STUDIES UPON BACTERIAL SPORES

I. THERMAL RESISTANCE AS AFFECTED BY AGE AND ENVIRONMENT

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Received for publication, February 13, 1925

INTRODUCTION

The resistance of bacterial spores to the destructive action of high temperatures has been a matter of general interest to bacteriologists for many years, but the significance of this resistance as a factor in practical home and industrial problems, particularly in the preservation of foods by canning, has but recently come to be appreciated.

The pioneer work of Russell (1895) thirty years ago, followed promptly by that of Prescott and Underwood (1897, 1898) established beyond question the relation of bacteria to food canning problems, and since that time numerous investigators have confirmed their conclusions and shown that successful food preservation is largely a problem of applied bacteriology. The findings of the early investigators were promptly evaluated and the processes involved in the canning of foods were modified to conform to the new information. Bacteriological investigations in this field were, however, for many years concerned primarily with the isolation and study of the microörganisms causing spoilage in canned foods, no thorough study being undertaken of the fundamental problems involved, particularly with respect to the thermal relations of bacterial spores.

Attention was forcibly drawn to this matter during the World War by heavy losses from valuable stocks of canned foods in warehouses, through spoilage, and particularly by outbreaks of

botulinus poisoning resulting from the use of insufficiently processed canned foods. Studies which these experiences initiated are still in progress.

For a thorough understanding of the problems involved in the present investigations, some familiarity with the observations of other workers upon bacterial spore resistance is essential. Sufficient space, however, is not available here for a detailed review of their reports, and so for the benefit of those who may care to look into the subject further, a condensed summary of their findings is appended at the end of this paper.

It is apparent that a number of influences are operative in determining the resistance of spores. In the reports of other workers it has been pointed out that the hydrogen-ion concentration of the medium in which the spores exist is one factor, and it has been suggested that the age of the spores may have something to do with their degree of resistance. One group of workers has made observations which indicate that possibly the amount of moisture present may be important, and that oil or other non-aqueous liquids may exert a protective influence. Others have observed that the kind of nutrient substances used in the cultivation of certain pathogenic forms apparently affects the resistance of the spores developing in the cultures, and the temperature of incubation has likewise been pointed out as an important factor.

Outside influences, however, have not been responsible for all the variations noted; for differences in resistance have been recorded where identical cultural conditions prevailed, and the opinion has been voiced that variation in resistance is an inherent property of spores which is not affected by conditions of environment.

It is seen, therefore, that the results of investigations by various workers are inconclusive, and further study of the subject is needed.[1] The matter is of immense practical importance as well as of considerable theoretical interest.

[1] Since the manuscript of this paper was completed the attention of the writer has been drawn to an important contribution to studies upon spore resistance by Esty and Williams (Jour. Infect. Dis., vol. 34, no. 5, p. 516–528), published

The problem of spore destruction is a complex one. The high resistance which many spores exhibit calls for rigorous treatment of the medium in which they are present to assure its sterilization. From the standpoint of the food canner this is a serious matter, for the processing required to destroy the troublesome spores often means injury to the appearance and quality of the canned product. Of particular significance, however, from both the practical and theoretical standpoints is the variation in resistance which they show. Not only do the spores of various species of bacteria show differences in their degree of resistance, but different strains of the same species often vary widely in this respect. Of still greater significance is the wide variation in resistance of the individual spores of a single strain. That these facts have an important bearing upon the occasional failure of hitherto successful practices in food canning can hardly be questioned; and until more is known concerning the factors which determine the resistance of bacterial spores there will always be uncertainty as to the outcome of particular canning procedures.

The investigations herein recorded were undertaken for the purpose of getting light upon certain phases of this important problem, and it is hoped that the results secured will not only be of assistance in removing much of the uncertainty that now accompanies food canning, but also help to a better understanding of the nature of bacterial spores.

EXPERIMENTAL PART

In preparation for a study of the factors affecting the variations in heat resistance of bacterial spores several matters had to be taken into consideration:

1. The selection of a suitable organism for the tests.
2. The development of a method that would assure maximum spore formation.

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just as the present investigations were being completed (May, 1924). It is a pleasure to note that although their investigations were headed in a slightly different direction, and different methods were employed in the determination of thermal death points, the results of the two pieces of work, so far as they cover the same ground, are mutually confirmatory.

3. The establishment of a definite point of departure for experiments through study of the spore cycle of the organism under observation.

4. Provision for the preparation and standardization of spore suspensions.

5. The development of a method for testing the thermal resistance of the spores.

THE ORGANISM

The organism selected for these studies was the *Bacillus mycoides*, one of the species of bacteria normally present in garden soil. It is commonly associated with the decomposition of organic matter and has frequently been isolated from canned foods. The strain of this organism used was one of 19 isolated from garden soils of 17 different states and was chosen because of the relatively high thermal resistance of its spores as shown by preliminary tests. This particular strain was isolated from Texas soil.

A number of characteristics of the *Bacillus mycoides* make it an especially favorable organism for studies of this sort: it is readily cultivated in the laboratory by ordinary laboratory technique, and grows rapidly; it forms spores readily and abundantly when proper environmental conditions exist; it has a striking and characteristic type of growth which facilitates the reading of tests and the detection of contamination; and it is non-pathogenic.

The morphological and physiological characteristics of the *Bacillus mycoides* are too well known to require consideration here. It is important to note, however, that an abundant oxygen supply is essential for spore formation, and that the optimum temperature for vegetative growth is 30°C.

MAXIMUM SPORE FORMATION

In the cultivation of *Bacillus mycoides* in the laboratory it is observed that spore formation takes place, most abundantly at least, at the surface of the medium. This being the case, provision for maximum aeration of the cultures in this work appeared necessary. Under laboratory cultivation it is also noted that this organism forms a thick felt-like scum or pellicle upon the surface

of liquid media, and a tough tenacious surface growth upon solid media, such as nutrient agar. So tough is this mass growth that it is impossible, except in very old cultures on solid media, to prepare an even suspension of the organism or its spores by ordinary agitation in liquid. To make thermal death point tests uniform suspensions of the spores are required. It was necessary, therefore, to provide for some means of separating the filaments or chains of cells at any stage in the development of the culture.

