STUDIES ON BACTERIAL SPORES

III. A CONTRIBUTION TO THE PHYSIOLOGY OF SPORE PRODUCTION IN BACILLUS MYCOIDES

B. C. BRUNSTETTER AND C. A. MAGOON

Division of Horticultural Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture

The first study of this series (Magoon (1926a)) reported the effect of age, temperature and humidity on the thermal resistance of spores of *B. mycoides*. These spores, washed free of metabolic products, were stored at 10°C., 20°C., and 30°C., under three conditions of humidity—over calcium oxide, over 50 per cent H_2SO_4, and over water. Samples from each of the nine sets of storage conditions were tested for thermal resistance (as measured by the minutes of heating at 100°C. required for 100 per cent destruction of spores) after one, thirty, sixty, ninety, one hundred and twenty, one hundred and fifty and one hundred and eighty days of storage, respectively. It was found that the thermal resistance of spores had increased during thirty days under all sets of storage conditions. Though changes after thirty days were rather irregular, it was outstanding that the slowest change in resistance took place at 10°C. over CaO. Considering the storage period as a whole, it was found that at 10°C. the thermal resistance of the spores increased regularly with the humidity. At 20°C. it was about the same over CaO and 50 per cent H_2SO_4 and was slightly greater over water, while at 30°C. it was maximum over 50 per cent H_2SO_4 and least over water. Under the three conditions of humidity the maximum thermal resistance was found at 20°C.

In the second report (Magoon (1926b)) experiments were described in which by cultivation and selection of survivors from successive thermal death-time tests, a strain of *B. mycoides* was

85

obtained whose spores had attained a resistance at least 25 times that of the original spores.

These results emphasized the fact that the thermal resistance of spores within a given bacterial species is not a fixed property and showed that if the spore problem is to be solved satisfactorily more information must be obtained in regard to the factors responsible for the resistance of spores to heat and for variations in that resistance; in other words, a thorough understanding of the biology of spore formation is required. Such a biological understanding is highly desirable as a foundation for a biochemical study of the process of spore formation and of the nature of the resistance of spores to heat.

Microscopic observation shows that the production of spores by vegetative cells follows a definite sequence of changes in the internal structure of the cell. It is necessary, therefore, if a satisfactory chemical or physical analysis is to be made of the factors responsible for the greater thermal resistance of the spores as compared with the vegetative cells, to secure samples of cells in these various physiological states in adequate amounts. This calls for the cultivation of the test organisms under as rigidly controlled environmental conditions as possible. In the present work an endeavor was made to provide for these needs.

The organism chosen for this study, as in the previous work, was *B. mycoides*. Its adaptability for the purpose is well known and considerable important information on the biology of this organism has been accumulated by workers both here and abroad since the earlier papers of this series were printed.

This paper reports the observed effect on the production of spores by *B. mycoides* of the following environmental factors: (a) Oxygen supply; and (b) the food supply. Preliminary results obtained on the effect of dissociation on the mechanism of spore production are presented, and in order to indicate the generality of the results obtained with *B. mycoides* some brief comparative studies with other aerobic spore-formers are described.

RÉSUMÉ OF THE LITERATURE

In reviewing the significant studies on the physiology of spore production by aerobes, the following factors are discussed: (1)

The oxygen supply; (2) the temperature; (3) the hydrion concentration; (4) the relative importance of the food supply and the concentration of metabolic products in the medium; (5) the water content of the vegetative cells, and (6) the tendency to "dissociate."

In 1877, Cohn first demonstrated that *B. subtilis* forms spores, which were much more resistant to heat than are vegetative cells, and Koch (1877) showed that the cause of anthrax was the spore-forming organism, *B. anthracis*. These discoveries, the first of which dealt a finishing blow to the theory of spontaneous generation, and the second of which, independently made by Pasteur (1858), threw light on a practical problem and clarified a pathological mystery, naturally focused attention on these two spore-formers. The result was that most of the early physiological studies were confined to *B. subtilis* and *B. anthracis*, and, with the exception of the work of Blau (1906) and of Wund (1906), who studied the effect of temperature and of oxygen, respectively, on spore-formation in a number of spore-formers, it is not too much to say that critical work on the factors concerned in aerobic spore production has been limited to only six or seven species. In spite of this, general "explanations" of spore formation based on one or two species have been advanced more than once.

Cohn and Koch in the studies just mentioned were the first to note that the free access of air to cultures was essential for the formation of spores by the organisms they studied. This was confirmed, among others, by Buchner (1890), who, however, did not ascribe to oxygen any specific influence on spore production by *B. anthracis*; he believed the effect of oxygen was merely to make vegetative growth possible.

Schreiber (1896), working with *B. subtilis*, *B. anthracis*, and *B. tumescens*, concluded that oxygen was essential for the formation of spores, apart from its effect on vegetative growth. He found that in tubed liquid media at 30°C., *B. anthracis*, which is non-motile, and grows at the bottom of media, must have a layer of liquid no higher than 15 mm. over it if spore production was to occur; under the same conditions, *B. subtilis* and *B. tumescens* formed spores irrespective of the height of the liquid. However, when the cotton stoppers were replaced by cork, he found

that *B. subtilis* must have a column of air at least 3 cm. high over the surface of the medium, and that *B. tumescens* required an air column at least 5 cm. high if spore production was to occur. With less than these amounts of oxygen, growth but not spore production took place.

Wund (1906), a student of A. Meyer, studied quantitatively the effect of the minimum, optimum and maximum oxygen concentration on spore germination, vegetative growth, and spore formation by a number of aerobic spore-formers, grown on glucose agar plates. He found, for example, the following data in the case of *B. mycoides.*

	OXYGEN PRESENT PER LITER, MGM.		
	Minimum	Optimum	Maximum
Spore germination.....................	4.3	70	1,336
Vegetative growth.....................	4.3	60	1,336
Spore formation.......................	6.8	276	1,336

Among the organisms studied, the highest minimum figure for spore formation was 130 mgm. of oxygen per liter (*B. tumescens,* *B. pumulos* and *B. ruminatus*). The remaining minimal oxygen concentrations for spore production were much lower, ranging from 20 to 3 mgm. of oxygen per liter. Wund's data show interesting differences among the aerobic spore-formers studied as regards their relation to oxygen. In general, the minima for spore germination and vegetative growth (which were practically the same) were lower than the minima for spore production. In view of the fact that the addition of glucose to a medium enables many aerobic spore-formers to grow at much lower oxygen concentrations than are otherwise possible (see data of Eisenberg (1918) and others whom he cites), it is quite probable that the minimal oxygen concentrations obtained by Wund would have been quite different, probably higher, if glucose had been absent from the culture medium.

Holzmüller (1909) studying the physiology of five strains of *B. mycoides,* as well as of four closely related forms, also found that oxygen was essential to spore formation. He performed the interesting experiment of taking samples for microscopic obser-

vations at intervals from a broth culture in which spore formation could not go to completion because of an insufficient supply of oxygen. The time required for spore formation in the new environment varied inversely with the age of the broth culture. Thus, when the broth culture was two days old, twenty hours were required by the sample to form spores, but when the broth culture was fourteen days old, only half an hour was required for spore formation.

It seems fairly definite that the biochemical transformations leading to spore formation within the cells of aerobes demand oxygen for their completion. However, there are important differences among aerobic spore-formers as regards their relations to oxygen.

It was early discovered that spore production occurred only within a given range of temperature. Koch (1877) found that *B. anthracis* formed spores at 35°C. in twenty hours, but that this process required seven days at 16°C., while no growth or spore formation occurred below 15°C. According to Cohn (1877), *B. subtilis* neither grows nor forms spores above 50°C., but both processes occur at 47°C. Brefeld (1881) noted that below 5°R. (6.2°C.) *B. subtilis* grows slowly but does not form spores.

Schreiber (1896) observed the following minimal temperature relations: *B. subtilis* grew at 8°C., but formed spores only at 10°C.; *B. tumescens* grew at 10°C., but formed spores only at 11°C.; *B. anthracis* grew at 12°C., but formed spores only at 14°C. He has recorded the interesting observation that *B. anthracis* was injured and spore formation adversely affected by transferring abruptly from 37°C. to 18°C.

Migula (1897, 1904–07) confirmed the observation that spore formation did not occur at all temperatures at which vegetative growth took place. He found that although *B. megatherium* still grew at 12°C. or a little less, dividing about every three to four hours, spores were not formed at this temperature after six weeks. He obtained good colony development of *B. subtilis* after a week at 4 to 8°C. but found no spore production. According to him, the temperature limits for the growth of *B. anthracis* are from 10°C. to 43°C.; though the optimum for growth is 37°C. the

optimum for spore formation is about 6° less.   He found that the various strains of B. anthracis differed considerably with regard to the minimum temperature limit for spore formation, one forming spores at 16°C., while for another strain the lower limit was 24°C.   Blau (1906) ascertained the maximal temperatures for spore germination and spore production for a number of aerobic spore-formers, and incidentally their optimal growth temperatures. The organisms were cultivated on glucose agar plates.   It is interesting to note that B. mycoides neither grew nor formed spores at 35°C., only 5 degrees higher than its optimum for germination, growth and spore formation.[1]   He further states that B. cohaerens formed no spores at 35°C., though it grew well at this temperature.   Itano and Neill (1918–19) noted that B. subtilis completed the spore cycle at 37°C. in much less time than at 25°C.

