5-17-1951

The Development of the Scholander Micro-Respirometer for Use in the Study of Metabolism in Bacterical Endospores

Phillip G. Crook

CROOK — THE SCHOLANDER MICRO-RESPIROMETER

THE DEVELOPMENT OF THE SCHOLANDER MICRO-RESPIROMETER FOR USE IN THE STUDY OF METABOLISM IN BACTERIAL ENDOSPORES

by

Philip G. Crook

A Thesis

in Partial Fulfillment of the

Requirements for the Degree of

Master of Science in Biology

The University of New Mexico
1951

This thesis, directed and approved by the candidate's committee, has been accepted by the Graduate Committee of the University of New Mexico in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

E. L. Castetter

DEAN

5/17/51

DATE

Thesis committee

L. B. Johnson

CHAIRMAN

Douglas B. Hamm

Kirk W. Clark

Acknowledgements

I would like to express my gratitude to Drs. R. B. Johnson and D. G. Humm for their helpful advice and suggestions during the course of this investigation.

TABLE OF CONTENTS

Introduction 1

Materials and Methods 4

Experimental Results 11

Discussion 19

Summary 24

Literature Cited 25

Figures

Number		Page
1.	Original Scholander Micro-respirometer.....	7
2.	Modified Device.....	7
3.	Anthrax heated to 65° C. for 30 and 90 minutes.	12
4.	Anthrax heated to 80° C. for 30 minutes after pasteurization.....	14
5.	Anthrax that has not been heated.....	16
6.	B. subtilis heated to 65° C. for 30 minutes....	18

Tables

Number		Page
I.	The effect of 0.5% Glucose in phosphate buffer on the respiration of B. subtilis.....	18

Introduction

It has generally been assumed that bacterial endospores represent a dormant stage exhibiting little, if any, metabolic activity and possessing no functional enzymes. Dubos (1947) states as follows:

> Although both the spores and vegetative cells of B. mycoides give the same analysis for ash, moisture, and protein, the spores lack enzymic activity.

He trys to account for this by hypothesizing a "tying up" of reactive groupings. In the same chapter he says:

> Finally, the fact that enzymes and other biological systems exist in an inactive and resistant form in these structures [spores], and become again active and susceptible during the obscure phenomona of spore germination, presents to the physiologist and biochemist problems of great originality and importance.

These are characteristic comments to be found in most current texts. In a search of the literature, remarkably few instances have been encountered in which any attempt has actually been made to determine the presence and/or extent of metabolism in a resting spore.

Ruehl (1923) placed spores in a variety of media and, after incubation, tested the substrate for change. He claims to have demonstrated gelatinase activity in some instances, but the lack of adequate controls (which consisted only of a final stain to determine the possible presence of vegetative forms) casts some doubt as to the

validity of these results.

Tarr (1933), following the lead of Cook (1932), was able to definitely demonstrate an active glucose dehydrogenase in the spores of several members of the Family Bacillaceae. He also carried out manometric determinations using a Barcroft differential apparatus and measured the aerobic metabolism of glucose by spores and corresponding vegetative cells. This system is most active at 40-50° C., with a pH optimum of 6.8. It was sensitive to 5% ethylurethane, saturated phenylurethane, and M/500 KCN. Hydrogen sulfide inhibited glucose utilization in vegetative cells but stimulated respiration in one strain of B. subtilis spores, but without effect on three others tested.

Keilin and Hartree (1947) attempted to confirm Tarr's work with respect to oxygen uptake in the presence of glucose, but failed to obtain as high results. The respiratory activity of spores was found by Cook to be 90% that of vegetative cells, while Tarr obtained a value of 35% to 40%, and Keilin and Hartree found the rate to be only 6%.

Both Tarr and Keilin and Hartree heat shocked spore suspensions to 80° C. for 30 minutes immediately prior to carrying out manometric experiments. This was purported to shorten the time required to obtain straight line

respiration curves.

In this paper, the Scholander micro-respirometer has been adapted, and techniques worked out, to permit its use in attacking the problems associated with endospore respiration.

