A STUDY OF THE GROWTH OF BACILLUS MEGATERIUM IN STEADY STATE CULTURE

by

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INTRODUCTION

The bacterial endospore has been observed, recognized, and studied for many years. These observations have covered many aspects of the cycle of spore existence. The various steps in this cycle have been outlined and defined very carefully by several workers. One of these classifications involved the time sequence as follows: vegetative cell; sporangium, containing a refractile spore; free endospore; germination; and outgrowth. Other workers have suggested such other phases as the forespore.

The present study was undertaken to attempt to study the transition between the first two stages mentioned above, namely that from the vegetative cell to the sporangium. It was hoped that some type of relationship between these two events other than the strictly temporal sequence could be elucidated. It has been suggested several times that some event or series of events which occur during the time of vegetative growth and multiplication of the culture is directly responsible for the initiation of the sporulation process. In other words it was thought by many workers that there was some type of "triggering" reaction or mechanism, which caused the cells of a particular culture to suspend the strict growth and multiplication of the vegetative phase and to begin to prepare a highly resistant resting cell form, the endospore. It was hoped that some evidence, either pro or con, could be found.

An apparatus, called the chemostat by its builders, Aaron Novick and Leo Szilard, was chosen as the method of growing and

studying cells just prior to sporulation and in preparation for that process. It was hoped that by using this method, a continuous supply of reproducible cells and thus reproducible results could be obtained.

The actual production of visible sporangia and free spores was followed by measurement of oxygen uptake in the Warburg apparatus on several of the cultures. It was intended to show the progression to completion of the reactions initiated and partially completed during the first non-visible steps of sporulation which previously had occurred in the chemostat growth vessel.

REVIEW OF LITERATURE

Sporulation

Endospore formation by the aerobic members of the genus Bacillus has been quite widely studied. Several excellent reviews (Cook 1932, Knaysi 1948) of this material, as well as the other stages of the spore cycle have been prepared. These reviewer's material in combination with the more recent literature on this general subject have been the main sources of the background upon which the present study was based.

There have been several hypotheses as to the reactions occurring during the sporulation process. The first, of three main hypotheses, was that the endospore is formed by the growth of a specialized granule in the bacterial protoplasm. (Koch 1896; de Bary 1887; and Ward 1895, in Bayne-Jones and Petrilli 1933.) A second is that the endospore was formed by the fusion of a

number of granules, some of which may be nuclear material. (Ernst 1888 and 1889; Babes 1889; Schaudinn 1902; and Dobell 1908, 1909, and 1911, in Bayne-Jones and Petrilli 1933.) The third hypothesis states that the endospore is formed by gradual condensation of the cell substance, possibly due to dehydration. (Matzuachita 1902 and von Daránÿi 1927 and 1930, in Bayne-Jones and Petrilli 1933.) These hypotheses were restated by Knaysi (1946). It has also been stated by Powell and Hunter (1953) that, "it is clear that sporulation involves concentration and possible de novo synthesis of cell material..." This latter suggestion has been modified and extended greatly by Foster and Perry (1954) and by Foster (1956). A system is suggested in which the vegetative cell proteins are broken down with the resulting amino acids being used in the synthesis of special heat resistant spore proteins. These new proteins are incorporated into the spore being formed. All of these hypotheses are of great importance to the study of the sporulation process. It has also been pointed out that certain elements and compounds are concentrated in rather large amounts within the forming spore. (Slepecky and Foster 1959.)

It has several times been suggested that the formation of the bacterial endospore was "triggered" in some fashion. (Bayne-Jones and Petrilli 1933, Knaysi 1948, and Hardwick and Foster 1952.) This "triggering" would be the result of some reaction or series of reactions in the vegetative cell which caused it to suspend the growth, division, and multiplication of the vegetative state and initiate the formation of a spore. This reaction or "triggering" would result in an irreversible committment to sporulation.

(Bayne-Jones and Petrilli 1933 and Foster 1956.) After such a reaction and the irreversible committment mentioned, the cells proceed to form the spores with which this paper is closely associated. As pointed out by the above authors, the sporulation process is completed within two hours after initiation. It is this fact which led to the assumption that the cells of Bacillus megaterium would produce spores in the chemostat growth vessel if environmental conditions were sufficient and a "triggering" were carried out.

The necessity of the presence of certain mineral elements in the environment of the sporulating cell has been pointed out by several workers. Among these necessary elements is manganese (Charney et al. 1951, Curran and Evans 1954, Fabian and Bryan 1933, and Slepecky and Foster 1959). Other metal requirements have been pointed out by several of the above authors (Charney et al., Curran and Evans, and Slepecky and Foster). All of these reports were taken into consideration during the choice of the growth medium to be used in the experiments to be carried out. The medium reported by Slepecky and Foster (1959) was used.

Several reports of antisporulation factors are also extant in the literature. Most of these reports are concerned with lipoidal or lipid like materials. Foster et al. (1950) reported such factors being present in complex organic media. They also state that such compounds may be removed by adsorption of the medium with Norite A. In a later paper, Hardwick et al. (1951) reported removal of apparently lipoidal or lipoprotein compounds by the continuous extraction with ethyl acetate, ethyl alcohol, or pyridine. These workers also

report that nonvolatile fatty acids showed greater inhibition than did volatile lipoidal fractions. By the addition of known fatty acids to their medium, they found that the greatest effect was produced with the use of C_{10}, C_{12}, C_{13}, and C_{14} acids. The statement is made that in casein hydrolysate peptone medium, lauric and myristic acids make up 15.8% of the antisporulation factors and are probably responsible for the antisporulation activity observed. Foster and Wynne (1948) showed the presence of some non-lipoidal inhibitors of sporulation. They further showed the inhibitory powers of unsaturated C_{18} fatty acids. The removal of these compounds by soluble starch was also discussed. The above stated facts made it essential that such compounds be rigidly excluded from the medium in use in the study. This fact made it doubly essential that a synthetic medium be employed for the growth of the cells of Bacillus megaterium.

Another environmental factor which has been widely and completely studied is that of oxygen requirements. The situation has been most forcefully stated by Knaysi (1945). He stated, "On the other hand, the necessity of oxygen for the quick and efficient formation of large numbers of endospores is one of the incontrovertible facts of bacteriology." Brunstetter and Magoon (1932) stated that, "...biochemical transformations in the cell leading to spore formation require oxygen." Foster and Heiligman (1949) reported the use of a continuous shaking machine for the uniform and homogeneous physiological conditions in their cultures. This use of some shaking device is widely accepted as a good means of

providing oxygen to a highly aerobic culture. Although short periods of anaerobic culturing do not irreversibly destroy the sporogenic properties of the cells, as reported by Hardwick and Foster (1952), a constant aerobic environment is strongly to be desired. Curran and Evans (1954) reported that shaking increased the number of spores in their experiments. Foster (1956) stated that mechanical shaking for homogeneity was almost universal at that time and that much limitation of aeration may occur without this precaution. Similar results are obtained by the use of the expedient of bubbling air through the growth medium at a rather rapid rate. Since the shaking of the chemostat apparatus was not feasible, it was decided to make use of a constant stream of air for the purpose of providing the necessary aerobic conditions for the growth and sporulation of the cells. This expedient also provided rapid and adequate mixing of the growing culture and maintained the population suspended throughout the medium.

Chemostat

The original description of the chemostat apparatus was made by its originators, Aaron Novick and Leo Szilard (1951). In this article, the authors presented a description and diagram of the apparatus. Also included in the paper were certain mathematical considerations of the operation of the apparatus and theory of growth obtained by its use. They stated that the apparatus was "a device for keeping a bacterial population growing at a reduced rate over an indefinite period of time."

The apparatus has been used primarily for the study of bacterial mutations. The first report of such a use of this particular apparatus was made by Novick and Szilard in 1950. In this series of experiments, the authors studied the mutation of the constantly growing cells to a faster growth rate or to a shorter generation time. As they state, the presence of a faster growing mutant causes the displacement of the parent culture and the eventual total supremacy of the mutant population. This material is also treated mathematically in an interesting and particularly applicable manner.

Kubitschek (1954) introduced certain variations in the basic design of the chemostat. His modifications were mainly concerned with the reservoir of nutrient medium and the means of delivering the growth medium into the growth vessel. The initiation of the use of a capillary tubing for the regulation of the flow of nutrients and the maintenance of the steady state were also discussed.

MATERIALS AND METHODS

Experimental Organism and Cultural Conditions

Endospores are, with a few exceptions, formed by the members of two genera of bacteria, the Bacillus and the Clostridium. The former genus, Bacillus, being aerobic lent itself to the methods of study to be used much more readily than did the anaerobic members of the genus Clostridium. It was decided that Bacillus megaterium should be the primary organism because of its large size, ease of growth, and the great amount of material extant in

the literature concerning this organism.

Maintenance cultures were carried on nutrient agar slants, which could be safely held for at least six months in screw capped test tubes. Inoculation of the chemostat growth chambers was made directly from one of these agar slants using a fairly large inoculating loop of organisms. The loop was used to inoculate a fresh slant after removal from the chemostat growth vessel.

It was thought desirable to use a completely synthetic growth medium in order that as many variables as possible could be eliminated or controlled. The use of such a synthetic medium of known composition also allowed the variation of one or more of the components to any desired concentration. Thus the effect of the overall composition or of the concentration of each of the components could be determined. It was thought that it might be possible to determine the effect of certain constituents such as manganese; the carbon source, sucrose; or the nitrogen source, NH_4, on the "triggering" or progress of the sporulation reaction of the cells.

A study of the literature revealed several possible choices of previously reported media. After further study, it was decided to use the medium of Slepecky and Foster (1959). This medium, designated as "synthetic sucrose" in this paper has a composition as follows:

Sucrose	1.0 gm.
$(NH_4)_2HPO_4$	1.0 gm.
KH_2PO_4	5.0 gm.
$MgSO_4$	0.2 gm.
NaCl	1.0 gm.
$CaCl_2$	5.0 mgm.

$MnSO_4$	7.0 mgm.
$ZnSO_4$	10.0 mgm.
$FeSO_4$	10.0 mgm.
Distilled Water	1.0 l.

The medium was prepared as three separate solutions which were added together to prepare the complete medium. The first of these solutions was a stock mineral salts solution made up by adding ten times the final concentration of the salts to one liter of distilled water, allowing each to dissolve before adding the next in order to prevent, as far as possible, the formation of a colloidal precipitate of the ingredients. The second solution contained the two phosphate compounds. The compounds were dissolved in 100 milliliters of distilled water, adjusted to a pH of 6.9 and then enough distilled water was added to make the final total volume of the solution 200 milliliters. The third solution contains the sucrose, magnesium sulfate, and sodium chloride dissolved in 600 milliliters of distilled water. In preparing the final medium, one combined 1.0 milliliter of the mineral salts stock solution, 60 milliliters of the sucrose containing solution, 19 milliliters of distilled water and after mixing them, autoclaved in the growth container. The phosphate buffer, 20 milliliters, was autoclaved separately in another container and added to the above growth vessel after cooling. This separate sterilization was necessitated by the fact that the mineral salts would precipitate out of solution as a phosphate compound if the buffer were present during autoclaving. In working with the chemostat, four liters of medium were prepared at one time and placed into the reservoir bottle.

