# THE INFLUENCE OF CATIONS ON AEROBIC SPOROGENESIS IN A LIQUID MEDIUM[1]

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Received for publication, March 2, 1933

## INTRODUCTION

The formation and function of bacterial spores have been the subject of numerous studies since spores were first observed by Perty in 1852. Cohn (1877) working with *Bacillus subtilis* observed the process of sporogenesis and germination and established the true nature of the bodies called spores. Since that time, numerous theories have been advanced by various workers to explain the cause and conditions leading to spore formation.

To date, the exact nature or process of spore formation has not been explained, but the moving pictures by Bayne-Jones (1932) do give us a visible picture of the formation of the spore. These pictures indicate that the actual process of sporogenesis is not long drawn out but rapid. It may require some time to establish the proper conditions but the act takes place quickly. It appears that granules are formed within the bacterium and immediately before the spore is formed these granules migrate to the opposite end from that in which the spore is formed.

As Brunstetter and Magoon (1932) have pointed out, there are a number of factors which influence sporogenesis. Only a brief summary of the more important factors will be possible here. The relation of oxygen supply to sporogenesis was noted by those who first worked with sporogenic aerobes. Cohn (1877)

[1] Journal Article number 149 (n.s.) from the Michigan Agricultural Experiment Station.

[2] Data from thesis presented in partial fulfillment of the requirements for the M.S. degree at Michigan State College by the junior author.

and Schreiber (1896) with *Bacillus subtilis*; Koch (1877), Buchner (1890) and Schreiber (1896) with *Bacillus anthracis*; Schreiber (1896) with *Bacillus tumescens*; Holzmuller (1909) and Brunstetter and Magoon (1932) with *Bacillus mycoides* observed the influence of oxygen on the sporogenesis of these organisms. Other workers, as Matzuschita (1902) and Wund (1906), made similar observations. The latter made a quantitative study of minimum, optimum and maximum oxygen requirements for spore formation and germination as well as vegetative growth for a number of sporogenic aerobic bacteria and found the minima for spore formation to be higher than for spore germination or vegetative growth.

The influence of the food supply on sporogenesis has long been recognized. Buchner (1890) advanced the theory that the stimulus of the organism to form spores comes when the nutrient material immediately surrounding the organism has become deficient. Considerable work has been done to confirm or disprove this theory. Among the more important work in this connection may be mentioned that of Osborne (1890), Stephanidis (1899), Schreiber (1896) and Holzmuller (1909). Of interest in this connection is the work of Tarr (1932) where he shows that media rich in amino acids inhibit sporogenesis. He concludes that endospore formation in aerobic bacilli bears an inverse relationship to the amount of available nutrient present and is not due to any toxic effect of added compounds or compounds already present in the medium.

Another theory that has been advanced to account for sporogenesis is the accumulation of metabolic products of the bacterial cells during growth. Turro (1891) was the first to advance this theory. He concluded that the carbohydrate compounds of the cell are utilized and the nitrogen compounds which are left are the chief constituents of the spore and induce sporogenesis. Migula (1897) likewise believed that sporogenesis was induced by accumulated metabolic products such as acids and alkalies and not by food exhaustion. Mellon and Anderson (1919) showed that, immunologically, the protein of the spore is different from that of the vegetative cell. Henrici (1928) found the follow-

ing factors had an influence on sporogenesis: the strength of medium, the amount of the inoculum and the particular point of the growth curve at which sporogenesis was determined. Churchman (1925) does not believe that sporogenesis is brought about by adverse conditions of environment and supports his contention by experiments with dye inhibition on spore formation.

The influence of temperature on spore formation has been recognized since the first work was done on this phase of the subject. Among the earlier and more important work in this connection may be mentioned that of Koch (1877), Cohn (1877), Brefeld (1881), Schreiber (1896), Migula (1897) (1904–07), Blau (1906) and Itano and Neill (1918–19). The work of these investigators showed that sporogenesis occurs only between certain well-defined limits of temperature.

The reaction of the medium is likewise important in sporogenesis as in all physiological processes. This phase of the subject has been investigated by Fitzgerald (1911), Itano and Neill (1918–19), Whitworth (1924), Leifson (1931) and Cook (1931). In general, the work of these investigators shows that excessively alkaline or acid media delay or inhibit spore formation. There likewise appears to be an optimum pH where spores are formed most abundantly.

