The purpose of this investigation was to compare some of the properties of a spore-forming mutant of *Bacillus cereus* 569R with the wild type. The strains were grown under identical conditions in liquid medium. The mutant, strain 45, was characterized with respect to growth rate, pH changes in the medium, morphological development, cell density during the growth cycle, the effect of oxygen deprivation on the time of spore formation, and the production of an extracellular protease.

Strain 45 was found to maintain a higher growth rate than 569R. The pH curves indicated that, in time, strain 45 was about 30 minutes ahead of wild type. Morphologically, strain 45 exhibited no long chain stage characteristic of early logarithmic growth in *B. cereus* 569R. Poly β hydroxybutyrate was accumulated before the sharp rise in pH in 45. This comparatively early synthesis suggested that acetate utilization and polymer synthesis may not be coordinately controlled.
in this mutant. Spore formation occurred two and one-half hours earlier in strain 45, and the spores were twice as large as spores of wild type. The production of protease in the mutant occurred earlier, and in higher concentration when compared with 569R.

The results of these experiments fell into two classes. The first class included growth rate, pH, and cell density shifts. These events occurred approximately one-half hour earlier in cultures of strain 45, and appeared to be correlated with the cell mass. The second class of results—the time of polymer synthesis, the period when the time of sporulation was sensitive to oxygen, the time of protease production, and the time of sporulation—appeared to be independent of cell mass, and occurred markedly earlier in strain 45. The very early appearance of these sporulation-related events in the culture cycle suggested that the mutant strain 45 may be catabolically derepressed for sporulation. Characterization of two asporogenous mutants of _B. cereus_ 569R was also undertaken.
A Comparison of Some Physiological Properties of
*Bacillus cereus* 569R with a Mutant Strain

by

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TABLE OF CONTENTS

INTRODUCTION 1

Cytological Development 1
Commitment to Sporulation 4
Developmental Stages 6
Cell Density Shifts 8
Physiology of Sporulation in B. cereus T 9
Extracellular Protease 18
Penicillinas 22
Phage and Lysogeny in Bacillus species 26
Genetics of Sporulation 29
Statement of Purpose 31

MATERIALS AND METHODS 33

Bacterial Cultures 33
Standard Culture Conditions 33
Media 34
Preparation of Stocks 36
Growth Curves 37
Dry Weight Curves 38
pH Curves 38
Morphological Studies 39
Renografin Density Gradients 39
Oxygen Uptake and Sporulation 40
Proteolytic Activity on Albumin Agar Plates 40
Spectrophotometric Determination of Protease 41
Inducibility of Penicillinase 42

RESULTS 45

Growth in Liquid Medium 45
Morphology of Development 50
Renografin Density Gradient Studies 59
Oxygen Uptake and Sporulation 64
Proteolytic Activity 68
Penicillinase 71
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Composition of SMCAY</td>
<td>34</td>
</tr>
<tr>
<td>2. Composition of G agar</td>
<td>35</td>
</tr>
<tr>
<td>3. Composition of Andrade Agar</td>
<td>36</td>
</tr>
<tr>
<td>4. Proteolytic activity on albumin agar plates</td>
<td>69</td>
</tr>
<tr>
<td>5. Assay for proteolytic activity with casein substrate</td>
<td>70</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Standard curve for protoelytic activity.</td>
<td>43</td>
</tr>
<tr>
<td>2. Growth of <em>B. cereus</em> 569R and mutants 45, U29, and U17 in SMGY.</td>
<td>46</td>
</tr>
<tr>
<td>3. Dry weight curves for <em>B. cereus</em> 569R and mutants 45, U29, and U17 in SMGY.</td>
<td>48</td>
</tr>
<tr>
<td>4. pH curves for <em>B. cereus</em> 569R and 45 in SMGY.</td>
<td>49</td>
</tr>
<tr>
<td>5. pH curves for mutants U29 and U17 in SMGY.</td>
<td>51</td>
</tr>
<tr>
<td>6. Morphological development of <em>B. cereus</em> 569R.</td>
<td>53</td>
</tr>
<tr>
<td>7. Morphological development of strain 45.</td>
<td>54</td>
</tr>
<tr>
<td>8. Free spore size for <em>B. cereus</em> 569R and mutant 45.</td>
<td>56</td>
</tr>
<tr>
<td>9. Morphological development of mutant U29.</td>
<td>57</td>
</tr>
<tr>
<td>10. Morphological development of mutant U17.</td>
<td>58</td>
</tr>
<tr>
<td>11. 46-60% Renografin density gradients of <em>B. cereus</em> 569R.</td>
<td>60</td>
</tr>
<tr>
<td>12. 46-60% Renografin density gradients of strain 45.</td>
<td>62</td>
</tr>
<tr>
<td>13. 46-60% Renografin density gradients of U29 and U17.</td>
<td>63</td>
</tr>
<tr>
<td>14. Oxygen uptake and spore formation in <em>B. cereus</em> 569R.</td>
<td>66</td>
</tr>
<tr>
<td>15. Oxygen uptake and spore formation in strain 45.</td>
<td>67</td>
</tr>
</tbody>
</table>
The study of the growth and development of bacterial populations involves an understanding of the interactions between cells, and between cells and their environment in culture. It has long been known that cultures proceed through various stages of development, and that such stages may depend on the substances the cells excrete into the medium, as well as on the environmental changes which occur as the constituents of the medium are used for growth. The spore-forming bacilli have a distinctive growth cycle which makes them good model systems for such studies. The growth cycle commences with the germination of the spores, proceeds through a vegetative stage, and terminates in sporulation. One such system, Bacillus cereus 569, was chosen for these investigations.

Cytological Development

Changes in cell structure during sporulation were first described by Young and Fitz-James (1959a, b) using B. cereus var. alesti. By electron microscopy, these researchers outlined the cytology of sporulation. Their results have been presented as seven stages in spore formation. In the vegetative cell, two nuclear bodies were seen with a septum forming at the equator of the cell at
division. After the last nuclear division preceding sporogenesis, the two nucleii condensed and formed an axial filament of chromatin. This process was referred to as stage I, and was considered to mark the beginning of sporulation. During stage II, a polar spore septum developed which included a portion of the chromatin. At stage III, there was a double layer of cytoplasmic membrane separating the spore chromatin from the rest of the cell. The spore envelope was visible, and spore cortex developed during stage IV. Stage V was characterized by coat formation, and refractility of the endospore as determined by phase microscopy. Stage VI was characterized by spore maturation, and spores of this stage were highly refractile when viewed with phase microscopy. The last stage, stage VII, was characterized by spores free of the sporangium (Murrell, 1967; Schaeffer, 1969). After stage III, the spore wall was shown to develop independently of the vegetative cell wall. Previous to this time, vegetative cell wall was required for sporulation as shown by the fact that protoplasts of vegetative cells at stages I and II would not sporulate, but protoplasts of cells at stage III would go on to form spores (Fitz-James, 1964).

During the development of *B. cereus* T, Nakata and Halvorson (1960) reported changes in cell appearance as observed with light microscopy. Logarithmic stage cells were filamentous and found in long chains with homologous cytoplasm. Older cells appeared shorter
and existed in shorter chains. Their cytoplasm was granular. Later in the culture cycle, phase dark prespores developed. With time, these prespores became refractile and were classified as endospores. Later still, these endospores lost their sporangia and became free spores. In our laboratory, similar morphological changes were observed with B. cereus 569R. A transition from the "long cell stage" accompanied by the granulation of cytoplasm occurred at about four to five hours. Shrinkage of the cell contents with a saturated solution of salt revealed that the protoplasts of "long" and "short" cells were of the same size, and that the "long" cells consisted of two protoplasts in one cell wall unit. Intracellular prespores were visible by ten hours, refractile endospores by 11.5 hours, and free spores by 13.5 hours.

Mesosomes have also been correlated with normal cell division and with the sporulation process. They may function in genome separation by providing a continuous connexion between the dividing chromatin and the cytoplasmic membrane. Newly synthesized cell wall appeared to be connected with the mesosomes, and they were seen to be closely associated with the spore septum (Ellar, Lundgren, and Slepecky, 1967).
Commitment to Sporulation

The concept of commitment to sporulation has been discussed by many workers. In most spore-forming cultures, it has been shown that sporulation was negligible as long as bacterial growth was logarithmic, and that as soon as vegetative growth declined, spore formation began (Foster, 1956). From chemostat experiments, Foster observed that cells could be grown vegetatively for many generations, and that sporulation was therefore not obligatory, but depended on the environmental conditions. In other experiments, aerobic bacilli were removed from a growth medium just prior to sporogenesis. These cells sporulated without further division even when fresh nutrients were furnished.

Vegetative cells of *B. cereus* T would sporulate after being diluted or placed in distilled water. This phenomenon was termed endotrophic sporulation by Halvorson (1963). The addition of glucose to cells of *B. cereus* T taken from a growing culture up to nine hours and resuspended in phosphate buffer was capable of suppressing spore formation. After nine hours, glucose was totally ineffective as an inhibitor. Foster (1956) interpreted these results as a change in sporulation potentiality from reversible to irreversible, and defined commitment to sporulation as that stage at which glucose could no longer retard spore formation. When cells of *B. cereus* T, prior to
cytoplasm granulation, were placed in a medium that would not support growth, lysis was observed. Granulated stage cells, however, were able to sporulate in this medium. It was noted that only cells which contained polyβ-hydroxybutyrate in sufficient amounts were capable of spore formation under these conditions.

An endogenous factor was found to be involved in the transition from non-granulated to granulated cells. This substance was of low molecular weight, and was absent from vegetative cells. When this factor was added to exponential phase cells, sporulation was initiated (Srinivasan and Halvorson, 1963; Halvorson, 1962). It was also observed that cells through stage II of sporulation were able to resume multiplication and growth (Schaeffer, 1969). Wright (1966) has observed that a differential process may have no true zero time at all; thus the concept of commitment may not describe a distinct event. In this laboratory, we have defined commitment to sporulation as being that time at which a cell can no longer divide vegetatively. Experiments with B. cereus 569R have led to the conclusion that commitment to sporulation occurred in this strain, if at all, just prior to endospore formation, since division of individual cells was observed until just before prespores were seen (Fraser, 1969).
Developmental Stages

Fraser and Baird (1968) studied the progressive changes in the culture cycle of *B. cereus* 569R. Using a salts-casamino acids medium, the stage of development of individual organisms or chains was classified with respect to four properties. They first studied the Gram reaction of cells and showed that the transition of cells from Gram negative to Gram positive in the culture was a simultaneous and relatively sudden shift. Since the cells were not in synchronous growth these results indicated that this shift was in response to an environmental change.