All experiments were carried out at 30°C. plus or minus 2°C.

Petroff-Hausser Counting Chamber

The enumeration of the bacterial suspensions was necessary in order to show the presence and maintenance of the steady state in the chemostat growth vessels. Among the many available methods of enumeration, direct counts and spectrophotometric determination of turbidity were considered the most feasible. Turbidity measurement was decided against because it would have required the construction of a special carrier and holder for the positioning of the growth vessel in the instrument. It was also felt that the length of time necessary to collect a one to two milliliter sample from the overflow tube of the growth vessel would not be reproducible enough nor controllable enough to warrant its use.

From the several direct counting methods in use, the counting chamber method was chosen. This method is generally considered to be the most accurate as well as one of the quickest. Wilson and Kullmann (1931) have reported that, "The direct count with the Petroff-Hausser or similar bacterial counting chambers proved to be the most accurate of all the methods investigated and its use whenever possible is advised." Smith (1925) also suggested the use of the counting chamber in bacterial enumeration and reported on their use at the Army Medical School.

In regard to the accuracy of the results obtained with the counting chamber, Wilson and Kullmann (1931) reported that, "Direct counts of Rhizobia by means of a Petroff-Hausser counting chamber can be made under laboratory conditions in such a manner that the variance is that due to chance alone." These workers further stated, however, that "...the accuracy of the count will

be dependent solely on the total number of organisms counted." For this reason a policy of counting the total 400 square ruled field of the chamber or of at least 200 cells in scattered areas of the ruled field was followed. These procedures were occasionally hindered by the formation of long rather tightly wound or folded chains during the continued growth of the cultures. This chain formation will be further discussed later in this paper.

The actual calculation of total number of cells from the number of cells counted was carried out according to the formula which follows:

$$\text{Bacteria per milliliter} = \frac{\text{Total bacteria counted X 20,000,000}}{\text{Number of small squares counted}}$$

The log of the number of bacterial cells per milliliter of the growth medium was then plotted against time in hours to obtain a curve indicating the growth of the culture. From the graphs, it was apparent that the steady state had been established and maintained. This fact could be easily seen from the horizontal nature and constant height of the curve. Thus the number of bacteria was constant over an extended period of time, within acceptable limits of error for sampling the growing culture and determination of numbers in the counting chamber.

Phase Contrast Microscope

All enumerations of bacterial suspensions were carried out by direct microscopic counts, as outlined above. These counts, as well as cultural examinations, were carried out using an American Optical Company Phase Contrast Microscope model 6TC-P4. The ease

of discrimination between spores, sporangia and vegetative cells was greatly increased by use of this apparatus. It has been shown that the highly refractile spore can be easily and quickly identified on the basis of its refractility alone, without recourse to other such time consuming processes as heat shocking and plating, or differential staining. In this paper, as in most other literature on this subject, a sporangium refers to a vegetative cell in which is contained the refractile spore about to be released and a spore is designated as a round to oval very highly refractile body. The spores produced at various stages in this study were always found to be typical of those of Bacillus megaterium. An electron micrograph of spores from one of these cultures is presented in Plate I. Special note should be made of the similarity of this micrograph to that of Mayall presented in Gunsalus and Stanier (1960) page 226. It will also be noted that this is the same typical form as the sections made by Mayall and Robinow, shown on page 218 and the replica preparations made by Fitz-James, presented on page 224 of Gunsalus and Stanier. The vegetative cell of Bacillus megaterium is rod shaped, 1.2 to 1.5 by 2.0 to 4.0 microns, having rounded ends, and occurring individually or in short chains. A sporangium of this organism refers to the still intact vegetative cell, containing a fully formed, already refractile spore. These latter forms must be differentiated from vegetative cells which contain a generalized area which will become the completed spore, the forespore, but which have not yet become refractile. For purposes of simplicity, the vegetative cells containing forespores have been lumped together with the vegetative cells proper.

EXPLANATION OF PLATE I

Electron micrograph of four spores of Bacillus megaterium growth in the chemostat. Magnification approximately 30,000.

PLATE I

Warburg Apparatus

Oxygen uptake was measured manometrically using common procedures outlined in Manometric Techniques (Unbreit et al., 1957.). The water bath was maintained at 30°C., the same temperature used for the growth of the cells in the incubated growth vessel. The center wells of the Warburg flasks contained 10% KOH to absorb the carbon dioxide in the air above the cell suspension and any carbon dioxide formed by the respiring cells. The shaking rate of the manometers was maintained at 120 oscillations per minute throughout the experiment with the exception of the time during which readings or resettings of the level of the manometer fluid were taking place. The total cumulative oxygen taken up by the cells was plotted against the elapsed time in hours.

Chemostat

Several types of apparatus for the continuous culturing of bacterial as well as other types of cells have been in existence for many years. Although they are represented by many varied names, these types of apparatus fall into two main categories, those which are externally controlled and those which are internally controlled. Internal control refers to the case in which the size of the population is determined by internal conditions such as turbidity, which activates the controlling systems and maintains a constant population size. External control on the other hand, depends on there being a constant rate of addition of nutrient materials with one of the nutrilites in a limiting amount.

In this case the size of the population is determined exclusively by the concentration of a single nutrient substance as it is added from a reservoir supply.

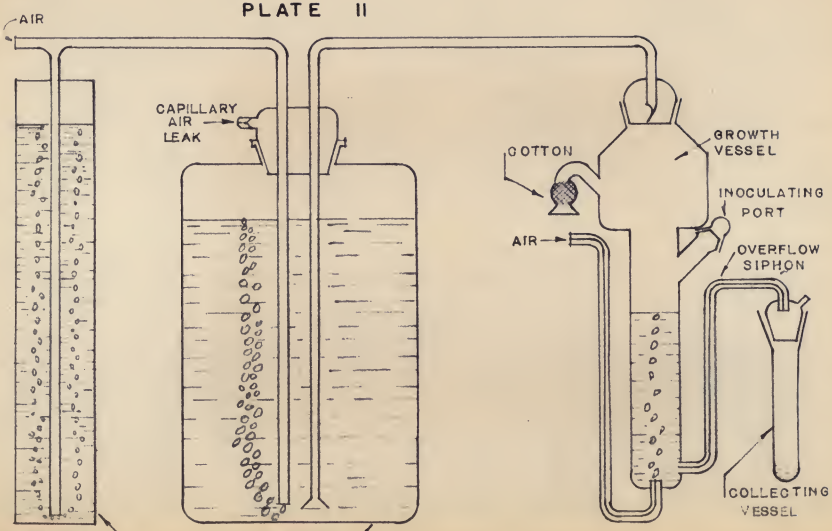
The chemostat, devised, built, and first discussed in the literature by Novick and Szilard (1951) was based on an external control system. (See Plates II and III for a diagram and photograph of the chemostat.) In their article (1950), the same authors discuss the use of the chemostat with a limiting amount of tryptophan in a synthetic medium on which a tryptophan requiring mutant of Escherichia coli was being grown. This and several other similar systems, involving the growth of metabolic mutants requiring some nutrilite, have been fairly widely reported. There have also been some cases of limiting the supply of carbon or nitrogen sources, mostly in internally controlled systems. In the project to be reported on here, it was decided to use a limitation of carbon source as the externally controlling factor. Using the synthetic sucrose medium outlined above, this limitation amounted to the variation of the concentration of sucrose from the basic 0.1% normally present, while keeping the flow rate into the apparatus constant.

Kubitschek, (1954), reported a modification of the basic chemostat apparatus of Novick and Szilard. (See Plates IV and V for a diagram and photograph of the modified chemostat.) This modification involved a slightly differing mode of transfer of medium from the reservoir to the growth vessel. The addition of a mariotte bottle as the reservoir and the use of a capillary tube above the dripping tube, allowed a great simplification of

EXPLANATION OF PLATE II

Diagram of the major divisions of the chemostat, showing the pressure regulator, reservoir bottle, and the growth vessel. After Novick and Szilard (1951).

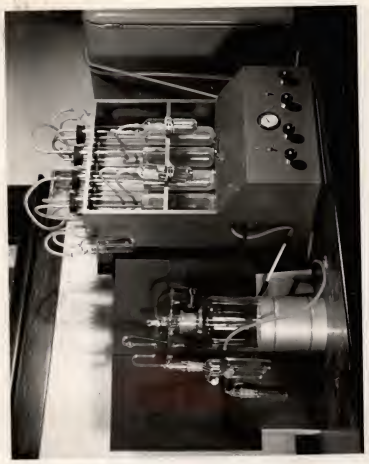
PLATE II


AIR
CAPILLARY AIR LEAK
COTTON
AIR
GROWTH VESSEL
INOCULATING PORT
OVERFLOW SIPHON
COLLECTING VESSEL
PRESSURE REGULATOR
RESERVOIR

EXPLANATION OF PLATE III

Photograph of the chemostat apparatus as prepared for operation.

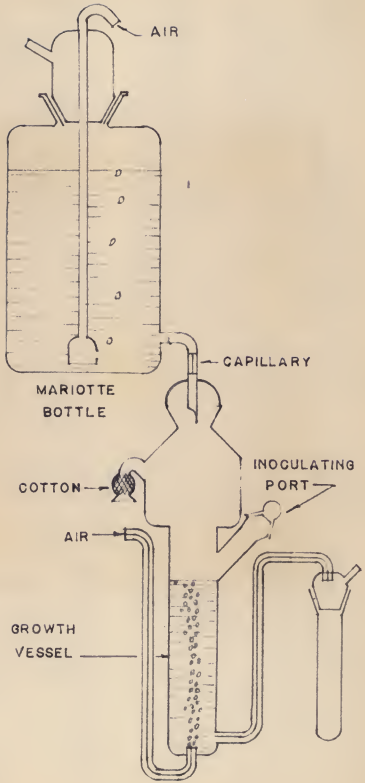
PLATE III



EXPLANATION OF PLATE IV

Diagram of the Kubitschek modification of the chemostat. Use of a mariotte bottle as the reservoir and insertion of a capillary tube to control flow rate are shown. Also present in the diagram is the growth vessel. After Kubitschek (1954).

PLATE IV


AIR
CAPILLARY
MARIOTTE
BOTTLE
INOCULATING
PORT
COTTON
AIR
GROWTH
VESSEL

EXPLANATION OF PLATE V

Photograph of the Kubitschek modification of the chemostat as prepared for operation.

