions which are independent of vegetative growth and may be interrupted at certain susceptible stages." The fourth, which is rather supplementary but not specific, is Schmidt's: "Sporulation is a function both of the environment and of cellular factors determining the reaction of the cell to a given environment."

These several hypotheses are not necessarily mutually exclusive; and it is not my purpose at this time to prove or disprove any of them. But, for the sake of this presentation and perhaps as a basis for discussion, let me assume that we are in agreement on the following concept: Sporulation is a normal metabolic process which will occur within the bacterial cell only when (1) the cell is of a sporogenous type; (2) the cell acquires the proper physiological condition; and (3) the cell is surrounded by the proper environment.

At this point I must emphasize that much of our information comes from observations made on growing cultures; and investigators have not always

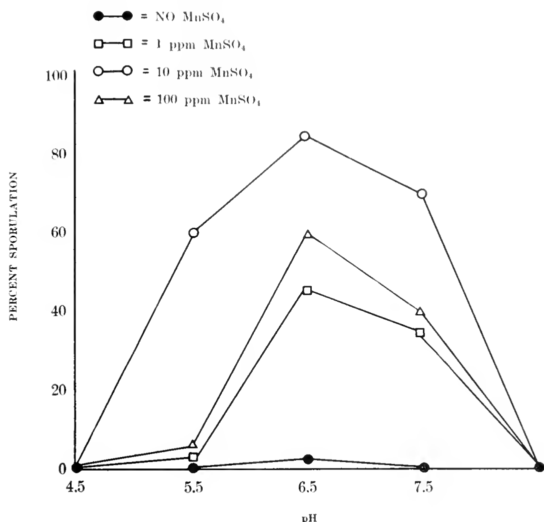

FIGURE 1.

distinguished between conditions which favor vegetative growth and conditions which specifically favor sporulation. The technique of replacement cultures, i.e., the procedure of producing the vegetative cells in a medium favorable for growth and then suspending these cells in distilled water or in a second modified medium for sporulation, provides a means of partially separating growth from sporulation. Using this technique, Foster and his colleagues, Grelet (1951, 1955), Tinelli (1955), and others have collected data which have been helpful to our understanding of this phenomenon.

Environmental factors affecting sporulation

I. Physical factors

(a) *Temperature.* There seems to be a general agreement that the optimum temperature for sporulation is close to that for growth, but the range is narrower.

(b) *pH.* Likewise the optimum pH for sporulation is similar to that for growth, but the range is narrower. Some of our own data substantiate this, as shown in Figs. 1 and 2. *Bacillus coagulans* [NCA strain 43P] was cultured on an agar slant medium, which, except for the agar, was chemically

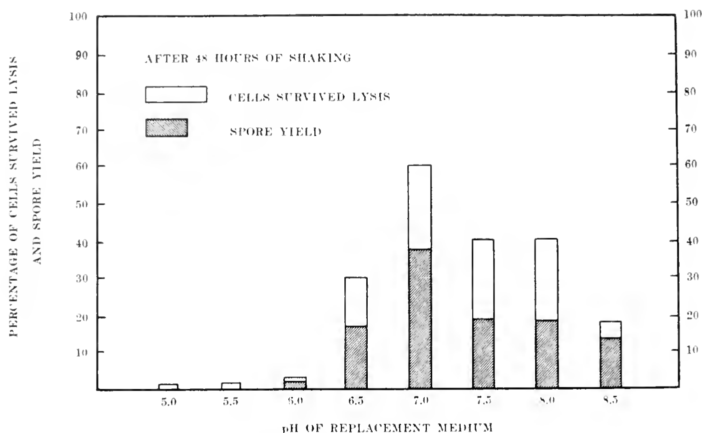

FIGURE 2.

defined (a modified Campbell and Williams [1953] medium). Sporulation was evaluated on stained smears from a 6-day old slant incubated at 45°C. It is apparent from data in Fig. 1 that maximum sporulation occurred at pH values close to 6.5, even though the organism appeared to grow equally well over the pH range 5 to 7.5. Fig. 1 shows also the effect of added manganese.

Fig. 2 shows the effect of pH on cells sporulated in replacement culture. The vegetative cells were obtained from a 10-hour thermoacidurans broth shake culture. After washing, the cells were suspended in M/20 phosphate buffer adjusted to the indicated pH level and placed on a rotary shaker at 45°C. In this case the degree of sporulation is represented as the "spore yield" in order to compensate for the lysis which invariably took place in replacement cultures with this organism. Minimum lysis and maximum sporulation occurred at pH 7.0. In our studies with Putrefactive Anaerobe 3679, maximum sporulation always occurred when the growth and sporulation media were adjusted to the pH range of 7.0 to 7.6. pH levels outside this range reduced sporulation even at levels where the vegetative growth was still good.

(c) *Oxygen requirements.* Knaysi in 1948 believed that oxygen was an absolute necessity for sporulation of the *Bacilli*. While admitting the lack of conclusive proof, he nevertheless stated that the need of oxygen in the quick and efficient formation of large numbers of spores is "one of the

incontrovertible facts of bacteriology." Since 1948, additional information has appeared. Roth *et al* (1955), using *Bacillus anthracis* and *Bacillus globigii*, determined that when cultures were initiated from a heat-shocked spore inoculum, an oxygenation rate of 0.7-1.0 mM O_2 L/min. was required for complete sporulation, whereas only 0.1-0.2 mM O_2/L m. was required for subcultures initiated from a culture at the peak of vegetative growth. Lower oxygenation rates markedly reduced the percentage of sporulation.

Tinelli (1955) has recently presented evidence that as a culture of *B. megatherium* approaches the sporulation stage, the rate of endogenous oxygen uptake progressively decreases. However, just before the cells enter the prespore stage, the rate markedly increases and, after a short time at this higher rate, again decreases and becomes quite low as sporulation proceeds to completion. This would suggest that, as the cells sporulate, they have an increased energy demand in order to sustain the endogenous changes leading to the formation of a spore.

II. Chemical factors.

(a) *Carbon sources.* From the literature, it is difficult to evaluate the effect of carbon sources on sporulation *per se* because there is no clear differentiation between the carbon requirements for growth and for sporulation. It is logical to assume that sufficient carbonaceous material must be present for reproduction and cell growth; and it must be of the kind to produce cells having sufficient energy reserve for sporogenesis. Foster and Heiligman (1949b) demonstrated that *B. cereus* grows well, but sporulates poorly in a glutamate-salts medium. The addition of 2 mg/ml of glucose increased growth by only 23 percent but increased sporulation by 2500 percent. It would appear that, in this case, glutamate alone was adequate as a carbon source for vegetative growth, but that cells produced in such a medium were lacking in energy reserves sufficient to cause a high degree of sporulation. The addition of the small amount of glucose corrected this condition without significantly increasing the level of vegetative growth.

There has been considerable speculation on the effect of glucose *per se*. Grelet (1951), who supports Knaysi's hypothesis that sporulation only occurs in healthy cells facing starvation, concludes that sporulation by *B. megaterium* does not occur, under his experimental conditions, unless the glucose is exhausted or omitted from the medium. However, if the nitrogen, sulfur, iron, or zinc is limiting, sporulation occurs in the presence of glucose. In his replacement cultures, the delayed addition of glucose failed to stop sporogenesis. Powell (1956) demonstrated that in a complex medium sporulation by *B. cereus* was complete even though up to 20 percent of the original carboydrate was still present. We have demonstrated that Putre-

factive Anaerobe 3679 sporulates in the presence of glucose; but in such cultures the spores germinate, and spore yields are low unless they are harvested at exactly the right time. Similar results were also observed by Halvorson (1956) and his co-workers. Hardwick and Foster (1952), using replacement culture techniques, presented rather convincing evidence that glucose affects sporulation of *B. mycoides*. The vegetative cells were produced in a glucose-glutamate salts medium; they were washed and resuspended in replacement medium in which the glucose effect was evaluated. The adverse effect of acidity resulting from glucose metabolism was overcome by using phosphate buffer as the replacement solution. When the glucose was added after five hours or less of shaking, sporulation was completely suppressed. Further delay in the addition of glucose progressively reduced the inhibition. When glucose was added after 9 or 10 hours, full sporulation took place. They concluded that the inhibitory effect of glucose was due to the fact that glucose metabolism successfully competed with the intracellular metabolism essential for sporogenesis. The early addition of glucose preempted the use of nitrogenous precursors for sporogenesis whereas delayed addition allowed sporogenesis to proceed to an irreversible stage. They substantiated the above assumption by demonstrating that the simultaneous addition of ammonia counteracted the glucose-inhibitory effect.

(b) *Nitrogen sources.* Hardwick and Foster (1952) have demonstrated that cells of *B. mycoides* produced in a medium that is low in nitrogenous material fail to sporulate when they are shaken in distilled water, whereas cells produced in a nitrogen-rich medium sporulate readily. They concluded that a cell impoverished in regard to its protein content loses its ability to sporulate. They considered these results as additional evidence that spore proteins are synthesized from cellular nitrogenous materials.

Other investigations have been directed toward the specific effect of various amino acids. Williams and Harper (1951) show that the omission of leucine from their chemically defined media reduced sporulation by *B. cereus*. Krask (1953) reported that methionine sulfoxide, an antagonist for the conversion of glutamic acid to glutamine, inhibited sporulation by *B. subtilis* in concentrations which did not appreciably affect the vegetative growth. He also cites unpublished data which indicated that more glutamic acid was required for maximum sporulation than for growth. Blair (1950) noted that the omission of methionine from a synthetic medium suppressed sporulation of *Clostridium botulinum*. In our studies with *B. coagulans*, we obtained the results presented in Table I. When the sulfur-containing amino acids (methionine and cystine) were left out of the medium, sporulation was markedly reduced. We thought, at that time, that we had demonstrated a sporulation requirement for methionine, as the amount of growth

TABLE I

Sulfur requirement for sporulation of *Bacillus coagulans* [43-P]

	Percent sporulation
1. Complete medium	75 - 90
2. [1] minus S-amino acids	5 - 10
3. [2] plus inorganic SO_4	70 - 90

appeared to be unaffected. However, we did one experiment too many. We re-examined the composition of our medium and noted that, when sulfur-containing amino acids were left out, the only sulfur in the medium other than the unknown amount which might be contributed by the agar was that contributed by the 10 ppm $MnSO_4$ which we had added to the medium to satisfy the manganese requirement. When we fortified the medium with inorganic sulfate and left out the methionine and cystine, the organism sporulated equally as well as in the complete medium. The complete data suggest that instead of a methionine requirement the organism actually has a sulfur requirement which is greater for sporulation than for growth. I think that these particular data serve to emphasize a more important point, namely, that when we investigate sporulation we are studying a phenomenon of the interdependent relationships and we must be cautious in drawing conclusions unless we know these relationships.

The role of alanine in stimulating or suppressing sporulation is controversial. In our laboratory, both in shake culture and in replacement culture, L-alanine exhibited some stimulatory effect towards the sporulation of *B. coagulans*, even though this compound is an active germination stimulant for spores of this organism. Foster and Heligman (1949a), using *B. cereus* cultured in a glutamate-salts medium, reported that the addition of L-alanine, DL-alanine, or β-alanine suppressed the stimulatory effect of glucose towards sporulation. This suppression of sporulation could be reversed by the addition of yeast or liver extract, leucine or isoleucine. Grelet (1955), working with different strains of *B. cereus* cultured in a glucose-amino acid-salt medium, demonstrated the following: (1) his strains were exacting to both glutamate and alanine while the other three amino acids—valine, leucine, and isoleucine—stimulated growth; (2) good sporulation always occurred when alanine in the complete medium became limiting; and (3) good sporulation was also provoked when the valine, leucine, or isoleucine became limiting even though excess alanine was still present in the medium.

(c) *Nutrilites.* The only data previously presented on the effect of a specific growth factor on sporulation is that of Williams and Harper (1951),

TABLE II
Effect of folic acid and related metabolites on sporulation of *Bacillus coagulans* [43-P]

	Percent sporulation
1. Complete medium	75 - 90
2. [1] minus folic acid	5 - 10
3. [2] plus PABA	75 - 90
4. [2] plus adenosine	25 - 50
5. [2] plus adenine	20 - 30

Inosine, guanine, uracil, and thymine no effect at 10 $\mu g/ml$.

the same paper referred to earlier. In their hands, sporulation was reduced when para-aminobenzoic acid was omitted from the medium. While they do not present specific data on the effect of the omission of this compound on the amount of growth, their discussion of the general results imply some reduction in the amount of growth.

We have obtained some evidence that *B. coagulans* requires more folic acid (or PABA) for sporulation than it does for growth. Typical data are presented in Table II. The complete medium is the synthetic medium containing agar which supports a high degree of sporulation as indicated by the first line. The omission of folic acid markedly reduces the percentage sporulation. The requirement for folic acid is fulfilled by PABA (line 3). As folic acid is considered to function in the synthesis of purines and pyrimidines, we have attempted to compensate for this function by the addition of various purine and pyrimidine bases in place of the folic acid or PABA. The addtion of adenosine or adenine produces a partial stimulation, but inosine, guanine, uracil, or thymine failed to exert a demonstrable effect. High concentrations (100 $\mu g/ml$) of guanine or uracil completely suppressed the growth of this organism in this medium.

(d) *Other factors.* There have been reports on other substances which do not readily fall into either of the above three categories, but which have stimulated sporulation under the conditions in which they were used. I have chosen to "lump" a few of these together.

Powell (1951) reported that oxalate (10 mM) stimulated spore formation by *B. megaterium* when added to a complex medium. Subsequently Powell and Hunter (1955) reported that on the same medium sporulation by *B. sphaericus* was stimulated by the addition of bicarbonate or α-ketoglutarate. They concluded that the higher CO_2 concentration furnished by these compounds was the stimulating factor. They also postulated that the

higher carbon dioxide concentration favors the production of dipicolinic acid, a major and specific constituent of spores.

We have obtained somewhat similar results (Amaha, Ordal, and Touba, 1956) when *B. coagulans* was cultured on thermoacidurans broth in shake culture. Essentially no spores were produced in the basal medium. The addition of malate, succinate, or *a*-keto-glutarate markedly stimulated sporogenesis, but the other compounds tested (citrate, fumarate, oxalacetate, pyruvate, or D-alanine) were without apparent effect.

In the time allotted for these remarks, I have had to leave out some pertinent data. I trust that these omissions will be adequately taken care of in the discussion to follow.

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Discussion

Arnold J. Lund

In my role as discusser of Dr. Ordal's paper, I appreciate that he included a category he termed "other factors," because this gives me the opportunity to describe a phenomenon we have been studying. I shall limit my remarks to describing our work.

In searching for a method of culturing the *Clostridium* species, known as Putrefactive Anaerobe (PA 3679), so as to obtain massed crops of "clean spores," we observed that culture filtrates of the organism had sporogenic activity.

The organism we used was an isolate from an agar plate colony of PA 3679 (ATCC 7955). It varied from the parent culture only insofar as it sporulated more freely than the parent culture, and on some mediums its spores were slightly smaller. Except for these variations, it has been culturally indistinguishable from the original strain. We have designated it PA 3679-h.

The sporogenic activity of the culture filtrates was first observed when using Difco Brain-Heart Infusion Broth with added 0.1% sodium thioglycollate as a culture medium for the organism. When the medium was inoculated at the level of 0.1% by volume with a spore suspension containing 1×10^5 spores per ml., then incubated at 37°C. for 5 days, the culture developed less than 1% spores for a total spore crop of less than 1×10^6 spores per ml. When, however, the cells were removed from the culture after only 18-20 hr. incubation and the medium was reinoculated at the original level and again incubated, the second cell crop yielded 70-90% spores, with a total population in excess of 1×10^7 spores per ml. This indicated to us that the growth of the first crop of cells had made the medium sporogenic. This sporogenic character of the culture filtrate must have resulted from one of the following: (1) exhaustion of essential nutrient for vegetative growth;

(2) removal of a sporulation inhibitor; (3) the synthesis of something the organism requires for sporulation.

When the culture filtrate was fortified with the dry ingredients of the medium at the level initially used in preparing the medium, the spore crop was increased over that of the non-refortified filtrate with the same percentage of sporulation. This seems to argue against the exhaustion of nutrients or the removal of inhibitors of sporulation as responsible for the increased sporulation in the culture filtrates. In light of the report by Hardwick, Guirard and Foster (1951) implicating saturated fatty acids as antisporulation factors, we used their methods for removing these antisporulation factors, namely treatment of the medium with charcoal, addition of soluble starch to the medium, and saponification of the dry ingredients of the medium, then neutralizing the soaps to convert them to the free fatty acids and extracting the material with fat solvents, in the manner described by Hilditch. None of these treatments enhanced the sporulation in the mediums treated.

Inasmuch as the evidence seemed to point toward the synthesis of some sporulation factor in the culture filtrates, work was directed toward characterization of the sporulation-enhancing property of the spent medium. A simple medium for growth of the cells was a broth composed of 2% trypticase (BBL) with 0.1% sodium thioglycollate. When this medium was exhausted, it could be refortified with the initial ingredients, and reinoculated to yield good sporulation. I might point out that, after much wasted time, we learned to our sorrow that "trypticase" is not always trypticase. Much of our early work was done with one lot of this peptone, and when some of that work was repeated at a much later time, we got different results. For example, spent medium, when refortified with 2% trypticase, yielded cultures with 70-90% sporulation and a total population of about 1 x 10^8 spores per ml. Four per cent fresh trypticase was used as a fresh medium control because this concentration of peptone was equivalent to the total concentration of trypticase used in the refortified culture filtrate. Under these conditions, the fresh 4% trypticase yielded less than 1% spores, in spite of good vegetative growth. Much later in this work, with a subsequent lot of trypticase, we observed that the 4% fresh control yielded very good sporulation, or about 10-20% of that of the spent refortified medium. This represented about 15% sporulation in the culture. On the advice of Dr. Vera of the Baltimore Biological Company, who suggested that the most probable difference between the various lots of trypticase was a difference in thiamine content, we routinely began adding 0.1 gamma of thiamine per ml of the medium. When such additions were made to the fresh 4% trypticase mediums, spore crops in the order of 3 x 10^7 spores per ml were obtained.

and variability between lots was removed. The percentage of sporulation was difficult to evaluate because of a concomitant lysis of the vegetative cells.

When thiamine was added to the spent medium or to the refortified spent medium, no increase in percentage of sporulation was observed. When 2% trypticase broth was enriched with up to 10 gamma of thiamine per ml of the medium, almost 100% incipient sporulation occurred, as is demonstrated by the fact that, at the end of 24 hours of incubation, almost every cell in the culture, as observed microscopically, was in the swollen prespore stage. These swollen prespores, however, never went on to form the refractile mature spores, but after 72 hrs. these forms disappeared and only vegetative cells could be seen. This inability to observe refractile spores in the 2% trypticase medium containing thiamine indicated that this medium was still deficient for sporulation. Efforts to overcome the deficiency by the addition of Mg, Mn, Fe, Co, Ca, vitamins, nucleic acid bases, glucose, and short-chain fatty acids were unsuccessful in improving the sporulation.

The sporogenic effect of the culture filtrates was not restricted to PA 3679-h, either with regard to production or response. *Clostridium botulinum* 62A and *Cl. aerofoetidum* ATCC 4894 grew with enhanced sporulation in the refortified spent medium from PA 3679-h. The culture filtrates from *Cl. botulinum* 62A, *Cl. aerofoetidum*, PA 3679, *Bacillus cereus*, and *Pseudomonas fluorescens* enhance, to varying degrees, the sporulation of PA 3679-h. PA 3679 sporulated very poorly in all culture filtrates studied.

When the replacement technique of Hardwick and Foster was used and PA 3679-h was grown in refortified spent medium, it became committed to sporulation, so that when the growth medium was replaced by a nonsporogenic medium such as 2% trypticase broth, before incipient sporulation could be observed, sporulation occurred in the replacement medium. No sporulation occurred when the growth medium was replaced by buffered thioglycollate solution or by a thioglycollate and glucose solution. This failure to sporulate in the absence of the peptone might not have been caused by a nutritional inadequacy; it might have been the result of an inadequate poising of the replacement solution in the absence of the peptone. This problem was not studied further.

Upon dialysis of the culture filtrates of PA 3679-h, only the dialysate was sporogenic when refortified with trypticase. The activity in the whole spent medium was stable to lyophilization and to heat. The activity could be removed from the whole spent medium by treatment with charcoal and by absorption on both strong anion (IR 400) and strong cation (IR 120) ion exchange resins, but the activity could not be recovered from either the

charcoal or these resins. Weak anion (IR 4B) and weak cation (IRC 50) did not remove the activity from the spent medium.

When solvent extraction methods were used, the activity could be extracted from the lyophilized spent medium by means of acidic ethanol or acidic aqueous acetone (80% acetone). Neutral ethanol seemed to destroy the activity, inasmuch as, after such extraction, neither soluble nor insoluble fractions were active, either individually or in combination.

The sporogenic activity of the whole culture filtrates of PA 3679-h was stable on storage in either acid or neutral conditions but, when stored under alkaline conditions in the refrigerator, the activity was lost. Purified active fractions diminished in activity when stored under refrigeration at pH 8.5.

A partially defined medium (Medium Q), containing 2% acid-hydrolyzed casein (NBC, "vitamin-free and salt-free"), biotin, thiamine, niacin, and pyridoxine, plus minerals and 0.1% sodium thioglycollate, supported growth of the organism. The spent medium from this growth appeared to be sporogenically active when refortified with trypticase at a 2% level, but was not active when refortified with the dry ingredient of the growth medium. It seems, therefore, that the organism needs something from the trypticase as well as something from the spent Medium Q. The fresh Medium Q to which trypticase was added at the same level yielded sporulation which was inferior to that of 4% trypticase broth. However, 2% trypticase broth, using either the sporogenic or nonsporogenic lots of trypticase and 2% vitamin-free acid-hydrolyzed casein with added thiamine, was superior to 4% trypticase broth, and equal to the trypticase refortified spent Medium Q.

Microbiological amino acid assays and two-dimensional paper chromatography showed that growth of PA 3679-h in medium Q exhausted the medium of phenylalanine, tyrosine, proline, serine, threonine, methionine, and arginine. Leucine, isoleucine, and valine were diminished. Glutamic acid, aspartic acid, and tryptophane were present in the spent medium in about the original concentration. Histidine, lysine, α-aminobutyric, γ-aminobutyric acids, and an unidentified ninhydrin positive material were synthesized during growth.

Displacement chromatography using Dowex 50 cation exchange resin, NaCl, $CaCl_2$, and NH_4OH as displacers showed that the activity of the spent medium was resident in those fractions at the end of the neutral amino acids and ahead of the fractions containing the basic amino acids. The active fractions contained the amino acids leucine, isoleucine and γ-aminobutyric acid. These fractions also contained an unidentified fluorescent material. On one occasion sporogenic activity extended beyond those fractions containing neutral ninhydrin positive materials. These active fractions also contained no basic amino acids.

On the basis of microbiological assay, paper chromatography, and column chromatography information, together with negative results obtained in the addition of specific amino acids or combinations of amino acids, we have fairly conclusive evidence that the amino acids of the original medium, and α- and γ-amino butyric acids, and diamino pimelic acid do not specifically enhance sporulation of PA 3679-h in 2% trypticase broth.

Inasmuch as 2% trypticase broth is virtually nonsporogenic, but mediums containing 4% and over of trypticase, 2% trypticase plus 2% acid digest of casein, or even 2% trypticase plus 2% casein, yield spores if adequate thiamine is present, it is apparent that casein contains some unidentified component which is required for sporulation. Growth in the higher concentrations of casein derivatives yields larger cell crops than the 2% trypticase with about 20% sporulation. When the spent 2% trypticase is refortified with trypticase, the sporulation reaches as high as 90%. This may in part be the result of the increased concentration of unidentified component of casein which is required for sporulation. It does not explain, however, the high sporulation rate obtained in the interrupted growth experiments, wherein the second crop of cells taken from a medium yields up to 90% spores; but if the original inoculum were allowed to grow and remain in the medium indefinitely, the cells would never produce many spores. Therefore, on the basis of the information obtained from the studies in progress, we believe that during growth the organism synthesizes and elaborates into the medium something which it requires for sporulation.

Informal Discussion

HARLYN HALVORSON: Dr. Ordal has given us an excellent review of the theories of sporulation. Inherent assumptions in all of these theories, especially the starvation theory defended by Grelet, involve the nature of precursors of spore macromolecules. Irreversible commitment to sporulation would involve either a redirection of *de novo* synthesis of macromolecules which are spore specific, or a modification of vegetative cell macromolecules prior to their incorporation into spores. Although Foster and Perry have indicated the former applies in the case of some spore proteins, it is not clear that this is a general phenomenon. A methodology for investigating *de novo* synthesis in the case of proteins and nucleic acids has been forthcoming from the studies of Gale, Mandelstam, Cowie and ourselves. Vegetative pools of amino acids, purines, pyrimidines and nucleotides can be specifically suppressed with a resulting restriction in protein synthesis, nucleic acid synthesis, or both. There are also an increasing number of mutants available in the *Bacillaceae* to aid in specifically controlling pool components. Also, by briefly exposing cells to C^{14} labeled pre-

cursors, specifically labeled pools can be prepared. A study of sporulation employing these controlled pools can provide valuable information not only to the nutritional requirements of spore formation but also to the extent to which vegetative macromolecules existing prior to the commitment to sporulation are incorporated into the spore.

CAMPBELL: Dr. Ordal, in connection with the effect of folic and para-amino benzoic acids on spore formation, have you tried replacing either one of these with Vitamin B-12?

ORDAL: Yes, but we found that B-12 cannot replace the folic acid requirement.

CAMPBELL: In connection with your findings that sulfate can replace methionine, it is of interest to note what we have found in the vegetative growth of *B. coagulans*. We thought we also had a methionine-requiring strain and tried to replace these sulphur amino acids with sulphate. This did not work unless we had B-12 present in the medium. With this organism we can leave out the methionine or other sulphur amino acids and give them sulphate plus B-12 and get good growth.

POWELL: We have not made any thorough study of the requirements of sporulation, but there are one or two things we have come up against that you may be able to explain to us. We find that all the strains of *B. megatherium*, *B. cereus* and *B. subtilis* we have looked at do not sporulate in tryptic broth, but three strains of *B. sphericus* and *B. fusiformis* sporulate with no trouble at all. The medium contains 500 milligrams of nitrogen per 100 ml so the growth of *B. sphericus* is not enough to exhaust the medium; nevertheless this organism has no trouble whatsoever in sporulating in tryptic digest broth. We made another interesting observation when we diluted the tryptic broth and added glucose and manganese. In this diluted digest broth we got very strong stimulation or sporulation of *B. cereus* provided glucose was present. We got very strong stimulation of *B. megatherium* in the presence and absence of glucose. We got stimulation of sporulation by manganese in the absence of glucose for *B. subtilis*. When we changed our batch of medium we couldn't repeat those results.

CHURCH: I would like to ask the people studying sporulation if the conditions under which the spores have grown affect their properties, in particular as regards their germination requirements?

MANDELS: I want to comment on the question raised by Dr. Church. We have done some work with fungus spores indicating that the nature of the medium upon which they are grown has a really terrific effect upon the viability of the spores produced. The spores grown on a sugar yeast extract medium, for example, lose their viability within a week when left in

the culture. I get essentially less than 1% germination, whereas cultures on filter paper agar, for example, may retain their viability for quite a number of months.

SUSSMAN: As another intruder in the bacterial scene, I should like to bring to your attention a more famous fungus, the *Neurospora*, a favorite laboratory animal in the botany department. In this case it turns out that the method of growth of these spores determines thermal lability in terms of response to activating heat treatment in two ways: it determines first the amount of heat that is required for activation, which is a term I reserve for the breaking of dormancy, and second to a heat treatment of shorter duration and less intensity which is required for the regeneration of sensitivity to furfural, which is an activation chemical. Now both the heat treatments, called heat shock by bacteriologists, and the furfural effect, which applies to some thermophilic bacteria, are relevant directly to the question of germination of bacterial spores.

Bacterial Spore Germination—Definitions and Methods of Study[1]

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DURING the past ten years considerable progress has been made in unraveling the complexities of bacterial spore germination (see the excellent reviews of Knaysi, 1948; Wynne, 1952; Schmidt, 1955; and Stedman, 1956 a, b). Unfortunately, there is as yet no general agreement as to the meaning of germination as applied to bacterial spores. Thus, this term has been used in a variety of ways depending on the aim of the investigation, the methods employed, and the preference of the investigator. The purpose of this paper is to briefly review the methods which have been used in studying spore germination and to arrive at a definition of this process which will be acceptable to most of the investigators in this field of study.

Criteria of Germination

Visible turbidity. The appearance of visible turbidity following the inoculation of a medium with spores was employed by early investigators as a criterion of germination. The objection to this method stems largely from the fact that it is qualitative in nature. Thus, it is of value only in establishing that some spores have or have not germinated, provided that the environmental conditions are suitable for the subsequent propagation of the vegetative cells. That this criterion of germination can be misleading is demonstrated by the findings of numerous investigators that spores can germinate under conditions which do not permit the germinated form to survive (see Wynne, 1952; Schmidt, 1955; and Stedman, 1955 a, b). Another drawback to this method of study is that the environmental conditions imposed upon germinating spores may not affect germination but may appreciably influence subsequent vegetative development (Evans and Curran, 1943; Wynne and Harrell, 1951; Fitz-James, 1956; Levinson and Hyatt, 1956; O'Brien and Campbell, 1956). Thus, this method of studying spore germination is of limited value and has been largely abandoned for more precise quantitative methods.

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Direct microscopic counts. Direct microscopic counts have been employed for quantitative studies of spore germination. Such procedures are tedious and are not readily adapted to studies with anaerobes. Furthermore, they lack precision since with some species it is extremely difficult to establish a microscopic criterion of germination (Fischoeder, 1909; Swann, 1924; and Cook, 1932).

Heat lability. One of the most accurate methods for determining spore germination utilizes the loss in heat resistance as a quantitative measure of the number of germinated spores. Briefly, the method consists of heating a spore suspension at a time-temperature relationship which is lethal to the germinating spores but not to ungerminated spores and plating the survivors. A control suspension of ungerminated spores is treated similarly and the percentage germination calculated. Another technique often employed is to heat the spores at the beginning of the germination experiment, followed by a second heating after incubation in a suitable medium. The difference in spore counts before and after incubation is used for calculating the percentage germination. Several precautions must be taken when using this method. These have been summarized by Wynne (1952) and need not be enumerated here. Some investigators have criticized this method on the basis that it is detailed and time consuming. However, the fact that a definite time-temperature relationship can be established as a reproducible end point of germination outweighs these disadvantages.

Stainability. Uptake of stains such as methylene blue has been used as a measurement of spore germination (Powell, 1950; Levinson and Sevag, 1953). In this method air-dried smears of spores are stained with an aqueous solution of methylene blue. Ungerminated, heat-resistant spores appear as unstained, refractile bodies, while germinated, heat-labile spores stain blue. The total number of spores and the number staining blue are counted and the percentage germination calculated.

Darkening under a phase contrast microscope. When observed under a dark phase contrast microscope, ungerminated spores appear as refractile bodies while germinated spores appear homogeneously black or very dark blue (Pulvertaft and Haynes, 1951). This technique of studying germination correlates well with stainability and heat lability methods. This method is also excellent for observing the changes which take place in the transition of germinated spores to actively dividing vegetative cells. It should be pointed out that spores which do not darken never develop into bacteria.

Decrease in optical density of spore suspensions. A very rapid quantitative method of measuring spore germination is that of following the decrease in optical density of spore suspensions at some specified wave length, such as 610 mμ (Powell, 1950, 1951; Hachisuka *et al.* 1954, 1955). Some inves-

tigators have employed a 470-530 mμ band filter (Levinson and Sevag, 1953), a 420 mμ filter (Church *et al*, 1954) or a 540-590 mμ filter (Mandels *et al.*, 1956) to follow the changes in optical density. This method measures the loss in refractility of spores and has been correlated with the appearance of translucent areas in the spores when observed by electron microscopy (Hachisuka *et al*, 1954, 1955). A high degree of correlation also exists between this technique and those which measure dye uptake, heat lability, and darkening under a phase contrast microscope. The major advantage of this method is the speed with which quantitative germination data can be obtained.

Manometric methods. Manometric methods, in conjunction with other methods, have been used to measure the effect of substances which stimulate the basal respiration of spores. The marked increase in respiratory activity of germinating spores has been shown to compare well with other methods of determining germination (Levinson and Sevag, 1953; Levinson and Hyatt, 1955, 1956; Hachisuka *et al*, 1956; Mandels *et al*, 1956). An excellent discussion of this technique has been presented recently by Levinson and Hyatt (1956).

Definitions of Germination

In early studies on spore germination, most workers defined germination as the entire transition from the resting spore to an actively dividing vegetative cell. This view is still held by some modern investigators. Thus, Knaysi (1951) defined germination of the endospore as "a process involving growth and [which] takes place under conditions that favor vegetative growth." Fitz-James (1954) defined germination as the period "extending from the resting stage of the 'mononculeate' spore to the vegetative or 'binucleate' cell." More recently Fitz-James (1956) has divided the germination process into two parts: "The initial activation of the resting spores into a respiring cell and the period of growth leading up to the formation of a young vegetative bacillus." Preuner (1951) suggested the term "prevegetative" for the developmental sequence leading up to the vegetative form.

Most investigators have recently defined germination in terms of some convenient and easily demonstrable stage. Thus, germination has been defined to include loss in heat resistance, stainability, decrease in optical density, darkening under a phase contrast microscope, marked increase in respiratory activity, emergence from the spore coat, or the beginning of cell division. Using stainability and increase in respiratory activity as criteria of germination, Levinson and Sevag (1953) divided germination into two phasés: (a) "pregermination" or the process occurring when the spore "becomes stainable and has started to consume oxygen, but before it has elongated and become typically bacillary in shape," and (b) "germination"

which they define as the emergence of the bacillus from the spore case. However, demonstration by Mandels, Levinson, and Hyatt (1956) of the rapid increase in respiratory activity coincident with stainability has recently led Levinson and Hyatt (1956) to accept the validity of the latter as a criterion of germination.