The second property that was studied was the ability of living cells to take up neutral red dye. These results showed that out-growing spores stained uniformly. During growth, individual cells reacted differently to the dye even within single chains. Cells adsorbing the dye appeared in several successive peaks which have been correlated with peaks of chain break-up and with peaks of lysis of a portion of the population during the growth cycle.

The third property considered was the electrophoretic distribution of cells through a pH gradient to indicate differences of electric charge on the cell membrane. These examinations revealed that four to six separate bands were present, rather than a continuous distribution of charges. The results indicated that there were subpopulations
of cells which differed in isoelectric points (surface charge) at all times in the culture cycle.

The last property investigated was the growth potential of individual cells or chains of cells isolated into microdrops under oil. Based on the ability of these chains or cells to divide and/or form spores under these conditions, five developmental stages of organisms were observed and correlated with time in the culture cycle. These stages included $\alpha$, found at two to four hours, which neither divided nor formed spores; $\beta$, found at three to six hours, which divided but formed no spores; $\gamma$, found at four and one-half to thirteen hours, which both divided and formed spores; $\delta$, found at seven and one-half to thirteen hours and comprising only a small percent of the population, which neither divided nor formed spores; and $\epsilon$, found at nine and one-half to thirteen hours, which did not divide, but formed spores in the original cells. This last group was defined as committed to sporulation. It is interesting to note that in every case where cells in the microdrops divided at all, they formed full clones of approximately 100 cells.

These researchers have concluded that, except for the Gram reaction, the tests indicated that at most times during the development of the culture, the population was a mixture of two or more subpopulations of cells in different stages of development.
Cell Density Shifts

Work in progress in this laboratory has indicated that characteristic cell densities are correlated with the developmental states of *B. cereus* 569R (Wise and Fraser, 1970). Three discrete bands of cells have been observed and related to the time of culture development using 46 to 60% Renografin density gradients. The first band, $\beta(1.215 \text{ g/cm}^2)$, was found in the early stages of growth. At this time, the cells appeared in long chains of approximately 16 cells per chain. After five hours, there was a shift in cell density to the $\gamma_1$ band ($1.205 \text{ g/cm}^2$). Cells in this band were characteristically in shorter chains of approximately eight cells per chain. This first density shift was gradual, and was also found to be reversible by dilution of the cells into fresh medium.

At approximately seven and one-half hours, a second density shift occurred. The shift was fairly sudden, being completed in one cell generation time or less. Cells in this $\gamma_2$ band were found floating on top of the gradient. Morphologically, the cells were found in even shorter chains of approximately four cells per chain, and contained granular material. This density shift was found to be irreversible by dilution of the cells.

Preliminary results indicated that the second density shift was due to a decrease in the number of ribosomes per cell. This
decrease resulted from the cessation of ribosomal RNA synthesis while DNA and protein synthesis continued. Cells at this stage were not considered committed to sporulation since they were able to divide and grow in fresh medium. It has been suggested, however, that the measure of cell density can provide a quantitative tool to assess the early stages of sporulation in B. cereus 569. These shifts in cell density have been correlated with the stages of development observed by Fraser and Baird (1968) as indicated by their symbol designation.

Physiology of Sporulation in B. cereus T

Bacillus cereus T has been extensively studied both physiologically and biochemically. This system has yielded valuable information through the use of the active culture technique in which cells appeared to be about the same physiological age, and thus produced spores rapidly and simultaneously. This technique was developed to reduce the number of ungerminated spores in a culture, and to improve the synchrony of sporulation. Approximately four culture transfers were made before inoculation of the active culture. At this time cells grew synchronously. Halvorson (1957) has claimed that this technique effectively separated vegetative growth from sporulation making it an important tool for studying the development of the culture.
Using a salts–yeast extract–glucose medium, Halvorson (1957) observed a characteristic pH curve during the growth cycle of B. cereus T in active culture. The initial pH of the culture was close to neutrality, but dropped to a minimum of around 5.4 by three to three and one-half hours. The pH then rose again to about 7.7 by five and one-half to six hours, and leveled off. The minimum in the pH curve corresponded to both the end of logarithmic growth in the culture as determined by optical density measurements and to the exhaustion of glucose from the medium. It has further been correlated with the morphological changes in the cells which mark the initial stages of sporulation. These changes, observed microscopically, involved a transition from cells that were filamentous, forming long chains, and having homogenous cytoplasm, to shorter cells, a breaking up of the chains, and the appearance of granulated cytoplasm (Nakata and Halvorson, 1960; Kominek and Halvorson, 1965). The pH pattern described above was in agreement with those reported by Knaysi (1945) for Bacillus mycoides, and has been shown to be fairly general among Bacillus species, with exceptions found in media lacking fermentable carbohydrates (Murrell, 1967). It has also been noted that if the carbohydrate concentration was high, and the pH of the culture fell very low, some cultures would fail to increase in pH and to sporulate (Bernlohr and Novelli, 1960).

Further investigations with Bacillus cereus T carried out by
Nakata and Halvorson (1960) revealed that the characteristic pH drop in the culture cycle was due to the accumulation of organic acids in the medium. Chromatographic analysis of the supernatant preparations from low pH cultures showed that only pyruvic and acetic acids were present. These acids accumulated during vegetative growth until the population was near maximal, when the pH had reached its minimum and glucose was exhausted from the medium. The pyruvic acid peak consistently reached its maximum about one-half hour prior to that of acetate, suggesting that pyruvate was the direct precursor of acetate. After the low pH point, both acids disappeared from the medium as the pH rose and sporulation proceeded. The authors suggested that the utilization of these acids is necessary for spore formation.

In further studies, Nakata and Halvorson (1960) collected supernatants from cultures in which sporulation was inhibited by α-picolinic acid or ethyl malonate. These supernatants were analyzed for pyruvic and acetic acids. The principal acid found was acetate. It was present in amounts comparable to those found in normal cultures if all the pyruvic acid had been converted to acetate at the pH minimum. Nakata (1963) looked at the accumulation of organic acids in buffered cultures and found that twice as much acetate was present in buffered medium when compared to the unbuffered medium.

The work of Gollakota (1959) further implicated these organic
acids in the sporulation process. He reported that α-picolinic acid, a dipicolinic acid analogue, completely inhibited sporulation of *B. cereus* T, but allowed normal vegetative growth. In these cultures, the pH decreased with logarithmic growth, but remained at the minimum level even after prolonged incubation. The inhibition of sporulation was reversible by the addition of various organic acids such as pyruvate, acetate, citrate, isocitrate, malate and others (Gollakota and Halvorson, 1960).

The route of glucose oxidation in this system has been explained by an operational hexose monophosphate shunt. Vegetative cells of *Bacillus cereus* T have been shown to contain a complete glycolytic system together with the enzymes of the hexose monophosphate oxidative pathway (Halvorson and Church, 1957; Doi, Halvorson, and Church, 1959). Goldman and Blumenthal (1960), and Blumenthal (1961) studied the radioactivity of CO$_2$ formed from specifically labeled $^{14}$C glucose and found that the labeling of the CO$_2$ in vegetative cells of *B. cereus* was almost exclusively that predicted for a glycolytic system. The accumulation of acetate and pyruvate as end products of glucose oxidation during vegetative growth suggested that a complete oxidative mechanism was not functioning in *B. cereus* T. In support of this suggestion, it was shown that normal vegetative growth continued in cultures where acetate utilization was blocked by α-picolinic acid. Microscopic examination of these cultures showed that the cells
were typically vegetative in morphology (Nakata and Halvorson, 1960; Gollakota and Halvorson, 1960). It was concluded that sporulation was inhibited by α-picolinic acid through its ability to block acetate metabolism. Presumably, α-picolinic acid prevented the synthesis of the enzymes necessary for acetate utilization.

This block in acetate metabolism was confirmed by showing that vegetative cells had only trace amounts of either fumarase or condensing enzyme, which is necessary for the entry of acetate into the TCA cycle (Halvorson, 1965; Goldman and Blumenthal, 1964). Further studies indicated that terminal pathways of acetate oxidation were absent from vegetative cells since only low amounts of radioactivity were found in CO₂ after incubation of vegetative cells with 6-¹⁴C glucose (Goldman and Blumenthal, 1960), or 2-¹⁴C acetate (Hanson, Srinivasan, and Halvorson, 1963b).

The metabolism of the organic acids formed during vegetative growth has been shown to be essential for spore formation. Hanson, Srinivasan, and Halvorson (1963a, b) studied the metabolism of acetate in vegetative and sporulating cultures of B. cereus and showed that less than 5% of the acetate available from glucose was used during vegetative growth. In sporulating cells, however, the acetic acid was oxidized to CO₂ via the TCA cycle and converted to poly β hydroxybutyrate. The utilization of acetate has been correlated with the rise in the pH of the culture, and also to the granulation of the
cells (Nakata, 1962). The induction of the enzyme system capable of oxidizing acetate has thus been shown to occur during the transition from vegetative growth to sporulation. The enzymes required for this utilization were shown to be adaptively formed, the adaptation being chloramphenicol sensitive, and occurring coincidentally with large increases in the levels of condensing enzyme, aconitase, succinic dehydrogenase, fumarase, and malic dehydrogenase (Nakata and Halvorson, 1960; Hanson, Srinivasan, and Halvorson, 1963a, b; Halvorson, 1965; Murrell, 1967).

Martin and Foster (1958) first suggested that the TCA cycle was involved in sporulation because the precursors of dipicolinic acid, which comprised 7 - 15% of the dry weight of spores, were drawn from TCA cycle intermediates. Hanson, Srinivasan, and Halvorson (1963a, b) supported this hypothesis by showing that during the pH minimum, only the TCA cycle was operative, and that acetate was then converted to polyβ-hydroxybutyrate and CO₂. Before and after this period, the cycle was not involved in metabolism as shown by the inability of fluoroacetate to inhibit growth and sporulation at these times. The fluoroacetate did not inhibit vegetative cells since they lacked the condensing enzyme needed to convert acetate to citrate. Fluoroacetate was not inhibitory to sporulation after the low pH period because the cells became impermeable to acetate.

Further evidence for a typical oxidative decarboxylation
mechanism in \textit{B. cereus} T was provided by Warburg experiments which showed that the ratio of the liberated CO$_2$ to oxygen taken up was 2:1, as would be expected for the oxidative decarboxylation of pyruvate (Nakata and Halvorson, 1960). Experiments with \textit{Bacillus subtilis} have shown that mutants which were found to be blocked in TCA cycle enzymes were unable to form spores, indicating the essential role of this cycle in sporulation (Hanson, Srinivasan, and Halvorson 1963b; Halvorson, 1965; Fortnagle and Freese, 1968).