PLATE V

the apparatus, both in glassware and in the hardware of regulating systems. Although both types of chemostat were available, it was decided that the Kubitschek modification should be used exclusively in this study. This decision was based on the greater mobility and greater adaptability of the Kubitschek model. All further references made in this paper to the chemostat should be assumed to be the Kubitschek modification of the apparatus of Novick and Szilard.

It was necessary to have a vigorous mixing taking place in the growth vessel in order to keep the majority of the bacterial cells and chains suspended. Since the genus Bacillus is highly aerobic, it was also necessary to provide some means of supplying large amounts of oxygen to the cells, to insure their growth at optimum conditions and rates. It was hoped that the oxygen supply could be kept high enough that it would not itself become a limiting factor in growth. In this way, the sucrose, controlled within known concentrations, would be the only limiting factor in the growth of the cell suspension. It was essential to this type of experiment that all growth limiting factors were known, kept constant, or controlled as the case required. The above mentioned mixing and aeration were carried out by the bubbling of a constant stream of air through the growing culture from bottom to top as shown in the diagram (Plate IV). This air stream was supplied by an aquarium pump. Also attached to the pump was a hose to the top of the mariotte bottle which kept a constant column of air in the central tube and a small release of air bubbles. This latter

attachment was required in the process of maintaining a constant head of pressure and, thereby, a constant rate of flowing of the nutrient medium into the growth vessel.

Several excellent reviews of the growth of bacteria in steady state culture have been prepared (Novick 1955 and James 1961). The former of these two has a good description of the various types of apparatus used in these studies. Novick also spends a portion of his time describing the mathematical considerations involved in evaluating studies making use of the chemostat, which he helped to develop. Several of the equations which he presents are particularly applicable to the study under consideration. Among these equations are that which relates to the growth law in a continuously diluted system. It is:

$$\frac{dN}{dt} = \alpha N - \frac{w}{V}N = \left(\alpha - \frac{w}{V}\right) N$$

where N = the number of bacteria per milliliter, t = the time, alpha = the mean division rate, w = the rate of flow of the liquid from the reservoir into the growth vessel and the simultaneous flow of culture from the growth vessel through the overflow siphon, and V = the volume of the growth vessel in milliliters. In this equation, the factor of $\frac{w}{V}$, if larger than alpha will result in the removal of more cells from the growth vessel than are replaced by division, and if smaller in magnitude than alpha will allow the cells to multiply more rapidly than they are washed from the growth vessel. In either case, the culture will reach a situation at which the cells are multiplying and being replaced at exactly the

same rate at which they are being washed out of the growth vessel. At this point, the bacterial population will be in a steady equilibrium state which will be maintained as long as the environmental conditions are maintained constant. A second pertinent equation is:

$$\frac{dN}{dt} = \alpha(c)N - \frac{w}{V}$$

This equation represents the situation in which the growth rate, alpha is a function of the concentration, c, of some limiting nutrient. This case is the one which is most applicable to the conditions of the experiments to be discussed in this paper. In all the experiments to be discussed, the carbon source, sucrose, was maintained in a limiting concentration. It is this equation, therefore, which describes the law of growth for the chemostat cultures.

RESULTS

The ideal situation in chemostat cultures is that in which the growing culture is maintained in a steady state. Under these conditions there is a constant composition of the contents of the growth vessel. This constant composition refers not only to the number of cells, but also to the concentration of nutrilites, which are of course being constantly removed by overflow as well as added from the reservoir, and to waste products of metabolism which are added by the growing cells and removed at the overflow. There is a constant volume of growing culture of a constant

composition.

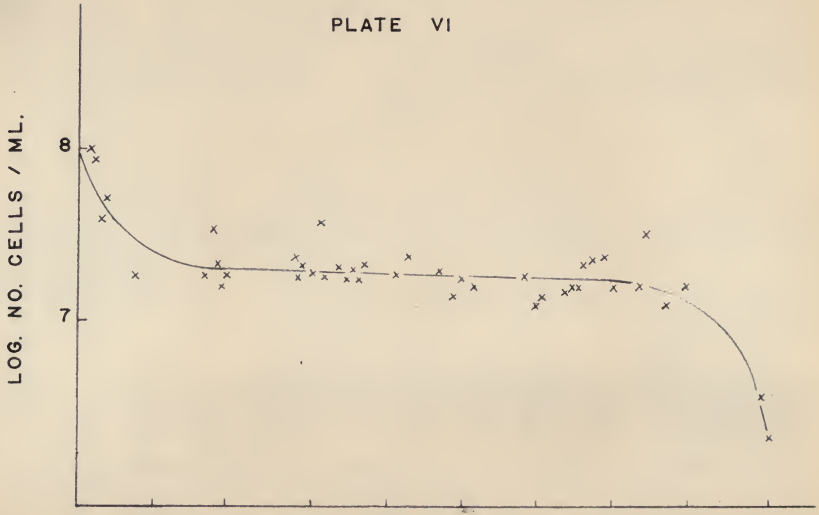
The constancy is most easily shown by the uniform number of organisms present in the growth vessel. One can represent this uniformity by the use of graphs such as that presented as Plate VI. In this case, the number of cells per milliliter obtained by calculations based on the direct microscopic count with the Petroff-Hausser Counting Chamber, was plotted against the time in hours after the initiation of the flowing of the growth medium from the reservoir into the growth vessel. In this as in all further cases, the cells were inoculated into the growth vessel and allowed to grow up to a medium turbidity as determined visually, before the flow of fresh medium into the growth vessel was begun. A study of this graph revealed that the number of cells was effectually invariant over a period of five days, before a contaminant which grew faster than the Bacillus megaterium took over the major proportion of the numbers in the culture and the run was ended. The numbers of cells, with only a few exceptions were between two and four times 10^7. This constancy is of course impossible to maintain in any other means of culturing such as batch culturing. The higher numbers which will be noted at the initial end of the graph were due to the fact that the turbidity was allowed to become greater than the steady state level before the flow of medium was begun.

If enumeration of a batch culture, as for example from a shake flask of the same medium and organism, had been begun at the same turbidity, it would have been found to be at about the end of the log phase of the growth curve. In this hypothetical

EXPLANATION OF PLATE VI

Graph representing the number of cells of *Bacillus megaterium* plotted against hours of culturing in the chemostat. The graph shows cells growing in the steady state.

PLATE VI



case, the cells would have begun to show forespores and to have been converted into sporangia within about five hours (Slepecky and Taylor, unpublished results). The further sampling of such a shake culture would have revealed that about 90% of the vegetative cells would have been replaced by free, mature spores within approximately fifteen hours after the beginning of sampling (Slepecky and Taylor). By the use of the chemostat, the culture of cells was made to continue growing steadily in the logarithmic phase for 115 hours. Throughout the 115 hours during which the above culture was being observed, there were no free spores, sporangia, nor even forespores observed in any of the samples removed from the growth vessel. This fact points up another important observation made in growing Bacillus megaterium in chemostat culture, namely non-sporulation in the constantly growing cultures. This is a case showing growth for 100 hours beyond the point at which one would normally have expected to find free spores present in batch cultures, without the appearance of the said spores. Thus the chemostat allows the cultivation of this aerobic spore-forming organism on the particular medium in use without the formation of spores as long as growth conditions are correct and contamination can be prevented.

Although there are several variations of small magnitude in the curve in question, these can be ascribed quite readily to sampling errors, inherent errors in the enumeration procedure, or to the possibilities of variation in flow rates or temperatures. This graph falls well within what might be expected and accepted

as steady state growth or culturing.

A second phenomenon, peculiar to the apparatus in use, is shown by Plate VII. In this case, the flow of medium was begun with a fairly low turbidity and allowed to increase until it should reach a steady state or equilibrium level. The run was begun with a three centimeter lenth of capillary inserted into the tube connecting the reservoir to the growth vessel. This length of capillary allowed quite a rapid flow of the medium and therefore a higher concentration of limiting nutrilite, sucrose, thereby allowing the culture to proceed farther along the growth curve and to reach a higher equilibrium point. In this case the similarity of the initial portion of the curve to a normal growth curve should be noticed. This similarity is based on the fact that it shows a normal logarithmic increase in numbers and a leveling off toward a stationary phase of growth. At the 37 hour sampling, forespores and sporangia were observed in several of the cells counted. The formation of the initial sporangia at this point during the growth phase is in very good agreement with the statements of Henrici (1928) in regard to the sporulation process. He states that spores are formed "...at the point of inflection between the log and stationary phases of the growth curve."

Since it was desired to maintain the culture in a presporulation phase, the length of the capillary was increased from three to six centimeters. This change caused a slowing of the rate of entry of fresh medium into the growth vessel. The consequent reduction in concentration of sucrose per unit volume

EXPLANATION OF PLATE VII

Graph representing the number of cells of *Bacillus megaterium* plotted against hours of culturing in the chemostat.

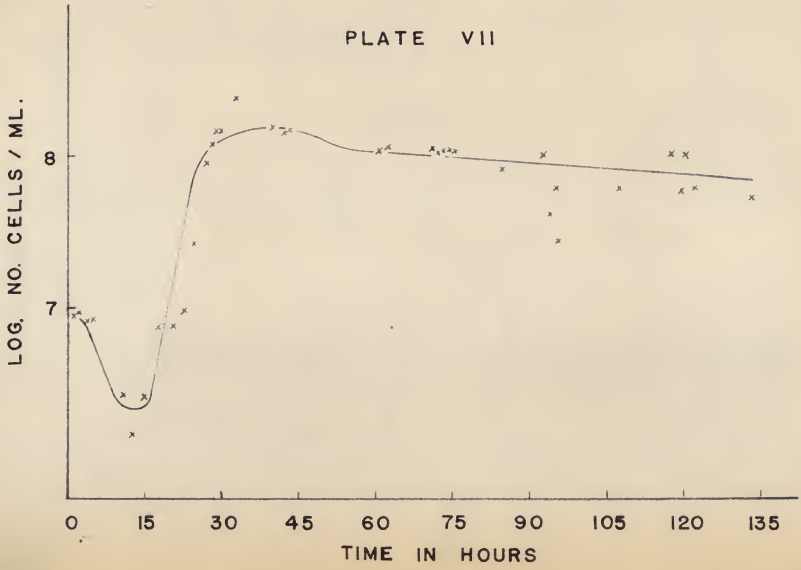


PLATE VII
LOG. NO. CELLS / ML.
8
7
0
15
30
45
60
75
90
105
120
135
TIME IN HOURS

available to the cells in the population, caused a reduction of the numbers of cells and effectually a retreat downward to a lower point of equilibrium on the normal growth curve. The lowered washout rate, which would have tended to increase the numbers of cells, was overcome by a much more greatly lowered growth rate or doubling time. In this graph is depicted the possibilities of adjusting a bacterial population to a desired point in the growth curve and maintaining it there for an elongated period of time. Also shown is the ability of the experimenter to vary the position of this equilibrium at will and thus to simulate any portion of the growth cycle of the cells, with the possible exception of lag and decline phases, which would not lend themselves to this type of apparatus.