Casman and Rettger (1931) found that the succinodehydrogenases of B. mycoides as well as those of a number of other spore-formers, were distinctly inhibited at the maximum temperatures of growth of these organisms.   The heat labilities of the paraphenylene diamine oxidases of B. cereus and of B. mycoides varied considerably, but as a rule were found to approximate their maximum temperatures of growth.   They made the interesting observation that B. mycoides, after exposure to the maximum temperature for growth for some time, lost the ability to grow at or near the surface of the agar but retained its ability to grow in the depths.

To summarize, it is clear that for some, and probably all, species of aerobic spore-formers the temperature range for spore production is narrower than the temperature range for growth; that is, growth may occur at temperatures too high or too low for the formation of spores.   The temperature limits for both growth and spore formation vary not only with the species but with the strain or variety of spore-former.

[1] "Strain differences" undoubtedly affect the level of the "maximum;" for example, Eisenberg (1918) reports strains of Bacillus mycoides which were "partly thermotolerant and partly thermophil," and which grew well at 55°.   Holzmüller (1909) also found that the maximum temperature for spore germination, growth and spore formation varied according to the strain of B. mycoides used.

Only a small amount of work seems to have been done on the effect of the hydrion concentration of the medium on the process of aerobic spore production. The older work consisted chiefly in adding acidic or basic substances in various concentrations to a broth medium, and comparing the time required for spore formation with the time required by the same organism in a control medium. The general conclusion was reached that spore formation takes place not only in a definite range of temperature and of oxygen concentration, but also within definite limits of acidity and alkalinity.

Closely connected with such experiments are those in which the effect on spore production of adding available carbohydrates to the medium was studied. Though usually in such experiments the resulting acidity was not measured, it is apparent that the effect on spore production of adding glucose to a peptone medium was largely due to the degree of acidity which developed. Schreiber (1896), and later Gärtner (1903), made the important point that the effect of the addition of carbohydrate to the medium depends on the amount of available nitrogen present. For example, the addition of glucose to a medium retards growth and spore formation only when the available nitrogen supply is scanty.

Itano and Neill (1918–19) found that the optimum reaction for the germination of *B. subtilis* spores was pH 7 to 8, that the limits for growth were pH 4.2 and pH 9.4, respectively, and that germination and completion of the spore cycle at 24°C. and 37°C. occurred between pH 5 and pH 10. "The spores can germinate when the pH value is 10, although after germination the vegetative cells multiply only to a very slight extent and soon pass into spores. The slight growth and multiplication of vegetative cells in broth of pH 10 suggest that the formation of endospores in this medium must be caused largely by the unfavorable reaction of the medium rather than by the accumulation of end products." They state that the automatic adjustment of the medium by the growing organism seems to play a rôle in the completion of the spore cycle.

B. anthracis, according to Whitworth (1924), can grow in

broth from pH 6.4 to more than pH 8.4, the optimum range being from pH 7.8 to pH 8.1   Delay in sporulation results from growth on excessively acid or alkaline agar.   In view of his data, he regarded it as very probable that the hydrion concentration of soils plays an important rôle in the life of any *B. anthracis* spores present.

Although it is generally recognized that spore production by aerobes occurs within the acidity range whose limits are approximately pH 5 and pH 10, it is evident that relatively few studies deal with this point and that much more work needs to be done. The question of the effect of hydrion concentration on the velocity of spore production is of general biological interest.   Investigations in this field deal essentially with the effect of hydrion concentration of the medium on the formation and storage of fat, protein, or glycogen in unicellular organisms.   Such storage of reserve material generally precedes spore formation; the kind of material stored depends, of course, on the nature of the organism.

These three factors, oxygen concentration, temperature, and hydrion concentration, affect spore production, not only directly but indirectly, by their effects on growth.   The closer cultural conditions are to the optimum with respect to these three environmental factors, the more rapid growth will be, and hence, after a given time period, twenty-four hours for example, the greater will be the utilization of the food supply and the greater will be the accumulation of metabolic products.   The relative importance of these last two factors as a general cause of aerobic spore production is uncertain, for the reason that relatively few sporeformers have been studied from this point of view, and, furthermore, the attempt to disentangle these two factors—a very difficult task—has not on the whole been successful.

In 1877 and 1881, Buchner (1890) conducted experiments on the cause of spore formation by *B. anthracis*, which led him to conclude that "Die physiologische Ursache der Sporenbildung liegt in dem eintretenden Mangel an Ernährungsmaterial."   Two types of experiments supported this view.   He found that by transferring *B. anthracis* from one nutrient solution to another

fresh nutrient solution at suitable intervals, vegetative growth without spore formation could be indefinitely maintained. This type of experiment is, however, equivocal as regards its interpretation, for, as Migula (1897) pointed out, failure of the vegetative cells to form spores could arise either from an excess of nutrient material or because transfer to a fresh nutrient solution was always made before the concentration of metabolic products became sufficient to cause spore formation.

The second type of experiment on which Buchner based his conclusion was his finding that transfer of well-nourished vegetative cells to distilled water (or 2 per cent sodium chloride solution) quickly led to complete spore formation (under suitable conditions of oxygen supply and temperature), while at the end of the same time vegetative cells placed in nutrient solution obtained from an old culture had not formed spores. In this experiment metabolic products seem to be excluded as a cause of spore formation by *B. anthracis*.

Buchner published these results in 1890 in a refutation of the theory of Lehmann (1890) as to the cause of spore formation by *B. anthracis*, which had appeared earlier in the year. Lehmann based his preliminary communication on data obtained by his student Osborne (1890). Osborne found that after increasing dilutions of broth cultures, inoculated with *B. anthracis*, had been aerated for a day and a half, growth and the absolute number of spores were directly proportional to the concentration of the medium. The number of spores was obtained by plating out the cultures after heating them for one hour at 65° to 70°C. Osborne concluded that spore formation is not favored in the slightest degree by dilute concentrations of the food supply.

Buchner criticised this experiment on the ground that it is not the absolute number of spores that is significant, but the number of spores relative to the number of vegetative cells. The degree to which a medium favors spore production, he believed, is given by the percentage of spores, which he called "the intensity of spore formation." This concept involves the idea of a velocity, which in turn involves the idea of a time interval. Buchner claimed that the time interval of a day and a half is too long and

that twenty-four hours, even twelve hours under the most favorable conditions, is sufficient.

Osborne also plated *B. anthracis* on nutrient agar medium on which repeated crops of *B. anthracis* had previously been grown. Since, under these conditions, only sparse growth with slight spore formation was obtained, while numerous spores were obtained on fresh medium with an abundant food supply, he inferred that a depleted food supply is attended by only slight spore formation. He seems to have neglected entirely the factor of metabolic products in his interpretation of the experiment.

Stephanidis (1899), another student of Lehmann, found that the rapidity with which spores of *B. anthracis* are formed is greater on poorer media on which growth is sparse. He then attempted to determine the relative production of spores formed on plated agar media whose content of meat extract varied from 5 to 0.02 per cent. The temperature of incubation was 37°C.; the time of incubation was not stated. He did not determine the percentage of spores but seems to have averaged the number of spores per chain of cells, counting 10 chains that seemed representative. From his data he concluded that the relative number, as well as the absolute number, of spores increased directly with the concentration of the medium. It is difficult to see how his figures are comparable, for he found that growth was directly proportional to the concentration of the medium, and presumably the length of the chains of cells also increased with the concentration of the medium.

Turro (1891) believed that *B. anthracis* forms spores as a result of the accumulation of its products of metabolism. He concluded that the carbohydrate compounds of the cell are oxidized in the presence of atmospheric oxygen and metabolic products; the nitrogen compounds of the cell, which are the chief constituents of the spore, remain and, as end-products, condition spore formation.

Schreiber (1896) confirmed Buchner's finding that the vegetative state of *B. anthracis* could be indefinitely prolonged by periodic transfer to fresh media; this was also true of *B. subtilis* and *B. tumescens*. He also found that when well-nourished vegeta-

tive cells of these three organisms were placed in distilled water or in solutions of various salts, under favorable conditions of temperature and oxygen supply, spore formation promptly occurred. In his opinion, spore formation was induced by the sudden hindering of growth following previous good nutrition of the vegetative cells. He considered it improbable that the accumulation of metabolic products could be the cause of spore formation, since transfer of well-nourished vegetative cells to nutrient media in which spore formation had previously occurred again and again always resulted in a retardation of such formation.