As a result of preliminary trials a method was developed which furnished ideal conditions for both the aeration of the cultures and the ready separation of the cells and spores as desired. The method was as follows:

Clean, fine quartz sand, such as is used in greenhouse experiments, was passed through a standard 40-mesh brass sieve, subjected to thorough washing, and then dried. After drying this was measured into the ordinary glass petri dishes, 15 by 100 mm. in dimensions, in 25-gram quantities, distributed to an even depth over the bottom of the plate, and then roasted in the dry air oven until completely sterile. This sand served as the substratum for the culture.

A satisfactory nutrient solution was next sought, and after repeated trials of different substances standard beef extract-peptone broth, made according to the formula recommended in the Manual of Methods for Pure Culture Study of Bacteria (1923) was selected as most suitable for the work.

In preparing these sand cultures a suspension of the spores from an old agar culture was made in a quantity of the sterilized nutrient broth, and brought to a boil in order to destroy all vegetative forms that might be present. With a sterile pipette, using aseptic precautions, just enough of this spore suspension was transferred to the sand plates exactly to saturate the sand, and the cultures thus prepared were placed in the incubator at 30°C.

It is seen that under these conditions the spores were suspended in a highly favorable nutrient medium, the fine particles of sand with their adsorbed layer of air assured maximum aeration, and the temperature of the incubator was the optimum growth temperature for this organism. Hourly examination by microscopic

technique of the grains of sand from these cultures early showed the chains of cells hugging closely the surfaces of the sand particles, and their very rapid development proved that optimum conditions for growth had been provided. Very prompt and abundant spore formation was likewise secured.

THE SPORE CYCLE

It was necessary at this point of the work to determine the length of the spore cycle of the organism under study in order that exact information might be had as to the age of the particular spores being used at any time, and especially that freshly formed spores might be subjected to thermal death point tests, so that a definite starting point for resistance studies might be established.

Accordingly, sand cultures were prepared from a suspension of spores in the manner described, and placed at 30°C. to incubate. At the end of one hour, and at hourly intervals thereafter, suspensions were prepared by shaking a small quantity of these sand cultures in tubes of sterile water. Microscopic examination was then made of these suspensions for evidence of vegetative growth and spore formation.

After preparation of specimens for microscopic examination a portion of the suspension was heated for one minute in a small tube immersed in boiling water and inoculations were made from this into nutrient broth. It was hoped in this way to determine the length of the germination period as well as to check upon the microscopic findings with respect to the length of the spore cycle. The cultural method failed to accomplish its purpose, however, as either the heating was not sufficiently long continued to destroy all vegetative cells or else the germination period of some of the spores equaled or exceeded the length of the spore cycle for other cells. The microscopic tests, however, proved satisfactory as they afforded an opportunity not only for determining the length of the spore cycle but also for observing the various stages passed through by the organism in the formation of spores.

The protocol of one of these experiments is given as follows:

Determination of length of spore cycle of Bacillus mycoides

Material: Sand cultures prepared by inoculating sterile sand with a suspension of the heat spores in nutrient broth.

Temperature of incubation: 30°C.

Date of test: November 26 and 27, 1923.

Microscopic findings:

TIME	INTERVAL	NOTES
	hours	
9:30 a.m.	At start	Spores only
10:30 a.m.	1	Spores only
11:30 a.m.	2	Spores only
12:30 p.m.	3	Spores only
1:30 p.m.	4	Vegetative growth, some spores
2:30 p.m.	5	Vegetative growth, some spores
3:30 p.m.	6	Vegetative growth, some spores
4:30 p.m.	7	Vegetative growth only
5:30 p.m.	8	Vegetative growth only
6:30 p.m.	9	Vegetative growth marked
7:30 p.m.	10	Vegetative growth marked
8:30 p.m.	11	Vegetative growth marked
9:30 p.m.	12	Vegetative growth abundant
10:30 p.m.	13	Vegetative growth abundant
11:30 p.m.	14	Vegetative growth abundant
12:30 a.m.	15	Vegetative growth very abundant
1:30 a.m.	16	Vegetative growth very abundant
2:30 a.m.	17	Cells show slight internal granulation
3:30 a.m.	18	Cells show marked granulation
4:30 a.m.	19	Granulation advanced
5:30 a.m.	20	Uniting of granules (advanced)
6:30 a.m.	21	Completed spores observed
7:30 a.m.	22	Abundant spores
8:30 a.m.	23	Very abundant spores
9:30 a.m.	24	Very abundant spores

It was thought that possibly the number of spores inoculated into the sterile sand in the preparation of cultures might have some influence upon the length of the spore cycle, as the accumulation of metabolic products is known to affect vegetative growth, and presumably might hasten or retard spore formation. Accordingly, other tests were made using cultures that were prepared by inoculating one set of sand plates with a full-strength

suspension of spores and another with the same diluted to one-tenth of its original strength.

The results of these comparative tests showed no perceptible differences in the progress of spore formation, the spore cycle being completed in both cases in twenty-one hours.

The length of the spore cycle among bacteria is not only of great importance, for the purposes of an investigation like the present; information in regard to this point is also necessary for an adequate application of bacteriological methods to practical problems. It has been the practice of bacteriologists for many years to sterilize certain kinds of culture media by what is known as the fractional sterilization method, which consists in heating the substance at 100°C. or less for a definite length of time on each of three successive days. With the development of home food canning the intermittent process of sterilization has come into wide use, particularly in the Southern States, and the experience of home canners, as well as of bacteriologists in the laboratory, has been that at times this method of treatment fails. An explanation for such failure is to be found in the spore cycle study which indicates that often too long an interval has been allowed to intervene between the first and second heat treatments; types of food destroying bacteria which have short spore cycles find time to return to the spore form again, and thus survive the second, and sometimes the third cooking.

