AN ABSTRACT OF THE THESIS OF

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Title: PROPERTIES OF RIBONUCLEIC ACID AND DEOXYRIBONUCLEIC ACID DURING DEVELOPMENTAL STAGES IN B. CEREUS 569

Abstract Approved: D. K. Fraser

Two different aspects of the life cycle of a spore-forming Bacillus are presented in this study. The developmental cycle in Bacillus cereus 569R involves a series of changes in cell states. Beginning with a germinating spore, several clearly distinguishable cell stages appear, leading ultimately back to a mature spore. Three of these stages are defined in terms of cell morphology, density of the organisms, and patterns of macromolecular synthesis which are found during different periods. The first period examined was the \( \beta \) stage, characterized by cells in long chains which band at a density of 1.215 g/cc in Renografin gradients and have a rapid rate of division and macromolecular synthesis. The cells next appear in the \( \gamma_1 \) state, and exhibit a slight decrease in cell density to 1.205 g/cc, a shorter average cell chain length and a slowed but continued logarithmic increase in macromolecular components. The final \( \gamma_2 \) stage, involves cessation of net RNA synthesis followed by a rapid decrease in cell density to 1.125 g/cc. Both the \( \beta \) and \( \gamma_1 \) cell stages are highly dependent on cell concentrations and the transition from the \( \beta \) to the \( \gamma_1 \) state is reversible. However, the \( \gamma_2 \) cells, although able to continue
division when diluted or placed in fresh medium do not return to a previous cell density. During the $\gamma_1$ to $\gamma_2$ transition, $\lambda$S RNA synthesis occurs in absence of ribosomal RNA synthesis. The permanence of the $\gamma_2$ cell state is presumed to be due to the inability of the cells to restore a $\gamma_1$ level of ribosomes per cell even though the $\gamma_2$ cells can divide repeatedly, Thus, the $\gamma_2$ cells may represent an irreversible commitment toward spore formation.

The second portion of this work involves experiments on the genome size and nuclear DNA equivalents of B. cereus 569R. Several genetic observations in our laboratory suggested that B. cereus 569 might be diploid. The estimation of genome size and its relation to the amount of DNA per spore was determined and the spores of B. cereus 569 are shown to have sufficient DNA to contain two complete genomes. This physical evidence, along with genetic evidence suggests that B. cereus 569 is diploid.
PROPERTIES OF RIBONUCLEIC ACID AND DEOXYRIBONUCLEIC ACID
DURING DEVELOPMENTAL STAGES IN P. CEREUS 569

BY
John Arnold Wise

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INTRODUCTION

This section of the thesis is not intended to provide an exhaustive review of the literature concerning development and sporulation in bacteria. Rather, the intent is to define an approach to the study of growth and development in microorganisms which has been used in this laboratory. It involves the consideration that populations of bacterial cells react in a variety of ways with their environment and with each other. A number of diverse observations are cited in an attempt to show the complexity of interactions that exist in populations of unicellular organisms, with some intent of correlating these observations to studies on Bacillus cereus 569.

Additional references will be cited with the results and discussion when appropriate. For further information on sporulation, the reader can consult several recent reviews on the subject (1-4).

The Developmental Cycle

The study of spore-forming bacteria provides a unique opportunity to investigate a process of differentiation at the cellular level. The spore represents a dormant state in which little or no metabolic activity occurs. The breaking of dormancy leads through a series of steps to outgrowth and formation of a vegetative cell. The cell can then divide repeatedly until environmental conditions invoke another series
of responses which lead back ultimately to the establishment of dormancy. This cycle necessarily involves the control of gene expression since different genes are required for the formation and germination of spores, than are needed for vegetative growth (2, J. C. Vary, personal communication).

In the next two sections, a brief description of the most extensively studied aspects of this developmental cycle are presented. First, the breaking of dormancy, or germination, is discussed. Then some aspects of sporulation are described.

Spore Germination

Germination of spores is an irreversible process involving the sequential loss of heat resistance, dipicolinic acid, impermeability to dyes, calcium, refractility, and optical density to visible light (20). Germination is a degradative process, which does not require protein or RNA synthesis, but appears to involve a number of pre-existing enzymes. The post-germinative development or outgrowth of spores, consists of swelling and emergence of a cell, followed by elongation and cell division. Outgrowth involves active biosynthesis of RNA and protein, and later DNA synthesis. If B. cereus spores are activated by treatment with heat or reducing agents and then placed in nutrient medium with adequate aeration, germination is rapid (5-10 minutes) and the subse-
quent outgrowth and cell divisions are synchronous for several generations.

The breaking of dormancy leading eventually to the vegetative state involves an ordered sequence of events. Ribosomes present in spores of *B. cereus* T were found to be defective when tested in poly-U directed, amino acid incorporating system (22). After germination, RNA synthesis begins and is necessary to activate the defective ribosomes. Several minutes later, protein synthesis commences.

Messenger RNA, which is not present in detectable levels in the spore, as well as ribosomal and transfer RNA species are synthesized at early times during outgrowth. This early ordering of biochemical events is following by a periodicity of enzyme synthesis during outgrowth. Enzymes such as α-glucosidase, alkaline phosphatase and histidase appeared sequentially at different times during outgrowth (20). This suggests that an ordered transcription occurs, and that the entire genome is not accessible to RNA polymerase once outgrowth begins. In addition, the pattern of enzyme synthesis observed during outgrowth was repeated over several division cycles in synchronous vegetative growth (23), suggesting that similar transcriptional and translational controls operate during both phases of development.

In summary, spore germination and outgrowth involve an ordered and uni-directional sequence of events which are irreversible, and in which the end result is a vegetative cell.
Spore Formation

Sporulation has been divided into seven stages according to cytological changes observed with the electron microscope (1). Stage I is characterized by the end of logarithmic growth and the condensation of the nuclear regions into an axial filament. Little or no net DNA synthesis occurs during Stage I. The beginning of septum formation at one end of the cell defines the beginning of Stage II. The completion of this forespore septum results in the segregation of the nuclear material into two compartments referred to as the mother cell and forespore cytoplasmic units. The forespore cytoplasm occupies a volume equivalent to approximately 20% of the vegetative cell and usually contains one-half the DNA (82).

During Stage III, the forespore protoplast is engulfed as a result of uni-directional growth of the cytoplasmic membrane toward the end of the cell. This results in a complete spore protoplast enclosed in its own membrane and distinctly free in the cytoplasm. The next three stages involve the synthesis of structural spore components. Cortex formation occurs in Stage IV, and dipicolinic acid (DPA) synthesis begins. After completion of the cortex, the spore coat appears (Stage V) accompanied by cystine-rich coat proteins which are partially responsible for radiation resistance of spores. Calcium uptake also occurs during Stage V. Spore maturation (Stage VI) is the result of the coat material becoming
denser leading to increased refractility of the spores and development of heat resistance. Finally, the mother cell disintegrates because of the action of lytic enzymes and the spore is liberated (Stage VII).

The described stages in sporulation occur sequentially and can be correlated with numerous biochemical events also appearing in an ordered manner. A large number of mutants have been obtained which are blocked at various cytological stages during sporogenesis (2, 83). If the block occurred at an early stage, biochemical events associated with later stages did not appear (83). Conversely, mutants blocked at later stages, produced all the enzymes normally found early in sporulation. Thus, spore formation appears to involve a linear sequence of events, each of which is dependent on those preceding it.

A map of the genes producing asporogeny in B. subtilis, shows them to be located throughout the chromosome with four main clusters appearing (85). The total number of cistrons which affect the ability of B. subtilis to form a heat-resistant spore has been estimated at approximately 100 (ref. 84) to approximately 800 (ref. 83). However, the exact genetic lesion responsible for asporogeny is known with certainty in very few mutants. A further complication with mutant studies is that all early stage asporogenous mutants and some later stage mutants exhibit unidirectional pleiotropic effects which prevent expression of subsequent biochemical and morphological events (83).
Commitment to Sporulation

The concept of commitment to sporulation has been discussed for some time. Briefly, it suggests that once cells initiate the sporulation process, they will continue to sporulate regardless of changes in medium or other manipulations (86). However, recent experiments have shown that transfer of B. cereus into fresh medium prior to the beginning of Stage III results in renewed growth and subsequent division of both the mother cell and forespore cytoplasm (87). When transferred after the completion of Stage II, the mother cell can renew division, but the forespore does not reproduce and continues to develop to a point intermediate between Stages II and III. Cells that have completed Stage III continue to develop and form mature spores. While these results do show a commitment to sporulation, it occurs well past the first stage of sporulation, and the commitment of the mother cell is independent of commitment of the forespore.

Recently it has been suggested (21) that sporulation is merely a specialized case of cell division. The two differ in that development of the forespore septum is acentric and there is usually no observable deposition of cell wall peptidoglycan between membrane layers of the septum (1, 87). However, dilution of Stage II cells of B. cereus (completed forespore septa) into fresh medium results in the transformation of the forespore septum into a division septum by accumulation of cell
An Integrated Study of the Developmental Cycle in *B. cereus* 569R

The events just described leading to and from the dormant state provide the research emphasis for most investigators. However, a bacterial species which can produce spores provides an opportunity to examine the transitions from a spore to a vegetative cell and back to a spore as a complete developmental cycle. One value of such an approach is the ability to study the changing properties of the cells and their environment as a function of cell number and time. Certainly in nature these organisms exist in a microenvironment and the numbers of cells and their metabolic activities affect this environment and lead to altered responses by the cells which in turn elicit more environmental changes. Thus, the state of the cells at any time is dependent on previous events, and a newly attained developmental state will influence subsequent changes.

*B. cereus* 569 was the organism chosen for studying the developmental cycle. To examine developing cultures, tests were used by which individual organisms, rather than the culture as a whole, could be identified. Fraser and Baird (14) have classified individual organisms into developmental or physiological states by 4 methods: (a) Gram reaction; (b) growth
potential as revealed by the microcultures derived from single organisms or chains of organisms isolated into microdrops; (c) ability of individual organisms to take up neutral red; (d) electrophoretic distribution of organisms through a pH gradient. With these techniques, the authors sought to determine whether organisms changed state in a gradual manner or in a sudden, quantal manner, and whether the population consisted of organisms which were all in one state of development at any given time or whether it was heterogeneous.

*B. cereus* 569 spores were inoculated into a defined growth medium and samples were removed at 5 minute intervals for Gram staining. All the organisms sampled before 3.5 hours were Gram-negative. During the period from 3.50 to 3.75 hours, all the organisms became uniformly more darkly stained, and after 3.75 hours all the organisms were Gram-positive.

The growth potential of the organisms revealed four distinct types of development and five cell states (*\( \alpha \) through *E*) were designated. The organisms in *\( \alpha \)* state appeared between 2 and 4 hours, were usually single and divided once, if at all. Chains in the *\( \beta \)* state were found between 4.5 and 9 hours and formed clones of about 100 organisms but no spores. Cells in the *\( \gamma \)* state also formed clones of about 100 organisms which in turn usually all formed spores. Organisms in the *\( \delta \)* state (7.5 - 13 hours) were a minority group which neither divided nor formed spores. Finally, *E* cells appeared between 9.5 and 13 hours and did not divide or produced spores. Thus, the presence of organisms in any state
appeared in overlapping peaks during development, and at any given time, the organisms were not usually in the same state.

The staining of cells with neutral red caused them to appear bright under a phase contrast microscope while unstained organisms were dark. During outgrowth cells stained uniformly. At later times individual cells reacted differently with the dye, and commonly some cells within a chain were bright while others were dark. Bright cells appeared in seven successive peaks during development and were correlated with times of chain break-up and cell lysis.

Organisms were also examined by an electrophoretic technique which determined their isoelectric points and not their electrophoretic mobility. The results showed that at any time there existed 4 to 6 groups of organisms which differed in isoelectric point, with lack of any continuous distribution of charges. Furthermore, bands varied in width and position at different times.

The authors concluded that at any time during development, the cultures consisted of at least 2 sub-populations of cells in different states of development. The future behavior of the organisms when placed in a microdrop had apparently already been determined by previous interactions in a changing environment. Finally, with the exception of the response to the Gram reaction, no organisms were found in transitions from state to state and therefore, the shifts must occur rapidly in individual organisms.
At least seven periods of lysis were also observed during the developmental cycle in *B. cereus* 569 (14). Microscopic observation showed some organisms were disintegrating or cell material was being extruded through the cell wall at one or more places. The lysis seemed to follow closely the peaks in appearance of bright organisms. Recently *B. cereus* 569 was shown to be carrying two different lysogenic phages (S. Haworth, personal communication). Several periods of phage release have been observed during growth and could account for at least some of the lytic phenomena. In addition, one of the phage has been shown to carry out generalized transduction of *B. cereus* 569 mutants (95). The role of phage in the developmental process is not certain, but the induction of cell lysis by phage or lysis from other causes adds a complicating factor to the developmental cycle.

The Complex Nature of Growth and Development

It has long been observed that when a bacterial culture was transferred to a new growth medium, a lag in cell division occurred. The duration of this lag was shown to be dependent on the inoculum size. Studies by Lankford, et al. (5) on a number of *Bacillus* cultures, revealed the presence of a compound in culture filtrates which would overcome the inoculum-dependent lag. This material was termed "schizokinin", and was shown to have specificity for the *Bacillus* sp. which produced it.
In reciprocal tests, some cross stimulation was observed, while one Bacillus produced a "schizokinen" which had a strong inhibitory effect on several other strains.

Byers, et al. (6) later identified the "schizokinens" as a class of Fe(III)-chelating secondary hydroxamates. A related class of compounds with similar growth stimulating properties for Arthrobacter were termed siderochromes. These authors postulate that all microbial cells may require an exogenous schizokinen for initiation and maintenance of cell division.