It is interesting to note that nearly all of the investigators who have proposed new criteria (or definitions) of germination have always attempted to correlate their techniques with loss in heat resistance of the spore. Thus, for practical purposes, loss in heat resistance has been the standard for comparison of other methods of studying germination. This is not surprising when one considers that the most outstanding physiological difference between spores and vegetative cells of any one organism is the heat stability of the former in contrast to the heat lability of the latter. Since a spore after loss of heat resistance can no longer be considered as being in the spore state, germination may be defined in terms of loss of heat resistance. Thus, *spore germination may be regarded as the change from a heat resistant spore to a heat labile entity which may not necessarily be a true vegetative cell.* The excellent correlation of other physiological and morphological changes with loss in heat resistance makes this definition sound from both the morophological and physiological viewpoint.

If we accept the definition proposed above for germination, what term should be used to designate the transition of the germinated form to the vegetative cell? In our studies (O'Brien and Campbell, 1956) we have used the term "outgrowth" to designate the stages occurring subsequent to germination. This general term includes the various steps listed by Levinson and Hyatt (1956) as swelling, emergence, elongation, and cell division.

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Discussion

E. S. Wynne

We agree with Dr. Campbell that development of turbidity is not a serviceable criterion of spore germination. As pointed out earlier (Wynne, 1952), a number of workers have reported germination without appreciable subsequent vegetative multiplication under a variety of experimental conditions, including unfavorable oxidation-reduction potential (Knight and Fildes, 1930) inadequate nutrients (Knaysi, 1945; Powell, 1951), and the presence of appropriate concentrations of penicillin (Wynne and Harrell, 1951) or streptomycin (Wynne *et al*, 1952). On the other hand, salts of C_{18} unsaturated fatty acids have been found to be potent inhibitors of germination, but much less effective against vegetative cell multiplication (Foster and Wynne, 1948). Finally, with putrefactive anaerobe No. 3679, the amount of germination occurring by the time of appearance of turbidity has been found to vary with incubation temperature (Mehl and Wynne, 1951).

In our laboratory, change in heat lability has been considered the most workable criterion of germination (Wynne and Foster, 1948a; Wynne, 1952). The first investigator to employ loss of heat stability as a criterion of germination appears to have been Fischoeder (1909), whose method was a modification of a technique devised by Weil (1901) but was subsequently discarded. Using spore suspensions of *B. anthracis*, Fischoeder removed aliquots at intervals during incubation and heated for 3 minutes at 80°C to kill any vegetative cells resulting from germination. Plating then yielded colonies representing only the heat-resistant ungerminated spores. Decrease in the number of spores from the level present before incubation was used to measure germination. Similar methods of study were employed by Evans and Curran (1943) in their classical work showing the importance of preheating spores before incubation for germination, and by Hills (1949). In our studies, percent germination rather than numbers of ungerminated spores has been used as a basis for quantitative measurement of germination, since with this standard it is felt there is less likelihood of attaching a false significance to small deviations (Wynne and Foster, 1948a; Wynne, 1952).

As detailed elsewhere, (Wynne and Foster, 1948a; Wynne, 1952), the method employed in our laboratory for studying germination with *Clostridia* has generally involved a pre-heating period of 5-20 minutes at 75° C for expelling dissolved oxygen and effecting any possible heat activation of spores. After incubation in appropriate media under anaerobic conditions, a second heating period of 20 minutes is employed to destroy units which

have become heat labile. The total heating time at 75 C in the control tubes without incubation is equalized with that of incubated tubes. Spore levels are determined by plating into a counting medium such as Yesair's pork infusion agar with 0.1% soluble starch and 0.5% BBL thioglycolate supplement, or the yeast extract starch bicarbonate agar recently described (Wynne, Schmieding and Daye, 1955), with an anaerobic overlay of 3-4 ml of the counting medium of 1.5% agar containing BBL thioglycolate supplement. Counts are considered to represent ungerminated spores. The number of ungerminated spores remaining after incubation is subtracted from the level of spores prior to incubation, and the difference is considered to be spores which have germinated. Percentage germination is then calculated by dividing the number of germinated spores by the spore level prior to incubation, or after incubation in water or buffer. Generally, duplicate or triplicate platings are made from triplicate tubes, with statistical analysis of the data for significance.

In testing a given agent or condition for possible effects on germination, it has been emphasized that the length of the incubation period is important (Wynne and Foster, 1948a; Wynne, 1952). For stimulatory effects it is desirable to use an incubation period sufficiently brief to insure relatively little germination in the controls. On the contrary, inhibitory effects are best shown with an incubation period of such length that germination in the controls is essentially complete. However, the incubation period must not be long enough for appreciable resporulation. Furthermore, it is essential that the test conditions be such that dormancy does not occur to any significant degree (Foster and Wynne, 1948; Wynne and Foster, 1948a; Wynne, 1952).

In testing a given agent or condition for possible effects on spore germination, it has been emphasized in previous publications (Wynne and Harrell, 1951; Wynne *et al*, 1952) that a change in level of recoverable spores may result from (1) an effect on germination, (2) a sporicidal effect, (3) inhibition of colony development in the counting medium, or (4) any combination of these. A sporicidal effect may be tested for by exposure to the agent or condition under consideration in an environment in which germination does not occur, e.g., by incubation in water or buffer. Inhibition of colony development in the counting medium may be detected by adding the agent tested directly to the counting medium. It is possible to test for sporicidal effect and/or inhibition of colony development by a single control consisting of spores in water or buffer containing the agent studied and incubated for the same time as parallel systems containing spores in (1) water or buffer, (2) germination medium, and (3) germination medium plus agent (Wynne and Harrell, 1951; Wynne *et al*, 1952).

The general method of study of spore germination just described has been illustrated in tables in a number of publications (Foster and Wynne, 1948; Wynne and Foster, 1948a, 1948b; Wynne and Harrell, 1951; Wynne, 1952; Wynne *et al*, 1952; Wynne *et al*, 1954). In germination of five strains of *Clostridia* in buffered glucose at 37°C, a post-incubation heating period to eliminate vegetative cells has proved unnecessary (Wynne *et al*, 1954). Germinated spores apparently become non-viable from nutritional inadequacy of the medium (Powell, 1951) or during dilution and plating procedures.

Recently in our laboratory considerable effort has been expended in the study of an unexpected phenomenon. When spores of mesophilic *Clostridium* are incubated at 75°C in glucose carmelized by overautoclaving, the level of recoverable spores falls rapidly (Wynne, Galyen and Mehl, 1955; Wynne and Galyen, 1956), with essentially no decrease in incubation in brain-heart infusion broth. This rapid decrease in heat resistant units is generally essentially complete in from 20 to 120 minutes, depending on the strain. Furthermore, the process appears to be logarithmic in 3 to 5 test organisms, with a definite lag period of from 2 to 15 minutes. Turbid spore suspensions incubated at 75°C in caramelized glucose have shown no detectable loss in optical density. The vegetative cells of the five test species do not grow at 50°C, and two of them do not grow at 48°C.

We have been unable to explain the rapid loss in heat-stable units occurring in overautoclaved glucose at 75°C on any basis other than the occurrence of germination, and considerable evidence has been secured that germination does in fact occur. These findings have been published in abstract form (Wynne, Galyen and Mehl, 1955; Wynne and Galyen, 1956) only, but detailed publication is contemplated.

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Informal Discussion

CHURCH: Our criteria of germination have been based on a thermal lability and we feel, too, that it is sound and safe procedure and a most practical one for use at this time. I wonder if what is occurring prior to germination, a state of activation such as we have recently observed in the case of glucose oxidation and which under certain conditions leads to germination, cannot also be included as a third state in the physiological development from the resting spore.

SUSSMAN: At the risk of being somewhat pedantic, I should like to say a little about what I consider to be the original concept of germination that developed in biology. This concept grew up around the fact that one could observe morphological changes which coincided with the disruption of dor-

mancy. This meant, therefore, that germination in the traditional sense referred to the morphological changes coincident with the breaking of the spore wall, perhaps with the protrusion of a germ tube; the excystment of certain animals would also fit in this category. Therefore, the stages between the rupture of dormancy by whatever means and the emerging of the vegetative mycelium, or vegetative animal, were all lumped together in the process called germination. Subsequent to this, refinements in physiological and biochemical techniques made it possible to study the transition from the dormant organism to the germinated one. Due to these refinements transition stages appeared, one of which Dr. Church and I have called the activated stage.

To me the sequence of stages as recently worked out by Church and Halvorson coincides very nicely with some of the observations in fungus systems with which I have worked. For example, in the dormant cells of some fungi one has a system where there are qualitative differences in their metabolism as compared with the vegetative organism. Immediately upon the application of a stimulus that will break dormancy, metabolic changes ensue before recognizable morphological ones appear, so that the traditional usage of the term "germinated" cannot be applied. Therefore, there are three stages which I see in general: There is the dormant cell; then there is the in-between stage which is not dormant and is not germinated, but which I prefer to call the activated stage, followed, of course, by the germinated cell. I should like to refer to the work of Church and Halvorson again, for I think that there is a strict parallel with the situation discussed for fungus spores. In the case of the spores with which these authors worked, if the heat-shocked cell is studied before it is confronted with what I would call an activating agent like sugar, many enzymatic changes occur. Here, then, is a cell in the never-never stage between the dormant one which shows restricted enzymatic activities and the germinating cell which shows the change in heat resistance: it is, therefore, a cell that is heat resistant but activated in the sense of metabolic activtity. Its heat resistance makes it a hybrid between the dormant and vegetative condition. For these reasons I suggest that consideration be given the concept that the stage intermediate between the dormant and germinating one be called the activated stage.

ROBINOW: I should like to make two comments. In experiments on the sensitivity of spores of *B. cereus* to X-rays, carried out with the late Douglas Lea and repeated over here, it was found that exposure of 1 and 1½ million roentgen units, although it suppressed outgrowth of viable bacilli, did not prevent the spores from losing their refractility and becoming stainable when transferred to a nutrient medium. I wonder whether others have made similar observations and, if so, whether the interval elapsing between the

onset of the first visible germination changes and the emergence of new vegetative bacilli from ruptured spore cases ought not to be subdivided into at least two periods with different sensitivity to damaging influences.

The other matter concerns the advisability or otherwise of dating germination of any kind of spore, the conidia of molds, for example, or those of bacteria, from (a) the moment of rupture of the spore case, or (b) from the completion of the first cell division. Concerning (a) one wants to remind oneself that vegetative spores such as the conidia of *Penicillium* or other imperfect fungi germinate directly without rupture of their wall. There is continuity here between the spore wall and the wall of the outgrowing hypha. It is different with ascosphores of *Penicillium* and with the endospores of bacteria. Here a new cell wall forms beneath the spore coat. On germination the coat bursts open and is later discarded by the growing organism. As to cell division (b) it must be pointed out that what in life appears as a single bacillus emerging from a spore case is, at least in *B. megatherium* and *B. subtilis,* often already fully divided into two cells with two, if not four, nuclear equivalents. The transverse septum separating the two cells is readily seen after suitable staining but not easily during life.

MANDELS: I would like to comment briefly on that. It seems to me that biologically the significant point of germination is a transition from a state of low metabolism to one of high. It seems to me that the germination of a bacterial spore should be considered to be those changes that occur along with this initial loss of heat resistance that occurs very rapidly along with an increase in respiratory activity. The fundamental thing seems to me to be this increase in metabolic activity. This is followed by swelling and rupture of the spore coat.

SUSSMAN: It seems to me that these experiments point to one of the deficiencies in the use of loss of heat stability as a criterion of germination. I should like to recount just briefly a few experiments with *Neurospora.* If one takes spores of this organism and treats them with a variety of poisons, one finds that none of these materials penetrate the intact spore as measured by techniques like manometry. Although these materials are removed from solution, they penetrate only at the time when a visible protrusion appears, signalizing the start of germination. With reference to the work that Dr. Halvorson's group is doing, *Neurospora* ascospores can be activated by heat, but if exposed to anaerobic conditions for an hour, the respiratory stimulation is lost and the rate diminishes to that of the dormant cell. The point is that although you get some of the types of metabolic stimulation that seem to be associated with the whole germination process, it can only go so far and no further. This means to me that the whole of the germination process has not really been accomplished and that there is only "partial" germination

under anaerobic conditions. In *Neurospora*, Goddard demonstrated that the spores could be reactivated at any time after anaerobic incubation by exposure to air so that the process is entirely reversible.

HARLYN HALVORSON: Although Dr. Foster has pointed out that resistance to heat probably does not play a major role of the natural selection of spores, heat resistance remains as our only criterion of dormant spores. Since we obviously require an operational definition of germination, until a clearer basis of natural selection is available, it seems clear that we should employ the loss of heat resistance as a measure of germination. Such a definition, however, does not describe the activation of respiratory enzymes in spores of *B. cereus* var. *terminalis* without the loss of heat resistance. In *B. megaterium* on the other hand, respiratory activity parallels loss of heat resistance. However, other dormant enzymes of the *B. cereus* var. *terminalis* type may be found. Therefore we should probably adopt the term "activation" to decribe this stage prior to germination.

MURTY: I wish to point out the difficulties of studying the effects of enzyme inhibitors on germination if an increase in metabolic rate and ability for outgrowth are included in the criteria of germination. As many of us already know, it is very difficult to inhibit germination as chacterized by decrease in optical density and ability to take up stain. Enzyme inhibitors like arsenate, cyanide, etc., fail to stop these changes in optical density and stainability of the spores. However, some of them do interfere with respiration and outgrowth. It is further complicated by the fact that one cannot always completely reverse the inhibition of respiration and outgrowth by washing. Furthermore, the medium in which germination is studied seems to affect the numbers of spores eventually outgrowing even when one washes the spores and adds agents to reverse the action of the inhibitors originally added. Thus there is a difference in the numbers of spores outgrowing, depending upon whether they are allowed to germinate first in a simple, chemically defined medium, often insufficient to support outgrowth, and then transferred to a complete medium, or in a complete medium in the first instance. I believe Dr. Powell also ran into a similar problem when studying the effect of mercuric chloride on germination. So it appears that it may not always be possible to use respiration and outgrowth as the criterion for germination and especially so when studying the effects of inhibitors.

Effect of Moisture Activity on Germination

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It is well known not only that water is necessary for biological activity, but that too low a level of availability may restrict or completely prevent such activity. For example, spore germination and vegetative growth of micro-organisms will not take place in a completely dry environment; furthermore, it is possible to restrict the water content in either moist solid materials or in solution to such an extent that germination and growth are completely inhibited. In porous solids control of moisture is a simple matter, and in solution a high concentration of some inert substance will limit the water availability.

Water availability, however, is not precisely measured by percentage of moisture, since biological materials vary widely in their chemical affinity for water; thus, the amount of so-called "bound water" is not the same for all materials at a given moisture percentage. The water availability would be more truly expressed by a quantity which represents the net capacity of the moisture present to act as liquid water. Such a quantity would be the relative humidity of an atmosphere which would be in equilibrium with the material, and can be easily determined by measuring the equilibrium vapor pressure as compared to that of pure water. This quantity, expressed either as a percentage or as a decimal fraction, is called the water or moisture activity. Thus spores of a given species of mold may show different minimum percentages of moisture required for germination on different foods, but the calculated moisture activities will be the same. Unfortunately this important quantity is often neglected in studies of moisture requirements.

The critical moisture activities for spore germination have been rather well explored for fungi (Gottlieb, 1950). Most species require from 70% to 100% moisture activity for normal germination, with a few species exhibiting feeble signs of germination at 62% (Snow, 1949). Most of the common molds fall into the 75%-100% range, 75% being the commonly accepted value for safe storage of agricultural products. These values are arrived at by microscopic observation of spores planted on solid materials or glass slides incubated in atmospheres of controlled relative humidity. Some of the powdery mildews, however, have been found to germinate at moisture activities of nearly zero (Semeniuk and Gilman, 1944), probably by virtue of a high internal osmotic pressure and therefore presumably a high imbibi-

tion capacity. Less information is available regarding vegetative growth, but for the species for which this has been determined, higher moisture activities are required than for germination of their spores.

Bacteria are known to be considerably more exacting in their moisture requirements for growth than the majority of fungi. About 40% moisture has been considered necessary for growth on meats (Fabian, 1951), corresponding to about 95% moisture activity, and this latter figure is often quoted as that below which bacteria in general will not grow. In a few growth studies on individual species (Scott, 1953; Christian and Scott, 1953) values in the neighborhood of 95%-97% have been found.

Moisture requirements for the germination of bacterial endospores have been almost entirely neglected. Bullock and Tallentire (1952) prepared mixtures of spores of *Bacillus subtilis* with peptone, lactose and kaolin, and exposed samples of these mixtures to atmospheres of 100% relative humidity. Samples were withdrawn at intervals for plate counts and determinations of moisture uptake. As moisture uptake in the peptone powders proceeded, a point was reached where the spores lost heat resistance but remained viable. At a second point some of the spores germinated and died (both spore and total counts dropped), while at a third spores germinated and multiplied. These findings would indicate that spores of bacteria, like those of fungi, require higher moisture activities for growth than for germination. In kaolin only the first of these phenomena occurred, while in lactose the second took place but almost simultaneously with the first. The percentages of moisture uptake for the first change are given and they are far apart, as would be expected from the radically different hygroscopicities of the materials; these workers chose well from the standpoint of a wide range of moisture affinities and nutrients, but they did not determine moisture activities, so their data do not help us in the determination of critical moisture levels for this organism. Their main conclusion was that the range between the second and third points might be put to use in ridding such products as pharmaceutical powders, in which they were interested, of at least some of their spores.

Waldhalm and Halvorson (1954) found that spores of *Bacillus cereus* var. *terminalis*, when dried and rehydrated by storing in an atmosphere of 100% relative humidity until equilibrium was reached, possessed a higher moisture activity than vegetative cells of the same organism similarly treated. This indicated that the spores had less affinity for water than the cells, in opposition to the theory that spores contain an abnormal amount of "bound water." In an extension of this work Halvorson and Waldhalm (1953) reported preliminary experiments in which they mixed spores with dried wheat flour and exposed the mixtures to moist atmospheres so that different moisture activities were created. The mixtures were incubated and sampled

for counts at intervals. They observed an initial significant drop in spore count, but no further drop over a period of several weeks. However, the highest moisture activity they reached was 75%, which they considered insufficient for germination: and they attributed their initial spore loss to local areas in which the moisture activities were sufficiently high. It may also be that some spores lose their heat resistance under these conditions without germinating, since products like sugar and flour containing spores are known to gradually lose spores on standing. Dried spores stored in our laboratory showed a slow decrease in viability with time, althought they exhibited complete germination as measured by decrease in optical density of their suspensions. Loss of viability may precede this change however, and transcluency may be induced in a nonviable spore by germinating nutrients.

We continued these studies by the same general method with the difference that we attempted to create the desired moisture levels internally by mixing liquid water directly with the spore-flour mixtures. Though we tried a number of devices, we were only partially successful. Bullock and Tallentire claim to have done this in their peptone-spore powders, but they do not give much detail as to how they obtained uniform distribution. We were not able to create a desired moisture level with good reproducibility, but by preparing a large number of powders we did obtain samples well covering the range we desired. Unfortunately these powders lost moisture activity on standing, and in no case were we able to demonstrate a significant trend in germination that could be correlated with moisture activity. Most of our samples showed an early drop in spore count and none thereafter, recalling the data of Halvorson and Waldhalm. This occurred regardless of the initial moisture activity. While the explanation of local areas of high moisture activity would be applicable here, due to the method of introducing water into the powders, it may also be that the conditions existing in our powders destroyed the viability of some spores, or at least rendered them heat sensitive without actual germination. Or they may have been so near to germination that this treatment was sufficient to nudge them over the top. Spores in a given population seem to exist in a variety of conditions, and therefore respond differently to a number of treatments, some proceeding toward germination from the fully dormant spore, and others toward loss of viability without germination, branching off from the germination pathway at an undetermined point. That dead spores may have some of the properties of germinated spores is indicated by the fact that one batch of autoclaved spores showed solid staining.

We went next to a microscopic method patterned after the one that was used successfully with mold spores. Because of their smaller size the morphological changes are difficult to follow microscopically in the early stages of

germination; though it has been done in liquid media with phase microscopy, this is not practical in dry mounts. We suspended the spores in a nutrient solution in the cold, prepared replicate smears of this suspension on slides, and dried them in a desiccator. The slides were then incubated in atmospheres in which relative humidity was controlled by sulfuric acid solutions, and withdrawn at intervals for staining. When nutrient broth was used as the suspending medium, vegetative cells were seen only rarely, indicating absence of germination. When the relative humidity of the atmosphere was 100%, these cells appeared in about 12 hours; in 24 hours the smear was almost solid with growth, in 48 hours extensive resporulation had occurred, while in a week (possibly sooner: this was never checked) much of the vegetative tissue had autolyzed and many of the new spores appeared relatively clean.

Under relative humidities slightly below 100%, the picture was the same up to the point of resporulation, which was not observed at reduced humidities. When the sulfuric acid used to maintain the atmosphere was more concentrated than 5% (98.4% relative humidity), it took longer for the vegetative cells to appear, some times as long as 8 days over 8% acid (97.3% relative humidity), and the final growth was much less dense. With concentrations of sulfuric acid greater than 8% no change was observed in the spores in an experimental period of 32 days.

When the suspending medium contained only L-alanine and adenosine, the solidly staining germinated spores disappeared, though vegetative cells were occasionally found. The same limits of humidity affected the germination in the same way as when nutrient broth was used.

To check these findings we prepared spore suspensions in the same two media as were used for the slides, but dissolved different amounts of sucrose in them to limit the moisture activity. Germination was followed by measuring the drop in optical density in a Klett Summerson colorimeter. In solutions containing up to and including 20% sucrose (98.6% moisture activity) the drop was the same as in the control tube containing no sucrose. In sucrose concentrations between 20%-30% this drop was less, while in concentrations above 30% (97.6% moisture activity), it became negligible. These moisture limits are in good agreement with those determined by means of smears (see Table I).

The storage history of the spores is not without effect on these phenomena. The experiments described so far were carried out with spores which had been stored for some time. A recently prepared batch of spores of *B. cereus* var. *terminalis* which had, however, been harvested and stored for a short time under refrigeration was slightly more tolerant to lowered moisture levels; it showed some germination in 32% sucrose (97.3% moisture activi-

TABLE I

Germination of Old Spores of *B. cereus* var. *terminalis* in sucrose

% Sucrose	Moisture Activity	Klett Drop
0	100.00	100
19	98.72	98
20	98.63	97
21	98.54	77
22	98.43	72
25	98.11	66
29	97.72	41
30	97.60	40
31	97.47	4
32	97.34	0

ty), whereas freshly prepared nonrefrigerated spores germinated in 35% sucrose (96.8% moisture activity). Spores of *Bacillus subtilis* and *Bacillus megatherium* were also prepared and tested by this technique to see if species differences existed. Unrefrigerated spores of *B. subtilis* ceased germination above 34% sucrose (97.0% moisture activity), while for *B. megatherium* the limit was 38% sucrose (96.3% moisture activity). Spores of *Clostridium botulinum* Type B were also examined, both in 2% thiotone water and in a solution containing L-alanine, L-phenylalanine, and L-arginine, which has been found to satisfy the germination requirements for this organism. In both media the highest concentration of sucrose supporting germination was 31% (97.5% moisture activity) (see Table II).

TABLE II

Moisture Limits for Germination

Organism	Condition	Moisture Limit	Method
B. cereus var. *terminalis*	Old	97.3	Slides
B. cereus var. *terminalis*	Old	97.6	Sucrose
B. cereus var. *terminalis*	Fresh, Ref'd	97.3	Sucrose
B. cereus var. *terminalis*	Unref'd	96.8	Sucrose
B. subtilis	Unref'd	97.0	Sucrose
B. megatherium	Unref'd	96.3	Sucrose
Cl. botulinum	Old	97.5	Sucrose

The limiting moisture levels for growth of these organisms was also investigated for comparison. The highest concentrations of sucrose which would support growth (increase in optical density in 48 hours) were 45% (94.7% moisture activity) for *B. cereus* var. *terminalis*, 46% (94.4% moisture activity) for *B. subtilis*, and 49% (93.7% moisture activity) for *B. megatherium*. For *Cl. botulinum*, using a trypticase-glucose medium, no growth could be demonstrated above 30% sucrose (97.6% moisture activity) (see Table III).

TABLE III

Moisture Limits for Growth

Organism	Moisture Limit
B. cereus var. *terminalis*	94.7
B. subtilis	94.4
B. megatherium	93.7
Cl. botulinum	97.6

Thus, for aerobic species of spore formers at least, higher moisture activities are required for germination than for growth. There appear to be slight species differences in the moisture requirements for germination among the aeobic species, but this is affected by their method of preparation and storage history, and if our idea of multiple stages of germination is correct, this can be verified only when we know for certain that we are dealing with fully dormant spores.

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Discussion

Morton M. Rayman

Mr. Chairman and members of the Conference: Dr. Beers has presented an excellent review of the important role of moisture relationships in bacterial spore germination and growth. You are to be congratulated. Dr. Beers. Research in this field is difficult. Very little systematic work has been published. Nevertheless. the need for establishing the critical moisture levels for germination exists. Not only is there an intrinsic interest. but also for the solving of practical problems: it is of particular importance in the preservation of certain food products as well as pharmaceutical products.

I shall confine my remarks mainly to some aspects of the moisture limitation problem aimed at preventing spores from germinating in food stuffs. Since the beginning of World War II the dehydrated food industry has expanded enormously. and hundreds of millions of pounds of dehydrated vegetables. milk. and eggs are now produced annually. Substantial amounts of dried or dehydrated fruits and cereal products are also processed today. Currently there is a trend toward the dehydation of precooked foods for convenience feeding. For all these products it is essential that the water content be reduced to a point at which germination and bacterial growth are restricted. In addition to the aforementioned items. there is a variety of nonsterile canned foods which are of special interest. These include canned white bread. canned steamed puddings. dehydrated sliced bacon. canned cheese spreads. and canned chocolate nut rolls.

I am glad to note that progress has been made in relating spore germination to the role of moisture. Certainly we do not yet have everything in sharp focus, but the outlined shadows are emerging. Perhaps a diagram will illustrate the general pattern. In Fig. 1 three zones of differing effective water concentrations are shown. From essentially 100 percent water downward to a limiting concentration *A*, one encounters a region in which germination and growth are normal and unrestricted. As dehydration proceeds within the second zone. between water concentrations *A* and *B*. delayed germination and restricted growth are met. In this range of moisture values the phenomenon of dormancy may occur. It further appears that below the critical water concentration *B* no germination or growth can take place.

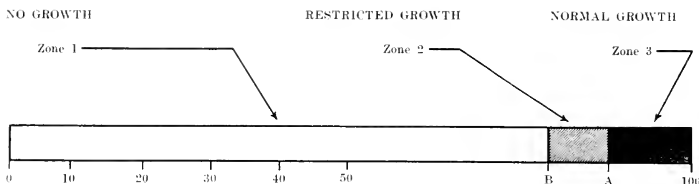

Fig. 1. Influence of Moisture on Spore Germination and Growth [Diagramatic].

Dr. Beers has reported that this point is believed to exist in the region of approximately 95 percent relative humidity for many bacterial spores and it is perhaps as low as 70 percent for molds. One research goal of the food bacteriologist is to locate and clearly define the limits of Zone 2 for foods and, particularly, the variation in the position of point *B*. It seems, at present, that this geometry may be shifted somewhat in accordance with other complex parameters. For a given species at least two factors may now be recognized on this issue, namely, (a) the pH of the medium, and (b) the age of the spores. Undoubtedly there are others.

Halvorson (1949) has employed the term "moisture activity" to refer in the thermodynamic sense to the effective concentration of water in the substrate. As determined by vapor pressure techniques, this activity is equivalent to a relative humidity measurement of the spores' environment at equilibrium conditions. For a given food one may expect to find a limiting moisture activity preventing spore germination and growth. An important bacteriological distinction should be made between the moisture activity of a substance and its total water content. Since the latter value includes some water which may be regarded as unavailable to the organism, it is necessary to know the relation between the total moisture content and the activity of the water in the substrate under consideration. Only recently have some of these relationships been worked out. Two illustrations are shown in Table I. It will be noted that for these different substrates the relationships differ. Whether the limiting moisture activities for the different foods are exactly the same, however, has not yet been proven. Some studies related to the moisture limitations on spore germination have been reported for canned bread (Halvorson, 1949; Kadavy and Dack, 1951; Ulrich and Halvorson, 1948-49; Wagenaar and Dack, 1954), beef liver paste (Williams, 1950 and 1953), canned cheese spreads (Wagenaar and Dack, 1955), and for such dehydrated meats as beef and pork (Segalove and Dack, 1951). Data on the germination of *Clostridium botulinum* spores for the two products shown in Table I will illustrate this point. In the experimental development of

TABLE I

Relationship of Total Water Content to Moisture Activity[1] for Different Substrates

White Bread, crumb[2]		*Beef Liver, paste*[3]	
Total Moisture (percent)	Moisture Activity (percent)	Total Moisture (percent)	Moisture Activity (percent)
38	96	60	97-100
33	94	50	96-98
30	92	45	94-96
28	90	40	90-95
27	88	35	90-95
25	86	32	90-95
22	82		
20	76		

[1]Approximate values are given.
[2]Data based on work of Halvorson, 1949.
[3]Data based on work of Williams, 1950.

canned white bread for the armed forces, it became necessary to establish bakery formulation limits for safety to have assurance that spores of *Cl. botulinum* in the flour would not germinate in the baked product. Investigations (Halvorson, 1949; Kadavy and Dack, 1951; Ulrich and Halvorson, 1948-49; Wagenaar and Dack, 1954) demonstrated that the control of moisture content when combined with pH control was effective in preventing toxin production in canned bread. In experimentally inoculated packs of canned bread at moisture levels above 36 percent and with the pH adjusted to 5.8, *botulinum* toxin was produced on storage of the cans at 32° C. (90° F.). While toxin formation occurred within 2 weeks of incubation in canned bread containing 40 percent moisture if the pH was set at 5.8, no toxin was found in a year's storage for bread at the same moisture level when the pH was adjusted to 4.8. Government specifications for canned bread, drafted after extensive experimentation, now require that the moisture content of the product shall not exceed 35.0 percent and the pH shall not exceed 4.8. A value of 35 percent for the total moisture in canned bread corresponds to about 93 to 95 percent moisture activity. Under these moisture conditions with added pH control a margin of safety is provided for this canned food to preclude spore germination.

In a report on a somewhat parallel investigation by Williams and Purnell (1950 and 1953) involving limiting growth of the same organism on another substrate, liver, the corresponding moisture activities and total moisture contents were measured. Dehydrated raw liver, inoculated with spores of *Cl. botulinum*, was made into pastes with varying amounts of water in the range of 32 to 60 percent total moisture content, and the extent of spore germination and growth after incubation at 37° C. was determined. Their results indicated that no increase in the viable count took place during 16 days' incubation when the total moisture content was 35 percent or less. However, at a moisture level of 40 percent, the growth was irregular and delayed. Good germination and growth were attained at total moisture values of 50 percent or higher. Thus the limiting concentration appeared to occur between 35 and 40 percent total moisture content. This corresponded to a roughly measured moisture activity of 90 to 95 percent. As may be noted from Table I, the hygrostatic technique employed for determining moisture activity did not distinguish between samples of 35 and 40 percent total moisture. As in the case of canned bread, control of pH in the liver paste was an important factor; thus when the pH of the paste was initially adjusted to the broad range of 6.8 to 7.5, irregularities in the results were smoothed out.

There are some indications that a slow aging effect which occurs during the laboratory storage of spore crops results in altered characteristics of the spores. Recent studies by Halvorson and Murty (1956) suggest that the moisture limits for germination of *Bacillus cereus* var. *terminalis* spores have shifted slightly lower over a period of about 10 months, even though the spores were stored in the frozen state. Dr. Beers has also reported that moisture limits for germination vary somewhat with the history of the spores. If it be true, as discussed earlier this afternoon, that vegetative cells require less moisture to permit growth than spores require for germination, then it might appear that aging spores are becoming less spore-like and more like vegetative cells. Perhaps such a phenomenon could assist in explaining the occasional spoilage which occurs during prolonged storage of canned foods arising from the altered germination requirements of long dormant spores.

One final comment may be of interest and it relates to the moisture-binding properties of different bacterial proteins. Waldhalm and Halvorson (1954) showed that the dehydrated spores and vegetative cells of *B. cereus* var. *terminalis* possess different affinities for water. Their work demonstrated that spores are less hygroscopic than vegetative cells and that the spores can give up their moisture to the cells if both are stored together in a closed system. When the difference in structure and physico-chemical properties of spore proteins and bacterial cell proteins is ultimately worked out, we may hope to

better understand and more effectively utilize the factors involved in these water relationships.

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Activators and Inhibitors of Germination

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Introduction

THIS aspect of spore germination has received a great amount of attention in the last ten years. Mr. Titus and I estimate that during this period of time there have been well over one hundred papers published containing sound and relevant information. Since it is obviously impossible to review adequately and correlate the published work during the allotted period of time, we decided that I would present some original data on activation and inhibition of spore germination, and that Mr. Titus would then present some comments and relevant references to the literature.

As was quite apparent in an earlier session, there is still some disagreement as to the definition of spore germination and as to the criteria to be used in determining that it has taken place. For the present we must admit that different criteria have been used in different laboratories and for different purposes and hope we may correlate the results satisfactorily until stronger definitions and criteria may be agreed upon.

In the work to be presented, the criterion of germination of a spore was the appearance of a colony on a plating medium, since this work was conducted in relation to spore counting and spore recovery techniques.

Procedure

Two suspensions were used in this work, 5230-5 and 5230-24. Culture 5230, a strain of *Bacillus subtilis*, was received in 1952 from Dr. H. R. Curran as #15u. Suspension 5230-5 was produced from growth on nutrient agar with 1 ppm manganese. Suspension 5230-24 was produced on the synthetic medium 5504. These suspensions did not contain more than 0.1% vegetative cells.

For plating two media have been used: Difco dextrose tryptone agar plus 0.5% Difco soluble starch, hereafter referred to as DT + ST, and medium 5504. The second medium was composed of 0.2% L-asparagine, 0.5% dextrose, the usual salts A and B, and 1.5% Difco agar. The medium was prepared and sterilized without the dextrose, which was added in the form of a concentrated, separately sterilized solution to the melted basal medium just prior to pouring plates. All other additives to either medium were also prepared in separately sterilized solutions and added to the melted media just prior to pouring plates.