The fact that one can keep a culture of organisms growing in steady state has been used by several previous workers. These workers have used such steady state cultures as sources for batches of cells to be used in other experiments. The different samples of cells taken from a chemostat can be assumed to be identical. This assumption was based on the fact of steady state with its previously mentioned constancy of composition.

The graph under discussion, in its middle phases, supports the previous graph in depicting a steady number of cells per unit of time elapsed. Here again the almost even and essentially horizontal nature of the middle portion of the curve shows the extent and nature of the equilibrium maintained.

The wider fluctuations in the later portions of the graph

are easily explained. A power failure caused the cessation of both the source of aeration and mixing in the growth vessel and the heating apparatus of the incubator housing the whole chemostat. The loss of aeration and the decrease in temperature of course caused a reduction in the rate of growth of the culture. Since the rate of flow of medium into the growth vessel and the rate of overflow and washout of the culture remained constant throughout, the total number of cells was decreased. The cells were washed out of the growth vessel faster than they were replaced by division, until a new equilibrium level was established.

It is interesting to note that, after the first of these fluctuations, the culture recovered and returned to its original equilibrium level. The return of aeration and the increase in temperature caused an increased growth rate expressed as a shorter generation or doubling time. This increase in the rate of division of the cells of the population while the inflow and washout rate remained constant led to an increase in numbers in the population until equilibrium was once again reached. Since the flow rates, temperature, and aeration were equivalent to those before the power failure, it was not surprising to find that the equilibrium point established was equal to that of the previous portion of the curve. This fact again bore out the steady state features of the apparatus. Still another means of regulating the level of equilibrium of the population is represented by the data graphed in Plate VIII. Once again the graph is one presenting the numbers of bacteria per milliliter of

EXPLANATION OF PLATE VIII

Graph showing the number of cells of Bacillus megaterium plotted against the hours of culturing. The graph shows regulation of the population size by the changing of the concentration of sucrose in the medium.

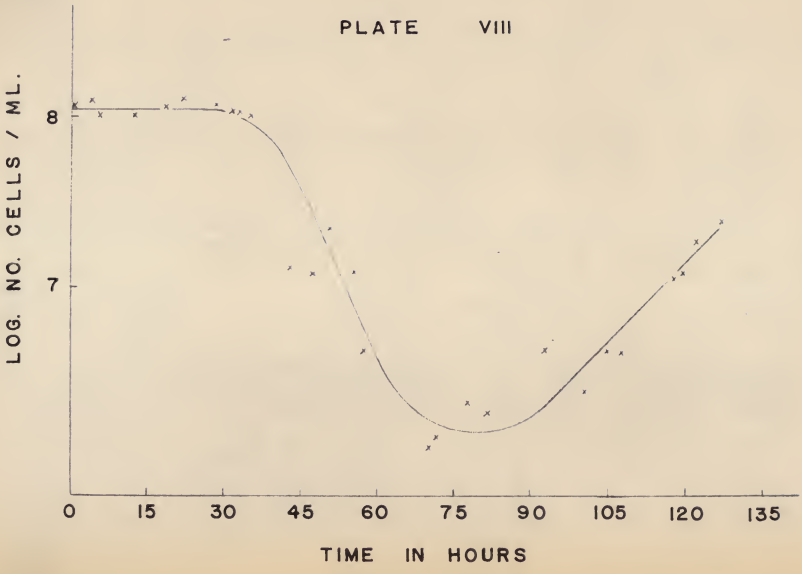


PLATE VIII
LOG. NO. CELLS / ML.
8
7
0
15
30
45
60
75
90
105
120
135
TIME IN HOURS

culture medium against the hours of growth since the beginning of medium flow. The chemostat growth vessel and dripping apparatus were connected to two separate reservoir bottles by a Y tube. A six centimeter length of capillary tubing was inserted into the system above the dripping apparatus and the experiment was begun. The initial inoculation was made into normally prepared synthetic sucrose medium as outlined above. The population in the growth vessel was established at an equilibrium level of about one to two times 10^8 cells per milliliter and maintained on 0.1% sucrose at that level for 32 hours. Once again the constancy of numbers was clearly shown by the horizontal nature of the graph.

At this time, the connecting tubing from the reservoir in use was clamped off and the medium from the other reservoir was allowed to begin entering the growth vessel. The medium in this second reservoir bottle contained synthetic medium made up containing only half the normal amount of sucrose, that is 0.05%. The curve clearly showed the profound influence of this decrease in available carbon. Once again the cells were being washed out of the growth vessel faster than their numbers were replaced by division. The numbers, therefore, decreased until a new equilibrium was instituted. A fairly stable population level was maintained for 40 hours.

The source of fresh nutrients was again switched from the 0.05% sucrose containing reservoir to the 0.1% reservoir after 105 total hours of growth in the chemostat. As was to be expected, the increase in available sucrose, being added at the

same rate as previously, caused an increase in numbers. The shape of the curve during this increase was again that of a logarithmic increase.

Although it was necessary to discontinue this experiment because of the entry of contamination, it is firmly believed that the culture would have been established at an equilibrium level equal to that at which it had been growing on the original 0.1% sucrose. This increase to the old equilibrium level would be exactly analogous to the initial establishment of equilibrium in the preceding graph, Plate VIII, or in any one of the chemostat experiments.

The first three graphs have shown the establishment and maintenance of a steady state culture and its manipulation. The setting of equilibrium levels by the two expedients of changing the length of capillary tubing regulating the flow rate of medium and the changing of the concentration of the limiting nutrilite in the inflowing medium have been outlined in detail.

A third method of regulation of equilibrium levels is available to the experimenter making use of the Kubitschek (1954) modification of the chemostat. This method of regulation is based upon the vertical distance between the reservoir bottle and the level of the capillary in the tubing connecting it to the growth vessel. The physical principle involved is that of the head of pressure caused by the force of gravity on the liquid in the reservoir. By increasing the height of the pressure head, that is increasing the distance between the bottom of the reservoir and the bottom of the capillary tube, one can increase the

rate of flow of the medium into the growth vessel. As with the other methods of regulation, an increase in medium inflow causes an increase in growth rates of the population and a consequent rising of numbers and a higher level of equilibrium.

Plate IX depicts a portion of a longer full experiment. The numbers had begun to decline as indicated in the first eight hours of the graph. In order to reverse this trend, and to bring the equilibrium back up to a convenient level for counting and accuracy of sampling, the growth vessel was raised about five inches while the level of the reservoir bottle was held constant. This movement effectively lessened the hydrostatic pressure of the system due to gravity and slowed the inflow of nutrient medium into the growth vessel from about 10 milliliters per hour to about eight milliliters per hour. The subsequent lowering of the overflow rate and the smaller numbers of cells being washed from the growth vessel while growth rate was maintained almost constant resulted in an increase in numbers. After a slight overshooting of the final level, the population was stabilized at an equilibrium level of about three times 10^7 cells per milliliter. Thus is shown the effect of changing the length of the pressure head in controlling the numbers of bacteria.

This method of control is closely related to the first method mentioned, namely the variation in the length of the capillary inserted into the medium delivery tube. In both of these cases, the method of control is based on the regulation of the rate of inflow of the fresh nutrient medium while other conditions remain essentially constant.

EXPLANATION OF PLATE IX

Graph showing the number of cells of Bacillus megaterium plotted against the hours of culturing. The graph shows the regulation of the population size by the variation of the pressure head.

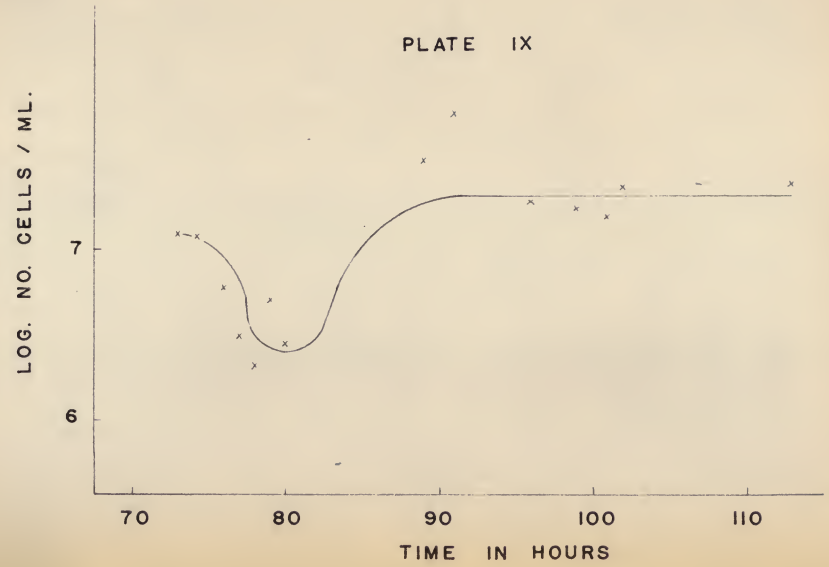


PLATE IX
LOG. NO. CELLS / ML.
7
6
70
80
90
100
110
TIME IN HOURS

When either the hydrostatic pressure or the length of capillary was changed, the result was a change in the flow rate of the incoming medium, as stated above. This change in flow rate had two separate and distinct effects on the culture in the growth vessel. The change in flow rate caused a variation in the concentration of the limiting nutrilite present in the growth vessel at any one time. The change in rate also caused an increase in the rate of outflow through the siphon or leveling device shown on the diagram of the apparatus. (See Plate IV.) If the flow rate increased, the effect of higher concentrations of sucrose was to increase the number of organisms in the growth vessel. Such an increase, however, also caused an increase in the number of cells washed from the growth vessel through the siphon overflow, thus tending to decrease the total number of cells in the growth vessel. These two effects and their results are in direct conflict and the final result is determined by a summation of the two separate effects. The effect of outflow of the cells generally had the greatest effect and is the most reliable indicator of what will occur as a result of a particular change in the controlling mechanisms.

The curve depicted in Plate X shows an experiment using a chemostat charged with 0.2% sucrose, that is, synthetic sucrose medium containing double the normal amount of sucrose. The turbidity was allowed to become quite high before the flow of nutrients was begun. After initiation of addition of nutrients from the reservoir, the turbidity and cell numbers dropped quite rapidly to an equilibrium level of about three times 10^7. It

EXPLANATION OF PLATE X

Graph presenting the numbers of cells per milliliter against the hours of culturing.