One of the main products of acetate metabolism by sporulating cells was shown to be poly $\beta$-hydroxybutyrate. Thus, a major role of acetate and subsequently of this polymer in \textit{B. cereus} T may be to provide carbon precursors and energy for sporulation (Nakata, 1966). After the end of logarithmic growth in \textit{B. cereus} T, at the pH minimum, polymer formation began. Accumulation of polymer proceeded for several hours, leveled off for a short time, and then continued to a maximum which occurred just before the onset of sporulation. At this point the polymer accounted for about 10% of the dry weight of the cells. Polymer content then decreased rapidly, and by the time mature spores were visible, it had completely disappeared.

Experiments with 2-$^{14}$C acetate indicated that acetate incorporation into polymer paralleled polymer formation during the initial rise in synthesis but, that after the lag period, little acetate incorporation occurred, indicating that later polymer synthesis was from a source
other than exogenous acetate (Kominek and Halvorson, 1965).

The inducible nature of the poly \( \beta \) hydroxybutyric acid synthetic mechanism was also investigated by Kominek and Halvorson (1965). It was shown that acetoacetyl Co A reductase, which catalyzes the reduction of acetoacetyl Co A to \( \beta \) hydroxybutyryl Co A, was absent from vegetative cells, but appeared in increasing concentrations in sporulating cells. Experiments with chloramphenicol and \( \alpha \)-picolinic acid, which inhibited the formation of acetoacetyl Co A reductase, indicated that this enzyme was necessary for polymer synthesis in this system.

The secondary increase in polymer concentration was found to be due to the incorporation of acetoin into poly \( \beta \) hydroxybutyrate. Acetoin is formed from pyruvate and oxidized to \( \text{CO}_2 \) and polymer via the 2, 3 butanediol cycle as described by Juni and Heym (1965a, b; 1957). Two enzymes which were necessary for the cycle to operate, 2, 3, butanediol dehydrogenase, and diacetyl reductase, were found in sporulating cells, but little activity was present in vegetative cells of \( \text{B. cereus} \) T. The formation of this enzyme system was found to be pH-dependent, with induction occurring only when the pH of the medium was less than 6.0. Cultures buffered at a higher pH (7.4) accumulated very little polymer, but were able to sporulate normally. Thus, the synthesis of large amounts of polymer was not necessary for sporulation, but when polymer was synthesized, it was entirely
used up before mature spores were formed. No poly β-hydroxybutyrate has been detected in mature spores (Nakata, 1963; Kominek and Halvorson, 1965; Murrell, 1967). Under conditions where large amounts of polymer were built up, and poly β-hydroxybutyrate was not exhausted from the medium for many hours, sporulation was delayed until the period of rapid polymer utilization (Slepecky and Law, 1961).

In many *Bacillus* species the oxygen supply had been shown to be critical for sporulation, and Knaysi (1945) has suggested that oxygen is necessary for the oxidation of the accumulated organic acids by the TCA cycle. Tinelli (1955a, b) observed lysis and lack of spore formation in cultures of *Bacillus megaterium* with insufficient aeration. With increased aeration, a rise in oxygen consumption was observed just before the appearance of spores. Halvorson (1957) also noted lysis of cultures of *B. cereus* T with insufficient aeration, and confirmed an oxygen demand for sporulation in this system.

In studying oxygen consumption of cultures with time, Halvorson (1957) found two peak periods of oxygen demand. The first peak occurred during early vegetative growth, and declined as the pH curve reached its minimum. This peak was attributed to the increase in cell population, and was not thought to represent a change in the oxygen demand per cell. The second peak in oxygen consumption occurred just after the low pH point. It was not due to an increase in cell population as determined by optical density measurements, but
was interpreted to be caused by an increase in the oxygen demand per cell, correlated with the induction of TCA cycle enzymes which metabolized the organic acids accumulated during vegetative growth. During this period there were morphological changes characteristic of the initial stages of sporulation in the cells. It was also noted that less oxygen was required for the later stages of sporulation.

These physiological stages observed in *B. cereus* T have been correlated with those morphological stages observed by Fraser and Baird (1968) in *Bacillus cereus* 569R. The peak of β stage cells appeared during logarithmic growth prior to the low pH point in the culture. At or just after the low pH point, the cells became thicker and the chains shorter, characteristic of the early γ stage. Late γ stage cells were recognized by the accumulation of granules of poly β-hydroxybutyrate. Thus, the sequence of events for 569R, under the conditions used in our laboratory, was similar to that observed in *B. cereus* T grown under Halvorson's conditions, except that a considerable amount of growth occurred in 569R during the γ stage after the low pH point, and the cells were still able to multiply if transferred to fresh medium, long after the optical density had ceased to rise.

**Extracellular Protease**

Spore forming bacteria have been observed to excrete a number of products into their culture medium. With *Bacillus* species the
substances reported in the literature were mainly exoenzymes and antibiotics. The production of an extracellular proteolytic enzyme, characteristically during the post logarithmic stage of growth, has been reported for many *Bacillus* species. Protease activity was first detected in high amounts when the growth rate of the culture was declining, and this activity was shown to reach a maximum and remain constant until free spores were visible. This activity was observed to result from de novo synthesis rather than from delayed excretion or activation of preformed enzyme. In no case has a cell-bound protease been reported (Bernlohr, 1964; Pollock, 1962; Schaeffer, 1969).

In studies with *B. licheniformis*, measurable quantities of protease appeared in the medium only during the stationary phase of growth (Bernlohr and Novelli, 1963). In *Bacillus subtilis*, a low rate of protease synthesis was observed during logarithmic growth. In the post logarithmic phase, a dramatic increase in protease levels was noted (Coleman, 1967).

It was originally believed that a single enzyme was responsible for proteolytic activity in sporeforming bacteria. However, Schaeffer (1969), has reported that *B. subtilis* and *B. licheniformis* were shown to produce two types of enzymes which appeared at the same time. They differed in that one enzyme was a metal enzyme, active at a neutral pH, whereas the other was an alkaline serine enzyme with
esterase activity. Only the neutral enzyme has been observed for _B. megaterium_ and _B. cereus_.

Neumark and Citri (1962) showed that incubation of _B. cereus_ 569 with high levels of amino acids inhibited extracellular protease. Amino acids had no effect on the activity or stability of the already formed protease. The authors concluded that a high level of amino acids repressed protease formation and that this repression was not the function of any single amino acid, but appeared to depend on the presence of a number of amino acids in sufficiently high concentration. The repressive effect was observed to be specific for L-amino acids. Levisohn and Aronson (1967) isolated mutants of _B. cereus_ T which were capable of sporulating in the presence of high levels of amino acids. These mutants were also found to form high levels of protease. The appearance of this activity was not caused by depletion of the amino acids from the medium since full activity was found in the presence of a mixture of 18 amino acids. Levisohn and Aronson also carried out reversion studies with these mutants which indicated that protease production, sporulation, and a purine requirement might be functionally linked.

The synthesis of protease was found to be induced at the beginning of sporulation. Asporogenous mutants of _B. subtilis_ which were blocked before stage I of sporulation were found to be incapable of producing proteolytic activity. Asporogenous mutants which were
able to reach stage II retained the ability to produce protease. A class of mutants which were protease ± were also isolated and found to be oligosporogenic (Murrell, 1967; Schaeffer, 1967; Schaeffer, 1969).

Through genetic studies, it was reported that asporogenous *B. subtilis* mutants which were also protease negative could be transformed to sporogeny with a concommitant transformation to protease production (Spizizen, Reilly, and Dahl, 1963; Spizizen, 1965).

Bernlohr (1964) observed that the yield of protease from cultures of *B. licheniformis* was a function of their sporulation characteristics. Under conditions supporting only inefficient sporulation, low levels of protease were observed. For prime sporulation conditions, high levels of this enzyme were noted. He also reported that the addition of glucose to cultures of this strain not only retarded the initiation of sporulation, but also delayed the appearance of protease activity in the medium (Freese and Fortnagel, 1967; Bott and Davidoff-Abelson, 1966).

All these observations strongly suggested that protease formation might be required for sporulation. However, the two functions, protease production, and sporulation, were not always related. Cases of asporogenous mutants capable of producing protease, and of spore formers exhibiting only low levels of protease production have been reported (Spizizen, 1965; Levisohn and Aronson, 1967).

The exact function of protease has not been determined.
Protease production has been correlated with the onset of a high rate of protein turnover occurring during post logarithmic growth (Mandelstam, 1960; Monro, 1961). It has been suggested that protease functions physiologically in sporulation metabolism possibly through degradation of vegetative cellular material for subsequent use, or in the release of spores (Bernlohr, 1964). Protease has also been postulated to be necessary for the hydrolysis of a specific protein into components which could initiate further steps in morphogenesis (Rogolsky, 1969b).

Penicillinases

Bacterial penicillinases have been shown to catalyse the hydrolysis of penicillin to the antibiotically inactive penicilloic acid. They hydrolysed the amide bond in the β-lactam ring of 6-amino penicillanic acid, 7-amino cephalosporanic acid, and/or their N-acetyl derivatives. Thus they are referred to as β-lactamases (Citri and Pollock, 1966). β-lactamase was first reported by Abraham and Chain (1940) and has since been found to be widely distributed in bacteria. In Bacillus species, this enzyme has been described for B. anthracis, B. cereus, B. licheniformis, B. megaterium, and B. subtilis. It was further determined that the penicillin resistance of bacterial cells and their ability to produce a β-lactamase were not closely correlated, suggesting that any
relationship was indirect. The \(\beta\)-lactamases were shown to be a heterogeneous group of proteins, and few generalizations could be made which applied to the entire group. Strains of bacteria were shown to vary widely in the amount of penicillinase characteristically produced, and in the extent to which its formation could be stimulated by the treatment of cells with penicillin (Ozer, 1968).

One species which has been well studied with respect to penicillinase production is \textit{B. cereus}. The penicillinase of \textit{B. cereus} was found to be similar to other exoenzymes. It was inducible rather than constitutive, had no cysteine, and was usually produced and secreted into the medium in large quantities after active growth of the organism had ended. It also had a high affinity for adsorption on washed cell walls of uninduced cells. It was shown to be different from most exoenzymes in that its substrate was not macromolecular, and in that there did not appear to be a barrier to the penetration of penicillin within the cell (Duthie, 1947; Pollock, 1962; Ozer, 1968).