By means of preliminary tests, diluted stock suspensions were prepared and standardized to give a mean count of approximately 100 spores per 0.1 ml on the DT + ST agar following a preheat of 15′ at 212°F. The incubation temperature was 85°F. The standardization to not over 100 colonies per plate and the use of lower incubation temperature were earlier found necessary in order to achieve maximum counts. In all tests, triplicate plates of each variable were poured. Plates were counted daily or at intervals of two or three days. Observations were continued as long as 20-23 days where continually increasing counts indicated.

Results

The results to be presented have been gathered over a considerable period of time. Since the incubation periods have not always been equal, it has been necessary, in order to present the results comparatively, to group the data somewhat according to incubation periods. The group periods adopted are 2-3, 5-6, 9-10, 15-17, and 20-23 days.

Table I shows the results of a heat activation test using both the complex and the synthetic media. On the complex medium 63% of the spores do not require heat activation. On the synthetic medium from 96 to 84% of the spores require heat activation depending upon the length of observation. Since 100% of the spores germinate and form colonies on the synthetic medium following heat activation, it is shown that the medium is adequate for colony development from a germinated spore. It is apparent, therefore, that chemical activation is taking place in the complex medium. Studies which have been conducted previously by this technique have very probably not measured the full requirements or effects of heat activation or perhaps have been unable to find heat activation because of the chemical activation produced by the plating medium.

Table II shows the effects of chemical activation in the synthetic medium. The control counts on the basal medium represent the range of 4 control

TABLE I

Heat activation of spore suspension 5230-5

Medium	Mean Spore Count per Plate Incubation Time in Days			
	2-3	5-6	9-10	15
DT+ST	63	70	70	x
DT+ST H*	100	98	x	x
5504[1]	0	4	5	16
5504 H*	49	103	x	x

* Heat activated spores

TABLE II

Chemical activation of spore suspension 5230-5

Medium	Mean Spore Count per Plate Incubation Time in Days				
	2-3	5-6	9-10	15-17	20-23
5504 (Range)	0	1-2	3-4	6-9	13-21
Yeast Extract 500*	61	58	58	59	x
50	54	57	56	59	x
10	22	43	46	47	x
L-alanine 50	46	58	x	59	x
5	53	58	x	58	x
Adenosine 10	1	3	5	x	19

* mcg/ml

experiments carried out at different times. The numbers following the substances represent micrograms per ml concentration. Yeast extract at 500 or 50 mcg activates about the same number of spores as the complex medium, 10 mcg is somewhat less effective. One mcg was without effect. Confirming the results obtained by other investigators with other procedures, it was found that L-alanine is an effective activator at 500 and 5 mcg concentration. With this suspension, adenosine at 10 mcg was ineffective.

Table III shows a sumary of heat and chemical activation of the two spore suspensions. In general, the behavior of the 5230-24 suspension was the same as the 5230-5, the main difference being that the 5230-24 suspension showed somewhat more rapid germination on the basal medium and a somewhat greater sensitivity to chemical activators. This table shows again

TABLE III

Heat and chemical activation of spore suspensions 5230-5 and 5230-24

Medium	Percentage of Maximum Count 5230-5	5230-24
DT+ST	65-70	85-90
DT+ST H*	100	100
5504	6-9	10-15
5504 H*	100	100
5504 + Yeast extract 500**	60-70	75-80
5504 + L-alanine 500	60-70	75-80

* Heat activated spores
** mcg/ml

TABLE IV
Chemical activation of spore suspension 5230-5 by L-amino acids in 5504 medium

Substance added*	Mean Spore Count per Plate Incubation Time in Days			
	2-3	5-6	9-10	20-23
None	0	1-2	3-4	13-21
L-alanine 50	57	71	75	75
D-alanine 50	0	2	32	13
DL-alanine 100	0	13	39	66
L-valine 100	36	60	68	64
DL-valine 200	19	30	55	60
β-alanine 100	10	21	47	59

* mfg/ml

that the 5230-5 suspension contains about 30-35% of spores that are not activated chemically by yeast extract, L-alanine, or the complex medium and therefore require heat activation. About 65-70% of the spores can be activated either by heat or chemical activation. With suspension 5230-24, the results are qualitatively the same but quantitatively slightly different.

Table IV shows the results of testing for chemical activation by various amino acids. It may be observed that L-alanine is effective and D-alanine is inactive. The effect of DL-alanine depends entirely upon the period of observation. D-alanine inhibits the activating effect of L-alanine for a considerable period of time but eventually the same final count is reached. L-valine has been found as effective as L-alanine; and D-valine inhibits some-

TABLE V
Chemical activation of spore suspension 5230-5 by L-amino acids in 5504 medium

Substance Added*	Mean Spore Count per Plate Incubation Time in Days				
	2-3	5-6	9-10	15-17	20-23
None	0	1-2	34	6-9	13-21
L-methionine	2	3	6	13	45
L-serine	2	5	15	23	39
L-threonine	2	3	6	13	19
L-leucine	x	2	4	17	28
L-alanine	x	3	5	16	29

* 100 mcg/ml

TABLE VI

Test for activation by substances other than amino acids. Suspension 5230-5

Substance Added*	Mean Spore Count per Plate Incubation Time in Days				
	2-3	5-6	9-10	15-17	20-23
None	0	1-2	3-4	6-9	13-21
NH_4Cl 1000	x	4	6	24	44
Na Pyruvate 100	x	1	4	25	37
Furfural 10	x	2	2	3	5
Mn 100	0	0	1	1	1

* mcg/ml

what the effect of L-valine, although this is eventually overcome. β-alanine also is an effective chemical activator although the rate of development of colonies is somewhat slower than with L-alanine or L-valine.

Table V shows the results of tests with several other L-amino acids. L-methionine and L-serine seem to be slightly active following a long lag period. L-threonine is inactive and L-leucine and L-arginine are very questionable as to activity.

Table VI shows tests for activation by some substances other than amino acids. NH_4Cl and Na pyruvate appear to show slight activity after a long lag. Furfural and Mn^{++} are inactive in the concentrations tested and, in fact, in comparison to the controls, appear to be somewhat inhibitory of the spontaneous germination.

Table VII shows a summary of activation results for both suspensions

TABLE VII

Activation by amino acids and other substances

Strong	Weak with Long Lag	Inactive
L-alanine	L-arginine	DL-methionine
β-alanine	L-leucine	DL-phenylalanine
L-valine	L-methionine	L-phenylalanine
	L-serine	L-glutamic acid
	NH_4Cl	DL-serine
	Na pyruvate	L-threonine
		Furfural
		Mn

Table VIII
Inhibition of L-amino acid activation by D-amino acids. Suspension 5230-5

Substance Added	Mean Spore Count per Plate Incubation Time in Days				
	2-3	5-6	9-10	15-17	20-23
5504	0	1-2	3-4	6-9	13-21
L-serine	2	5	15	23	39
DL-serine	0	1	2	3	3
L-valine	36	60	68	x	64
DL-valine	19	30	55	58	60
L-alanine	51	71	75	x	75
DL-alanine	0	13	39	x	66

5230-5 and 5230-24. As strong activators we find only L-alanine, β-alanine and L-valine. The inactive substances include DL-methionine, DL-phenylalanine, L-phenylalanine, L-glutamic acid, DL-serine, L-threonine, furfural and Mn^{++}. Some substances are listed as weak with a long lag. Further consideration of the data of these experiments suggests that L-arginine, L-leucine, NH_4Cl and Na pyruvate should be transferred to the inactive column, leaving only L-methionine and L-serine in the group showing weak but definite activation.

Table VIII shows the inhibition of L-amino acid activation by D-amino acids. As noted before, the inhibition of L-alanine by D-alanine and L-valine by D-valine is overcome by prolonged incubation. The inhibition of L-serine by D-serine appears to be of a more extended nature. In fact,

Table IX
Inhibition of activation by D-amino acids

Activator	Inhibitor	Mean Spore Count per Plate Incubation Time in Days				
		2-3	5-6	9-10	15-17	20-23
L-alanine	D-valine	49	73	74	x	x
	D-serine	1	55	58	x	x
B-alanine	D-alanine	1	2	2	2	4
	D-valine	0	2	5	9	16
	D-serine	0	1	2	x	x
L-valine	D-alanine	1	1	1	3	7
	D-serine	0	1	2	x	x

Table X
Inhibition by D-amino acids

	D-alanine		D-valine		D-serine	
	5	24	5	24	5	24
L-alanine	±	+	—	—	—	—
β-alanine	+	+	+	+	+	+
L-valine	+	+	±	±	+	+
DT + ST	+	+	—	—	—	—
Heat activation	—	—	—	—	—	—

— = No inhibition + = Inhibition

even the spontaneous germination appears to be somewhat inhibited by D-serine.

Table IX shows some experiments on what I have termed cross-blocking by D-amino acids. In these tests L-alanine, L-valine and β-alanine have been used as activators with D-alanine, D-valine and D-serine as inhibitors. It may be seen that neither D-valine or D-serine inhibit the activation by L-alanine, all three D-amino acids inhibit activation by β-alanine, while D-alanine and D-serine inhibit activation by L-valine.

Table X summarizes the inhibiting effect of D-amino acids for both spore suspensions. The ± symbol is used with both the L-alanine — D-alanine and L-valine — D-valine systems since it was shown that these inhibitions were overcome on prolonged incubation. It also been found that D-alanine, but not D-valine or D-serine, inhibits the chemical activation produced by the DT+ST medium. This result, together with the fact that neither D-valine or D-serine inhibits L-alanine, may suggest that the chemical activator in the DT+ST medium is L-alanine. Also, as is shown in this table, the D-amino acids fail to inhibit the development of heat activated spores on the basal 5504 medium, suggesting that the mechanism of heat activation is different from and unrelated to the mode of activation by L-amino acids.

In addition to activation by heat, a further phenomenon of interest is the deactivation which may occur during the storage of heat-activated spores. This was first pointed out by Curran and Evans in 1946. They found that spores of several members of the genus *Bacillus* were activated by preheating and that a substantial proportion lost activation and were apparently non-viable following storage at 98°F. Post-storage preheating led to partial or complete reactivation, depending upon the culture used. Preheating and storage in distilled water had a different effect on activation and deactivation than similar treatment in 1% glucose solution.

Table XI shows some results upon the deactivation following preheating

TABLE XI
Spore deactivation following preheating and storage

Suspension	Storage Temp. °F	Mean Plate Count Storage Time in Days 0	5	10	14
5230-5	70	100	35	8	6
	60	100	91	54	46
	50	100	96	50	40
5230-24	70	100	58	20	13
	60	100	52	16	11
	50	100	99	56	38

for 15 minutes at 212°F and storage at various temperatures. The suspensions were plated both on the DT+ST medium and the 5504 medium. No significant differences between the mean counts on the two media were observed. Therefore, recovery of the deactivated spores was not improved by the presence of activators in the DT+ST medium. In other experiments it also was found that the inclusion of L-alanine in the 5504 medium did not improve recovery of deactivated spores. Plotting the logarithm of the mean spore counts against the time of storage gives an approximately straight line relationship within the limits of accuracy of the few experiments available. A difference may be noted between the two suspensions. With 5230-5, the deactivation at 70°F was much more rapid than at 60°F and 50°F. With 5230-24 deactivation at both 70°F and 60°F was much more rapid than at 50°F. Each suspension seems to have its own particular pattern of behavior. It should also be mentioned that in experiments with both suspensions conducted only at 70°F storage, the logarithmic relationship did not seem to hold for storage periods longer than 10-14 days, since a small number, approximately 10% of the population, will continue to germinate even after 20 days' storage.

These experiments are quite preliminary attempts to establish the pattern of deactivation during storage to serve as a guide to experiments on heat and chemical reactivation. The results, however, together with those of Curran and Evans, seem definitely to contra-indicate the routine preheating prior to storage of suspensions to be retained over a period of time. Preheating for activation or for the removal of viable vegetative cells should be used only where it is a known and controlled variable.

Discussion

Dudley S. Titus

A review of the literature dealing with spore germination stimulants leads

to the conclusion that we are gathering data which, up to the present time at least, do not lend themselvs to many overall generalizations. There are, however, certain factors which seem to be necessary in the majority of cases, in order for germination to proceed rapidly and completely. The most obvious of these factors are heat activation, the presence of L-alanine, and a riboside.

The heat activation of spores prior to incubation with various chemical stimulants often results in a more complete or more rapid germination. This characteristic response seems to be more common among the aerobic spore formers than among the anaerobes. Six out of nine species of aerobic spore formers, for which optimal germination conditions have been recorded, have been reported in at least one instance to require heat activation. Furthermore, in some cases where heat activation was either not used or not required, the spore suspensions tested had been pasteurized during their preparation. Although Dr. Schmidt and several others have reported that the effect of heat activation is lost rapidly, it may be possible that pasteurization of spores of some species may produce a more permanent change which makes subsequent heating unnecessary. In other cases where heat activation was not used, the spore suspensions were cleaned of vegetative cells by exposure to sonic oscillation (Heiligman, Desrosier, and Broumand, 1956). The effect of sonic oscillation on these spores has apparently not been reported, but in view of the reports which were reviewed by Rahn (1945), that sonic oscillation is an effective lethal agent, it may be that mild sonic treatment sufficient to destroy vegetative cells, but not spores, has an effect similar or even identical to that of mild heat treatments.

The stimulatory action of mild heat on germination is apparently not a permanent characteristic. Church and Halvorson (1956) reported that fresh spores of *Bacillus cereus* var. *terminalis*, after heat activation, germinated in the presence of L-alanine and adenosine. After 5 years of aging, however, these spores germinated rapidly and completely without heating in the presence of *either* L-alanine *or* adenosine, *or* after heat activation alone.

Heat activation, in addition to providing more rapid and complete germination, may also alter the germination requirements. Levinson and Sevag (1953) found that spores of *Bacillus megaterium* germinated without heating in a L-alanine-adenosine-glucose-glutamate-salts medium, but after heat activation only glucose and L-alanine were required. Powell and Hunter (1955) reported that unheated spores of *Bacillus subtilis* required L-alanine, adenosine, glucose, and tyrosine for optimum germination, while heated spores required only glucose and adenosine. In addition to those cases reported in the literature where heating is not beneficial, in our laboratory Amaha (1956) found that unheated spores of *B. coagulans* germinated rapidly and completely

in the presence of L-alanine alone. These spores were less than six months old, had been stored at refrigerator temperatures, had never been subjected to heating of any kind, and were regarded as free of vegetative cells.

Although it seems that spores of some aerobic strains have no heat requirement for germination, it is quite obvious that heat plays a vital role in the germination of many, if not the majority of aerobic spores. I heartily agree with Dr. Schmidt that spore suspensions should be subjected to heat only where it is a known and controllable variable.

A specific germination requirement for L-alanine is even more prevalent among the aerobic spore formers than is the requirement for heat activation, although several exceptions have been recorded. Knaysi (1945) reported that spores of *B. mycoides* germinated in the presence of glucose and acetate, although germination was apparently relatively low and inconsistent. Pulvertaft and Haynes (1951) found that spores of *Bacillus cereus* and *B. subtilis* germinated in the presence of adenosine alone. These determinations were apparently made in the presence of autolyzed vegetative cells, however, and the possible presence of L-alanine and/or other stimulants cannot be discounted. Heiligman *et al* (1956a) and Hachisuka and coworkers (1955a) also reported germination of aerobic spores in the absence of L-alanine.

A requirement for the presence of adenosine for optimum germination has been reported in several instances, although this requirement does not appear to be as specific or as widespread as does the requirement for L-alanine. Other ribosides seem to be able to replace adenosine, and Powell and Hunter (1955) reported that inosine provided better germination than did adenosine.

Several workers have reported that glucose, either alone or in combination with other compounds, stimulates germination of some aerobic spores. Heiligman *et al* (1956a) found that spores of *Bacillus coagulans*, *Bacillus globigii*, and the anaerobic spore former, PA 3679, required only glucose or another energy yielding organic carbon source for germination. Hachisuka *et al* (1955a, 1955b) reported that glucose and L-asparagine or DL-isoleucine were sufficient for complete germination of *B. subtilis* spores, but only after the glucose (or other sugar) had been caramelized to a yellowish brown color.

The requirements for optimum germination of the anaerobic spore formers has not been investigated as thoroughly as has been the case with the aerobes. Wynne (1956) found that glucose autoclaved at a pH above 5.0 caused germination of spores of several *Clostridia* strains. As mentioned earlier, Heiligman *et al* (1956a) found that PA 3679 required only glucose for germination. Hitzman, Zoha, and Halvorson (1955) reported that L-phenylalanine, L-arginine, and L-alanine were required for optimum germination of heated spores of *Clostridium botulinum* type B and *Clostridium roseum*. Brown (1956) found that spores of two strains of PA 3679 germinated com-

pletely in the presence of M/180 ethylene diamine tetraacetic acid. It appeared that EDTA was acting solely as a chelating agent. It is interesting to note that at concentrations of EDTA above M/10 no germination occurred.

In summary, there appear to be three general systems for the germination of spores. The most common system is one containing an amino acid, usually L-alanine, a riboside, and occasionally glucose. In some instances, more than one amino acid is required. A second system which has been reported frequently is one in which glucose or another energy yielding carbon source, in the absence of any nitrogen source, is sufficient for germination. The third method requires only the presence of the proper concentration of a chelating agent. Heat activation appears to be either stimulatory or makes the germination requirements simpler in most aerobic spores and in some anaerobic spores. It is not possible to say whether the three generalized systems outlined here are really different or not, since we have not as yet been able to ascertain the function of these stimulants in the germination process.

The literature regarding germination inhibitors is not extensive. Foster and Wynne (1948) found that unsaturated fatty acids inhibited germination of anaerobic spores. Roth and Halvorson (1952) reported that this inhibition occurred only when the fatty acids were rancid, and implicated peroxides as the active agents. Powell (1950) found that oxine (10mM) and 2,3-dimercaptopropanol (BAL) (10mM) completely inhibited germination of *B. subtilis* spores. BAL inhibition could be partially overcome by soluble salts of Zn, Mg, Cu, or Fe. Exposure to $HgCl_2$ (5%) also caused complete inhibition which could be partially reversed by L-alanine and glucose. Powell (1951) reported that typical respiratory enzymes inhibitors such as NaF, cyanide, 2,4-dinitrophenol, and iodoacetate had no inhibitory effect on germination. On the other hand, Heiligman *et al* (1956b) found that 2,4-DNP caused essentially complete inhibition and azide, NaF, IOAc, arsenate and arsenite caused at least partial inhibition. Malonic acid, however, did not inhibit the stimulatory action of citric or succinic acids. Hachisuka *et al* (1956) reported that $NaHCO_3$ and Na_2CO_3 inhibited germination of *B. subtilis* spores. Hills (1949) found that glycine, DL-methionine, DL-cysteine, or DL-valine at relatively high concentrations inhibited the action of L-alanine.

The most consistent inhibitory action reported is that of D-alanine inhibiting the action of L-alanine. Even this has exceptions, however, since Lawrence (1956) reported that when spores were heated in the presence of the substrates, D-alanine could *replace* L-alanine as a germination stimulant for *B. cereus* var. *terminalis* spores.

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Informal Discussion

CAMPBELL: I would like to comment on the effect of L-alanine on germination and the relationship of this to colony counting as noted by Dr. Schmidt. If spores of *B. terminalis* are placed in a medium which supports outgrowth, the addition of alanine gives almost 100% germination in 5 to 10 minutes, as evidenced by darkening under a dark phase contrast microscope. If you follow the changes that occur over an 8 hour period, you find that only 20 to 30% of the germinated spores give rise to vegetative cells. The remaining 70 to 80% do not swell, elongate, or divide. However, in this 8 hour period the vegetative cells have formed heat stable spores again. This complicates the problem of counting colonies since the count will fluctuate as the heat stable spores are formed.

MURTY: With regard to germination requirements, I wish to bring to your notice what our experience has been with spores of *B. terminalis*. These requirements can vary widely with the method of preparation and storage—the so-called history of the spores—even when the same organism is used. As a consequence, we do not call a suspension of spores clean and fresh unless it satisfies the requirements that have been originally worked out for this organism. Even freshly produced spore suspensions can differ in germination requirements if something went wrong with the washing or the medium. We could not prevent germination by any of the so-called enzyme inhibitors like arsenate, cyanide, etc. Furthermore, the spores would germinate even under strictly anaerobic conditions. Heavy metal ions like copper, chromium, or mercury, and the organic mercurials like phenylmercuric acetate and phenylmercuric benzoate are the only ones we know of that can inhibit germination. This inhibition could be reversed partially by washing and adding -SH reagents. Church and Halvorson found that spores of *B. terminalis*, which do not specifically respond to glucose, do so only under certain conditions such as prior activation of the spores. One then wonders if at least the first phase of germination, which cannot be inhibited by any of the inhibitors of glycolysis or the TCA cycle or by anaerobiosis, is an energy requiring process. We are inclined to believe that it is not an energy requiring process. We found that, during germination, a large part of the dipicolinic acid of the spores was released into the medium and that DPA was also released on autoclaving the spores. We are therefore inclined to believe that the first phase of germination is essentially some sort of a depolymerization process, a view also shared by Powell and coworkers.

HARLYN HALVORSON: Spore germination has been described as an exothermic reaction. The supporting evidence generally involves the ability of spores to germinate either spontaneously or under conditions where energy

yielding reactions were not clearly demonstrated; e.g. in the presence of L-alanine and adenosine.

In the latter case, these germinating agents can no longer be considered as energetically inactive. Adenosine is at least partially degraded by the action of adenosine deaminase and ribosidase (Powell and Hunter, 1955; and Lawrence, 1955). Also L-alanine is converted to pyruvate which is metabolized via a tricarboxylic acid cycle (Halvorson and Church, this symposium). Several carbohydrates which also serve as germinating agents are actively oxidized. It is therefore likely that other germinating agents may also serve as energy donors.

Activation, which can lead to spontaneous germination, also results in an activation of endogenous carbon reserves (Murty and Church, this symposium). In this case as well, energy requirements for germination may be supplied via endogenous metabolism which can be appreciable under certain circumstances. One might imagine that the failure of energy inhibitors (cyanide, dinitrophenol or azide) to interfere with germination would also argue against such metabolism being involved. However, before interpreting these negative results, I should like to comment on our experience with pyruvate. At pH 7 pyruvate is not a germinating agent. If one incubates spores with pyruvate at pH 4 (where pyruvate is largely undissociated), germination occurs after the pH is raised to 7. The failure of inhibitors at pH 7 to block germination, whereas they are active in inhibiting carbohydrate metabolism in extracts of spores, may merely be due to their failure to enter the spore. The permeability of these agents should be more critically examined in order to interpret the exothermic nature of germination.

STUMBO: You speak of organisms losing their heat resistance. How much heat resistance do they lose; and, is the transition from a resting state to the heat-labile state that you refer to an instantaneous thing? Would you not expect to find spores which, shall we say, are half undressed? And maybe if you used, instead of 20 minutes, 30 minutes at 75° or 10 minutes at 75°, you would get a different answer with respect to heat sensitivity. I would be amazed if you didn't. Should this prove to be the case, I think you can expect variations with species. It seems to me you are going to have to define very carefully the time-temperature relationship you are talking about. I think you are going to have to define the relationship with respect to species, and possibly with respect to the history of the spores that you are talking about in order to define heat sensitivity. I can't agree that you can arbitrarily pick 20 minutes at 75° or 20 minutes at 80°, or some other relationship, and say all the spores that resist this are heat stable and all the spores that don't are heat sensitive. I am sure you can find some spores

that, in the so-called resting state, are killed by some of these time-temperature relationships, no matter how carefully they are selected.

To define germination in terms of loss of heat resistance implies that heat resistance is a constant characteristic which can be defined by some given time-temperature relationship—but it isn't.

BROWN: Getting back to the germination process, I wonder if any of you have considered the possibility that this could be the removal of an inhibitor that permits the germination process to proceed. We have recently completed some work that seems to indicate this and with your permission I would like to briefly outline it on the board.

We used Dr. Lund's mutant strain of Putrefactive Anaerobe Number 3679 that was described yesterday. The spores were grown in the spent medium, treated with lysozyme to remove the vegetative cells, and washed 10 to 14 times with sterile distilled water using centrifugation to separate the spores from the wash water.

Four methods were used to measure spore germination. The methods used were: optical density changes as measured with a spectrophotometer; refractivity changes as measured under the phase microscope; differences in staining properties; and the loss of heat resistance. The loss of heat resistance due to germination was determined by exposing the treated spores to a temperature of 97°C for 10 minutes. The number of surviving spores was determined by a dilution count procedure using eugon agar as the recovery medium. The count was then determined from the tables of Halvorson and Ziegler. Statistical methods were employed to study the relationship between the 4 measurements of spore germination. The correlation coefficients were highly significant at the 1% level but the relationship was not high enough to permit the easier measurements to be used in lieu of the viable count. Consequently, the viable count was used in all of the experiments as a measure of spore germination.

A wide variety of organic compounds were tested for their ability to germinate the mutant strain spores. Of the compounds tested, none were able to change the refractivity of the spores during a 30-minute interval. It was considered possible that a metal inhibitor could be blocking the germination process. Germination of the mutant PA 3679 spores was accomplished by treatment with ethylene diamine tetraacetic acid (EDTA) at an optimum concentration (M/180) when the clean spore suspension was adjusted to a constant turbidity with the colorimeter. When the concentration of EDTA was too high (M/10) no germination occurred. Other chelating agents were tested but only the EDTA type compounds were effective.

It was hypothesized that treatment of a spore suspension with stronger solutions of EDTA (M/10) resulted in the removal of both an inhibitor and a

metal activator required for the enzymatic reactions leading to germination. Experiments were conducted to test this hypothesis. An analysis of the metal change during spore germination with EDTA was made with an emission spectrograph and the information from this analysis was used to study the process in more detail. It was found that copper, iron, magnesium, manganese and calcium were removed by the EDTA.

The affinity constants of the various metals with EDTA were used to prepare EDTA metal complexes in an attempt to determine the metal inhibitor that was being removed during the germination process. If the prepared metal-EDTA complex was lower in the series (affinity constant), theoretically there would be an exchange reaction taking place and germination would occur. The results from experiments using this technique indicated that the metal inhibitor was probably a member of the "light" group of elements.

A study of environmental conditions affecting the germination process by EDTA indicated a very rapid reaction that could occur between pH of 5.0 and 9.4. The germination process by EDTA was almost completely stopped at 0° C. It is probable that at this temperature the chelating ability of the EDTA was not markedly reduced but the subsequent enzymatic reactions associated with germination were adversely affected by the decrease in temperature. The effect of inhibitors on spore germination by EDTA indicated that sodium azide, 2,-4,-dinitrophenol and iodoacetate had essentially no effect on the process.

A study of the metal inhibition suggested that it could be the beryllium inhibition of an alkaline phosphatase. It is logical that phosphatase activity could take place as one of the initial reactions because it is an energy yielding mechanism. Efforts to detect beryllium in or on the spore indicated that it was not present to the extent of 1 ppm or greater. Beryllium was able to block the germination process by EDTA when added at a concentration of 6.5×10^{-3} M.

Treatment of the spores with excess EDTA followed by washing to remove the EDTA and adding back various metals in an attempt to cause the germination process to proceed were essentially negative. However, magnesium gave slightly positive results. The failure to germinate such spores by the addition of various metal ions was probably due to the disturbance of a metal complex, the disruption of which was irreversible.

Phosphatase activity was demonstrated in a spore extract obtained by crushing the spores with glass beads. A rate of reaction was calculated for the phosphatase enzyme when pyrophosphate was used as the substrate. The rate of splitting was 19 μg of P/mg dry wt. of spores/hr. The phosphatase reaction was stopped by the addition of beryllium sulfate.

Chemical Changes Occurring During Spore Germination

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In our studies of spore germination, we have taken loss of heat resistance as the criterion of germination (Wynne and Foster, 1948). Early studies showed that loss of heat resistance was associated with more readily measurable changes such as decreased refractive index and increased permeability to stains (Powell, 1950). In subsequent studies we therefore assessed germination by nephelometry or by examination of stained films. We found that while the above changes were taking place the germinating spore excreted solid material equivalent to 30 percent of its dry weight into the medium, at the same time swelling slightly (Powell and Strange, 1953). This excretory process occurred when germination took place in a medium containing only the specific germination stimulant, or in a nutrient tryptic digest broth, or spontaneously in water suspension as a result of heat activation (Powell and Strange, 1953). The composition of the excreted material, the "germination exudate," was remarkably similar in all the *Bacillus* species we examined.

Composition of the Germination Exudate

The most striking common property of germination exudates was their strong and characteristic ultraviolet absorption. The substance responsible for this absorption was isolated from germination exudates of *B. megaterium* and identified (Powell, 1953) as the calcium salt of pyridine-2:6-dicarboxylic acid (calcium dipicolinate). Calcium dipicolinate accounted for 50 to 60 percent of the dry weight of germination exudates and therefore represented approximately 15 percent of the dry weight of resting spores. We have observed the ultraviolet absorption spectrum of calcium dipicolinate in extracts from disintegrated resting spores of *B. megaterium*, *B. subtilis*, *B. cereus*, *B. polymyxa*, *B. sphaericus*, and also of *Clostridium sporogenes*, *Cl. tetani*, *Cl. histolyticum*, and *Cl. septicum*.

The remainder of the germination exudate consisted of free amino acids, peptides, and small amounts of protein. A non-dialyzable peptide of characteristic constitution and of molecular weight in the region of 10,000 was invariably present (Strange and Powell, 1954). This peptide represented

10 to 15 percent of the total germination exudate. Its constituents were $\alpha\epsilon$-diaminopimelic acid (DAP), alanine, glutamic acid, and hexosamines occurring in the molecular ratio of 1:3:1:8. Non-dialyzable peptides of very similar constitution were present in extracts from disintegrated resting spores of *Bacillus* species (Strange and Powell, 1954). The remaining constituents of the germination exudate have not yet been fully investigated; they appear to be mainly peptides of lower molecular weight and of different composition from the non-dialyzable fraction.

The Origin of the Constituents of the Germination Exudate

At the moment, we do not know how calcium dipicolinate is distributed within the resting spore. An X-ray examination of crystalline calcium dipicolinate and of air-dried resting spores of *B. megaterium* was kindly carried out for us by Dr. M. M. Bluhm and Dr. J. C. Kendrew of the Cavendish Laboratory, Cambridge. They obtained a characteristic crystalline-powder diagram with calcium dipicolinate, but could find no evidence of a crystalline structure in resting spores. It seems likely that calcium dipicolinate is incorporated within the spore protoplasm through chelate linkages of calcium with protein, and that this arrangement may contribute to the heat-resisting properties of the intact resting spore.

The non-dialyzable peptide containing DAP and hexosamines appears to be derived from the spore coat (Strange and Dark, 1956). Spore coat preparations from *B. megaterium* obtained by mechanical disintegration still contained considerable amounts, and those from *B. subtilis* smaller amounts of bound hexosamine. This was slowly released in the form of the DAP-hexosamine peptide when the spore coat preparations were incubated in distilled water or buffer solutions. In *B. cereus* it appeared that the peptide was readily and completely released during mechanical disintegration.

Independent evidence for the location of the DAP-hexosamine peptide on the spore surface has recently been obtained from electrophoretic studies (Douglas, 1955). From a comparison of the electrophoretic behavior of resting spores of *B. megaterium* and *B. subtilis*, it was concluded that these spores had different surface compositions. It was tentatively suggested that the surface layer of the *B. subtilis* spore contained polysaccharide, whereas that of *B. megaterium* contained lipid possibly complexed with protein. In order to provide "standards of reference" to assist in the interpretation of results, the electrophoretic behavior of inert particles coated with various types of protein, lipid, and polysaccharide was recently studied (Douglas and Shaw, 1956). In this series was included a preparation of the DAP-hexosamine peptide isolated from germination exudates of *B. megaterium*.

The behavior of inert particles coated with the spore peptide was very similar to that of *B. subtilis* spores. The technique of "ion spectra" (DeJong, 1949) was also applied to particles coated with the spore peptide and to *B. subtilis* spores, and further similarities between the two surfaces were indicated. *B. megaterium* spores gave a cation spectrum which suggested a possibility for their surface composition other than the lipid-protein complex first suggested. The cation spectrum obtained with *B. megaterium* spores closely resembled that of inert particles coated with a polyglutamic acid preparation isolated from *B. anthracis*. It is now suggested that the surface layer of the *B. megaterium* spore could be composed of the same type of material as appears to be present at the surface of *B. subtilis* spores, but orientated differently so that more carboxyl groups are presented to the aqueous medium.

The Mechanism of Release of the DAP-hexosamine Peptide from the Germinating Spore

Lysozyme accelerated the release of the DAP-hexosamine peptide from spore coat preparations of *B. megaterium* (Strange and Dark, 1956). It was suggested that a lysozyme-like lytic system might be responsible for the release of the peptide during germination, and that the activation of this system might be one of the first stages of the germination process. Evidence in favor of this possibility has recently been obtained. The DAP and hexosamine content of whole cells of *B. cereus* was fairly constant during growth and sporulation, but during sporulation there was a change in distribution of DAP and hexosamine between the soluble and insoluble fractions of the disintegrated cell. DAP and hexosamine were found in the insoluble fraction of vegetative cells and in the soluble fraction of sporulating cells and spores. Extracts of sporulating cells and spores contained a lytic system capable of releasing the DAP-hexosamine peptide from the insoluble fraction of vegetative cells. This lytic system resembled lysozyme in its mode of action and its heat stability (Strange and Dark, 1956). A similar lytic system was present in extracts from spores of an avirulent strain of *B. anthracis*. The insoluble fraction from spores of *B. megaterium* showed moderate activity although none could be demonstrated in the soluble fraction (Strange and Dark, 1956).

Conclusions

In contact with certain simple substances, and under conditions which may be totally unsuitable for growth, the resting spore germinates, i.e., it loses its heat-resisting properties, becomes permeable to stains, and swells slightly. While these changes are occurring calcium dipicolinate is excreted and a non-dialyzable peptide of characteristic constitution is released from the spore coat. As far as we can judge, these changes take place simultaneously. In

our speculations as to the structure of the resting spore and the changes which occur during germination, we have suggested that the protoplasm of the resting spore is a highly condensed "waterproofed" system stabilized by the incorporation of calcium dipicolinate and possibly by the constitution of the spore coat (Powell and Strange, 1956; Powell and Hunter, 1956). We have also suggested that during germination hydration of this structure and a process akin to depolymerization occurs, reactive enzyme groups are freed, and the spore becomes capable of metabolism and growth. These views do not differ esentially from the very early suggestions made by Lewith (1889). Evidence has recently been obtained (Powell and Strange, 1956; Strange and Dark, 1957) that spores contain a lytic system, similar to lysozyme, capable of releasing the DAP-alanine-glutamic acid-hexosamine peptide from spore coat preparations. It is possible that the activation of this enzyme and its attack on the spore coat may be the "key" reactions of the germination process. To suggest a mechanism for this activation and its connection with the specific germination requirement in a given *Bacillus* species we find to be an imaginative exercise far beyond our powers.