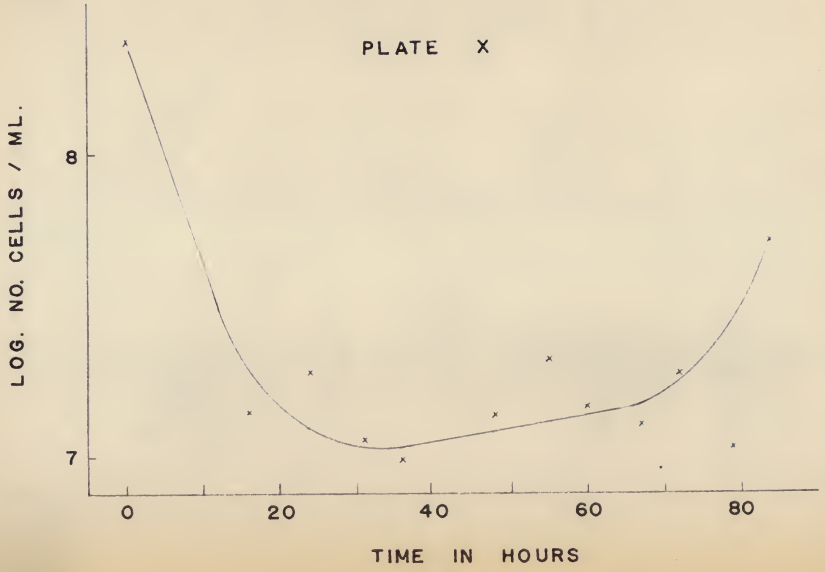


PLATE X
LOG. NO. CELLS / ML.
8
7
0
20
40
60
80
TIME IN HOURS

should be pointed out that this level is about the same as the level normally maintained on 0.1% sucrose in the previously presented graphs. This level of growth was maintained for about 30 hours.

A second reservoir containing 0.1% synthetic sucrose medium was attached to the above chemostat by means of a Y tube. The composition of the inflowing medium was changed from 0.2% to 0.1% sucrose after the sampling at 48 hours. It will be immediately recognized that the population maintained essentially the same equilibrium level, three times 10^7, after the change as it had before the change. This maintenance of the same equilibrium for an additional 30 hours is of particular interest.

The explanation of this result seems to involve the concentration of the sucrose in the medium. It appears that above a certain level, apparently between 0.1 and 0.2%, the concentration of sucrose was no longer a limiting factor in the growth of the population in the growth vessel. If the concentration of sucrose was above its limiting value for the rate of flow being maintained, that is, if the concentration of sucrose was such that it was not entirely used by the cells during their growth as quickly as more was added by the inflow of fresh medium, the number of cells and their growth rate was determined exclusively by the rate of inflow of medium. There was also a simultaneous overflow of spent culture fluid containing part of the bacterial population. In this case in particular, the sucrose concentration was not limiting and the rate of overflow was maintained

constant, thereby allowing the numbers to be determined by the overflow rate exclusively. As mentioned, this rate was held constant and thus the numbers were also held constant.

This experiment points up the fact that one must be aware of the conditions of his experiment and be able to control them. It was necessary to vary the limiting concentration alone while maintaining the flow rate constant. Because of the results described for this experiment it was decided that 0.2% sucrose could not be used in further experiments. This left a range of variation in concentration of sucrose from 0.1% downward to be explored.

In pursuance of the study of varying concentration of the sucrose in the medium, concentrations of 0.05% and 0.025% were tried. Reference to Plate VIII will show that the use of 0.05% sucrose concentration was within the limiting range and that it allowed good growth of the organisms under the conditions used in these experiments. In all cases in which 0.025% sucrose was used, however, the cells did not grow in a satisfactory manner. It was assumed that this concentration would be appropriate for a limiting parameter if the cells would grow well. The fact that the cells in the growth vessel never reached a turbidity acceptable to counting within limits of accuracy by the use of the Petroff-Hausser Counting Chamber, was rather surprising. The cells of Bacillus megaterium when inoculated into the growth vessel containing 0.025% synthetic sucrose with adequate aeration and no inflow of fresh medium nor washout of any of the culture, would begin to grow giving a slight visible turbidity.

In no case, however, did the turbidity reach a level which would warrant the beginning of inflow of medium. The numbers were so low that the flow when started only served to wash the already present cells from the growth vessel leaving no cells at all. Therefore the concentration was found to be best between 0.05% and 0.1% for the purposes of the study underway.

Since sporulation and a "triggering" thereof were involved in the study being reported, it was necessary to show that the organism in use, Bacillus megaterium, would indeed form spores under the conditions in use in the chemostat. This problem was approached in the following manner. A chemostat was set up having 0.1% synthetic sucrose in the reservoir bottle. The growth vessel was inoculated and the cells allowed to grow up to a visible turbidity. The flow of fresh nutrient medium was then initiated. The population was allowed to establish a steady state of growth. Plate XI shows the results of the experiment in question. The steady level of about two times 10^7 cells per milliliter of culture medium was maintained for 26 hours to establish the fact that the culture was indeed in the steady state, that the cells were growing logrithmically, and that no sporangia of the cells were present in the cells at that point. With these initial conditions established, it was possible to initiate the desired variations in conditions. The inflow of fresh medium was precluded by the clamping off of the tube connecting the reservoir bottle and the growth vessel. This procedure also stopped the washout of cells through the overflow siphon. Thus the cells present in the growth vessel were left

EXPLANATION OF PLATE XI

Growth and sporulation of Bacillus megaterium is presented in this graph of number of cells per milliliter versus the hours of culturing.

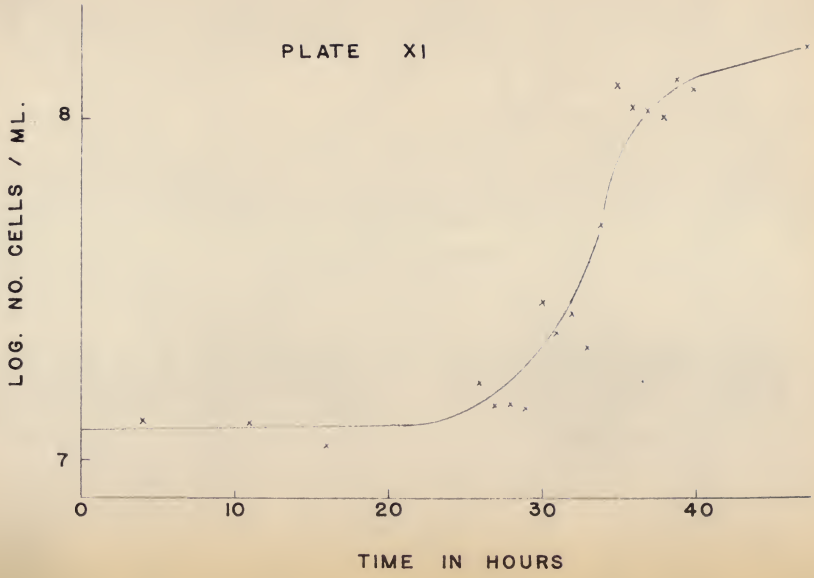


PLATE XI
LOG. NO. CELLS / ML.
8
7
0
10
20
30
40
TIME IN HOURS

to continue growth as a batch culture under the same conditions of aeration, temperature, and enclosing environment as pertained in the other experiments. The only difference in conditions was the lack of replacement of nutrients and the washout of waste products with a part of the culture. Sampling and enumeration were continued at regular intervals using the same procedures which were used to obtain the information for the steady state portions of the previous experiments.

The graph reveals that the numbers increased approximately logarithmically, leveling off at a level of about two times 10^8 cells per milliliter. From this point, the numbers rose gradually until the 48th hour, at which point dark forespores were first observed. Thus it was shown that the cells would proceed to the first stages of the production of spores in the chemostat environment in a manner analogous to that of a batch culture.

Another experiment concerned with this same problem was undertaken subsequently. The results, presented in Plate XII, are similar to those shown in the previous plate. The numbers again increased logarithmically from an initial low level and then levelled off. The levelling occurred at about one and one half times 10^8 in this case. The vegetative cells began showing the characteristic areas known as forespores after 58 to 59 hours. The forespores are recognized in the phase contrast microscope as dark areas in the cells which are in a central to para-central location. These dark areas later become the refractile spores.

EXPLANATION OF PLATE XII

Number of cells is plotted against hours of culturing.
The point of sporulation of the cells is indicated.

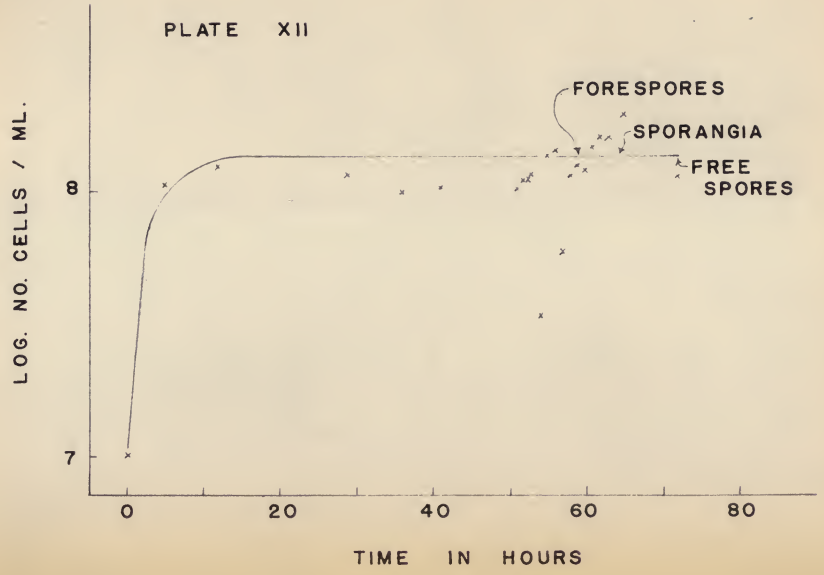


PLATE XII
FORESPORES
SPORANGIA
FREE SPORES
LOG. NO. CELLS / ML.
8
7
0
20
40
60
80
TIME IN HOURS

In this experiment it was possible to follow the further events of the progress of the culture from forespores to free spores. At the 62 hour sample it was observed that some of the vegetative cells contained the first refractile areas. These areas, not yet mature spores, only showed a fraction of the total refractility which they would eventually attain. In the 63 and 64 hour samples, all of the forespores had begun to take on varying degrees of refractility. The final sample shown on this graph, which was taken after 73 hours of culturing, showed that a great proportion of the cells were in the stage which is designated as the sporangium. There were also some free spores observed as having been released from the vegetative cells. This experiment confirmed the results of the previous one and extended them to include observation of the presence of sporangia and even free spores.