That the composition of the media upon which the bacteria are grown is important in sporogenesis is shown by the work of Schreiber (1896), Gartner (1903), Noguchi (1907–08), Fitzgerald (1911), Hall (1922) and Brunstetter and Magoon (1932). The presence or absence of certain materials in the media either stimulates or inhibits the formation of spores.

In Hadley's (1927) excellent and comprehensive treatise on microbic dissociation may be found many references to the literature dealing with dissociation of aerobic sporogenic bacteria such as *B. anthracis*, *B. subtilis*, *B. mycoides*, *B. megatherium*, *B. ramosus*, etc. In many instances the influence of dissociation on sporogenesis is not specifically studied, yet it is sometimes noted that some of the variants or atypical forms lose their ability to produce spores either temporarily or permanently. This no doubt accounts for some of the asporogenous strains of

*B. anthracis*, which have been noted by Lehmann (1888), Behring (1889), Roux (1890) and of *B. mycoides*, Nadson and Adamovic (1910).

The factors so far considered which enter into the physiology of sporogenesis have been: Oxygen, pH, temperature, abundance of food supply, type of food, metabolic products and microbic dissociation. Daranyi (1927) in his work with *B. anthracis*, *B. cylindrosporus* and *B. subtilis* has shown that still another factor must be taken into account. He considers the decrease of the water content of the organism the most important factor favoring spore production. In his experiments spores of *B. anthracis* that were dried for two days in a desiccator and then placed on fresh agar slants and incubated for eighteen hours produced 70 per cent spores. The control which was seeded with an eighteen-hour slant culture after incubation at the same temperature and for the same length of time only produced one per cent spores. He believes that Buchner's theory may be explained on the basis that as the organisms become older their water content decreases thereby causing spore formation.

It is apparent from this brief and incomplete review of the literature that sporogenesis is influenced by a number of factors, some of which are interrelated and exert a greater influence on spore formation under a given set of conditions than others. If the conditions are reversed, however, then another factor or factors may be of greater importance.

## HISTORY OF CULTURES

The four aerobic spore-forming organisms used in this study were *Bacillus subtilis*, *Bacillus cereus*, *Bacillus mesentericus* and *Bacillus megatherium*. The culture of *Bacillus cereus* furnished by Doctor L. F. Rettger of Yale University had been isolated from a hay infusion. The others had been isolated from hay and soil infusions, and were from our own stock collection. Each culture was plated out by the loop dilution method and transfers made from well-isolated typical colonies. To insure pure cultures the above transfers were identified according to Bergey's manual of Determinative Bacteriology, third edition (1930).

## METHOD

The study of sporogenesis necessitated using a solid medium where the culture could be obtained in a vegetative stage for subsequent inoculations into the liquid medium containing various molalities of the salt under study. It was also essential that a liquid medium be used which of itself did not stimulate spore formation. Preliminary work confirmed the "spore cycle" of sporogenic organisms, as determined by Magoon (1926). Thus, twenty-four hours was taken as the time for these organisms to go from the spore stage through the vegetative stage and back into the spore stage again. Eighteen to twenty hours' incubation was taken as the time for the organisms to go into the spore stage if the inoculum was in the vegetative stage. Application was therefore made of the "spore cycle" in this study.

A variety of slanted media were inoculated with a culture of each one of the four organisms; these cultures had been transferred previously at several twelve-hour intervals to insure only vegetative growth. Observations for spores were made at intervals by making spore stains of the smears made from the various media. Two of the media studied proved interesting and useful; glucose agar stimulated each one of the four organisms to almost 100 per cent spore formation in about twenty hours, while on the beef liver infusion agar introduced by Stafseth (1920) and further developed by Huddleson and others (1927) the cultures formed very few spores within the same time limit. The liver infusion agar was therefore adopted to maintain the cultures used in this work. Various liquid media were similarly studied, including plain broth, Dolloff's medium (1926), Leifson's medium (1931), and Hotchkiss' medium (1923) consisting of 1 per cent Bacto-peptone. Williams (1930) (1931) recommended 1 per cent peptone as a good medium for studying the ratio of spores to vegetative cells. Of these media the 1 per cent peptone medium used by Hotchkiss in her work was adopted as it did not stimulate spore formation of the cultures studied within eighteen hours. All media were sterilized by autoclaving for twenty minutes at 15 pounds pressure. The solid media were adjusted so that the final pH after autoclaving was 6.6. The pH of the distilled

water used in making the 1 per cent peptone medium was such that the final pH of the medium was near 6.6 after sterilization.