Materials and Methods

The organisms used in these experiments were B. subtilis, strain P.C.I. 220, obtained from the Federal Security Agency, and a strain of B. cereus var anthraces, avirulent to guinea pigs, obtained from the Army Medical Department Research and Graduate School culture collection. The first was chosen because it is a standard strain and is the same species that has predominantly been used in previous work. The anthrax bacillus was chosen because of the characteristic autolytic propensities of the sporangium in this species. This increases the ease with which one can obtain spore suspensions free from cellular debris and potentially assimilable nitrogen sources which would permit germination.

The organisms were grown on a nutrient agar containing 0.25% peptone, 0.15% beef extract, and 2% agar in distilled water. In practice, half quantities of commercial dehydrated Bact-Nutrient Agar were suspended in water with sufficient additional agar-agar to give 2% concentration of that ingredient. The reaction of the medium was adjusted with NaOH to give a final pH of 7.2.

Fifty cubic centimeter portions of the above medium were dispensed in 16 oz. prescription bottles and autoclaved 15 minutes at 121° C. Following sterilization,

the bottles were put on their flat sides and the medium allowed to solidify. This gave a surface area of about 112 cm^2. for growth of the organisms. The prepared bottles were placed in a 37° C. incubator for 36 hrs. to check for sterility and to allow them to dry.

Stock cultures of the organisms were kept in screw cap vials of nutrient agar. These slants were not placed under oil but instead were incubated until sporulation occurred and then held at about 5° C. Inoculation of the growing bottles was accomplished by streaking the entire surface of the medium with a loop.

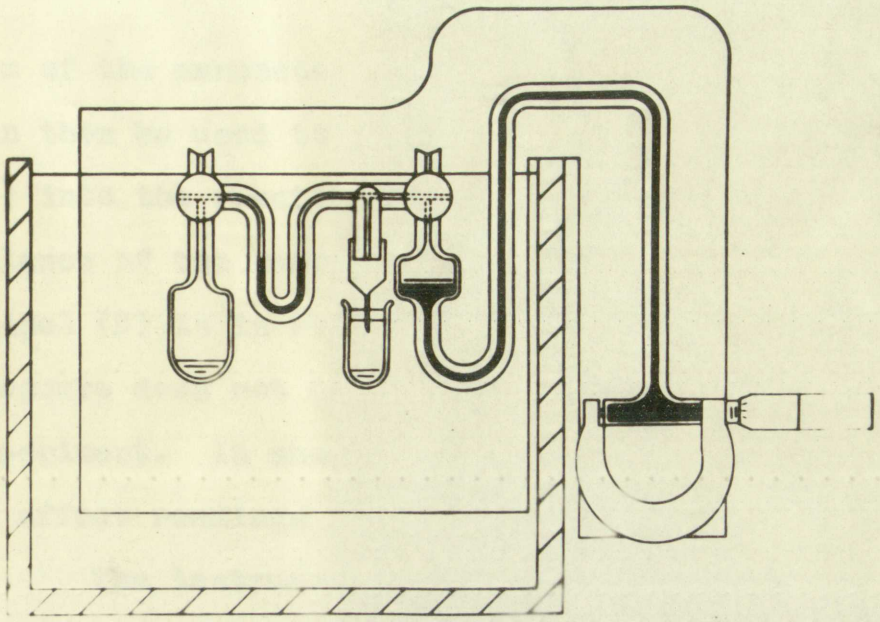
The bottles were then incubated at 32° C. for 2 to 3 weeks and held at room temperature prior to actual use. Tarr (1933) has shown that under these circumstances virtually 100% of the cells sporulate.

The spores were harvested before each experiment by adding approximately 5 cc. of distilled water to the surface of the agar culture and allowing it to stand for 5 minutes. At the end of this time, almost complete suspension was effected by tilting the bottle back and forth. No attempt was made to scrub the surface, thus obviating the possibility of dislodging particles of agar that would be carried through subsequent centrifugations. The suspensions obtained were forcibly ejected from a fine bore pipette several times to produce a homogenous mixture.