Quite wide variations will be noted in the later phases of this curve. Most of these variations were apparently caused by the beginning of the formation of bubbles at the surface of the growth medium in the growth vessel. This problem of foaming is quite acute in this type of apparatus, and is definitely to be avoided if at all possible. The formation of bubbles causes a concentration of the bacterial cells and waste products, especially those of high molecular weights, at the surface of the bubbles. The cells being thus concentrated are removed from the culture and not detected in the sampling and enumeration. Chances for contamination are also increased when excessive foaming occurs. The foam level may rise to a level in contact

with non-sterile parts such as the cotton in the pressure equilizing vent or through some of the ground glass joints which serve to connect the various sections of the apparatus together. Although the contamination problem is more severe in stationary or shaking batch cultures, there is still a definite hazard attached to foaming in the chemostat. The surface of the bubbles also provides a very good surface for the denaturation of proteins and could lead to an impairment of the viability of the cells, as by causing damage to the protein portions of the cytoplasmic membrane. The possibility of foaming was one of the criteria taken into account in the choice of the medium to be used. Synthetic sucrose, being of simple formula and having no added protein supplements nor any high molecular weight compounds in its makeup, had little predisposition to foaming. The medium was tested and found to be almost free from foam even when a good growth of bacteria was present. This one experiment is the only case of excess foaming observed throughout the duration of the study. The probable cause of the foaming was thought to be the release of high molecular weight substances, mostly protein in nature into the medium, either from the cells in the process of forming spores, or from the remains of the vegetative cells which had already been lysed in the process of releasing their mature spores into the environment. This increase in high molecular weight compounds could easily account for the foaming observed.

Another possible explanation of the fluctuations in the curve is that the incoming stream of air, which serves the

purpose of mixing the culture in order to keep the individual cells and chains of cells in suspension in the liquid, had become too slow to accomplish this purpose. It is possible that the chains and clumps of cells were becoming larger due to the accumulation of capsular material would serve the purpose of cementing the cells together. In either case, a certain amount of cellular material was observed to have settled out of the medium and to have collected at the bottom of the growth vessel. The case in question was probably due to a combination of both the foaming and the settling out of some of the clumps of cells. These explanations are far more probable than that the total number of cells per milliliter actually did change as greatly and as rapidly as would be indicated by the graph presented.

These two experiments, support the basic hypothesis of this study, namely that sporulation will occur in this apparatus and that its use is valid for the study of the reactions and occurrences preceding the formation of the visible spores. It was acceptible to believe that the formation of spores, if "triggered" and carried forward, would be completed and visibly detectable in the apparatus in use.

Plate XIII presents a graph that contains some very pertinent material. Although the curve is far from smooth or near the theoretical curve to be hoped for, it does have several important features. The initial irregular nature of the curve is probably explained by clumping and inefficient mixing of the culture in the growth vessel. There is, however, a general plateau at about 10^8 cells per milliliter which represents the

EXPLANATION OF PLATE XIII

Sporulation, germination and reinstitution of the steady state of Bacillus megaterium is presented. The number of cells per milliliter was plotted against hours of culturing.

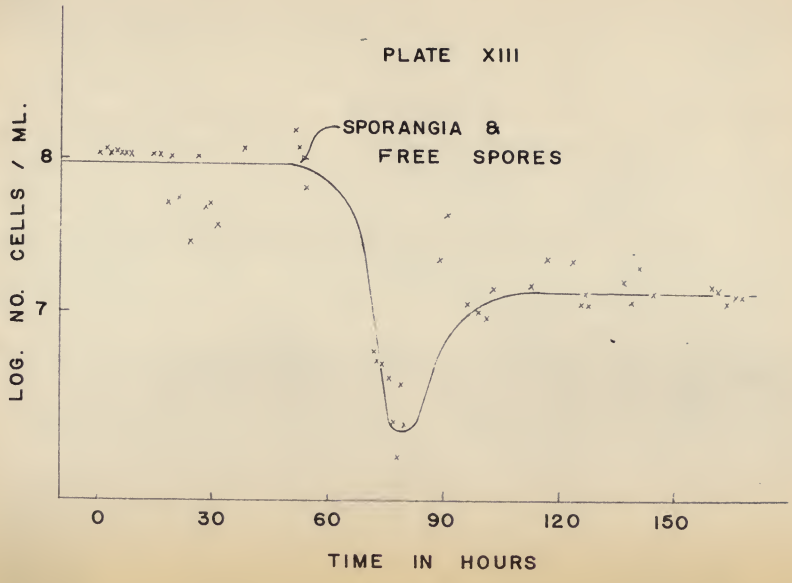


PLATE XIII
SPORANGIA &
FREE SPORES
LOG. NO. CELLS / ML.
8
7
0
30
60
90
120
150
TIME IN HOURS

steady state level being maintained. This level is apparent through the 38th hour.

On taking the 50 hour sample, it was noted that the capillary tube had become blocked by portions of a flocculent precipitate which had formed in the medium because of the association into complexes of some of the mineral elements. Such a colloidal type of precipitate was always observed in the medium but seldom caused any difficulty. The plugging of the capillary caused a stoppage of the flow of nutrients into the growth vessel. The culture was, therefore, essentially a batch culture for a period of as long as twelve hours. This period of closed culturing is exactly comparable to portions of the last two experiments described. It is assumed that the cells had approximately ten hours in which to proceed with the formation of their spores without being washed from the growth vessel as would normally have occurred within that time.

The capillary tubing was carefully freed of the particles which had blocked its orifice. The apparatus was not opened during this process. This reinitiation of the inflow of the fresh nutrient medium from the reservoir broke off the period of batch culturing. The numbers of cells in subsequent samplings declined swiftly as would be predicted from the washing out of cells which were no longer dividing in a logarithmic fashion. When the cells began formation of spores, the metabolism of the cell was completely switched over to the formation of the spore compounds and completely away from the synthesis of cellular components, according to Foster (1956). This absolute

switch explains why the spores are formed at the previously mentioned "point of inflection between the logarithmic and stationary phases of the growth curve." It also explains why the growth curve of sporulating organisms levels off into a plateau consistant with the stationary phase and shows no decline phase as long as the spores do not regerminate. The change-over of the entire metabolism of the cell explains quite well the decline in numbers. The cells being "committed" to the formation of spores, are no longer capable of division because of the lack of synthetic mechanisms involved in vegetative growth. The absence of division in a culture, part of which was being washed from the growth vessel by the constant inflow of fresh medium caused a steady decrease in numbers.

The sample taken at 51 hours showed the presence of forespores and sporangia among the cells present. After 52 hours, some free spores were observed along with large numbers of sporangia. At this time adjustments in the height of the growth vessel relative to that of the reservoir were made in an effort to allow entry of enough fresh medium to remove waste products and broken portions of sporulated cells, which might include substances inhibitory to vegetative growth. After a period of time of about four hours, the height was again adjusted so that the spores and sporangia would not be completely washed from the growth vessel before regermination could occur. As indicated on the curve spores were present from 52 to 68 hours. At the time of the 69 hour sampling, the first appearance of new vegetative cells was noted as occurring. This fact indicated

that the spores were beginning to germinate and the population to return completely to the vegetative state. More and more vegetative cells, in proportion to spores, were found until 72 hours, at which time very few spores were found and 73 hours at which time no spores at all were observed in the sample taken.

It will be noted on the graph that the numbers of cells in the population still continued to drop until the 78 hour sampling. This is due to the fact that the new cells were still in the lag phase which is always present immediately after the germinative process. After a short period during which the cells were in lag and therefore not reproducing as quickly as they were being washed from the growth vessel, the numbers began again to rise. The graph shows a typical logarithmic increase in the numbers. The population increased over a period of time, finally levelling off at a density of about three times 10^7 cells per milliliter. This level is considerably lower than the steady state level maintained previous to the sporulation sequence. The reason for this is that the height of the pressure head had been varied during the above mentioned period of time. The growth vessel was at this time farther from the reservoir than previously, thereby allowing a greater washout rate and a decrease in the equilibrium level of the population.

This experiment is important in corroborating both the fact that the vegetative cells will form spores in the chemostat and also the fact that the spores will germinate in the synthetic sucrose medium contained in the growth vessel of the chemostat and be converted into a steady state vegetative culture. Similar

results had been obtained when a spore inoculum was used. In that case the chemostat was inoculated with a milliliter of a spore suspension and followed by the usual methods of sampling and enumeration. It was found that the spores would germinate and that after outgrowth, the vegetative cells would establish a steady state culture. This latter fact could be used in future studies of germinative processes and their environmental dependence.

An experiment was run to determine the oxygen uptake of the cells in the steady state population in the chemostat growth vessel. The determination was made using standard techniques with Warburg manometers and water bath. All materials, with the exception of the cells, needed for the Warburg determination were prepared and measured into the flasks. A sample of the steady state population was removed from the growth vessel using a sterile pipette. Manometers one through five received respectively 0.1, 0.25, 0.5, 1.0, and 2.0 milliliters of the culture medium and its included cells exactly as taken from the growth vessel. The volumes in the Warburg flasks were made up to 3.2 milliliters with buffer solution of the same composition and pH as that which is a component of the synthetic sucrose medium. The flasks were fitted to the manometers and readings were begun after a fifteen minute equilibration period. The Warburg water bath was maintained at 30°C, plus or minus one degree, the same temperature as that used in the incubator containing the chemostat. This was done for the sake of continuity of conditions. All transfers of cells were made as quickly as possible

in order to produce as little shock to the culture as possible. It was hoped that this expedient would subvert the return of the cells to the lag phase of growth. Enumeration of the remainder of the sample from the chemostat showed that the sample removed contained approximately 10^8 cells per milliliter.

The effect of placing a sample of the continuously growing culture into the Warburg flasks was that of converting the cells to the conditions of a batch culture. The results would therefore be expected to parallel those of Plates XI and XII. The cells would be expected to grow logarithmically and to start to show visible signs of sporulation at the period of entering the stationary phase of the growth curve. A plot of the total cummulative oxygen uptake during this period of time against the total elapsed time of measurement would be expected to give a similar S-shaped curve.

Plate XIV presents the data from this experiment. The total oxygen taken up by the cells is plotted against the total time elapsed in hours. The family of curves resulting are numbered from the bottom to the top in order to coincide with the above mentioned amounts of sample culture added to the Warburg flask. We can see that the increasing size of sample, from bottom to top, each with its larger number of cells has an increased rate of oxygen uptake. This result is only to be expected. Numbers four and five will be the only two flasks discussed in detail. It can be seen that number four levels off fairly rapidly after an initial increase over the base level which is maintained almost unchanged by the other three flasks. Number five continues

EXPLANATION OF PLATE XIV

Graph representing cummulative oxygen uptake by cells of *Bacillus megaterium* plotted against hours of measurement.