On the other hand Migula (1897) stated that the induction of spore formation is mainly conditioned, not by the exhaustion of the food supply, but by the accumulation of metabolic products such as acids or alkalies. He believed that direct proof of this statement was afforded by the following experiment: When dry meat extract or dry peptone was added to a broth culture of *B. anthracis* shortly before spore formation, preparation for sporulation was not prevented though the food supply was greatly increased. If, however, at the same time, he added water with the dry nutrients no spore formation but resumption of growth took place; often, simply diluting with water had the same result. Decreasing the concentration of metabolic products permitted growth.

Holzmüller (1909), working with the "*B. mycoides* group," checked Buchner as regards the indefinite prolongation of the vegetative state by periodic transfer to fresh media and also with respect to his conclusion that the cause of spore formation was the exhaustion of the food supply. Vegetative cells in a good state of nutrition (two-day agar plate culture) were transferred to distilled water and to a fresh nutrient agar plate medium. After twenty-four hours the cells in distilled water had nearly all sporulated; there were no spores, but vigorous vegetative growth, on the nutrient agar plate.

Henrici (1928) made microscopic observations on spore formation by *B. cohaerens* on full strength nutrient and quarter-strength nutrient agar slants, each strength inoculated with a heavy and a light (1:50) spore suspension. Spore formation proceeded more

rapidly in the quarter-strength agar media than in the full-strength media, regardless of the number of spores inoculated, and more rapidly in the heavily seeded than in the lightly seeded cultures. He inferred that the rate of spore formation is determined not by the concentration of cells alone but by the ratio of the population density to the concentration of nutrient material. He also found that spore formation by *B. megatherium* commenced practically at the point of inflection between the logarithmic growth phase and the resting phase, and that it then proceeded at a practically constant rate for some time, later decreasing in rate.

It will be noted that investigation of the relative importance of the nutrient value of the medium and of the concentration of metabolic products in the medium in inducing spore production has been confined to a few organisms only. That the relative importance of these two factors depends partly at least on the nature of the organism is shown by work reported in this paper. It is therefore highly desirable that many more organisms be studied from this point of view. The terms "food supply" and "metabolic products" are, of course, blanket terms. The cultivation of aerobic spore-formers on synthetic culture media seems not as yet to have been successful. The specific food factors essential for growth and spore formation are unknown. Likewise the metabolic products formed in complex media are largely unknown. The effect of the few known metabolic products, such as hydroxyl ion or ammonia ion, on spore production has not so far been investigated under controlled conditions.

Recently Daranyi (1927), who studied *B. anthracis*, *B. subtilis*, and *B. cylindrosporus*, has introduced a new factor in the study of spore formation. According to him, the most important influence favoring spore production is the decrease of the water content of the bacillus, an "entquellung" of the cell colloids. This occurs under natural conditions when the bacillus ages, "aging" involving a loss of water. Buchner's theory, he believes, holds to the extent that with the cessation of cell-division, occurring when the food supply becomes a limiting factor, the bacilli become older and thereby the water content decreases. Daranyi was able to

induce an earlier spore production on agar by previously dehydrating the spores used as inoculum; for example, the spores of a virulent strain of *B. anthracis* that had been dried for two days in a desiccator produced 70 per cent spores after eighteen hours incubation on a freshly prepared agar slant, while on the control, inoculated with an eighteen-hour agar slant growth, only 1 per cent spores were produced. after eighteen hours incubation at the same temperature.

That the same kind of organism is not constant in its response to environmental conditions as regards spore production, and that any given response depends, partly at least, on its previous cultural history, are shown by the work of investigators who have succeeded in obtaining permanently asporogenous strains from sporogenous strains of aerobic spore-formers.

Lehmann (1888) found a fully virulent but asporogenous strain of *B. anthracis* among a number of old cultures at the Berlin Hygienic Institute. Behring (1889) obtained two asporogenous strains of *B. anthracis* by growing them for several months on gelatin agar medium containing, in the one case, 1 per cent hydrochloric acid, and in the other, rosolic acid. Roux (1890) obtained asporogenous strains of *B. anthracis* by cultivating it in broth containing small amounts of phenol (less than 1 to 1666). This procedure was confirmed by Migula (1897) for non-motile, but not for motile, spore-formers. Phisalix (1892) cultivated *B. anthracis* at 42°C. to get it in the asporogenous state. Nadson and Adamovic (1910) cultivated *B. mycoides* on a meat-peptone gelatin medium; the old liquefied gelatin medium was added after sterilization to an equal volume of an agar or bouillon medium containing constituents, with the exception of water, in double amount. Grown on such a medium, *B. mycoides* changed, they reported, beyond recognition; the cultures resembled those of Actinomycetes. The properties of gelatin-liquefaction and sporulation were lost.

The loss of the property of spore production seems to be part of the large and complex problem of microbic dissociation. No attempt will be made to present a complete consideration of the work dealing with dissociation phenomena in aerobic spore-

3661

formers. A few illustrations from recent literature, however, to make clear the point that dissociation profoundly affects aerobic spore-formers, will be presented.

Löhnis and Smith (1923), studying the genus *Azotobacter*, have described two of the seven cell types found as small sporulating rods and large sporulating rods. The former type is identical with *B. terminalis* Mig., *B. fusiformis* A.M. et Gottheil and in the case of *A. chroococcum* and *A. Beijerinkii*, with *B. pumilis* A.M. et Gottheil. The latter form, which produced white, yellow and brown colonies, was identical with *B. luteus* Baker et Smith, *B. petasites* A.M. et Gottheil, *B. malabarensis* Löhnis et Pillai and *B. danicus* Löhnis et Westermann. They state that all cell types were transformed into each other.

Cunningham (1931) stated that he had obtained two aerobic spore-formers as variants from the anaerobic spore-former, *B. saccharobutyricus* von Kelcki (*Clostridium butyricum Prazmowski*). One of these variants was identical with *B. cereus* Frankland and the other corresponded to *B. sphaericus* Meyer.

Haag (1926) reported a study of a culture named *Bac. viridiglucescens* Sack. He found that after this organism had been incubated two to three weeks on agar plates, each grayish-yellow round colony had become surrounded with a white marginal growth. These secondary colonies contained small oval spores and large oval or round vegetative cells. Streak cultures of the marginal growth showed thin rods (originating from the spores) and thick, plump oval cells. The thin rods, which showed good spore formation, were identical with *B. mesentericus* in all points. The thick, plump organism corresponded to *B. megatherium* De Bary. It exhibited extremely slow spore formation.

Two very interesting studies of dissociation in *B. mycoides* have been made by Oesterle and Stahl (1929). Their results (independently conducted but reported together) will be considered separately.

Oesterle studied three strains of *B. mycoides* in three media: (1) germ-free soil extract, (2) germ-free filtrate of "Faülflüssigkeit," and (3) broth exposed to sunlight and also to ultraviolet light. Typical rods of *B. mycoides*, incubated for several months in the

first two of these media, generally autolyzed; numerous small granules (Kugelformen) were present. These, after some passages on agar and in broth, grew to rods, which afforded smooth, soft agar colonies (S-form). From these colonies there appeared, under conditions not yet established, typical *B. mycoides* outgrowths. Typical *B. mycoides* could be cultivated from these outgrowths. The S-form was motile, growing singly or in groups of two, and formed spores.

Exposure of broth cultures of typical *B. mycoides* to direct sunlight for a week resulted in the formation of granules which passed through kieselguhr filters and reverted after several months to typical *B. mycoides* after passing through a succession of intermediate forms.

When broth cultures were exposed to dosaged illumination with ultraviolet light, typical *B. mycoides* was transformed to granules, thread forms, and small Gram-negative rod-like gonidia. These three forms after a number of transfers eventually reverted to typical *B. mycoides*.

Stahl found that typical *B. mycoides* disintegrated to filterable forms under the influence of sodium chloride and sodium chloramine Heyden and of bichloride of mercury. The atypical forms thus produced reverted by way of a coccus stage and smooth colony forms to typical *B. mycoides*. He stated that "the degeneration forms noted in the literature many times are in part forms capable of development from the developmental cycle of *B. mycoides.*" In his opinion, the species *B. effusus*, *B. olfactorius*, and *B. nanus*, described and pictured by Holzmüller, belong with great probability to the developmental cycle of *B. mycoides*.

Nyberg (1929) cultivated a number of strains of *B. mycoides* in liquid media for two months; from these old cultures a number of interesting variants were obtained. These variants, as well as those from other spore-formers, showed in colony form and cultural characteristics, all transitions between *B. mycoides*, *B. mesentericus*, and *B. subtilis*.

Though the effect of dissociation on the mechanism of spore formation is not specifically considered in these reports, it is very plain that spore production in general is profoundly affected by

dissociation changes. It is outstanding that during the transition from the "typical" to an atypical form there is little or no spore production. Apparently a variant from the typical form may or may not be a spore-former, though it may later change into a spore-former. It seems plain from recent work of the type here cited that studies of the biology of old cultures will clarify relations in the classification of spore-formers, which are at present obscure.