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Discussion

Jackson W. Foster

You have all heard about carrying coals to Newcastle. Well, that is how I feel in commenting on spore germination work done by Dr. Powell. Perhaps henceforth this kind of action may not inappropriately be regarded as carrying spores to Porton. My predicament is even worse than most of you realize, when it is considered that the problem of germination has been scarcely touched in my laboratory for the last five years.

However, I could not sit through the series of excellent presentations we have been privileged to hear up to this point without deriving some ideas of a discussable nature. Consequently, my comments are based mainly on certain points brought out in the previous papers and discussions. I am, so to speak, "playing it by ear." and if it appears that I am digressing from Dr. Powell's work *per se*, discussion of which is my formal assignment, I trust that you will regard what I have to say as being at least germane to this conference.

I view with detachment what appears to be an onrushing enthusiasm on the part of some of the participants here to formulate definitions of the process of germination. We ought to reflect whether we may be somewhat premature in this desire. My own feeling is that we have a great deal more to learn about the germination act before we start attaching monikers to processes which currently are wholly mysterious. It is well and good to have a glossary of terms which denote features which are largely, if not exclusively of operational significance at this time. The operational aspect is what was implied, and no more, by use of the terms "germination outgrowth" and "germination heat lability" or their equivalents, in some of the preceding presentations. I think we know all too little about the nature of these processes and it behooves this group of experts to deliberate the matter seriously before deciding to recommend formal adoption of such terms and subsequent incorporation into the scientific literature in this field. Any pronouncement by this group is likely to be taken as "official." and I for one feel we run the serious hazard of imposing an unwanted and unnecessary legacy upon subsequent workers. As the phenomena we would purport to describe now become less and less mysterious, I rather suspect that what will happen is that we and our successors will be faced with the problem of con-

triving to get around names preordained but superfluous and inconsistent with ultimately clarified mechanisms.

Another consequence I think we shall encounter, if we rush into this thing hastily, is the danger of implying by a proposed terminology things we don't intend at this stage. I strongly suspect that the uninitiated will infer from such definitions that the germination process is a discontinuous state and that names arbitrarily designating various stages stand for a series of individual and discrete events. To my mind, this is extremely far-fetched. Germination at the present state of information is essentially a conceptual and operational matter; and despite the lack of a series of specific definitions, we certainly have little difficulty in communicating information and ideas on the subject. Without doubt, more precise definitions of various stages based on the very limited information and organisms studied thus far will belie the situation as it is studied further and in more organisms.

The germination process has to be regarded as a continuous event, and a focal point of any objective for description of the phenomenon is what happens when the process is projected back to the prime event, namely at point zero. Something happens there and it progresses—imperceptibly in the beginning—until the gross change is large enough to recognize. At this point we call it germination, a perfectly satisfactory designation, so long as we recognize it as a mere culmination of an integrated series of previous transformations. Until we can agree that these integrations will be qualitatively and quantitatively distinguishable in any one species, and the same for all species, any rigid set of definitions will have become obsolete before it is established.

To reflect further on the nature of the germination process, it may well be that what we currently regard as features of germination are only peripheral phenomena distantly removed in physiological time from the initial qualitative change. Point zero in the back-extrapolated curve will probably provide the ultimate solution of germination in the sense of what I like to think of as the "prime event." If we assemble all information bearing on the issue, there is very little which is inconsistent with the idea that germination in itself is a process identical with or akin to induced enzyme synthesis. It is too lengthy a task to take the time here to justify this view in detail, and I merely submit the following in a general way.

Much like what we find in induced enzyme systems, germination requires specific inducers and energy. I think it not without serious significance for the whole problem of germination that the most effective inducers happen to be substances closely tied in with the general function of making energy available, e.g. glucose and adenosine (or inosine). Of course, the question now comes up, what about alanine? I have purposely refrained from in-

cluding it because, although its effect is striking, it appears that alanine induction and alanine racemase are not common to all spores. For the moment, it is not unreasonable to believe that alanine *per se* is peripheral and that perhaps via pyruvic acid it, like adenosine and glucose, may well have to do with the availability of energy at the zero focal point.

If we visualize, provisionally, that the process is a kind of adaptive synthesis(es), then we have to look for some kind of synthetic activity, it possibly being the initial meaningful step in the metamorphosis of a spore cell to a vegetative cell. There are some data that tend to argue against this. One of the most imposing facts which might be hastily mustered against this idea is the rapidity of germination under optimal conditions. But the idea of synthesis being involved here should not be exempted, because no matter how few minutes it takes to observe the gross changes we characterize as germination, we know there must have taken place prior to that changes undetected by techniques thus far employed. Certainly synthetic activity could well be initiated within seconds or less of the triggering effect; and if we judge from turnover numbers of known enzymes and from the fact that the newly synthesized molecules need constitute but a minute portion of the spore cell walls—and may be autocatalytically synthesized at that—I see no reason why synthesis could not be the main theme commencing from point zero. Induced synthesis can be very rapid. In our laboratory a pseudomonad gave a definite response manometrically, indicative of synthesis of an induced enzyme system, less than five minutes after addition of the specific inducer. Needless to say, a lot of enzyme molecules must have been synthesized prior to the time their activity could be detected manometrically. One of the last experiments on germination in our laboratory was done over five years ago by Dr. W. A. Hardwick. His preliminary experiment showed the uptake and fixation of P^{32}- or S^{35}-methionine in spores of species of *Bacillus* within a very minute of their being placed in a germination medium. Of special interest is the fact that the fixation, indicative of an active metabolism, was easily detected well before the great majority of spores had lost their heat resistance and without the appearance of anything remotely resembling vegetative cells. Unfortunately this experiment was not designed to ascertain just how early in germination phosphate fixation could be detected.

Another experiment that Dr. Hardwick did in my laboratory also illustrates a very early intraspore turnover well before germination, e.g. appearance of vegetative cells. It supports the idea of very early synthesis by showing how intracellular substances originate—in this case a pool of amino acids, which presumably are precursors for *de novo* syntheses. S^{35}-labelled spores of *B. cereus* were obtained by growth in a medium in which $Na_2S^{35}O_4$ was

the sole source of sulfur. Aliquots of well washed spores were analyzed for free S^{35}-methionine in the following treatments: (A) ungerminated spores (at zero time), (B) after shaking 15 minutes in a germination solution, (C) after shaking 50 minutes in the germination solution, (D) after shaking 50 minutes in plain buffer. Previous experiments showed that about 90 percent of the spores had lost heat resistance in 50 minutes, but that nothing suggesting growth or change in morphology took place in the germination medium. All the treatments had 0.5 mg unlabelled DL-methionine per ml as a pool to trap labelled methionine. Total radioactivity was measured in the isolated methionine after boiling the cells in 50 percent ethanol. Residual labelled sulfate was eliminated by addition of carrier Na_2SO_4 and preparation with $Ba(OH)_2$. The methionine was purified as a band by paper chromatography, and the counts made on eluted material corresponding to methionine (Rf=0.8) as judged by reactions with ninhydrin and with platinic iodide. The methionine from the respective treatments had the following counts per minute (corrected for background): (A) 2.6; (B) 17.4; (C) 62.1; (D) 4.4. Thus, after 50 minutes in the germination solution, there was 24 times as much free methionine as existed in ungerminated spores, and 14 times as much as controls incubated in the non-germination (buffer) medium. This experiment proves that a pool of free amino acids is formed from spore proteins very early in the germination process. Unquestionably this pool is reutilized for subsequent syntheses during germination. Thus, there is evidence that intraspore turnover may occur very early in the germination process and I would like to leave this phase of the discussion with the reflection that most of the phenomena that we have observed up to now are probably peripheral to the key phenomenon of activation of the intraspore turnover.

Further in connection with the proposal to subdivide germination terminology is the matter of heat resistance, or resistance in general, of the spore. Dr. Stumbo has already touched on an important point in this relation. I already had something in mind to submit to your consideration. That heat resistance is universally regarded as the distinguishing characteristic of a spore is evidenced by its reiteration time and again during this conference. I have a feeling we would be much closer to being able to provide a true definition of germination—to provide a legal definition of the phenomenon as it were—if we really knew what a spore means to a bacterium in nature. I can understand how the measurement of heat resistance has evolved to become the stereotyped criterion. It obviously is a manageable technique and gives reproducible results. However, from the standpoint of characterizing the spore functionally, and that is what we accomplish when we define a cell in terms of heat resistance or lability, we ought not overlook asking

ourselves what this heat resistance means to the organism in nature. I am reluctant to believe these organisms are apt to be exposed in nature to the temperatures we impose in the laboratory as the criterion for the physiological nature of spores. One can only conclude we are using a technique which admittedly is handy, but which nevertheless is quite beyond any plan that nature has in mind producing spores. Resistance is an obvious selective mechanism, to be sure, but it is difficult to visualize as a selective mechanism resistance of the order of magnitude commonly and arbitrarily employed in the laboratory.

There are legions of microorganisms which contrive to survive in nature by mechanisms clearly not involving either heat resistance or spore formation. Non-sporeforming bacteria have evolved in nature side by side with sporeformers; consequently it is quite clear that spore resistance is not a necessary requirement for survival. Are we truly characterizing the natural physiological meaning of the spore by assigning resistance to it? In fungi and in actinomycetes we have spore structures unquestionably analogous to those in bacteria, yet their heat resistance comes nowhere near the order of resistance typical of bacterial spores, at least as we evaluate them in the laboratory. The temperature tolerance of fungal and actinomycetal condiospores by and large is only a few degrees above that of the corresponding mycelium. Furthermore, there is some evidenec in favor of the conclusion that not all bacterial spores formed in nature are truly (heat) resistant. Certainly, the loss of the remarkably high resistance which we set up as a laboratory parameter of germination should not be taken as characteristic of these spores in nature.

Most of our laboratory cultures are highly selected populations, having been cultivated on laboratory media, heat shocked, pasteurized, refrigerated, and only God knows what else. Even in such spore types, highly selected for resistance, we note an unmistakable tendency to generate spores which do not possess the resistance characteristic of the other individuals in the clone. It would be intriguing to have experiments that could shed light on the problem of natural populations of spores (in soil, for example) and the distribution of heat resistance. I dare say that a surprisingly large proportion of the bacterial spores extant in nature, not being selected for heat resistance, would fail the bacteriologist's test for a spore. Is it possible that through our laboratory technology we have engendered a concept of a monster spore instead of a typical spore?

Among certain of the preceding speakers I noted differences of opinion as to what characteristics, other than heat resistance, distinguish ungerminated versus germinated spores. It occurred to me that an even more elementary question which would have to be answered satisfactorily is: what

properties are common to all spores? We have all had experience enough to agree that not all the spores, even in a single clone, are resistant; so resistance *per se* could not be accepted without considerable rationalization. Furthermore, not all bacterial spores are germinatable by methods by which the bacteriologist can detect germination, so we cannot use as a definition of a spore: a cell that can germinate. Also, not all bacteria we ordinarily regard as competent in this matter do form spores under conditions which are physiologically homogeneous and where other clonal members do sporulate. Hence, conditions for sporulation have no absolute implications for our definition. Not all spores are heat activatable, and those which are respond differently to different temperatures. I will admit that I thought the Illinois group had a clue to the answer when they came up with the alanine-alanine racemase story. But they did too many experiments and the hope for general applicability of this feature is not as sustaining now as it appeared to be when that work was first reported.

One could extend this list, but presently, and I advisedly use the term "presently," the only things I know to be common to all bacterial spores are (1) they are produced inside another cell, and (2) they all contain dipicolinic acid. The first of these requires no additional comment. What about dipicolinic acid, which so far as my knowledge goes, has not been found absent in any of the aerobic or anaerobic spores thus far tested. It being present in surprisingly high percentage in the resistant form of the bacterial cell and undetectable in the non-resistant vegetative form, and the fact that it has not been recorded as occurring in any other biological system, make it very easy to ascribe a preponderant, if not compelling, importance to this compound in the underlying mechanisms of resistance. Although I see no serious objection to this assumption, it is nevertheless worth emphasizing to you the state of our information on this subject by the reminder that the evidence for a direct role of dipicolinic acid in resistance can only be regarded as circumstantial. Other possibilities, such as its representing a metabolite of amino acid metabolism similar to the analogous pipecolic acid in other systems, could be eliminated if we had more specific information on the function of dipicolinic acid. Are there any other substances, unique or common, present in significant amounts in spores? A host of other questions could be asked, but they devolve to one: this biologically unique compound probably has some special significance for the known properties of spores.

The work of Dr. Curran and of Dr. Powell, indicating an unusual concentration of calcium in spores, naturally focusses attention upon a relation between this cation and dipicolinic acid. This question became all the more attractive when work in Dr. Powell's laboratory suggested that dipicolinic

acid was excreted during germination as the calcium salt or chelation complex of dipicolinic acid. In our work with endotrophic sporulation no calcium was present, of course, during sporogenesis, and this prompted an analysis of endotrophic spores for dipicolinic acid and for calcium. As suspected, the calcium content was far less than the molar equivalent of the dipicolinic acid present in those spores. Thus, the bulk of the dipicolinic acid was not present as the calcium salt. Inasmuch as these spores survived pasteurization as readily as spores from a complete growth medium, we can only conclude that resistance is not dependent on all the dipicolinic acid being present as the calcium salt. I think this experiment summarizes the final point I have to make, namely, that our solution to the problem of resistance lies not necessarily on what we may find in the spore, but on what are the minimal prerequisites for the distinctive attributes of a spore and spore resistance.

Cytological Changes Occurring During Germination

C. F. Robinow

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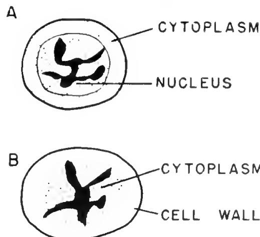
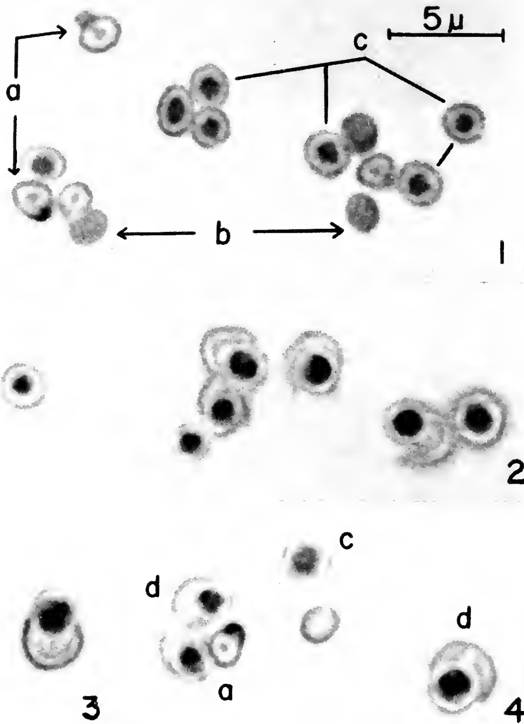
I AM very interested in spores and grateful for the opportunity to attend this meeting but I have not lately worked with spores and have only fragments of fresh cytological studies to offer.

One of these, illustrated by Figs. 1-4, concerns the chromatin of *B. megaterium* spores. This is one of the few constituents of spores on whose spatial distribution we have fairly reliable information. In *B. cereus* and *B. megaterium* it is arranged in a shape which in optical section gives the impression of a wreath or irregular ring of chromatin encircling the interior of the spore at some little distance inward from the spore membrane. That is how the chromatin is seen alike in sectioned, resting spores (Robinow, 1953), in spores cracked open and rendered stainable by bombardment with ballottini glass beads (Fitz-James, 1953), and in spores fixed during the first few minutes after transfer to fresh nutrient medium (Robinow, 1953, Figs. 27, 28). What is inside the circle of chromatin? Two different answers suggest themselves of which it is not immediately possible to say which is the right one.

Is there a relatively large, closed round nucleus in the spore filled with nucleoplasm and chromatin and surrounded by a thin shell of cytoplasm? Or are things the other way around, with the spores filled with cytoplasm in which is embedded, near its periphery, an open net- or basket-work of a few coarse chromatin strands? This ambiguity persists into the early phases of germination. The chromatin is then no longer a mere shell or circle but assumes the shape of variously arranged beaded bars; but, superficially, the similarity between the spore interior and an ordinary nucleus is, if anything, greater than it is in the resting stage. This situation is illus-



Text Fig. 1.



Figs. 1-4. Spores of *B. megaterium* germinating at 37° C. on yeast extract glucose agar. Fixed with acetic alcohol, stained by the Feulgen procedure and afterwards mounted in aceto-carmine.

1. Fixed 10 minutes after transfer. (a) Resting spores which have extruded their chromatin during the Feulgen-hydrolysis. (b) Spores which have failed to germinate and were probably not viable. (c) Spores with stained interior which have begun to germinate.

2-4. Fixed 35 minutes after transfer. Many spore cases already ruptured (d). (a) and (c) as above. The chromatin is now in the shape of Feulgen-positive bars and granules embedded in a basophil matrix. Spores like those in Fig. 3 at (d) in Fig. 4 look deceptively like cells with a neat, relatively large ordinary nucleus but must probably be interpreted in the manner of text Fig. 1 (compare with Figs. 27, 28 of Robinow 1955).

strated in Figs. 1-4 on Plate I and, crudely simplified, in text Fig. 1. The electron microscopy of sections of resting spores (Chapman, 1956, Robinow, 1953) and the behaviour of the chromatin during and after its first division (Robinow, 1956a, b) suggests that the second interpretation is the correct one and that the chromatin structures lie directly in the cytoplasm which is for some reason at this stage always unduly basophilic. This is clearly brought out by Figs. 2, 3 and 4, Plate I. In most of the spores in these pictures there is a deeply stained matrix between and around the Feulgen-positive elements. In this instance the stain is Feulgen followed by acetocarmine, but the same intense basophilia of the central region is also seen in germinating spores that had been hydrolyzed and stained with Giemsa, although one would expect hydrolysis to have removed all but the nuclear basophilia. The material in question has not yet been characterized. It disappears rapidly as growth of the young vegetative forms gets under way.

Perhaps these few remarks suffice to indicate that there are several morphological problems of spore germination waiting to be solved by cytochemistry and electron microscopy.

(Also shown were photomicrographs illustrating the behaviour of the chromatin during spore formation in *B. megaterium*, recently published elsewhere [Robinow, 1956a] and a series of pictures of *Metabacterium polyspora).*

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Discussion

P. C. Fitz-James

I should like to begin this discussion by showing first some chemical data which fit the pictures of germinating spores that Dr. Robinow has just presented.

Fig. 1 is taken from published material (Fitz-James, 1955) and shows

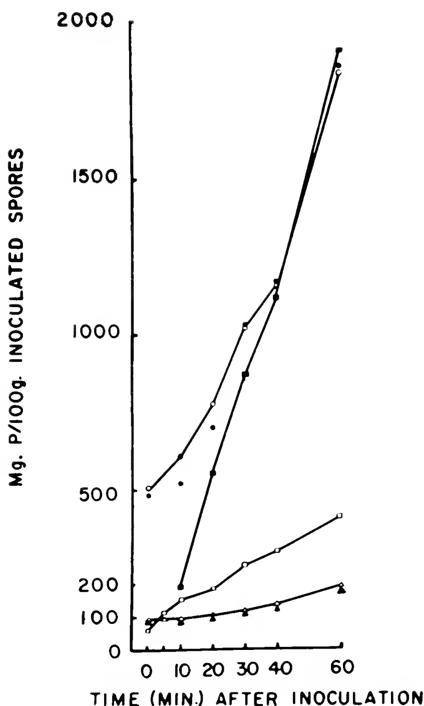

Fig. 1. The phosphorus fractions of *B. cereus* germinating in semi-synthetic medium. P as mg/100 g inoculated spores.

■, P uptake
□, Acid-Soluble P.
○, RNA-P (method of Schneider, 1945).
●, RNA-P (method of Schmidt & Thannhauser, 1945).
△, DNA-P (method of Schneider, 1945)
▲, DNA-P (method of Schmidt & Thannhauser, 1945).

the rapid and early rise of ribosenucleic acid phosphorus (RNA-P) which occurs following the initial awakening of the resting spores. In this particular study the spores (*B. cereus*) began to lose their refractility $2\frac{1}{2}$ minutes after inoculation and were 90% non-refractile by 6 minutes. The medium here was sufficient to insure rapid growth into vegetative forms. In other studies, medium containing only alanine, adenosine and glucose

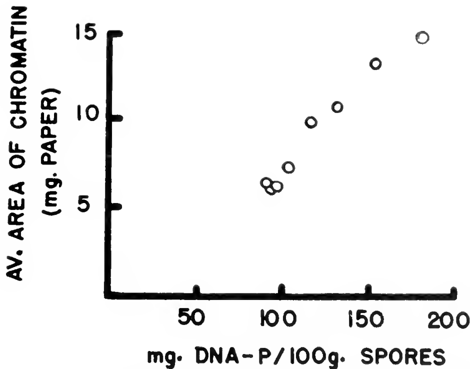

Fig. 2. The relation between basophilic nuclear material (chromatin) and DNA-P during the rapid germination of *B. cereus*.

showed no nucleic acid synthesis but only a rise in acid-soluble phosphorus concomitant with a fall in phosphorous of the residue fraction. The two points I wish to bring up here are the change in rate of RNA synthesis which occurred just as or before the desoxyribose nucleic acid (DNA) began to rise and the steady rise in DNA-P. The break in the rate of RNA synthesis was found in all experiments in which germination and growth was sufficiently synchronous and could be used as an indication of synchrony. Even the most synchronous studies however always showed a steady rise in DNA. This rise in DNA was found to be proportional to the amount of stainable chromatin estimated by the technique of projecting and tracing microphotographs and weighing the paper images of the chromatin (Fig. 2). During the period of study chosen the amount of DNA found in the spore had doubled and the number of nuclear bodies had increased accordingly (Fig. 3).

This indicated that the nuclear body of the bacillary cell tended to have a constant DNA content and that during germination reduplication and division went on at the same time and was complete when the original amount of DNA had been doubled. It also suggests that the spore contains the smallest functioning amount of DNA and indicates to me the biological goal of a sporulating cell—to parcel in an insulated chamber the functioning unit of chromatin.

Some more recent studies conducted jointly with Miss Young have permitted further examination of the average concentration of DNA/spore of a number of species and indicated the influence common laboratory media might have on the nucleic acid content as well as on the size of the spore.

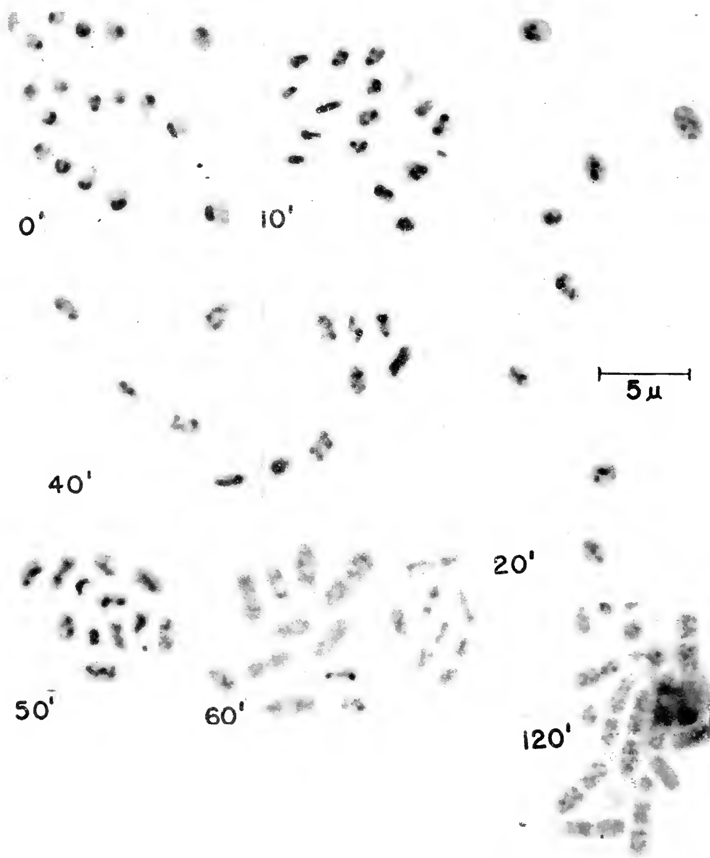

Fig. 3. The chromatin of germinating *B. cereus* spores fixed with osmium, hydrolysed with N HCl (60°, 8-10 minutes) and stained with SO_2-azure A. Magnification as indicated.

The different species now presented are considered as varieties of *B. cereus.* The varieties, *sotto, thuringiensis* and *alesti*, are strains in which each sporulating cell produces a crystal as well as a spore (Hannay, 1953; Hannay and Fitz-James, 1955), which crystals are toxic to insect larvae (Angus, 1954; Toumanoff *et al.* 1955).

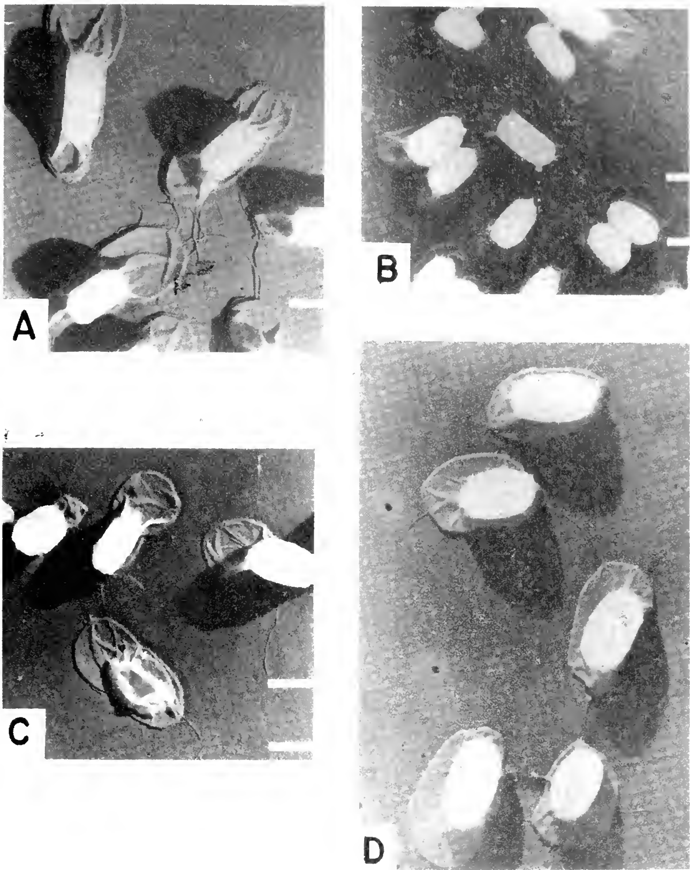

Fig. 4. Electron micrographs of shadowcast spores showing the marked differences in size. Magnification is the same in each: marker = 1 micron.

a. *B. cereus.*

b. *B. cereus* var. *sotto.*

c. *B. cereus* var. *thuringiensis.*

d. *B. cereus* var. *alesti.*

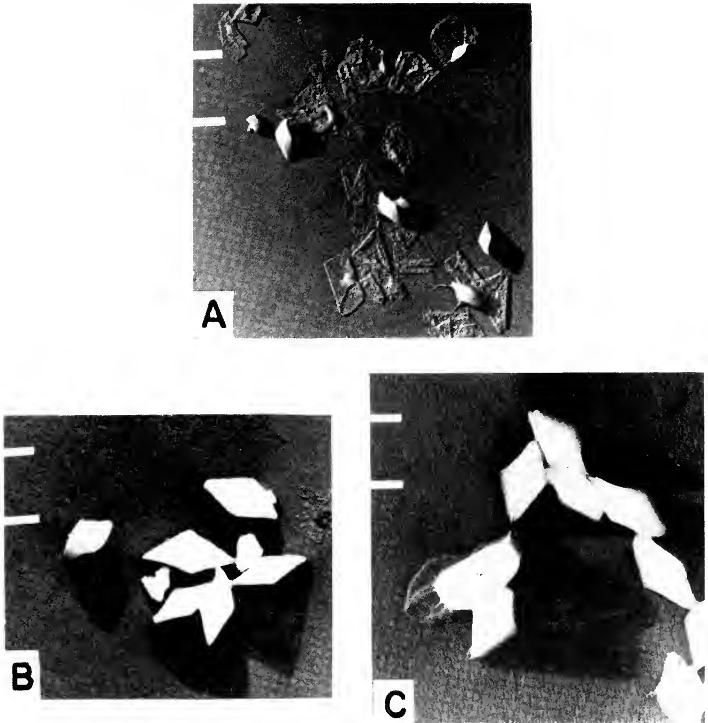

Fig. 5. Electron micrographs of shadowcast parasporal protein crystals showing variation in size.

a. *B. cereus* var. *sotto* (containing cell wall debris).

b. *B. cereus* var. *thuringiensis.*

c. *B. cereus* var. *alesti.*

Table I shows the influence of media on the size and composition of the spores of these varieties. The agar of Howie and Cruickshank (1940) is much better supplied with minerals, is higher in phosphorus but lower in nitrogen than the nutrient agar (Difco). The higher nitrogen content of nutrient agar is reflected in the greater content of N/spore and in the slightly greater average weight/spore. The nutrient agar however produced spores of a smaller average volume and of a lower RNA content. When grown on nutrient agar, our common *B. cereus* control contained 13% ; var. *sotto* (+)

TABLE I

The influence of medium on the size and composition of the spores *Bacillus cereus* varieties

Organism	Medium	Av. Wt. (10^{-12}g.)	Av. Spore Vol. (μ^3x10^{-2})	RNA-P/ Spore (10^{-16}g.)	DNA-P/ Spore (10^{-16}g.)	N/ Spore (10^{-16}g.)
B. cereus	N.A.*	1.40	–	31.0	9.6	1500
	H&C+	0.98	23.9#	35.6	9.8	1000
B. cereus var. *sotto* (+)	N.A.	0.77	8.2	21.5	10.9	776
	H&C	0.74	15.0	33.5	11.3	712
B. cereus var. *sotto* (—)	H&C	0.80	15.4	36.0	11.6	848
B. cereus var. *thuringiensis*	N.A.	1.02	–	34.0	12.6	1120
	H&C	0.93	20.0	42.0	12.1	995

* = Nutrient agar (Difco).
\+ = The agar medium of Howie and Cruickshank, supplemented with 0.5% casamino acids Difco.
\# = The previously published (ref. 2) value of 1.04 μ^3 for the volume of the spores of *B. cereus* is erroneously high by the factor of 5.
(+) = crystal bearing parent strain
(−) = non-crystal producing mutant
N = Nitrogen

27%, and var. *thuringiensis* 19% less RNA/spore than the same varieties grown on the agar of Howie and Cruickshank. It should be mentioned here that the spores grown on nutrient agar showed greater variation in size and were much less stable to storage as wet suspensions than were those grown on Howie and Cruickshank medium. The DNA content/spore however was not influenced by the medium and seemed to be a constant for each species. Variety *sotto* (—) is one of several non-crystal forming variants which were selected from platings of the crystal-bearing variety of *sotto*. The colony characteristics of the *sotto* (—) were identical to those of the *sotto* (+), the only distinguishing feature being the inability of the rod to produce a crystal with its spore. The DNA content and volume of the spores of these two *sotto* variants were also identical. The greater N content of the spores from these cells which do not have a crystal is perhaps to be expected.

Electron micrographs of clean spores of four of the groups gathered in Table I are shown in Fig. 4, and from this type of picture reasonably accurate calculations of spore volume could be made.

In grouping these data on the crystal bearing strains of *B. cereus* we also found that the largest spores were those which contained the greatest amount of DNA (Table II). Thus for any given species there may be a minimum space into which that cell's chromatin may be arranged.

TABLE II
Comparison of spore and crystal size with spore DNA

Organism	DNA-P/Spore (10^{-16}g.)	Spore Volume (μ^3x10^2)	Crystal Volume (μ^3x10^2)
B. cereus	9.8	23.9	—
B. cereus var. *sotto* (+)	11.3	15.0	2.2
B. cereus var. *sotto* (—)	11.6	15.4	—
B. cereus var. *thuringiensis*	12.1	20.0	10.1
B. cereus var. *alesti*	21.0	61.0	23.3

It was also evident here from studies of average crystal size (Fig. 5) and average crystal volume (Table II) that those varieties with the smallest spores and least amount of DNA also produced the smallest crystals. This relationship suggests a possible origin for the crystal protein.

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Informal Discussion

SUSSMAN: I would like to address my question to either Dr. Robinow or Dr. Fitz-James. Have the cytological stages in the development of the spores been tested with DNA-ase, or by extraction with hot perchloric acid, in order to determine whether the material that stains Feulgen-positive is really DNA?

FITZ-JAMES: Every state except the transient basophilic stage. Shortly after

that there is a sharp break in the RNA rise and shortly after this the synthesis of DNA begins.

ROBINOW: Fitz-James has made it very probable by direct chemical determination that there is DNA in the Feulgen-positive material which resting spores extrude above the cell surface when they are treated for a few minutes with N/1-N/3 nitric or N/1 hydrochloric acid at room temperature. His experiments made use of the fact that the material which *B. cereus* spores extrude when they are treated with N/3 nitric acid containing 0.1% potassium permanganate is soluble in low concentrations of sodium acetate in water. Fitz-James showed that the dissolution of the "chromatinic side body" went hand in hand with the gradual loss of DNA from the treated spores and the accumulation of DNA from the treated spores and the accumulation of DNA in the washing fluid.

WITTER: I want to ask Dr. Robinow if the extrusion of chromatin under the influence of hydrochloric acid can be correlated with changes in optical density and also how the ability to react in this way is correlated with the various phases of germination.