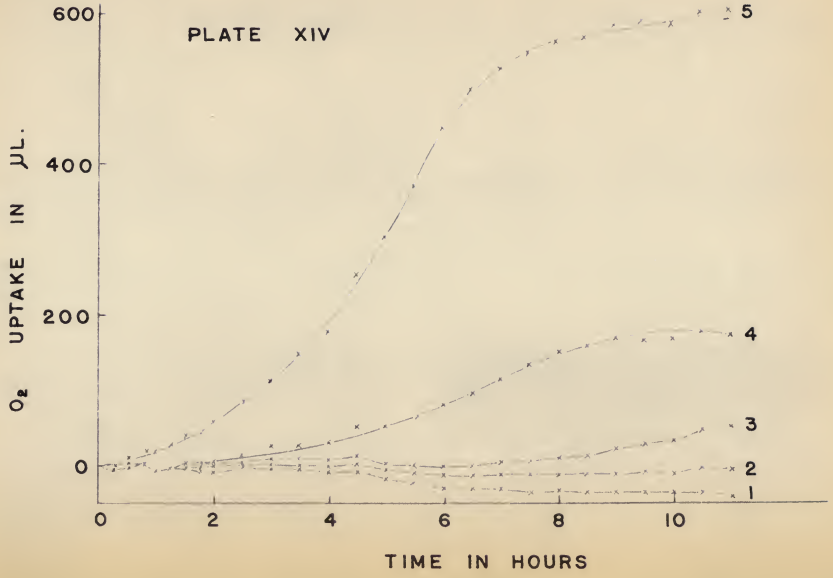


PLATE XIV
600
400
200
0
O_2 UPTAKE IN µL.
0
2
4
6
8
10
TIME IN HOURS
5
4
3
2
1

to increase quite a bit more rapidly and increases in rate, as determined from the slope of the curve. It can be seen from this plate that respiration in flask number five had levelled off after about six hours and was almost entirely horizontal after nine hours. This graph would indicate that the cells, after a short lag phase during which oxygen uptake increased only slowly, went into a logarithmic phase during which the total respiration was increased by the increasing numbers of cells in the population. Thereafter, the cell population had produced an environmental condition that caused it to enter a stationary growth phase. This fact is well depicted by the shape of the oxygen uptake curve. The oxygen uptake per unit time became almost nothing at this period, indicating that the cells were either dead or in a resting type of situation. The curve is indeed a close parallel of the growth curve presented in Plate XI.

It might have been expected that the cells would have started to form spores at about this stage of growth. This fact would be predicted from the parallelism to the curve of growth in Plate XI as well as the decline in oxygen uptake. The decline in uptake would indicate that the cells were rearranging their metabolism and synthesizing constituents necessary in the process of forming a spore. It would be assumed that this process required little respiratory energy. Unfortunately no visible evidence of free spores, sporangia, nor forespores was detected in any of several samplings made from the Warburg flasks over a period of time. The flasks were maintained and kept shaking for aeration for 32 hours and sampled regularly, but no

spores were detected. It is possible that some undetected factor in the environment of the culture in the Warburg flask precluded the formation of spores.

The cells of the chemostat culture, at least in higher concentrations, did behave as would have been predicted from previous knowledge and comparison with the results of the experiment shown in Plate XI. A careful study of the reason for the non-sporulation of the cultures in the Warburg flasks might allow a repeat experiment to be successful in the production of spores from cells of Bacillus megaterium under the conditions reported for this experiment. It might also be informative to try a similar experiment using a larger sample of cells from the chemostat and determine if they would sporulate. Another interesting possibility would be the addition of additional fresh nutrient medium, or of additional sucrose alone to the sample of cells when it is added to the Warburg flask. It is probable that such an addition would prolong the duration of the increase in respiration and delay the beginning of the stationary phase. The final production of spores might also be stimulated and a better result obtained than that presented in this experiment.

DISCUSSION

The basic question under study was the presence or absence of a mechanism to "trigger" the sporulation of the vegetative cells of Bacillus megaterium. Several methods have previously been used to approach this and similar problems. These have been mentioned in the Review of Literature.

In the study, here being reported, the approach was somewhat different from those previously used. It was hoped that by maintaining a culture of the vegetative cells growing in a steady state condition at a point just previous to the formation of the first visible signs of sporulation and varying some of the conditions, applicable information might be obtained. The hypothesis was that a variation in the conditions which affected the "triggering" would either prolong or shorten the time necessary for the first appearance of the spores. A lengthening of this time would probably not be discovered by the method under study, but a shortening should have caused the appearance of spores, sporangia, or at least forespores in the vegetative cells of the culture.

Samples were regularly taken, observed, and enumerated as outlined above in order to determine the steady state, if present, and the possible presence of the sporangia or spores. It has been pointed out in the section of this paper which is concerned with results, that forespores, sporangia, and free spores were observed to be formed in the growth vessel on several occasions. In none of these cases, however, did the appearance of these forms occur during the uninterrupted steady state growth of the cells. The only appearances observed were after a shorter or longer period of cessation of the flow of fresh nutrient medium into the growth vessel. The resulting type of growth was therefore at least partially that of a batch culture.

There are numerous possibilities for variation in the course of searching for a "triggering"mechanism. With the medium in

use in this study, it would be possible to vary the concentrations of the various constituents. This method was actually made use of in several of the experiments reported in the previous section. The sucrose concentration was varied in an attempt to ascertain whether the carbon source and in particular its relative concentration would have a marked effect on the sporulation process of a steady state culture. As noted, the appearance of spores was not detected when the concentration of sucrose was reduced by one half. Neither a further reduction in concentration nor an increase in concentration were found to be feasible.

It would also have been possible to vary the concentrations of the nitrogen source, or various of the mineral salts which were present in the complete medium. Although time did not permit the pursuance of these possible courses, further studies employing these parameters and the chemostat apparatus would be of definite interest and possibly of great value.

A further possible variation in the medium would include the addition of other compounds not normally present in the medium. Such additions could be of value in studying both the stimulation and the inhibition of the sporulation process. Thus the addition of certain fatty acids (Hardwick and Foster 1952) (Hardwick, et al. 1951) and related compounds might be expected to preclude the formation and maturation of spores in the cells, as is the case in certain organic media studied (Foster, et al., 1950). One would, however, expect the above cells to maintain a steady state situation of vegetative cells. Other additions might be used to attempt to stimulate sporulation. Such additions

might include further mineral elements such as copper, cobalt, and silicon (Kolodziej and Slepecky unpublished results).

A complete changing of the carbon source or nitrogen source might also be attempted. Such a change might involve the switch from sucrose to a mixture of glucose and acetate. The change might cause an increased speed of appearance of the spores. The nitrogen source could similarly be changed to a more complex type. A combination of both of the above elements might be attempted by the substitution of some of the smaller amino acids as both carbon and nitrogen sources. Among the possible candidates for such a procedure would be glutamic and aspartic acids, alanine, and glycine. Many possibilities are obviously open for further studies along this line.

The early portions of the study under consideration boiled down to a perfection of the methods and procedures involved in the use of the apparatus itself. Among the factors involved in this portion of the study were the methods of preparation of the medium and its combination in the reservoir bottle with the least possible chance of contamination. The final system settled upon was to autoclave all but the phosphate buffer directly in the reservoir bottle. The separately sterilized buffer was then added, while all of the ingredients were still hot. The center tube and ground glass attachments of the mariotte bottle were inserted and seated firmly in place immediately. All of these operations were carried out as rapidly as possible and without any undue movements of the apparatus or disturbances of the air.

A further possible source of contamination was from the stream of air which was constantly circulated through the growth chamber. It was necessary to prevent the entry of any organisms with the incoming air. The solution of this problem was fairly easily accomplished. The air from the aquarium pump was bubbled through a 1:1000 mercuric chloride solution. The bubbles were kept small by the use of a sintered glass bubbling tube. The air was subsequently passed into the growth vessel through a tube plugged with pyrex glass wool as a further safeguard.

The formation of a colloidal precipitate, principally of iron salts, led to a further modification. It was necessary to place a loose plug of pyrex glass wool in the end of the delivery tube of the mariotte bottle. With this plug in place, the problem of the blocking of the capillary tube was circumvented. After institution of this procedure, there were no cases of the interruption of the flow of fresh medium due to the blocking of the capillary tube aperture.

A further consideration of the non-observance of spore formation in the logarithmically growing, steady state periods of growth is in order here. The chemostat growth vessel holds approximately twenty-two milliliters of culture medium. This amount is reduced to about twenty milliliters when aeration is under way. During steady state growth then, there is twenty milliliters of culture medium containing the growing cells. The rate of addition of fresh medium used to maintain the cells in the steady state and a population density of about two times

10^8 cells per milliliter was approximately ten milliliters per hour. There was a simultaneous overflow of an equal amount of the culture from the siphon overflow. The growth vessel, therefore, had one half of its total contents replaced every hour. It can be readily seen statistically that one half of the cells of the population are removed every hour also. In the first hour, one half of the original population is washed from the growth vessel. During the second hour, one half of the remaining members, or one fourth of the original population will be washed from the growth vessel. These cells will also be replaced by an equal number of cells, formed by division from the other cells. These latter cells, however, play no significant role in the discussion under way. In the third hour, one half of the remaining members of the original starting population will again be washed from the growth vessel. It is easily seen that the number of members of the original population left in the growth vessel after a given number of hours is $(\frac{1}{2})^n$ where n = the number of hours since the beginning of observation. This factor is important in a consideration of the appearance of free spores in the growth vessel.

Study of Plates XI and XII will show that the length of time from the cessation of medium inflow to the appearance of the first forespores varied from nine to twenty-two hours depending on the level of the steady state population. By substituting these figures in the formula derived above, it will be seen that in the first case one cell in 512 would be from the original population. Since at most 400 cells were counted in

each sampling, the possibilities of seeing one of the members of the original population and that the cell observed was one of the few which had formed a spore in this minimum time period, would be very slight. In the second case mentioned above, the odds are even more definitely opposed to the observation of a spore. In this case, one cell in 4,194,304 would be a member of the original population. Since the population at the beginning time of the calculation was approximately 30,000,000, only slightly more than seven cells of the original population would remain per milliliter of culture medium or a total of 140 cells in the entire growth vessel. It is obvious that the possibility of finding one of these cells within the one fiftieth of a milliliter used in the Petroff-Hausser Counting Chamber is too small to be considered. This calculation shows the reason that spores were never detected in the growing populations in the growth vessel.

I should point out that the first case discussed, that in which nine hours were required for the appearance of spores was that which started at the most often used population density. Thus this case is the one most closely resembling most of the material presented here. Since evidence for a "triggering" mechanism was being sought, it was assumed that the providing of more nearly optimum conditions for spore formation, a "triggering", would cause a shortened time to elapse before the appearance of spores. If this time were shortened, the number of cells of the original population remaining would increase to an amount which would have definite possibilities of observation. For example,

if the time were shortened by only two hours, one in every 128 rather than one in every 512 would be from the original population. This change would greatly increase the probabilities of observing one of the cells and that the cell in question would be one which contained a visibly detectable stage of sporulation. It seems, therefore, that the methods in use in this study were applicable to the problem and might have been expected to give results under these conditions.

