THE INFLUENCE OF CATIONS ON
AEROBIC SPORE FORMATION
IN A LIQUID MEDIUM

THESIS FOR THE DEGREE OF M. S.

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Cations

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A Dissertation submitted to the Graduate Faculty in candidacy for the Degree of Master of Science.

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THESIS

Introduction.

Physiological studies as to the nature of spores have received considerable attention since the organism Bacillus anthracis was first isolated and demonstrated to have the ability to form spores. The greatest amount of research thus far has concerned itself with a study of the activities and properties of these spores.

The exact nature of the processes which take place within the organism immediately before the formation of the spore are not very definite at the present time. Schreiber (17) states that an organism which has the inherent property of forming spores may do so at any time; in other words the number of rapid transfers without sporulation does not affect its ability to form spores later. Thus we can see that this ability is a definite criterion of the spore forming organisms.

In general a spore is considered as a condensed, dehydrated protoplasmic mass consisting chiefly of nuclear material. Investigators have analyzed the spores chemically and report that they consist chiefly of proteins. Mellon and Anderson (16) studied the protein of the spore and the protein of the vegetative stage immunologically and state that the two proteins are different. To date the exact nature or process of spore formation is not explained, but the moving pictures by Bayne-Jones (1) do give us a visible picture of the formation of the spore. These pictures indicate that the actual process of spore formation is not a long-drawn out process but a rapid one. It may require some time to establish the proper conditions but the physiological 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.

Magoon (15) in some earlier work concludes that spore formation is a normal process and the spore is a result of the union of granules which are formed within the organism. Thus we can readily see that the exact process of spore formation is vague and not fully explained.

German investigators have done some work in studying spore formation in bacteria, especially of B. anthracis. The views, as to the reason for spore formation, of these early investigators are indicated by the two theories advanced by Buckner (5) (6) and Turro (19). Buckner states that the stimulus of the organism to form spores comes when the nutrient material has become deficient immediately surrounding the organism. Working with the bacillus of anthrax he states that by the renewal of this nutrient material, in the local area, before the organism has reached a certain point the organism can be held to countless generations of only vegetative cells.

Turro presents the view that spore formation of B. anthracis is due to the accumulation of products of metabolic activity.

Schreiber presents a view, partially in accord with both of the above, in which he gives the impression that the deficiency of nutrient material can not be a direct cause but can be considered as the inciting cause of spore formation. He also demonstrated that oxygen of the air is a specific and necessary condition for spore formation in the case of aerobic spore forming organisms.

The work which follows the above investigations is limited more to the study of the reaction of the spore to various physical and chemical factors. Work typical of the above is on spores of the anaerobic organisms as the clostridia group, since their importance in the food and meat canning industry is indeed very great.

In more recent work Williams (20) (21) shows that various concentrations of peptone do exert slightly different influences and he recommends using a one per cent peptone solution in studying the ratio of spores to vegetative cells.

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 cerus* was very kindly furnished by Doctor L. F. Rettger of Yale University and was isolated by him from

a hay infusion. The other three cultures were obtained from the stock collection of the Department of Bacteriology at Michigan State College having been isolated earlier from soil and hay infusions. 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 (2).

Method.

The study of spore formation 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 spore forming organisms, as determined by Magoon (15). Thus twenty-four hours were 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 mediums 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 all vegetative growth. Observations for spores were made at intervals by making spore stains of the smears made from the various mediums. Two of the mediums studied proved interesting and useful; dextrose 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 (18) and further developed by Huddleson (13) the cultures formed very few spores within the same time limit as the dextrose agar. The liver infusion agar was therefore adopted to maintain the cultures used in this work. Various liquid mediums were similarly studied including plain broth, Dolloff's medium (8) Leifsons medium (14) and Hotchkiss medium (12) consisting of one per cent Bacto-peptone. Of these the one per cent peptone medium used by Hotchkiss in her work was adopted as it of itself did not stimulate spore formation of the cultures studied within eighteen hours. All mediums were sterilized by autoclaving for twenty minutes at fifteen pounds pressure. The solid mediums were adjusted so final pH after autoclaving was 6.6. The pH of the distilled water used in making the one per cent peptone medium was such that 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, wrinsed in tap water followed by distilled water and sterilized in dry heat at 180°C. for three hours.