ROBINOW: My own experience has been only with the affinity of spores for stains. The extrusion response is given only by spores that are refractile and non-staining. Spores that have become transparent and readily stainable after being placed on a suitable nutrient medium no longer extrude their chromatin when challenged with nitric or hydrochloric acid at room temperature or at 60°C.

Enzymes Active in the Intact Spore

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Cornell University, Geneva, New York

IN WORKING with spore enzymes, one of the requirements is clean spore suspensions. Unfortunately there is no accepted criterion of cleanliness. However, it is generally recognized that a large number of washings with water, buffers or acids are necessary to eliminate contaminating material from the medium or debris from the sporangium, and that care must be exercised to prevent germination and destruction of enzymes that may be present in the spore proper.

In at least four cases, studies with intact spores have demonstrated enzymatic activity which does not appear to be attributable to vegetative residue or to the presence of germinated spores. These enzymes are: (1) alanine racemase, (2) catalase, (3) adenosine deaminase, and (4) nucleoside ribosidase. In addition, there are indications of other active systems which I hope will be discussed by the workers involved.

Alanine racemase. The report of Stewart and Halvorson (1953) on alanine racemase was the first clear-cut demonstration of an active enzyme in bacterial spores. Resting spores of *Bacillus terminalis* (a strain of *B. cereus*) formed a maximum of 140 micromoles of D-alanine from the L-isomer per hour per milligram at pH 8.5. Eight strains of seven species of *Bacillus* were tested and activity was found in all strains, ranging from a Q of 10 to a Q of 84 μM/hr/mg. The enzyme was specific for alanine, and in another paper by the same authors (1954) active extracts were shown to require pyridoxal phosphate. It was further shown that the enzyme in whole spores and in particulate fragments was stable at 80°C for 2 hours, and resistant to pepsin digestion. By sonic vibration and differential centrifugation, a "soluble", heat- and pepsin-sensitive enzyme was obtained from the spores. The implication here is that combination of certain groups of the enzyme with groups of the larger particles of a certain minimum size results in a stable but active enzyme. I wonder whether dipicolinic acid plays any part here.

In a later paper, Church and others (1954) demonstrated that the activity of this enzyme is not essential for germination. *B. globigii* spores, which

[1]Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 1092, Sept. 16, 1957.

lack the enzyme, require L-alanine for germination. At a pH of 11.3, the enzyme is inactive, but *B. terminalis* spores would still germinate in the presence of alanine and adenosine. At present, it is not known what role, if any, this enzyme plays in the economy of the spore. I believe Dr. Church has evidence that alanine is deaminated to yield pyruvic acid and ammonia in the presence of whole spores or spore extracts. I would also like to ask about the peak activity of the racemase which was reported to occur in young vegetative cells, about 4 hours old.

Catalase. In a number of cases, spores have been washed until they exhibit no catalase activity. At this point, they have been considered clean. With *B. terminalis* spores such a point was not reached. Even after prolonged washing, the level of activity remained constant, suggesting that the enzyme was an integral part of the cell (Lawrence and Halvorson, 1954). It was found that the enzyme activity of the intact spore was completely stable for 10-20 minutes at 80°C, while vegetative catalase was destroyed by 3 minutes at this temperature. 0.01 molar concentrations of KF, NaN_3 or NaCN produced 50-60% inhibition, and 0.02M Na ethylenediaminetetraacetate had no effect. It was found that by disrupting the spores the specific activity increased, and a portion of this activity was then sensitive to 5 minutes at 80°C. As Foster pointed out (1956) it is not known whether the catalase activities of spores and of vegetative cells are caused by identical enzymes. W. G. Murrell, in his Ph.D. thesis at the University of Oxford (1952) also found a heat-stable catalase in intact spores of *B. subtilis.*

Adenosine deaminase. Powell and Hunter (1956) noted that for their strains of *B. cereus* and *B. anthracis* inosine was a more effective germination stimulant than was adenosine. This suggested to them that inosine might be, in fact, the active compound, and that adenosine might be a precursor of inosine for these spores. The presence and activity of the deaminase was verified by noting the change in UV absorption (peak shifts from 260 to 250 millimicrons) and by measuring the ammonia liberated. No oxidation nor transamination was detected. The enzyme in the intact spore was heat resistant, surviving several hours at 60°C, but in extracts and homogenates the activity was destroyed in 15 minutes at this temperature. Cytidine was attacked at nearly the rate of adenosine, but in spore extracts, adenine, guanine, guanosine and cytosine were not deaminated. It is of interest to note that there was no correlation between the rate of deamination and the rate of germination in adenosine. Neither a preheating of the spores at 60°C for 2 hours, nor the addition of L-alanine plus L-tyrosine had any effect on the rate of deamination, although the germination in adenosine alone was remarkably stimulated by 1-hour heat treatment. These workers found that the enzyme was also present in germinated cells in a heat sensitive

form. It should be noted that this enzyme has been demonstrated only in extracts of spores of *B. terminalis.*

Nucleoside ribosidase. It has been found (Lawrence, 1955; Powell and Hunter, 1956) that spores of a number of species of aerobic bacteria will cleave adenosine and other ribosides, presumably by a hydrolytic process, into the free base and the free sugar. The specificity for nucleosides appears to vary from strain to strain. Strains of *B. cereus* and *B. anthracis* attacked adenosine and inosine at equal rates, feebly attacked guanosine, and were inactive toward xanthosine and cytidine. In contrast to this, spores of the *terminalis* variety of *B. cereus* attacked adenosine, guanosine, inosine, xanthosine, adenylic acid, cytidine and uridine. It has since been found that 2-aminoadenosine and purine riboside are also cleaved, but desoxyinosine, desoxyadenosine, desoxyguanosine and adenine-9-β-D-glucoside are not (Lawrence, unpublished).

In all cases, the enzyme was remarkably heat stable, resisting 100°C for an hour or more. Cell free preparations also retained activity. I believe that workers here at Illinois may have further information on cell-free preparations of *B. terminalis.*

The role of this enzyme in germination is not clear. It may play an important part in those spores which require adenosine for rapid germination since it has been found that *B. terminalis, B. polymyxa, B. anthracis,* and *B. cereus* all possess riboside activity. The enzyme is not present in spores of *B. globigii.* This species does not require adenosine for rapid germination.

Other enzymes. Recent work by several people has indicated the presence of other active enzymes in spores. There is a question in my mind as to where to draw the line between dormant and active enzymes in intact spores—heat treatment of spores activates the glucose dehydrogenase system, and apparently these spores are intact, at least they appear so microscopically. Also, mere aging of lyophilized spores alters their response to various germinating agents—does this indicate a low level of activity of cellular enzymes? Does this bear any relationship to the proteolytic enzymes of extracts of spores, which were demonstrated by Levinson? Last, I would like to mention diaphorase, apparently found only in spore extracts; and also the oxidation of p-phenylenediamine and hydroquinone, mentioned by Dr. Powell; and the alanine deaminase found by Church. I have no doubt that in the near future spores will be found to possess other active systems also.

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Discussion

Herbert M. Nakata

The importance of using clean spore suspensions and the necessity of specifying the age and history of the spores used in germination studies have been emphasized and discussed. Similar emphasis is encouraged when using spore suspensions for enzymatic investigations. With the increased number of publications in recent years regarding spore enzymes, it has become apparent that some general criteria must be founded and accepted to minimize misinterpretation of data, results, and conclusions by others. Therefore, it is my intention to suggest and discuss briefly some aspects that we feel lead toward the realization of such useful criteria whereby enzymes classified as being active in the dormant intact spore can be differentiated from the other categories listed in the program.

(*A*) The first of these suggested criteria deals with the need for controlling both the physical and chemical environments of the experimental spore suspensions during the time measurements are made. That is, the environment must be such that the enzyme activity occurs under conditions precluding the possibility of spore germination. The term germination, as I will use it, refers to the loss of heat resistance, refractility, and the sensitivity to stains. In recent years, much emphasis has been placed on this criterion and several methods, such as the use of (1) high temperatures, (2) pH's unfavorable to germination, (3) substrates that are not conducive to germination, and (4) germination inhibitors, have been suggested as some ways in which complications due to undesirable germination can be avoided.

(1) *Use of high temperatures.* Among these methods, the use of high temperatures has been proven satisfactory by Dr. Lawrence (1955 a, b) and in our laboratory for the study of the heat-resistant nucleoside ribosidase

in the spores of *B. terminalis,* as germination was not evident in this species when incubated with the specific germination nutrients above 65°C.

(2) *Use of pH's unfavorable to germination.* The use of a pH that is unfavorable to the germination of a particular species may also be successful, providing it is employed in instances where the pH range for germination is rather acute, thereby allowing the selection of a pH which will yet be feasible for some enzymatic studies (Harrell and Halvorson, 1955).

(3) *Use of substrates not conducive to germination.* (Stewart and Halvorson, 1953). Special care must be exercised where the substrate added may be conducive to germination. This is of particular importance when aged spores are employed to study a reaction which necessitates the addition of a substrate that partially fulfills the normal germination requirement of that species. As an illustration, a significant proportion of aged *B. terminalis* spores has been observed to germinate in either L-alanine or adenosine alone, although fresh spore suspensions—that is, spores harvested and stored less than a week—of this species require the presence of both compounds before germination is evident and complete. Several other similar illustrations were mentioned in yesterday's session. Consequently, one who is concerned with enzymatic studies employing dormant intact spores should be reminded to check the reaction mixture to determine whether or not germination has occurred during experimentation.

(4) *Use of germination inhibitors.* (Murty and Halvorson, 1957a; Levinson and Sevag, 1953; Powell, 1950). In regard to the use of specific inhibitors of germination, some of those discussed in last night's session, or others, may prove quite useful, providing they do not interfere with the enzymatic system under consideration.

(*B*) As a second criterion, enzymes classified in this category must display activities under conditions where the possibility of activating dormant enzymes in the intact spore is unlikely. This stipulation has become imperative with the discovery of dormant enzyme systems—so-called because their activities are normally absent or undetectable in the fresh dormant intact spores and require some means of activation before measurements can be made (Church and Halvorson, 1956; Murty and Halvorson, 1957b). It has therefore become essential that one consider this possibility of activation, which is referred to as ungerminated spores that display heretofore undetected enzymatic behavior, as a direct result of some activating stimulus, or stimuli. These physiological changes at this time appear to be somewhat analogous to that occurring in spores during storage. Among the practices which consequently should be avoided when studying enzymes active in the dormant intact spore are: (1) heat shocking, particularly prolonged heat

shocking; (2) the addition of activating substances or the combination of heat shocking with activating substances; and (3) prolonged storage.

(1) *Prolonged heat shocking.* The effect of prolonged heat shocking has been shown by several investigators (Powell and Hunter, 1955; Murty and Halvorson, 1957b; Church and Halvorson, 1956) to alter the physiological behavior of spores without germination. As an example, the germination requirement of *B. terminalis* has been observed to change where L-alanine no longer is a requisite for the germination of these heat-treated spores. Heat shocking of spore suspensions prior to disintegration has also been shown to have various influences on the activities of enzymes found in the subsequent spore-free extracts. In one of the more recent studies, Dr. Krask (1956) has reported a 2-fold increase in acetokinase-like activity in *B. terminalis* after 15 minutes of heat shocking prior to disintegration and a 16-fold increase after 1 hour. This and other similar reports well indicate, then, that some sort of activation is occurring during prolonged heat shocking. These findings, correlated with the evidence that dipicolinic acid is gradually released during heat shocking (Harrell, 1956) may have direct bearing on the activation of some enzymatic systems in intact spores.

(2) *Addition of activating substances.* Oftentimes enzymatic activation occurs unnoticed to the investigator since small amounts of a particular substance may be all that is necessary for the activation of some systems. This has been well demonstrated by the glucose oxidizing system in the spores of *B. terminalis*, where trace quantities of L-alanine together with prolonged heat shocking were responsible for the activation of an enzyme system previously dormant or undetectable in the untreated spores. This will be discussed in greater detail by Dr. Murty and Dr. Church in the following papers.

(*C*) Thirdly, a few of the past investigations have revealed enzymes in spore homogenates and spore-free extracts prepared from dormant spores. and these enzymes, although undetected in the dormant intact spores, were assumed to be actively present prior to disintegration. Unless the enzymatic activity can be demonstrated with intact spores under conditions satisfying the suggested criterion, or one similar to it, the validity of these assumptions is open to criticism. Enzymes that may be active in spore homogenates and extracts are not necessarily active in the dormant whole spore. During ultrasonic or mechanical disruption a variety of substances are released from the spores which conceivably can result in the following: (1) the germination of unbroken spores in the homogenates, whose subsequent activities may be erroneously attributed to the ungerminated spores, or (2) the activation of enzymes previously undetectable in the intact spores but highly active in the subsequent extracts as a result of the disintegration process. Viable counts on the extracts are strongly recommended to rule out the first possibility.

Among the enzymes described by Dr. Lawrence as being active in the intact spores, two in particular have received considerable attention—namely the alanine racemase and the nucleoside ribosidase. Besides adequately satisfying the general criteria suggested for this group of enzymes, they have been shown to have several characteristics in common which may be applicable for other enzymes yet to be reported. (1) Each of these enzymes has been demonstrated to occur in much greater amounts in spores than in the homologous vegetative cells. Although some reports have indicated that the vegetative cells of *B. cereus* were completely devoid of ribosidase activity, we have been able to demonstrate some heat-labile ribosidase in the vegetative cell of *B. terminalis* (Nakata, 1956). Perhaps this discrepancy could be explained by the difference in the age and species of the cultures used in each case. (2) The racemase, ribosidase, and also the catalase were found to be extremely heat resistant in the intact spores, in germinated spores, and in the spore-free extracts (Stewart and Halvorson, 1953, 1954; Lawrence, 1955a,b; Nakata, 1956). In the case of the racemase and ribosidase, the enzymes were observed to be intimately associated with a particulate fraction which presumably is responsible for their thermal-resistant properties. Although no particulate fraction has been demonstrated for the heat-stable catalase, it too may be similarly protected with particles of varying magnitudes so that they are not separable during centrifugation.

Insofar as the adenosine deaminase is concerned, we have found no conclusive evidence to place it among the enzymes active in the dormant intact spores. Aside from its being present in large amounts in the vegetative cells as well as in the spores, it is heat sensitive and non-particulate. From the data available, there appears to be no evidence that adenosine deamination occurs in the absence of germination and under conditions where activation of the enzyme in the intact spore is unlikely.

We have noted that when using fresh spores of *B. terminalis* no germination is evident during incubation with adenosine alone at 37° C, thus providing a system where the adenosine deaminase could be studied with dormant resting spores (unpublished data). Employing the optimal conditions for the deaminase as reported by Powell and Hunter (1956), we have observed the fate of adenosine using samples of unheated spores, spores heat shocked 15 minutes and 60 minutes at 65° C. The only product of adenosine noted in each case was adenine, indicating the absence of deamination in the dormant intact spores as well as in the heat-treated spores. Subsequent examination of the incubation mixture revealed the absence of germination in all cases. However, deamination of adenosine to inosine was found to occur with aged spores or under conditions favoring germination, thus prompting the

question: can the apparently dormant deaminase be activated prior to germination or is germination *per se* the activating step?

Perhaps one of the most controversial questions encountered in reference to these thermal-resistant enzymes is whether these catalysts are biological. Because they are particulate in nature, one finds it difficult to demonstrate normal protein behavior using methods generally employed for common enzymes. No doubt the strongest evidence in support of the enzymatic nature of these catalysts is that autoclaving completely destroys activity in 15 minutes. Also, a general procedure has been described by Stewart and Halvorson (1954) by which a suspension of particulate alanine racemase was separated into a soluble, heat-labile fraction and a particulate, heat-resistant fraction by ultrasonication and subsequent centrifugation. Further evidence has shown that the soluble heat-labile fraction was susceptible to pepsin digestion whereas the particulate fraction was completely stable. Results analogous to that reported for the racemase have been indicated in our laboratory with the nucleoside ribosidase of *terminalis* (Nakata, 1956).

The effects of the heavy metals Cr^{++}, Cu^{++}, and Hg^{++} in trace amounts (10^{-3} M) were also studied and observed to completely inhibit ribosidase and racemase activities, further supporting an enzymatic system (unpublished data).

The significance of the alanine racemase system in *B. terminalis* was investigated by Church *et al* (1954) and by Harrell and Halvorson (1955) who reported it to play no active part in the germination process. The significance of the nucleoside ribosidase and the adenosine deaminase systems, however, to my knowledge, has not as yet been defined. Dr. Lawrence (1955a), working with the ribosidase in whole spores and spore extracts of *B. terminalis*, has demonstrated the adenosine cleavage to adenine and free ribose. Although the recovery of ribose as compared with the adenosine cleaved was low at 37°, no enzymes were detected in spore extracts which metabolized the liberated ribose. Inosine, the deaminated product of adenosine, was not implicated in his studies.

Dr. Powell, working with germinated spores of *B. cereus*, also observed low ribose recovery when these spores were incubated with adenosine alone, but noted the disappearance of adenosine with formation of adenine and hypoxanthine (Powell and Hunter, 1956). When these germinated spores were heated 15 minutes at 60° prior to incubation with adenosine, expected ribose values were obtained. The interpretation that ribose was metabolized by the germinated spores was questionable since analysis of the homogenates failed to reveal such enzymes.

Considering these and the data obtained in our laboratory with fresh *B. terminalis* spores, a more feasible explanation is suggested. The spores

used by Dr. Lawrence were undoubtedly activated intact spores, perhaps due to aging during storage, where both the ribosidase and the deaminase systems were active. Then the incubation of these spores or their extracts with adenosine resulted in its cleavage to adenine and ribose, the latter measured in his particular case. Simultaneously, adenosine was probably deaminated to inosine, which evidently was not followed in this particular experiment.

Both of these enzymes were apparently active in the germinated *B. cereus* spores employed by Dr. Powell and co-workers, as hypoxanthine, the product of a ribosidic cleavage of inosine, was observed together with adenine, which reportedly was not capable of deamination. Heating the germinated spores gave expected ribose liberation from adenosine because the deaminase, being heat sensitive, was inactivated, leaving only the ribosidase system operative.

At this time, we feel that evidence is insufficient to permit intelligent speculation on the actual significance of the ribosidase system. These observations only suggest that, like the alanine racemase system, it plays no active part in the germination process.

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Informal Discussion

MANDELS: I would like to make a few comments relating to the remarks of Dr. Lawrence on the enzymatic activity of spores. In our studies with fungus spores, we have found the intact, viable spores to possess fairly high enzymatic activity—the enzymes we have worked with being invertase, an atypical ascorbic acid oxidase, and a sulfhydryl oxidase. The activity of these enzymes is much higher than could possibly be necessary for metabolic requirements of the cell. Furthermore, we can completely inactivate these enzymes without impairing the metabolic activity of the cell. Thus, it is quite possible that there are enzymatically active proteins in spores, and possibly vegetative cells, too, that have no particular catalytic function essential to the metabolism of the cell. The enzymes I have mentioned are apparently localized on the external surface of the plasma membrane of the spore. Their primary function may thus be as structural proteins in the membrane.

FITZ-JAMES: I should like to say a word about cleaning spores and light microscopy as a criterion of cleanliness. The light microscope will detect the presence of larger pieces of vegetative debris, but one really needs an electron microscope to decide the purity of a spore preparation. In trying to clean the spore of crystallophoric strains of *B. cereus.* we at first felt quite satisfied from observations with the light microscope that repeated alkali and water washings of spore-crystal mixtures were giving us clean spores. The electron microscope, however, showed they were coated with variable amounts of fibrous crystal protein, and further cleaning with acid and alkali was necessary to free spores completely of crystal protein.

HARLYN HALVORSON. The enzyme content and biochemical capabilities of spores indicate that they are physiologically more akin to vegetative cells than

was previously thought. One interesting feature of the latter has been their ability to alter their phenotype through induced enzyme synthesis. Several experiments illustrate also that the enzyme pattern of the spore is not limited to those required for the maintenance and breaking of dormancy. This was first indicated by Martin Pollock who studied the rate of induced penicillinase synthesis during germination of spores obtained from penicillin induced vegetative cells and non-induced cells. The former produced penicillinase more rapidly than the latter, indicating that the penicillinase-forming system was carried through the spore state. Dr. Knox Harrell (unpublished results) in my laboratory studied this same problem and found that extracts of spores produced from penicillin-induced cells contained penicillinase. The intact spores were inactive on penicillin. It seems unlikely that this enzyme plays a role in dormancy or germination. These results further suggest that some of the differences in germination requirements observed in the same or related spore species are a reflection of the differences in enzyme patterns of the vegetative cells which are carried into the spore cytoplasm at the time of sporulation.

MURTY: At the time we were running these experiments it was unfortunate that I could not get any inhibitor other than p-fluorophenyl alanine. However, we shall try other inhibitors also. I do not know if the inhibition of p-fluorophenyl alanine can be reversed by phenyl alanine. However, we know that the activation by L-alanine is completely antagonized by D-alanine, a phenomenon also observed by Murrell.

GERHARDT: I am very strongly reminded of and would like to point up to the group the sometimes very close analogy between what one sees here in spores and in some of the earlier, and now continuing, very elegant experiments of J. Gordin Kaplan, with whom many of you may not be familiar. His observations of the heat stable catalase of yeast and its activation on the rupture or solvent extraction of the yeast cell, which he refers to as the Euler effect, may be comparable to what one often sees in spores. I think the interesting point which he has demonstrated, and which may serve as a model for experiments here, is his recreation of the heat stability of yeast catalase by absorption of the isolated enzymes on lipid and I understand more recently on RNA. That is, he has been able to activate the enzyme and then deactivate it at will in an artificial system. One wonders here if one could activate one of these spore enzymes by heat, possibly create a model system with dipicolinic acid, and then deactivate the enzyme by absorption on that system.

Enzymes Dormant in the Intact Spore

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IN CONFORMITY with the views expressed earlier by Mr. Nakata and others, I shall restrict my discussion to enzyme systems which are dormant in the intact resting spore but which can be activated without disturbing in any way the gross morphology of the spore or stimulating germination. In other words, I shall discuss only those enzymes whose activity can be measured in the intact spore in the absence of germination, the ways of activation of these enzymes, the changes occurring during the process of activation, and the probable mechanism of their dormancy and stability to heat while dormant in the intact spore.

To my knowledge the only enzymes meeting the above criteria are the oxidative enzymes. Working with spores of a strain of *Bacillus subtilis* which specifically required L-alanine, Murrell (1955) found that when a suspension of the spores was incubated with L-alanine, the decrease in the viable count of heat-stable spores was inversely proportional to the strength of the spore suspension. When dense suspensions were incubated with glucose alone, oxygen uptake could barely be measured, but on addition of L-alanine to the systems, there was a marked increase in the oxygen uptake in the absence of any detectable germination of the spores. Spencer and Powell (1952) observed a similar synergism between oxidation of glucose and L-alanine. D-alanine, added before or at the same time as the L-isomer, inhibited the action of L-alanine. Adenosine and tyrosine had no such stimulatory action. Murrell further observed that the time and order of addition of the substrates had a profound effect on the oxidation of glucose.

When L-alanine was added first and glucose added as the second substrate, the rate of oxidation of glucose decreased with increasing time of incubation of the spores with L-alanine alone. When glucose was added first and L-alanine added as the second substrate, the rate of oxidation was stimulated to the same extent each time alanine was added. The effect of pretreatment of the spores was more complex.

When the spores were treated with alanine, washed and tested with glucose alone, the rate of oxidation was the same as with untreated spores. Tested with glucose and L-alanine the rate of oxidation was considerably less than with untreated spores.

Spores treated with L-alanine and glucose and washed. Tested with glucose alone the rate of oxidation was the same as with untreated spores except for a

slight carry-over of activity in the beginning. Tested with glucose and L-alanine, the lag period was reduced, but there was no decrease in the rate of oxidation.

Murrell concluded that L-alanine had to be present for the oxidation of glucose and that the rate of oxidation was a function of both the number of spores and the concentration of L-alanine.

Church and Halvorson (1955, 1956), working with aged spores of *B. cereus* var. *terminalis*, found that when the spores were heated at 65° from 60 to 90 minutes, they rapidly oxidized glucose in the absence of any detectable germination of the spores. They found that the spores so activated could also oxidize gluconate, 2-ketogluconate and pyruvate, but I shall leave the discussion of these to Dr. Church. However, freshly grown spores either failed to oxidize glucose or oxidized glucose at an extremely low rate under similar conditions. They further observed that adenosine had the same effect as heat shock in aged spores. The heat activation decayed with time, but activity could be regained by a second heat shock, although the degree of restoration decreased with the time elapsed between the first and second heat treatments.

We have obtained essentially similar results. Freshly grown or aged spores of *B. terminalis* germinated rapidly and completely, but failed to grow when incubated with the germinating nutrients under anaerobic conditions, an observation independently made also by Roth with other aerobic spores. Dense suspensions of spores of *B. terminalis* failed to germinate completely under anaerobic conditions, even when incubated for a long period with large amounts of the nutrients supplemented with glucose. The resulting mixture of germinated and ungerminated spores was washed free of the nutrients, and by differential centrifugation a suspension relatively rich in ungerminated spores was obtained. When heat-shocked to kill the germinated spores, this suspension would actively oxidize glucose. This led us to believe that it might be possible to activate the glucose-oxidizing enzymes of the spores in the absence of germination, by heating a thick suspension of the spores in the presence of small amounts of the germinating nutrients. This was indeed found to be the case, and the relations between the amount of enzymes so activated, the duration of heating, the number of spores, the concentration of nutrients, the time of addition of nutrients, etc., are given below.

Results. The amount of enzymes activated seemed to be a function of the duration of heat shock and the concentration of nutrients. With a low concentration of nutrients the limiting activity of the enzymes is reached in 7 hours of heating (Table I).

With a fixed concentration of nutrients, the amount of enzymes activated became a function of both the duration of heating and strength of the spore

TABLE I

Effect of duration of heat shock and concentration of nutrients on oxidation of glucose by spores of *B. terminalis*

Duration of heat shock in hours - 65 C	Concentration of Nutrients			
	26 μg ml L-alanine 6 μg ml adenosine		65 μg ml L-alanine 15 μg ml adenosine	
	Endog.	Exog.	Endog.	Exog.
3	10	25	—	—
4	12	30	12	30
5	11	43	21	75
7	21	64	13	113
8	24	66	69	150

Strength of spore suspension—6 mg dry wt/ml
Substrate—Glucose 0.5 mg/ml μ l oxygen uptake in 30 min.

suspension. A dilute spore suspension (1 mg dry wt/ml) required a minimum of 9 hours, but even then the amount of glucose oxidizing enzymes activated, as judged by the difference between the exogenous and endogenous, was very low. Furthermore, the endogenous oxidation decreased with increasing strength of the spore suspension and duration of heat shock (Table II).

L-alanine alone with heat was effective in activating the enzymes. Heat shock with adenosine alone was ineffective. Heat shock with L-alanine and adenosine together was no more effective than with L-alanine alone. However,

TABLE II

Effect of duration of heat shock and strength of spore suspension on oxidation of glucose by spores of *B. terminalis*

Duration of heat shock in hours 65°C	Concentration of spores mg dry wt/ml					
	15 mg/ml		6 mg/ml		1 mg/ml	
	Endog.	Exog.	Endog.	Exog.	Endog.	Exog
3	15	18	10	25	—	—
4	10	30	12	30	—	—
5	4	61	11	43	—	—
7	—	—	21	64	—	—
8	—	—	24	66	—	—
9	—	—	—	—	20	32

Nutrients—26 μg/ml L-alanine, 6 μg/ml adenosine
Substrate—Glucose 0.5 m/ml μ l oxygen uptake in 30 min.

Table III

Effect of time of addition of nutrients on oxidation of glucose by heat shocked spores of *B. terminalis*

Time of addition	Nutrients			
	26 μg/ml L-alanine		6 μg/ml adenosine	
	Endogenous	Exogenous	Endogenous	Exogenous
Before heat shock	4	60	—	—
After heat shock	High oxygen uptake; partial germination			

Strength of spore suspension—15 mg dry wt/ml
Substrate—Glucose 0.5 mg/ml
Duration of heat shock—5 hrs. at 65°C μ 1 oxygen uptake in 30 minutes

the L-alanine had to be added before heat shock. If L-alanine or adenosine was added after heat shock, the spores germinated partially and, as a consequence, both the endogenous and exogenous oxidation were very high (Table III).

The enzymes are not active immediately after heat shock, but only after 40-45 min. incubation at 30°C following the heat shock. However, the L-alanine could be washed out immediately after heat shock or later without any significant change in activity. In contrast, germinated spores lost nearly all their glucose-oxidizing activity on washing (Table IV).

P-fluorophenylalanine prevented the heat activation of these enzymes when added along with L-alanine before heat shock, but had no effect when added after heat shock. Furthermore, p-fluorophenylalanine had no effect on germination of the spores in the presence of the normal quantities of the nutrients (Table V).

When a relatively dilute, activated suspension of spores (6 mg/ml) was

Table IV

Effect of washing on oxidation of glucose by heat shocked spores of *B. terminalis*

Time of washing	Endogenous	Exogenous
No washing	4	66
Immediately after heat shock	6	70
40 minutes after heat shock	8	60

Germinated spores lose nearly all glucose-oxidizing activity on washing.
Duration of heat shock—5 hrs. at 65°C
Strength of spore suspension—15 mg dry wt/ml
Nutrients—26 μg/ml L-alanine; 6 μg/ml adenosine
Substrate—Glucose 0.5 mg/ml μ 1 oxygen uptake in 30 minutes

TABLE V

Effect of p-fluorophenylalanine on the enzymatic activity of heat shocked spores of *B. terminalis*

Time of addition of p-fluorophenylalanine	Endogenous	Exogenous
Before heat shock	0	8
Immediately after heat shock	4	61
60 mintues after heat shock	4	54

Strength of spore suspension—15 mg dry wt/ml
Duration of heat shock—5 hrs. at 65°C. Substrate—Glucose 0.5 mg/ml
Nutrients—26 μg/ml L-alanine: 6 μg/ml adenosine
p-fluorophenylalanine—1.0 x 10^{-2}M μ 1 oxygen uptake in 30 minutes

heated 1 hour at 65° to inactivate the active glucose-oxidizing enzymes, and equilibrated at 30° for 40-45 min., there was no significant decrease in the activity. Thus, it appeared that the glucose-oxidizing enzymes were stable to heat, but this was not the case when a more concentrated suspension (15 mg/ml) was used. During storage in the frozen state for almost a year, the spores have undergone some changes that obviate the need for alanine in the activation of the enzymes. When a dense suspension (15 mg/ml) of such aged spores, activated by heat alone or by heat in the presence of L-alanine, was heated again 1 hour at 65°, the activity was completely lost. However, the activity could be regained by adding more alanine, before the second heat shock, although in the case of the suspension activated by heat alone the first time, a much longer second heat shock was required (Table VI).

TABLE VI

Effect of duration of heating, strength of spore suspension, and time of addition of nutrients on the enzymatic activity of heat activated spores of *B. terminalis*

Duration of second heat shock 65°C	No Time 15 mg/ml		Before 1st heat shock only 6 mg/ml		Before 1st heat shock only 15 mg/ml		Before 2nd heat shock only 15 mg/ml		Before each heat shock 15 mg/ml	
	End.	Exog.	End.	Exog.	End.	Exog.	End.	Exog.	End.	Exog.
0	4	32	11	43	4	61	4	32	4	61
1 hr.	0	0	6	30	0	0	0	0	10	60
5 hr.	0	0	—	—	0	0	16	58	—	—

Duration of first heat shock—5 hrs. at 65°C
Nutrients—26 μg/ml L-alanine: 6 μg ml adenosine
Substrate—Glucose 0.5 mg/ml μ 1 oxygen uptake in 30 minutes

Discussion. The results obtained by Murrell, Church and Halvorson and us lead to some common conclusions. It is evident that the oxidative enzymes normally dormant in the intact spores can be activated in the absence of any detectable germination.

The mode of activation may vary with the age and history of the spores. As has already been pointed out, we were able to detect small amounts of alanine in the washings from aged spores and this probably explains the activation of these enzymes by heat alone as noted by Church and Halvorson and by us. So it is safe to conclude that L-alanine is responsible for the activation of the enzymes.

Once the spores have been activated, inhibitors have no effect, although the activation seems to decay with time. The rate of oxidation of glucose is a function of the number of spores and the concentration of L-alanine, the concentration of L-alanine being limiting in the case of dense suspensions.

However, there are some significant differences also. While in the case of spores of *B. subtilis* the presence of L-alanine is necessary for the maximum rate of oxidation of glucose, in the case of *B. terminalis* spores the L-alanine can be washed out after heat activation without any loss of activity.

The decrease in the capacity of *B. subtilis* spores pretreated with alanine to oxidize glucose and L-alanine is similar to the loss in glucose-oxidizing activity noted on washing germinated spores of *B. terminalis*. While in the case of germinated *B. terminalis* spores, the loss of activity on washing may be due to washing out part of the cofactors involved, the situation with the spores of *B. subtilis* is considerably more complicated.

Murrell has noted a decrease in activity with increasing time of incubation of spores in L-alanine alone before adding glucose as the second substrate. Church and Halvorson have noted that the restoration of activity of *B. terminalis* spores on second heat shock fell off with the time elapsed after the first heat activation. In the case of dense spore suspensions we found that alanine had to be added before the second heat shock for the enzymes to be activated, suggesting the possibility of alanine being metabolized during the activation or oxidation of glucose. All these observations can probably be explained on the simple assumption that the activated spores were also metabolizing L-alanine. This would suggest that we should look more carefully for an enzyme metabolizing alanine in the activated spores. As Mr. Nakata has pointed out earlier, the adenosine deaminase may be another enzyme one should look for in activated spores.

The release of alanine during storage suggests the possibility of proteolytic activity, and with the proper techniques of activation one may be able to demonstrate the presence of proteolytic enzymes in the intact spores as proteolytic enzymes have been demonstrated in spore-free extracts.

Means of Activation. Depending upon the species, strain, history, etc., the dormant enzymes of the intact spore may be activated in the absence of any detectable germination by treatment with small amounts of appropriate germinating nutrients, and/or heating.