METHODS AND APPARATUS

The culture vessels used in the beginning of this work consisted of test tubes of 75 cc. capacity, fitted with two-hole rubber stoppers, carrying straight inlet and outlet tubes. (For convenience this type of aeration apparatus will be referred to as "straight culture vessel.") Compressed air was passed through a solution of $KMnO_4$ to a manifold, from which connections were made through needle valves to the culture vessels. The air pressure was regulated by a mercury head, $2\frac{1}{2}$ to 3 inches high. With this arrangement difficulty was experienced in securing uniform, uninterrupted bubbling, and as a result marked variations in the amount of growth and in spore production after twenty-four hour's aeration were often noted.

Early in this work it was realized that a more uniform environment for the cultivation of bacteria could be secured by aeration in liquid media than by the use of solid substrates. The advantages to be derived have been discussed by the writers in a previous publication (Magoon and Brunstetter (1930)), in which a culture vessel designed to secure efficient aeration and based on the principle of the gas scrubber has been described in detail. (This second type of aeration apparatus will be designated as the "spiral culture vessel.")

It should be emphasized that with both forms of apparatus contamination was prevented not only by passing the air through a solution of $KMnO_4$ but also through a cotton plug before it entered the culture media.

As a consequence of the uniformity of distribution of food material and metabolic products in efficiently aerated cultures,

quantitative counts of the percentage of spores formed in a given medium after twenty-four hours may be undertaken without the variability of results that may be expected in unaerated liquid media or on plates or slants of agar media. The percentage of spores was considered an adequate measure of the "intensity factor" of the spore-producing power of a given cultural environment, which is what is desired. Consequently, the number of spores of a given culture, which may be regarded as a measure of the "capacity factor" of its spore-producing power, was not determined.

The strain of *B. mycoides* employed for most of this work was isolated from Texas soil in 1922 and was chosen because of the high thermal resistance of its spores. Several single cell cultures from this strain were made by means of the Chambers micromanipulator and were used as sources for subsequent transplants in this work. This strain gave cultural reactions in all respects identical with those regarded as typical of *B. mycoides* Flügge. The other strains used were isolated in 1931 from soils secured from various parts of the country. The strains are designated by the names of the experiment stations from which they came.[2]

A number of unsuccessful attempts were made to secure a synthetic medium on which *B. mycoides* would grow vigorously. Consequently, a peptone medium, Difco bacto-peptone, was used throughout the investigation.

All incubations were made at 30°C.

EXPERIMENTAL

The effect of aeration on the growth of B. mycoides in 2 per cent peptone solution

Early in the investigation it was found that aeration of peptone cultures of *B. mycoides* greatly increased the amount of growth obtained in twenty-four hours. As an illustration, the following experiment is presented: Four spiral culture vessels were sterilized and 70 cc. of sterile 2 per cent peptone were transferred to each

---

[2] These soils had been collected in 1918 and were stored in the laboratory for thirteen years.

vessel. After inoculating each medium with 0.1 cc. of a spore suspension of B. mycoides, the vessels were connected to the aeration apparatus and vigorously aerated for a minute to secure an even distribution of the inocula. The air supply was then shut off from two vessels and in the other two vessels aeration was adjusted at the rate of 60 to 70 bubbles per minute. After twenty-four hours, 30 cc. from each culture were centrifuged until the supernatant liquid was clear and the volumes of the sediments were read. The average for the controls was 0.06 cc.; the average for the aerated cultures was 0.60 cc.

The inference from this result, of course, is that in an aerated medium of about 0.5 per cent peptone solution, exhaustion of the food supply will occur much sooner than in a medium of the same composition unaerated, and also that there will be a correspondingly greater accumulation of metabolic products. The great advantage of aerating a liquid medium is that growth is not then limited by an insufficient oxygen supply, with the result that the effect of the factors of food supply and metabolic products on spore production may be much more profitably studied. Another advantage, perhaps no less important when dealing with an organism like B. mycoides, which grows in unstirred liquid media almost entirely on the surface as a pellicle, is that bubbling air through the medium prevents localized growth, so that the concentration of the food supply as well as that of products of metabolism is uniform throughout the medium. The use of aeration is, of course, not new in the analysis of the physiology of aerobic spore formation; it has been used, for example, by Buchner, Osborne, and by Stephanidis. So far as the application to aerobic spore-formers like B. mycoides goes, its importance in studying spore formation can not be over-emphasized.

The effect of the concentration of peptone in unaerated cultures on spore production

Peptone solutions of the following concentrations were prepared, tubed, and sterilized: 2, 1, 0.5, 0.25, and 0.12 per cent. After inoculation with equal volumes of a spore suspension of B. mycoides they were incubated for forty-three hours; smears were

then made, using pellicle material only, for determinations of the percentages of spores, using three preparations from as many tubes for each concentration.

It was found that less than 1 per cent of spores was produced under these conditions in any concentration of peptone. This result, often repeated, shows that the effect of the peptone concentrations on spore production is obscured under these conditions.

The effect of the concentration of peptone on spore production in aerated cultures of B. mycoides

Peptone solutions were made in the following concentrations and sterilized: 5, 2, 1.5, 1.0, 0.75, 0.50 and 0.25 per cent. After sterilizing the straight culture vessels, 25 cc. of each solution were transferred to them; the media were then inoculated with equal amounts of spores of B. mycoides from a sand culture. The culture vessels were then connected to the aeration apparatus and vigorously aerated for twenty-four hours. Duplicate tubes of each concentration were employed. After twenty-four hours the tubes were disconnected, the nature of the growth noted, smears prepared for microscopic examination, and 15 cc. of the cultures centrifuged for ten minutes. At the end of this time, the growth was completely thrown down. The volumes of the sediments obtained were then noted. This means of recording growth is relatively crude, yet it nevertheless gives an approximate measure of the yield of cells obtained for a given concentration. Plate counts or direct microscopic counts do not give accurate results under these conditions because growth occurs in clumps whose size increases with the concentration of the medium. The results of four such runs, in which there were duplicate cultures for each concentration, are presented in table 1 and figure 1.

It will be noted that the peptone concentrations have been expressed not in the form of percentages, but as milligrams of peptone initially present in the medium. This gives the total amount of nutrient present in the environment and allows the results to be compared with subsequent runs, in which the spiral culture vessels were used, each containing 70 cc. of peptone solution.

The figure shows clearly, first, that with an adequate oxygen supply the amount of growth was directly proportional to the amount of peptone present in the culture (a fact which had been noted by previous investigators); second, that the number of spores relative to the number of vegetative cells increased as the amount of peptone present in the culture decreased.

TABLE 1

Effect of the amount of peptone in the medium on growth and spore production by B. mycoides after aeration for twenty-four hours

RUN NUMBER	MILLIGRAMS OF PEPTONE INITIALLY PRESENT IN 25 CC. OF MEDIUM														INOCULA— NUMBER OF SPORES
	1,250		500		375		250		187		125		63		
	Yield	Spores	Yield	Spores	Yield	Spores	Yield	Spores	Yield	Spores	Yield	Spores	Yield	Spores	
	cc.	per cent	cc.	per cent	cc.	per cent	cc.	per cent	cc.	per cent	cc.	per cent	cc.	per cent	
I ⎰		0	0.4	0	0.3	6	0.2	80	0.2	95	0.1	95	0.05	100	⎱ 9,500,000
⎱		0	0.4	23	0.4	40	0.2	50	0.2	90	0.1	90	0.05	95	⎰
II ⎰		0	0.4	0	0.25	35	0.2	42	0.15	45	0.1	99			⎱ 4,900,000
⎱		0	0.4	0	0.25	52	0.2	60	0.15	80	0.1	90			⎰
III ⎰	0.6	0	0.4	0	0.4	32	0.2	78			0.1	95	0.08	99	⎱ 6,200,000
⎱	0.6	0	0.4	41	0.4	56	0.2	70			0.1	99	0.04	99	⎰
IV ⎰	0.6	0	0.4	0	0.25	9	0.2	80			0.1	52	0.05	90	⎱ 6,900,000
⎱	0.6	0	0.4	0	0.35	55	0.2	63			0.1	90	0.05	95	⎰
Mean..	0.6	0	0.4	8	0.33	36	0.2	65	0.13	78	0.1	88	0.05	98	

The figures for the percentage of spores often vary considerably for duplicate cultures containing the same amount of peptone. This was generally due to the fact that the rate of aeration in one of the cultures was by accident markedly slower than in the duplicate culture. This slower aeration was usually accompanied by a lower production of spores.

In a second series of runs, the rate of aeration was varied as well as the concentration of peptone, since previous observations had demonstrated the great importance of the oxygen supply, as a

factor not only in the production of growth but of spores in a peptone solution of a given concentration.