\textit{B. cereus} 569 was shown to be inducible for penicillinase. The pH optimum was close to neutrality, and the \(\beta\)-lactamase was inactive and precipitated at pH 5.0. The reaction kinetics of this induction showed the reaction to be zero order in the presence of saturating concentrations of substrate (Henry and Housewright, 1947; Banfield, 1967). This strain formed only traces of penicillinase until treated with penicillin, after which high yields of \(\beta\)-lactamase in a stable
extracellular form were observed (LePage, Morgan and Campbell, 1946; Pollock and Kramer, 1958).

In studying the β-lactamase of _B. cereus_, it was observed that not all penicillinase activity could be neutralized with antiserum prepared against the exoenzyme. For 569 penicillinase, 80 - 90% was found free in solution and was inactivated by this antiserum. This fraction was termed the α form of β-lactamase. Of the rest of the enzyme, 30 - 50% was neutralizable by the exoenzyme antiserum, but was cell-bound. This fraction was termed the β form of the enzyme. A third fraction was found to be non-neutralizable by exoenzyme antiserum, and was also cell-bound. The unneutralizable fraction was not decreased by disintegration of the cells, although a large portion of the cell-bound enzyme was released into solution. This third fraction was termed γ-penicillinase. It was found to be inducible, and was present in the same proportion in constitutive mutants as in the wild type (Pollock, 1956; Pollock, 1962; Citri and Pollock, 1966).

Localization of γ-penicillinase was studied by Sheinin (1959). The results showed that, in contrast to adsorbed exoenzyme, the γ penicillinase remained attached to the cytoplasmic membrane after treatment with the cell-wall lytic enzyme of Strange and Dark (1957). γ-penicillinase was obtained in solution only when cell membranes were disintegrated by sonication or with solvents.

In high salt concentrations or urea, the α form of the enzyme
was converted to the $\gamma$ form without loss of activity (Citri, Garber and Sela, 1960). There has been some evidence suggesting that the $\gamma$ form is a precursor of the $\alpha$ form. Nascent penicillinase on ribosomes has been found to be iodine sensitive suggesting that it might be in the $\gamma$ form. It has also been noted that neutralizing antiserum against $\alpha$ enzyme increased the activity of the ribosomnal enzyme. The true nature of the relationship between the $\alpha$ and $\gamma$ forms of penicillinase is not yet clearly understood (Duerksen and O'Conner, 1963; Ozer, 1968).

Penicillinases from 569 and from a constitutive mutant were found to be indistinguishable serologically, and thus a single mutational event affected regulation of the enzyme, but not its structure (Sneath, 1955; Benedict, Schmidt and Coghill, 1945; Kogut, Pollock and Tridgell, 1956; Bernstein, Nickerson, and Day, 1967).

Yip, Shah, and Day (1964) and Collins (1964) have suggested that penicillinase was produced in the absence of penicillin during particular stages in the life cycle of individual cells of some bacteria. The $\beta$-lactamase activity in $\textit{B. cereus}$ was shown to vary considerably with the stage in growth of the uninduced cultures (Foldes, and Merety, 1960). Ozer (1968) looked at this correlation, and found that penicillinase activity per viable cell was lowest during logarithmic growth, and highest during sporulation in $\textit{B. cereus}$ 569. For both inducible and constitutive strains, a higher accumulation of $\beta$-lactamase was
observed at sporulation. These findings suggested a normal derepression of penicillinase during sporulation for this strain.

In further studies by Ozer, a cellular inducer, soluble peptidoglycan, was isolated. The increased accumulation of penicillinase appeared to be related to the appearance of this soluble peptidoglycan which was normally produced during the sporulation. This inducer was found to be identical with the spore peptide reported by Powell and Strange (1953) and Strange and Powell (1954). In mutants which were penicillinase negative and oligosporogenous, addition of exogenous penicillinase resulted in a stimulation of sporulation.

The precise reaction by which penicillinase may participate in sporulation has not been elucidated. However, it was suggested that penicillinase might be involved in cell wall metabolism during sporulation. Various other workers have also suggested a correlation between antibiotic production in cells and sporulation (Pollock, 1962; Balassa, Ionesco, and Schaeffer, 1963; Schaeffer, 1967).

Phage and Lysogeny in Bacillus species

The existence of bacteriophage for Bacillus species was originally reported by d'Herelle (1922). In 1930, Cowles observed that in B. megaterium 899, bacteriophage could be produced by cultures derived from heat-treated spores, and that virtually every colony arising from these heated spores produced phage. These results
demonstrated true lysogeny for *Bacillus* species.

In experiments with *B. cereus* and *B. anthracis*, Cowles found that survivors of non-lysogenic strains exposed to phage-formed spores, and that when these spores were heated to temperatures which destroyed free phage, the germinating cultures contained phage, but repeated cycles of sporulation, heating, and growth eliminated the phage from these cultures. This situation demonstrated that unstably lysogenic cells probably segregated non-lysogenic progeny.

Bott and Strauss (1965) studied *B. subtilis* carrying phage SP10. When such a culture was grown overnight in phage antiserum, some clones were found to be incapable of producing phage. This work indicated that SP10 did not establish a lysogenic relationship in *B. subtilis* which could be maintained in the absence of extrinsic bacteriophage. This state was called pseudo-lysogeny. Romig and Borodetsky (1961) isolated phage SP13 from *B. subtilis* and found an unstable lysogeny. Phage SP10 for *B. subtilis*, isolated by Thorne (1962), was found to carry out generalized transduction, and to be more stable than phage SP13 in *B. subtilis* W23. Stable lysogeny was reported for *B. subtilis* infected with the defective phage PBSx (Ionesco, Ryter, and Schaeffer, 1964). Rutberg (1969) has reported a phage, φ105, which was temperate and lysogenized *B. subtilis* 168.

There are relatively few studies on bacteriophages for *B. cereus*. McCloy (1958) isolated three phage from *B. cereus* W which
were serologically identical, but differed in plaque morphology. One of these phages, α, was found to be temperate. Thorne (1968) has isolated a non-temperate phage, CP-51, which carried out generalized transduction in *Bacillus cereus* 569. *B. cereus* 569 has been shown to be inducible for phage by treatment with mytomycin C (Saz, 1969; Baird, 1969). It has not yet been shown whether this relationship represents true lysogeny. Both turbid and clear plaques have been observed from platings of cultures. These differences in morphology indicated that there were two types of phage present in this strain.

Preliminary work in this laboratory (Haworth and Fraser, 1969) has shown that *B. cereus* 569R releases phage spontaneously at characteristic times during the growth cycle in SMCA medium. Two peaks of phage release were observed at four and one-half and at eight hours respectively, with titers reaching approximately $10^5$/ml during these peaks. Both clear and turbid plaque-formers were noted and the peaks for each morphological type occurred at the same times in the culture cycle.

It has been suggested that lysogeny in *B. cereus* is related to sporogenesis (Saz, 1969). This relationship must be a complex one, if indeed it exists, since several asporogenous mutants carrying phage have been isolated in our laboratory, and conversely some spore-forming strains have been found to be phage sensitive.
Genetics of Sporulation

Relatively little has been published on the genetics of sporogenesis. The information that has been reported is based on studies with asporogenous mutants. Many of these mutants have been classified cytologically by electron microscopy, and it has been shown that mutational blocks at all stages of sporulation could be found in *B. subtilis* and *B. cereus* (Schaeffer, 1969).

Schaeffer, Ionesco, and Jacob (1959) demonstrated that the ability to form spores could be restored to asporogenous mutants by transformation. Through transformation studies, Spizizen (1965) found that the characteristics of protease production, antibacterial activity, competency, and sporogenesis in *B. subtilis* all appeared to be controlled by closely linked genes. Takahashi (1961) showed that transduction in *B. subtilis* could restore spore-forming ability to asporogenous mutants. Mapping by transduction showed that these spore markers were not located in one closely linked group. He suggested that several independent biochemical processes operated in spore formation, and that by blocking any one such process, the formation of mature spores could be prevented (Takahashi, 1965).

Murrell (1967) hypothesized that sequential gene action occurred during sporulation. This hypothesis was supported by studies with sporulation mutants of *B. subtilis* (Balassa, 1968). Balassa suggested
that if the sporulation block was early in the process, most or all sporulation-dependent characteristics would be absent. Conversely, the later the block occurred, the more of these characters would be expected to be retained. He examined ten sporulation characters in randomly selected asporogenic mutants to look at the frequency of negative phenotypes. He found that the earliest event appeared to be the production of gelatinase, and the latest, the production of elastase. The frequency of mutation for these ten characters corresponded to a temporal sequence of events during sporulation. Further investigation of these ten characters by computer analysis confirmed the idea that a block early in the process produced a block in all later products, and thus allowed an ordering of the characters into a linear sequence which was identical to that obtained by studies of the mutational frequencies.

More recent experiments (Rogolsky, 1969a, b) with transformation and PBS1 transduction of *B. subtilis* revealed localized genetic sites for asporogeny on the chromosome. Loci for asporogeny were found in four gene clusters; three of these clusters were extensive. The first linkage group, located between cys A and ery at the proximal end of the chromosomal map, extended approximately 27 map units. Mutations within this linkage group blocked the production of bacteriocin which was necessary for the early stages of morphogenesis. The second linkage group was found to the right of *ura* on the map, extended 47 map units, and controlled sporulation functions present
after protease and bacteriocin production and before dipicolinic acid synthesis. The third linkage group was found to the left of lys-2, measured about 55 map units, and had a locus near the terminal end which obstructed both protease and bacteriocin production when defective.

Jacob, Schaeffer and Wollman (1960) speculated that the genetic information for sporulation was located episomally. However, attempts to cure cells of sporogenesis by standard treatments for curing episomes gave unreliable results. Further, the fact that four gene clusters have been established for sporogenesis in Bacillus subtilis suggested that all spore genes could not be located on an episome (Schaeffer, 1969).

Statement of Purpose

One mutant of B. cereus 569R which was of particular interest in this laboratory was designated strain 45. It was originally isolated as a revertant of an asporogenous mutant derived from the N-methyl-N'-nitro-N-nitrosoguanidine treatment of 569R. On G medium, asporogenous colonies of 569R appeared white and translucent when viewed through a dissecting microscope. Some asporogenous colonies developed dark, opaque sectors which were characteristic of spore-forming cells. A number of spore-forming revertants were isolated from these sectors. Strain 45 was a stable revertant isolated in this
manner. Such revertants, including 45, formed spores which appeared to be approximately twice as large as those of \textit{B. cereus} 569R. Strain 45 formed spores several hours sooner than wild type under identical growth conditions. It typically accumulated larger amounts of poly \(\beta\) hydroxybutyrate than wild type, and polymer appeared sooner in the culture cycle. This mutant was shown to be mitomycin C inducible for only one type of phage, producing plaques similar to the turbid plaque type produced by wild type 569R.