Nature of Activation. Inasmuch as the oxidative enzymes as well as other enzymes dormant in the intact spore are active in spore-free extracts, one begins to wonder seriously about the nature of the activation process and the causes for dormancy and heat stability. That considerations of permeability alone cannot fully explain the nature of the activation process is evident when one recalls that, in some cases at least, the activity of the extracts was increased by heat shocking the spores prior to rupture. We therefore started looking for other changes during heat activation—particularly the release of dipicolinic acid from the spores. The total dipicolinic acid content of the spores was 6.6-7.0% on a dry weight basis. We have noted a partial release of dipicolinic acid during heating with alanine, and subsequent incubation at room temperature and oxidation of glucose, on germination and on autoclaving (Table VII). Partial release of dipicolinic acid has also been observed by Harrell (1956) during the heat activation of aged spores of *B. terminalis*. On complete germination or autoclaving, most of the dipicolinic acid of the spores is liberated into the medium and, presumably, the total oxidative enzymes associated with growth from spores are completely activated and rendered sensitive to heat, inasmuch as the germinated spores become nonviable even on 15 min. exposure to 65°C. In marked contrast, no decrease in the viable count of heat-stable spores could be detected by

TABLE VII

Dipicolinic acid content of *B. terminalis* spores and release of dipicolinic acid on germination and activation by heat shock in the presence of nutrients

Time	% of dipicolinic acid released
Immediately after heat shock	2.5
1 hr. after heat shock	5.2
After 30 minutes of oxidation of glucose (90 minutes after heat shock)	
30 minutes after germination	65 - 70
On autoclaving	80 - 85

Strength of spore suspension—15 mg dry wt/ml
Nutrients—26 μg/ml L-alanine; 6 μg/ml adenosine
Substrate—Glucose 0.5 mg/ml
Duration of heat shock—5 hrs. at 65°C
Dipicolinic acid content of spores on dry wt—6.6%

Murrell, Church or us in the spores actively oxidizing glucose. It is evident that some of the spores have undergone a change without a simultaneous loss in heat stability. The spore is also relatively rich in calcium. The above findings stimulate us to speculate that the dipicolinic acid is present in the spore in the form of a polymer and that the polymer produces during its formation an inclusion complex with the enzyme, thus protecting it from heat injury, in a manner similar to the inclusion complexes described by Schlenk and coworkers (1950, 1955). Upon germination or by other means of releasing dipicolinic acid from the polymer, the enzymes are released so that they become active and heat sensitive. The first phase of germination would then consist of depolymerization of such a complex—a view also shared by Powell, Strange and coworkers (1956). Such a view would also probably explain the failure of most enzyme inhibitors to prevent germination of the spores, and also the ability of aerobic spores to germinate under anaerobic conditions.

The activation of the oxidative enzymes may then, in all probability, be compared to partial germination of the spore, consisting in a partial release of dipicolinic acid resulting in activation of only a part of the enzymes associated with growth. It is conceivable that the amount of enzyme so activated is such a small part of the total enzymes of the spore that enough is left to enable the spore to grow upon germination.

There was a time when ungerminated spores were believed not to contain any enzymes, but the presence of active enzymes in ungerminated spores is now beyond any doubt. One may perhaps venture to state that in all probability the ungerminated spore contains most of the enzymes found in the homologous vegetative forms. It is not too much to hope that with improved techniques many more active enzymes may be demonstrated in the ungerminated spore in the years to come. A study of the biochemical changes involved in early phases of germination or the late phases of sporulation may one day provide the answer to the interesting problem of the dormancy and heat stability of the bacterial spore.

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Discussion

Brooks D. Church

Activation of Glucose Oxidation in Spores of *Bacillus cereus* var. *terminalis*

Dr. Murty has discussed enzyme activation in spores of *B. cereus* var. *terminalis* after treatment of the spores with heat and L-alanine. Only in spores aged two years or longer was he able to activate glucose oxidation with heat alone. In no instance was he able to activate the enzyme in the absence of heat.

Activation of the glucose-oxidizing enzyme in these spores (Church and Halvorson, 1955, 1956) was carried out by the use of heat or with adenosine in the absence of heat. The several types of enzyme activation studied by Murty and by us, together with the effect of spore age, may be related to initial steps in a common series of reactions involving glucose. I feel that a discussion of spore enzyme activation in the light of our combined information may be helpful toward a better understanding of the activation mechanism. The following experiments are reviewed as an aid in this discussion.

Under conditions of heat shock normally employed for germination studies, 15 minutes at 65°C, clean spores failed to demonstrate uptake of oxygen in the presence or absence of glucose. However, when the heat shocking period was extended, active endogenous respiration and exogenous glucose oxidation were observed. The results indicated that 60 min. were required for optimal activation. An analysis of the reaction showed that for each mole of glucose oxidized 1.1 moles of CO_2 were formed and 1.4 moles of oxygen utilized. The rate of oxygen uptake was not increased when the

heat activation period was extended for longer than 90 minutes. Murty reported that the activation of glucose oxidation in the same strain of spores as used here required considerably longer time at 65°C. This difference in their work and ours may be due to difference in age and the method of preparation of the spores.

In order to further characterize the extent of the enzyme-activated system, the oxidative capacity of the activated spore was examined with other carbohydrates. The results indicated that gluconate, 2-ketogluconate, and pyruvate were oxidized by the heat-activated spore, in addition to glucose. However, hexosediphosphate, fructose-6-phosphate, maltose, ribose, arabinose, lactose, and fructose were not oxidized. As will be detailed by Dr. Harlyn Halvorson, these results are consistent with the demonstration in spore extracts of a non-phosphorylated and a phosphorylated shunt pathway and an active tricarboxylic acid cycle for glucose metabolism.

The oxidative capacity of heat-activated spores towards glucose remains optimal for at least 2 hours after activation. However, the activation was transient and steadily diminished during prolonged storage at 5°C. Following 24 hour storage after heat-activation, the spore retained only 15% of its glucose-oxidizing capacity. However, the results indicated that the glucose-oxidizing capacity, which was largely lost during 24 hour storage of heat-activated spores, was fully restored by a second heat shock treatment. When the originally heat-activated spores were stored for periods longer than 24 hours the extent of reactivation decreased. After one week of storage at 5°C the glucose oxidation was irreversibly lost. The ultimately irreversible loss in heat-activated spores may be due to a slow catabolism of some endogenous component required for metabolic activity. Some of the previous difficulty in demonstrating glucose oxidation may thus be related to a prior heat-shock treatment followed by a prolonged storage period.

The level of glucose oxidation by a spore preparation was also influenced by the age of the spores. Freshly harvested, heat-activated spores had a negligible endogenous respiration and a Q_{O_2} (N) of 6 on glucose. The optimal endogenous (Q_{O_2}(N)=5) and glucose oxidation (Q_{O_2}(N)=30) was found in the same spore preparation which had been aged for at least 4 months at -20°C after harvesting. Storage up to 4 years did not increase the respiratory level of the spore preparation. In both fresh and aged spores, heat activation was required for respiratory activity.

In spores of *B. cereus* var. *terminalis* glucose acted both as a substrate for oxidation and as an agent for germination. The ability of glucose to act under certain conditions as a substrate but not as a germinating agent was shown when glucose was initially added at concentrations of less than 10^{-3} M. However, when this concentration of glucose was added after a pro-

longed period of endogenous metabolism, a slow germination paralleled the oxidation of glucose. The lowering of the glucose concentration requirement for germination was probably due to a metabolic contribution to germination from the endogenous reserves. However, at higher concentrations glucose acted both as a substrate for oxidation and as a germinating agent.

The glucose oxidation results indicated that this system was clearly dormant in the intact spore and required heat-activation for its expression. More direct evidence for the existence of the glucose-oxidizing system in the intact spores was the demonstration of glucose oxidation in DPN supplemented extracts of spores. This raised the question: did rupturing the spore completely remove the dormancy of the glucose-oxidizing system? Thus a comparison was made of glucose oxidation by spore extracts prepared from unheated dormant and heat-activated spores either by sonic disintegration or by grinding. It was clear that only part of the activity was observed in extracts of dormant spores. Heat activation prior to rupture increased the activity in subsequent extracts by 250%. This difference could not be attributed to a difference in rupture of the spores. Thus the activity observed in extracts of unheated spores must be in a dormant state in intact spores; otherwise its activity would be detected by the procedures we used. Physical disruption thus liberated a part of the respiratory system.

We have seen previously that the age of the spore prior to activation also influenced the glucose-oxidizing capacity. The increase in this capacity during aging could be caused by either a change in the mechanism controlling dormany, or by a quantitative increase in the activity of some component of the system itself. An examination was made of the activity of extracts of heat-activated spores of various ages. Extracts of freshly harvested spores supplemented with DPN were as active as those of aged spores. Thus the reason that heat-activated freshly prepared spores did not oxidize glucose was probably not a modification in the dormancy-regulating mechanism. On the other hand, it seemed probable that aging led to a quantitative increase in activity in the system either through loss of an inhibitor and/or production of some cofactor such as DPN. The observations of Swartz *et al* (1956) on heat-activated enzyme systems in bacteria may represent an example of the former.

A comparison of glucose oxidation of germinated spores and non-germinated but heat-activated spore preparations was made. The experiment was undertaken to establish whether heat-activation in the absence of germination brought about any quantitative alteration in the enzyme system. The results indicated that germination only slightly increased the rate of glucose oxidation over that of the heat-activated spores. At the same time it

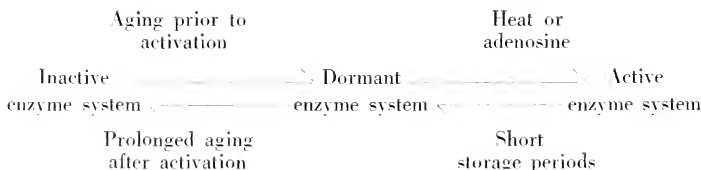
was of interest to note that the endogenous respiration of spore reserves was higher in heat-activated than in germinated spores.

We have previously shown that spores can be heat-activated to oxidize glucose without germinating. These findings suggested that in the alanine-adenosine germinating system activation of glucose oxidation preceded germination. Previous studies by us demonstrated that unheated spores of *B. cereus* var. *terminalis* would not germinate in the presence of adenosine and low concentrations of glucose. The ability of unheated spores in such a mixture to activate glucose oxidation in the absence of germination was observed and reported earlier (Church and Halvorson, 1956). If either higher concentrations of glucose were added or a heat-activation applied after 95-minutes of incubation, a rapid germination was observed. The rate of this oxidation is again related to the age of the spore.

The biochemical basis of the process of germination is ultimately restricted to the enzymatic constitution of the dormant spore. An understanding of the state of dormancy and its loss during germination requires knowledge not only of the enzymatic pattern of the spore but also of the factors controlling enzymatic activity *in vivo*.

The spore enzymes described thus far fall into two groups: one whose activity is recognizable in the intact dormant spore, and the other requiring rupture of the spore. Racemase, adenosinedeaminase, ribosidase, and a heat resistant catalase are examples of the former; pyrophosphatase, glutamic-aspartic transaminase, and heat sensitive catalase belong to the latter group. The glucose-oxidizing system described in our work and in the work of Murrell (1955), and Murty and Halvorson (1956) falls into still another class of dormant enzymes whose activity *in vivo* requires appropriate activation. Since activation precedes germination, a study of the activation process itself should provide a useful approach to the overall problem of germination.

The properties of the glucose-oxidizing system in intact spores described here can be diagrammatically represented in the following manner:



Thus the enzyme system may be either inactive, dormant, or active in the intact spore. The dormant enzyme system was that glucose-oxidizing ca-

pacity which was seen in intact spores following proper activation. The inactive enzyme system was that whose activity was only recognized in disrupted spore preparations. This condition was found in freshly harvested spores and in aged, heat-activated spores which had undergone prolonged storage.

The mechanism of glucose oxidation by spores of *B. cereus* var. *terminalis* largely followed a non-phosphorylated shunt pathway, being initiated by a DPN-linked glucose dehydrogenase. A deficiency in TPN or some other part in the electron transport system, either through metabolic loss or poisoning, would readily produce the inactive respiratory state observed. Synthesis of these cofactors or a destruction of an inhibitor during aging of the freshly harvested spores would elevate the glucose oxidizing capacity of the intact spore.

The conversion of the dormant enzyme system to an active system presents a different problem. In the case of glucose oxidation, activation was achieved by either prolonged heat treatment or by adenosine under conditions precluding germination. Since both of these are stimulatory to the germination of this strain (Stewart and Halvorson, 1953), the reactions involving heat or adenosine activation of glucose oxidation may be common to those reactions responsible for germination. It is not unlikely that other germinating agents, such as alanine, may also serve as activating agents for dormant enzymes.

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Informal Discussion

LAWRENCE: I have obtained some data which may bear on the possible relationship of dipicolinic acid to heat resistance, as mentioned by Dr. Murty.

Following Dr. Foster's work, I extracted some *B. terminalis* spores with 1 *N* sulfuric acid for 17 hours at room temperature. Assay of the resulting supernatant fluid showed that some ninhydrin positive material had been removed, as well as dipicolinic acid corresponding to 10% of the original dry weight of the spores.

This treatment killed the spores; that is, they would not now form colonies nor germinate with L-alanine and adenosine using the optical procedure. However, the treated spores retained 50% of their initial adenosine ribosidase activity. This remaining activity was no longer resistant to heat; after 30 minutes at 100°C the activity decreased from a Q of 5 to a Q of 0.6 micrograms ribose produced per mg spores per minute. This should be compared to the heat resistance of the enzyme in untreated spores, which retains nearly full activity after 30 minutes at 100°C.

Two things had been done to the spores by the acid treatment: practically all of the dipicolinic acid was removed, and heat resistance of the adenosine ribosidase was eliminated.

HARLYN HALVORSON: The rapidity and specificity of the germination requirements suggest a trigger mechanism for the breaking of dormancy. As has been previously indicated, germination is associated with a burst of enzymatic activity from DPN- and TPN-linked dehydrogenases as well as ATP-linked kinases and probably ADP-linked energy yielding reactions. One attractive regulatory mechanism to control their activities could be the following: If these cofactors were entirely present in the dormant spore in the form of DPNH, TPNH and ATP, respiratory activity could be suppressed due to a lack of H acceptors for the dehydrogenases and to a deficiency in phosphate acceptors (ADP). Heat activation or the proper addition of germination requirements may enable the oxidation of DPNH and TPNH and the hydrolysis of ATP. I wonder if Dr. Powell would like to comment on this.

SUSSMAN: In response to the points raised by Dr. Halvorson, I'd like to say that the dormant ascospores of that ubiquitous and fascinating organism, *Neurospora,* contain more ATP than any fungal cell of my acquaintance. On the other hand, as soon as the spores are activated there is a precipitous decline in ATP accompanied by an approximately stoichiometric rise in ADP concentration. This is interesting in view of what Dr. Halvorson said about a compound associated with energy transactions being "plugged in," as it were, into the manifold jobs of the cell at the time when it is needed most. There seems to be definite selective advantage in an arrangement like this that mobilizes such substances for use when dormancy is broken. The second point I should like to make is concerned with the very interesting series of four papers we are discussing. It looks more and

more like there is a surface phenomenon, or a phenomenon connected with the cell surface, which may be a primary reaction in activation. I should like to discuss an observation made with *Neurospora* by Dr. David Goddard who suggested that the key activating step may be attributable to the presence or absence of the enzyme pyruvic carboxylase. In our work we discovered that if we made grindates of the dormant cells, we got thorough decarboxylation of pyruvate: and almost as much of the enzyme is found in extracts of dormant spores as those from activated cells. However, if one added the pyruvate, as Goddard did, to the intact spore, no decarboxylation occurred. The resolution of these disparate observations seems to me to be an experiment in which we showed that lysozyme added to the dormant spore "activated" the pyruvate carboxylase to the extent that pyruvate was not decarboxylated by the dormant cells. The analogy that I would like to draw is that here we might have the stripping off of a masking substance or layer from an enzyme associated with activated spores, and a concomitant activation of an enzyme which may be required for germination.

Non-Oxidative Enzymes of Spore Extracts

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Many of the non-oxidative enzymes have already been mentioned, so I shall limit my discussion to those non-oxidative enzymes of spore extracts with which I have had recent personal experience. This limitation, you will discover, is very confining.

It is not unlikely that some of these enzymes may actually exist in an inactive condition within the spore, and that the process of grinding, etc., involved in preparation of extracts entirely distorts the true picture of the state of affairs within the spore. This warning should temper considerably your gullibility in accepting any theory which I may propound. Furthermore, you must realize that the evidence for some of these theories is far from overwhelming. Indeed, I am most grateful for this opportunity to speak more or less irresponsibly on a subject over which I have been mulling for some time. I have a great many slides but since each will appear only briefly, I trust you will bear with them.

In spite of the low metabolic activity of the spores, we felt rather early in our work that spores, as living things, must have some enzymes. One of the first enzymes with which we worked was the glutamic-aspartic transaminase of *Bacillus megaterium* spore extracts (Levinson and Sevag, 1954a). This enzyme (catalyzing the reaction: α-ketoglutarate + aspartate $\rightleftharpoons$ glutamate + oxalacetate) was estimated by the measurement of oxalacetate production (Fig. 1). Briefly, the main finding was that, on a mg protein N basis, the glutamic-aspartic transaminase was as active in extracts of spores as in vegetative cell extracts of *B. megaterium.*

This finding—that is, the mere presence of enzyme in the spores—encouraged us to go on. However, before proceeding with the story, I must backtrack somewhat. Some of you may recall that we had shown a stimulation of germination and respiration of *B. megaterium* spores by manganous ion (Fig. 2). Incidentally, this figure gives an indication of the close relationship between germination, respiration, and turbidity of spore suspensions. Cobalt and zinc also had a stimulating effect on germination and respiration, but other cations, such as Mg^{++}, Ca^{++}, Cu^{++}, and Fe^{++} did not (Levinson and Sevag, 1953).

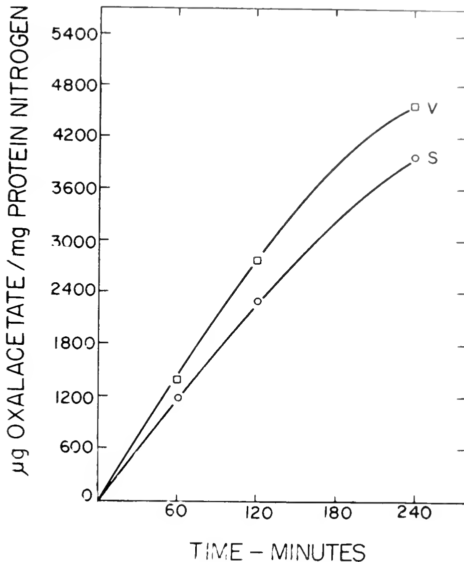


Fig. 1. Oxalacetate produced from α-ketoglutarate and aspartate via glutamic-aspartic transaminase of extracts of spores and vegetative cells of *B. megaterium* (Levinson and Sevag, 1954a).

We have had an idea that the effect of manganese is to activate an enzyme, which, within the spore, can produce substances useful to the spore in its germination. We have obtained certain indirect evidence supporting this point of view, which, by the way, is contradictory to the ideas expressed by Pulvertaft and Haynes (1951), who postulated the necessity for a specific excitant for spore germination. An extract of ground spores is stimulatory to germination of intact spores. The dialyzed extract is relatively inactive, but the material which passes through the dialysis bag is somewhat more active than was the original extract (Table I). In line with our theory, but not excluding other possibilities, we have postulated that this phenomenon may be due to continued production of the hypothesized essential principle during the course of the dialysis.

Spores will, as we all know, germinate in the absence of added manganese. However, it was determined by spectrographic techniques (Cohen and Wiener, 1954) that our spores, grown in the liver fraction medium suggested by Foster and Heiligman (1949), contained from 50 to 100 ppm of

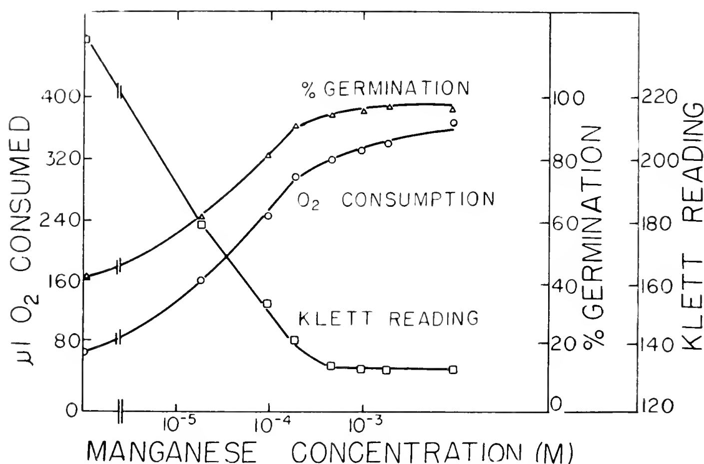


Fig. 2. The effect of manganese concentration on germination, oxygen uptake, and turbidity of spore suspensions. Indicated Klett readings represent turbidities. Spores were not heated. Manganese was as $MnSO_4$. Medium was composed of ammonium and potassium acetates, pH 6.6, 0.05M each; and glucose, 0.025M. Reaction period was 140 min. at 30°C (Levinson and Sevag, 1953).

manganese—an amount perhaps sufficient for activation of an enzyme necessary for the production of substances required for spore germination.

The list of enzymes activated by manganese (Table II) is incomplete and varied, and appears at first glance to be quite hopeless because of its very length and diversity. We thought it significant, however, that cobalt and zinc, which are peptidase activators, also stimulate *B. megaterium* spore germination. It may also be of significance that some of the proteolytic en-

TABLE I

Effect of spore extract on oxygen uptake of intact spores

Extract fraction	*O_2 uptake (μl)*
Whole extract	45
Dialyzable	105
Non-dialyzable	22
No extract	9

3.0 mg spores. Glucose 0.025M, 120 minutes incubation, 30°C. Potassium and ammonium acetates 0.15M, pH 6.8.

TABLE II

Some biological systems in which manganese is involved

System	Reference
1. Utilization of an enzyme precursor to benzoic acid oxidation.	Bernheim, 1954
2. Bacteriophage activity.	Rountree, 1955
3. Xylose isomerase of *Pasteurella pestis*.	Slein, 1955
4. Aldolase of *Brucella suis*.	Gary *et al*, 1955
5. Glutamine synthesis by *Micrococcus pyogenes*.	Fry, 1955
6. Activation of reproduction, sugar consumption, and respiration of *Saccharomyces cerevisiae*.	Lima, 1954
7. Enolase activation.	Malmström, 1953
8. Incorporation of formate into serine by pigeon liver extracts.	Sakami, 1955
9. Cysteine desulfurase of *Escherichia coli*.	Binkley, 1943
10. β-aspartokinase activation.	Black and Wright, 1955
11. Peptidase activation.	Smith, 1951

zymes are most active at elevated temperatures, and this may have something to do with the phenomenon of heat activation.

Hills (1949, 1950) has reported that L-alanine, a possible hydrolytic product of peptidases, is essential for the germination of *B. anthracis* and *B. subtilis* spores, and we have found, as have many others, that the germination of *B. megaterium* spores is much enhanced with L-alanine.

Another point in regard to the enhancement of germination by L-alanine is that spores immersed in L-alanine solution reach their maximal degree of germination practically instantaneously. When manganese is the stimulant, germination increases with the time of incubation (i.e., there is a definite lag). For example, a spore suspension which has germinated 60 per cent after 2 hours in manganese, or in L-alanine, will have reached this percentage within five to ten minutes in L-alanine, but it may take as long as 1.5 hours for this percentage of germination to be reached with manganese. This seems to indicate a more direct action of L-alanine than of manganese in germination.

Specifically, I believe that the spore creates its own stimulus for germination (providing a source of energy is available). No externally supplied excitant is needed. We have proposed that manganese acts, at least in part,

REACTION MIXTURE	G+X			G+X+Mn			G	X	AX	X+Mn	G+AX	G+AX+Mn
DAYS	1	2	3	1	2	3	3					

Fig. 3. Tracing of paper partition chromatogram showing hydrolysis of gelatin by spore extract. G = gelatin (1.0 per cent); X = spore extract (equivalent to 2.7 mg ground spores per ml reaction system); AX = autoclaved spore extract; Mn = manganese (10 ppm) as manganous sulfate. Mixtures chromatogrammed after 1, 2, and 3 days of incubation at 30°C (Levinson and Sevag, 1954b).

to activate protease or peptidase of the spores, which acting on spore material can produce amino acids or simple peptides which in turn stimulate the spore to germination. The site of action of these amino acids is another related problem which I am not prepared to discuss just now, but perhaps the L-alanine acts as Dr. Murty (this Symposium) has indicated.

We have some evidence that the spores of *B. megaterium* do contain proteolytic enzymes (Levinson and Sevag, 1954b). Using 1.0 per cent solutions of dialyzed gelatin and egg albumin as substrates, we have demonstrated the presence of proteolytic enzymes in spore extracts by several methods.

(1) *Paper partition chromatography for the detection of amino acids.* With gelatin as the substrate (Fig. 3), amino acids resulting from protein hydrolysis were observable after incubation of the protein with spore extract. The colors were much more intense when manganese was used, and in addition, the liberation of the amino acids from gelatin was accelerated with manganese. Much the same general picture is seen with egg albumin as the substrate (Fig. 4). Without manganese, there was no hydrolysis in

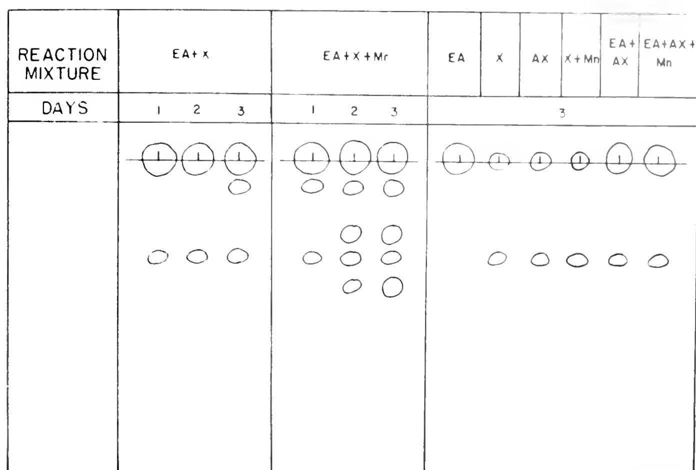


Fig. 4. Tracing of paper partition chromatogram showing hydrolysis of egg albumin by spore extract. EA = egg albumin (1.0 per cent): other conditions as for Fig. 3.

two days; but with manganese, definite signs of liberation of amino acids were observable in this time.

(2) *A colorimetric method based on the reaction of free amino groups with ninhydrin.* The proteinaceous substrate-enzyme mixture was incubated, treated with ninhydrin, and the resulting color was read in a Klett colorimeter. By this method, too, a breakdown of the protein substrate is evident, and manganese is decidedly stimulatory to this breakdown (Fig. 5). The question arises here as to whether manganese is an artifact. That is, is it possi-

Table III

Effect of manganese on ninhydrin reaction

"Amino Acid"	Manganese	Klett*
Sodium Glutamate	+	188
	—	185
Bacto Peptone	+	22
	—	25
DL-Alanine	+	104
	—	112

* The Klett reading indicates the intensity of the ninhydrin color.

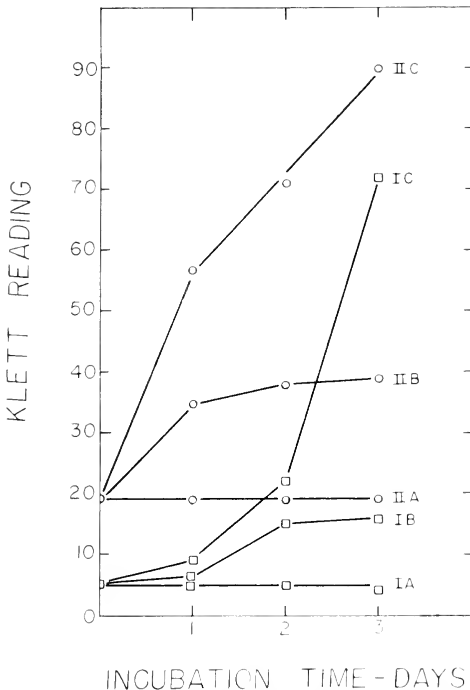


Fig. 5. Colorimetric estimation of proteolysis by spore extract. Curves labelled I represent data with gelatin as substrate. Curves labelled II represent data with egg albumin. A = autoclaved spore extract; B = spore extract; C = spore extract and manganese. Spore extract equivalent to 2.7 mg ground spores per ml reaction system. Incubation at 30°C (Levinson and Sevag, 1954b).

ble that the addition of manganese to *any* peptide or amino acid mixture might increase the color developed with ninhydrin? Such does not appear to be the case (Table III).

(3) *Viscosimetric method.* Changes in the viscosity of gelatin also give indication of proteolytic activity. All such experiments indicated that the rate of loss of viscosity with manganese was about 1.5 times that without Mn^{++} (Fig. 6).

Other pertinent information developed from the use of the uncentrifuged ground spores or homogenates. Incubation of the homogenates without the

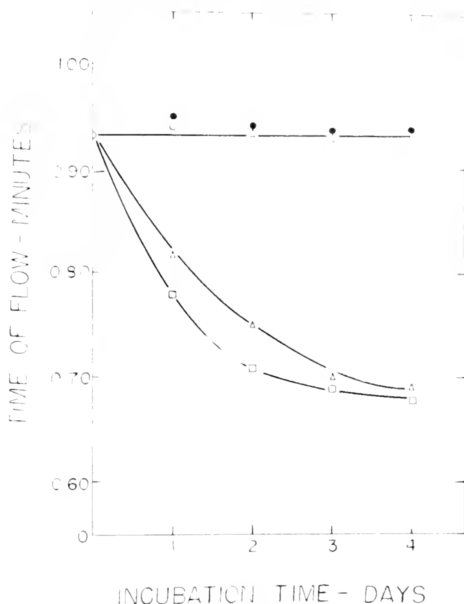


Fig. 6. Viscosimetric demonstration of gelatin hydrolysis by spore extracts. Open circles, gelatin and autoclaved extract; triangles, gelatin and spore extract; squares, gelatin and spore extract with manganese. Incubation at 30°C.

addition of other protein, resulted in an increase in the intensity of the ninhydrin color (Fig. 7). Not only that, but manganese accelerated and increased the proteolysis. This seems to us to be highly significant, since it could be evidence that the spore material itself can serve as substrate for the proteolytic enzymes of the spores. You may recall that in the proteolysis of gelatin and egg albumin by spore extract (Fig. 5) the incubation time was in days, and the Klett reading reached 90 in three days. In the case of these spore homogenates, however, the incubation period is in hours and the Klett readings go much higher. We attribute this faster proteolysis to the substrate's being, in this case, homologous for the enzymes concerned, thus permitting the breakdown to proceed much faster and to a greater extent than did the breakdown of the heterologous egg albumin and gelatin substrates.

As I have previously shown, spore extracts contain substances which

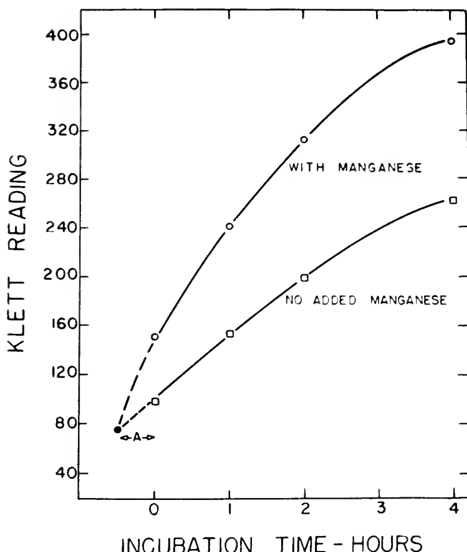


Fig. 7. Proteolysis in spore homogenates. Each ml of homogenate was derived from 30 mg ground spores. Ninhydrin color of homogenate developed after centrifugation following various times of incubation at 37°C. The closed circle indicates the estimated time of addition of manganous sulfate to one of the aliquots, and the distance, A, represents the time required for centrifuging. The dashed portions of the curves are, therefore, somewhat approximate. The magnitude of the Klett readings in comparison to those of Fig. 5 is due partly to the greater amount of spore material used, with consequently greater amounts of enzyme and substrate, but in greater measure, perhaps, to the homologous nature of the substrate (Levinson and Sevag, 1954b).

stimulate the germination of intact spores (Table I). These stimulating substances are dialyzable, as are amino acids. Chromatograms show the presence of several amino acids in the extracts—alanine and glutamic acid being the most noticeable. If we measure oxygen consumption as a criterion of germination, we find (Fig. 8) that we get similar curves of activity as a function of concentration when we use dialyzate of extract as when we use L-alanine. The stimulation obtained from 100 μg of extract solids was roughly equivalent to that obtained from 10 μg of L-alanine. Actually our extracts contain only about 5 μg L-alanine per 100 μg of extract solids. Thus, it is probable that the entire story of manganese stimulation does

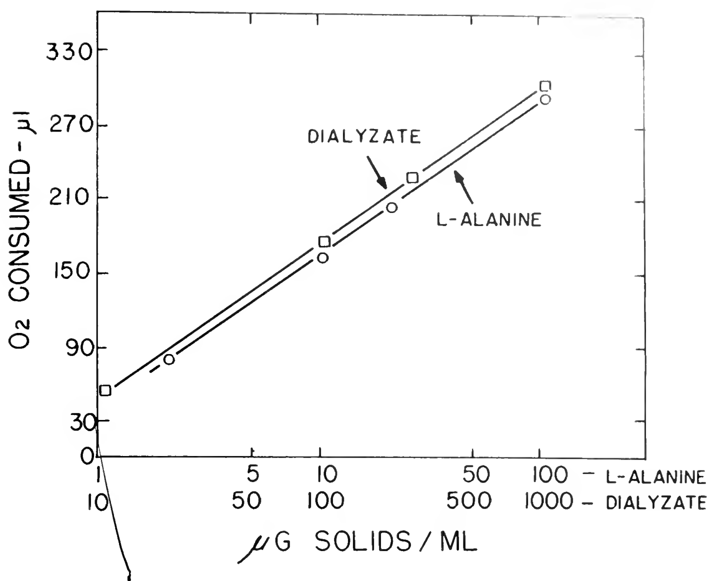


Fig. 8. The influence of L-alanine and of dialyzate of spore extract on oxygen uptake of intact spores. Oxygen uptake after 150 min. at 30°C in a medium containing ammonium and potassium acetates, 0.05M each, pH 6.8, and glucose, 0.025M.

not lie in the production of L-alanine. Indeed, we are sure of this on the basis of other experiments using D-alanine as an inhibitor of L-alanine activity (Levinson and Hyatt, 1955). We do feel, however, that these experiments gave us some insight into the problems involved in spore germination.