The inoculum for the first run came from the ninth daily transfer in aerated 4 per cent peptone solution; the inoculum for run 2, from the eleventh transfer, and the inoculum for run 3, from the thirteenth daily transfer. In this series of runs the spiral culture vessels were used, each containing 70 cc. of peptone solution; the experiment set up was such that the rate of aeration
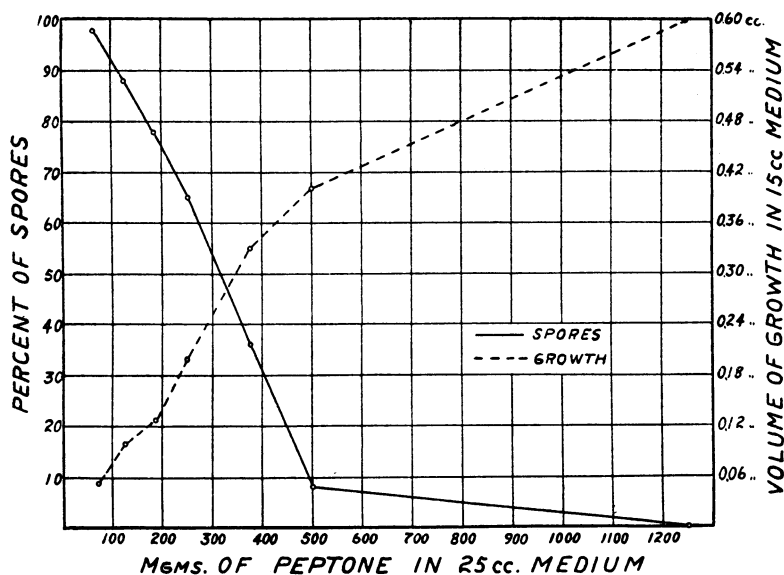

FIG. 1. PERCENTAGE OF SPORES AND VOLUME OF GROWTH OF Bacillus mycoides IN AERATED PEPTONE SOLUTIONS AFTER TWENTY-FOUR HOURS' INCUBATION AT 30°C. AS FUNCTIONS OF THE AMOUNT OF PEPTONE PRESENT IN THE MEDIUM

could be accurately maintained throughout the twenty-four hours. The concentrations of peptone used were 1.0, 0.5, 0.25, 0.12 and 0.06 per cent. Each tube was inoculated with 0.1 cc. of a 4 per cent peptone culture aerated for eighteen hours; the cells were all in the vegetative state. After incubation all the tubes were adjusted to aerate approximately at the same rate. At the end of twenty-four hours, smears were prepared from each tube for the determination of the percentage of spores and for cell counts.

In this way three successive runs were made in which the degree of aeration was 60, 150, and 250 to 300 bubbles per minute, respectively. The concentration of peptone, as in the first series of runs, is expressed in milligrams of peptone present in the medium (in this case 70 cc.).

The nature of the vegetative growth of B. *mycoides* changes after repeated transfer in aerated broth or peptone media. As previously stated, growth in peptone solutions inoculated with spores occurs in clumps. If such a clump is transferred into a fresh peptone or broth solution, growth after four or five daily

TABLE 2

Growth and spore production by B. mycoides as functions of the amount of peptone in the medium and the rate of aeration: incubated twenty-four hours

RUN NUM-BER	RATE OF AERA-TION BUB-BLES PER MIN-UTE	MILLIGRAMS OF PEPTONE INITIALLY PRESENT IN 70 CC. OF MEDIUM									
		700		350		175		88		44	
		Spores	Number of cells cubic centimeter	Spores	Number of cells cubic centimeter	Spores	Number of cells cubic centimeter	Spores	Number of cells cubic centimeter	Spores	Number of cells cubic centimeter
		per cent		per cent		per cent		per cent		per cent	
1	60	0	137,500,000	26	70,300,000	41	65,500,000	55	25,300,000	93	20,100,000
2	150	11	128,100,000	36	117,200,000	61	40,900,000	78	32,700,000	94	17,900,000
3	250-300	24	171,900,000	61	85,900,000	76	59,700,000	94	39,700,000	95	15,600,000

Inocula (number of vegetative cells per cubic centimeter): Run 1, 29,700,000; Run 2, 75,700,000; Run 3, 11,000,000.

transfers no longer is present in the form of clumps but is uniform throughout the medium, so that a representative sample for microscopic cell counts may easily be taken.

The data in table 2 generally checked the figures of the first series of runs with respect to the increased growth obtained with an increased food supply, and also the increase in the percentage of spores with a decreased food supply.

The fundamental importance of the oxygen supply in spore production by B. *mycoides* is demonstrated by the increased spore production with an increased rate of aeration for any given peptone concentration. This is illustrated in the curves of figure 2.

Of particular interest are the slopes of all three curves for spore production, comparing the cultures containing 88 and 44 mgm. of peptone, respectively. It will be seen that where the rate of aeration was 60 bubbles per minute, the percentage of spores dropped sharply; where the rate of aeration was 150 bubbles per minute, the percentage of spores dropped less sharply, and
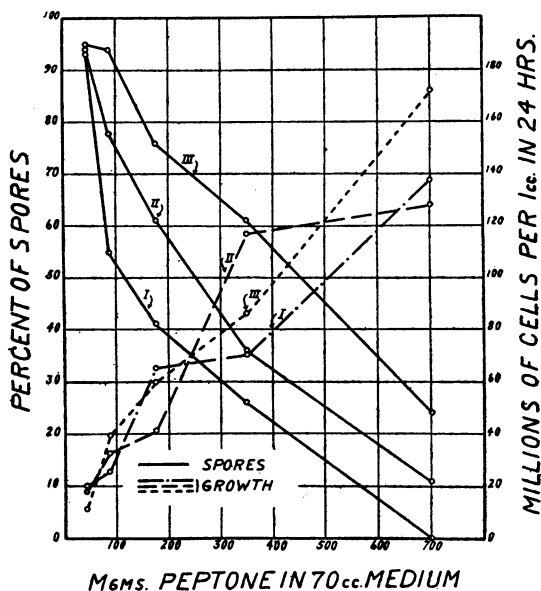

FIG. 2. PERCENTAGE OF SPORES AND VOLUME OF GROWTH OF *Bacillus mycoides* IN AERATED PEPTONE SOLUTIONS AFTER TWENTY-FOUR HOURS' INCUBATION AT 30°C. AS FUNCTIONS OF THE AMOUNT OF PEPTONE PRESENT IN THE MEDIUM AND THE DEGREE OF AERATION

I, aeration at the rate of 60 bubbles per minute; II, aeration at the rate of 150 bubbles per minute; III, aeration at the rate of 250 to 300 bubbles per minute.

where the aeration was 250 to 300 bubbles per minute the drop was only slight. The slopes of the curves for spore production at the higher concentrations show much smaller differences, the percentage of spores varying with the amount of peptone present but the slopes remaining roughly the same.

The interpretation of these growth and spore production curves seems to be that the amount of growth is determined primarily

by the amount of food present; the greater the nutrient supply the more abundant the growth, at least within the limits employed in the present experiments. As indicated by the intersections of the growth curves, the effect of the rate of aeration on the growth obtained is slight, if present, and is within the experimental error of estimating growth. In the case of spore production, on the other hand, two factors are clearly operative, namely, the food supply and the degree of aeration. With increased food supply the percentage of spores produced is progressively decreased, but this decrease is offset to a marked degree by an increase in the rate of aeration. If high spore production is desired the degree of aeration must be increased where higher food concentrations are used.

The importance of the rate of aeration can be demonstrated strikingly by the addition of methylene blue, so as to make a 0.005 per cent solution, to twenty-four-hour aerated cultures of *B. mycoides*. When aeration is discontinued, reduction of the dye with discoloration of the medium occurs in a few minutes. The rate of aeration required to restore the color of the medium throughout depends on the amount of growth, which in turn depends mainly on the amount of peptone in solution. It has been found, for example, that an aeration rate of 100 bubbles a minute was necessary to reoxidize completely the methylene blue in a culture aerated for twenty-four hours in 3 per cent peptone solution.

It will be noted that the size of the inoculum was variable, not only from run to run, but within a given run. Although in a given run the same volume of suspension of spores or vegetative cells was employed to inoculate solutions of decreasing amounts of peptone, the number of cells per milligram of peptone doubled as the amount of peptone in the medium was halved. Thus, in Run 1 of the second series, where the inoculum was 29,700,000 cells in 70 cc. of medium, there were 42,000 cells per milligram of peptone in the 1 per cent peptone solution, but in the 0.5 per cent peptone solution, which received the same volume of cell suspension, there were 85,000 cells per milligram of peptone. The question then arises as to what extent the relative spore produc-

tion after twenty-four hours aeration was influenced by variation in the number of organisms per milligram of peptone initially present.