The early appearance of poly \(\beta\) hydroxybutyrate and of spore formation with strain 45 led us to hypothesize that this mutant might be missing a growth stage which shortened its growth cycle. The purpose of this research was to characterize strain 45, both morphologically and physiologically in order to investigate its growth cycle, and to compare its properties with those of wild type 569R and two asporogenous mutants of \textit{B. cereus} 569R.

The asporogenous mutants employed were designated U17 and U29. Both were isolated by \(N\)-methyl-\(N'\)-nitro-\(N\)-nitrosoguanidine treatment of wild type \textit{B. cereus} 569R. Both required exogenous uracil for growth. These mutants differed, however, in cell morphology and in the ability to accumulate polymer. Both strains were found to be phage sensitive, and could not be induced by mitomycin C.
MATERIALS AND METHODS

Bacterial Cultures

*Bacillus cereus* 569R was obtained from the culture collection of Dr. Curtis B. Thorne, Department of Microbiology, University of Massachusetts, and has been maintained in this laboratory. The mutant strain 45 was isolated in this laboratory as a revertant of an asporogenous, uracil-requiring mutant of 569R. Strains U17 and U29 were isolated in this laboratory from cultures of 569R after treatment with N-methyl-N'-nitro-N-nitrosoguanidine.

Standard Culture Conditions

For all growth cycle experiments the culture conditions were identical. Washed, heat-shocked suspensions of spores of 569R or 45 were heated at 65°C for 15 minutes. A 250 ml Erlenmeyer flask containing 50 ml of SMCAY was inoculated with 0.1 ml of one of these spore stocks. These spore stocks were assayed and found to contain approximately 2 X 10^9 spores per ml. The flasks were shaken at 34°C on a rotary shaker to incubate the cultures. For the two asporogenous strains, U29 and U17, overnight colonies from G agar plates were used to inoculate 50 ml of SMCAY in 250 ml Erlenmeyer flasks. Cells from the colonies were suspended in the medium to a
Klett reading of 3.0. These cultures were also incubated by shaking at 34°C on a rotary shaker. When large samples were to be removed, duplicate cultures were used.

**Media**

SMCAY growth medium was routinely used to study the culture cycles of the strains under investigation. Its composition is given in Table 1. G agar was used to maintain the asporogenous strains of *B. cereus* 569R. Its composition is listed in Table 2.

**Table 1. Composition of SMCAY**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin Free Casamino Acids</td>
<td>5.00 gm</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.25 gm</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.25 gm</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.10 gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.00 mg</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>5.00 mg</td>
</tr>
<tr>
<td>MnSO$_4$·4H$_2$O</td>
<td>5.00 mg</td>
</tr>
<tr>
<td>Concentrated HCl</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1.00 gm</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O$^b$</td>
<td>80.00 mg</td>
</tr>
<tr>
<td>Glucose$^b$</td>
<td>2.00 gm</td>
</tr>
</tbody>
</table>

$^a$For SMCA medium, yeast extract was omitted.

$^b$Added aseptically after autoclaving.
Table 2. Composition of G agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO$_4 \cdot 7$H$_2$O</td>
<td>500 μg</td>
</tr>
<tr>
<td>CuSO$_4 \cdot 5$H$_2$O</td>
<td>5 mg</td>
</tr>
<tr>
<td>ZnSO$_4 \cdot 7$H$_2$O</td>
<td>5 mg</td>
</tr>
<tr>
<td>MnSO$_4 \cdot H_2$O</td>
<td>50 mg</td>
</tr>
<tr>
<td>MgSO$_4 \cdot 7$H$_2$O</td>
<td>400 mg</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>2 gm</td>
</tr>
<tr>
<td>CaCl$_2 \cdot 2$H$_2$O</td>
<td>80 mg</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>500 mg</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>2 gm</td>
</tr>
<tr>
<td>Glucose$^a$</td>
<td>1 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>20 gm</td>
</tr>
</tbody>
</table>

$^a$Added aseptically after autoclaving.

Bovine Serum Albumin Agar plates for protease activity determinations were prepared as follows. 100 ml of 1.5% Noble agar was autoclaved separately. 100 ml of 2% bovine serum albumin (fraction V, Armour) was filter sterilized. The sterile albumin was added to the hot agar, and a milky precipitate was formed. The solution could be heated over water to 75° C if only a small amount of precipitate formed initially. One ml of 19% NaCl was added to this solution, and the agar was poured into plates and allowed to solidify and dry before use.

Andradé agar was used in penicillinase induction studies. The components of this medium are listed in Table 3. Andradé indicator
was composed of five grams of acid fuchsin, 1 N NaOH, and approximately 150 to 180 ml distilled water per liter. The acid fuchsin was dissolved in the distilled water, NaOH was added, and the solution was allowed to shake at room temperature for 24 hours. After this period, the indicator was straw colored, and ready for use.

Table 3. Composition of Andradé Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>2.5 gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>Andradé Indicator</td>
<td>40.0 ml</td>
</tr>
<tr>
<td></td>
<td>pH 7.2</td>
</tr>
</tbody>
</table>

Preparation of Stocks

Spore stocks of *B. cereus* 569R and 45 were prepared by the following method. *B. cereus* spores of 569R or 45 were inoculated into SMCAY and grown overnight at 34° C on a rotary shaker. The cultures were checked microscopically for sporulation, and the spores, when 100% were free of the sporangia, were harvested by centrifugation for ten minutes at 6,500 rpm. The pellets of spores were then resuspended in distilled water, and washed three times with sterile distilled water. The washed spores were resuspended in ten ml of sterile distilled water and heat shocked for one hour in a 65° C water
bath. Following this treatment, the spores were washed three more times as above, resuspended in distilled water, and stored at 4°C. Viable spore counts were determined by diluting the spores in distilled water and plating appropriate dilutions on nutrient agar plates. Colonies were counted after overnight incubation at 30°C.

For long term storage, frozen glycerinated cultures were made of the two asporogenous strains U29 and U17. Cultures of U29 and U17 were grown up to the middle of logarithmic phase in SMCAY. Approximately two ml of these cultures were transferred to sterile screw capped vials. Several vials were made for each strain. To each vial, 10% volume/volume sterile glycerol was added. The cultures were quick frozen in acetone and dry ice, and stored at 0°C. For short term stocks, U29 and U17 were maintained on plates of G agar. Colonies were restreaked on fresh plates every two to three days.

Growth Curves

In these experiments, 250 ml Erlenmeyer side arm flasks containing 50 ml SMCAY each were inoculated with 0.1 ml of a $2 \times 10^9$ spores/ml stock of either 569R or 45, or to a Klett reading of 3.0 with overnight colonies of U29 or U17. The cultures were incubated under standard conditions, and readings were taken every 30 minutes for twelve hours using a Klett-Somerson Photoelectric Colorimeter.
with filter #66, which reads in the range of 640 to 700 \(\mu\)m.

**Dry Weight Curves**

Standard SMCAV cultures were used for these determinations. Cultures were made in quadruplicate. Beginning at two hours, 15 ml samples were removed every hour. Cells were centrifuged at 6,500 rpm for ten minutes, and resuspended in two ml of distilled water. To pre-weighed planchettes, 0.5 ml of the resuspended cells were added. Samples were all made in triplicate. The planchettes were placed in a 60°C oven overnight to dry. The samples were then allowed to equilibrate to room temperature. They were then reweighed to determine the dry weight of the 15 ml samples. A Metler balance reading out to five decimal places was used to weigh the planchettes.

**pH Curves**

Standard SMCAV cultures of each of the four strains were used for determining the pH of the cultures at intervals during growth. Samples of two ml each were removed from the cultures every 30 minutes. The pH was determined using a Beckman Zeromatic single probe electrode pH meter, and the results were plotted against time.
Morphological Studies

Using standard SMCAY cultures and standard culture conditions, the morphological changes during growth were observed in *B. cereus* 569R, and mutants 45, U29, and U17. Samples were taken from the cultures at times when morphological changes occurred in the wild type strain. The state of the three mutants at those times was also observed. The samples were observed microscopically with a Zeiss phase contrast microscope. Photographic records of the observations were made with a Polaroid camera attached to the microscope.

Renografin Density Gradients

For studies of cell density with time, the four strains being investigated were grown in standard SMCAY cultures under standard conditions. Samples of 15 to 20 ml were removed at 30 minute intervals from three and one-half to six hours for strains 569R and 45, and at three, four and one-half, and six and one-half hours for the asporogenous strains. These samples were centrifuged at 10,000 rpm for eight to ten minutes, and the pellets stored in ice. The pellets were resuspended in 0.5 ml SMCA just before use. Samples of 0.2 to 0.4 ml of each suspension were layered on 46 to 60% Renografin density gradients. The gradients were centrifuged for 45 minutes in a Beckman model L2 ultracentrifuge using a SW 50 L
rotor at 20,000 rpm at 4° C. The gradients were removed after centrifugation and observed for the distribution of the cells in the Renografin.

Oxygen Uptake and Sporulation

*B. cereus* 569R and the mutant 45 were grown in standard SMCAY cultures under standard conditions. Several flasks were inoculated with each strain, and at various times, one of the flasks was removed from the shaker, but not from the incubator, for a period of one hour. After this one hour interval, the flasks were returned to the shaker to continue incubation until the end of the culture cycle. Control flasks for each strain were those which were never removed from the shaker during the culture cycle. Near the end of the culture cycle, when endospores were forming, cells from all the flasks were compared to the controls microscopically to determine the stage of sporulation that had been reached in each flask. A Zeiss phase contrast microscope was used for these observations, and pictures were taken of the results with a Polaroid camera attached to the microscope.

Proteolytic Activity on Albumin Agar Plates

Cells of 569R, 45, U29, and U17 from standard SMCAY cultures at five or ten hours were spotted on bovine serum albumin plates and
incubated at 30° C for 24 hours. Zones of clearing on the cloudy albumin plates indicated the presence of proteolytic activity. Duplicate plates were spread with 0.1 ml of 100 mg/ml streptomycin (Calbiochem) before the cells were spotted. This procedure inhibited growth of the cells, and was used as an indication of whether the cells were capable of producing protease at the age they were plated, or only after further growth.