Cytokinins, long known to promote cell division, growth, and morphogenesis in plants, have recently been found in yeast and E. coli (7). They were present in the tRNA fractions and have been shown to consist of minor bases in the tRNA hydrolysates. A stimulatory growth reaction has been elicited in bacteria by cytokinins, but whether they have true biological significance in bacteria is not known (13).

Cell concentrations also appear to be important in the developmental cycle. It has frequently been observed that high concentrations of spores germinate less well than low ones (16). In B. cereus it was found that $10^8$ spores per ml yielded only 10% germination in a nutrient medium while $10^6$ spores per ml resulted in over 90% germination. The nature of this self-inhibition is not certain, but some activation treatments such as heat may exert their effects at least partially by the neutralization of self-inhibitors (19). With B. cereus 569, the initial number of spores
present influences outgrowth and cell division of the population. Thus, with high spore concentrations, increased growth inhibition and lysis of outgrowing cells was observed (Fraser, unpublished). The $\alpha$ cells represent an early cell type which cannot divide and may result from inhibitory substances released in the medium. This type of auto-inhibition appears to control development by limiting the number of cells which are able to survive and undergo further division.

The presence of sporulation may similarly be influenced by cell numbers. When early sporulating cells of $B. \text{cereus}$ T were transferred to non-growth medium, the cell concentration influenced the ability of cells to sporulate (J. Wise and B. D. Church, unpublished). If cells were suspended in the same volume of non-growth medium ($2-3 \times 10^9 \text{cells/ml}$) nearly all the cells formed spores. However, early sporulating cells in the same non-growth medium at $3 \times 10^7 \text{cells/ml}$ produced less than 10% spores and lysis of the cells was noted. This observation suggests the possibility that extracellular factors may be involved in spore formation. Such sporulation factors or sporogens have been isolated from sporulating bacteria and appear to induce sporulation in log phase cultures (11, 12).

Many low molecular weight compounds are also released from cells during spore formation. At least 20 significant peaks of 280 nm absorbing material could be obtained by column separation of supernatant fluids from sporulating cultures of $B. \text{cereus}$ T (J. Wise and H. Orin Halvorson, unpublished). The presence of these unidentified compounds must be
considered when examining the developmental process. In addition, several proteins appear in the sporulation supernatant including a protease, ribonuclease, $\alpha$-amylase and several peptide antibiotics (4). None of these extracellular materials have a known function in sporulation, but their amounts cannot be accounted for by cell lysis. The finding that one asporogenous mutant can induce sporogenesis in another by a cross-feeding reaction (Fraser, unpublished) definitely suggests a role for extracellular substances in spore formation.

In Neurospora a number of observations have been made concerning extracellular compounds and their relation to circadian rhythms and sporulation. A diffusible compound has been shown to promote synchronous asexual sporulation in Neurospora colonies. However, a mutation, apparently at a single site, abolishes synchrony, but does not affect sporulation (8). The circadian rhythm in the growth of Neurospora was shown to be coordinated by a diffusible compound(s). If diffusion was inhibited by bisecting a colony with a coverslip, the 2 groups of cells exhibited separate, non-coordinated circadian rhythms (9). Another compound, termed a sporogen, could stimulate sporulation in Neurospora when added to non-sporulating cultures (10).

An additional complication in studying growth cycles in microorganisms involves the possibility that cell-cell interactions may be of importance. Indeed, cell clumping is common in Bacillus sp. and has been observed in at least 2 stages of the $B.\,cereus$ 569 cell cycle (14). Also, genetic
exchange has been shown to occur in *B. cereus* 569, possibly by a mechanism which requires cell contact between genetically distinct cell chains (15). In a number of respects *B. cereus* is not unlike some strains of *Actinomycetes*, which tend to grow in long hyphal chains that clump extensively, and the clumping promotes DNA exchange between chains (69).

The importance of these cell-cell interactions during a growth cycle is not clear. However, some fungi require a period of cell contact prior to stationary phase in order for sporulation to occur. (G. B. Calleja, personal communication and 18). In this case, cell clumping was shown to be reversible and specific, i.e., only cells which were derived from a floc (clump) could participate in reflocculation. Similar specificity has been observed when strains of sporogenous and asporogenous *B. cereus* 569 were grown in mixed culture, and clumps of spore-forming cells contained few, if any asporogenous chains (Fraser, unpublished). In addition, Calleja found that the specificity of flocculation resided in extracellular proteins, the removal of which rendered the cells incompetent for reflocculation without adding back the protein (18).

The above observations on microbial growth and development do not represent a complete survey of the subject nor do they provide any unifying picture of the mechanisms operating during cell cycles. However, it is felt that these diverse, and sometimes poorly defined aspects of population growth emphasize the complexity of interactions occurring between cells and their environment. One must take into account products
from one cell affecting other cells. It appears that in many cases threshold levels of factors accumulate which can cause rather sudden changes in the entire cell population. Some of these factors could be considered analogous to the hormones present in higher organisms. In studies of developmental cycles in bacterial systems, cell-environment interactions and cell-cell interactions should be considered. And wherever possible, developmental changes should be examined on an individual cell basis for a clearer understanding of the state of cells at any given time during development.

Statement of Purpose

The research presented in this thesis concerns two different aspects of the life cycle of *B. cereus* 569R. The first involves studies on various cell stages occurring during growth and sporulation of *B. cereus* 569R. These stages are defined in terms of cell morphology, density of the organisms, and patterns of macromolecular synthesis which are found during different periods. In addition, the changes from one stage to another are examined for the influence of cell numbers on the reversibility or permanence of these changes.

The second aspect of this work involves experiments on the genome size and nuclear DNA equivalents of *B. cereus* 569R. Several genetic observations in our laboratory suggested that *B. cereus* 569 might be diploid. The estimation of genome size and its relation to the amount
of DNA per spore was determined in order to obtain supporting physical evidence for diploidy of this organism.
MATERIALS AND METHODS

Bacterial Strains

Bacillus cereus 569R - A rough variant of NRRL 569 obtained from C. B. Thorne.

Bacillus megaterium 899 - From the collection of A. Wolf, obtained through H. O. Halvorson.

Bacillus subtilis 168 - Obtained from J. Spizizen.

Escherichia coli B/r - From the laboratory collection of D. K. Fraser.

Growth Media

B. cereus 569R was routinely cultured in a liquid defined medium containing: Vitamin Free Casamino Acids (Difco) 5.0 gm/l, K₂HPO₄ (0.25 gm/l), KH₂PO₄ (0.25 gm/l), MgSO₄ (0.1 gm/l), NaCl (5.0 mg/l), FeSO₄·7H₂O (5.0 mg/l), MnSO₄·4H₂O (5 mg/l). Glucose (2.0 gm/l) was added aseptically after autoclaving. This medium is designated SMCA.

For making spore stocks, yeast extract (1.0 gm/l) and CaCl₂·2H₂O (0.08 gm/l) were added to SMCA.

Viable counts were done on nutrient agar (Difco).

B. megaterium 899 and B. subtilis 168 were grown in penassay broth (Difco). Spores from both of the above were obtained by plating on
potato-dextrose agar and incubating the plates at 34°C for 4 days.

Spore Stocks

After release from sporangia, spores of *B. cereus* 569R were harvested, washed repeatedly in distilled water (at least ten times) at 4°C until all microscopically visible debris and whole cells were removed. The clean, washed spores were suspended in distilled water (3 x 10^{10}/ml), distributed in 0.5 ml aliquots to screw cap tubes and frozen at -20°C. A working stock solution of spores was obtained by thawing a tube, diluting ten fold with distilled water (1.5 x 10^{9}/ml) and storing the spore suspension at 4°C.

Spores to be used for DNA determinations were scraped from plates, washed twice with distilled water, treated at 37°C with lysozyme, (200 μg/ml, 30 min.) followed by DNase (10 μg/ml) and RNase (50 μg/ml) for 30 minutes. The spores were washed again with distilled water, centrifuged through 65% Renografin and the spore pellets washed an additional 5 times with distilled water. Spore counts were made with a Petroff-Hauser counting chamber using triplicate dilutions in 0.1% sodium lauryl sulfate (to disperse spore clumps).

Growth Conditions

A sample of a spore stock solution was heat shocked (65°C for 15
min.) and added to 50 ml of SMCA in a 250 ml erlenmeyer flask to give a final concentration of 3-5 x 10^6 spores/ml. The flask was then aerated by shaking at 34°C. When larger volumes were required (i.e. for multiple samples) the desired volume of SMCA was first inoculated with 3-5 x 10^6 spores/ml and 50 ml aliquots distributed to 250 ml flasks. For most experiments, a replacement technique was used in which, for example, a 10 ml sample removed from the experimental flask was immediately replaced with a 10 ml sample from a supplementary flask and the contents of the second flask discarded. Therefore, if twenty 10 ml samples were required over a period of several hours, twenty 50 ml cultures were started, thus insuring a constant volume in all flasks, but also avoiding some of the variation observed when sampling from multiple flasks.

When large volumes of cells were required, but when constant conditions from experiment to experiment were not critical, 2.8 liter fernbach flasks containing one liter of SMCA were used, and inoculated with heat shocked spores as before.

Continuous culture experiments were carried out using a simple turbidostat. An SMCA grown culture (50 ml) at a desired cell stage was placed in the reservoir (total capacity-100 ml) and the turbidity of the culture was maintained by constantly dripping in fresh SMCA at the required rate. The reservoir was placed in a water bath (34°C) and an air-driven magnetic stirrer was used to agitate the liquid in the reservoir while a tube extending to the bottom of the reservoir provided filter-sterilized
air. Finally, an outflow tube was placed with its orifice at the 50 ml level and the air pressure in the reservoir forced media out as it rose above the 50 ml level, as well as providing an exit for the pumped-in air.

Growth Measurements

Turbidity of cultures was measured using a Klett-Summerson colorimeter with a 660 nm filter (red).

The pH profile of B. cereus 569R cultures growing in SMCA was highly reproducible, and therefore provided an accurate means for determining growth stages.

Total cell counts were done with a Petroff-Hauser counting chamber. During early growth stages (β cells) it was necessary to plasmolyze the cells with saturated NaCl to accurately count the number of cells per chain.

Dry weight determinations were done in triplicate. Samples (10 ml) from cultures were centrifuged at 10,000 rpm for 15 min. in a Sorvall refrigerated centrifuge and the pellets were resuspended in 2 ml of distilled water. Three 0.5 ml aliquots were pipetted onto preweighed aluminum planchets, dried overnight at 70°C and weighed.
Cell Density Measurements

Renografin-76 is a non-toxic, low viscosity, high density solution composed of 76% N,N'-diacetyl-3,5-diamino-2,4,6-triiodobenzoate in buffer. This material was first used by Tamir and Gilvarg (24) to separate E. coli from B. megaterium cells and to separate cells from spores. It has been used successfully in our laboratory to distinguish stages of development in B. cereus 569R.

Renografin-76 was considered to be a 100% solution. Density gradients of 46-60% or 25-40% were routinely used to determine the density of cells in culture. Linear 4.8 ml gradients were made using a gradient maker as described by McConkey (25). The higher density solution was introduced at 2.35 ml while 2.45 ml of the lowest density material was used. A polystaltic pump (Buchler Inst.) was used to pump the solution into the gradient tube. A bent, motor driven wire was present in the chamber containing the highest density solution, and served as a mixer.

Gradients were placed at 4°C for at least one hour before layering with a 0.2 ml sample containing $5 \times 10^7$ cells. The cells were suspended in Renografin at a concentration one-half that of the low concentration in the gradient (i.e. 23% for a 46-60% gradient).

Gradients were centrifuged in a Beckman L2 ultracentrifuge at 4°C using a SW50 rotor. After 1 hr. at 22,000 rpm, the tubes were either examined visually or the gradients were quantitated by puncturing the
bottom of the tube, collecting drop fractions and plating the samples on nutrient agar.

Renografin absorbs strongly at 260 nm and therefore, the density in any gradient fraction could be routinely determined by diluting and reading the absorbance at 260 nm. By previously comparing the O.D. 260 to the absolute density by weight of fractions from a gradient, the O.D. 260 can be used as a direct measure of density.

Cell Fractionation for Chemical Assays

Samples (40 ml) were centrifuged at 10,000 rpm for 10 min. at 4°C. The cell pellets were suspended in two ml cold distilled water. Ten ml of ice-cold 6% perchloric acid (PCA) were added to each sample, and after mixing thoroughly, placed in ice for at least 15 minutes. The solutions were centrifuged at 5,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet suspended in two ml of 6% PCA. The samples were heated at 90°C for 15 minutes, the tubes cooled and centrifuged at 5,000 rpm for 10 min. while still warm. The pellets were washed once with 2 ml of ice-cold 6% PCA and the wash combined with the above supernatant fractions for the assay of RNA and DNA. The pellet was dissolved in two ml of 1 N NaOH for protein determination.

DNA was determined by the procedure of Burton (26) using deoxyadenosine as a standard. RNA was assayed by the orcinol reaction (27)
using D-ribose as a standard. For the estimation of protein, the method of Lowry et al (28) was used with bovine serum albumin as a standard. The above determinations were done in duplicate for all samples.

The DNA content of spores was estimated by a procedure similar to that of Dennis and Wake (29). Clean washed spore samples (1-3 x 10^{10} in 0.5 ml) were treated with ten ml of 5% trichloroacetic acid (TCA) at 0°C for 30 min. After centrifuging, the pellet was extracted twice with three ml of 5% TCA at 90°C for ten minutes. The combined extracts were made up to ten ml with 5% TCA and the deoxypentose measured by the method of Keck (30) using deoxyadenosine as a standard.