All glassware used was of Pyrex type, cleaned by soaking in cleaning solution overnight, rinsed in tap water followed by distilled water and sterilized in dry heat at 180°C. for three hours. The chloride salts, whose effect on sporogenesis in the 1 per cent peptone medium was studied, can be classed for convenience into four groups according to valence. The univalent salts used were NaCl, KCl, $NH_4Cl$, and LiCl, also sodium lactate; the bivalent salts used were $MgCl_2$, $MnCl_2 \cdot 4H_2O$, $BaCl_2$, $CoCl_2 \cdot 6H_2O$, $PbCl_2$ and $NiCl_2$; the trivalent salts used were $AlCl_3$, $CeCl_3$, $FeCl_3 \cdot 6H_2O$, and the quadrivalent salt used was $SnCl_4$. All of the salts were Baker's analyzed products.

Stock solutions of a definite molality of these salts were prepared in sterile distilled water and tested for sterility. The desired amount of the salt solution was added to 5 grams of 2 per cent peptone medium and made up to 10 grams with sterile distilled water. Thus, each tube contained 10 grams of 1 per cent peptone with a definite molality of the salt under study.

In making a determination, a very small amount of the aerobic sporogenic bacterial culture used was transferred with a needle from the liver infusion agar slant into 10 cc. of 1 per cent peptone medium and incubated for eighteen to twenty hours at room temperature. One cubic centimeter of each of these spore-free cultures was added to a series of tubes containing the desired molal concentration of each salt, also to a tube containing 10 cc. of 1 per cent peptone which served as a control. These tubes were then incubated at room temperature for eighteen to twenty hours after which the number of bacteria per cubic centimeter and the per cent spores present were determined by using Breed's (1911) (1918) method for direct microscopic counts. Ordinary microscopic slides were marked off into 1 sq. cm. areas, using a diamond point. From each of the tubes containing concentrations of the salt under study 0.01 cc. amounts of the uniform suspensions were placed on the slide and spread evenly over a 1 sq. cm. area. These were allowed to air-dry and then stained by Anjeszky's spore stain method which gives a red spore and a blue sporangium.

Hydrogen ion determinations were made electrometrically, using a Leeds and Northrup potentiometer in conjunction with a saturated calomel cell and a quinhydrone electrode.

Preliminary determinations were made with each salt and each one of the four organisms to determine the range of stimulation, if any, and the point of decrease due to the toxicity of that molality of salt. When this range was found, "Three molalities" were selected to be used in the final study of the salt under consideration. The three molalities selected were (*a*) the one which gave maximum stimulation, (*b*) a molality at the point between the maximum viability and no growth due to toxic effect of the salt and (*c*) a molality at a point lower than that of the maximum viability due to insufficient stimulation caused by the low salt concentration. Determinations were made in all cases employing these "three molalities" and a control. Not less than four separate determinations were made on each series of molalities of each salt, before continuing to the next salt. Each figure in the tables, therefore, represents the average of not less than four determinations. The separate determinations paralleled each other very closely with no wide variations.

## RESULTS

### *1. Influence of univalent cations in combination with chlorine*

For convenience the salts under study were taken up in groups according to valence. The univalent salts were studied first. The "three molalities" were selected by preliminary determinations. Four series of molalities plus controls were prepared and each series inoculated with each of the four organisms.

Table 1 shows the influence of NaCl on viability and sporulation for each of the four aerobic sporogenic organisms studied. These data show that NaCl in molal concentration 0.25 gave maximum stimulation for growth and also maximum stimulation for sporogenesis. The molal concentration below the maximum showed a stimulation over the control but not as great as that of the maximum. The molality above the maximum definitely showed a decrease of stimulation due to the approach to the point of toxicity of the NaCl. The pH ranged from 6.4 to 7.45.