PREPARATION AND STANDARDIZATION OF SPORE SUSPENSIONS

The preparation of uniform spore suspensions was made easy by the loose sand substratum. This, while it furnished optimum conditions for vegetative growth and abundant spore formation, made possible the separation of the filaments and cells so that an even and satisfactory suspension could be obtained in a very few moments by merely shaking a small quantity of the sand culture in a tube of sterile water.

In order to eliminate the possible effects of residual nutrient substances and metabolic products of the culture in the thermal death point tests the spores were washed twice with sterile distilled water, and a final suspension made of the washed spores.

This was accomplished as follows: About 20 cc. of sterile distilled water was transferred aseptically to a sterile 20 by 150 mm. culture tube. By means of a small flat-edged metal scoop, freshly flamed, a quantity of the sand culture was introduced into the tube, the cotton plug returned, and the tube agitated until a sufficient number of spores had been freed into the liquid. The resulting even suspension was then transferred by means of a sterile pipette to a sterile centrifuge tube and centrifuged at high speed to throw down the spores. The supernatant liquid was then pipetted off and more sterile water added to the tube in such a way as to bring the spores into suspension again. Centrifuging was repeated and the clear liquid pipetted away as before. This repeated washing in relatively large quantities of sterile distilled water removed from the spores all substances which might affect their thermal death points in the succeeding tests. The washed spores were then suspended again in sterile distilled water and the suspension standardized by its opacity, following the method described by Brown and Kirwan (1915).

METHOD FOR TESTING THERMAL RESISTANCE OF SPORES

For the accurate determination of thermal death points it is essential that the application of heat be instantaneous, or as nearly so as possible, in order that all may receive identical treatment. Failure in this respect may be responsible for conflicting results or may lead to erroneous conclusions. It is also highly desirable, if not absolutely necessary, that all treated spores be given an opportunity for germination, for the culturing of a small portion, such as a loopful or drop, from a treated suspension of spores is likely to give approximate results only. For these reasons it was not considered best to follow the methods of Bigelow and Esty (1919) which have hitherto been considered especially desirable for work of this sort. The tubes with which these workers made their tests were 7 mm. in internal diameter and 250 mm. long, with walls 1 mm. thick. It takes a perceptible time for heat to pass through 1 mm. of glass and still more for an equilibrium of temperature to be reached in a column of liquid 7 mm. in diameter. It was decided, therefore, that thin walled capillary

tubes should be used to contain the spore suspension to be treated, and that these should be introduced entire into the culture tubes for sterility tests.

It was realized that capillary tubes had the disadvantage of holding but small quantities of the suspension, but the fact that the number of spores in the suspension could be made as large as desired seemed to dispose of this objection.

Owing to the fact that the hydrogen-ion concentration of liquids is altered when heated in soft glass tubes, due to the dissolving out of alkalies, as shown by Esty and Cathcart (1921), "Pyrex," a very hard and insoluble glass was chosen for this work. Tubing having an internal diameter of 4 mm. was drawn out to capillaries of 1 to 1.5 mm. in internal diameter, these cut into lengths of 9 to 10 cm. and the ends sealed immediately in the flame to keep the interior sterile until needed for use.

The spore suspension having been prepared and standardized in the manner described, a quantity was transferred with aseptic precautions to a small sterile shell vial held at a convenient angle by inserting the base in a lump of modeling clay.

The capillary tubes were then charged with the spore suspension according to the following technique: Tubes sufficient in number to meet the requirements of the test were placed in a glass stender dish and covered with alcohol. As each was needed it was picked out with freshly flamed forceps, the alcohol burned off to sterilize the outside, and the sealed tips clipped off by means of a special clipping instrument freshly sterilized in the flame. One end of the tube was then dipped into the spore suspension which rose in the tube by capillary attraction. When sufficient liquid had been taken up the tube was removed, the liquid centered in the tube so as to leave a free space at each end, and the tips of the tube sealed immediately in the flame.

As rapidly as they were filled and sealed the tubes were dropped into a vessel containing cold potassium bichromate-sulphuric acid cleaning solution. This was done to kill any spores adhering to the outside of the tube which had not been destroyed at the time the tips were sealed. They were allowed to remain in this solution until all the tubes were charged. The cleaning solution

was then drained away and the tubes washed in clean cold water. When washed the tubes were transferred to a dish of fresh alcohol kept cold by placing on crushed ice. This precaution was taken to prevent any tendency of the spores to germinate, which might have affected their resistance.

The tests were made in series of fives, that is, five tubes were used in making a resistance test at each time interval.

The source of heat was an electrically heated and controlled constant temperature oil bath, fitted with a motor driven stirring device, which assured a uniform temperature throughout the bath. "Wesson" oil was the heating medium used.

To make the exposure, five of the suspension-charged tubes were removed from the cold alcohol with sterile forceps and transferred to a small aluminum holder, and the receptacle immersed in the hot oil. Both the immersion and the removal were performed as rapidly as possible and the length of exposure timed by the watch.

At the conclusion of the heat exposure the tubes were immediately transferred to a 4-ounce salt-mouth glass stoppered bottle containing fresh acetone, which dissolved off the oil. By means of sterile forceps they were then removed to fresh alcohol to keep them sterile until inoculations into nutrient media could be made. This, in most instances, was done at once, and in no case did the time exceed more than two or three minutes.

The sterility tests were made by flaming the plug and mouth of the culture tube in the usual way and then introducing the capillary tube containing the spore suspension. In preparing the capillary tubes for this inoculation they were withdrawn from the alcohol with sterile forceps and, without flaming, one sealed tip was removed with the freshly flamed clipper. The open end was then inverted over the mouth of the culture tube from which the cotton plug had been removed, and the upper tip of the capillary snipped off. At the same instant the capillary tube was released and dropped into the nutrient broth of the culture tube. The plug was then replaced, and after making certain that the contents of the capillary had been forced up into the medium by the bubble of air formed when the tube touched the medium, the culture was ready for incubation.