Spectrophotometric Determination of Protease

A spectrophotometric method for the assay of proteolytic activity based on that of Kunitz (1947) was used to observe protease production with time in the four strains under investigation. The strains were grown in standard SMCA Y cultures, under standard conditions, and two ml samples were removed every two hours. The samples were centrifuged at 6,500 rpm for ten minutes to remove the cells. One ml of each supernatant sample was placed in a boiling water bath for 15 minutes to destroy any protease activity. These boiled samples served as controls. The second ml was used to determine the proteolytic activity. For the assay, one ml of 2.5% Hammerstein casein (Mann) in Sorensen's buffer pH 7.6 was placed in each assay tube and incubated at 35° C in a water bath for five minutes. One ml of protease standard at various concentrations, a one ml sample of boiled supernatant, or a one ml sample of supernatant was added to
each tube. The tubes were shaken to mix the contents, and incubated at 35° C for 20 minutes. The reaction was terminated by the addition of three ml of 5% trichloroacetic acid to each tube. After this addition, the contents of the tubes were mixed well, and the tubes were allowed to stand at room temperature for at least one hour. The precipitate which formed was removed by filtration through Whatman #1 filter paper. The filtrate was measured at 280 mµ on a Zeiss PM Q II spectrophotometer for trichloroacetic acid insoluble material. The readings from the boiled samples were subtracted from the corresponding unboiled supernatant sample to give the value due to proteolytic activity alone. The standard curve obtained by this method with known amounts of protease (Sigma), is shown in Figure 1.

Inducibility of Penicillinase

The inducibility of penicillinase was determined on Andrade agar plates. These plates were streaked with cells of each strain, and allowed to incubate at 30° C for 24 hours. After this period, a solution of 5% penicillin (Nutritional Biochemicals Corporation) was pipetted onto the colonies. If penicillinase was present, there was an immediate reaction of the Andrade indicator, which is pH sensitive. When penicillin is attacked by penicillinase, peniciloic acid is formed which causes the color of the indicator to change to bright red. If penicillinase was induced by the presence of the penicillin, then this
Figure 1. Standard curve for proteolytic activity.
red color appeared only after several hours had elapsed. If the cells were not able to be induced by penicillin at all, the red color never appeared. Thus the use of these plates allowed a distinction between constitutive, inducible, and non-penicillinase producers.
RESULTS

Growth in Liquid Medium

The growth curves of the four strains of \textit{Bacillus cereus} investigated, 569R, and mutants 45, U29, and U17, in SMCAY are shown in Figure 2. Equivalent inocula of 569R and 45 were used, as determined by spore counts of the spore stocks. A lag of approximately two hours was characteristically observed for both spore formers 569R and 45 before logarithmic growth began. The initial drop in Klett units for 569R and 45 was due to the loss of refractility of the germinating spores, and was not observed with the asporogenous strains inoculated with growing cultures. Logarithmic growth was ending by four hours for both 569R and 45. Strain 45 maintained a consistently higher optical density than 569R throughout the culture cycle. The rise in Klett readings during the stationary phase of growth was caused by the development of refractile endospores in the sporulating cultures. This rise was first noted for strain 45 at about eight hours. Cultures of strain 569R did not show refractility until approximately ten and one-half hours.

The two asporogenous strains were routinely inoculated from overnight colonies grown on plates of G agar. Cultures were always inoculated to a Klett reading of 3.0. This procedure gave fairly
Figure 2. Growth of \textit{B. cereus} 569R and mutants 45, U29 and U17 in SMGAY.
reproducible times for events in the growth cycle. A lag of approximately one hour was typical under standard growth conditions. In some cases, a longer lag in growth of these strains was observed. This occurred when the viability of the cells used as the inoculum was lower than usual. Logarithmic growth was over by four hours for both asporogenous strains. Neither U29 nor U17 was ever observed to reach readings higher than 100 Klett units during the twelve hour growth cycle. U17 rarely exceeded 50 Klett units for this period. Late in the culture cycle, U17 was observed to show a decline in the level of Klett readings due to the lysis of a portion of the cells.

Because of the difficulties involved in making cell counts of chain-forming bacteria with accuracy, a dry weight curve was determined for all four strains. From this curve, shown in Figure 3, it was observed that strain 45 maintained a higher amount of growth, that is, a higher dry weight, during the culture cycle than 569R. A plateau in dry weight increase was seen at nine hours for both 569R and 45. The asporogenous strains, U29 and U17, showed little increase in dry weight throughout the growth cycle. Lysis was reflected in the curve for U17 by a decrease in the dry weight.

The pH curves of these strains were determined to observe whether they showed the same kind of shape found by Halvorson (1957) with B. cereus T. in active culture. Figure 4 shows the results for the two spore forming strains. Both strains reached a pH minimum by
Figure 3. Dry weight curves for B. cereus 569R and mutants 45, U29 and U17 in SMCAY.
Figure 4. pH curves for *B. cereus* 569R and 45 in SMGAY.
three and one-half to four hours in SMCAy, although the pH fell more rapidly for strain 45. The absolute value of the minimum was lower for strain 45; however, in some experiments, both strains reached a minimum of 4.8. When the pH began to rise after the minimum, a shelf in the curve was observed. This plateau occurred at approximately five to five and one-half hours for 569R, and at about four and one-half to five hours for 45. These plateaus were present in all pH experiments run, although in some experiments the plateaus were more pronounced than in others. After these plateaus, the pH rose rapidly, and by six to six and one-half hours, the pH was higher than the initial pH of the culture. Figure 5 shows the pH curves of the two asporogenous strains. The culture of U29 reached its minimum pH at three and one-half hours, while the pH fell more slowly with U17 and the minimum was not reached until four and one-half hours. The pH failed to rise with both of these asporogenous cultures during the remainder of the culture cycle. After the pH minimum, swollen cells were seen in cultures of U29, and lysis was apparent in cultures of U17.

Morphology of Development

Phase contrast photographs of cultures of 569R, 45, U29, and U17 grown for equivalent lengths of time in SMCAy are shown in Figures 6, 7, 9, and 10 respectively. These morphological changes
Figure 5. pH curves for mutants U29 and U17 in SMCAI.
have been correlated with those reported by Fraser and Baird (1968).

Newly germinated spores of 569R (Figure 6a) existed singly rather than in chains and were characteristic of the α stage. Figure 6b shows β stage cells. They were typically found in long chains, and the divisions between cells in a chain were difficult to see. The γ₁ stage cells, after the pH minimum are shown in Figure 6c. Chain breakup was occurring at this time, and the divisions between cells in a chain were becoming more apparent. Figure 6d shows γ₂ stage cells. Shorter chains were in evidence at this time, and the divisions between cells in a chain were clear. At this stage, phase bright granules of poly β hydroxybutyrate were visible. Figure 6e, ten hours, shows the further accumulation of polymer and the beginning of endospore formation. Prespores were not refractile with phase microscopy at this time. Figure 6f shows that phase-bright endospores were present by 12 hours.

Figure 7 shows the development of strain 45 at these same times. The α stage cells observed upon germination of 45 (Figure 7a) were somewhat larger than those of 569R. In Figure 7b, early vegetative cells are shown. Unlike those of wild type, cells of 45 were never found in the long chains characteristic of the β stage in 569R (Figure 6b). In appearance, these cells were similar to the γ₁ stage cells of wild type (Figure 6c). Figure 7c shows the development of phase bright granules of poly β hydroxybutyrate in 45.
Figure 6. Morphological development of \textit{B. cereus} 569R.

a. Newly germinated spores at one hour.
b. Logarithmic stage cells at two and one-half hours.
c. Early \textgamma stage cells at five hours.
d. Late \textgamma stage cells at seven and one-half hours.
e. Cells at ten hours.
f. Endospore development at twelve hours.
Figure 7. Morphological development of strain 45.

a. Newly germinated spores at one hour.
b. Early logarithmic phase cells at two and one-half hours.
c. Five hour cells showing polymer accumulation.
d. Seven and one-half hour cells.
e. Nine and one-half hour cells showing endospore formation.
f. Free spores at eleven and one-half hours.
Polymer formation commenced at an earlier time in cultures of 45 than in cultures of 569R, and the granules appeared to be in higher concentrations than those found in wild type. The appearance of the cells of 45 (Figure 7c, five hours) was similar to that observed for \(\gamma_2\) stage cells of 569R (Figure 6d, seven hours). In Figure 7d, the localization of polymer in one end of the cells was noted. This phenomenon preceded prespore formation. Figure 7e showed phase bright endospore formation. Free spores were observed by the end of the culture cycle as shown in Figure 7f. As can be seen from Figure 8, free spores of strain 45 appeared to be approximately twice as large as the free spores found in cultures of 569R.

Although the asporogenous mutants showed similarities in growth and pH patterns, morphological differences were evident. For U29, long chains characteristic of the \(\beta\) stage in 569R were infrequent early in the growth cycle. Short chains with the divisions between cells in a chain clearly visible were typical as shown in Figure 9a, and b. This type of growth was similar to that of \(\gamma_1\) stage cells in 569R. Cells shown in Figure 9c were beginning to produce polymer granules. Figure 9d showed the further accumulation of polymer by the cells. At this time, swollen cells began to appear. In Figures 9e and f, more swollen and distorted cells were apparent. No spore formation was noted in the twelve hour period of observation.

The development of cultures of U17 is shown in Figure 10. Cells
Figure 8. Free spore size for *B. cereus* 569R and Mutant 45.

a. Strain 45 at 12 hours.
b. Strain 569R at 13.5 hours.
Figure 9. Morphological development of mutant U29.

a. One hour cells.
b. Early logarithmic phase cells at two and one-half hours.
c. Five hour cells showing the beginning of polymer formation.
d. Seven and one-half hour cells.
e. Nine and one-half hour cells.
f. Eleven and one-half hour cells showing some swollen forms.
Figure 10. Morphological development of mutant U17.

a. One hour cells
b. Early logarithmic cells at two and one-half hours.
c. Five hour cells.
d. Seven and one-half hour cells.
e. Nine and one-half hour cells.
f. Eleven and one-half hour cells showing some polymer formation.
early in the growth cycle appeared in long chains typical of the β stage in 569R. Cells of this type were seen in Figure 10a and b. Throughout the culture cycle, these long chains were found to persist. No chain breakup was noted. Little polymer was accumulated by this strain, and that which was formed appeared late in the culture cycle, as seen in Figure 10d and e. Lysis occurred late in the culture cycle also, as shown in Figure 10f.