TABLE 1

*Influence of univalent cations of chloride on sporogenesis in a liquid medium*

| MOLALITY | B. SUBTILIS | | pH | B. CEREUS | | pH | B. MESENTERICUS | | pH | B. MEGATHERIUM | | pH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bacteria per cubic centimeter | Per cent spores | | Bacteria per cubic centimeter | Per cent spores | | Bacteria per cubic centimeter | Per cent spores | | Bacteria per cubic centimeter | Per cent spores | |
| **Sodium chloride** | | | | | | | | | | | | |
| 0.5 | 7.1 | 1.6 | 6.5 | 7.5 | 0.48 | 7.3 | 8.0 | 0.1 | 7.15 | 5.4 | 2.1 | 7.2 |
| 0.25 | 8.3 | 5.01 | 6.4 | 11.3 | 3.36 | 7.3 | 8.2 | 4.71 | 7.3 | 7.7 | 4.25 | 7.2 |
| 0.05 | 8.0 | 2.05 | 6.5 | 8.1 | — | 7.3 | 7.6 | 4.76 | 7.25 | 5.7 | 0.05 | 7.1 |
| Control | 7.7 | — | 6.45 | 7.1 | — | 7.25 | 6.0 | — | 7.1 | 5.4 | — | 7.1 |
| **Ammonium chloride** | | | | | | | | | | | | |
| 0.5 | 2.3 | — | 6.45 | 6.0 | 0.2 | 6.45 | 8.0 | — | 6.6 | 3.1 | — | 6.55 |
| 0.25 | 8.0 | 1.2 | 6.3 | 7.3 | 1.1 | 6.55 | 8.8 | 0.28 | 6.6 | 5.6 | 0.2 | 6.65 |
| 0.125 | 6.0 | 1.1 | 6.35 | 8.0 | — | 6.8 | 8.5 | — | 6.8 | 4.9 | 0.1 | 7.05 |
| Control | 6.0 | — | 6.85 | 6.5 | — | 7.3 | 6.0 | — | 7.0 | 5.0 | — | 7.1 |
| **Potassium chloride** | | | | | | | | | | | | |
| 0.5 | 5.5 | — | 6.4 | 7.6 | 0.15 | 7.15 | 9.5 | — | 7.2 | 7.3 | — | 7.4 |
| 0.25 | 6.2 | 0.27 | 6.4 | 8.3 | 0.4 | 7.3 | 10.0 | 0.18 | 7.2 | 7.8 | 0.2 | 7.35 |
| 0.125 | 5.8 | — | 6.4 | 9.0 | — | 7.3 | 9.9 | — | 7.25 | 7.2 | — | 7.35 |
| Control | 6.0 | — | 6.3 | 10.0 | — | 7.35 | 10.0 | — | 7.25 | 7.5 | — | 7.4 |
| **Lithium chloride** | | | | | | | | | | | | |
| 0.25 | 5.0 | — | 7.2 | 6.0 | — | 7.15 | 5.7 | — | 7.5 | 5.0 | — | 7.6 |
| 0.125 | 7.7 | 1.6 | 7.3 | 6.1 | 1.12 | 7.5 | 6.9 | 7.8 | 7.5 | 6.8 | 2.8 | 7.6 |
| 0.05 | 6.9 | — | 7.3 | 6.4 | — | 7.4 | 6.7 | 2.2 | 7.5 | 6.4 | — | 7.6 |
| Control | 8.0 | — | 7.35 | 7.8 | — | 7.5 | 7.9 | — | 7.65 | 7.0 | — | 7.6 |
| **Sodium lactate** | | | | | | | | | | | | |
| 0.5 | 3.5 | — | 5.0 | 4.0 | — | 7.1 | 3.1 | — | 7.3 | 4.0 | — | 7.3 |
| 0.25 | 7.1 | 3.5 | 5.8 | 6.2 | 1.85 | 7.1 | 6.0 | 1.1 | 7.3 | 7.0 | 1.4 | 7.3 |
| 0.05 | 6.7 | 0.35 | 6.0 | 5.8 | — | 7.3 | 5.7 | 0.65 | 7.1 | 7.0 | — | 7.1 |
| Control | 6.8 | — | 6.2 | 5.4 | — | 7.3 | 5.5 | — | 7.1 | 6.3 | — | 7.1 |

— = absence of spores.