These operations, which may perhaps appear complicated from the description, were in reality very simple, and rapidly performed. In all cases the spores exposed to the heat treatment were placed under optimum conditions for germination within five minutes of the time the tubes were withdrawn from the oil bath.

The determination of the thermal death point consisted simply in the incubation of these cultures at 30°C., noting the point at which germination ceased, as indicated by the last positive and the first negative growths in the culture tubes.

It might seem that with so many operations entering into the tests the danger of contamination would be very great and the interpretation of results, therefore, difficult. As a matter of fact, contamination did not occur in 1 per cent of the tests and the results were clear-cut and definite. As was earlier pointed out, contamination when present was easy to detect because of the very unusual and characteristic growth of *Bacillus mycoides* in broth cultures made in this way.

This technique has the merit of assuring uniform and instantaneous exposure of all spores to the heat, and of giving to each the opportunity to germinate.

STUDY OF FACTORS AFFECTING VARIATION IN RESISTANCE OF SPORES

The relation of the hydrogen-ion concentration of the medium to the destruction of bacterial spores has already been given consideration at the hands of Buchanan and his collaborators (1918), Esty and Cathcart (1921), and Bigelow and Cathcart (1921), and it was decided, therefore to eliminate this subject from the present study.

Because so many as yet unknown elements enter into the matter of the relation of food supply, protective colloids, metabolic products, etc., to the resistance of spores it seemed desirable, in so far as possible, to eliminate these factors also from the present investigation.

The influences that might possibly affect the resistance of spores which it was decided to investigate at this time were those of the age of the spores and the environmental factors of temperature

and humidity up to the time of the tests. The plan pursued was as follows:

Sand substratum spore cultures were prepared as previously described, with the modification that sterile clay tops were substituted for the glass covers of the petri dishes. This was done to facilitate the regulation of the humidity of the cultures within the storage chambers. These cultures were incubated at 30°C. for two days to allow for maximum spore formation, as indicated by the spore cycle tests.

At the end of this period of incubation a thermal resistance test upon the young spores, twenty-four to thirty hours old, was performed to establish a point of departure in the determination of thermal resistance variations. The remaining cultures were grouped into series for storage under environmental conditions as follows:

Temperatures—ice-box (approximately 10°), 20° and 30°C. (optimum growth temperature)

Humidities—over a dehydrating agent (CaO), over carefully adjusted sulphuric acid solution (50 per cent humidity), and in water-saturated atmosphere (100 per cent humidity)

The age factor was to be studied by resistance tests made at intervals of thirty days upon each of these series of cultures.

As arranged, series of sand cultures were stored at each of the three temperatures mentioned, in humidity chambers whose atmospheres had moisture contents of approximately zero per cent, 50 per cent, and 100 per cent respectively. Resistance tests performed at thirty-day intervals upon cultures stored under these sets of conditions made possible a study of the influence of age under nine distinct situations, and corresponding studies upon the factors of temperature and humidity.

The results of these tests are presented in tables 1 to 9.

DISCUSSION OF TABLES

Age factor

That age is a factor in determining the resistance of the spores of *Bacillus mycoides* to heat is abundantly shown by the foregoing

results of thermal death time tests. In no case did the resistance of the spores remain constant throughout the period of the tests, or even approximately so. The nearest approach to constancy was observed in the results shown in table 1, but even here perceptible change occurred.

In some instances the degree of resistance increased rapidly during the early days of the test period and then dropped again;

TABLE 1

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held in a desiccator over calcium oxide in an icebox at a temperature of approximately 10°C., for from thirty to one hundred and eighty days. Exposures in these and succeeding tests made in oil bath maintained at 100°C. Suspension in these and succeeding tests contained 150 million spores per cubic centimeter

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	+++++	No test	+++++	+++++	+++++
1	+++−−	+++−−	+++++	+++++	+++++	+++++	+++++
1½	+−−−−	−−−−−	++++0	No test	+++++	++−−0	++−−−
2	−−−−−	−−−−−	++−−−	++−−−	−−−−−	+−−−−	+−−−−
3	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	+−−−−	−−−−−
4	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

Note: Signs used apply similarly to all succeeding tables.

+ indicates positive growth in culture tube following exposure to heat of the spores under test.

− indicates no growth in culture tube.

0 indicates that the test was lost by accident.

It is seen that in a dry, cold environment there was but slight change in resistance of the spores during the entire test period, though there took place a slight increase after the first thirty days of storage.

later to be followed by another increase. The degree of resistance attained in a number of cases was two or three times as great as that of the young spores.

With several series the resistance curve rose abruptly during the first thirty days and then became horizontal, or practically so. Several showed a gradual increase in the resistance of the spores for as long as sixty days.

Temperature factor

Glancing back over the tables it will be noted that in every case, regardless of humidity, and almost without regard to the age of the spores, the greatest resistance was developed at the temperature of 20°C., indicating that a temperature somewhat

TABLE 2

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held in a dessicator over calcium oxide at a temperature of 20°C., for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	No test	+++++	+++++	+++++	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
3	−−−−−	+++++	+++++	++++−	++++−	+++++	+++++
4	−−−−−	+++−−	++++−	−−−−−	−−−−−	++−−−	++++0
5	−−−−−	+−−−−	++++−	−−−−−	−−−−−	+−−−−	+++−0
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	+−−−−
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
8	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

When held in a very dry atmosphere and at a moderate temperature marked changes occurred in the thermal resistance of the spores. Within the first thirty days of storage the thermal death time rose from two to six minutes, indicating a threefold increase in resistance over that of the original spores. This degree of resistance was maintained for another thirty days, and then showed a decided decline. At one hundred fifty days the resistance began to increase again, and at one hundred and eighty days was highest during the period of the tests. The significance of this fluctuation is not known.

below the optimum for vegetative growth, and in most cases well above the minimum, seems to be most favorable for change in spore resistance, at least in the case of *Bacillus mycoides*.