It has been noted in several places in this paper that the cells had a tendency to form long chains and for these chains to become quite intertwined. The fact that these agglomerations of cells were formed did not cause the loss of the steady state condition of the population. In all cases in which this type of growth occurred, the cell enumeration was made by counting each cell, not the groups of cells. In this manner, the total number of cells was still obtained. The fact that the numbers were not affected should be explained. Theoretically and actually, one half of the cells present in the culture will be washed from the growth vessel during the amount of time necessary for all of the cells to divide once, one generation time. The question of which cell is washed out at any given time is purely a statistical one. Whatever cell is present at the point of exit into the overflow siphon will be the cell which is washed out. The large agglomerations of cells are as likely to be washed from the growth vessel as are single cells. The fact that the cells are in these convoluted chains rather than dispersed singly means only that it requires less of the

agglomerations than of single cell units present to account for the same total number of cells. The fact that there are fewer of these units and that they are more spacially separated leads to the conclusion that fewer of the units are washed from the growth vessel per unit of time. Since each agglomeration contains a large number of cells, however, not as many need be washed out in order to maintain a constant number of cells in the population. This description explains why the number of cells remained constant, in the steady state, while the cells became joined into large agglomerated groups.

The above mentioned phenomenon of clumping of chains of cells is a common characteristic of Bacillus megaterium when it is grown in sucrose as a carbon and energy source. It would have been possible to circumvent this situation by substituting some other carbon source such as glucose. Such a substitution would, however, have raised other problems. Among these problems are: lack of such luxurient growth, lack of equally rapid growth, need for such an addition as acetate, and lack of as large an amount of material in the literature which was applicable. The fact that the clumping had little or no effect on the steady state nature of the growth was a deciding factor in the choice.

It has been noted that 0.2% concentrations of sucrose were not used because they were not limiting in the system. It would probably have been possible to use this concentration if the length of the pressure head from the reservoir to the capillary tubing had been increased sufficiently. This increase

would have been one to several feet. It would have been necessary to construct some special shelving to support the reservoir. Such an increase in pressure head would have resulted in a greatly increased rate of flow of nutrient medium into the growth vessel, thereby, causing the sucrose to again become limiting in concentration. It is also possible that the other components of the synthetic sucrose medium would then have been inadequate for the growth and sporulation of the cells at the increased flow and washout rates. Since the balance is so delicate, it was decided not to attempt the use of this particular concentration of sucrose at all. The use of the 0.025% sucrose concentration was ruled out because of its failure to support sufficient growth, as reported above.

It might be pointed out in conclusion that many other types of experiments could be attempted along the lines presented here. There are many possible variations on this theme including any number of possible variations in composition of the growth medium. Also amenable to variation, are such factors as the pH, temperature, and aeration. All in all it seems that this is a fruitful line of research for problems of this sort.

SUMMARY

A procedure was worked out so that Bacillus megaterium could be grown in steady state culture in the chemostat.

The assumption of this steady state condition was shown by graphic representations of the data obtained. The fact that the number of cells was maintained constant, a continuous outflow of

the cells being just balanced by a multiplication of the remaining members of the population, proved a steady state of growth.

It was shown that cells of Bacillus megaterium could be prevented from sporulating for at least 100 hours. It is probably true that this non-sporulation could be extended for as long as desired if the cultural conditions were maintained correctly and if contamination could be avoided.

Various methods of controlling the population density of the steady state culture were outlined and discussed.

The formation and germination of spores of Bacillus megaterium in the chemostat growth vessel was shown. Sporulation occurred after conversion of the growth vessel's contents to a batch culture. Germination was best shown by the use of a spore inoculum which was converted to a steady state vegetative culture.

Cummulative oxygen uptake of the steady state cells was shown to increase logarithmically and then to level off after ten hours.

Further studies with this organism and apparatus were suggested.

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BIBLIOGRAPHY

Bayne-Jones, Stanhope and Alexander Petrilli.
Cytological changes during the formation of the endospore in Bacillus megaterium. J. Bacteriol. 25:261-275. 1933.

Brunstetter, B. C. and C. A. Magoon.
Studies on bacterial spores. III. A contribution to the physiology of spore production in Bacillus mycoides. J. Bacteriol. 24:85-122. 1932.

Cook, R. P.
Bacterial spores. Biol. Rev. Cambridge Phil. Soc. 7: 1-23. 1932.

Charney, Jesse, W. P. Fisher, and C. P. Hegarty.
Manganese as an essential element for sporulation in the genus Bacillus. J. Bacteriol. 62:145-148. 1951.

Curran, Harold R. and Fred R. Evans.
The influence of iron or manganese upon the formation of spores by mesophilic aerobes in fluid organic media. J. Bacteriol. 67:489-497. 1954.

Fabian, F. W. and C. S. Bryan.
The influence of cations on aerobic sporogenesis in a liquid medium. J. Bacteriol. 26:543-558. 1933.

Foster, J. W.
Morphogenesis in bacteria: some aspects of spore formation. Quart. Rev. Biol. 31:102-118. 1956.

Foster, J. W., W. A. Hardwick, and Beverly Guirard.
Antisporulation factors in complex organic media. I. Growth and sporulation studies on Bacillus larvae. J. Bacteriol. 59:463-470. 1950.

Foster, Jackson W. and Fred Heiligman.
Mineral deficiencies in complex organic media as limiting factors in the sporulation of aerobic bacilli. J. Bacteriol. 57:613-615. 1949.

_____.
Biochemical factors influencing sporulation in a strain of Bacillus cereus. J. Bacteriol. 57:639-646. 1949.

Foster, J. W. and Jerome J. Perry.
Intracellular events occurring during endotrophic sporulation in Bacillus mycoides. J. Bacteriol. 67:295-302. 1954.

Foster, Jackson W. and E. Staten Wynne.
The problem of "dormancy" in bacterial spores. J. Bacteriol. 55:623-625. 1948.

Gollakota, Krishnamurty G. and H. Orin Halvorson.
Biochemical changes occurring during sporulation of Bacillus cereus:J. Bacteriol. 79:1-8. 1960.

Gunsalus, I. C. and Roger Y. Stanier.
The Bacteria. Volume I. New York:Academic Press. 1960.

Hardwick, W. A., Beverly Guirard, and J. W. Foster.
Antisporulation factors in complex organic media. II. Saturated fatty acids as antisporulation factors. J. Bacteriol. 61:145-151. 1951.

Hardwick, W. A. and J. W. Foster.
On the nature of sporogenesis in some aerobic bacteria. J. Gen. Physiol. 35:907-927. 1952.

_____.
Enzymatic changes during sporogenesis in some aerobic bacteria. J. Bacteriol. 65:355-360. 1953.

Henrici, A. T.
Morphologic variation and the rate of growth of bacteria. Springfield, Illinois:Charles C. Thomas. 1928.

Knaysi, Georges.
A study of some environmental factors which control endospore formation by a strain of Bacillus mycoides. J. Bacteriol. 49:473-493. 1945.

_____.
The endospore of Bacteria. Bacteriol. Rev. 12:19-77. 1948.

_____.
On the process of sporulation in a strain of Bacillus cereus. J. Bacteriol. 51:187-197. 1946.

Kubitschek, Herbert E.
Modifications of the chemostat. J. Bacteriol. 67:254-255. 1954.

Novick, Aaron and Leo Szilard.
Experiments with the chemostat on spontaneous mutations of Bacteria. Proc. Nat'l. Acad. Sci. 36:708-719. 1950.

_____.
Description of the chemostat. Science 112:715-716. 1951.

Perry, Jerome J., and J. W. Foster.
Non-involvement of lysis during sporulation of Bacillus mycoides in distilled water. J. Gen. Physiol. 37:401-409. 1954.

Powell, J. F.
The sporulation and germination of a strain of Bacillus megaterium. J. Gen. Microbiol. 5:993-1000. 1951.

Powell, Joan F. and J. R. Hunter.
Sporulation in distilled water. J. Gen. Physiol. 36:601-606. 1953.

Powell, Joan F. and R. E. Strange.
Biochemical changes occurring during sporulation in Bacillus species. Biochem. J. 63:661-668. 1956.

Slepecky, Ralph and J. W. Foster.
Alterations in metal content of spores of Bacillus megaterium and the effect on some spore properties. J. Bacteriol. 78:117-123. 1959.

Umbreit, W. W., R. H. Burris, and J. W. Stauffer.
Manometric techniques. Minneapolis, Minnesota:Burgess Publishing Co. 1957.

Young, I. Elizabeth and Philip C. Fitz-James.
Chemical and morphological studies of spore formation. I. The formation of spores in Bacillus cereus. J. Biophys. Biochem. Cytol. 6:467-481. 1959.

A STUDY OF THE GROWTH OF BACILLUS MEGATERIUM IN STEADY STATE CULTURE

by

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ABSTRACT

The main principles under study were that of growth and sporulation of the organism Bacillus megaterium in a steady state culture. The growth was shown to assume a true steady state nature. This quality of the growth in the chemostat was shown to coincide with the theoretical predictions of the type of growth to be expected in the apparatus. The results were also shown to be analogous to the results obtained by other workers. Thus it was shown that Bacillus megaterium may be grown in the chemostat successfully and that it will assume the steady state.

That the vegetative cells of the organism will in truth form spores in the apparatus was also beyond doubt. Several instances of the formation of spores in the growth chamber of the apparatus were outlined. It might be mentioned parenthetically that the spores of the organism were also shown to germinate, grow out, and establish a steady state growth in the apparatus.

A demonstration of various methods of controlling the level of growth was made. It was pointed out and demonstrated by graphic representations of experimental data that the number of organisms per milliliter of growth medium may be controlled by a change in the pressure head in the reservoir and feed system, by the length of the capillary interposed in the tubing conducting medium from the reservoir to the growth vessel, or by the regulation of the concentration of the limiting growth

factor of the medium in the reservoir. Such regulation has at least partially been treated mathematically. Reference has also been made to similar treatment of experimental data in the literature.

Another interesting point shown throughout this study is that a culture of Bacillus megaterium may be maintained growing without sporulating. Although the medium was sufficient in all respects to support the formation of spores by the vegetative cells of the culture, the actual formation could be precluded. The method of keeping the cells growing logarithmically and with a rather rapid overflow rate caused the culture to remain in the vegetative stage. Never was a spore, a sporangium, nor even a forespore observed during a period of steady state growth.

The results of an experiment designed to study the oxygen uptake of a sample of cells from the steady state culture have been presented. In this section, it was shown that the cells grow as a batch culture in the Warburg flask with a logarithmic increase in the uptake of oxygen due to the increasing numbers of cells in the population. A stationary phase is later reached in which the cell respiration falls to almost nothing. It is suggested that this may be a period of reorganization of the cellular constituents requiring little respiratory energy, depending on stored compounds, and involved in the sporulation process.

The overall results of the experiments and results reported here seem to indicate that the method reported is indeed fruitful and should be followed up. It is believed that the results

obtained do have a bearing on the sporulation process in Bacillus megaterium and that they dovetail quite nicely with the material in the literature concerning this particular problem.