Bacteria in terms of millions.

The influence of $NH_4Cl$ is shown by the data in table 1. The pH's throughout were lower than in the case of NaCl but still well within the favorable range for growth and sporulation. Table 1 demonstrates the influence exerted by KCl. The pH's were slightly higher than with $NH_4Cl$ and were practically the same as those for NaCl. In the case of both $NH_4Cl$ and KCl the maximum point of spore formation was at a molality of 0.25 which coincides with that found for NaCl.

In recent years LiCl has found many applications in bacteriology. This makes it a very interesting salt to study in this problem. The data in table 1 show that it exerted a stimulating influence similar to that exerted by the other salts. However, its maximum point for stimulating sporogenesis was at a lower molality (0.125) than was the maximum for the other univalent salts (0.25).

The univalent salts thus far studied were chlorine salts of high dissociation constants. It, therefore, seemed advisable to study the influence of a substance like sodium lactate with a different anion and a high dissociation constant. The influence of sodium lactate, as shown by the data in table 1, was identical to that of the other univalent salts which have the maximum point of stimulation at molality of 0.25. The pH determinations also indicated that it was within the favorable range.

### *2. Influence of pH*

To determine the influence exerted by pH in a medium otherwise favorable to viability and sporogenesis the following experiment was performed. One per cent peptone medium containing a molality of 0.25 of NaCl was prepared and equal amounts were adjusted to various pH's. The pH's to which the medium was adjusted were 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 and covered the pH range found in the study of these salts. The data in table 2 show that there is a slight variation in a favorable medium between the four organisms studied at the same pH. They also show that each one of the organisms had a wide range of pH before a noticeable effect was produced upon the formation of spores. On this basis the pH of the medium, when various molalities of

salt were added, was considered favorable if it came between the range of pH 5.0 and 7.5.

*3. Influence of bivalent cations in combination with chlorine*

Attention was next turned to the bivalent salts as a group. The data indicate that $MgCl_2$ does not exert a very noticeable stimulating effect on growth and reproduction of the organism as evidenced by the count. Although the pH's were within the favorable range for spore formation, $MgCl_2$ did not stimulate the production of spores.

TABLE 2

*Influence of pH on spore formation in a liquid medium at the optimum molality (0.25) of NaCl*

| pH | B. SUBTILIS | | B. CEREUS | | B. MESENTERICUS | | B. MEGATHERIUM | |
|---|---|---|---|---|---|---|---|---|
| | Bacteria per cubic centimeter | Per cent spores | Bacteria per cubic centimeter | Per cent spores | Bacteria per cubic centimeter | Per cent spores | Bacteria per cubic centimeter | Per cent spores |
| 7.5 | 3.6 | 7.0 | 4.8 | 3.5 | 5.0 | 5.0 | 3.8 | 3.0 |
| 7.0 | 3.7 | 8.0 | 5.0 | 3.5 | 5.4 | 5.0 | 4.1 | 3.0 |
| 6.5 | 4.4 | 8.0 | 6.0 | 4.0 | 5.6 | 5.0 | 4.9 | 3.5 |
| 6.0 | 4.8 | 8.5 | 6.4 | 5.0 | 7.0 | 6.0 | 5.0 | 4.2 |
| 5.5 | 5.2 | 9.2 | 5.4 | 5.5 | 6.8 | 7.0 | 5.0 | 4.5 |
| 5.0 | 5.0 | 8.0 | 4.2 | 5.0 | 6.0 | 6.0 | 4.9 | 4.0 |

Bacteria are in terms of millions.

The influence exerted by $MnCl_2 \cdot 4H_2O$ and $BaCl_2$, is identical to that of $MgCl_2$. Preliminary work did not indicate any molality of these bivalent salts under study, which stimulated reproduction of the bacteria, and so the molalities of salt used in the experiments were arbitrarily decided upon.

The three more toxic bivalent salts chosen for study were $CoCl_2 \cdot 6H_2O$, $PbCl_2$ and $NiCl_2$. Although slightly different molalities of salt were used in each case, the influence exerted by them can be considered at the same time. The toxicity of these three salts for the bacteria was definitely shown by the great decrease in number of organisms in the molalities of the salt as compared to the numbers of organisms in the control tube. The pH range was favorable and yet in none of the bivalent salts

was any stimulation exerted on the organisms to cause them to form spores.