Determination of RNA Synthesis

RNA synthesis in *B. cereus* 569R was measured using the radioactive isotope $^{32}\text{P}$ as inorganic orthophosphate.

For measuring the rate of RNA synthesis during growth (31), two ml samples were removed from a 50 ml culture at intervals, exposed to $^{32}\text{P}$ for 10 minutes, mixed with an equal volume of ice-cold 20% TCA and held at 0°C for at least 30 minutes. The samples were divided in half, and the acid-precipitable material was collected on each of two filters which had been pre-washed with 10% TCA at 0°C. The precipitate retained on the two filters was washed three times with 10% TCA at 0°C and one time with ethyl alcohol at 0°C.
The following lipid extractions were performed with solvents maintained at 40 to 45°C: twice with 70% ethyl alcohol, twice with ethyl alcohol-diethyl ether (1:1), and once with diethyl ether.

The two filters were then removed and air dried, and one was counted for total $^{32}$P. The second filter was placed upside-down in a 20 ml beaker, and a two ml amount of 0.5 N NaOH at 27°C was added and the beaker kept at 37°C for exactly 40 minutes. The alkaline hydrolysis was terminated by immersing the beaker in ice and adding 0.6 ml of 50% TCA (final conc. 5% TCA).

After 30 minutes or more at 0°C the contents of the beaker, including the disintegrating filter, were poured onto a new filter and the beaker, the old filter and the acid-insoluble DNA and protein were washed three or more times with 5% TCA at 0°C. A final wash with diethyl ether was used to remove the TCA and the filter was dried and counted. The difference in counts between the filter that was subjected to alkaline hydrolysis and the one which was not, represents the total $^{32}$P in the RNA fraction.

The $^{32}$P was counted by placing the dried, acid-free filters in scintillation vials containing 10 ml of the following mixture: 4 gm of 2,5-diphenyloxazole (PPO) and 100 mg of dimethyl 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in one liter of toluene. Radioactivities were measured with a two-channel, tri-Carb liquid scintillation spectrometer (model 3314; Packard Instrument Co., Inc.).
RNA Extraction

Thirty ml culture samples were pulse-labeled with $^{32}$P for 30 min., the samples were chilled, the cells harvested by centrifugation, and washed by suspending in 0.15 M-sodium acetate (pH 6.5). After recentrifugation the cells were suspended in two ml 0.15M-sodium acetate and broken with an Eaton press (32). Breakage, as determined by microscopic observation, was never less than 80% under these conditions.

RNA was isolated from B. cereus 569R by the method of Kirby (33) as modified by Bishop et al. (34). The broken cell suspension was made up to a volume of 10 ml with 0.15 M-sodium acetate and 0.5 gm of triisopropynaphthalene sulfonate (Eastman Organic Chemicals) was added and the mixture shaken at 4°C for five minutes. Ten ml of a phenol-cresol mixture (500 gm phenol, 70 ml redistilled M-cresol, 0.5 gm 8-hydroxyquinoline, and 55 ml aqueous 0.15 M NaCl) were then added and the mixture shaken at 4°C for 10 minutes. The mixture was centrifuged at 8,000 x g for 10 minutes at 4°C, and the upper aqueous phase was carefully removed. Sodium chloride was added to a concentration of 0.5 M and the solution was re-extracted with 10 ml of phenol-cresol mixture at 4°C. The aqueous phase from the second extraction was mixed with 2 volumes of cold ethanol and the nucleic acids allowed to precipitate at -20°C for one hour. The precipitate was recovered by centrifugation at 12,000 x g for 30 minutes, washed twice with 75% ethanol and once with
ethanol, dried by evacuation and finally dissolved in 0.1M sodium acetate (pH 5.2) plus 0.05 M NaCl. The RNA fractions were then resolved on sucrose gradients or by polyacrylamide gel electrophoresis.

Characterization of RNA on Sucrose Gradients

Sucrose gradients were formed in the same manner as previously described for Renografin gradients (25). Linear gradients of 15% to 30% sucrose in 0.1M sodium acetate (pH 5.2) plus 0.05 M NaCl were layered with 100–200 µg of RNA in a volume of 0.2 ml, and centrifuged in an SW 50L rotor at 37,000 rpm for 10 hours at 4°C. Gradients were analyzed by puncturing the bottom of the tubes and assaying the drop fractions for absorbance at 260 nm and for radioactivity.

DNA Extraction:

Four to five grams of wet packed B. cereus 569R cells were suspended to a total volume of 10 ml in Tris buffer containing 0.1M NaCl, 0.05M ethylenediaminetetraacetate (EDTA), 0.05M Tris-HCl buffer (pH 8.2). Cell breakage was accomplished with the Eaton press or by alumina grinding, and the broken cells were homogenized into a total of 50 ml of Tris buffer. B. subtilis 168 and B. megaterium 899 were lysed by suspending two to three grams of cells in 50 ml of Tris buffer, and treating with 300 µg/ml of lysozyme at 37°C for 30 minutes.
DNA was prepared by a modification of the method of Berns and Thomas (35) as used by Brenner et al. (36). To the 50 ml lysates were added self-digested (65°C, 30 min.) pronase at 50 μg/ml, and sodium lauryl sulfate (SLS) to a final concentration of 0.5%, and the suspensions were incubated at 37°C overnight. *E. coli* B/r was also processed in this manner, but SLS and pronase were sufficient to produce maximum lysis, and prior cell breakage was unnecessary. After overnight incubation, the SLS concentration was increased to 1% and an equal volume of buffer saturated phenol was added to the suspension. The phenol and aqueous layers were well mixed and then gently shaken for several minutes. The mixture was centrifuged, the aqueous phase collected, and sodium perchlorate was added to a 1M concentration. An equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added and the mixture was shaken and centrifuged. The aqueous phase was recovered, and the chloroform treatment was repeated once or twice until the interphase material was largely removed. Two volumes of cold 95% ethanol were added to the aqueous phase and the resultant DNA precipitate was loosely spooled around a glass rod, washed in ethanol and suspended in SSC/100 (SSC=0.15 M NaCl and 0.015 M sodium citrate). After the addition of NaCl to 0.1 M final concentration, the DNA was repeatedly (three to four times) precipitated with 95% ethanol and suspended in SSC/100 until the precipitate was translucent. The DNA solution was made 0.1M to NaCl and 0.05 M to EDTA and Tris buffer, and
was incubated with 50 μg/ml of pancreatic ribonuclease at 60°C for one hour. SLS was added to 0.5% and the DNA was incubated overnight at 37°C in the presence of 50 μg/ml of pronase. The SLS concentration was then increased to 1%, and the DNA was again treated once with phenol and twice with chloroform-isoamyl alcohol. After several 95% ethanol precipitations, the DNA was finally dissolved in 0.12M phosphate buffer (an equimolar mixture of NaH₂PO₄ and Na₂HPO₄, pH 6.8) to a final concentration of 1 to 2 mg/ml.

DNA was isolated from spores of *B. cereus* 569R by germinating heat-shocked spores in non-growth media containing 1 mg/ml of D-alanine, 0.1 mg/ml Adenosine, and SMCA salts. After one hour at 34°C, the spores were 95% germinated, and were harvested and washed with 0.15M NaCl plus 0.1M EDTA, pH 8.0. The germinated, washed spores (4-6 gm) were suspended in 50 ml of 0.15M TCA (pH 8.0, prepared by adding NaOH to TCA) and shaken for 30 to 40 minutes at room temperature (37). Five ml of 10x Tris buffer was added and SLS was added to a final concentration of 2%, and the suspension heated at 60°C for 15 minutes. An equal volume of phenol saturated with Tris buffer was added, and the emulsion was shaken at 45°C for 30 to 40 minutes. After centrifugation, the aqueous phase was made 1 M with sodium perchlorate and further steps in DNA extraction and purification were carried out as previously described.
DNA Shearing

Concentrated preparations of purified DNA (1-2 mg/ml) were sheared by passing the solutions twice thru a needle value under pressure of 40,000 psi. Such a high pressure pump is commercially available and we are grateful to Dr. R. Simon for the use of the one in his laboratory. After shearing the DNA solution was filtered thru a cellulose acetate filter and frozen for storage at -20°C.

Reassociation of DNA

The methods for the reassociation of sheared, denatured DNA were those of Britten and Kohne (73, 74).

Reassociation reactions were carried out by heat denaturing samples containing between 40 and 100 µg/ml of sheared DNA in 0.12 M phosphate buffer, pH 6.8. After heating at 100°C for 10 minutes, the samples were immediately transferred to pre-warmed cuvettes (60°C) in the temperature controlled chamber of a Gilford 2000 spectrophotometer. The cuvettes were sealed and the reassociation of the DNA was followed spectrophotometrically at 260 nm. Each run consisted of a sample of Bacillus sp. DNA at 2 concentrations plus E. coli B/r DNA which served as a standard. At least 3 independently purified and sheared DNA samples from each organism were run at 2 concentrations against E. coli B/r DNA.
Determination of Genome Size From Reassociation Values

The reassociation rate of denatured DNA follows closely second-order kinetics (73, 90) and the rate of the reaction can be used as a measure of genome size. The rate is dependent upon the number of collisions between fragments of complementary nucleotide sequence. Thus, the number of complimentary fragments present per microgram of DNA solution is a function of the original size of the genome. If the fragment size is constant, an organism whose DNA content is $10^7$ daltons will have 100 times as many fragments of a single register as an organism with $10^9$ daltons of DNA. Therefore, under identical conditions, DNA from the larger organism will take 100 times as long to reassociate.

A convenient way to examine reassociation reactions is to plot the function of DNA reassociated vs. $C_0t$ (moles nucleotides x seconds per liter). The $C_0t$ values represent units of nucleotide pair formation, which are a product of DNA concentration and time:

$$C_0t = (\text{OD}_{260}/\mu g \text{ of DNA}) \times (\mu g \text{ of DNA/ml})/2 \times \text{hours of incubation}.$$  

This can be simplified to:

$$C_0t = \frac{\text{OD}_{260} \times \text{hours}}{2}$$

Thus, a $C_0t$ of 1 results from incubating DNA at concentrations of 83 $\mu g/ml$ for 1 hour, where the $\text{OD}_{260}$ of denatured DNA is 0.024/$\mu g$ (73).
The value for the half-reaction ($C_{\text{o}t}/2$) can be used as a measure of the genome size (73). The values of $C_{\text{o}t}/2$ for DNA from the organisms used in this study were compared to the $C_{\text{o}t}/2$ of *E. coli* B/r DNA. Assuming the size of *E. coli* chromosome to be $4.5 \times 10^6$ nucleotide pairs (72, 49), *B. cereus* 569, *B. subtilis* 168, and *B. megaterium* 899 were determined.
RESULTS

Growth Characteristics of *B. cereus* 569R

The growth of *B. cereus* 569R in batch culture is considered to be a reflection of a number of parameters; some constant (volume, temperature, aeration), and others continually changing (nutrient supply, pH, oxidation-reduction potential of the medium, accumulation of various extracellular factors, etc.). By choosing a single set of constant parameters for each experiment, the changes observed during growth and development follow a temporal and highly reproducible pattern (14).

For *B. cereus* 569R, 50 ml SMCA medium was inoculated with $5 \times 10^6$ spores/ml and incubated with aeration at 34°C. Other details concerning growth conditions are described in the Methods Section. Events are recorded in hours relative to 0 time (inoculation). This provides a reference time for stages prior to early sporulation and also avoids the use of the highly ambiguous convention of $t_0$, $t_1$, etc., for sporulation stages (1).

Changes in Cell Morphology

The first property examined in a developing culture was that of cell morphology. Cell samples were examined at intervals under a phase contrast microscope and observations were recorded with a Polaroid camera.
At an early time in growth (up to 5 hr.) the cells appeared as long chains, containing what appeared to be four cell units per chain (Figure 1A). These cell types were designated $\beta$ cells. When the suspension was saturated with NaCl, the membranes shrank away from the cell walls (plasmolysis) and the $\beta$ cells were shown to actually consist of an average of 16 cells per chain (Figure 1B).

At a later time, a distinct morphological change in the cells was observed. The cell chains became shorter, and due to rounding of the cells at the ends, the cell septa became more distinct ($\delta_1$ cells). Figure 1C shows typical four unit chains at 7.5 hours, which contained an average of eight protoplasts after plasmolysis.

After 7.75 hours, further chain break-up occurred and the cells appeared in two unit chains, typical of $\delta_2$ cells. Figure 1D shows nine hour cells which had an average of four cells per chain and were beginning to show signs of granule formation.

The designation of $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$ to describe cell types was adopted by Fraser and Baird (14) and originally referred to the growth potential of single organisms or chains isolated in microdrops under oil. This convention is adopted here and the morphological features just described can be correlated quite well with these stages. However, the $\gamma$ stage was further sub-divided into $\gamma_1$, and $\gamma_2$ mainly on the basis of cell density changes which will be described in the next section.
Figure 1. Morphological Development of *B. cereus* 369R.

1A. 5.0 hour β cells.

1B. 5.0 hour β cells exposed to saturated NaCl.

1C. 7.5 hour γ1 cells.

1D. 9.0 hour γ2 cells.
The developmental changes in B. cereus 569R are further depicted in Figure 2. By 10.5 hours, the cells had begun to clump and large concentrations of granules were seen within the cells (Figure 2A). Figure 2B shows pre-spores and slightly refractile spores which were beginning to appear in most cells at 13 hours. One hour later, Figure 2C shows spores with increased refractility and by 15 hours (Figure 2D) the major portion of the cells contained highly refractile spores. Events occurring after 7.75 hours were quite synchronous, with the entire cell population undergoing the same biochemical or morphological changes within 30 to 45 minutes.