Humidity factor

To evaluate the direct influence of humidity upon change in spore resistance is extremely difficult if not impossible. In com-

TABLE 3

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held in a dessicator over calcium oxide at a temperature of 30°C., for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	++++0	+++++
1½	+−−−−	+++++	No test	No test	No test	+++++	++−−−
2	−−−−−	+++++	+++++	+++++	+++++	++++−	−−−−−
3	−−−−−	−−−−−	+−−−−	−−−−−	++−−−	−−−−−	−−−−−
4	−−−−−	−−−−−	−−−−−	−−−−−	+−−−0	−−−−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

In a dry but warm atmosphere (temperature optimum for vegetative growth) it is seen that a decided increase in resistance to heat developed in the spores during the first thirty days. This increase, while not as great as in those held at 20°C., was never-the-less marked, and was maintained for five months without marked alterations. At the one hundred and eighty-day test the resistance had fallen back to its original position, or essentially so.

TABLE 4

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at icebox temperature (approximately 10°C.) in an atmosphere having a 50 per cent humidity, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	+++++	++++N
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+−−−−	+++++	+++++	+++++	+++++
3	−−−−−	++++−	−−−−−	+++++	+++−−	+++++	+++++
4	−−−−−	−−−−−	−−−−−	−−−−−	++−−−	+++−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	++−−−	−−−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

In a cold atmosphere, half saturated with moisture the spores showed a prompt and marked increase in resistance. In thirty days the degree of resistance had doubled. At the sixty-day period there was an apparent decline, but the results of the succeeding tests indicate that some uncontrolled factor influenced the results of the sixty-day tests. The highest resistance was reached at the time of the one hundred and fifty-day tests.

bination with other factors, such as temperature, it is observed to be very important, as may be seen in the case of the series of spore cultures held at the temperature of the ice-box. It is apparent that the lack of moisture had a direct restraining influence upon the change in resistance of spores held over calcium oxide in the chamber at ice-box temperature as shown in table 1. Here it will be noted that no increase occurred during the first thirty

TABLE 5

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 20°C. in an atmosphere having a humidity of 50 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	++++0
1	+++−−	+++++	No test	+++++	++++0	++++0	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
3	−−−−−	+++++	++++0	+++++	+++++	+++++	+++++
4	−−−−−	++0 0 0	+++−−	+−−−−	++++−	−−−−−	++++−
5	−−−−−	++−−−	−−−−−	−−−−−	−−−−−	−−−−−	++−−−
6	−−−−−	+−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
7	−−−−−	++−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
8	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
9	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
10	−−−−−	−−−−−	−−−−−	−−−−	−−−−−	−−−−−	−−−−−

Under conditions of medium temperature and humidity the spores showed marked increase in resistance. At thirty days evidences of an especially high resistance are noted in the case of some spores. The high degree of resistance attained for the majority of the spores was retained throughout the period of the tests.

days, although spores held at ice-box temperature but in atmospheres having humidity values of 50 and 100 per cent respectively showed a very marked increase in resistance during this period.

On the other hand, high humidity combined with low temperature resulted in as great changes as those which developed under either high humidity-optimum growth temperature or low humidity-optimum growth temperature conditions.

TABLE 6

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 30°C. in an atmosphere having an humidity of 50 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	++++0
1	+++−−	+++++	+++++	+++++	+++++	+++++	++++0
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++−−
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+−−−−
3	−−−−−	++++0	+++++	+++++	+−−−−	++++−	−−−−−
4	−−−−−	++−−−	+++−−	+−−−−	−−−−−	++++−	++−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

In a warm, moderately humid environment the spores, as shown by these results, developed a pronounced increase in resistance within the first thirty days. Some irregularity in the shape of the thermal death time curve is noted, and at the time of the one hundred and eighty-day tests the resistance seems to have decreased considerably.

TABLE 7

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at the temperature of the icebox (approximately 10°C.) in an atmosphere having a humidity of 100 per cent, for from thirty to one hundred and fifty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	No test	+++++	
1	+++−−	+++++	+++++	+++++	+++++	+++++	No ma-
1½	+−−−−	++++0	No test	No test	+++0 0	+++++	terial
2	−−−−−	++++−	+++++	+++++	+++++	+++++	for 180-
3	−−−−−	+++−−	+++++	++++−	++−−−	+++++	day
4	−−−−−	+−−−−	++−−−	−−−−−	−−−−−	++−−−	tests
5	−−−−−	−−−−−	+−−−−	−−−−−	−−−−−	−−−−−	
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	

In a cold, moisture-saturated atmosphere the resistance of these spores continued to increase steadily for sixty days. After six months of storage the resistance was still more than twice that of spores twenty-four to thirty hours old.

TABLE 8

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 30°C. in an atmosphere having an humidity of 100 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	++++0	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	+++++	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
3	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
4	−−−−−	++++−	++++0	+++−0	++++−	++++−	+++++
5	−−−−−	−−−−−	++++−	−−−−−	+++++	+−−−0	++−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

When held in a moderately warm, moisture-saturated environment the resistance of the spores increased in the same fashion as shown in table 7, but to a slightly greater degree, and the high degree of resistance attained was still retained at the time of the one hundred and eighty-day tests.

TABLE 9

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 30°C. in an atmosphere having an humidity of 100 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	++++0	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++−−
2	−−−−−	+++++	+++++	+−−−−	+++++	−−−−−	+−−−−
3	−−−−−	++++−	++−−−	−−−−−	+++++	−−−−−	−−−−−
4	−−−−−	−−−−−	−−−−−	−−−−−	++++−	−−−−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

With maximum moisture, and optimum growth temperature supplied the spores of this series showed considerable variation in their resistance. The thermal death time curve was quite irregular.

GENERAL DISCUSSION

Having now observed the alteration in thermal resistance of bacterial spores under different conditions as regards age, temperature and humidity, we come to ask ourselves the reasons for these changes.

For many years bacteriologists have held the opinion that spore formation among bacteria represents the response of the organism to an unfavorable environment, and that the spore once formed remains dormant or inactive until a return of conditions favorable for vegetative growth.