If the stimulation of germination of spores by spore extracts is due in appreciable part to the products of hydrolysis of spore protein, then one would expect more stimulation from extracts derived from incubated homogenates than from extracts made immediately after the preparation of the homogenate. Fig. 9 shows that this is the case.

Thus:

1. Spore extracts exert proteolytic activity.
2. Spore material can, under certain conditions, act as substrate for this activity.

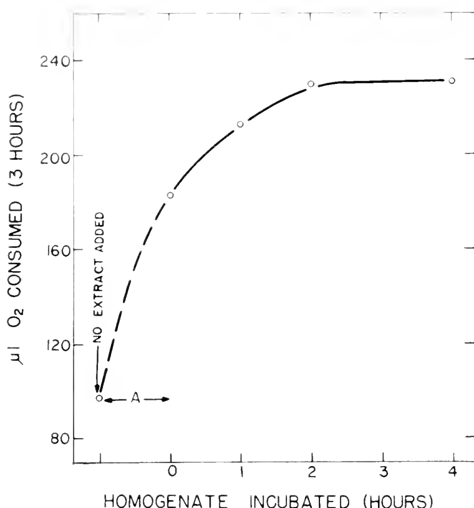


Fig. 9. Influence of time of incubation of homogenates on stimulation of oxygen consumption by extracts derived from them. Extracts prepared by centrifuging homogenates which had been incubated at 37°C for various periods of time. Reaction systems for oxygen consumption measurements contained ammonium and potassium acetates (pH 6.8, 0.05M each): glucose (0.025M): intact spores (3.0 mg): and extract equivalent to that derived from 2.5 mg spores.

3. Products of this hydrolysis of spore protein—L-alanine, for example—are capable of marked stimulation of spore germination.
4. Manganese stimulates both proteolysis and spore germination.

More recently, and none of this is published, we have been working with the enzyme pyrophosphatase in extracts of the spores of *B. megaterium*. As we test it, the enzyme catalyses the hydrolysis of sodium pyrophosphate to give orthophosphate:

$$Na_4P_2O_7 + H_2O \rightarrow 2\ Na_2HPO_4$$

There is not really much that I can say about the reaction. Its main interest to me lies in the observation (Fig. 10) that the activity of the enzyme is dependent on manganese. Without the addition of manganous ion we find either no activity or an extremely low activity, but with 5×10^{-4}M manganese we find nearly maximal activity. Cobalt and zinc also show a small amount of activation. The pH optimum (Fig. 11) for this manganese-activated pyro-

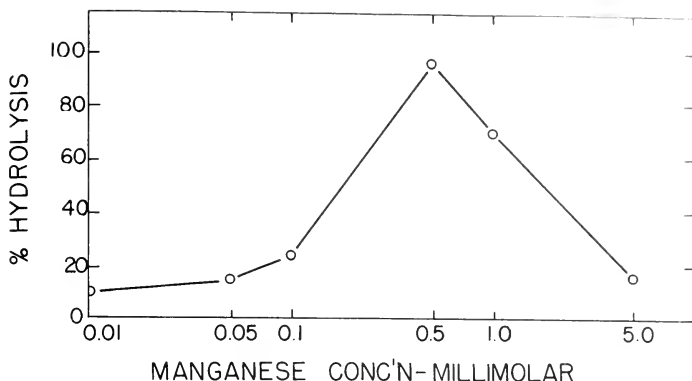


Fig. 10. The effect of manganous sulfate on pyrophosphatase activity of *B. megaterium* spore extract. Reaction systems contained 0.25 ml. of spore extract prepared in Mickle Disintegrator (25 mg spores per ml extract): 0.05 ml veronal buffer, 0.05M; 0.25 ml $Na_4P_2O_7$ (final concentration, 0.001M); and 1.0 ml of manganous sulfate. Reaction stopped by addition of 0.2 ml 100% tricholoroacetic acid after incubation for 30 min. at 37° C.

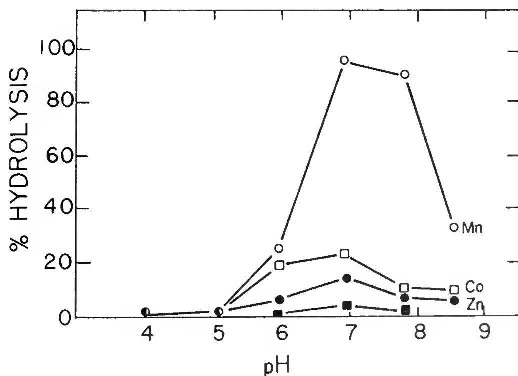
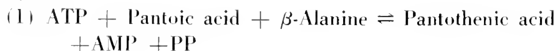


Fig. 11. pH-activity curve for spore extract pyrophosphatase. Conditions as for Fig. 10, except that acetate buffer (0.05M) was used at pH 4, 5, and 6. Divalent metal ions, as sulfates, in a final concentration of 5 x 10^{-4} M.

phosphatase is near neutrality, setting it off from the cobalt-activated acid pyrophosphatase and the magnesium-activated alkaline pyrophosphatase extracted from *Streptococcus faecalis* (Oginsky and Rumbaugh, 1955), both from the standpoint of the metal requirement and the pH optimum.

We have gotten all sorts of relationships in substrate concentration, enzyme concentration, etc., but these are not really significant now. I should like, though, to indulge in some wild conjectures as to the means by which this enzyme could be involved in spore germination.

This involves two important reactions. The first reaction is concerned with the synthesis of coenzyme A, and the second is concerned with transacetylation.



(1) ATP + Pantoic acid + β-Alanine $\rightleftharpoons$ Pantothenic acid + AMP + PP

(2) Ad. P$\sim$PP + CoASH + Acetate $\rightleftharpoons$ Ad. P + CoAS$\sim$Ac + PP

Inorganic pyrophosphate is said by Dr. Fritz Lipmann (1954) to be a product in both reactions. In the presence of pyrophosphatase, the reactions will be forced to the right, due to the disappearance of the pyrophosphate, and the production of CoA and of acetyl CoA will be increased. These increases might result in more rapid utilization of glucose, with increased oxygen uptake. If these reactions can be shown to exist in spores, or in germinating spores, we may have a relationship between the requirements for manganese in spore germination and in pyrophosphatase activation. The possibility exists, and this is totally unsupported, that manganese acts as an activator of proteolytic enzymes resulting in the production of β-alanine, or of L-alanine convertible to β-alanine. This would fill a requirement for the production of coenzyme A. In addition, manganese would serve as a pyrophosphatase activator keeping the equilibrium of these reactions to the right. It is well known that the products of an enzymatic reaction can inhibit the specific enzyme involved in the reaction. Thus, α-amylase is strongly inhibited by α-maltose; fructose and glucose markedly inhibit invertase; cellulase is inhibited by cellobiose. Since inorganic phosphate is a product of the enzymatic hydrolysis of pyrophosphate, we might expect phosphate to inhibit pyrophosphatase. This indicates where we might get a glimmer of understanding of the oft-reported inhibition of germination by inorganic phosphate, i.e. through inhibition of pyrophosphatase. There is a suggestion of the actual involvement of pyrophosphatase in germination (Fig. 12), since after spores have germinated (i.e. become stainable), there is a great reduction in the amount of demonstrable pyrophosphatase. Extract of resting spores produces 1,350 μg of orthophosphate P per mg protein N in 10 minutes. Germinated spore extract produces 220 μg orthophosphate P in the same time, or about 16% as much as resting spore extract. It may

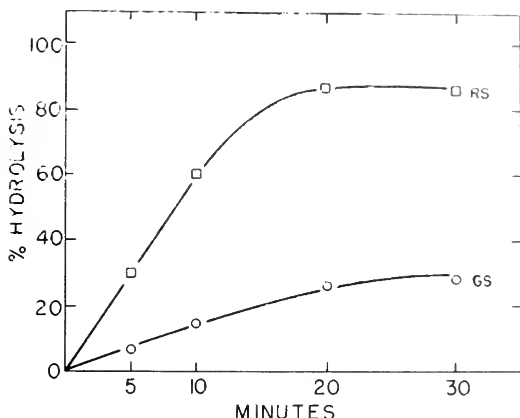


Fig. 12. Pyrophosphatase of extracts of resting spores (RS) and of germinated spores (GS). Spores ground in Mickle Disintegrator. RS ground after heating at 50°C for 15 min. GS prepared by incubation of RS with 0.025 M glucose for 1.5 hr. at 30°C (81% germination) followed by grinding. All mixtures contained 0.25 ml extract, 5 x 10^{-4}M manganous sulfate, 10^{-3} M $Na_4P_2O_7$, and veronal buffer at pH 7.0 in a total volume of 2.0 ml. Reaction stopped at indicated time by addition of 0.2 ml 100% trichloroacetic acid. 100% hydrolysis is calculated to yield 124.1 μg P.

be of significance that the extract of the so-called germinated spores was actually derived from spores which had germinated 81%, or to put it another way, 19% of whose spores had not germinated. Could this signify that only the resting spores had appreciable amounts of pyrophosphatase, and that the small amount of activity evidenced by the extracts of germinated spores was due to residual resting spores? This appears to be quite an unusual situation, and one which I believe is worth further investigation.

The spore coats of *B. megaterium* are rich in phosphorus which is acid and alkali insoluble. Fitz-James (1955) estimates that this P fraction is about 60% of the total P of *B. megaterium* spores. It is possible that this insoluble residue forms a lattice work making the spore coat impermeable to nutrients. The breakdown, or partial breakdown, of this lattice work through the mediation of the manganese-activated pyrophosphatase would permit nutrients to enter the spore and to participate in the biochemical events necessary for germination of the spore.

I realize that I have neglected some important non-oxidative enzymes. Perhaps Dr. Krask will touch on these. I hope that when the time for gen-

eral discussion arrives we'll have comments on possible implications of the pyrophosphatase.

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Discussion

Bernard J. Krask

Dr. Levinson has reported a glutamo-asparto transaminase and has also suggested an important role for Mn^{++} in relation to protease and pyrophosphatase activity of spores. Current studies in our laboratory on enzymes from resting spores of *B. cereus* var. *terminalis* amplify the apparent importance of glutamic acid and Mn^{++} to sporulation and germination. Three of the enzymes under study bear some relationship to Dr. Levinson's proposals while the fourth is of interest because of its stimulation by adenine nucleotides and adenosine and its utilization of a substrate present in the spore itself. The paucity of enzymes discovered in spores as compared to those recognized in the vegetative cell, however, makes the particular task of separating and relating these few spore enzymes to the processes of vegetation, sporulation and germination a difficult one. Further, an enzyme found in a spore may not be specifically related to sporulation or germination—it is possible that an enzyme may be transferred from vegetative cell to vegetative cell through the spore without any relation to sporulation or germination proper. The suggested relationships of the following enzymes to either sporulation or germination must therefore be speculative and await further study to prove their specific roles in these processes.

(1) *Glutamine synthetase (GS)*

$$\text{Glutamic acid} + NH_3 + ATP \overset{Mg}{\rightleftharpoons} \text{Glutamine} + ADP + P_i$$

The amidation of glutamic acid to glutamine through glutamine synthetase occurs in spore homogenates, spore-free extracts, and vegetative cells of *B. cereus.* The enzyme was studied by substituting hydroxylamine for NH_3 and determining glutamine as glutamohydroxamic acid (GHA). The spore enzyme has a pH optimum of 7.2 and requires Mg^{++} and ATP. Mn^{++} could not be tested satisfactorily since precipitates form at low Mn^{++} concentrations in homogenate preparations. Mn^{++} does, however, satisfy the metal ion requirement of GS from other bacterial sources (Fry, 1954).

This enzyme is of particular interest because early studies in our laboratory on the sporulation of *B. subtilis* in a simple glucose-glutamic acid-salts medium led to the postulate that this reaction was involved in sporulation (Krask, 1953). Sporulation of *B. subtilis* was dependent on the glutamic acid concentration. The inclusion of methionine sulfoxide, a known inhibitor of the conversion of glutamic acid to glutamine, resulted in an inhibition of sporulation without affecting growth. These results were the stimulus for an investigation of enzymes metabolizing glutamic acid.

(2) *Glutamotransferase (GTF)*

$$\text{Glutamine} + NH_2OH \overset{\text{Mn, ATP}}{\rightleftharpoons} \text{Glutamohydroxamic acid (GHA)}$$

The verification of the synthesase reaction in spores and cells indicated that glutamine might be related to spore formation. Of the many reactions involving glutamine, one in particular—catalysis by glutamotransferase—appeared attractive in terms of a relationship to synthetic processes. Glutamotransferase catalyzes the enzymatic exchange of the amide group of glutamine with hydroxylamine to form glutamohydroxamic acid (Waelsch *et al*, 1950; Grossowicz *et al.* 1950). The reaction may be considered as the transfer of the gamma-glutamyl radical from glutamine to an acceptor hydroxylamine. Waelsch and his coworkers ascribe particular importance to this reaction in peptide synthesis. They suggest that the reaction of glutamine with hydroxylamine is only a model for a biologically significant reaction in which the gamma-glutamyl radical combines with amino acids to form gamma-glutamyl peptides.

This enzyme occurs in spore homogenates and extracts and in the vegetative cells. The spore enzyme has a pH optimum of 7.4-7.45 and is almost completely inactive at pH 6.0 and 9.0. In contrast to GTF from other sources, spore GTF is satisfied by Mn^{++} and ATP only. Mg^{++} and Co^{++} are inactive as is ADP and AMP. It is of interest that Williams and Thorne (1954) and Williams *et al* (1955) found a type of GTF in exoenzyme preparations of *B. subtilis* cells which catalyzed transamidation reactions between glutamine and amino acids and transpeptidation reactions between gamma-glutamylglutamic acid to form gamma-glutamyl peptides of greater chain

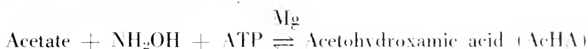
length. The latter activities occurred at pH 8.8 while that of specific GTF activity occurred at pH 6.5.

(3) *(X)—Transferase (XTF)*

$$(X) + NH_2OH \overset{\text{Adenosine}}{\rightleftharpoons} (X)\text{- Hydroxamic acid (XHA)} + NH_3$$

In our course of study on GTF it was noted that a reaction occurred with hydroxylamine in the absence of glutamine. This unidentified transferase (XTF) occurs only in the spore homogenate or debris and reacts with a bound non-dialyzable substrate in the spore. Further, the reaction required no metal ions and was stimulated by ATP, ADP, AMP and adenosine—optimal stimulation occurred with adenosine. ATP was the least effective of the nucleotides while adenine was completely inactive. The pH range of the enzyme is broad with an apparent acid optimum. The stimulation by adenosine and activity with a substrate proper to the spore itself suggests this enzyme is functional in germination.

(4) *Acetokinase-like enzyme*



$$\text{Acetate} + NH_2OH + ATP \overset{Mg}{\rightleftharpoons} \text{Acetohydroxamic acid (AcHA)}$$

The fourth enzyme studied has been tentatively called an acetokinase-like enzyme but is in all probability an aceto-CoA-kinase as described by Jones and Lipmann (1955). The reaction of acetate with hydroxylamine to form acetohydroxamic acid (AcHA) can only occur through the formation of acetyl phosphate or acetyl-CoA which then react with hydroxlamine. This reaction occurs in spore homogenates and vegetative cells and requires ATP and Mg^{++}. The reaction appears to be independent of added CoA, but in the light of Murrell's studies (1955) demonstrating CoA in spores and our results of extract studies it is probably a CoA mediated reaction.

Preliminary studies also show that the spore homogenates contain ATP-ase.

While the studies of these enzymes were concerned primarily with a characterization of their properties, the following tables demonstrate their occurrence, their relative activity, and the effect of heat shock on this activity. All comparisons are on the basis of 20 mg dry weight or its equivalent.

Table I demonstrates the occurrence and relative activities of the enzymes. The activity associated with the intact spores may be questionable since heat shock for 30 minutes had no effect, although, as will be demonstrated, heat shock prior to disruption has a marked effect on the enzymes. The activity of all the enzymes becomes manifest upon disruption for one hour. The unidentified transferase appears only in the disrupted spores and is absent from a ten hour intact vegetative cell suspension.

TABLE I

The enzymatic activities of intact spores, vegetative cells and treated spores

	System			
	GS	XTF†	GTF	AcHA
	μM GHA	μM XHA	μM GHA	μM AcHA
Intact spores	0.04	0	0.06	0.11
Intact spores HS 30*	0.03	0	0.06	0.09
Disrupted spores	4.58	1.18	4.19	1.19
Vegetative cell	12.66	0	3.18	3.82

* Minutes of heat shock at 65°C
† Activity in presence of AMP

Table II compares the activities in the extracts and debris from disrupted homogenates. It is apparent that GS activity is associated with both fractions while, as expected because of non-dialyzable, assumed bound substrate, XTF activity is associated only with the debris fraction. The total recovery of GTF activity is less than expected and suggests that other factors may be necessary for GTF activity. The loss of kinase activity upon separation of the homogenate indicates that a necessary cofactor (CoA?) may be in the extract while the enzyme is in the particulate fraction.

Table III compares the effect of heat shock prior to disruption on enzyme activity to that of unheated disrupted spores. Perhaps the most striking change occurs in the acetokinase-like activity which becomes apparent after only 15 minutes of heat shock and is markedly increased by one hour heat shock. GTF and XTF activity decrease markedly while GS activity is relatively unaffected. It is possible that XTF activity decreases not because of enzyme inactivation but because of substrate inactivation. Experiments in which spores were heat shocked for one hour and subsequently disrupted for one half-hour and one hour showed similar effects on the activities, thus indicating that the effect on the enzymes was a thermal effect in the intact spore and was not simply a result of disintegration.

These results suggest that the differential effects attained through heat shock on enzymes in the intact spore may approximate the conditions which

TABLE II

The enzymatic activities of spore extracts and particulate fractions

	System			
	GS	XTF*	GTF	AcHA
	μM GHA	μM XHA	μM GHA	μM AcHA
Disrupted spores	4.58	1.18	4.19	1.19
Spore free extract	3.41	0.03	0.89	0
Resuspended particles	1.08	0.89	0.92	0.26

* Activity in presence of AMP

TABLE III

The effect of heat shock prior to disruption upon enzymatic activity

	System			
	GS	XTF†	GTF	AcHA
	μM GHA	μM XHA	μM GHA	μM AcHA
Disrupted spores	4.58	1.19	4.19	1.19
HS 15*—disrupted	4.18	1.37	1.79	2.76
HS 60*—disrupted	3.96	0.74	1.33	18.75

* Minutes of Heat Shock at 65°C.
† Activity in presence of AMP

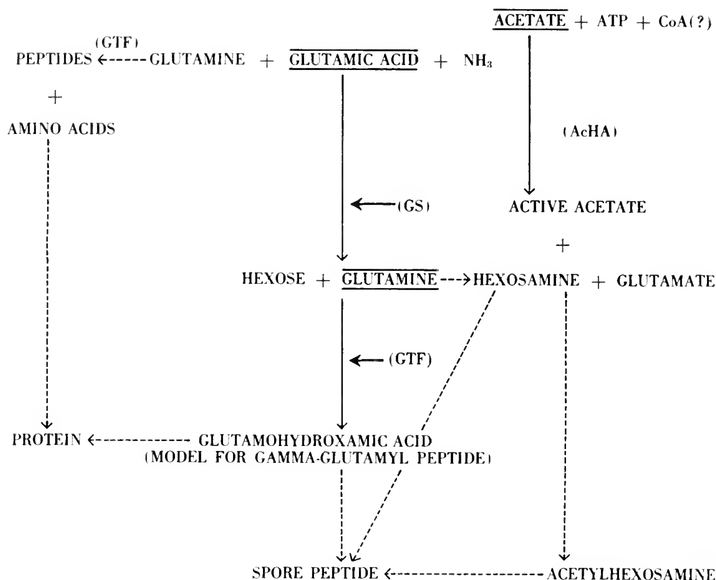
occur naturally in germination without heat shock and also may explain why heat shock appears to be stimulatory to germination. It is possible that the intact resting spore consists of a self-regulating system which may be composed of components inhibitory to germination, thus maintaining a resting state. Heat shock may remove such inhibition normally destroyed by some such proteolytic mechanisms suggested by Dr. Levinson. Recent work by Swartz *et al* (1956) on enzymes of *Proteus vulgaris* which were found in extracts after boiling for 2 minutes and which retained 50% activity after 15 minutes boiling was explained by the discovery of an inhibitor, apparently a protein, destroyed by heat. Of interest, in view of Dr. Levinson's suggestions, is the fact that one of the heat-activated enzymes was a pyrophosphatase.

The results of the heat shock experiments might also furnish a means of determining whether an enzyme is involved in germination or sporulation. Heat-lability in the intact spore could indicate a relationship to sporulation rather than germination, while heat-stability could indicate either a specific role in germination or a general role in all the cell processes. A differential activation would certainly indicate an important function in germination.

Fig. 1 represents some possible functions of these enzymes in the synthesis of a spore peptide recently studied and characterized by Strange and Powell (1954) and Strange and Dark (1956). The peptide is found in the spores of *B. cereus* and two other species and is extruded during germination. It is associated with the spore coat, and its components are glutamic acid, alanine, acetyl glucosamine, alpha-epsilon diaminopimelic acid, and an unidentified hexosamine. Powell and Strange have suggested that the enzymatic depolymeryzation or detachment of this peptide from the spore coat may be one of the first stages of germination.

It should be emphasized that the role of these enzymes in the synthesis and hydrolysis of the spore peptide is at present only speculative. Nevertheless, it is attractive in that the suggested functions relate the enzymes both to sporulation and germination.

In respect to peptide synthesis, the synthetase (GS) and glutamotransferase (GTF) offers a means of synthesis of a possible peptide component as well as



*Dotted lines represent reactions not demonstrated in spores but shown in part to occur in bacteria. Solid lines represent reactions discussed in text.

Fig. 1.

Possible functions of GTF, GS, XTF and the acetokinase-like enzyme in sporulation and germination*

the peptide itself. Since the peptide components were determined after acid hydrolysis, it is possible that the peptide linkages occurred through gamma-glutamyl radicals, and that glutamine rather than glutamic acid is a peptide component. The absolute Mn^{++} requirement for GTF is also of significance because of the apparent importance of this metal ion to sporulation. In respect to functions other than those related to the particular spore peptide, GTF may be related generally to peptide and protein synthesis.

The synthesis of active acetate, while without doubt of great significance to the synthetic processes probably occurring in germination, as demonstrated by activation through heat shock, can also be related to the synthesis of a peptide component. The formation of hexosamine and acetyl hexosamine from glutamine, hexose and acetyl CoA has been demonstrated in *Neurospora*

crassa by Leloir and Cardini (1953). The presence of the synthetase and kinase systems in spores suggests that the amination of hexose by glutamine and its subsequent acetylation should be demonstrable and such studies are now in progress.

While the above mentioned enzymes can be related to spore peptide synthesis, the unidentified transferase has properties which suggest that it could function in peptide hydrolysis. The stimulation by adenosine particularly suggests a function in germination. The identification of the substrate for XTF activity with the spore peptide, while entirely speculative, has many features which indicate a plausible relationship: (1) the peptide is non-dialyzable as is the substrate of XTF; (2) the peptide is made up of components with which a transferase- hydroxylamine system could react; (3) the peptide is absent from vegetative cells as is XTF activity; (4) the range of liberation extends from alkaline to acid pH as does the range of XHA formation; and (5) the liberation or depolymerization of the peptide is probably enzymatic.

Further significance of the relationship of the peptide to XTF activity can be found in recent work of Meister *et al* (1955). These workers have demonstrated that enzymes catalyzing transferase reactions with hydroxylamine also catalyze the hydrolysis of these substrates. The authors suggested that the transfer, hydrolysis and synthesis reactions are catalyzed by the same enzyme. This idea, together with the demonstrated activity of XTF as a transferase and its probable relationship to germination, suggests that XTF may be the enzyme responsible for the spore peptide depolymerization. This concept should be susceptible to direct experimental test. If it proves incorrect, two important problems still remain: (1) what is the substrate in the XTF system, and (2) what enzyme is responsible for peptide depolymerization?

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Informal Discussion

POWELL: We would like to add to the list of non-oxidative enzymes present in spore extracts a $\alpha\varepsilon$-diaminopimelic acid (DAP) decarboxylase which we have demonstrated in spores of *B. sphaericus* (Powell and Strange, 1957). The enzyme is heat-stable in intact resting spores but becomes heat-labile during germination or mechanical disintegration. The DAP decarboxylase activity of vegetative cells of *B. sphaericus* is very much greater than that of spores but is completely heat-labile in intact cells.

I find the work that Dr. Krask has reported extremely interesting, especially in view of the implication that the attack of lytic systems on the spore coat, splitting off peptide or breaking down polymerized phosphate, may bring about the first stages of the germination process. One of the most interesting properties of the intact resting spore is its resistance to enzymes. I mentioned earlier the work of Douglas who is studying the electrophoretic behaviour of resting spores before and after treatment with various enzymes in an attempt to get some information about their surface structure. He finds small changes in the electrophoretic behaviour of resting spores after treatment with certain enzymes e.g. lysozyme (Douglas and Parker, 1957); but none of the enzymes tested produced the characteristic cytological changes which occur during germination. We have recently described (Powell and Strange, 1956) a lysozyme-like intra-cellular lytic system which appears in sporulating cells and spores, and we have suggested that the activation of this system may bring about the first stages of the germination process. When resting spores are incubated with partially purified highly active preparations of this lytic enzyme, however, no germination occurs (Powell, unpublished). I would suggest that this may mean that

attack by the lytic enzyme is not, in fact, the first stage of the germination process or that in intact spores the enzyme substrate is inaccessible or present in some "protected" form. It may also follow that the function of the germination stimulant may be to modify the substrate so as to make it susceptible to attack by the lytic system.

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Oxidative Enzymes of Bacterial Spore Extracts

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UNTIL recent years we were faced with a contradiction in attempting to understand the biochemical basis of dormancy and the breaking of dormancy in bacterial spores which were believed to be nearly devoid of enzymatic activity. Previous papers given at this symposium have presented increasing evidence for an extended constitutive enzyme pattern of the bacterial spore. However, our evidence is still too meager to afford a biochemical explanation of the various cytological and physiological changes occuring during germination. One approach to the problem is to compare the enzyme patterns of vegetative cells, dormant spores, and spores in different stages of germination. We have heard here of dormant or inactive enzymes. Do, for example, changes in enzymatic activities following heat activation and germination activate these dormant enzymes, or does the appearance of new activities represent a limited protein synthesis? It is clear in certain cases, as outlined by Dr. Murty and Dr. Church, that activation of enzymes occurs. However, what is the extent to which quantitative (constitutive) and qualitative (induced) protein synthesis takes place? In both cases, we would expect energy yielding reactions and carbohydrate metabolism to provide the carbon skeletons for amino acid synthesis. An examination of the oxidative metabolism in spore extracts provides an excellent starting point for such a study.

Carbohydrate oxidation

Claims for respiratory activity of bacterial spores have been advanced by a number of different investigators (Brody, 1955; Keilin and Hartree, 1947; Tarr, 1933); however, failure to check the possibility that this metabolic activity was due to contaminating vegetative enzymes or germinated spores makes one question these claims. Employing well cleaned spores and micro respirometer techniques, Crook (1952) was barely able to detect respiration either with or without glucose. Crook's failure to observe the dormant glucose-oxidizing system in resting spores was probably due to insufficient activation. When aged spores are sufficiently activated by heat shock, adenosine (Church and Halvorson, 1956), or heat shock and L-alanine (Murrell, 1955; Murty and Halvorson, 1956), an active glucose oxidation can be demonstrated in the absence of detectable loss of heat resistance.

The activation of glucose oxidation by heat in clean spores of *Bacillus*

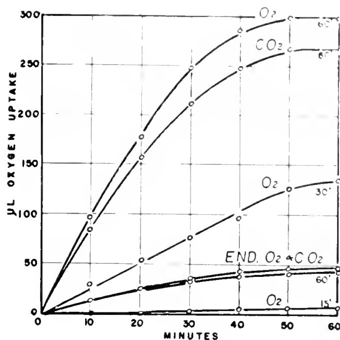

Fig. 1. The activation of glucose oxidation by heat. Clean spores of *B. cereus* var. *terminalis* were heat shocked at 65C, cooled and tested for respiratory activity by the two cup method. Warburg flask contents: Main compartment, 30 mg spores in 1 ml 0.067 M phosphate buffer, pH 7.2; center well, 0.2 ml 30 per cent KOH where appropriate; side arm, 1 mg glucose. Total volume 2.1 ml. Reaction run at 30C under atmospheric oxygen.

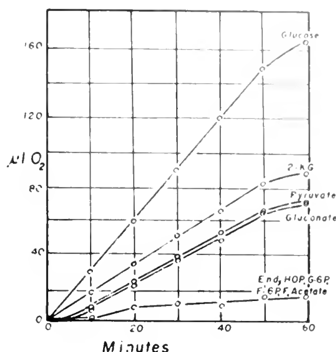

Fig. 2. Oxidative capacities of activated spores. See legend Fig. 1 for details. Substrates were added at a level of 5μmole ml.

cereus var. *terminalis* is shown in Fig. 1. The oxidation capacities of activated spores are shown in Fig. 2. In addition to glucose, these spores rapidly oxidize 2-keto gluconate (2KG), pyruvate, and gluconate. Glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose (F), hexose diphosphate (HDP), and acetate are not attacked. Thus, activated spores are excellent material with which to elucidate the pathways of glucose metabolism which furnish biologically useful energy and intermediates for biosynthesis during germination.

Since the approach to an understanding of the metabolic pathways operative in activated spores required measurements of single-step reactions of a metabolic chain, experiments were performed with crude spore extracts prepared by the following procedure: Twenty grams of superbrite glass beads were suspended in 20 ml of 0.067M phosphate buffer at pH 7.4 with two grams of dry spores. The mixture was ground in a Waring blender at

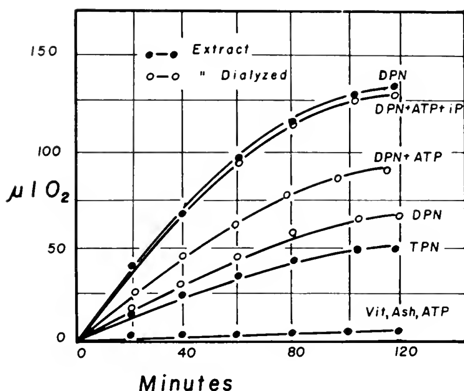

Fig. 3. Cofactor requirements for glucose oxidation by spore extracts. See text for preparation of extract. Extract was dialyzed overnight against water at 5C. The Warburg flask contents: sidearm, 1 mg of glucose, and 5×10^{-5} M DPN, TPN, or 0.2 ml of 0.05 M ATP where indicated; center well, 0.2 ml 30 per cent KOH: main compartment, 1 ml of enzyme preparation, 10 μmole inorganic phosphate, spore ash and vitamins where indicated. Total volume 2.1 ml. Reaction was run at 30C under atmospheric oxygen.

5C. for 30 minutes and the debris removed by centrifugation at 10,000 times gravity for 20 minutes. The supernate so obtained was free of spores. Approximately 50 per cent of the spores were ruptured by this treatment.

The cofactors required for optimal glucose oxidation by crude spore extracts are shown in Fig. 3. Optimal activity was observed in the presence of yeast extract. This requirement could be fully replaced by diphosphopyridine nucleotide (DPN) and partially by triphosphopyridine nucleotide (TPN). However, when the extracts were dialyzed, only 65 per cent of the activity was obtained in the presence of DPN. In these dialyzed preparations a partial requirement for both ATP and inorganic phosphate was observed.

The oxidative capacities of crude spore extracts supplemented with TPN or DPN are shown in Fig. 4. Glucose and pyruvate are actively oxidized in the presence of DPN. Gluconate and 2KG oxidations are TPN dependent. On the other hand, members of the Embden-Meyerhoff glycolytic system (G-6-P, F-6-P, F and HDP), as well as arabinose and ribose, are invariably inactive.

A kinetic analysis of the glucose oxidation by crude spore extracts is shown in Fig. 5. It is clear that an active system is present capable of converting glucose to pyruvate. The low recoveries of pyruvate are partially

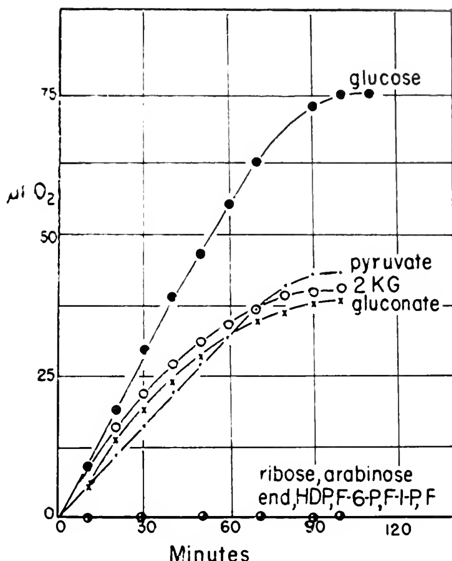

Fig. 4. Oxidate capacity of spore extracts. See legend Fig. 3 for details. Substrates were added at a level of 5 μmole/ml. DPN was added to flasks containing glucose and pyruvate; TPN to flasks containing gluconate and 2KG. All others received both TPN and DPN.

explained by the presence of an active pyruvate-oxidizing system in the extract. When pyruvate oxidation is suppressed (Edwaros *et al*, 1956) by the addition of the thiamine antagonist, Bis-1, 3-beta ethylhexyl-5-methyl amino hexahydropyrimidine (W-1435), higher recoveries of pyruvate are observed from glucose oxidation.