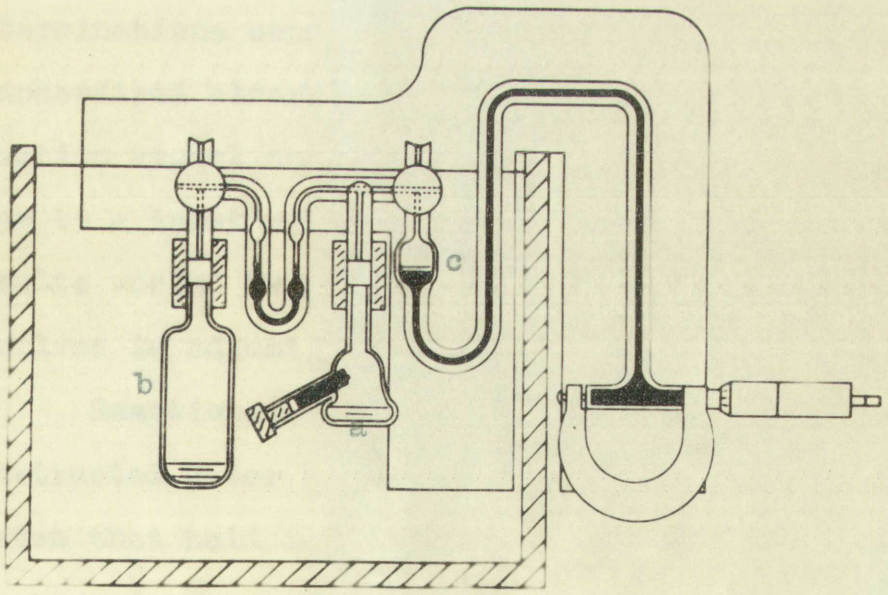
The spores were washed three times by centrifugation and resuspended in distilled water. Following this, they were taken up in physiological saline and pasteurized by heating at 65° C. for 30 minutes. The suspension was then quickly cooled to room temperature, the spores sedimented by centrifugation, washed once in distilled water, and finally suspended in the particular medium to be used in the experiment.

Oxygen consumption was studied by means of a modified Scholander micro-respirometer. The original apparatus (Scholander and Edwards, 1942) had several shortcomings that made impractical its application to such problems as are dealt with in this investigation. The details of construction of the modified device are to be found in figure 1, and the original device is shown in figure 2. The design of both the reaction vessel and the compensating vessel was altered, as well as their method of attachment. Bulbs were placed in the manometer to permit the apparatus to remain untended for longer periods of time.

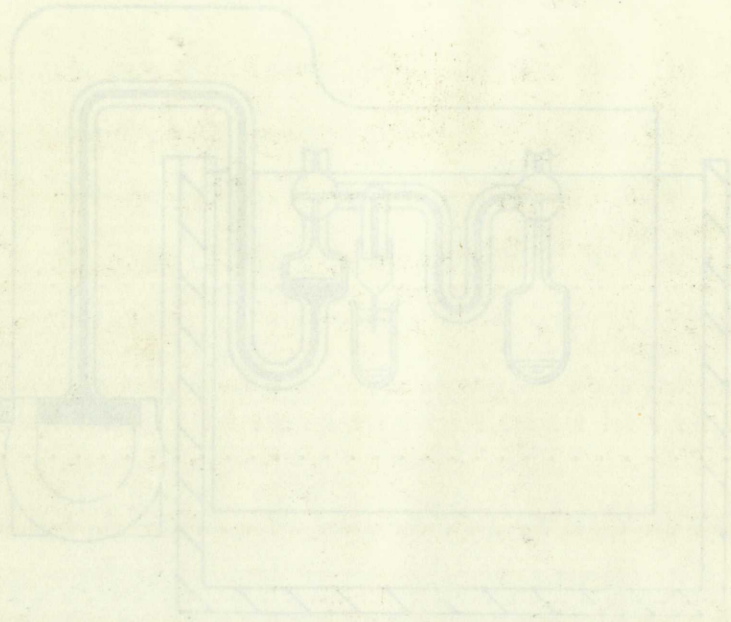
In the modified form, the reaction vessel (A) is equipped with a side arm in which is placed a roll of filter paper impregnated with a KOH solution to absorb CO_2 evolved by the system under investigation. As oxygen is utilized, the column of Brodie's solution in the right