That abundant spore formation takes place at temperatures best adapted for vegetative growth, among some species of bacteria at least, has been often observed and has been shown again in the present work with *Bacillus mycoides*. Unfavorable temperature conditions, therefore, do not determine spore formation. Insufficient food supply cannot be the controlling factor because spore formation and vegetative growth proceed side by side. Our own experiments, earlier mentioned, indicate also that the concentration of metabolic products cannot be the determining influence, for spore formation took place as quickly in a dilute suspension of organisms as in one containing ten times as many. Absence of moisture is not responsible for the formation of spores, for every bacteriologist knows that spores form in liquid as well as on solid media.

If, then, neither food supply, moisture, temperature, nor metabolic products which are the factors commonly supposed to affect bacterial activities, control spore formation, may we not look upon this phenomenon of spore formation as one of the normal phases in the ordinary existence of at least certain of the bacteria, which proves of vital importance to the species in that it enables some individuals to survive adverse conditions? May we not look upon the spore, from the standpoint of the bacteria, as a most fortunate provision against adversity rather than as a product of it?

This idea has been furthered by the analysis of the present experimental findings which prove that spores rarely, if ever, are dormant. Increase in resistance under a wide range of conditions

has been observed. This change has been found to take place most rapidly under conditions apparently not the most likely to injure the vitality of the cell but rather under what might be termed "temperate" conditions—conditions suited to positive but rather sluggish vital activities normal to the spore form.

Plant physiologists and plant chemists have come to know that the seed which used to be considered dormant is in reality undergoing fundamental internal alterations even though the change cannot be detected from its external appearance. In like manner also, tubers and other storage organs of plants which during the winter period have been supposed to be in a dormant condition have been found to have undergone great changes internally.

We are coming, therefore, to a realization that strict dormancy among living forms rarely, if ever, exists and that what has been interpreted as such is merely a retardation in the rate of vital activities.

In the light of evidence at hand it seems reasonable to conclude that variations in resistance of bacterial spores under differing environmental conditions are the results of normal spore transformations which are retarded by some and possibly accelerated by other conditions of environment.

The results of the present investigation are of general scientific interest and of practical significance.

That virulence among pathogenic bacteria may be altered in either direction by modifications in cultural practices has long been known. That virus once formed may be weakened by certain storage treatments, as is done in the preparation of the attenuated virus of rabies for the Pasteur treatment, has likewise long been known and put to practical use in the practice of human and of veterinary medicine. It has been learned also that under certain conditions of cultivation a spore-forming organism may be prevented from forming spores normally, or if doing so, may be made to form spores possessing different degrees of resistance.

The assumption has been that once a spore has been formed its resistance is a fixed property. That this idea must be abandoned seems evident, for what has hitherto been looked upon as a constant has been shown to be a variable.

The results of the present study are of practical significance in two fields: First, that which is concerned with disinfection and disinfectants; and the second, that which involves food preservation.

A few only of the disease-producing bacteria are of spore forming types, but those which possess this property are extremely dangerous. It is of importance, therefore, that all methods of disinfection and all disinfectants be selected and applied in such a way as to take into account not only the variations in the resistance of different strains of these organisms but also the changes in resistance almost certainly possible in all spores.

Of particular importance is this matter as it applies to the testing and standardization of chemical disinfectants. Germicidal values are determined, in many instances at least, by the ability of the substance in different dilutions to destroy the spores of *Bacillus anthracis* or other pathogenic spore-forming bacteria. It is absolutely essential for safety that the resistance of the spores used in these tests be as uniform as possible, and the alterations in resistance occurring under storage as well as under cultural conditions must be taken into account.

The significance of these findings as they concern food preservation lies in the importance which thermal death points of bacterial spores have in determining the temperatures and time periods of the processing, or cooking of the food in the can.

The canning industry is rapidly getting away from rule-of-thumb practices and more and more is basing its operations upon the findings of research laboratory workers. The rate of heat penetration into the cans of food during the processing has been extensively studied; the relations of the character of the food and the character of the pack to ease of sterilization have been investigated; bacteria causing food spoilage have been isolated and subjected to careful observation; and the thermal death points of numerous highly resistant spore-forming types have been determined. Upon the basis of these findings new processing schedules have been developed and the results of careful laboratory experiments are being applied in the industry.

This has been done under the assumption that the thermal

death points observed for particular organisms represented fixed values. From the present findings we are led to conclude that these values are not constant, and unless a considerable margin of safety is provided for in the standardization of food processing schedules the new practices, at times, may be expected to fail. In determining the thermal death points that are to serve as the basis of new processing schedules bacteriologists must be as certain as possible that the resistance shown by the test spores represents the highest degree attainable by them.

SUMMARY

The object of the present investigation was to throw light upon the important subject of thermal resistance of bacterial spores, with special reference to changes in resistance which have been observed to occur, and which it is thought may have an important bearing upon the problem of food preservation.

The organism selected for the study was the *Bacillus mycoides* which, because of its cultural and physiological characteristics, is particularly well adapted to the work.

The experiments were confined primarily to a determination of the influence of age and of the environmental factors of temperature and humidity upon variations in thermal resistance of spores, and data were obtained at thirty-day intervals upon spores stored under nine different sets of environmental conditions. A study of the spore cycle of the organism under test; the development of special cultural methods in order to provide for maximum spore formation and the preparation of satisfactory spore suspensions; and the development of a more satisfactory technique for making the resistance tests, were necessary parts of the investigation.

The results of the tests may be summarized briefly as follows:

1. In a cold, dry environment there was practically no change in the resistance of the spores during the first thirty days of storage, but a small increase was manifest by the time of the sixty-day tests which was maintained for the remainder of the test period.

2. When held in a very dry atmosphere and at a moderate temperature marked changes occurred. Within the first thirty days of storage the thermal death time rose from two to six minutes, indicating a threefold increase in resistance over that of the original spores. This degree of resistance was maintained for another thirty days, and then showed a decided decline. At one hundred and fifty days the resistance began to increase again, and at one hundred and eighty days was at a maximum point. The significance of this fluctuation is not known.