The chloride salts, whose effect on spore formation in the one per cent peptone medium were studied, can be classed for convenience into four groups according to valence. The monovalent salts used were $NaCl$, KCl, NH_4Cl, $LiCl$, sodium lactate; the divalent salts were $MgCl_2$, $MnCl_2\ 4H_2O$, $BaCl_2$, $CoCl_2\ 6H_2O$, $PbCl_2$ and $NiCl_2$; the tri-valent salts were $AlCl_3$, $CeCl_3$, $FeCl_3\ 6H_2O$ and the tetra-valent salts $SnCl_4$. 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 five cubic centimeters of two per cent peptone medium and made up to 10 c.c. with sterile distilled water. Thus each tube contained 10 c.c. of one per cent peptone containing a definite molality of the salt under study.

In making a determination, a very small amount of the aerobic spore formers used were transferred with a needle from the liver infusion agar slant into 10 c.c. of one 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 c.c. of one 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 per cent spores present were determined by using Breed's (3) (4) method for direct microscopic counts. Ordinary microscopic slides were marked off into one square centimeter areas, using a diamond point. From each of the tubes containing concentrations of the salt under study 0.01 c.c. amounts of the uniform suspensions were placed on the slide and spread evenly over a one square centimeter area. These were allowed to air-dry and then stained by Anjeszky's spore stain method (11) 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.

I. Influence of monovalent cations in combination with chlorine.

For convenience the salts under study were taken up in groups according to valence. The monovalent 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 one of the four organisms.

Table I gives the influence of NaCl exerted on the viability and sporulation on each one of the four aerobic spore-forming organisms under consideration. These data show that NaCl in molal concentration 0.25 gave maximum stimulation for viability and also maximum stimulation for spore formation. The point or molal concentration below the maximum showed a stimulation over the control and an influence that definitely marks the molal concentra tion 0.25 as maximum. The molality above the maximum definitely shows a decrease of stimulation due to the approach of the point of toxicity of the NaCl. The pH determinations were made electrometrically and come within the range of 6.5 to 7.45.

2. Influence of pH

To determine the influence exerted by pH in a medium otherwise favorable to viability and spore formation 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. It also shows that each one of the organisms has a wide range of pH before a noticeable effect is 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.

The influence of NH_4Cl is shown by the data in Table 3. The pH's throughout were lower than in the case of NaCl but still well in the favorable range for growth and sporulation. Table 4 demonstrates the influence exerted by KCl. The pH's were slightly higher than with NH_4Cl and compare very favorably with those found with

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 and thus makes it a very interesting salt to study in this problem. The data in Table 5 show that it exerted a similar influence as the other salts insofar as exerting a stimulation of processes is concerned. Its maximum point of spore formation was at a lower molality (0.125) than was the maximum for the other monovalent salts (0.25).

The monovalent salts thus far studied were salts of high dissociation constants. It, therefore, seemed advisable to study the influence of a substance like sodium lactate with a very low dissociation constant. The influence of sodium lactate, as shown by the data in Table 6, was identical to the other monovalent salts which have the maximum point of stimulation at molality of 0.25. The pH determinations also indicate that it was within the favorable range.

3. Influence of di-valent cations in combination with chlorine.

Following the study of the above monovalent salts attention was turned to the di-valent salts as a group. The data in Table 7 indicates that $MgCl_2$ does not exert a very noticeable stimulating effect on growth and reproduction of the organism as is 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.

The influence exerted by $MnCl_2$ $4H_2O$ and $BaCl_2$, as shown by the data in Tables 8 and 9, are identical to $MgCl_2$. Preliminary work did not indicate any molality, of these di-valent 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 di-valent salts chosen for study were $CoCl_2$ $6H_2O$, $PbCl_2$ 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 on the bacteria was definitely shown by the great decrease in numbers of organisms in the molalities of the salt as compared to the numbers of organisms in the control tube. Upon further studying the results in Tables 10, 11 and 12 we also find that the pH range was favorable and yet in none of the di-valent salts did we have any stimulation exerted on the organism to cause or allow it to form spores.

Table 1. Influence of NaCl on Viability and on Aerobic Spore-formation in a Liquid Medium.