The writers believe that under the experimental conditions employed, the ratio of the population density to the food supply at the beginning of a run is of little or no importance. For a given concentration of peptone, aeration accelerates growth to such an extent that by the end of twenty-four hours the number of cells per milligram of peptone is practically constant and further growth is restrained, due either to the exhaustion of the food supply or to the accumulation of metabolic products, depending on the concentration of the peptone.

An examination of figure 2 shows that any effect of a changed ratio of cell population to the food supply is of much less importance than the effect of a change in rate of aeration. The amount of growth in media containing 700 mgm. of peptone, for example, varied inversely with the inocula. The second run, which received the greatest inoculum, showed the greatest amount of growth in only two of the five concentrations.

However, the importance of the size of the inoculum on the relative number of spores formed in a series of aerated peptone solutions of decreasing concentration would very probably be outstanding if the runs were to be ended at twelve or eighteen hours, before the end of the logarithmic period of growth.

Previous investigators have pointed out that the absolute number of spores increases with increased concentration of medium. When the percentages of spores in table 2 are multiplied by the number of cells per cubic centimeter, a rough measure of the total number of spores is obtained. It will be noted that the maximum number of spores per cubic centimeter produced in Run 1 was in the medium containing 175 mgm. of peptone. Increasing the amount of peptone resulted in a decrease in the absolute, as well as the relative number of spores. Similarly, in the second and third runs, the maximal absolute numbers of spores were in the medium containing 350 mgm. of peptone. With an increase in food supply above this amount the absolute number of spores decreased.

The effect of extending the time of aeration on spore production

In the preceding experiments the peptone cultures had been incubated for twenty-four hours only. This short incubation period leaves unanswered the interesting question as to whether prolonged aeration at a rapid rate would eventually bring about

TABLE 3

Spore production by B. mycoides after long-continued aeration in peptone solutions

MILLIGRAMS OF PEPTONE IN MEDIUM (70 cc.)	RUN STARTED	AERATION PERIOD	STRAIN OF B. MYCOIDES	SPORES
		hours		*per cent*
2,800	October 13, 1930	264	Texas	0
1,400	December 11, 1930	144	Texas	6
1,400	January 16, 1931	22	Texas	0
		70		1
700*	December 17, 1930	24	Texas	0 / 0
		48		0 / 2
		72		11 / 16
		96		3 / 10
		168		7 / 18
		240		41 / 85
1,400	March 19, 1931	192	South Carolina	3
		192	Oklahoma	3
		192	Virginia	9
		192	Maine	17
		192	West Virginia	31

* The figures for spore production here represent counts on duplicate tubes. All spore counts are averages, each based on counts of two smears.

complete spore production in the higher concentrations of peptone, where heavy growth is obtained. This question is of importance from the standpoint of obtaining spores in quantities sufficient for chemical analysis.

The experiments performed in this connection are summarized in table 3.

The fact that no spores were observed at any time in eleven days in the medium containing 2800 mgm. of peptone (4 per cent) might mean that the cell population, though normal, had not yet exhausted the food supply or that metabolic products had injured the cells so that they were incapable of sustained growth or of forming spores. That the latter was the case is shown, first by the marked decrease in the number of bacilli after the third day of aeration and a corresponding increase in the amount of granulation of the cells that had occurred, and, second by the occurrence of dissociation. The culture was plated out on the eleventh day; in addition to typical *B. mycoides* the plates showed a variant, designated M 1 for convenience, which grew in compact, non-mycelioid colonies with sprawling out-runners of growth that curved at the end in a manner similar to typical colonies of *B. mycoides*. The growth, unlike that of typical *B. mycoides*, was non-adherent to the medium. This variant apparently is similar to that found by Nyberg (1929) (see Abb. 5, I–c, and Abb. 7, III–c).

DISSOCIATION AS A FACTOR IN SPORE PRODUCTION BY B. MYCOIDES

It was pointed out in the introduction that there was abundant evidence in the literature that certain aerobic spore-formers may undergo profound changes in morphology and colony form. However, the degree to which the mechanism of spore production is thereby altered is only scantily and indirectly indicated by past investigations.

The writers have found that dissociative changes play a very important part in the response of *B. mycoides* to aerated peptone solutions of decreasing concentrations. A culture undergoing dissociation may be totally unresponsive to the concentration of the medium as regards spore production. This is illustrated by the following experiment: A spore suspension was prepared from an old agar slant culture of the Texas strain of *B. mycoides*. It should be explained that this culture was only a few transfers removed from a culture obtained by the isolation of a single spore of *B. mycoides*. The suspension was used to inoculate 4 per cent peptone, which was aerated for twenty-four hours. Daily trans-

fers in 4 per cent peptone were made, with the exception that transfer No. 6 was aerated for sixty-five instead of twenty-four hours (the culture was negative for spores and showed rather extensive autolysis). At intervals the twenty-four-hour culture was used to inoculate a series of peptone concentrations (in the spiral culture vessels); after twenty-four hours' aeration the cultures were then examined for spore production.

The results are given in table 4.

When the stock culture had been transferred every twenty-four hours for four days, subcultures in aerated peptone solutions of decreasing concentrations showed subnormal spore production; after the seventh transfer of the stock culture, subcultures in dilute aerated peptone solutions failed to produce spores.

TABLE 4

Effect of decreasing concentrations of peptone on spore production by B. mycoides

RUN NUMBER	NUMBER OF TRANSFERS IN AERATED 4 PER CENT PEPTONE SOLUTION	MILLIGRAMS OF PEPTONE INITIALLY PRESENT IN THE MEDIUM				
		700	350	175	88	44
		Percentage of spores after twenty-four hours' aeration				
1	4	0	2	0	43	53
2	7	0	0	0	0	0
3	10	0	0	0	0	1
4	14	0	0	0	0	0

In the first run the low spore production can be explained by the low rate of aeration (20 to 40 bubbles per minute) with the possible exception of the culture containing 44 mgm. of peptone; in this case aeration was started at 30 bubbles per minute, but by the following morning had risen to 180 bubbles per minute. In the second and fourth runs the rate of aeration was 60 bubbles per minute; the third run was aerated at the rate of 150 bubbles per minute.

The stock culture was plated out after the ninth transfer in 4 per cent peptone. The majority of the forty-eight-hour colonies on nutrient agar (see fig. 3) were definitely "atypical"; that is, they did not show the coarse mycelioid growth characteristic of B. mycoides; they corresponded to the "M 1" type of variant

already noted. A number of small, smooth, white colonies were also present.

The different types of colonies showed an abundance of spores after four days' incubation, indicating that the variants produced were not asporogenous. Microscopic examination of the smears
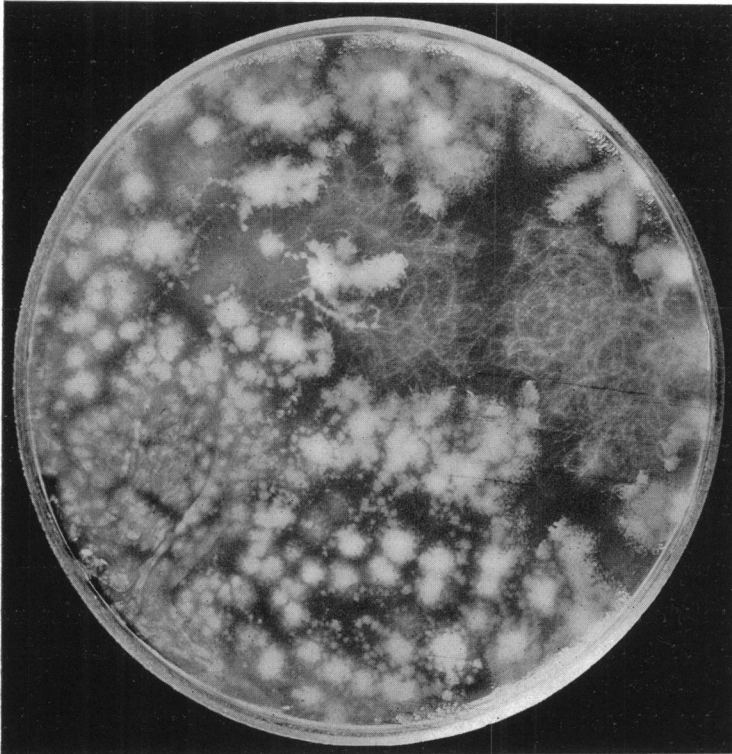

FIG. 3. AN M₁" TYPE VARIANT OBTAINED FROM TYPICAL *Bacillus mycoides* AFTER 9 CONSECUTIVE TRANSFERS IN 4 PER CENT PEPTONE SOLUTIONS
Plate incubated for forty-eight hours at 30°C.

of the second, third and fourth runs showed that in general the cells presented a finely granular appearance and took the stain lightly. It will be remembered that in experiments summarized in table 2, the peptone solutions were inoculated with a culture of *B. mycoides* which also had been transferred daily in 4 per cent

peptone solution; in the case of the first run, the inocula came from the ninth daily transfer. However, no dissociative phenomena or lowered spore production were noted.