3. In a dry but warm atmosphere (temperature optimum for vegetative growth) a decided increase in resistance to heat developed in the spores during the first thirty days. This increase, while not as great as in those held at 20°C., was marked, and was maintained for five months without material alterations. At the time of the one hundred and eighty-day tests the resistance had fallen back to nearly its original position.

4. In a cold atmosphere, half saturated with moisture the spores developed a prompt and marked increase in resistance, the degree of resistance doubling within the first thirty days. The highest point in this series was reached at the time of the one hundred and fifty-day tests.

5. A pronounced increase in resistance resulted when the spores were held under conditions of medium temperature and humidity. An especially high resistance was shown by some of the spores at the time of the thirty-day tests and a considerably increased resistance for the majority of the spores was not only attained but also retained throughout the period of the tests.

6. In a warm, moderately humid environment the spores developed a marked increase in resistance within the first thirty days. The death time curve for the entire period of the tests, however, was somewhat irregular.

7. In a cold, moisture-saturated atmosphere the resistance of the spores continued to increase steadily for sixty days, and after six months of storage was still more than twice that of spores twenty-four to thirty hours old.

8. When held in a moderately warm, moisture-saturated environment the resistance of the spores increased in the same

fashion as the preceding but was slightly less pronounced. The high degree attained, however, was still retained at the time of the one hundred and eighty-day tests.

9. With maximum moisture and optimum growth temperature supplied considerable variation in resistance was noted. The thermal death time curve was quite irregular.

CONCLUSIONS

A careful analysis of the experimental data presented leads to the following conclusions:

1. The bacterial spore is not dormant under ordinary conditions, as has commonly been supposed, but is instead sluggishly active.

2. The resistance of spores to heat is not a fixed property but a variable one, the degree of resistance being influenced by age, the temperature and humidity of the environment, and possibly other factors.

3. The highest resistance to heat develops under conditions of moderate temperature and humidity, and is probably reached by the time the spores are sixty days old. Spores of different species of bacteria may be expected to vary somewhat in this respect.

4. Change in resistance takes place most slowly when spores are dry and cold, but low temperature accompanied by high humidity results in the development of a high degree of resistance.

5. In determining the thermal death points of spores that are to serve as the basis of processing schedules for canned foods the bacteriologist must take into account the change in resistance of spores under various conditions, and be as certain as possible that the resistance shown by the test spores represents the highest degree attainable by them.

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APPENDIX

The following is a condensed résumé of the literature bearing upon the subject of bacterial spore resistance:

1888 Globig (1888) found that spores of the "potato bacillus" withstood exposure in streaming steam at 100°C. for 5 to 6½ hours; and in superheated steam at 113 to 116°C. for 25 minutes; at 112 to 123°C. for 10 minutes; at 126°C. for 3 minutes; and at 127°C. for 2 minutes. Death instantaneous at 130°C.

1889 Esmarch (1889) found that resistance of spores of *B. anthracis* was affected by nutrient medium used.

Geppert (1889) reported variation in resistance of spores of *B. anthracis* even when grown under identical cultural conditions.

Fraenkel (1889) confirmed the findings of Geppert (1889) and concluded that variation in resistance is an inherent property of different strains and not affected by conditions of environment.

1895 Miquel and Zattraye (1895) found that moist spores of *B. subtilis* in cultures 3 to 4 weeks old survived exposure for 15 minutes at 104.4 to 108.3°C.; 30 minutes at 102.8 to 106.3°C.; 1 hour at 102.9 to 104.7°C.; and 4 hours at 99.7 to 100.1°C.

Christen (1895) described organisms whose spores resisted 100°C. for periods of 4 to 16 hours; 105°C. for 1 to 4 hours; 110°C. for 1 to 2 hours; 115 to 116°C. for ½ to 2 hours; and 130°C. for 5 minutes.

1896 Cambier (1896) found that spores of soil organisms exposed to dry heat survived after 3½ hours treatment at 110.7°C., but were killed in 1½ hours at 124°C.; and in 15 minutes at 138°C. In air-dry soil the spore survived 111°C. for 4½ hours; 136.9°C. for 3 hours; 156.5°C. for 2 hours; and 180°C. for 35 minutes. All were destroyed in 5 minutes at 200°C.

1897 Kronig and Paul (1897) confirmed the findings of Geppert (1889).

Van Ermengem (1897) reported that the spores of *B. botulinus* were only slightly resistant to heat, being destroyed in 1 hour at 80°C.

1899 Weil (1899) confirmed the work of Esmarch (1889) and noted that temperature of incubation appeared to affect resistance of spores.

1902 von Wahl (1902) found 2 hours insufficient time to sterilize carrots, asparagus, and peas packed in glass containers when exposed to streaming steam at 100°C. Spores of an organism in carrots survived 3½ hours processing in water at 100°C.

1904 Neide (1904) described 3 organisms the thermal death points of whose spores varied from 15 to 38 minutes at 100°C.

1905 Blau (1905) reported the thermal death times for spores of various bacteria exposed to temperature of boiling water as follows: *B. subtilis*, 175–180 minutes; *B. robustus*, 450 to 480 minutes; *B. calidus*, 450 to 480 minutes; *B. cylindricus*, 1140 to 1200 minutes; and *B. tostus*, 1140 to 1200 minutes.

1908 von Hibler (1908) secured constancy in the resistance of spores of *B. anthracis* by using cultures that were "neither too old nor too young." Acidity and long storage in incubator found to affect resistance of spores.

1915 Shanly (1915) studied heat resistance of spores of 23 different species. The least resistant, *B. cereus*, survived exposure to 75°C. for 1 hour but was

killed was 80°C. for same period. Others, identified only by numbers, survived after 1 hour at 100°C. Variation in resistance of different strains of same species noted.

1918 Buchanan, Thompson, Orr and Bruett (1918) gave particular attention to effect of preliminary canning operations upon thermal death points. They also determined the relation of the hydrogen-ion concentration of the product to heat resistance of bacteria and their spores.