The study of carbohydrate metabolism by microorganisms over the past two decades has revealed a number of diverse pathways by which pyruvate can be formed from glucose (Gunsalus *et al*, 1955). The three major pathways for this are outlined in Fig. 6. The historical and widely distributed Embden-Meyerhoff pathways of glycolysis operate in lactobacilli, streptococci and members of the coli-aerogenes group. However, in obligate aerobes and in some faculative aerobes, such as pseudomonads and acetocbacter, carbohydrate oxidation follows different pathways. The first alternate pathway is the hexose monophosphate (HMP) oxidative route, which diverges at the level of G-6P; the 6-phospho—gluconate (6-P-G) in converted to pyru-

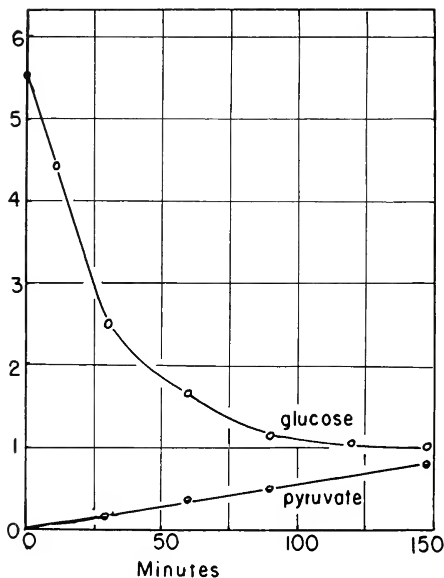

Fig. 5. Pyruvate formation during glucose oxidation by spore extracts. See legend Fig. 3 for details. Flask contained 5 x 10^{-5} M DPN. After deproteinization glucose was determined by a reducing method and pyruvate by the Friedman and Haugen (1942) procedure.

vate by either dehydration to 2-keto-3-desoxy-6-phosphogluconate (2KDPG) and cleavage to pyruvate and D-glyceraldehyde-3-phosphate, or by oxidation to a mixture of ribose-5-phosphate and ribulose-5-phosphate, which are then converted to sedoheptulose-7-phosphate, F-6-P, G-6-P and D-glyceraldehyde-3-phosphate. The second alternate pathway involves a direct oxidation of glucose to gluconate prior to phosphorylation. Gluconate is oxidized to 2KG and phosphorylated to 2K6PG which by an undefined pathway is converted to 2 moles of pyruvate.

Absence of an Embden-Meyerhoff glycolytic system

The absence of a functional glycolytic system in the activated spore, or in spore extracts, seems evident from the following observations. Activated spores or spore extracts were incapable of fermenting glucose, G-1-P, F-1-P, or HDP. Furthermore, the oxidation of glucose by transcelucent spores was resistant to NaF, a normal inhibitor of glycolysis (Hatchisuka *et al*, 1956). An analysis of the end products of glucose oxidation by spore extracts failed

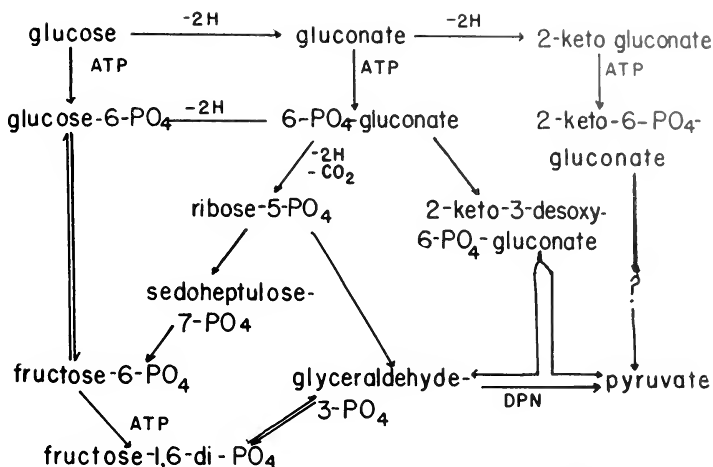

Fig. 6. Pathways of pyruvate formation from glucose.

to reveal the presence of phosphate esters. These extracts were also devoid of hexokinase and phosphohexokinase activities (Church, 1955). In addition there was no detectable phosphorylation of fructose, arabinose, ribose or G-6-P. The absence of a functional aldolase and triose-phosphate-dehydrogenase followed from the failure to detect TPN or DPN reduction in the presence of F-1, 6-di P, or F-6-P.

Oxidative pathway

To further define the oxidative pathways operative in spores, a characterization of the end products of glucose oxidation by spore extracts was undertaken. A chromatographic analysis of the deproteinized reaction mixture is shown in Fig. 7. The primary end products are gluconate with traces of 2KG, 2K6PG and pyruvate. A further identification of 2KG was obtained by eluting it from the chromatogram and condensing it with O-phenylene diamine. The spectrum of 2-ketogluconate-O-phenylenediamine complex was identical to that obtained with a synthetic sample (Lanning and Cohen, 1951).

The first recognized product of glucose oxidation by these extracts was gluconate. This could arise either by direct oxidation of glucose or by the action of hexokinase, G-6-P dehydrogenase, and a dephosphorylation of 6-P-G. The latter route could not be the explanation because of the absence of both hexokinase and a system of dephosphorylating 6-P-G. Furthermore,

CONTROL EXTRACT SPORES

Glucose-6-PO_4 (purple)
2-keto gluconate (grenish-yellow)
6-PO_4-2-keto gluconate (violet)
Pyruvate (red)
Gluconate (purple)
Glucose (purple)

Fig. 7. Detection of end products of glucose oxidation by chromatography. The reaction mixture of the experiment of Fig. 5 was deproteinized after 60 minutes incubation. The chromatogram containing the reaction mixture and knowns was run in an ethanol: methanol: H_2O(45: 45: 10) system. After drying, the paper was developed by the method of DeLey (1953).

the initial oxidation is not phosphate-dependent (Fig. 8). Dialyzed preparations suspended in a phosphate-free glycylglycene buffer contain an active DPN-linked glucose dehydrogenase (Fig. 9). This enzyme is soluble, since the total activity following centrifugation at 140,000 times gravity remains in the supernatant fraction (Table I). In contrast with the glucose dehydrogenase of *Pseudomonas fluorescens* (Wood, 1955), which is particulate and uses cytochromes b and c as hydrogen acceptors, the dehydrogenase of spores, as that in liver, utilizes DPN as the hydrogen acceptor.

The active G-6-P dehydrogenase in spore extracts (Fig. 10) is of the usual microbial variety employing TPN as the hydrogen acceptor (DeLey, 1955). From the studies of Cori and Lipmann (1952) and Brodie and Lipmann (1955) it is probable that the reaction here proceeds in two steps: (a) an oxidation of the pyranose ring of glucose-6-phosphate to 6-phospho-δ-gluconolactone and (b) a hydrolysis by a delactonizing enzyme to 6-P-G.

The reduction of DPN or TPN is not stimulated by the addition of phosphate. These findings are similar to those of Hachisuka *et al* (1956) who observed that the oxidation of glucose to gluconate in transclucent spores was not phosphate-dependent. It is interesting that although at least several of the enzymes of the HMP pathway, G-6-P dehydrogenase and a TPN

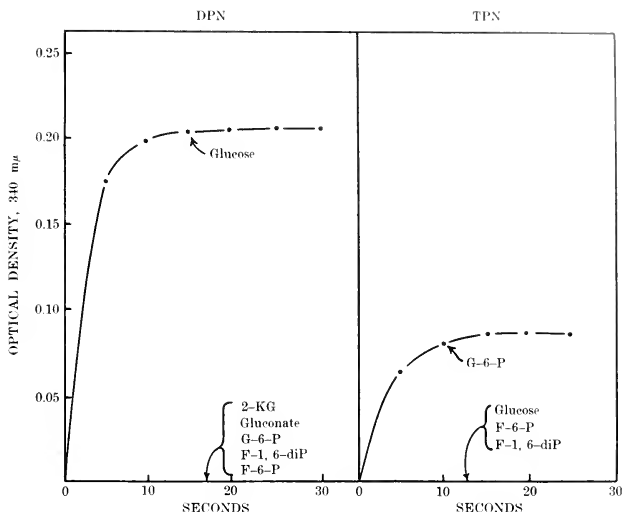

Fig. 8. Reduction of DPN and TPN by spore extracts. The Beckman cuvets contained 1 ml of M glycylglycine buffer, pH 7.4, 6.7×10^{-5} M DPN or TPN, 1.67×10^{-3} M substrates, 1 ml of dialyzed spore extract and distilled water to make a final volume of 3.0 ml. The temperature was 25C. The reduction was measured at 340 mμ.

TABLE 1

Distribution of glucose dehydrogenase after sonic rupture of the spore

Sample	Dehydrogenase[1]	Nitrogen[2]	Specific activity
Original extract	86	0.93	92
Supernate 140,000 xg	85	0.80	106
Residue 140,000 xg	0	0.12	0

[1] μl O_2/ml/hr
[2] mgN/ml

Warburg flask contents: side arm, 1 mg glucose and 5×10^{-5} M DPN: center well; 0.2 ml 30 per cent KOH: main compartment: 1 ml of enzyme preparation and sufficient 0.067 M phosphate buffer, pH 7.2, to bring the flask contents to 2.0 ml. Reaction was run at 30C under atmospheric oxygen.

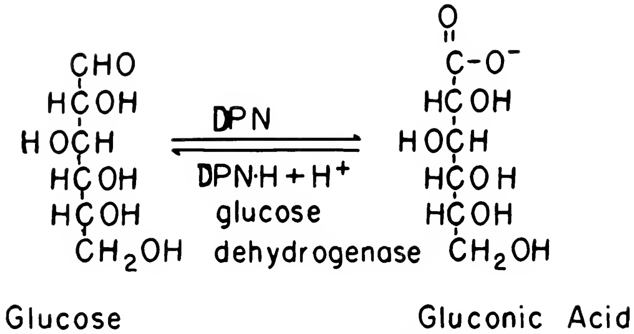

Fig. 9. Direct oxidation of glucose.

linked 6-P-G oxidizing system are present, they are not functional due to an absence of hexokinase activity. Although the mechanism of 6-P-G oxidation has not been studied, an analysis of the end products indicates that pentose is one of the end products. On the other hand, vegetative cells of members of the family of the *Bacillaceae* contain all of the enzymes of the HMP system up to the stage of sedoheptulose phosphate formation (DeLey, 1955).

Activated spores and extracts readily oxidize both gluconate and 2KG. The chromatographic analysis of glucose oxidation (Fig. 7) indicates that gluconate is converted to 2KG, as shown in Fig. 11. Alternatively one might expect a phosphorylation of gluconate via gluconokinase. However, a careful analysis of gluconokinase activity in these extracts by the method of Brody (1955) was negative.

2KG is phosphorylated by a typical magnesium-requiring kinase (Table

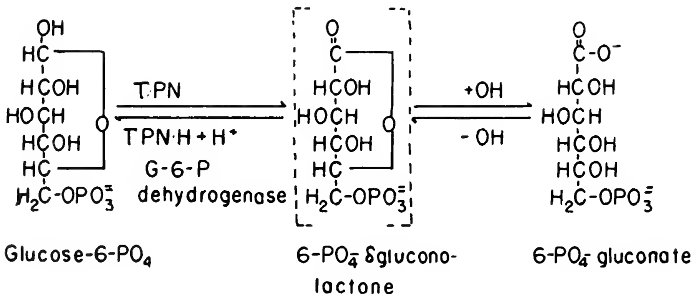

Fig. 10. Oxidation of glucose-6-phosphate.

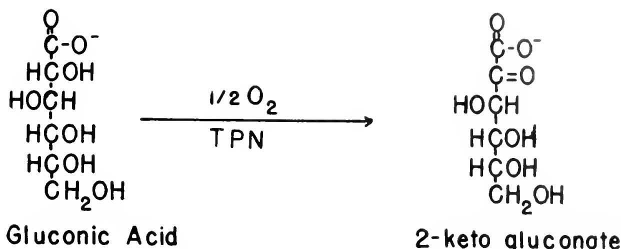

Fig. 11. Oxidation of gluconic acid.

II) similar to the inducible 2-ketogluconokinase found in *Aerobacter cloacae* by DeLey (1953) and *P. fluorescens* by Narrod and Wood (1954). The reaction is shown in Fig. 12. The involvement of ATP in this reaction may be the explanation for the phosphate stimulation of glucose oxidation previously observed in dialyzed extracts. The reactions leading to pyruvate formation from 2K6PG are little understood. Wood (1955) has suggested that this may occur in *P. fluorescens* via dehydration and cleavage reactions for 2-K6PG of the Entner-Doudoroff type leading to 2. 4 diketo-3-desoxy-6 phosphoguconate, which is cleaved to pyruvate and 3-phosphoglycerate.

Pyruvate oxidation

The metabolism of trioses represents the second phase of oxidative activity of spore extracts. The presence of a system leading to pyruvate from glucose as well as the oxidation of pyruvate (see Fig. 4) provides a basis

TABLE II

The stoichiometry of 2-keto gluconate phosphorylation by spore extracts of *B. cereus* var. *terminalis*

Reactant	μ moles		
	initially	final	change
2-keto gluconate	5.2	0.3	−4.9
acid-labile - P	13.2	8.8	−4.4
acid-stable - P	0.0	4.3	4.3

The incubation mixture contained 1 ml of spore extract, 0.2 ml of 0.05 M ATP, 1 ml of 2-keto gluconate, 0.008 M $MgCl_2$ and sufficient 0.1 M glycylglycene buffer pH 7.4 to give a final volume of 2.0 ml. After 20 minutes incubation at 30C the reaction was deproteinized and analyzed for 2-ketogluconate and acid-labile and acid-stable P.

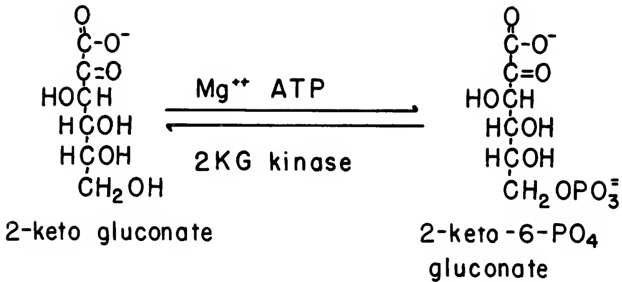

Fig. 12. 2-keto-6-phosphogluconate formation.

for a rich supply of energy and biosynthetic intermediates through decarboxylation, carboxylation, clastic and aldehyde transfer reactions.

The entire system for pyruvate oxidation was associated with a particulate fraction which was completely sedimented at 140,000 times gravity. Rapid pyruvate oxidation occurred with the addition of DPN, ATP, cocarboxylase and $MnCl_2$. When the reaction mixture is sparked with oxalacetate (OAA), an active oxidation of acetate, succinate, fumerate and OAA by these particles was observed (Fig. 13). There was no detectable oxidation of citrate, cis-aconitate or malate. Succinate oxidation was competitively inhibited by its analog, malonate, indicating the presence of succinic dehydrogenase. On the other hand Hardwick and Foster (1953) observed oxidative activity towards malate, succinate, α-ketoglutarate, and pyruvate in extracts of vegetative cells but not of spores of *Bacillus mycoides*. Their failure to detect oxidative activity in spore extracts was probably due to an insufficient endogenous supply of OAA to spark the reaction.

The cofactor requirements and oxidative capacity of these particules suggest that pyruvate is oxidized by the classical reactions of trioses known to be present in vegetative cells of *Bacillaceae*. Pyruvate is probably oxidatively decarboxylated to an active acetate which is further metabolized via either a dicarboxylic acid or tricarboxylic acid cycle. Although extracts do not actively oxidize the tricarboxylic acids, we feel that the results are too preliminary to favor a dicarboxylic acid cycle. Krask (this symposium) has also shown the presence of an active CoA-kinase or acetokinase in extracts of these spores.

It also seems likely that pyruvate may be derived from alanine. The L-alanine requirements for spore germination may be spared by pyruvate. Recently, Falcone (1955) reported the production of H_2O_2 and pyruvate from alanine by intact spores. We have also observed that intact spores of

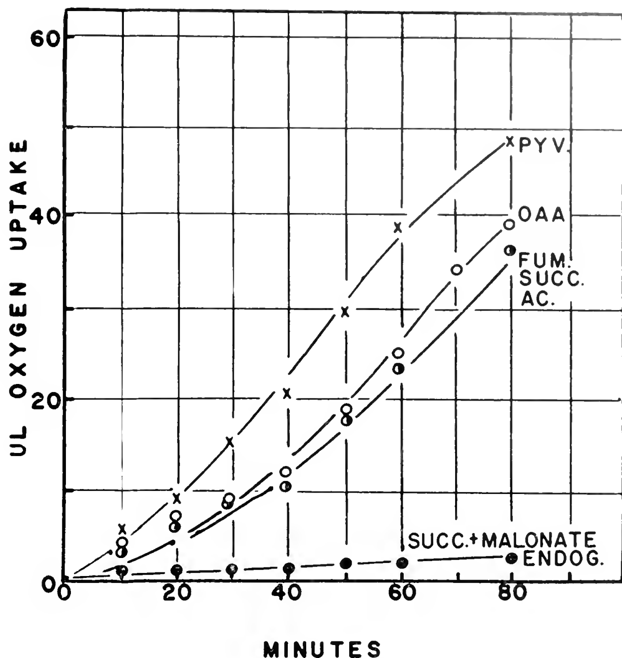

Fig. 13. Oxidation of various substrates by particles from spore extracts. A dialyzed spore extract (see Fig. 3) was centrifuged at 140,000 times gravity for 1 hour. The pellet was reconcentrated at one-eighth its original volume in 0.067 M phosphate buffer, pH 7.1. The Warburg flask contents: side arm, 25μ moles of substrate and 0.05μ mole of OAA; center well, 0.2 ml 30 per cent KOH; main compartment, 1 ml enzyme preparation, 8×10^{-3} M $MnCl_2$, 0.2 ml of 0.05 M ATP, 5×10^{-3} M DPN, 10^{-4} M cocarboxylase, and sufficient phosphate buffer to a final cell volume of 2.0 ml. The gas phase was air and the temperature 30C.

B. cereus var. *terminalis* actively deaminate alanine but not glycine or phenylalanine (Table III). When the pyruvate oxidation present in these spores is suppressed by W-1435, quantitative recoveries of pyruvate are obtained from alanine. W-1435 also inhibits spore germination in the presence of alanine. Thiamine, which reverses the inhibition of pyruvate oxidation by W-1435 (Edwards *et al*, 1956), restores partial germination in the presence of alanine. Dialyzed extracts of these spores require pyridoxal phosphate

TABLE III

Accumulation of pyruvate during the deamination of alanine by intact spores in the presence of an inhibitor of pyruvate oxidation

Substrate 5 μM	NH_3 μM	Pyruvate μM	Germination Per cent
Control	0	0	0
glycine	0	0	0
L-phenylalanine	0	0	0
L-alanine	5.0	0	100
L-alanine + W-1455	5.0	5.2	3
L-alanine + W-1455 + thiamine	4.6	2.5	41

The incubation mixture contained 100 mg of spores, thiamine at a final concentration of 6.6×10^{-5}M, W-1455 at a final concentration of 5×10^{-5}M and 0.067M phosphate buffer pH 7.0 to a final volume of 1.5 ml. After 60 min. incubation, NH_3 was determined by distillation and pyruvate after centrifugation by the method of Friedman and Haugen (1942). Germination was measured by the uptake of an aqueous methylene blue.

(B_6P) for the formation of NH_3 and pyruvate from D- or L-alanine. Since these extracts contain an active alanine racemase (Stewart and Halvorson, 1953), the deamination of both D- and L-alanine may represent a non-specificity on the part of the deaminase system or coupled reaction with alanine racemase. The presence of catalase in these extracts (Lawrence and Halvorson, 1954) precluded an examination for the H_2O_2 observed by Falcone. Presumably these two reactions are similar.

NH_3, H_2O_2, and pyruvate can be directly formed from alanine by the action of either D- or L-amino acid oxidase (Meister, 1955). Since these require either flavin adenine dinucleotide or riboflavin phosphate but not B_6P as cofactors, they differ from the requirements of the above system. Alternatively the observed reaction might be mediated by a B_6P activated glutamic-alanine transaminase (Meister, 1955) followed by a deamination of glutamate. Although vegetative cells of the *Bacillaceae* contain active glutamic-aspartic (Hardwick and Foster, 1953; Keynon *et al.* 1954; Levinson and Sevag, 1954; Meister, 1955) and glutamic-alanine (Harwick and Foster, 1953; Levinson and Sevag, 1954; Meister, 1955) transaminases, only glutamic-aspartic transaminase has been reported in spore extracts of *Bacillus megatherium* (Levinson and Sevag, 1954). Hardwick and Foster (1953) were unable to detect glutamic-alanine transaminase activity in spore extracts of *B. mycoides.* We have confirmed this observation employing extracts of *B. cereus* var. *terminalis* spores which actively deaminate alanine. This unique reaction can be formulated as follows: (Fig. 14).

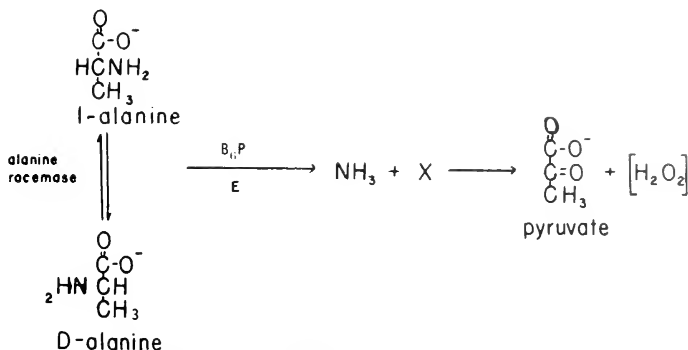

Fig. 14. Formation of NH_3 and pyruvate from alanine.

Fig. 15 summarizes the reactions demonstrated thus far. Pyruvate is formed either from glucose via gluconate, 2KG and 2K6PG, or from alanine. Pyruvate is oxidized probably via acetate to a TCA or DCA cycle, or metabolized by other clastic type reactions. Although the extracts contain G-6-P dehydrogenase and a system for oxidizing 6-P-G, the HMP pathway is not operative. The evidence here and elsewhere (Hachisuka *et al*, 1956) also eliminates a functional glycolytic system. The presence of a glycolytic system as well as further members of the HMP in vegetative cells of the *Bacillaceae* (Dedonder, 1952; DeLey, 1953; Keynan *et al*, 1954) must mean that these as well as other enzyme systems develop during germination.

Significance of oxidative capacity

The existence of a metabolic sequence leading to pyruvate formation as well as a cycle for triose oxidation provides a basis for the production of the C skeletons for amino acid synthesis: alanine from pyruvate, the aspartic acid family from OAA, and the glutamic acid family from 2-keto glutarate (Roberts *et al*, 1955). A glutamic-aspartic transaminase has been demonstrated in spores of *B. megatherium* (Levinson and Sevag, 1951). The existence of other amino acid synthesizing systems, active or dormant in the spore, when coupled with the energy yielding reactions demonstrated above, would permit a sufficient supply of amino acids required for protein and enzyme synthesis during the early stages of germination.

Qualitative changes, such as those occurring during germination, resemble induced enzyme synthesis in many respects. One of the interesting features of the latter is that in most cases studied thus far induced synthesis

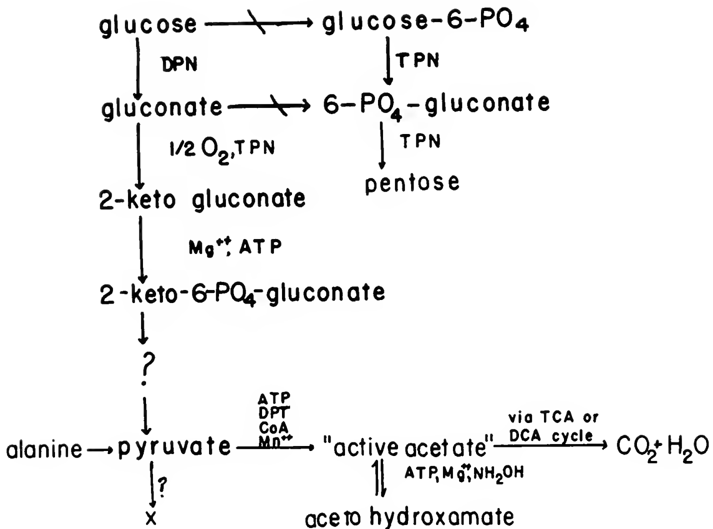

Fig. 15. Pathways of carbohydrate metabolism in spores of *B. cereus* var. *terminalis.*

is inhibited by the presence of glucose (Stanier, 1951). The mechanism of such inhibitions, called "glucose effects," is little understood. Bacterial spores, on the other hand, germinate in the presence of glucose and, therefore, presumably escape such "glucose effects." One possible explanation is that in systems demonstrating "glucose effects" glycolytic or HMP pathways are usually operative. If the inhibition is actually from some product of either of these two pathways, it would be lacking in the glucose metabolism of the spores. Such an escape of "glucose effects" is of selective value to the spore, which must obviously increase at least part of its enzymic patterns during germination.

The enzyme reactions presented here also suggest a biochemical relationship between the germinating agents for *B. cereus* var. *terminalis.* In the presence of adenosine, additions of alanine, high levels of glucose, or pyruvate stimulate germination (Church, 1955). The glucose dehydrogenase has a high K_s value, thus requiring high levels of glucose to saturate the system. Both glucose and alanine serve as precursors of pyruvate. An *in vivo* inhibitor of pyruvate oxidation by spores inhibits their normal germination in the presence of L-alanine and adenosine. The inhibition of both

germination and pyruvate oxidation is reversed by thiamine. The simplest interpretation of these results is that pyruvate or a product of pyruvate derived either from glucose, alanine, or endogenous reserves is required for germination.

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Discussion

Joan F. Powell

Dr. Halvorson has described enzyme systems present in resting spores of *B. cereus* which oxidize glucose to gluconate and pyruvate. These systems appear to be DPN- or TPN-dependent and it seems likely that the terminal stages of the oxidation proceed through flavoprotein and cytochrome systems. We found that spore extracts of *B. subtilis* and *B. megatherium* contained both free and combined flavin adenine dinucleotide (FAD) and we demonstrated the presence of a diaphorase-like system in these extracts (Spencer and Powell, 1951). Vegetative cells contained roughly three to four times as much FAD as spores on a basis of cell dry weight. They also had a much higher diaphorase activity than spores.

Keilin and Hartree (1949) reported that although spores of *B. subtilis* contained considerable amounts of unidentified haematin compounds, their cytochrome content was only 6% of that of vegetative cells. Chaix and Roncoli (1950) observed the development from an atypical to a "classical" cytochrome spectrum during the growth of *B. subtilis*. We attempted to demonstrate the presence of a cytochrome-cytochrome oxidase system in spores of *B. subtilis* and *B. megatherium* using methods depending on catalyzed

oxidations, but obtained results which were difficult to interpret (Powell, J. F. unpublished results. Resting spores of these organisms tested intact and mechanically disintegrated catalyzed the oxidation of p-phenylene diamine (Qo_2 37° ≏ 60) and hydroquinone (Qo_2 25° ≏ 30). There was, however, no stimulation of this oxidation by added cytochrome C. The oxidation catalyst of *B. subtilis* was unaffected by heating at 60° for 15 min., whereas that of *B. megatherium* was 75% destroyed. Spores of *B. subtilis* showed no change in oxidative activity after germination in L-alanine solution and the catalyst remained heat stable. Spores of *B. subtilis* catalyzed the oxidation of ascorbic acid and of reduced cytochrome C. Disintegrated spores were rather more active than spore extracts in the oxidation of reduced cytochrome C. On a dry weight basis, disintegrated vegetative cells appeared to be roughly ten times as active as disintegrated spores.

These results, though rather scattered and incomplete, suggest that spores contain a DPN-linked flavoprotein system and possibly a cytochrome system of relatively low activity compared with that of vegetative cells. The germination of spores of *B. subtilis* and *B. megatherium* was not inhibited by cyanide or azide (Powell, J. F., unpublished results; Powell, J. F., 1951) and the rate of glucose oxidation by these germinated spores was considerably less sensitive to cyanide than that of the corresponding vegetative cells (Spencer and Powell, 1951). It therefore appears that the constitution of the cytochrome system and its function change during sporulation and revert during the development of the germinated spore into a growing vegetative cell. Such changes in cytochrome constitution during the sporulation cycle might be very profitably studied using the spectrophotometric techniques developed by Chance (1952) and already applied to bacteria by Smith (1954).

It appears that these changes in the terminal oxidation systems may be paralleled by changes in the pathway of glucose oxidation. Dr. Halvorson has pointed out that vegetative cells of *Bacillus* species possess glycolytic activity as well as components of the phosphorylitic shunt which appear to be absent from resting spores. These systems must therefore fade out at sporulation and re-appear as the spore germinates and grows.

It is interesting to speculate on the connection between these changes in ezymic activity of the cell and the initiation of the sporulation process. Cantino (1956) in his studies on the sporulation of the water mold *Blastocladiella* has shown that the weakly-functional tricarboxylic acid cycle in this organism disappears at sporulation and reappears at the start of the next generation. The initiation of sporulation and the accumulation of intermediates in the tricarboxylic acid cycle could be induced by the addition of bicarbonate to the growth medium. It seems likely that similar studies with

sporulating bacteria would provide some ideas as to how the cyclical changes in their enzymic constitution are brought about.

I was interested in an observation recently reported by Falcone (1955) that spores of *B. subtilis* oxidized L-alanine producing hydrogen peroxide, and that hydrogen peroxide itself stimulated loss of heat resistance. We have not been able to repeat this observation with our strain of *B. subtilis*. It seems desirable that other strains of *B. subtilis* should be tested.

We wondered, at one stage, whether there was a burst of synthesis of CoI during germination and attempted to demonstrate this. We assayed CoI in extracts from disintegrated resting and germinated spores of *B. subtilis* in the usual way using *Haemophilus parainfluenzae* (Powell, J. F., unpublished results). In duplicate experiments we found that the CoI content of extracts from germinated spores was actually lower than that from resting spores.

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Supplemental Notes

By Joan F. Powell

Effect of manganese on sporulation of laboratory strains of *Bacillus cereus*, *B. subtilis* and *B. megatherium*

The effect of glucose (10^{-2}M) and manganese (10^{-4}M) addition on sporulation in shaken tryptic meat digest medium (100 mg nitrogen/100 ml) was tested. All the above organisms grew well but showed no signs of sporulation in the test medium with no additions or with added glucose.

Manganese addition stimulated almost complete sporulation of *B. cereus* in the presence but not in the absence of glucose, of *B. subtilis* in the absence but not in the presence of glucose, and of *B. megatherium* both in the presence and absence of glucose. These results were not always repeatable with different batches of meat digest medium prepared in an identical manner and of equivalent nitrogen content. For example, in four other batches of medium, only in one batch was sporulation of *B. cereus* stimulated by addition of manganese and glucose. In all batches of medium tested, *B. sphaericus* sporulated well in the absence of any additions.

Oxidations catalyzed by spores of *B. subtilis* (laboratory strain and NCTC 85) and *B. megatherium* (laboratory strain)

Resting spores of *B. subtilis* and *B. megatherium* tested intact and mechanically disintegrated catalyzed the oxidation of p-phenylenediamine (Qo_2 37° ≏ 60) and hydroquinone (Qo_2 25° ≏ 30). There was no stimulation of this oxidation by added cytochrome C. The oxidation catalyst of *B. subtilis* was unaffected by heating at 60° for 15 min. whereas that of *B. megatherium* was 75% destroyed. The *B. megatherium* catalyst also differed from that of *B. subtilis* in its comparative insensitivity to low concentrations of cyanide. Thus, with p-phenylenediamine substrate, the *B. subtilis* catalyst was 45% and 67% inhibited by 10^{-5} and 2 x 10^{-5} M. KCN, whereas that of *B. megatherium* was unaffected by 10^{-4}M.KCN although 95% inhibited by 2 x 10^{-4}M.KCN. The nature of the oxidation catalysis is still obscure: it seems likely that contamination with invisible vegetative debris would account for the heat-labile catalysis of *B. megatherium* spores.

Further tests were made with spores of *B. subtilis.* These spores showed no change in oxidative activity after germination in L-alanine, and the catalyst remained heat-stable. *B. subtilis* spores also catalyzed the oxidation of ascorbic acid and of reduced sytrochrome C. To follow oxidation of the latter

substrate, a 10^{10}/ml spore suspension in M/20 phosphate pH 7.3 was disintegrated, and 1 ml of the suspension (≡ 5.5 mg dry wt) added to 5 ml of a 6×10^{-5}M solution of reduced ($Na_2S_2O_4$) cytochrome C in M/20 phosphate pH 7.3. After 15 min. at room temperature there was a marked color change from pink to orange and the absorption band at 550 mμ had almost disappeared. During this time there was no appreciable change in a control containing no spores. After centrifuging, the activity of the supernatant was considerably less active than that of the suspension. The supernatant was added to an equal volume of 4×10^{-5} M reduced cytochrome C and the decrease in absorption at 550 mμ followed in a Unicam spectrophotometer. The oxidation was slow (50% substrate oxidized in 20 min.) but definite. In a control containing supernatant heated at 100° for 10 min., oxidation was extremely slow, i.e., less than 50% in 60 min. The more active uncentrifuged suspension could not be used for these measurements, being far too turbid. Disintegrated germinated spores showed approximately the same activity as disintegrated resting spores whereas disintegrated vegetative cells appeared, on a dry wt. basis, to be roughly ten times as active in the oxidation of reduced cytochrome C.

Comparison of the Coenzyme I content of extracts from resting and germinated spores of *B. subtilis* (laboratory strain)

The method of assay described by Gingrich and Schlenk (1944. J. Bact. *47:* 535) was used with *H. parainfluenzae* NCCT 4101 as test organism. The standard CoI preparation was supplied by Schwarz Laboratories as 50% pure CoI. On this basis, it permitted barely visible growth at 0.0017 γ CoI/ml. The amount of growth increased linearly in the range used, i.e., 0.01-0.05γ/ml. Resting and germinated (L-alanine) spores of *B. subtilis* were disintegrated, centrifuged and the extract sterilized by filtration. The sterile extract was added to the basal medium. There appeared to be no increased synthesis of CoI during germination: in fact, in duplicate experiments, the CoI content of extracts from germinated spores was lower than that from resting spores.

CoI content (γ) of extract from 10^{10} spores

	Resting (5.5 mg dry wt.)	Germinated (3.9 mg dry wt.)
Exp. I	0.13	0.08
Exp. II	0.14	0.10