The writers believe that the different results obtained after repeated transfer in 4 per cent peptone in these two series of runs illustrate the different resistance to dissociative changes displayed by different cultures of the same organism. In the one case, repeated transfer in aerated 4 per cent peptone solution did not affect *B. mycoides*; in the other case, such cultivation induced dissociation which profoundly affected the mechanism of spore production. The reason for this difference is at present unknown. The importance of understanding this difference is obvious, as it

TABLE 5

Spore production by the M 2 variant of B. mycoides

EXPERIMENT NUMBER	AERATED FOR	MILLIGRAMS OF PEPTONE PRESENT IN THE MEDIUM				
		1,400	700	350	175	87
		Percentage of spores after aeration				
	hours					
I	24	36	29	39	9	11
II	24	10	10	0	4	0
	48	17	8	3	28	1
	72	14	7	1	38	8

is highly desirable in a chemical study of the thermal resistance of spores to cultivate the test organism under conditions which insure a stable strain.

A smooth variant of *B. mycoides* frequently encountered in our studies, has the following characteristics: It is a small, slender bacillus forming spores centrally, possessing active motility, growing singly or in short chains of two or three cells and forming on nutrient agar white or yellowish white, smooth, slightly raised colonies, at first round and entire but later forming an indented border zone. This variant, designated for convenience the "M 2" type, is similar in many characteristics to a smooth type described by Oesterle and Stahl (1929).

The results on the mode of spore production by this variant grown in aerated peptone solutions of varying concentrations are given in table 5.

The results of these two runs, while not checking closely, and, as far as the second run is concerned, lacking regularity, show clearly that, contrary to the results obtained with the typical, or rough, type of *B. mycoides*, there is here a decrease of spore production with a decreased concentration of medium. This smooth type of *B. mycoides* shows increased growth with increased concentration of peptone, so that in higher concentrations of peptone there is a greater concentration of metabolic products. In this case, then, metabolic products instead of hindering spore production seemed to favor it.

SPORE PRODUCTION BY AEROBES OTHER THAN B. MYCOIDES

In order to learn whether the results obtained with *B. mycoides* were general, a number of other aerobic spore-formers were studied.

As in the case of *B. mycoides*, peptone solutions of various concentrations were prepared, sterilized and transferred to spiral aeration vessels. After aeration for twenty-four hours at 30°C., at a rate of about 60 bubbles per minute, the vessels were disconnected and smears were prepared from each culture. Quantitative determinations of spore production were made from each smear.

No spores in any concentration of peptone were found in aerated cultures of *B. aterrimus*, *B. brevis*, *B. circulans*, *B. mesentericus*, *B. fluorescens*, *B. globigii*, *B. laterosporus*, *B. lacticolus*, *B. panis*, or *B. subtilis*. No growth after twenty-four hours' aeration was noted in the cultures of *B. laterosporus* and *B. panis*; fair growth, without spore formation, occurred in the subsequent twenty-four hours' aeration of these cultures.

B. tumescens also showed poor growth. After twenty-four hours' aeration of a series of peptone cultures ranging in concentration from 2 per cent to 0.03 per cent, the only growth that occurred was in the 1 per cent peptone culture, which contained 64 per cent spores. The media, with the exception of the 2 per cent and 1 per cent peptone cultures, were reinoculated and reaerated; after the next twenty-four hours' aeration only the 0.5 per cent peptone culture showed visible signs of growth. No

spores were present in this growth. After a further aeration for twenty-four hours the 0.06 per cent peptone culture showed fairly heavy growth, while the 0.25 per cent and 0.03 per cent peptone cultures showed but slight growth. No spores were found in these cultures.

In a second experiment, medium turbidity after twenty-four hour's aeration was found only in the 2 per cent and 1 per cent cultures. The other cultures (4, 0.5, 0.25, 0.12 and 0.06 per cent peptone) showed no visible signs of growth. There were no spores in the 2 and 1 per cent peptone cultures.

In the case of B. sphaericus, the first experiment was negative. In the second experiment, 0.8 per cent spores were found in the 4 per cent peptone culture, 2.8 per cent spores in the 2 per cent, and 0.7 per cent spores in the 1 per cent peptone cultures. Cultures having a lower percentage of peptone were negative for spores.

Two experiments with B. simplex were negative except that two dilute concentrations of peptone gave 4 and 6 per cent spores, respectively.

The data obtained in experiments dealing with organisms which gave positive results are presented in table 6.

It will be noted that in quite a few instances the experimental results do not check. This lack of concordance, together with finding more than one type of bacillus in the same peptone cultures, encountered in the cases of B. asterosporus, B. adhaerens, B. globigii, B. lactimorbus, and B. subtilis, plus the evidences in the literature, is the basis of the writers' belief that with most if not all the spore-formers studied, the relation between the available food supply and the percentage of spores after twenty-four hours' aeration is complicated by the factor of dissociation. If cultures in the process of dissociation are unable to produce spores, a possible explanation of the negative results obtained with a number of spore-formers is that cultures of these organisms were, under the experimental conditions employed, undergoing dissociation. At the time these comparative tests were made, the importance of dissociation as a factor in spore production was not realized. As a result, clear-cut evidence of dissociative changes in cultures of these organisms was neither sought nor obtained.

TABLE 6

Relative spore production as a function of the amount of peptone present in the medium; cultures aerated for 24 hours

ORGANISM	EXPERIMENT NUMBER	MILLIGRAMS OF PEPTONE INITIALLY PRESENT IN THE MEDIUM — Percentage of spores																		
		2,800	1,500	1,400	750	700	500	375	350	250	183	175	125	88	62	44	38	31	16	8
B. adhaerens	I						0			0			0		7			1	59	
	II	0		3		69			72			80		81		72					
B. albolactis	I						0								95				95	86
	II	0		16		97			93			93		98		97					
B. cereus	I						0			90–100			90–100		90–100			90–100		
	II	0		15		31			99			96		97						
B. cohaerens	I						0			1			1		3			2	12	
	II	0		0		0			0			0		0						
B. fusiformis	I						79			72			66		58			23	68	3
	II	79		85		84			83			69		64		35				
B. graveolens	I						27			18			16		12			13		0
	II	3		26		4			0.1			0.6		2		37				
B. lactis	I	79		5		7			14			27			15	13				
B. malabarensis	I		0		0			61			99					100				
B. megatherium	I	0		0		0				46			50						13	
	II	7		26		54										0				
	III		0		74			0			36						37			
B. mesentericus niger	I						8			5			96		94			97	80	71
	II	0		0		0			0			2		3		1				
	III		0		0			6			0					0				

However, it should be noted that since the experimental conditions were designed to duplicate those dealing with *B. mycoides*, optimum conditions for sporulation were quite probably lacking in some cases. For example, *B. subtilis* might have responded differently if a higher temperature or higher concentrations of peptone had been used. Probably an examination of the cultures after forty-eight or seventy-two hours of aeration would have revealed a decided effect of the amount of food supply on the percentage of spores produced.

The results obtained with *B. fusiformis* are of particular interest: the two experiments agree in showing that here spore production decreased in general as the amount of food supply in the medium decreased. This result is the opposite of that obtained with typical *B. mycoides* and similar to that obtained with the smooth variant of *B. mycoides*.

Recent observations indicate that *B. fusiformis*, like *B. mycoides* can undergo dissociation; whether the results of these two experiments hold true of one or all the types of *B. fusiformis* can be decided only by further investigation. The important point is that there are aerobic spore-formers which produce spores in quite different fashion from *B. mycoides* and that consequently no general explanation of the mechanism of spore formation seems possible at present.

DISCUSSION

The term "food supply" is used in this paper in a broad sense as including both organic and inorganic nutrients. The relative importance of these two types of constituents in the nutrition of *B. mycoides* can not at present be decided. Numerous attempts were made in this investigation to obtain a synthetic medium in which to grow *B. mycoides*, but they were all unsuccessful. Apparently, complex organic substances of a structure at present unknown are required. The possibility must not be neglected, however, that as the concentration of peptone is decreased the amount of elements such as zinc and copper and iron may become limiting factors. It is undoubtedly true that the supply of phosphorus in organic and inorganic form is a limiting factor. How-

ever, it is not the only influence concerned, since experiments have shown that the addition of phosphates in the form of NaH_2PO_4 to 0.25 per cent peptone (aerated) does not prevent the production of 80 per cent or more spores in twenty-four hours. The lack of material essential for the synthesis of protoplasm in the lower concentrations of peptone serves to initiate in the vegetative cells of *B. mycoides* internal changes resulting in spore formation. In this case the vegetative cell as such does not seem to be able to enter into a prolonged resting state; this seems to be possible with the smooth variant of *B. mycoides* and with other species of spore-formers such as *B. fusiformis*.