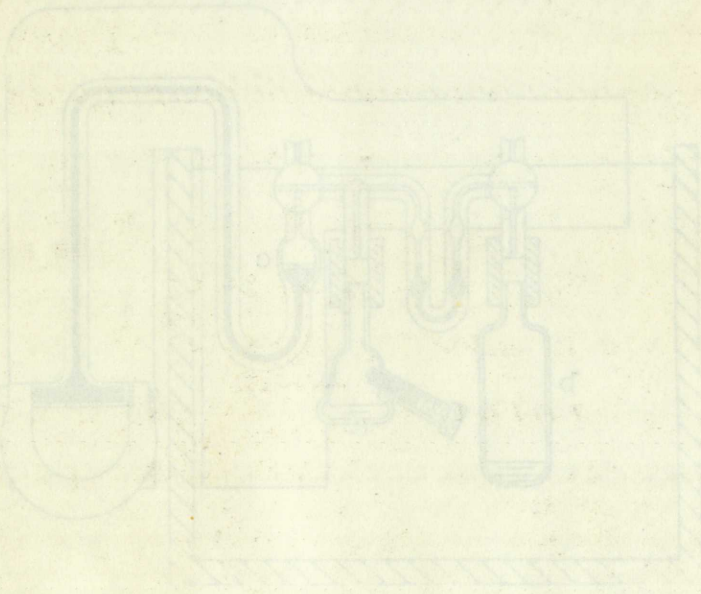


Original Scholander Micro-respirometer



Modified device





arm of the manometer is pulled upward. The micrometer can then be used to displace air from the air reservoir (C) into the reaction vessel and so restore the pressure balance of the manometer. The so-called compensating vessel (B) is in reality an artificial atmosphere whose pressure does not change during the course of the experiment. It should be as large as possible in order to affect readings of the manometer as little as possible.

The instrument is calibrated by finding the volume displaced by the advancing spindle of the micrometer. The volume displacement in the micrometer used was 0.29 uL. per division on the barrel scale which caused an easily observable shift in the manometer level. This value was taken as the sensitivity of the instrument. The accuracy of the method was tested in a series of determinations conducted by Butterfield (1951), in which standardized bicarbonate solutions were placed in the reaction vessel and acid added from a side arm, giving rise to a known volume of gas. He found that the observed results varied from 2% to 5% as a result of personal error involved in adjusting the level of the manometer.

Reactions were carried out in a specially constructed water bath incorporating a balanced heating system that held the temperature to plus or minus 0.01° C. The temperature used in all experiments was 32° C.

The suspending medium for the spores was M/5 phosphate buffer at pH 7.0. In experiments calling for glucose, this was added to the above to provide a 0.5% concentration. The total volume of spore suspension used in an experiment was approximately 0.5 cc., in a reaction vessel of 5 cc. capacity. The atmosphere was, in all cases, air. Evolved CO_2 was absorbed in 0.25 cc. of a 5% KOH solution taken up in a filter paper roll placed in the side arm of the reaction vessel.

Following introduction of the apparatus into the water bath, a period of 15 minutes with both stopcocks open to the atmosphere proved to be sufficient time for temperature equilibration to take place. The stopcocks were then moved to the closed position shown in figure 1 until the end of the experiment at which time both were opened simultaneously. Readings were usually made at intervals of one hour.