*4. Influence of trivalent cations in combination with chlorine*

The influence exerted by the three trivalent salts, $AlCl_3$, $CeCl_3$, and $FeCl_3 \cdot 6H_2O$, was likewise negative for sporogenesis. Here again, as in the case of the bivalent salts, there was no stimulation of the bacteria to reproduce or to form spores although the reaction of the medium was favorable.

*5. Influence of a quadrivalent cation in combination with chlorine*

The influence exerted by $SnCl_4$ on sporogenesis for each of the four organisms was likewise negative. It is noted that this salt does exert stimulating action on the organisms as far as reproduction is concerned, and although the pH range encountered is within the optimum there were no spores formed.

Since all the data for bi-, tri- and quadrivalent cations were negative, they are not included in the tables presented here.

## DISCUSSION

Most of the work done on spores deals with the spores after they are formed and their reactions to various external factors. The problem herein presented deals with the factors which induce the formation of the spores. From such a viewpoint it seemed important to know the correlation between numbers of organisms and the percentage of these organisms forming spores. The degree to which a medium favors sporogenesis is best determined in terms of the per cent of spores to vegetative cells. This gives what Buchner called "the intensity of spore formation." If the intensity factor is considered, two factors are taken into consideration, velocity and the time interval, both of which are important in determining the influence of any substance on sporogenesis. Preliminary experiments showed a very close correlation between the heat resistance method and the spore staining method of demonstrating the presence of spores. It is for this reason that the spore stain was adopted as the criterion of the presence of spores.

Leifson (1931) states that, in his experiments with certain anaerobic organisms, he found a slightly acid medium more favorable for sporulation than an alkaline one. The results in this paper indicate that for the four aerobic sporogenic organisms there is a considerable range of pH where sporogenesis can take place. This confirms Itano and Neill's (1918) (1919) results for *B. subtilis* and is contrary to the work reported by Cook (1931) where he states that growth and the formation of spores in *B. subtilis* were noted only at a pH between 6.0 and 7.0. A comparatively wide range (6.4 to 7.5) is herein reported and an optimum is indicated in an acid medium.

The exact nature of the influence exhibited by the salts included in this study is not clear. The results secured with cations of the univalent salts seem to correlate with the theory of sporulation as expressed by Buchner. Since, at the optimum molality (0.25 in the case of all univalent salts studied, except LiCl and 0.125 in that case) we do have an increase in number of organisms over control, a "local" exhaustion of food material may incite the organisms to form spores. Nevertheless in some of the other salts, as $SnCl_4$, we again have an optimum of bacterial viability at a definite molality and the pH is within the optimal range, but in this case no spores were formed. If the accumulation of metabolic products were the essential factor causing spore formation, we would again expect $SnCl_4$ to form spores in molality of 0.00005. The results herein presented confirm those of Magoon (1926) where he states that neither insufficient food, comparable to local exhaustion of nutrients, nor the accumulation of metabolic products cause spore formation.

Fitzgerald (1911) in her work with *A. capsulatus* reported that 0.5 to 5.0 per cent NaCl did not exert any appreciable stimulation of spore formation as compared to the spore formation obtained in the peptone bouillon used as a basal medium. It is a known fact that the bouillon contains some NaCl; this salt is no doubt one of the factors responsible for the spore formation in her controls. The NaCl added when she studied this salt may have exerted a toxic effect due to presence of too much salt. Thus, the percentage of spores would be almost the same as when no additional salt was added. In our study, NaCl as all other

cations of univalent chloride salts gave a definite and consistent stimulation of spore formation.