Both the amount of available food and the amount of metabolic products in the environment are of great importance in determining the extent of sporulation by *B. mycoides* under the conditions of these experiments. With relatively low concentrations of peptone the supply of one or more constituents essential to growth is exhausted before the accumulation of metabolic products retards growth and injures the vegetative cells. When the concentration of peptone is increased above 1 per cent (considering a volume of 70 cc. medium) the accumulation of metabolic products in the course of growth becomes the more important factor; if their concentration is sufficient, the vegetative cells are so altered that only a part of the population is capable of sporulation.

Measurements of the hydrion concentration of peptone cultures of *B. mycoides* aerated for twenty-four hours show that the alkalinity of the cultures parallels the amount of growth present, and increases with the amount of peptone in the medium. The maximum alkalinity attained was pH 8.1 (a twenty-four-hour, aerated, 4 per cent peptone culture), and while no systematic study of the effect of hydrion concentration has yet been made, it does not appear that the alkalinity of the medium is of much importance. When the aeration of cultures is extended beyond twenty-four hours, the alkalinity very probably has a much greater effect. Data on the hydrion concentrations of peptone cultures of the other spore-formers studied show that in these cases also the alkalinity in the course of twenty-four hours' aeration does not appear to have much effect on spore production.

In the case of organisms of the type of *B. fusiformis*, which produced an increased percentage of spores as the amount of peptone in the medium (aerated for twenty-four hours) increased, the accumulation of metabolic products seemed to be of much more importance than the food supply. With an increased food supply there was noted increased growth, which involves the production of a greater amount of metabolic products; correlated with this there was a greater spore production.

In view of the fact that the effect of the amount of peptone in the medium on spore production (after aeration for twenty-four hours) varied not only with different organisms, but apparently with the same organism according to its past cultural history, it is obvious that no general explanation—or even description—of the mechanism of spore production is possible at the present time. This apparent lack of a general mechanism as studied under the conditions selected in this investigation seems to offer a possibility of classifying the members of the Bacillaceae on a physiological basis. Any such study should be preceded by thorough investigations of dissociation phenomena of the various spore-formers concerned.

There is needed in the study of thermal resistance of spores a method for obtaining spores of a known and uniform state of resistance to heat or to chemicals. It is recognized by workers in this field that the same disinfectant tested on one lot of spores of a given organism may yield results quite different from those obtained with the same disinfectant under the same conditions on a second lot of spores. Whether the present technique in producing spores will yield material of a uniform degree of resistance (for a stable stock strain) remains to be seen.

In view of the facts that dissociation can alter the mechanism of spore formation of *B. mycoides* and that sporogenous variants of *B. mycoides* may be readily produced, the suggestion that the heat-resistant organisms responsible for food spoilage are, in the words of Morrison and Rettger (1930) "possibly super-resistant varieties of some common aerobic spore-forming organism because of the tendency to dissociation," is extremely interesting. They cite the work of Kelley (1926), who designated the causal

agents of three types of spoilage in evaporated milk as atypical strains of *B. cereus*, *B. simplex* and *B. megatherium*, respectively. They themselves regard the aerobic spore-former which they isolated from cans of spoiled evaporated milk as a relatively stable variant of *B. vulgatus*. Whether a systematic study of the spores of a number of variants of a given spore-former will reveal consistent differences in their thermal resistance can only be decided by future investigations.

CONCLUSION

Studies on the production of spores by *Bacillus mycoides* Flügge showed that when this organism was grown in solutions of peptone of various concentrations and with different degrees of aeration the percentage of spores, as determined after twenty-four hours of incubation, increased as the concentration of peptone decreased and was consistently higher as the degree of aeration was increased.

The volume of growth increased with increase in the amount of peptone present but seemed to be unaffected by the degree of aeration above the minimum used in these experiments.

Dissociation may profoundly alter the mechanism of spore production; a culture of *B. mycoides* undergoing dissociation did not produce spores in any concentration of aerated peptone after twenty-four hours' incubation; also, it has been found that a variant of *B. mycoides* giving smooth, compact colonies on nutrient agar produced an increased percentage of spores as the amount of peptone in the medium was increased.

Studies of spore production in other aerobic forms gave variable results; some responded in a manner similar to *B. mycoides*; others either produced no spores at all under the conditions of the experiment or, as in the case of *B. fusiformis*, produced greater percentages of spores as the concentration of the peptone solutions was increased.

In the discussion and explanation of spore production by aerobes one must consider not only the kind of organism but also its physiological state.

REFERENCES

BEHRING, E. 1889 Zeit. f. Hygiene, 7, 171–185.

BLAU, O. 1906 Centbl. f. Bakt., II Abt., 15, 97–143.

BREFELD, O. 1881 Bot. Untersuch. (Brefeld's), 4.

BUCHNER, H. 1890 Centbl. f. Bakt., I Abt., Orig., 8, 1–6.

CASMAN, E. P., AND RETTGER, L. F. 1931 Jour. Bact., 21, 27.

COHN, F. 1877 Beiträge zur Biologie der Pflanzen, 2, 249–276.

CUNNINGHAM, A. 1931 Centbl. f. Bakt., II Abt., 82, 481–494.

DARANYI, J. V. 1927 Centbl. f. Bakt., II Abt., 71, 353–7.

EISENBERG, P. 1918 Centbl. f. Bakt., I Abt., Orig., 82, 209–217.

GÄRTNER, A. 1903 Über den Einfluss des Nährmaterials auf die Entwickelung
    und die Sporenbildung des Milzbrandbacillus. Festschrift zum sechzi-
    gsten Geburtstage von Robert Koch. Jena, Gustav Fischer, 661–690.

HAAG, F. E. 1926 Centbl. f. Bakt., II Abt., 69, 4–14.

HENRICI, A. T. 1928 Morphologic variation and the rate of growth of bacteria.
    C. C. Thomas, Springfield, Ill.

HOLZMÜLLER, K. 1909 Centbl. f. Bakt., II Abt., 23, 304–354.

ITANO, A., AND NEILL, J. 1918–1919 Jour. Gen. Physiol., 1, 421–428.

KELLEY, C. D. 1926 Proc. and Trans. Roy. Soc. Canada, 20, 387–394.

KOCH, R. 1877 Beiträge zur Biologie der Pflanzen, 2, 277–310.

LEHMANN, K. B. 1888 München. Ges. f. Morphol. u. Physiol., Sitzangsber, 3,
    82–91.

LEHMANN, K. B. 1890 Würz. Phys. Med., Sitzangsber, 34–37.

LÖHNIS, F., AND SMITH, N. R. 1923 Jour. Agr. Res., 33, 401–432.

MAGOON, C. A. 1926a Jour. Bact., 11, 253–283.

MAGOON, C. A. 1926b Jour. Inf. Dis., 38, 429–439.

MAGOON, C. A., AND BRUNSTETTER, B. C. 1930 Jour. Bact., 19, 415–22.

MIGULA, W. 1897 System der Bakterien. Band I. Allgemeiner Teil. Jena,
    Gustav Fischer.

MIGULA, W. 1904–1907 In LAFAR, W.: Handbuch der Technischen Mykologie,
    Band I. Gustav Fischer, Jena.

MORRISON, E. W., AND RETTGER, L. F. 1930 Jour. Bact., 20, 299–311.

NADSON, G. A., AND ADAMOVIC, S. M. 1910 Bull. du Jardin Imper. Botan. de
    St. Petersburg, T. 10, 1910, 154–165. Abst. in Centbl. f. Bakt., II
    Abt., 30, 247, 1911; 31, 287–8, 1912.

NYBERG, C. 1929 Acta. Soc. Medic. Fennicae, T. 12, fasc. 2 u.3.

OESTERLE, P., AND STAHL, C. A. 1929 Centbl. f. Bakt., II Abt., 79, 1–25.

OSBORNE, A. 1890 Arch. f. Hygiene, 11, 51–59.

PASTEUR, L. 1858 Ann. chim. phys., 52, 404–418.

PHISALIX, C. 1892 Cited by MIGULA (1897) Bull. Med., 6, 533.

ROUX, E. 1890 Annales de l'Inst. Pasteur, 4, 25.

SCHREIBER, O. 1896 Centbl. f. Bakt., I Abt., 20, 353–429.

STEPHANIDIS, P. 1899 Arch. f. Hyg., 35, 1–10.

TURRO, R. 1891 Gaceta medica Catalana, No. 3–4. Abst. Centbl. f. Bakt.,
    10, 91–92. Cited by SCHREIBER (1896).

WHITWORTH, S. H. 1924 The influence of hydrogen-ion concentration on the
    biology of the anthrax organism. Thesis presented to the University
    of Zurich, 1924.

WUND, M. 1906 Centbl. f. Bakt., I Abt., Orig., 42, 97–101, 193–202, 289–296,
    385–393, 481–488, 577–588, 673–688.