At the termination of each experiment, the contents of the reaction vessel were quantitatively transferred to a ddigestion flask and a micro-Kjeldahl determination made to measure the amount of spores used in terms of nitrogen content. The digestion mixture consisted of 4 cc. of solution A (1 gm. $CuSO_4$, 10 gm. K_2SO_4, 0.2 gm. sucrose, and 5 cc. conc. H_2SO_4 made up to 100 cc. with distilled water) and 1 cc. of solution B (1 gm. of selenium dissolved

in 100 cc. of conc. H_2SO_4). The distillate was caught in 2% boric acid and titrated with HCl to an endpoint with a brom cresol green-methyl red indicator (10 cc. of BCG plus 2 cc. of MR, both 0.1% alcoholic solutions). This technique was standardized with $(NH_4)_2SO_4$, with a maximum deviation of 0.005 mgm. of nitrogen on a 0.4 mgm. sample.

In order to convert the mgms. of nitrogen so obtained to mgms. dry weight of organisms, known amounts of dried organisms were tested for nitrogen content and the following values found:

B. subtilis	11.1 % N
B. cereus var anthraces	12.8 % N

To determine the possibility of germination in the course of an experiment, checks were made on a control experiment and at the end of all regular determinations, using bright and dark phase microscopy. There is a definite change in refractivity associated with germination which never occurred in the experiments reported herein.

Experimental Results

The effect of various temperatures on the endogenous respiration of bacterial endospores is shown in figures 3, 4, 5, and 6.

The curves shown in figure 3 were obtained following standard pasteurization procedures at 65° C. (as described previously). Although earlier experiments have been excluded because the time intervals at which readings were made were too far apart to permit accurate plotting, no attempt at selection in the reported results was made. Initial respiratory levels varied over a range of 2 µL./hr./mgm. dry weight of organisms. This was always followed by an increase in activity, which reached a peak 1 to 4 hours later. Respiration then decreased to a post-stimulation low, rose again slightly, and entered on a constant level of oxygen consumption. This level was reached between 8 and 12 hours after the start of the experiment.

Despite the wide variation at the start of the curves, the level line respirations in all experiments agree to within plus or minus 0.175 µL. Thus divergence lies well within the experimental error of the determinations. The Q_{O_2} (µL./hr./mgm. dry wt. of organisms) for anthrax spores, under these circumstances, appears to