Renografin Density Gradient Studies

Cultures of 569R, 45, U29, and U17 grown in SMCA Y were examined for shifts in cell density during the growth cycle. In wild type 569R (Figure 11) early logarithmic phase cells at and prior to the pH minimum showed a density of $1.215 \text{ gm/cm}^2$. These cells were in the β stage, and their characteristic band in the density gradients was thus termed the β band. A shift to a lower density was observed at the time corresponding to the shelf in the pH curve after the pH minimum. The cells were then at a density of $1.205 \text{ gm/cm}^2$. Since these cells were in the early γ stage, the band was designated the $\gamma_1$ band. A second shift in density occurred after the pH shelf in the later γ stage. This band was designated the $\gamma_2$ band, and the cells in this band were found floating on top of the gradient. When cells of this stage were banded in less dense 25 to 40%, Renografin density gradients, it was shown that the $\gamma_2$ band had a density of
Figure 11. 46-60% Renografin density gradients of *B. cereus* 569R.
1.125 gm/cm². The corresponding results are given for strain 45 in Figure 12.

For strain 569R, the first shift, β to γ₁, occurred between three and one-half and five hours. This shift was relatively slow. The second shift occurred by six hours and was comparatively sudden. In contrast, the shift for cells of 45 from the β to the γ₁ band had occurred by four and one-half hours, and the second density shift was complete by five and one-half hours. It would appear that cultures of strain 45 undergo these characteristic shifts in cell density approximately thirty minutes before similar shifts are observed in the wild type cultures under the same growth conditions. In both cases, the γ₁ to γ₂ shift occurred just after the shelf in the pH curve, and the dry weight of the cells per ml of culture were nearly the same.

Density gradients of the asporogenous mutants of 569R were run at three different times to observe the patterns of cell densities. The results appear in Figure 13. For U29, a shift in cell density from a β-like band to a γ₂-like band was seen with time. However, these shifts were incomplete, and intermediate bands of varying intensities were observed at all times. No distinct γ₁-like band was noted at any of the three times observed for U29. Cells were distributed from the β-like to the γ₂-like band at all three times. In cultures of strain U17, the same kinds of multiple bands were seen. At three hours a homogenous band was observed which was of a higher density
Figure 12. 46-60% Renografin density gradients of strain 45.
Figure 13. 46–60% Renografin density gradients of U29 and U17.
than the β band of 569R. The most intense band progressed from a low to a higher density with time, but several other bands were observed at four and one-half and six and one-half hours. These asporogenous strains investigated did not show the sharp shifts in cell density with time noted for the two spore forming strains. The populations of the asporogenous mutants were heterogeneous with respect to cell density at the times observed.

Oxygen Uptake and Sporulation

Experiments were performed with 569R and strain 45 to determine the times, if any, during the culture cycle when the absence of sufficient oxygen would retard the time of the appearance of spores in the cultures. In these studies cultures were subjected to lowered oxygen levels by removing them from the shakers, thus stopping aeration, for an interval of one hour at various times in the culture cycle. After one hour, the cultures were again aerated. These cultures were compared to a control culture which was aerated continuously throughout the culture cycle. Using B. cereus 569R, a flask was removed from the shaker for a period of one hour at one of the following times; three, four, five, five and one-half, seven, or eight hours. At 11 and 12 hours, samples from each of these cultures, and from the control, were compared for spore formation microscopically. A photographic record was made of the results. Figure
14 shows the effects of a temporary lack of sufficient oxygen on the time of spore formation in 569R. Cultures removed from the shaker at three and four hours formed spores at the same time as the control. At 11 hours, endospores were visible and beginning to become refractile as shown in Figure 14a. At 12 hours, all the endospores were refractile in these two cultures and in the control as shown in Figure 14b. For cultures removed from the shaker at five, five and one-half, seven, and eight hours, microscopic observation showed that no endospores were visible at 11 hours. These results are shown in Figure 14c and e, and reflect a retardation in spore formation as compared with the control. At 12 hours, endospore formation was seen in these flasks, but refractility was variable, as seen in Figure 14d and f. After eight hours, lack of aeration for one hour appeared to have less effect on the time of spore formation.

The results of a similar experiment with strain 45 are shown in Figure 15. In this experiment, cultures of strain 45 were removed from the shaker for a period of one hour at each of the following times: three, four, five, or six hours. These cultures were compared to a control with continuous aeration for spore formation at eight and one-half hours. Microscopic examination at this time showed that cultures not aerated at three, four, and five hours showed some endospore formation at eight and one-half hours, but none of these endospores was refractile. In contrast, the control culture
Figure 14. Oxygen uptake and spore formation in *B. cereus* 569R.

a. Spore development at 11 hours in the control flask and in flasks removed from the shaker at three and four hours.
b. Spore development at 12 hours for the control flask and for flasks removed from the shaker at three and four hours.
c. Spore development at 11 hours in flasks removed from the shaker at five and five and one-half hours.
d. Spore development at 12 hours in flasks removed from the shaker at five and five and one-half hours.
e. Spore development at 11 hours in flasks removed from the shaker at seven and eight hours.
f. Spore development at 12 hours in flasks removed from the shaker at seven and eight hours.
Figure 15. Oxygen uptake and spore formation in strain 45.

a. Spore development at eight and one-half hours in the flask removed from the shaker at three hours.
b. Spore development at eight and one-half hours in the flask removed from the shaker at four hours.
c. Spore development at eight and one-half hours in the flask removed from the shaker at five hours.
d. Spore development at eight and one-half hours in the flask removed from the shaker at six hours.
e. Spore development at eight and one-half hours in the control flask.
showed phase bright endospores at this time. These results are seen in Figure 15a, b, and e. The flask not aerated at six hours showed the development of spores comparable to the control at eight and one-half hours, as shown in Figure 15d. Microscopic examination of the six hour flask at 13.5 hours showed that free spores were beginning to appear. By this time, the spores in the control culture were almost all free. Thus, the flasks removed from the shaker at three, four, and five hours were greatly retarded in the time of spore formation when compared with a continuously aerated culture. The six hour flask was not so severely affected.

The differences in the times when sufficient oxygen was critical to the time of spore formation were evident for these two strains. In strain 45, oxygen uptake was critical as early as three hours, even before the low pH point, and appeared to be less important by six hours. In cultures of 569R, oxygen uptake did not appear to be of great importance until about five hours, after the low pH point. After eight hours, aeration again became less important to the time of spore formation.

Proteolytic Activity

Since these studies indicated that strain 45 might be skipping a developmental stage, or at least might be exhibiting a shortened developmental period compared with wild type 569R, it seemed
possible that production of late products might occur earlier in the culture cycle for 45 than for 569R. One such late product which was investigated was the formation of an extra-cellular proteolytic enzyme. Preliminary experiments employing albumin agar plates were used to determine the presence of protease in these strains. When protease was made by the cells, a zone of clearing on the cloudy agar plates was seen. To determine whether the cells originally plated were producing protease, or whether growth was necessary for protease production, streptomycin was added to one set of albumin plates. The purpose of the streptomycin was to restrict further growth of the cells. The results of this experiment are given in Table 4.

Table 4. Proteolytic activity on albumin agar plates

<table>
<thead>
<tr>
<th>Time of plating</th>
<th>Strain</th>
<th>Clearing on albumin agar</th>
<th>Clearing on albumin + streptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 hours</td>
<td>569R</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>U29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>U17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10 hours</td>
<td>569R</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>U29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>U17</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Cells of 569R and 45 plated on albumin agar at five hours were able to produce zones of clearing, but no clearing was noted from cells plated at five hours on the albumin + streptomycin plates.
Cells of 569R and 45 plated at ten hours on both albumin and albumin + streptomycin plates were able to produce zones of clearing. For U29 and U17, no proteolytic activity was observed for cells plated at five or ten hours on albumin plates with or without streptomycin. These results indicated that 569R and 45 produced a protease later than five hours, whereas U29 and U17 appeared to be incapable of proteolytic activity even at ten hours by this method.

A spectrophotometric assay was employed to determine the time and amount of proteolytic activity in these strains. In this assay, casein was used as the substrate, and the cleavage of this substrate into small molecular weight products which were perchloric acid-soluble and measurable at 280 m\(\mu\) served as the basis for the determination of protease activity. The results of this experiment are shown in Table 5.

Table 5. Assay for proteolytic activity with casein substrate.

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>569R</th>
<th>45</th>
<th>U29</th>
<th>U17</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.026</td>
<td>0.005</td>
<td>0.010</td>
<td>0.015</td>
</tr>
<tr>
<td>4</td>
<td>0.012</td>
<td>0.024</td>
<td>0.014</td>
<td>0.018</td>
</tr>
<tr>
<td>6</td>
<td>0.032</td>
<td>0.442</td>
<td>0.017</td>
<td>0.006</td>
</tr>
<tr>
<td>8</td>
<td>0.045</td>
<td>0.829</td>
<td>0.036</td>
<td>0.056</td>
</tr>
<tr>
<td>10</td>
<td>0.105</td>
<td>0.249</td>
<td>0.046</td>
<td>0.044</td>
</tr>
<tr>
<td>11</td>
<td>0.112</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0.127</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Strain 569R produced increased amounts of protease beginning at about ten hours, and production increased to the end of the culture cycle. In strain 45, a large increase in proteolytic activity was observed at about six hours, and after eight hours, the level of activity began to decrease. U29 and U17 showed no appreciable increases in activity throughout the cycle. U17 activity rose slightly late in the cycle, but this rise was correlated with lysis of some of the cells which could lead to the liberation of intracellular enzymes capable of degrading casein.

Penicillinase

Several experiments were carried out in an attempt to study the time of appearance of penicillinase in cultures of B. cereus 569R, and mutants 45, U29, and U17. The first step was to classify the strains as constitutive penicillinase producers, inducible producers, or non-producers. To determine this, cells were streaked on Andrade agar plates and allowed to grow overnight. The plates were then flooded with a 5% solution of penicillin G. The immediate appearance of red zones surrounding the colonies is typical of constitutive strains. The appearance of red zones after a delay indicates inducible strains, and the absence of any red zones indicates that the colonies are non-producers. Plates of 569R were found to exhibit red zones three hours after exposure to penicillin. Colonies of strain 45
revealed red zones approximately four hours after exposure to penicillin. Colonies of U29 showed red halos about five hours after exposure to penicillin. However, these strains were classified as inducible producers. After twelve hours, colonies of U17 showed no red zones, indicating that little if any penicilloic acid was formed, and thus suggesting that this strain does not produce penicillinase.

Attempts to measure the presence of penicillinase with time in uninduced cultures by Citri's (1958) method were not successful. The levels of penicillinase present in the uninduced cultures were too low to be measured by this technique.