Changes in Cell Density

The density of cells was measured at intervals during growth using Renografin density gradients. Linear gradients of 46 to 60% were selected because cells at early growth stages banded near the middle of such gradients, and the gradients were shallow enough to resolve slight density changes that might occur.

Cells from cultures up to 5.5 hours placed on Renografin gradients were found in a narrow band at a density of 1.215 g/cc. The appearance of 5.0 hour cells (α cells) banded to equilibrium in a 46-60% gradient is shown diagramatically in Figure 3. The α cells maintained a constant density up to 5.5 hours, at which time the cell population underwent a slight and gradual decrease in density to 1.205 g/cc. Cells found at the
Figure 2. Morphological Development of *B. cereus* 569R.

2A. 10.5 hour cells with granules.

2B. 13 hour cells showing pre-spores.

2C. 14 hour cells showing spores with increased refracility.

2D. 15 hour cells containing highly refractile, fully heat resistant spores.
new density were designated $\gamma_1$ (Figure 3), and consisted of shorter chains, with more pronounced septa as previously described. The transition from the $\beta$ cell stage to the $\gamma_1$ stage was complete at approximately 6.5 hours. The reason for this density change has not been determined, but presumably is due to permeability changes in the cells as partially reflected by the change in the ability of the cells to take-up acetate and pyruvate at this time (38; see next section).

As growth progressed, a second density shift was observed. At 7.75 hours the cells underwent a pronounced decrease in density, which caused the cells to float on top of 46 to 60% gradient (Figure 3). When the same cells, designated $\delta_2$, were banded in a 25 to 40% gradient they were found to have a density of 1.125 g/cc as shown in Figure 4. Unlike the $\beta$ to $\gamma_1$ transition, the shift from the $\gamma_1$ stage to the $\delta_2$ cell stage was quite rapid, and was completed in less than 45 minutes. The rapid and dramatic decrease in density indicates a quantitative alteration in cell components, most likely involving the ribosome fraction. Evidence for a decrease in the number of ribosomes per cell at 7.75 hours will be presented in a later section.

Further density changes occurred, starting at about 10.5 hours. Although only two of them are shown in Figure 4, several other density shifts were observed at later times. These changes all involved increases in cell density and most likely reflect the synthesis of spore structural components. Recently, Spudich and Kornberg (39) have correlated late
Figure 3. 46-60% Renografin Density Gradients of *B. cereus* 569R.
Renografin Density Gradients
25→40%

Figure 4. 25-40% Renografin Density Gradients of B. cereus 569R.
density changes with the synthesis of specific spore components during sporulation of B. subtilis.

Growth Measurements

The growth of B. cereus 569R in SMCA was followed turbidimetrically. Figure 5 shows the changes in Klett readings taken at intervals. Typical logarithmic growth was noted up to 5.5 hours (Klett = 60) after which time the turbidity reflected a more complex growth curve. The shape of the curve was probably due to the slow transition of $\beta$ to $\gamma_1$ cells, which involved break-up of the chains into smaller units. Also, cell lysis is known to occur periodically (14; S. Haworth, unpublished) and could account in part for this type of growth.

When the growth was measured by dry weight increase the Klett curve and the dry weight increase were parallel up to 5.25 hours (Figure 7). However, at 5.25 hours an abrupt change in growth rate was observed. The new, and slower growth rate was also logarithmic, suggesting that the Klett readings in this case do not give a true measurement of growth.

The changes in pH were also measured and the results are presented in Figure 5. The pH of the culture medium gradually decreased until it reached a low point of 4.75 to 4.80 at 5.5 hours. The pH then began to increase and a slight plateau was consistently observed at about 7.5 hours and a pH value of 5.35 to 5.42. At 7.75 hours, a rapid increase
Figure 5. Growth and pH Changes During Development of 
*B. cereus* 569R
200
Klett

100
pH

"riffle

6

"r

"r

198
6

282
232

Time in hours

\( \beta \) stage \( \gamma_1 \) stage \( \gamma_2 \) stage
in the pH occurred, followed by a slower increase after about one hour which continued into late sporulation to a pH of 7.3 to 7.4.

This type of pH curve has been observed with most sporulating bacilli when grown in a glucose containing medium. Nakata and Halvorson (40) showed that acetate and pyruvate accumulated in the medium as glucose was utilized by *B. cereus* T, and exhaustion of glucose was reflected by the low pH point. Then acetate and pyruvate were metabolized via a newly induced TCA cycle (38), and caused the pH to rise. Later studies by Kominek and Halvorson (41) indicated that acetate and pyruvate were used largely for the synthesis of poly-β-hydroxybutyrate polymers which appeared in the cells as large granules (see Figure 2A). These polymers were later degraded and the carbon structures used for spore components.

In the present study, no attempt was made to determine when glucose was depleted from the medium or what levels of acetate and pyruvate might be present at any time interval. The observed pH curve in these studies may reflect metabolic changes similar or identical to those found in *B. cereus* T. However, such things as differences in the composition of the medium or its buffering capacity would be expected to alter the pH curve, but have little effect on the metabolic events which occur. For these experiments we were interested in pH changes only because they provided an easy means to follow developmental changes, and the actual cause of the pH change was not considered.
The pH curve was used routinely for monitoring growth and development in *B. cereus* 569R. The arrows in Figure 5 indicate the low pH point which coincides with the shift from the $\beta$ to the $\gamma_1$ stage, and the time of rapid pH increase at 7.75 hours which corresponds to the change from the $\gamma_1$ to the $\gamma_2$ stage. Thus, the pH of a culture could be measured and the precise stage in development ascertained. Furthermore, small differences in times of development from experiment to experiment, due to such factors as the age of the inoculating spores or slight variations in medium, could be standardized by alignment of the pH curves.

Environmental Effects on Development

The developmental stages just described for *B. cereus* 569R are probably due to complex interactions between the cells and their environment. Changes in cell state may result from the accumulation of stimulatory or inhibitory compounds which reach critical levels in the medium. Also, the surrounding environment may trigger intracellular changes, including cell division, which further influence the interaction of cells with the medium.

One might then ask whether the previous growth history of a culture will influence its ability to undergo further changes in development? Second, are the organisms in one developmental phase able to revert to
a previous state when the environmental conditions are altered; i.e. is development irreversible? And finally, what influence does the cell concentration have on the transition from one culture phase to another?

To examine these questions, a turbidostat was designed so that cultures could be maintained at critical cell concentrations by the constant influx of fresh medium at a prescribed rate. Since Renografin density gradients provided a means of quantitatively measuring changes in cell stages, the organisms in continuous culture could be examined at intervals to determine what phase transitions would occur, if any, when cells from one stage were maintained at a high or low cell concentration.

For these experiments, 50 ml cultures were grown in SMCA for 5.0, 7.0, and 8.5 hours to provide $\beta$ cells, $\delta_1$ cells and $\delta_2$ cells respectively. The cells from each stage were adjusted to concentrations of $5 \times 10^7$ or $1 \times 10^9$ ml and placed in the continuous culture vessel. Fresh SMCA medium was then dripped in at a rate sufficient to maintain a constant optical density in the growth chamber.

To obtain $\beta$ cells at the desired concentration of $1 \times 10^9$ cells/ml it was necessary to rapidly sediment the cells from eight to nine 50 ml cultures and resuspend the cells in a total of 50 ml of 5.0 hour medium (obtained by taking a 5.0 hour culture and removing the cells). Similarly, a concentration of $5 \times 10^7 \beta$ cells/ml was achieved by about a 2 fold dilution of a 5.0 hour culture with 5.0 hour medium. Cultures
at 7.0 hours and 8.5 hours were found to contain $6 \times 10^8 \gamma_1$ cells/ml and $2.5 \times 10^9 \gamma_2$ cells/ml respectively, and appropriate concentrations and dilutions were made in 7.0 hour or 8.5 hour medium.

At 0 time, when the cell suspensions were introduced into the turbidostat chamber, a cell sample was examined on a Renografin gradient and samples were obtained for gradient analysis at intervals thereafter. Also, the generation time was easily determined by measuring the time necessary to displace one volume (50 ml) from the growth chamber.

The results of these experiments are summarized in Figure 6. When $\beta$ cells were maintained at a cell concentration of $5 \times 10^7$/ml, they divided at a rate of 1 generation per 35-40 minutes, similar to that of $\beta$ cells in batch culture. Furthermore, $\beta$ cells at that concentration were maintained up to 100 generations with no appearance of cells possessing a $\gamma_1$ cell density. However, when $\beta$ cells were maintained at $1 \times 10^9$ cells/ml, the entire cell population was converted to cells of $\gamma_1$ density within 3 to 4 generations.

The transition from $\beta$ to $\gamma_1$ was found to be dependent on the cell concentration. Likewise, the maintenance of $\gamma_1$ stage was concentration dependent (Figure 6). Cultures of $\gamma_1$ at $5 \times 10^7$/ml quickly reverted to the $\beta$ cell density and growth rate, while cells at $1 \times 10^9$/ml were converted to $\gamma_2$ cells within 3 generations. The $\gamma_1$ cells could be maintained at a cell concentration of $6-8 \times 10^8$/ml, but only with considerable
Figure 6. Influence of Cell Concentrations on the Maintenance of Developmental Stages.
difficulty. This was primarily due to lack of automatic regulation of the turbidostat, resulting in fluctuations of Klett readings by as much as 20-25 units. These variations in cell concentrations were of little consequence in most cases, but indicated that cultures of \( \gamma_1 \) cells could not vary over more than about a 2 fold concentration range and still be maintained as \( \gamma_1 \) cells. The generation time of \( \gamma_1 \) cells was about 60-65 minutes, also similar to the \( \gamma_1 \) stage in 50 ml batch cultures.

The \( \gamma_2 \) cells, unlike cells from the previous stages, gave the same growth response regardless of cell concentration. Even when diluted 50 fold to 5 x 10^7 cells/ml, \( \gamma_2 \) cells did not revert to an early growth stage for at least 25 to 30 generations. Although \( \beta \) cells began to appear in significant numbers after 20 generations, it is questionable whether the \( \gamma_2 \) cells ever give rise to \( \beta \) cells. Since the \( \gamma_2 \) cells divided approximately every 70 minutes, the late appearance of \( \beta \) cells was most likely due to the presence of mutant cells which were growing at a much faster rate than the \( \gamma_2 \) cells.

Another interesting property of \( \gamma_2 \) cells was their ability to grow in continuous culture, even when cells from cultures as late as 10 hours were used. This observation challenges the rather well established concept that commitment to sporulation occurred shortly after the onset of the first sporulation events (86). Loosely defined, commitment is the point at which metabolism is so definitely channeled in the direction of sporulation, it can no longer be reversed by the addition of a full
growth medium. Commitment was generally believed to occur shortly after the TCA cycle enzymes were activated (38). However, our results indicate that cells have the potential for further cell division more than 2 hours after the TCA cycle becomes operative. Furthermore, in B. cereus 569R cell division has ceased prior to 9.0 hours, but cells obtained up to 10 hours apparently can resume division. Similar results have recently been reported for B. cereus (87) and B. subtilis (59).

These results at least demonstrate that the simplistic view of sporulation as an "all or nothing" response to the environment is in error. It also appears that cell concentrations are critical in maintaining the \( \beta \) cells and \( \gamma \) cells, but the \( \delta \) state, once achieved, is not reversible and is independent of cell concentration.

This approach, although used only to a limited extent in these experiments, provides an interesting tool for the study of developmental stages. For instance, to determine if cell concentration effects are actually due to accumulation of extracellular substances, further experiments using continuous cultures and feeding in "used" growth medium from different stages could be done. Mutants blocked in various phases of development could also be used in feeding experiments, to determine if certain classes of mutants can overcome their developmental block in the presence of factors from other mutants or from wild type cells.
RNA Synthesis During Development

The large decrease in cell density from $\gamma_1$ to $\delta_2$ stage and the rapidity with which it occurred prompted further investigation of this transition. One possible explanation for the density shift could be the accumulation of poly-$\beta$-hydroxybutyrate polymers in cells, causing the cells to become lighter in density. However, this is unlikely because this compound has been shown to be synthesized over a period of several hours, which should result in a gradual change in cell density over a period of time. But the density shift from $\gamma_1$ to $\delta_2$ was abrupt, and was complete within 45 minutes.

Owing to its high density and relative abundance in the cell, changes in RNA content could have a general effect on the cell density. More specifically, a decrease in ribosomal RNA, which comprises the bulk of the RNA fraction in cells, could lead to a decrease in cell density. If ribosomal RNA synthesis ceased at 7.75 hours while cell division continued, a decrease in the number of ribosomes per cell would result. Cells containing fewer ribosomes should be lighter in density, which is the direction of the observed density shift. To examine this hypothesis, RNA synthesis during development of B. cereus 569R was measured. Samples were taken at intervals and the RNA determined by the orcinol reaction (27). Figure 7 shows the increase of RNA in the culture with time of growth. Increase in dry weight is also included for comparison. Up
to 5.25 hours, the RNA increased at a rate parallel to growth rate of the culture, as measured by dry weight or Klett increase (see also Figure 5). At 5.25 hours, RNA increased at a new rate of the dry weight increase. Net RNA synthesis ceased abruptly at 7.75 hours, and the total RNA appeared to decrease slightly. While the increase in RNA stopped, the dry weight continued to increase at the same rate for 30 to 45 minutes, and thereafter further increase continued at a diminished rate. The increase in total protein as measured by the method of Lowry et al. (28) (curve not shown) followed almost exactly the increase in dry weight.