1919 Bruett (1919) found that scalding followed by chilling is not an effective means of spore destruction.

Burke (1919) reported that while exposure to a temperature of 100°C. may not kill spores of *B. botulinus* their vitality is weakened so that germination is delayed. Spores of this organism survived 3½ hours boiling at 100°C., and 5 hours boiling was considered insufficient to sterilize. Fractional sterilization, because of delayed germination, was held of doubtful value.

Normington (1919) in studies of organisms isolated from cold-packed canned peas found that all withstood 10 to 15 pounds steam pressure in the autoclave for 10 to 20 minutes.

Bigelow and Esty (1919) developed improved method for determining spore resistance. Spores of one strain destroyed only after 16 hours boiling at 100°C.; 100 minutes at 110°C.; 50 minutes at 115°C.; 10 minutes at 120°C.; and 4 minutes at 125°C. Initial number of spores and H-ion concentration of medium were found to affect the time required to sterilize.

Esty and Williams (1919) reported resistance tests upon organisms isolated from canned foods. Considerable variation was noted in resistance, ranging from 1½ to 17 hours at 100°C. True thermophiles were found most resistant.

Thom, Edmondson and Giltner (1919) found spores of *B. botulinus* from canned asparagus survived steaming at 116°C. for 15 minutes, and 100°C. for 1 hour.

Dickson, Burke and Ward (1919) reported upon 8 strains of *B. botulinus*. All survived 3 hours heating at 90°C.; 7 of them 3 hours at 95°C.; and 6 of them 2 hours at 100°C. Addition of 5 per cent of lemon juice to meduim did not prevent growth or formation of toxin but lowered the death point of spores. Spores treated in presence of animal and vegetable protein.

1920 Bigelow and Esty (1920) studied relationship of temperature and time to spore destruction in thermophiles from spoiled canned foods. Destruction of spores in one culture as follows: 1320 minutes in boiling water at 100°C.; 690 minutes at 105°; 225 minutes at 110°; 84 minutes at 115°; 23 minutes at 120°; 8 minutes at 125°; 3.5 minutes at 130°; 1.5 minutes at 135°; and 1 minute at 140°C. Nineteen thermophiles studied showed same time-temperature relationships.

Fenger, Cram and Rudnick (1920) found thermal death points of five organisms isolated from ligatures and sutures, when heated in non-aqueous liquids, to lie between 150 and 160°C. for 1 hour.

Donk (1920) found that spores of a thermophile isolated from canned corn required 17 hours to kill when heated in corn broth at 100°C. with 12,500

spores per 1 cc.; and 11 minutes when heated at 120°C. with 50,000 spores per 1 cc.

1921 Weiss (1921) found that free spores of *B. botulinus* were killed within five hours at 100°C.; 40 minutes at 105°C.; and 6 minutes at 120°C. Young moist spores, more resistant than old, and spores 1 month old found three times as resistant as those 5 months old. More resistant individuals changed more rapidly than less resistant ones. Thermal resistance of emulsions of young spores increased as concentration of emulsion increased. Sodium chloride lowered thermal resistance. Increase in H-ion concentration on acid side of neutrality, and of hydroxyl-ions on alkaline side lowered resistance of spores. Work on spores of *B. botulinus*. In another paper same author (1921) showed relation of H-ion concentration, consistency of food material, concentration of sirup present, etc., to thermal death point of *B. botulinus;* acid foods requiring less severe treatment to sterilize than non-acid substances.

Bigelow (1921) gave the thermal death times of 15 typical thermophiles when exposed to different temperatures as follows: At 100°C., between 788 and 834 minutes; at 105°, between 383 and 405 minutes; at 110°, between 117 and 122 minutes; at 115°, between 40 and 44 minutes; at 120°, between 11 and 12 minutes; at 125°, between 3.9 and 4.6 minutes; at 130°, between 1.7 and 2.2 minutes; at 135°, between 0.7 and 0.9 minutes; and at 140°C., between 0.6 and 0.9 minutes.

1922 Dickson and his associates (1922) noted marked variations in resistance of spores of *B. botulinus*. Tests on 40 strains made at 100°C. showed survival period varying from thirty minutes to six hours. About 95 per cent spores were destroyed in short time, some of remainder highly resistant. Spores from old cultures more resistant than those from young, and resistance greater in neutral media than in acid of alkaline media. Noted delay in germination, in one case for 330 days.

Esty and Meyer (1922) found spores of *B. botulinus* more resistant than practically any other anaerobe, requiring 4 minutes at 120°C. or 330 minutes at 100°C. to kill. Spores in juices of 17 different kinds of canned foods showed variation in resistance of from less than 10 minutes to 230 minutes at 100°C.

Tanner and Dack (1922) studied resistance of spores of *B. botulinus* to dry heat. Spores survived 110°C. after 2 hours; 140°C. from 15 to 60 minutes; and 160 to 180°C. from 5 to 15 minutes.

1923 Esty (1923) found resistance of 112 strains of *B. botulinus* to vary from 3 to 75 minutes at 105°C. Maximum resistance given as 330 minutes at 100°C.; 110 minutes at 105°C.; 33 minutes at 110°C.; 11 minutes at 115°C. and 4 minutes at 120°C. Greater resistance shown in heavy suspensions than in light.

Wyant and Tweed (1923) found that for 8 organisms isolated from "flat-sour" canned peas sterilization was accomplished at above 80°C. and below 110°C. after 10 minutes heating.

Tanner and McCrea (1923) reported resistance of spores of *B. botulinus* sealed in tubes exhausted to 17 mm. Spores destroyed within 5 hours at 100°C.; 2 hours at 105°C.; 1½ hours at 110°C.; 40 minutes at 115°C.

and 10 minutes at 120°C. Longer time required to destroy spores of same age in open tubes than in tubes exhausted and sealed.

1924 Esty (1924) made inoculation experiments upon peas and corn using suspensions containing different numbers of spores. Showed that spoilage in canned product was materially increased when number of spores was increased, due to especially resistant individual spores in suspensions.