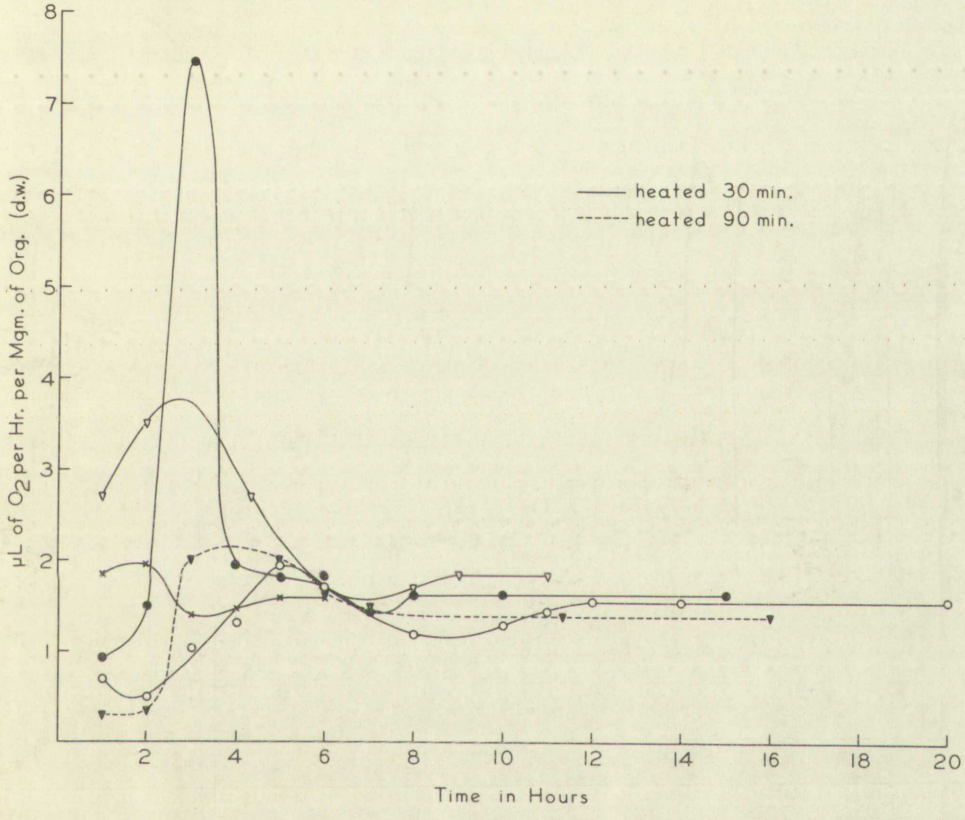


Figure 3. Anthrax heated to 65°C. for 30 and 90 minutes.

be about 1.7.

One experiment, in which the spore suspension was heated at 65° C. in phosphate buffer for an additional hour, the same general curve is observed, but without the period of recovery usually noted. Viability counts, utilizing standard plating procedures, indicated that only about 50% of the spores remained active following this treatment. Consequently, Q_{O_2} values obtained in this determination were multiplied by 2 to permit direct comparison with other curves.

Figure 4 presents the results, in graphic form, of three experiments in which spores were heated to 80° C. for 30 minutes after having been pasteurized and suspended in phosphate buffer. Viability was again seriously affected and only 20% of the spores remained active following the treatment. There was also an extension of the time required for germination to take place. At the end of 24 hours, no growth was observed on nutrient agar plates seeded with treated spores and incubated at 32° C. After 48 hours, however, there was copious growth and apparently complete germination. A recovery period is not illustrated by these curves, although characteristics are like those obtained at 65° C. and there appears to be better agreement in the initial extent of respiration. The level of respiration in the case of one very young