To better correlate these events to cell division, samples of the same hot PCA extract used for RNA determination were used to measure DNA by the diphenylamine reaction (26). Figure 8 shows that DNA synthesis continued at an unaltered rate until 8.5 hours, after which time very little increase occurred. Thus, DNA synthesis continued for a full 45 minutes after RNA increase stopped. The DNA curve will be discussed further in the section on DNA synthesis. Cell increase, as measured by total cell counts, was also shown to continue sometime after 8.5 hours. Therefore, it appeared that cells continued to divide for roughly one generation after net RNA synthesis had ceased.

Rate of RNA Synthesis

The synthesis of RNA was examined more closely by pulse labeling
Figure 7. RNA Synthesis and Dry Weight Increase During Development
Figure S. DNA Synthesis and Dry Weight Increase During Development
The graph shows the relative amounts of Dry wt. and DNA over time, with time in hours on the x-axis and relative amounts on the y-axis.
the RNA fraction with $^{32}$P. One ml samples were taken at intervals, exposed to $^{32}$P for 10 minutes, and the RNA fraction examined for radioactivity. The results, expressed as counts per minute per milligram of protein, are shown in Figure 9. The rate of incorporation of $^{32}$P into RNA was fairly constant up to 7.75 hours, when related to the amount of protein or the dry weight of the culture. After 7.75 hours, approximately a 4 fold decrease in the rate of $^{32}$P incorporation was observed. Although the apparent rate of RNA synthesis was diminished, $^{32}$P continued to appear in the RNA fraction well past 10 hours. This suggests that the cells continued to actively synthesize RNA well into sporulation, although at a reduced rate after 7.75 hours.

These results with $^{32}$P labeling must be interpreted with some caution. Since no attempt was made to reduce the amount of phosphate in the growth medium or to starve cells for phosphate prior to $^{32}$P exposure, the observed uptake of $^{32}$P into nucleic acid was not rapid. A lag of several minutes was usually noted before appreciable $^{32}$P appeared in the RNA. Thus, it appears that extracellular phosphate was not readily exchangeable with internal phosphate pools. When pulse labeling during a series of developmental stages, a serious problem arises concerning the "availability" of $^{32}$P to the cells in different growth phases. If cells become less permeable to inorganic phosphate at specific times during development, then this could significantly alter the apparent rate of RNA synthesis as measured by $^{32}$P incorporation.
Figure 9. Rate of RNA Synthesis During Development
A double labeling experiment using \(^{32}\text{P}\) and \(^{33}\text{P}\) was carried out to check the permeability of cells to inorganic phosphate at various times. \(^{33}\text{P}\) is a radioactive isotope of phosphorous which has a much weaker emission (0.235 meV) than \(^{32}\text{P}\) (1.7 meV) and therefore can be counted simultaneously with \(^{32}\text{P}\). About \(5 \times 10^6\) cpm/ml of \(^{33}\text{P}\) was added to a culture at 4.0 hours and at intervals, 1.0 ml samples were withdrawn and pulsed for 10 minutes with \(1 \times 10^6\) cpm/ml of \(^{32}\text{P}\) as previously described. When the RNA fraction was examined the ratio of \(^{33}\text{P}\) to \(^{32}\text{P}\) was about 10/1 before or after 7.75 hours. Unfortunately the degree of variation in \(^{33}\text{P}/^{32}\text{P}\) ratio in RNA isolated at different times did not allow meaningful conclusions to be drawn about changes in permeability to inorganic phosphate. Such changes in permeability to inorganic phosphate and other compounds most likely occur. However, the relatively uniform ratio of \(^{33}\text{P}/^{32}\text{P}\) in RNA suggests that extracellular phosphate was available for RNA synthesis, even during the transition from \(Y_1\) to \(Y_2\) cells.

A second and probably more meaningful evaluation of possible permeability changes at 7.75 hours, deals with the uptake of \(^{32}\text{P}\) into the DNA fraction. Since DNA synthesis continued for some time after RNA synthesis stopped, the observation that \(^{32}\text{P}\) incorporation into DNA after 7.75 hours proceeded at an undiminished rate, must mean that \(^{32}\text{P}\) could enter an intracellular pool and therefore, would most likely be available for RNA synthesis.
Characterization of RNA

The specific classes of RNA were next examined to determine if the synthesis of ribosomal RNA might be preferentially affected after 7.75 hours. Cultures in SMCA were labeled with $^{32}$P for 30 minutes and the extracted RNA was placed on 15-30% sucrose gradients. After centrifugation at 37,000 rpm for 10 hours, the gradients were fractionated and analyzed for optical density at 260 nm and for radioactivity. The fractionated RNA from a culture pulsed between 7.25 hours and 7.75 hours is shown in Figure 10A. The O. D. profile shows the major classes of RNA to be 23s, 16s and 4s. The ratio of 23s to 16s was less than the theoretical value of 2.0 and indicates that some degradation of RNA has occurred during extraction. This may also be reflected by the 260 nm absorbing material in the 5-10s region. The newly synthesized $^{32}$P containing RNA was found to coincide with the 23s, 16s, and 4s fractions, again with additional material in the 5-10s region of the gradient.

When the RNA from an 8.0 to 8.5 hour pulse was examined (Figure 10B), the same total RNA profile was seen, but an increase in degraded RNA appears. This degradation was probably the result of increased nuclease activity known to occur at this time (43), and also is reflected by the slight decrease in total RNA after 7.75 hours (Figure 7). However, it can be seen that there was very little newly synthesized RNA in the 23s or 16s fractions. The radioactivity in the 4s region is difficult to
Figure 10. Characterization of RNA on 15 to 30% Sucrose gradients.

10A. Pulsed with $^{32}$P between 7.25 and 7.75 hours. Optical density at 260 nm, $^{32}$P counts per minute.

10B. Pulsed with $^{32}$P between 8.0 and 8.5 hours. Optical density at 260 nm, $^{32}$P counts per minute.
interpret because of contamination by other RNA material.

From these results, it is clear that the ribosomal fraction of RNA was no longer synthesized after 7.75 hours. Whether appreciable 4s RNA was synthesized is not certain because of the large amounts of degraded RNA. To further examine the 4s material, the same RNA samples used above for sucrose gradient analysis were applied to 10% polyacrylamide gels.

After electrophoresis, the gels were scanned at 260 nm and then sliced. The slices were counted for $^{32}\text{P}$ and the results are presented in Figure 11. On 10% gels, the 5s ribosomal RNA was clearly separated from the 4s transfer RNA. The larger RNA species remain at the origin. The newly synthesized RNA from the 7.25 to 7.75 hours pulse showed both 4s and 5s species of RNA. The RNA from the 8.0 to 8.5 hour period contained significant but slightly diminished amounts of newly synthesized 4s RNA, while very little 5s RNA appeared to be made during this time.

The results on RNA synthesis in B. cereus 569R indicate that net increase in RNA ceases at 7.75 hours. Furthermore, the synthesis of 23s, 16s, and 5s species of RNA are selectively shut off while 4s RNA synthesis and presumably messenger RNA synthesis continue. This non-coordinant control of stable RNA species is in agreement with the finding of Doi and Igarashi (44) that the tRNA fraction of B. subtilis increases from 19% of the total RNA in vegetative cells to 35% of the total in late sporulating cells. Also, the cessation of ribosomal RNA synthesis with
Figure 11. Characterization of RNA on 10% Polyacrylamide Gels.

11A. Pulsed with $^{32}\text{P}$ between 7.25 and 7.75 hours. 
    optical density at 260 nm. 
    $^{32}\text{P}$ counts per minute.

11B. Pulsed with $^{32}\text{P}$ between 8.0 and 8.5 hours. 
    optical density at 260 nm. 
    $^{32}\text{P}$ counts per minute.
the continued growth and cell division of the culture would lead to a depletion of ribosomes per cell, and most likely account for the rapid decrease in cell density observed in the transition of \( \text{8} \), cells to the \( \text{82} \) stage.

DNA Synthesis During Development

The increase in DNA was measured by the method of Burton (26). Figure 8 shows that the increase in DNA was similar to the dry weight increase, but a number of shoulders appeared between 5 and 7 hours. These plateaus were found to be fairly reproducible but probably do not represent any type of synchrony of DNA replication. More likely, the apparent pauses in DNA synthesis were due to the loss of DNA from the cells. Since periods of cell lysis are known to occur during various growth stages (14; S. Haworth, personal communication) DNA could be lost in this manner. However, the lack of similar fluctuations in the dry weight curve indicated that appreciable lysis did not occur. Another explanation is that the cells were excreting significant amounts of DNA into the medium at the specified times. Extrusion of material from \textit{B. cereus} 569R cells has been observed previously (14). The identification of cells leaking nucleic acids was also observed using acridine dyes (D. K. Fraser and J. Baird, unpublished). This phenomenon has been reported in \textit{B. subtilis} where apparently complete genomes were released and the ordered uptake of genetic markers by competent cells occurred (45). In addition Aubert
et al. (46) found up to 40% of the DNA from sporulating *B. subtilis* cultures was released into the medium.

No attempt was made to examine *B. cereus* 569R cultures for extracellular DNA. The replication of DNA during development should also be studied more closely. Unfortunately, no suitable thymine auxotrophs are available for this use. Attempts at monitoring DNA synthesis by means of $^{32}$P were less than satisfactory due to the inadequate incorporation of $^{32}$P into the DNA fraction using the required short pulses.

Diploidy in *B. cereus* 569

The idea that *B. cereus* might be diploid has evolved from several sources. They are discussed in the following paragraphs. But, before considering this evidence, the term diploid when applied to bacteria requires clarification. Generally bacteria contain only a single set of genetic information, termed a genome, and are therefore considered to be haploid organisms. At least in *E. coli*, the genome is present as one continuous double-stranded DNA molecule. The genome of the bacterial cell exists as a diffuse nuclear region or nuclear body. However, under most growth conditions bacteria have more than one nuclear region per cell. But the nuclear bodies are genetically complete and segregate independently of each other at cell division.

When using the word diploid in reference to *B. cereus* 569, we imply
that two complete genomes are present in each nuclear body in a cell, and that these two genomes do not readily segregate from each other during growth and cell division. The two genomes may or may not be genetically identical.

Several lines of evidence have indicated that some Bacillus sp. may contain more than one genome per spore. Early studies by Woese (88) on the inactivation of spores by X-rays showed multiple target kinetics for B. cereus and B. megaterium while B. subtilis gave single target kinetics. Fitz-James and Young (77) examined the DNA contents of spores from species of Bacillus and showed that the average amount of spore DNA in B. megaterium was twice that of B. subtilis while B. cereus contained three to four times this amount. More recently, genetic studies (76, 91) and autoradiography of segregating chromosomes in microcolonies (92, 93) have firmly established that B. subtilis spores contain just one complete genome. On the other hand, the segregation patterns of chromosomes within microcolonies developing from single B. megaterium spores, are consistent with the presence of two genomes per spore (92).

The suggestion of more than one genome per spore in B. megaterium and possibly B. cereus, along with several observations in our laboratory, led us to suspect that a duplicate set of genes may be present in each nuclear body during at least part of the developmental cycle of B. cereus 569. First, it is quite difficult to obtain stable mutants of B. cereus 569 regardless of the mutagen or the means of selection.
Most presumptive mutants continue to segregate wild type colonies at a high frequency even after repeated cloning of single spore isolates. Second, although periods of DNA uptake have been demonstrated in B. cereus 569 (ref. 94), no transformation has been observed. Finally, genetic crosses with mutant strains of B. cereus 569 produce heterozygous colonies, the majority of which contain all the markers of both parents, while a few contain the parental genome together with a recombinant type (Fraser, et al., 15). Again, these heterozygous colonies are persistent in their ability to give rise to colonies of a second phenotype.

DNA Content of Spores

The amount of DNA in a spore represents one half or less of the total DNA in a vegetative cell (3). Electron microscopic examination of the sporulation process revealed that only one of the nuclei in a sporulating cell was incorporated into the mature spore (1, 70). Furthermore, B. subtilis spores are known to contain only a completed genome, with no partially replicated material present (76). Thus, the examination of DNA from bacterial spores provides an opportunity to look at a single nuclear equivalent of DNA.

Spores of B. cereus 569 were analyzed for their DNA contents. Spores from B. megaterium 899 and B. subtilis 168 were included for comparison. Table 1 shows the average amount of DNA contained in single spores from
these organisms. The value of 10.7 x 10^9 daltons for *B. cereus* 569 is in close agreement with the 10 x 10^9 daltons/spore obtained for *B. cereus* T (43). The other spore values obtained also fall in the range reported for DNA contents of *B. megaterium* (9.8-12.5 x 10^{-15} g) (ref. 71, 77) and *B. subtilis* (5.0-5.4 x 10^{-15} g) (ref. 29, 77).

Genome Size of *B. cereus* 569

The genome size of *B. cereus* 569 was next determined to establish a minimum size for the genetic composition of this organism. Britten and Kohne (73, 74) have used the rate of reassociation of sheared, denatured DNA as a means of determining the complexity of DNA from a variety of sources. Since the bacterial genome was found to contain very little, if any repeated sequences of DNA (73), the reassociation rate can be used as a direct measurement of genome size (74, 75).