Molality	*B. subtilis* bacteria per c.c.	*B. subtilis* %spores	*B. subtilis* pH	*B. cereus* bacteria per c.c.	*B. cereus* %spores	*B. cereus* pH	*B. mesentericus* bacteria per c.c.	*B. mesentericus* %spores	*B. mesentericus* pH	*B. megatherium* bacteria per c.c.	*B. megatherium* %spores	*B. megatherium* pH
0.5	7,081,600	1.6	6.5	7,531,600	0.48	7.3	7,974,800	0.1	7.15	5,388,400	2.1	7.2
0.25	8,350,800	5.01	6.4	11,279,500	3.36	7.3	8,161,000	4.71	7.3	7,703,200	4.25	7.2
0.05	8,580,500	2.05	6.5	8,125,000	-	7.3	7,619,500	0.76	7.25	5,657,200	0.05	7.1
control	7,687,800	-	7.45	7,150,400	-	7.25	6,304,000	-	7.1	5,389,800	-	7.1

Table 2. Influence of pH on Spore Formation in a Liquid Medium at the Optimum Molality (0.25) of NaCl.

pH	*B. subtilis* bacteria per c.c.	*B. subtilis* % spores	*B. cereus* bacteria per c.c.	*B. cereus* % spores	*B. mesentericus* bacteria per c.c.	*B. mesentericus* % spores	*B. megatherium* bacteria per c.c.	*B. megatherium* % spores
7.5	3,650,000	7.0	4,774,000	3.5	5,020,000	5.0	3,850,000	3.0
7.0	3,740,000	8.0	5,040,000	3.5	5,410,000	5.0	4,010,000	3.0
6.5	4,414,000	8.0	5,980,000	4.0	5,620,000	5.0	4,894,000	3.5
6.0	4,774,000	8.5	6,410,000	5.0	6,960,000	6.0	5,020,000	4.2
5.5	5,210,000	9.2	5,420,000	5.5	6,820,000	7.0	5,000,000	4.5
5.0	4,980,000	8.0	4,210,000	5.0	5,984,000	6.0	4,964,000	4.0

Table 3. Influence of NH_4Cl on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	B. subtilis bacteria per c.c.	B. subtilis % spores	pH	B. cereus bacteria per c.c.	B. cereus % spores	pH	B. mesentericus bacteria per c.c.	B. mesentericus % spores	pH	B. megatherium bacteria per c.c.	B. megatherium % spores	pH
0.5	2,335,000	-	6.45	6,003,500	0.2	6.45	8,716,000	-	6.6	3,111,700	-	6.55
0.25	7,944,000	1.2	6.3	7,242,700	1.1	6.55	8,348,000	0.28	6.6	5,590,000	0.2	6.65
0.125	6,010,500	1.1	6.35	8,020,500	-	6.8	8,563,500	-	6.8	4,900,500	0.1	7.05
control	8,370,500	-	6.85	6,490,000	-	7.3	9,443,500	-	7.0	5,070,000	-	7.1

Table 4. Influence of KCl on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH
0.5	5,507,000	-	6.4	7,655,000	0.15	7.15	9,524,500	-	7.2	7,353,500	-	7.4
0.25	6,176,000	0.27	6.4	8,241,500	0.4	7.3	9,947,500	0.18	7.2	7,768,700	0.2	7.35
0.125	5,886,000	-	6.4	9,002,500	-	7.3	9,906,000	-	7.25	7,279,700	-	7.35
control	6,890,200	-	6.3	10,263,000	-	7.35	10,614,000	-	7.35	7,504,000	-	7.4

Table 5. Influence of LiCl on Viability and on Aerobic Spore Formation in a Liquid Medium.

	B. subtilis			*B. cereus*			*B. mesentericus*			*B. megatherium*		
Molality	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH
0.25	5,779,250	-	7.2	6,293,600	-	7.15	5,751,000	-	7.5	5,052,333	-	7.6
0.125	7,671,700	1.6	7.3	6,164,250	1.12	7.5	6,862,000	7.8	7.5	6,028,700	2.8	7.6
0.05	6,910,000	-	7.3	6,420,000	-	7.4	6,720,000	2.2	7.5	6,402,000	-	7.6
Control	8,048,700	-	7.35	7,802,000	-	7.5	7,944,250	-	7.65	7,083,000	-	7.6

Table 6. Influence of Sodium lactate on Viability and on Aerobic Spore Formation in a Liquid Medium

	B. subtilis			*B. cereus*			*B. mesentericus*			*B. megatherium*		
Molality	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH
0.5	3,523,000	-	5.0	4,005,000	-	7.1	3,115,000	-	7.3	4,384,000	-	7.3
0.25	7,132,000	3.5	5.8	6,193,000	1.85	7.1	5,971,000	1.1	7.3	6,956,000	1.4	7.3
0.05	6,725,000	0.35	6.0	5,850,000	-	7.3	5,716,000	0.65	7.1	6,997,000	-	7.1
Control	6,816,000	-	6.2	5,460,000	-	7.3	5,502,000	-	7.1	6,392,000	-	7.1