Cultures of these strains were measured with time for differences in the levels of inducibility of penicillinase. However, the results of these experiments were inconsistent from run to run, and no conclusions could be drawn.
DISCUSSION

The spore forming bacilli have been studied by many workers; however, the main emphasis of their research has centered on studies of the germination process or the sporulation process. Little has been reported concerning the integrated development of such cultures from germination through vegetative growth and sporulation. Our laboratory has been studying *B. cereus* from this point of view, and several interesting aspects of its growth have been reported (Fraser and Baird, 1968).

A group of spore forming mutants of *B. cereus* 569R were isolated as revertants of asporogenous mutants. These mutants were of interest because they maintained a faster growth rate, sporulated sooner under identical conditions, and formed spores which were approximately twice as large as those of wild type 569R. A study of the growth cycle of such a mutant, strain 45, and the correlation of these findings with those for wild type was undertaken.

Morphological studies indicated that strain 45 exhibited no long-chain \( \beta \) stage comparable to that seen in the wild type. In *B. cereus* 569R, the long-chain \( \beta \) stage was associated with a high growth rate. This correlation suggested that growth may have outdistanced cell wall formation, thus leading to long chains. Only a few divisions between cells in these long chains were visible microscopically,
unless the chains were treated with a saturated solution of NaCl to shrink the cytoplasm from the cell walls. Thus, in the β stage, two or more protoplasts usually occupied a single cell wall unit. The β to γ₁ density shift was associated with a transition from the long-chain stage to a short-chain stage in B. cereus 569R. During this chain break-up, the ends of the cells in the chains became more rounded and the divisions between cells in the chains were clearly visible microscopically. The chain break-up and β to γ₁ cell density shift were associated with a slower growth rate, and to a lowered rate of synthesis of DNA, RNA, and protein (Wise and Fraser, 1970).

Although the long-chain β stage was never observed in strain 45, these cells did exhibit the same cell density as β stage cells of 569R during early logarithmic growth. Some difference in the mode of growth of 45 may be responsible for the inability of these cells to remain in chains even during a period of high growth rate which exceeds even that of 569R. It is possible that cell wall synthesis may be involved. Since strain 45 characteristically formed double sized spores, it seemed possible that 45 may contain a double complement of genetic material. If so, then gene dosage effects might possibly influence growth in such a way that cell wall synthesis would not fall behind cytoplasm division even at high growth rates, and long chains would not be observed.

The second rise in pH, following the shelf in the pH curve, has
been correlated with the shift in cell density from $\gamma_1$ to $\gamma_2$ at about six hours in wild type 569R, and it also marks the commencement of poly $\beta$ hydroxybutyric acid synthesis in the cells. This density shift and the pH rise were found to be associated in strain 45, although these events occurred approximately 30 minutes earlier with strain 45 than with wild type 569R, due, apparently, to the faster growth rate of this strain. The $\gamma_1$ to $\gamma_2$ cell density shift occurred relatively rapidly, and represents a depletion of the number of ribosomes per cell, while cell division continues (Wise and Fraser, 1970). In strain 45, however, the formation of poly $\beta$ hydroxybutyrate appeared to precede the $\gamma_1$ to $\gamma_2$ density shift. This observation suggests that there may be some kind of permeability control for acetate in strain 45, and that poly $\beta$-hydroxybutyric acid synthesis and acetate utilization may not be coordinately controlled in this strain. This same kind of phenomenon was observed in the asporogenic mutant U29. This strain formed large quantities of poly $\beta$ hydroxybutyric acid, but the pH of the culture, after reaching its minimum, never rose.

A study of oxygen uptake revealed that oxygen concentration was critical for spore formation in the wild type 569R between five and eight hours. In strain 45, the critical period of oxygen demand for sporulation appeared much earlier, from three to six hours. This very early oxygen demand in cultures of 45 may be explained partly, but not wholly, by the fact that there were a greater number of 45
cells in culture at any time when compared with 569R. Oxygen was thus depleted from the medium more quickly, and a higher oxygen demand would be expected. In terms of optical density, cultures of 45 were approximately one hour ahead of wild type.

The production of higher levels of protease occurred at about ten hours in wild type 569R. This production was associated with the beginning of prespore formation. In strain 45, high levels of protease were observed by six hours. This event preceded the formation of prespores in each strain by one and one-half to two hours. The levels of protease observed in strain 45 at its peak production, even when considered on the basis of protease produced per cell were a good deal higher than those observed for B. *cereus* 569R.

From these results, it appears that we are dealing with two independently controlled sets of phenomena. On the one hand, the density shifts and the pH curves seem to correlate closely with the cell mass of the culture. On the other hand, polymer synthesis, sensitivity to oxygen privation, protease production, and spore formation are independent of the cell mass, and appear markedly earlier in cultures of 45 than they do in cultures of wild type 569R.

Schaeffer (1968) has suggested that since the processes of sporulation in spore forming cultures are repressed by glucose in the presence of a utilizable nitrogen source, catabolic repression could be responsible for the triggering and regulation of sporulation.
such a scheme, derepression for sporulation would occur when glucose had been depleted from the growth medium. If strain 45 is thought of as being derepressed genetically the early appearance of sporulation-related events might be explained. A functional Krebs cycle has been shown to be dispensible for vegetative growth of spore forming cultures, but indispensable for sporulation. Repression of the synthesis of enzymes for the TCA cycle has been shown when a glucose catabolite and an organic nitrogen source are present. The early appearance of an oxygen demand for sporulation in strain 45 could reflect an early switch of metabolism to allow sporulation-directed processes. The early appearance of such sporulation-related events as poly β hydroxybutyric acid synthesis and protease production would tend to support such a suggestion. The appearance of free spores by ten hours as opposed to 13.5 hours for wild type spores is further positive evidence.

For a mutant derepressed catabolically, one might expect earlier sporulation, and a lack of coordination of events such as pH and density shifts with sporulation-related events such as polymer and protease production. In such a mutant it is also possible that there would be a lack of control over the amounts of products produced, and the relatively large amounts of polymer accumulated and protease produced in strain 45 would be explained.

The study of two asporogenic mutants, and a comparison of
their behavior with that of 569R and 45 was also undertaken. The results indicate that neither U29 nor U17 grew well under the standard culture conditions. While the optical density of these cultures increased, the cell mass was less than that of 569R at corresponding times. Initially U29 grew at a rate comparable to that of 569R or 45; U17 always grew at a slower rate. Strain U29 occasionally produced abortive spores during the late stages of growth, whereas in U17 such an event was never observed. Neither strain produced increased amounts of protease, and U29, like 569R and 45, was found to be inducible for penicillinase production while U17 was found to be a non-producer.

Both mutants gave similar pH curves, with the pH never rising after a minimum value was reached. Although the pH did not rise, U29 produced large amounts of poly β hydroxybutyric acid, indicating that perhaps acetate utilization and polymer synthesis were not under coordinate control in this mutant. Strain U17 produced very small amounts of polymer late in the culture cycle.

We initially thought that these mutants might be blocked in the transition from β to γ metabolism, since the pH failed to rise. U17 maintained a β stage morphology throughout its growth. However, U29 exhibits some characteristics ordinarily found in the later stages of 569R development. A study of the density shift patterns of these mutants revealed that there were no orderly sequences of density
shifts. A variety of density bands were observed at a given time. In U29, when several bands of cell densities were observed, nearly all the cells were shown to contain poly β hydroxybutyric acid. Such results suggest that there is no regulation over cell density in these mutants such as is seen in the spore forming strains. It would also appear that sequential shifts in cell density do not seem to be necessary for events such as polymer synthesis to occur.

The mutant studies indicate that the various events under investigation may not necessarily occur sequentially, although they do in cultures of the wild type B. cereus 569R. It might be of interest to isolate mutants blocked at the β to γ1 cell density transition, and to study what events toward sporulation such mutants could carry out.
SUMMARY

The purpose of this research has been to characterize a spore-forming mutant of *Bacillus cereus* 569R and to compare it with the wild type. Two asporogenous mutants of *B. cereus* 569R have also been examined.

The spore-forming mutant, strain 45, was first examined for its growth in SMCAE liquid medium. Its growth rate was found to be higher than that of wild type 569R, and its pH curve, although similar, was approximately 30 minutes ahead in time of the curve for 569R.

A study of the morphological development of strain 45 revealed the absence of the long chain early logarithmic stage characteristic of cultures of 569R. The presence of poly β hydroxybutyrate in cultures of 45 was noted several hours earlier than in cultures of the wild type. This polymer was also found to be present in higher concentrations in strain 45. The fact that polymer was seen in cells of strain 45 prior to the sharp rise in pH has suggested that acetate utilization and polymer formation may not be coordinately controlled in this mutant. The formation of spores occurred approximately two and one-half hours earlier in cultures of 45. It was also noted that spores of strain 45 were about twice as large as typical spores of 569R.
Renografin density gradients were used to determine the cell densities, and the times of the density shifts in the culture cycle. Like the wild type, strain 45 showed the \( \beta \) to \( \gamma_1 \) and the \( \gamma_1 \) to \( \gamma_2 \) cell density shifts. In strain 45, these shifts occurred approximately 30 minutes earlier than in cultures of wild type 569R.

Studies of the effect of oxygen privation on the time of spore formation in 569R and 45 were undertaken. In 569R oxygen demand was critical to the time of spore formation between five and eight hours. In strain 45, the critical period appeared to be from three to six hours. Thus oxygen was critical to the time of spore formation in 45 even before the pH minimum was reached.

An assay of the culture supernatants of 45 and 569R for proteolytic activity revealed that 45 produced protease in much higher concentrations than were found in the wild type. Cultures of 45 showed their highest proteolytic activity at eight hours, while the activity did not appear in significant amounts in wild type cultures until ten hours.

The results of these experiments indicated that two independently controlled sets of phenomena were present. One group of results, the density shifts and pH curves, appeared to be correlated with the cell mass of the culture. The time of polymer synthesis, sensitivity to oxygen, protease production, and sporulation appeared to be independent of cell mass. For this last set of data, the time of
appearance was earlier by about two hours in strain 45. These findings have suggested to us that strain 45 may be catabolically derepressed for sporulation. The early appearance of these sporulation-related events would tend to support such a hypothesis.

A study of two asporogenic mutants of B. cereus 569R, U29 and U17, showed that neither strain grew well under the standard growth conditions. U29 formed polymer, and occasional abortive spores. U17 remained in the long chain state throughout its culture cycle. For both strains, the pH never rose after reaching its minimum value. Both strains showed heterogeneity in Renografin density gradients, and neither strain produced increased amounts of protease late in the culture cycle.


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