Sheared, denatured DNA was allowed to reassociate at 60°C in 0.12M phosphate buffer. The reaction was monitored by following the decrease in optical density at 260 nm. The rate of reassociation can be conveniently expressed as the product of DNA concentration and time (C_0 t;22). Values of C_0 t at half completion of the reaction, C_0 t/2, were used to compare the genomes of the 3 Bacillus sp. and *E. coli* B/r. The genome size of *E. coli* was used as a reference, and was taken to be 3.0 x 10^9 daltons (49, 72).
<table>
<thead>
<tr>
<th>Organism</th>
<th>spores/ml (a)</th>
<th>DNA/ml (b)</th>
<th>DNA/spore</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>grams</td>
<td>daltons</td>
</tr>
<tr>
<td>B. cereus 569</td>
<td>$1.3 \times 10^{10}$</td>
<td>230 µg</td>
<td>$17.7 \times 10^{-15}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10.7 \times 10^{9}$</td>
</tr>
<tr>
<td>B. megaterium 899</td>
<td>$2.2 \times 10^{10}$</td>
<td>237 µg</td>
<td>$10.8 \times 10^{-15}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$6.5 \times 10^{9}$</td>
</tr>
<tr>
<td>B. subtilis 168</td>
<td>$1.8 \times 10^{10}$</td>
<td>86 µg</td>
<td>$4.8 \times 10^{-15}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$2.9 \times 10^{9}$</td>
</tr>
</tbody>
</table>

(a) average of triplicate counts using a Petroff-Hauser counting chamber

(b) measured by the indole method of Keck (30)
Table 2 shows the Ct values and the approximate molecular weights of the unique sequences of DNA present in each organism. None of the reassociation reactions deviated significantly from second-order kinetics, indicating the absence of large numbers of repeated sequences. It is interesting to note the variation in genome sizes exhibited by the 3 spore-formers. B. megaterium 899 appears to have a genome of 3.4 x 10^9 daltons which is slightly larger than the 2.7 x 10^9 daltons for B. subtilis 168. The genome size of B. cereus 569, 5.5 x 10^9 daltons, is twice that of B. subtilis 168. When the genome size is compared to the spore DNA values in Table 1, B. cereus 569 spores contain sufficient DNA to accommodate 2 complete genomes. B. megaterium 899 spores could also contain 2 genomes, while the genome size for B. subtilis 168 would only allow one genome per spore.

The E. coli genome size of 3.0 x 10^9 daltons (49, 72) used for this investigation, apparently is not without controversy (75, 78). However, the same value was obtained when calibrated against a reassociation reaction of T4 DNA (73, 74, 79) assumed to have a genome of 1.3 x 10^8 daltons. The C0t/2 value of 7.25 obtained for E. coli B/r DNA was less than the value of 9.0 reported by others (73, 79) and was presumably the result of slightly larger DNA fragments produced by shearing at 40,000 rather than 50,000 psi. Using 50,000 psi sheared DNA, a genome size for B. subtilis of 3.0 x 10^9 daltons was reported (80).
<table>
<thead>
<tr>
<th>Organism</th>
<th>$C_{o}t/2$ (a)</th>
<th>approximate no. (b) nucleotide pairs</th>
<th>mol. wt. (daltons)</th>
<th>genomes/spore</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus 569</td>
<td>$13.25 + 0.85$ (c)</td>
<td>$8.3 \times 10^6$</td>
<td>$5.5 \times 10^9$</td>
<td>2</td>
</tr>
<tr>
<td>B. megaterium 899</td>
<td>$8.5 + 0.70$</td>
<td>$5.2 \times 10^6$</td>
<td>$3.4 \times 10^9$</td>
<td>2</td>
</tr>
<tr>
<td>B. subtilis 168</td>
<td>$6.5 + 0.90$</td>
<td>$4.1 \times 10^6$</td>
<td>$2.7 \times 10^9$</td>
<td>1</td>
</tr>
<tr>
<td>E. coli B/r</td>
<td>$7.25 + 0.75$</td>
<td>$4.5 \times 10^6$</td>
<td>$3.0 \times 10^9$</td>
<td>-</td>
</tr>
</tbody>
</table>

(a) expressed as mole nucleotide DNA times seconds/liter (M x sec/liter) - Britten and Kohne (74)

(b) relation of $C_{o}t/2$ to nucleotide pairs derived by Britten and Kohne (74) using E. coli genome size as a reference (72)

(c) represents average reassociation values from both spore and vegetative cell DNA.
It is significant that *B. subtilis*, established to be haploid* by several types of evidence (88, 76, 91-93), also appears haploid when the spore DNA content is compared to the genome size. While genetic information is not available for *B. megaterium*, the X-ray inactivation of spores (88) and the autoradiography studies (92) suggest the presence of 2 genomes per spore, again consistent with the genome size measurements presented here. Thus, the presence of sufficient DNA in *B. cereus* 569 spores to accommodate 2 complete genomes supports our conclusion on that *B. cereus* is diploid.

Criteria for Diploidy

It should be pointed out that comparison of genome size with spore DNA content does not provide sufficient evidence to establish diploidy. Recently, Carlton and Helinski (83) reported that DNA from *B. megaterium* contained up to 40% small, heterogenous circular forms. Although the study indicated that many spore-forming strains lacked significant quantities of these circular DNA forms, their presence would complicate inter-

*There are two contradictions to this conclusion. One concerns the finding of Aubert, et al. (46) that 25% of the spores obtained from *B. subtilis* contain 2 chromosomes per spore. A more serious objection comes from Yoshikawa's autoradiography studies (81) showing *B. subtilis* spores to contain 4 conserved units of DNA. This is in opposition to the results of others (92, 93) which indicate 2 segregating units are present in *B. subtilis* spores. In view of our genome size measurements and those of Yehle and Brenner (80) it is clear that *B. subtilis* 168 spores can contain only a single set of genetic information and the meaning of Yoshikawa's results are uncertain.
pretations about the number of genomes per spore. The DNA's used for these experiments were not examined in cesium chloride-ethidium bromide gradients and the existence of circular DNA elements in B. cereus 569 cannot be entirely ruled out. However, no deviation of the DNA reassociation kinetics from second-order was observed, as might be expected if B. cereus 569 contained a large portion of its total DNA as non-chromosomal elements.

The autoradiographic analysis of segregating units from single spores can also be misleading. The presence of 2 complete genomes per spore may be due only to the mechanism of segregation or packaging of DNA during sporulation in some strains. Such diploid nuclei in spores would produce 4 segregating units, but informational segregation would also occur. True diploidy implies that the 2 genomes present in each nuclei do not normally segregate from each other during cell division. A heterozygote with 2 genetically distinguishable genomes per spore, would produce 4 segregating units as measured by autoradiography, but no genetic segregation would occur. Thus, to establish that B. megaterium is truly diploid will require genetic analysis.

The genetic evidence presented in the Appendix (see section on Phenotypic Segregation) is very pertinent to an argument for diploidy in B. cereus 569. Unfortunately, next to nothing is known about the genetic map of B. cereus. However, none of the markers used in this study appear to be closely linked (Fraser, unpublished). This, coupled with the ability to recover the markers as a unit after segregation,
regardless of the set of genetic characters used, makes the involvement of an episome unlikely. Furthermore, Pollock and Fleming (62) have recently reported a similar type of segregation in which unstable penicillinase negative cells of *B. cereus* 569 could be converted to fully penicillinase constitutive cells by treatment with heat or chloramphenicol. The above observations suggest that 2 alleles of each genetic marker involved can be present in the nuclei of spores or cells, and that the units containing these genes (possibly whole genomes) do not readily segregate from each other.

These genetic studies, in combination with the finding that the *B. cereus* 569 spore contains sufficient DNA to accommodate 2 complete genomes, indicates that *B. cereus* 569 may be diploid. Further studies on the segregation phenomenon as well as the genetics of this organism will be required for conclusive proof.
DISCUSSION

The developmental stages in \textit{B. cereus} 569R were clearly defined by morphology, cell density, and the rate of macromolecular synthesis. The morphological changes are probably due to alterations in cell wall structure. Other changes reported in developing cultures could also be a reflection of cell wall changes. Differences in the staining properties and electrophoretic mobility of cells observed at different times \cite{14} could be due in part to cell wall alterations. Certainly, the observation with the entire cell population changes from gram-negative to gram-positive between 3.50 and 3.75 hours \cite{14} is indicative of a cell wall change.

The changes in cell wall structure could be due to changing patterns of cell wall biosynthesis. One instance of such a biosynthetic change occurs during sporulation, when an altered peptidoglycan appears in the forespore \cite{4}. The nature of the change during sporulation is not certain. However, the degree of cross linking between muramyl-tetrapeptides or the amount of L-alanyl residues which are substituted on N-acetyl muramic acid would affect not only the rigidity of the cell wall but would result in a change in the number of free carboxyl groups. An alteration in charge of the cell wall would account for the staining properties and electrophoretic mobilities of the cells.

The biosynthetic pattern for cell walls need not be changed to find a difference in cell wall structure. An endopeptidase and other enzymes
known to be present in Bacillus sp. could alter the cell wall quite rapidly, without requiring cell wall synthesis. One degradative enzyme, an autolysin, exhibits changes in activity between early and late log phase (47). An alteration of existing cell wall material rather than a change in newly synthesized walls may be necessary to cause the rapid shift from gram-negative to gram-positive in B. cereus 569 (14).

In addition to possibly altering the cell shape and nature of the septum region, cell wall enzymes could account for periods of observed cell lysis (14) and times of increased cell fragility. Alternately, the lysis could be due to the presence of phage which are released at several times during the developmental cycle (S. Haworth, personal communication).

The cell density changes provide a simple way to quantitatively evaluate development in B. cereus 569R. It is not clear what changes in the cells account for the $\beta$ to $\gamma_1$ density shift. Differences in amounts of cell constituents such as lipids could possibly alter the density. However, the $\beta$ to $\gamma_1$ change occurred quite rapidly on an individual cell basis. Although the entire population required over an hour to complete the shift, no intermediate density bands were observed. This suggests a type of threshold response to the environment exhibited by single cells or single cell chains. Such all or nothing responses have been proposed by Fraser and Baird (14) to explain the changes in growth potential of cells at different times. Precluding quantitative
changes in molecular constituents, the slight shift in density from $\beta$ to $\gamma_1$ stage could be due to permeability or surface changes. No further enlightenment on these possibilities can be provided at this time.

The density shift from $\gamma_1$ to $\gamma_2$ was more extensively studied. It is hypothesized that the turn off of ribosomal RNA synthesis in the presence of further cell growth, causes a reduction in the RNA per cell. The decrease in the ratio of RNA to protein in the $\gamma_2$ cells then causes a decrease in cell density. Although this shift was quite rapid for the cell population, between 7.75 and 8.5 hours cells appeared in a broad diffuse band between the 1.205 g/cc density regions and the top of the gradient. Therefore, unlike the $\beta$ to $\gamma_1$ shift, the change in density from $\gamma_1$ to $\gamma_2$ was gradual when related to the individual cells, and is consistent with a gradual depletion of the RNA per cell.

Density differences due to RNA content of cells were recently reported in E. coli (48). Density gradients were used as a means of isolating mutants which had altered control of RNA synthesis. Net RNA synthesis in E. coli normally stops when a required amino acid is removed from the growth medium (stringent control). However, certain relaxed control mutants are known to continue RNA synthesis at an unaltered rate after amino acid deprivation (49). Furthermore, relaxed mutants which were amino acid starved were shown to band at a higher density than a similarly starved stringent strain. To obtain additional RC$^{rel}$ mutants, stringent cells were starved for an essential amino acid and the cells
then subjected to density gradient analysis. Cells which banded below the major band were isolated and examined for relaxed RNA control. A number of mutants were obtained by this method, indicating that alterations in RNA synthesis can cause measurable density changes in cells.

Another instance of density changes in cultures was noted in competent cultures of *B. subtilis* (50, 51). In this case, cells in late log phase were found in 2 bands in a Renografin gradient. The minor band appeared late in growth and contained 90-95% of the competent cells from the population. However, the reason for appearance of this lighter density band, which constituted only a minor portion of the total cells, was not determined.

Conditions of the growth medium at the onset of sporulation (i.e. depletion of nutrients) has been compared to a step-down condition (52). When cells are transferred from a rich growth medium to a minimal medium, net RNA synthesis stops for a period of time and then resumes at a new reduced rate. Although this is probably an oversimplified concept for the initial stages of sporulation, it deserves further comment.

One of the interesting findings from this research concerns the non-coordinant synthesis of the stable RNA species during $\xi_1$ to $\xi_2$ transition. The finding by Doi and Igarshi (44) that late sporulating cells of *B. subtilis* contain 35% of their total RNA as tRNA while vegetative cells contain 19-20%, would be compatible with this type of synthesis. However, a similar change in *B. megaterium* was not seen, with vegetative
cells and spores containing 15% and 21% of tRNA respectively (53).

Maaløe and Kjeldgaard (52) found that E. coli or S. typhimurium had altered ratios of ribosomal RNA to tRNA at different growth rates. At rapid growth rates there was 5 times as much rRNA as tRNA but at the slowest growth rate, the amount of tRNA was now 2.5 times the rRNA content per cell. The authors make the general assumption that rRNA and tRNA synthesis are coordinately controlled, but they did not examine the RNA species during shift experiments. The changes in rRNA/tRNA ratios could be due to independent rates of synthesis or selective degradation of rRNA could occur. The latter possibility will be discussed further.

A preliminary report of what appears to be a genuine non-coordinate control was found for E. coli (54). In this case ribosomal RNA synthesis proceeded in the absence of tRNA synthesis.

When interpreting results concerning non-coordinate control of stable RNA species, a degree of caution is necessary. One must rule out the possibility that both rRNA and tRNA are synthesized at the same rate, but one species is rapidly degraded. Certainly, the increase in tRNA relative to rRNA at slow growth rates could be due to turnover of the ribosomes, which has been observed during periods of slow growth or starvation (55). In addition, turnover of ribosomal RNA has been shown to occur late into sporulation (56).