Table 7. Influence of $MgCl_2$ on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	*B. subtilis* bacteria per c.c.	*B. subtilis* % spores	pH	*B. cereus* bacteria per c.c.	*B. cereus* % spores	pH	*B. mesentericus* bacteria per c.c.	*B. mesentericus* % spores	pH	*B. megatherium* bacteria per c.c.	*B. megatherium* % spores	pH
0.25	4,532,600	-	7.2	9,168,500	-	7.4	10,615,500	-	7.6	10,837,500	-	7.55
0.05	7,613,000	-	7.5	10,675,000	-	7.6	11,106,000	-	7.55	10,960,000	-	7.55
0.025	8,728,000	-	7.1	10,341,500	-	7.6	11,633,750	-	7.55	10,815,000	-	7.5
Control	10,788,750	-	6.9	11,246,500	-	7.6	11,561,000	-	7.5	11,307,500	-	7.5

Table 8. Influence of $MnCl_2\ 4H_2O$ on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	*B. subtilis* bacteria per c.c.	*B. subtilis* % spores	pH	*B. cereus* bacteria per c.c.	*B. cereus* % spores	pH	*B. mesentericus* bacteria per c.c.	*B. mesentericus* % spores	pH	*B. megatherium* bacteria per c.c.	*B. megatherium* % spores	pH
0.05	3,642,600	-	6.45	997,300	-	6.55	3,656,666	-	6.5	2,223,333	-	6.6
0.025	4,037,333	-	6.55	2,161,000	-	6.55	4,862,000	-	6.55	2,406,666	-	6.6
0.0125	3,786,600	-	6.55	2,260,333	-	6.6	4,610,666	-	6.75	2,210,666	-	6.8
Control	9,307,333	-	6.5	11,710,000	-	6.65	9,351,333	-	6.8	9,354,333	-	7.1

Table 9. Influence of $BaCl_2$ on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	B. subtilis bacteria per c.c.	B. subtilis % spores	pH	B. cereus bacteria per c.c.	B. cereus % spores	pH	B. mesentericus bacteria per c.c.	B. mesentericus % spores	pH	B. megatherium bacteria per c.c.	B. megatherium % spores	pH
0.05	3,688,666	-	5.0	6,410,000	-	7.2	5,406,666	-	7.3	6,046,666	-	7.3
0.025	8,099,000	-	5.4	8,436,000	-	7.25	8,086,000	-	7.3	6,869,000	-	7.25
0.0125	11,025,000	-	6.0	9,584,000	-	7.5	8,631,333	-	7.5	7,986,000	-	7.25
Control	13,267,666	-	6.5	10,192,000	-	7.55	10,206,000	-	7.55	8,761,333	-	7.5

Table 10. Influence of $CoCl_2\ 6H_2O$ on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	B. subtilis bacteria per c.c.	B. subtilis % spores	pH	B. cereus bacteria per c.c.	B. cereus % spores	pH	B. mesentericus bacteria per c.c.	B. mesentericus % spores	pH	B. megatherium bacteria per c.c.	B. megatherium % spores	pH
0.005	2,306,000	-	5.5	1,890,500	-	6.5	2,141,000	-	6.8	1,562,500	-	6.5
0.0001	4,777,777	-	5.4	5,071,500	-	6.5	6,479,000	-	6.5	7,679,000	-	6.65
0.00005	6,493,000	-	6.1	7,807,250	-	6.55	10,066,000	-	6.5	9,639,000	-	6.8
Control	9,087,000	-	6.5	9,902,500	-	6.9	11,118,000	-	6.5	10,563,000	-	7.0

Table 11. Influence of $PbCl_2$ on Viability and on Aerobic Spore Formation in a Liquid Medium.