From a study of the data presented it is quite evident that the cations of the salts studied fall into two groups: (*a*) the cations of the univalent salts which stimulate sporogenesis and (*b*) the cations of bi-, tri- and quadrivalent salts which as a group do not stimulate sporogenesis. The formation of spores was most abundant at the point of maximum viability of the organisms. Since there are so many factors that influence sporogenesis, as oxygen concentration, temperature, pH, metabolic products, food concentration, dissociation, water content of the cell colloids of the bacteria, it is impossible to determine the importance of any one factor without taking into consideration all the others. In this work all these factors were controlled as far as possible. Optimum conditions for growth without sporogenesis were maintained. The only variable was in known and definite amounts of the different salts added to the media. Just why the univalent salts stimulated sporogenesis while the bi-, tri- and quadrivalent salts did not remains to be explained. One might consider it to be due to their influence on the permeability of the bacterial cell. Osterhout (1915, 1915 and 1922) assumed a specific increase of permeability of plant cells as due to Na and a decrease as due to Ca. However, if one considers Daranyi's results in the light of this explanation, one finds that the two are not in agreement. According to Daranyi (1927) the most important influence favoring spore production is a decrease in the water content of the bacteria. If Na increases and Ca decreases the permeability of bacterial cells, then one should find greater spore production in the medium containing Ca, which is not the case. Furthermore, Winslow and Dolloff (1928) have shown that cations exert a primary effect upon the bacterial cell which is qualitatively the same for all cations but quantitatively different for each cation. In the presence of dilute solutions of salts permeability is increased and in the presence of concentrated solutions of salts it is decreased.

Henrici (1928) in his work with *B. megatherium* found that sporogenesis commenced practically at the point of inflection between the logarithmic growth phase of the culture and its

resting phase, after which it proceeded at a practically constant rate for sometime thereafter. This could hardly be the case in the experiments reported here since the salts were all used in concentrations which gave the same physiological effect on the bacteria at different points on the viability curve.

Falk (1923) presented a review of the literature up to 1923, upon the theories proposed to explain the physiological activity, exerted by the ions. He presents the view that to understand the rôles played by electrolytes in metabolic or physiologic processes of protoplasm there must be advancement and application of colloidal chemistry. The idea then presents itself that, of the salts studied, the cations may fall into a series where the univalent ions form different colloidal complexes with the protein of the bacteria or medium than do the other salts. The nature of the physiological action of the cations would then be determined by the influences of these complexes on the bacteria. If such is the case the ions might fall into a series such as the Hofmeister series but the action of the salts studied does not definitely follow this or any other series. It would be interesting indeed to study more ions, keeping in mind a definite series of activity which has been applied in colloidal chemistry.

A comparison of the molality of the various salts studied at the point of maximum stimulation with those secured by Hotchkiss (1923) shows a remarkable agreement. In her work a non-spore-forming, Gram-negative organism, *Escherichia coli*, was used; while in this work four Gram-positive spore forming organisms were used. The remarkable correlation between the two groups of widely different species with respect to the molality at which maximum stimulation occurred would indicate that the stimulating effect of the various cations upon bacterial viability is not confined to any one species but is of general physiological significance in bacteriology.

## CONCLUSIONS

1. Cations of the univalent chloride salts, NaCl, LiCl, $NH_4Cl$, KCl and also sodium lactate, exerted a distinct stimulating influence on aerobic spore formation in a liquid medium.

2. Cations of the bivalent chloride salts, $MgCl_2$, $MnCl_2 \cdot 4H_2O$, $BaCl_2$, $CoCl_2 \cdot 6H_2O$, $PbCl_2$ and $NiCl_2$; of the trivalent chloride salts $ALCl_3$, $CeCl_3$ and $FeCl_3 \cdot 6H_2O$; and of a quadrivalent chloride salt, $SnCl_4$, had no influence on stimulation of spore formation in aerobic bacteria in a liquid medium.

3. Spore formation was most abundant at the point of maximum stimulation of viability. This would indicate that sporogenesis is not due to a deficiency of nutrient materials or to an accumulation of metabolic products as has been postulated in the past. It would appear that it is a physiological process occurring when certain conditions are present.

4. The pH of the medium studied did not materially effect the formation of spores within a favorable growing range pH 5.0 to 7.5. However, an acid reaction was slightly more favorable for their production.

5. The molal concentration of the different salts at which maximum stimulation occurred for the four organisms studied, *B. cereus*, *B. subtilis*, *B. mesentericus* and *B. megatherium*, corresponds to a remarkable degree with those for *E. coli* as previously determined by other investigators. This work, therefore, confirms and extends our knowledge of the physiological effect of cations upon bacterial viability.

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