In developing systems an additional complication may exist when the results of Woese and Bleyman (57) are considered. They found that germinating spores of B. subtilis produced mainly ribosomal RNA, but
it was of a precursor variety and not mature rRNA. When the germinating spores were treated with actinomycin D nearly all precursor rRNA broke down. However, in the vegetative cell, most if not all precursor rRNA converted to the mature rRNA in the presence of actinomycin D (58). These results could be interpreted to mean that precursor rRNA formed during germination was structurally different from that formed in vegetative cells, or that other conditions prevailed at this time which enhanced the degradation of precursor rRNA. In either case, such possibilities could occur during sporulation as well. Examination of RNA produced during short radioactive pulses at the time of the $\delta_1$ to $\delta_2$ transition, is needed to establish conclusively whether rRNA can be made in the absence of rRNA synthesis.

Another interesting aspect of the $\delta_1$ to $\delta_2$ transition was the inability of $\delta_2$ cells to "revert" to an earlier stage. Even cultures in which cell division had been stopped for more than one hour, could resume DNA synthesis and cell division, but they remained at the $\delta_2$ cell density. It has been postulated that cells in early phases of sporulation are expressing an altered phenotype merely because of the growth conditions, and when these cells are supplied with fresh medium, they now express phenotype characters typical of vegetative cells (4, 59). Results with $\delta_2$ cells, however, suggest a more permanent change in cells when sporulation events commence. The data correlating the density change with RNA synthesis, could be interpreted to mean that the cells are not capable
of restoring the previous levels of ribosomes per cell, even at low cell concentrations in fresh medium. It is not clear why shift-up conditions would not cause the expected increase in ribosomal RNA synthesis.

The above observations may be pertinent to microcycle sporogenesis. Under selected conditions, spores can be induced to grow out into single cells which do not divide, but form another spore (60, 61). But spores obtained from microcycle could not undergo a second microcycle sporulation. DNA was shown to increase 2 to 3 fold during microcycle, but there was no net RNA synthesis. Possibly, a control for RNA synthesis during germination and outgrowth is similar to that seen in Δ2 cells. Since only a portion of the ribosomes in a sporulating cell are embodied in the spore, lack of ribosome formation during microcycle would further reduce the available ribosomes for the microcycle spore. Thus, further microcycles may be prevented by depletion of ribosomes to a critical level.

DNA synthesis in B. cereus 569 is of interest for several reasons. Since it appears that B. cereus 569 is diploid, at least under certain conditions, one wonders if there is a haploid stage during development. The formation of spores apparently does not involve a reduction to haploidy, but it could occur at other times. The conversion phenomenon observed at low cell concentrations (see appendix) may be the result of at least temporary reduction to a haploid state and should be examined further.
The mechanism of DNA replication in such an organism should also be examined. Does the presence of 2 genomes imply that both genomes are replicated simultaneously, or is some form of sequential replication involved? The answers to these questions of course, depend on the physical structure assumed by 2 genomes in a nucleus. If they are joined end to end in a concatenate, then replication may be straight forward. However, this type of structure would complicate the conversion phenomenon, since segregation would require a physical breakage of the DNA molecule.

Recently, Pollock and Fleming (62) reported nearly 100% conversion of an unstable penicillinase negative culture to a penicillinase constitutive one. This conversion was readily induced at 56°C or by chloramphenicol. The authors suggest that a protein repressor is involved in controlling the expression of the gene for penicillinase. In view of our results, the above strain may be a heterozygous diploid, which is not expressing one genome. A similar proposal must be made to explain our results with heterozygous strains, since some of the markers contained in the heterozygotes are dominant but apparently not expressed. This would imply that one of the 2 genomes is completely inactivated under usual conditions. Special growth conditions (heat, chloramphenicol, low cell numbers) then cause a "switching" of gene expression from one genome to another, or a physical segregation of the 2 genetically different genomes. These very interesting observations must be
investigated further before any meaningful models can be presented concerning this type of gene control.

An interesting mutant of *B. cereus* 569, named strain 45, has been studied in this laboratory. This mutant was isolated after mutagenesis of *B. cereus* 569R and was found to produce spores nearly twice as large as normal spores. Studies by Brantner (63) showed that under identical conditions, 45 grew more rapidly than the parent, did not exhibit an early long chain stage, reached developmental stages sooner, and sporulated 2.5 hours earlier than *B. cereus* 569R. It was speculated that strain 45 might represent a 'diploid' *B. cereus* 569R (i.e. tetraploid).

However, when the DNA content of the spores was recently examined, 45 was found to contain 1.5 times as much DNA as *B. cereus* 569R spores. The possibility exists that the spores contain 2 partially replicated chromosomes (3 gene equivalents) or that one half of the spores contained 4 genomes while the other half contained only 2. This latter possibility seems more feasible especially since strain 45 spores were found in 2 density bands in Renografin gradients. A mechanism for the production of 2 such classes of spores in equal amounts is not readily apparent. A third possibility is that each spore contains 3 complete genomes. Although not aesthetically appealing, the presence of 3 genomes per nucleus should not provide any special problems for replication or segregation.
Questions concerning diploidy in bacteria may be more readily resolved with another system. A mutant of *E. coli* termed P6 which was induced by camphor treatment, was reported to have increased cell size, weighed 3 times more, contained 3 times more DNA and RNA per cell, and was more resistant to X-rays than the parent strain (64, 65). More recent studies indicated that P6 had twice as much DNA per nuclear body as did the parent strain (66). Genetic evidence indicates that markers from several areas in the genetic map can be introduced into a recipient P6 strain. Heterozygotes which are selected from the cross can be shown to segregate the markers from both parents as a unit (M. J. Kvetkas, personal communication). The P6 mutant then looks like a diploid strain, with 2 genomes per nucleus that do not readily segregate from each other. Study of the control of replication and genetic studies of diploid strains may be more feasible with P6 than with *B. cereus* 569. However, *B. cereus* 569 represents a naturally occurring form of diploidy which may prove different from the artificially induced diploidy in *E. coli* P6, especially in view of the previous discussion on gene control.

Another case of diploidy in bacteria may be found in the streptomycetes. The similarity between *B. cereus* and *Streptomyces* appears quite striking. Both are soil microorganisms, both grow in chains which readily clump, and both form spores. A period of DNA uptake has been observed in *Streptomyces*, but measurable levels of transformation were not seen (67). DNA uptake also occurs in *B. cereus* 569R (68) and likewise,
transformation has not been detected. Genetic exchange in *Streptomyces*
involves cell contact, and large portions if not all of the genome can
be transferred (69). A type of genetic exchange recently found by
Fraser, Baird, and Kleeman (15) in *B. cereus* 569 has properties similar
to the *Streptomyces* system. Finally, genetic studies with *Streptomyces*
have frequently revealed unexpected segregation of dominant markers from
a strain with a recessive phenotype (G. Germaine, personal communication).
Also, after genetic crosses in *Streptomyces*, the segregation patterns
of parental markers are in many cases very complex, and cannot be explained
merely by the growth of cells in chains. Thus, the *Streptomycetes*
are very suspect of being diploid organisms, and future studies should be
pursued in this light.

It is interesting that *B. megaterium* also appears to be diploid,
while *B. subtilis* is haploid. Both *B. cereus* and *B. megaterium* are
rather closely related in a number of respects. Their sporulation and
germination properties are similar, and they both have DNA G-C contents
of 36-37%. In contrast, *B. subtilis* DNA has a G-C content of 43% and
is morphologically and genetically distinct from *B. megaterium* and
*B. cereus*. This brings up the question of why *B. cereus* 569 has almost
2 times the number of unique DNA sequences that are found in *B. megaterium*?
The same question applies to a slightly lesser extent in the case of
*B. subtilis* or *E. coli*. Why should *B. cereus* 569 require twice the
-genetic information of *B. megaterium* 859 or *B. subtilis* 168 when it can
apparently perform no unique tricks? Finally, what selective advantage would result in the persistence of a B. cereus cell which must synthesize approximately 4 times as much DNA per generation as a B. subtilis cell?

Speedy resolution of the above questions are not anticipated.
SUMMARY

Three developmental stages were defined by the density of cells in Renografin gradients, and the transitions from one developmental stage to another were examined quantitatively. The change from the \( \beta \) to \( \gamma_1 \) stage was found to be slow (1.5 hours) for the cell population as a whole, but rapid for individual cells since no cells intermediate in density between \( \beta \) and \( \gamma_1 \) were found. In contrast, the \( \gamma_1 \) to \( \gamma_2 \) stage transition was found to be rapid for the whole culture (30 min.) but gradual for individual cells in the population. Continuous culture studies showed that the \( \beta \) to \( \gamma_1 \) shift was reversible and dependent on cell concentrations. The \( \gamma_1 \) to \( \gamma_2 \) transition was also dependent on cell numbers but once the \( \gamma_2 \) cell state was achieved, it was maintained regardless of cell concentration.

When the patterns of macromolecular synthesis, were examined during development, net RNA synthesis was observed to stop abruptly at the time of the \( \gamma_1 \) to \( \gamma_2 \) shift, while DNA synthesis and protein synthesis continued at an undiminished rate for nearly one hour. Further characterization of the RNA synthesized during the \( \gamma_1 \) to \( \gamma_2 \) transition showed a selective inhibition of ribosomal RNA synthesis, and the great decrease in cell density from the \( \gamma_1 \) to \( \gamma_2 \) cell state was attributed to the depletion of total RNA per cell. The permanence of the \( \gamma_2 \) cell state must result from the inability of these cells to restore the \( \gamma_1 \) level of ribosomes per cell. Thus, the \( \gamma_2 \) cell state may represent an irreversible commitment of these cells toward spore formation.

Another aspect of this study was concerned with establishing that \textit{B. cereus} 569 might be diploid. Genetic studies were compatible with a
diploid nature for *B. cereus* 569 and physical studies further substantiated this hypothesis. The genome size of *B. cereus* 569 was established by determining the rate of reassociation of sheared, denatured DNA. A half reassociation value (Cot/2) of 13.25 was obtained which corresponds to a genome size of $5.5 \times 10^9$ daltons. The amount of DNA per spore was found to be $10.7 \times 10^9$ daltons, and thus, the spore contains sufficient DNA to accommodate two complete genomes.


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Diploidy of B. cereus 569

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Several lines of evidence have indicated that some Bacillus sp. may contain more than one genome per spore. Early studies by Woese (4) on the inactivation of spores by X-rays showed multiple target kinetics for B. cereus and B. megaterium while B. subtilis gave single target kinetics. Fitz-James and young (5) examined the DNA contents of spores from species of Bacillus and showed that the average amount of spore DNA in B. megaterium was twice that of B. subtilis while some strains of B. cereus contained three to four times this amount. More recently, genetic studies (6, 7) and autoradiography of segregating chromosomes in microcolonies (8, 9) have firmly established that B. subtilis spores contain just one complete genome. In contrast, the segregation patterns of chromosomes within microcolonies developing from single B. megaterium spores are consistent with the presence of two genomes per spore (8).

The suggestion of more than one genome per spore in B. megaterium and possibly B. cereus, along with several observations in our laboratory led us to suspect that a duplicate set of genes may be present in each nuclear body during at least part of the developmental cycle of B. cereus 569. First, it is quite difficult to obtain stable mutants of B. cereus 569 regardless of the mutagen or the means of selection. Most presumptive mutants continue to segregate wild type colonies at a high frequency even after repeated cloning of single spore isolates. Second, although periods of DNA uptake have been demonstrated in B. cereus 569 (ref. 2), no transformation has been observed. Since phage DNA retains biological activity
after uptake into these cells, their inability to undergo transformation could suggest a more complex genetic or physiological nature for these organisms. Finally, genetic crosses with mutant strains of *B. cereus* 569 produce heterozygous colonies, the majority of which contain all the markers of both parents, while a few contain the parental genome together with a recombinant type (Fraser, et al. 3). Again, these heterozygous colonies are persistent in their ability to give rise to colonies of a second phenotype.

We would like to report studies on several multiple mutant strains of *B. cereus* 569 that appear to contain two complete, but genetically distinguishable genomes. These genomes usually segregate as a unit, but under selected conditions apparently segregate away from each other at high frequency. We suggest that these strains are heterozygous diploid and that the segregants are homozygous diploid. In further support of this hypothesis, we examined the genome size by measuring the rate of reassociation of sheared, denatured DNA (10), and the results show that *B. cereus* 569 spores contain enough DNA to accommodate two complete genomes.

**Phenotypic Segregation**

During the isolation of genetically-labelled strains of *Bacillus cereus* 569, many "unstable" isolates are obtained. In spite of repeated
streaking, it frequently seems impossible to obtain these strains in a pure state.

A spore stock of an unstable strain of phenotype SP$^+$ THY$^-$ YE$^+$ PEN$^{\text{mag}}$ AM$^R$ (isolated in the laboratory of Dr. Martin Pollock at the University of Edinburgh) was streaked on Andrade fuchsin indicator medium (11), and the plate incubated overnight at 30°C, to give small colonies. One such colony was picked into SMCAY medium with added thymine (20 ug/ml) and grown at 37°C with shaking. At intervals, samples of the culture were removed, diluted and plated for single colonies on Andrade medium. The plates were incubated at 30°C. They were then scored by replication onto minimal plates, plates of Andrade agar with added yeast extract, and plates with aminopterin, after which the master plates were flooded with 5% penicillin solution to score for penicillinase production. Magno colonies (constitutively producing a large amount of penicillinase) turned red within 30 seconds; inducible colonies remained white for several hours. Figure 1 shows the changes in numbers of red and white colonies during the growth of the culture. Some white colonies appeared even at the earliest plating, since the inoculating colony contained some cells of the inducible genotype (direct streaking confirmed this fact). The white colonies were all of the phenotype SP$^+$ (AUX$^-$) YE$^-$ PEN$^{\text{ind}}$ AM$^S$. The YE$^-$ phenotype indicates that this strain forms no colonies at all on Andrade medium to which a 1:100 dilution of 20% yeast extract had been added. Thus, they were different from the parental
phenotype in at least four markers (the thymine requirement could not be
determined in the presence of the unknown auxotrophic requirement).
Restreaking showed few if any variants, with no reversion to the magno
phenotype.