	B. subtilis			B. cereus			B. mesentericus			B. megatherium		
Molality	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH
0.001	3,229,660	-	5.9	4,824,000	-	6.5	8,527,333	-	7.3	8,411,333	-	7.3
0.00005	7,533,666	-	6.15	9,134,000	-	7.1	10,005,333	-	7.4	10,437,333	-	7.4
0.00001	7,451,666	-	6.2	9,836,666	-	7.15	12,816,000	-	7.4	11,557,300	-	7.4
Control	10,857,666	-	6.45	11,480,666	-	7.15	13,562,666	-	7.4	12,602,000	-	7.4

Table 12. Influence of $NiCl_2$ on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH	bacteria per c.c.	% spores	pH
0.001	6,926,666	-	6.25	8,882,666	-	7.3	8,462,666	-	7.3	7,763,666	-	7.35
0.0001	13,336,666	-	6.3	11,380,666	-	7.4	13,354,666	-	7.4	11,992,000	-	7.4
0.00001	14,599,666	-	6.35	12,357,333	-	7.45	15,553,666	-	7.5	12,633,333	-	7.45
control	16,262,000	-	6.45	13,046,666	-	7.5	15,409,600	-	7.45	15,376,666	-	7.55

4. Influence of tri-valent cations in combination with chlorine.

Tables 13, 14, and 15 give the results of the influence exerted by the three tri-valent salts: $AlCl_3$, $CeCl_3$, and $FeCl_3 \, 6H_2O$. Here again, as in the case of the di-valent salts, there was no stimulation of bacteria to reproduce or form spores although the reaction of the medium was favorable.

5. Influence of a tetra-valent cation in combination with chlorine.

Table 16 shows the influence exerted by $SnCl_4$ on each one of the four organisms. 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.

Table 13. Influence of $AlCl_3$ on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	*B. subtilis* bacteria per c.c.	*B. subtilis* % spores	pH	*B. cereus* bacteria per c.c.	*B. cereus* % spores	pH	*B. mesentericus* bacteria per c.c.	*B. mesentericus* % spores	pH	*B. megatherium* bacteria per c.c.	*B. megatherium* % spores	pH
0.0002	8,810,333	-	6.65	8,440,000	-	7.15	9,381,333	-	7.15	10,103,333	-	7.15
0.0001	12,014,600	-	6.6	11,170,000	-	7.1	11,873,333	-	7.15	12,118,666	-	7.15
0.00005	13,262,666	-	6.25	12,066,666	-	7.1	12,832,666	-	7.1	14,685,333	-	7.2
Control	15,190,000	-	6.25	13,993,333	-	7.15	14,763,333	-	7.1	15,238,300	-	7.4

Table 14. Influence of $CeCl_3$ on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	*B. subtilis* bacteria per c.c.	*B. subtilis* % spores	pH	*B. cereus* bacteria per c.c.	*B. cereus* % spores	pH	*B. mesentericus* bacteria per c.c.	*B. mesentericus* % spores	pH	*B. megatherium* bacteria per c.c.	*B. megatherium* % spores	pH
0.00005	8,210,000	-	6.2	10,620,000	-	6.7	11,910,000	-	6.7	12,020,000	-	6.8
0.00001	9,086,000	-	6.25	11,080,000	-	6.8	13,020,000	-	6.9	13,791,000	-	6.9
0.000005	10,210,000	-	6.25	12,320,000	-	6.69	13,890,000	-	7.15	14,620,000	-	7.15
Control	11,755,000	-	6.3	12,920,000	-	7.1	14,465,000	-	7.25	14,896,000	-	7.25

Table 15. Influence of $FeCl_3$ $6H_2O$ on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	*B. subtilis* bacteria per c.c.	% spores	pH	*B. cereus* bacteria per c.c.	% spores	pH	*B. mesentericus* bacteria per c.c.	spores	pH	*B. megatherium* bacteria per c.c.	% spores	pH
0.0005	4,813,000	-	6.1	5,771,000	-	7.3	5,501,666	-	7.2	6,266,000	-	7.3
0.0001	10,785,666	-	6.1	13,208,000	-	7.3	11,102,000	-	7.25	11,453,333	-	7.3
0.00005	13,794,000	-	6.5	13,014,000	-	7.25	13,679,333	-	7.15	12,833,333	-	7.25
Control	15,156,666	-	6.75	15,298,666	-	7.25	14,900,000	-	7.15	13,974,000	-	7.2

Table 16. Influence of $SnCl_4$ on Viability and on Aerobic Spore Formation in a Liquid Medium.

Molality	*B. subtilis* bacteria per c.c.	% spores	pH	*B. cereus* bacteria per c.c.	% spores	pH	*B. mesentericus* bacteria per c.c.	spores	pH	*B. megatherium* bacteria per c.c.	spores	pH
0.0001	7,761,666	-	5.9	6,743,000	-	6.55	6,550,000	-	6.9	8,727,333	-	6.6
0.00005	12,767,000	-	5.9	10,859,333	-	6.75	11,719,666	-	6.8	12,866,666	-	6.75
0.00001	11,902,000	-	6.1	10,357,333	-	6.75	11,256,000	-	6.65	11,551,000	-	6.8
control	12,008,000	-	6.35	10,387,000	-	6.9	10,788,666	-	6.65	11,078,000	-	6.8

Discussion.

The four organisms chosen to be used in this study are representative of the aerobic spore forming group: B. subtilis, B. cereus, B. mesentericus, and B. megatherium. The influence exerted by the various salts was quite constant as far as the four organisms are concerned. Slight individual variations were obtained which are explained by the fact that we were dealing with three different species of bacteria that can be expected to exhibit such slight variations one from the other.

The most common method to demonstrate the presence of spores is the heat resistence of a culture. The greatest amount of literature concerning 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. As such it seemed very important to know the correlation between numbers of organisms and per cent of spores formed. Preliminary experiments showed a very close correlation between the heat resistance method and spore staining method of demonstrating the presence of spores. It is for these reasons that the spore stain was adopted as the criterion of the presence of spores.

Leifson (14) 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 spore forming organisms there is a considerable range of pH where sporulation can take place. This is in part contrary to the work reported by Cook (7) where he states that growth and spore formation of B. subtilis was noted only at a pH between 6.0 and 7.0. A comparative wide range is herein reported and an optimum is indicated in an acid medium.

The exact nature, of the influence as exhibited by the salts included in this study, is not clear. The cations of the monovalent salts seem to correlate with the theory of sporulation as expressed by Buckner. Since at the optimum molality, 0.25 in the case of all monovalent salts studied except LiCl and at 0.125 in that case, we do have an increase in number of organisms over control and thus a "local" exhaustion of food material may incite the organisms to form spores. Nevertheless in some of the other salts as $SnCl_4$ (table 16) we again have an optimum of bacterial viability at a definite molality and the pH is within the optimal range, but here no spores were formed. If the accumulation of metabolic products were the essential factors causing spore formation we would again expect $SnCl_4$ to form spores in molality of 0.00005. The results herein presented confirm the results of Magoon (15) where he states that neither insufficient food, comparable to local exhaustion of nutrients, nor the accumulation of metabolic products cause spore formation.

Fitzgerald (10) having worked with aerogenes bacilli, reports that NaCl did not exert any appreciable stimulation of spore formation as compared to the spore formation obtained in the peptone bouillon be used as a basal medium. It is a known fact that the bouillon contains some NaCl; this salt is no doubt responsible for the spore formation in his controls. The NaCl added when he studied this salt may have exerted a toxic effect due to presence of too much salt. Thus the per centage of spores would be almost the same as when no additional salt was added. In this study NaCl as all other cations of monovalent 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 monovalent salts which stimulate spore formation and (b) the cations of di, tri and tetravalent salts which as a group do not stimulate spore formation. The formation of spores was most abundant at the point of maximum viability of the organisms. This fact indicates that spore formation is a normal physiological process which occurs under the most favorable conditions of growth and viability.

Falk (9) made 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 better understand the roles 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 monovalent ions are the most active precipitants or coagulants and thus cause the organisms to form spores. 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 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, of the molality at which maximum stimulation occurred, between the two groups of widely different species would indicate that the stimulating effect of the various cations upon bacterial viability was not confined to any one species but was of general physiological significance to bacteriology.

Conclusions.

1. Cations of monovalent chloride salts as - $NaCl$, $LiCl$, NH_4Cl, KCl and sodium lactate - exerted a distinct influence of stimulation on aerobic spore formation in a liquid medium.

2. Cations of divalent chloride salts as $MgCl_2$, $MnCl_2\ 4H_2O$, $BaCl_2$, $CoCl_2\ 6H_2O$, $PbCl_2$ and $NiCl_2$; of trivalent chloride salts as $AlCl_3$, $CeCl_3$ and $FeCl_3\ 6H_2O$; and of a tetravalent chloride salts as $SnCl_4$, 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 spore formation 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 normal physiological process occurring under the most favorable conditions.

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

5. The determination of spores by staining compares very favorably with the heat method as a means of determining the presence or absence of spores.

6. 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*, correspond 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.

7. The results of this work would indicate that there is a relationship between valence and spore formation.

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