The percentage of white inducible colonies increased rapidly during
the growth of the culture from 1 to 3 hours, reaching 75% of the total.
The generation time for \textit{B. cereus} 569 under these growth conditions is
about 35-40 minutes. Yet the rate of increase in PEN\textsuperscript{ind} cells between
the interval of 1 to 2 hours would require a doubling time of 12-13 minutes.
Therefore, the only possible explanation for the rapid increase in the
new cell type is that cells of the original phenotype were giving rise
to cells of the new phenotype, probably by segregation.

After three hours of culture growth, the percentage of white colonies
suddenly dropped to about 20% of the total. The figures suggest that
they died, since there was no overall increase in the total number of
viable cells during this time. Lysis of one \textit{B. cereus} strain by mixed
growth with another has been a frequent observation in our work (Fraser,
unpublished). When isolated and grown alone under the same conditions
(medium containing yeast extract), the PEN\textsuperscript{ind} strain did not grow excep-
tionally rapidly (doubling time 35 min.), and did not revert to a
PEN\textsuperscript{mag} phenotype.

Repetition of this experiment with a number of PEN\textsuperscript{mag} colonies from
the same strain has always given the same result. We have not observed
any stable PEN\textsuperscript{mag} segregants nor any unstable PEN\textsuperscript{ind} segregants.

A similar segregation pattern was observed for an unstable strain isolated in our laboratory, SP\textsuperscript{-} GUA\textsuperscript{-} YE\textsuperscript{+} PEN\textsuperscript{ind} (Figure 2). The strain was maintained on a slant; otherwise the technique and materials were identical, with replication onto minimal plates containing appropriate additions. SP\textsuperscript{-} and SP\textsuperscript{+} colonies are readily distinguishable on Andrade agar plates, the SP\textsuperscript{+} colonies being opaque white, and SP\textsuperscript{-} colonies more translucent.

The main features of the segregation are identical to those of the case shown in Figure 1. Between 1 and 3 hours there is a rapid increase in colonies of the segregant type, SP\textsuperscript{+} (AUX\textsuperscript{-}) YE\textsuperscript{-} PEN\textsuperscript{ind}. Again lysis of the segregated strain occurred between 3 and 4 hours of culture development. SP\textsuperscript{+} colonies were stable, and all SP\textsuperscript{-} colonies tested gave mixed cultures.

A third example of segregation was observed with a strain of phenotype SP\textsuperscript{-} GUA\textsuperscript{-} TRY\textsuperscript{+} PEN\textsuperscript{meso} STR\textsuperscript{8}. In this case the segregated type proved to be SP\textsuperscript{+} GUA\textsuperscript{+} TRY\textsuperscript{-} PEN\textsuperscript{ind} STR\textsuperscript{R}, so that five genetic markers were altered in each segregation event.

Preliminary tests show that rapid segregation of unstable strains is probably dependent on growth in glucose-containing medium with ample aeration and low cell concentrations.

Neither unstable strains nor their stable derivatives differ from normal \textit{B. cereus} strains in any microscopically observable way. There-
fore, we thought it possible that normal \textit{B. cereus} wild-type cells might be diploid, and that unstable strains might be heterozygous diploid segregating progeny which had lost one of the two genome types, but probably were now homozygous diploid.

**DNA Content of Spores**

The amount of DNA in a spore represents one half or less of the total DNA in a vegetative cell (12). Electron microscopic examination of the sporulation process revealed that only one of the nuclei in a sporulating cell was incorporated into the mature spore (13). Furthermore, \textit{B. subtilis} spores are known to contain only a completed genome, with no partially replicated material present (6). Thus, the examination of DNA from bacterial spores provides an opportunity to look at single nuclear equivalent of DNA.

Spores of \textit{B. cereus} 569 were analyzed for their DNA contents. Spores from \textit{B. megaterium} 899 and \textit{B. subtilis} 168 were included for comparison. Table 1 shows the average amount of DNA contained in single spores from the organisms. The value of $10.7 \times 10^9$ daltons for \textit{B. cereus} 569 is in close agreement with the $10 \times 10^9$ daltons/spore obtained for \textit{B. cereus} T (14). The other spore values obtained also fall in the range reported for DNA contents of \textit{B. megaterium} (9.8-12.5 $\times 10^{-15} g$) (ref. 5, 15) and \textit{B. subtilis} (5.0-5.4 $\times 10^{-15} g$) (ref. 5, 16).
Genome Size of B. cereus 569

The genome size of B. cereus 569 was next determined to establish a minimum size for the genetic composition of this organism. Britten and Kohne (10, 22) have used the rate of reassociation of sheared, denatured DNA as a means of determining the complexity of DNA from a variety of sources. Since the bacterial genome was found to contain very little, if any, repeated sequences of DNA (22), the reassociation rate can be used as a direct measurement of genome size (10, 23).

Sheared, denatured DNA was allowed to reassociate at 60°C in 0.12M phosphate buffer. The reaction was monitored by following the decrease in optical density at 260 nm. The rate of reassociation can be conveniently expressed as the product of DNA concentration and time (Cot) (ref. 22). Values of Cot at half completion of the reaction, Cot/2, were used to compare the genomes of the 3 Bacillus sp. and E. coli B/r. The genome size of E. coli was used as a reference, and was taken to be 3.0 x 10^9 daltons (20, 21).

Table 2 shows the Cot values and the approximate molecular weights of the unique sequences of DNA present in each organism. None of the reassociation reactions deviated significantly from second-order kinetics, indicating the absence of large numbers of repeated sequences. It is interesting to note the variation in genome sizes exhibited by the 3 spore-formers. B. megaterium 899 appeared to have a genome 3.4 x 10^9
daltons which is slightly larger than the $2.7 \times 10^9$ daltons for *B. subtilis* 168. The genome size of *B. cereus* 569, $5.5 \times 10^9$ daltons, is twice that of *B. subtilis* 168. When the genome size is compared to the spore DNA values in Table 1, *B. cereus* 569 spores contain sufficient DNA to accommodate 2 complete genomes. *B. megaterium* 899 spores could also contain 2 genomes, while the genome for *B. subtilis* 168 would only allow little more than one genome per spore.

The *E. coli* genome size of $3.0 \times 10^9$ daltons (20, 21) used for this investigation apparently is not without controversy (23, 26). However, the same value was also obtained when calibrated against a reassociation reaction of T4 DNA (10, 22, 24) assumed to have a genome of $1.3 \times 10^8$ daltons (27). The $C_0 t/2$ value of 7.25 obtained for *E. coli* B/r DNA was less than the value of 9.0 reported by others (10, 24) and was presumably the result of slightly larger DNA fragments produced by shearing at 40,000 rather than 50,000 psi. Using 50,000 psi sheared DNA, a genome size for *B. subtilis* 168 of $3.0 \times 10^9$ daltons was reported (25).

It is significant that *B. subtilis*, established to be haploid* by several types of evidence (4, 6-9), also appears haploid when the spore

*There are two contradictions to this conclusion. One concerns the finding of Aubert, et al. (28) that 25% of the spores obtained from *B. subtilis* contain 2 chromosomes per spore. A more serious objection comes from Yoshikawa's autoradiography studies (31) showing *B. subtilis* spores to contain 4 conserved units of DNA. This is in opposition to the results of others (8, 9) which indicate 2 segregating units are present in *B. subtilis* spores. In view of our genome size measurements and those of Yehle and Brenner (25) it is clear that *B. subtilis* 168 spores can contain only a single set of genetic information and the meaning of Yoshikawa's results are uncertain.*
DNA content is compared to the genome size. While genetic information is not available for *B. megaterium*, the X-ray inactivation of spores (4) and the autoradiography studies (8) suggest the presence of 2 genomes per spore, again consistent with the genome size measurements presented here. Thus, the presence of sufficient DNA in *B. cereus* 569 spores to accommodate 2 complete genomes supports our conclusion that *B. cereus* 569 is diploid.

Criteria for Diploidy

It should be pointed out that comparison of genome size with spore DNA content does not provide sufficient evidence to establish diploidy. Recently, Carlton and Helinski (29) reported that DNA from *B. megaterium* contained up to 40% small, heterogenous circular forms. Although the study indicated that many spore-forming strains lacked significant quantities of these circular DNA forms, their presence would complicate interpretations about the number of genomes per spore. The DNA's used for these experiments were not examined in cesium chloride-ethidium bromide gradients and the existence of circular DNA elements in *B. cereus* 569 cannot be entirely ruled out. However, no deviation of the DNA reassociation kinetics from second-order was observed, as might be expected if *B. cereus* 569 contained a large portion of its total DNA as non-chromosomal elements.

The autoradiographic analysis of segregating units from single spores can also be misleading. The presence of 2 complete genomes per spore may be due only to the mechanism of segregation or packaging of DNA during
sporulation in some strains. Such diploid nuclei in spores would produce 4 segregating units, but informational segregation would also occur. True diploidy implies that the 2 genomes present in each nuclei do not normally segregate from each other during cell division. A heterozygote with 2 genetically distinguishable genomes per spore, would produce 4 segregating units as measured by autoradiography, but no genetic segregation would occur. Thus, to establish that *B. megaterium* is truly diploid will require genetic analysis.

The genetic evidence presented in this paper is very pertinent to an argument for diploidy in *B. cereus* 569. Unfortunately, next to nothing is known about the genetic map of *B. cereus*. However, none of the markers used in this study appear to be closely linked (Fraser, unpublished). This, coupled with the ability to recover the markers as a unit after segregation, regardless of the set of genetic characters used, makes the involvement of an episome unlikely. Furthermore, Pollack and Fleming (30) have recently reported a similar type of segregation in which unstable penicillinase negative cells of *B. cereus* 569 could be converted to fully penicillinase constitutive cells by treatment with heat or chloramphenicol. The above observations suggest that 2 alleles of each genetic marker involved can be present in the nuclei of spores or cells, and that the units containing these genes (possibly whole genomes) do not readily segregate from each other.

These genetic studies, in combination with the finding that the
B. cereus 569 spore contains sufficient DNA to accommodate 2 complete genomes, indicates that B. cereus 569 may be diploid. Further studies on the segregation phenomenon as well as the genetics of this organism will be required for conclusive proof.
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Figure 1. Phenotypic Segregation of Penicillinase Inducible (PEN$_{ind}$) cells from a Penicillinase Constitutive (PEN$_{mag}$) Culture.

--- total cfu

○-○ penicillinase constitutive cells

○-○ penicillinase inducible cells
Figure 2. Phenotypic Segregation of Sp$^+$ Cells from an Sp$^-$ Culture.

--- total cfu

- Sp$^-$ cells

- Sp$^+$ cells
COLONY FORMING UNITS / ml

HOURS
<table>
<thead>
<tr>
<th>Organisms</th>
<th>spores/ml (^{(a)})</th>
<th>DNA/ml (^{(b)})</th>
<th>DNA/spore (\text{grams})</th>
<th>DNA/spore (\text{daltons})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em> 569</td>
<td>(1.3 \times 10^{10})</td>
<td>230 ug</td>
<td>(17.7 \times 10^{-15})</td>
<td>(10.7 \times 10^9)</td>
</tr>
<tr>
<td><em>B. megaterium</em> 899</td>
<td>(2.2 \times 10^{10})</td>
<td>237 ug</td>
<td>(10.8 \times 10^{-15})</td>
<td>(6.5 \times 10^9)</td>
</tr>
<tr>
<td><em>B. subtilis</em> 168</td>
<td>(1.8 \times 10^{10})</td>
<td>86 ug</td>
<td>(4.8 \times 10^{-15})</td>
<td>(2.9 \times 10^9)</td>
</tr>
</tbody>
</table>

\(^{(a)}\) average of triplicate counts using a Petroff-Hauser counting chamber

\(^{(b)}\) measured by the indole method of Keck (17)
<table>
<thead>
<tr>
<th>Organism</th>
<th>$C_{o} t/2^{(a)}$</th>
<th>approximate no. (b)</th>
<th>mol. wt. (daltons)</th>
<th>Genomes/spore</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus 569</td>
<td>13.25 ± 0.85</td>
<td>$8.3 \times 10^6$</td>
<td>$5.5 \times 10^9$</td>
<td>2</td>
</tr>
<tr>
<td>B. megaterium 899</td>
<td>8.5 ± 0.70</td>
<td>$5.2 \times 10^6$</td>
<td>$3.4 \times 10^9$</td>
<td>2</td>
</tr>
<tr>
<td>B. subtilis 168</td>
<td>6.5 ± 0.90</td>
<td>$4.1 \times 10^6$</td>
<td>$2.7 \times 10^9$</td>
<td>1</td>
</tr>
<tr>
<td>E. coli B/r</td>
<td>7.25 ± 0.75</td>
<td>$4.5 \times 10^6$</td>
<td>$3.0 \times 10^9$</td>
<td>-</td>
</tr>
</tbody>
</table>

(a) expressed as mole nucleotide DNA times seconds/liter ($M \times \text{sec/liter}$) - Britten and Kohne (10)

(b) relation of $C_{o} t/2$ to nucleotide pairs derived by Britten and Kohne (10) using E. coli genome size as a reference (20)

(c) represents average reassociation values from both spore and vegetative cell DNA.