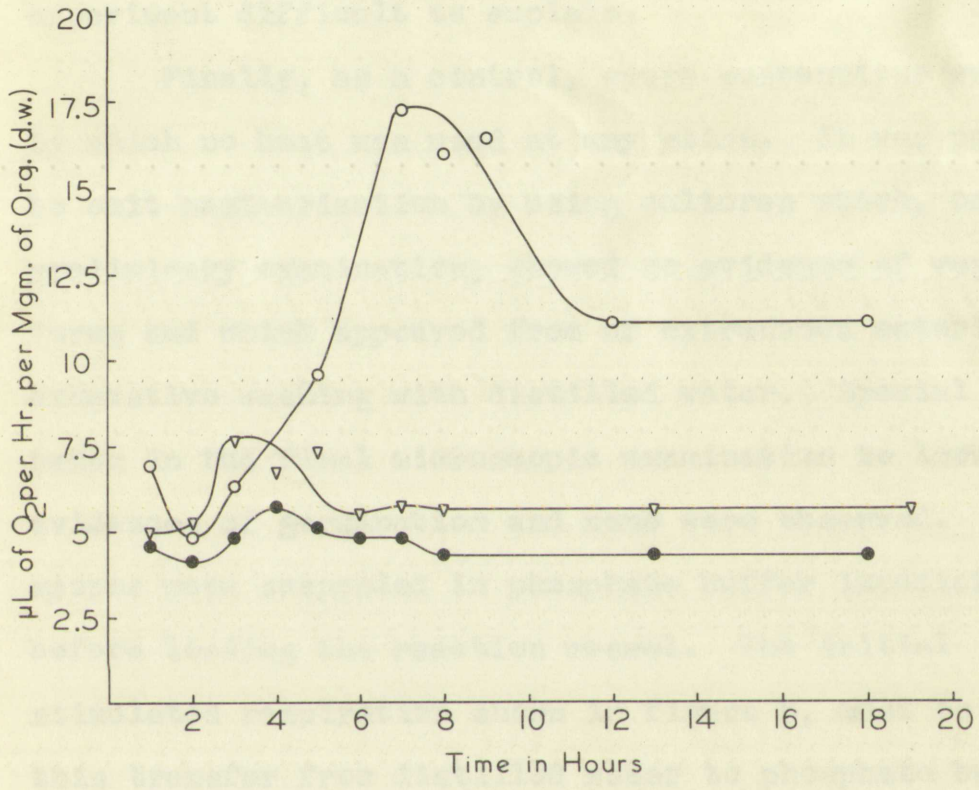
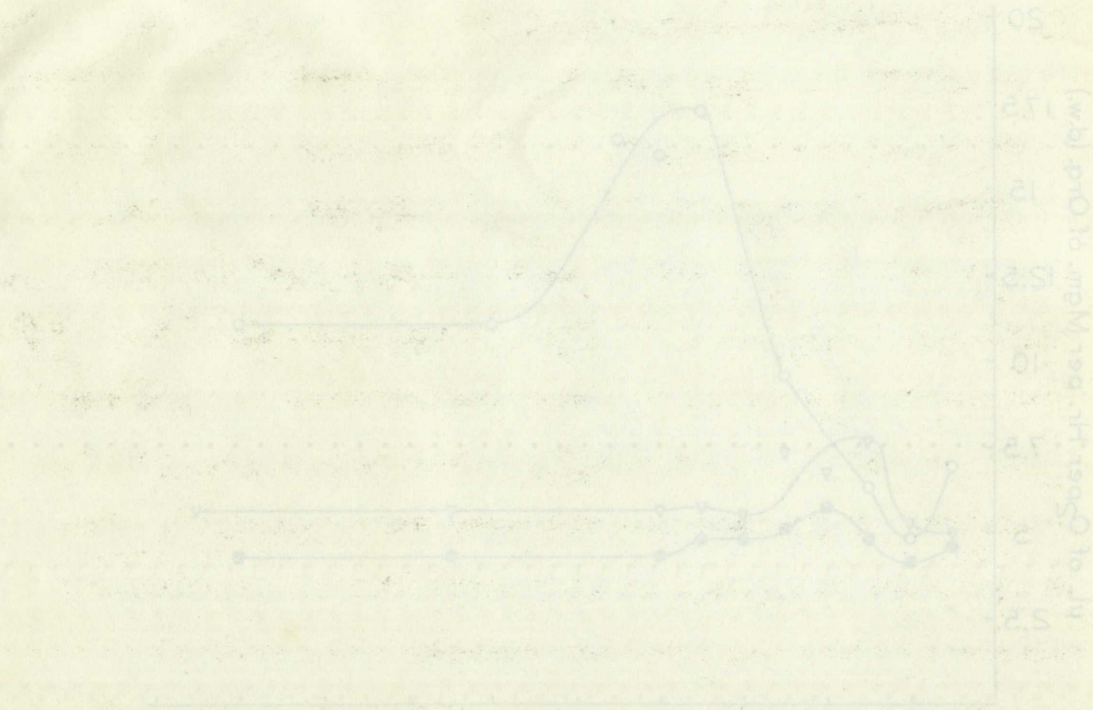


Figure 4. Anthrax heated to 80°C. for 30 minutes after pasteurization.



culture (11 days) rose to a much higher peak than the other two, and established a level line oxygen uptake a full 1.1 uL. above the others. This, plus the fact that it took a full 7 hours to reach the peak, makes this experiment difficult to explain.

Finally, as a control, spore suspensions were made in which no heat was used at any point. It was possible to omit pasteurization by using cultures which, on preliminary examination, showed no evidence of vegetative forms and which appeared free of extraneous material after exhaustive washing with distilled water. Special care was taken in the final microscopic examination to look for evidences of germination and none were observed. The spores were suspended in phosphate buffer immediately before loading the reaction vessel. The initial stimulated respiration shown in figure 5, must be due to this transfer from distilled water to phosphate buffer. Level line respiration at an oxygen uptake that may be considered very nearly true endogenous respiration, a Q_{O_2} of 0.35, occurred after 6 hours.

The results so far reported were obtained from experiments involving B. cereus var anthraces. There is not adequate evidence to determine whether or not the same conclusions hold for B. subtilis. If they do, the slopes of the curve may be different although the general

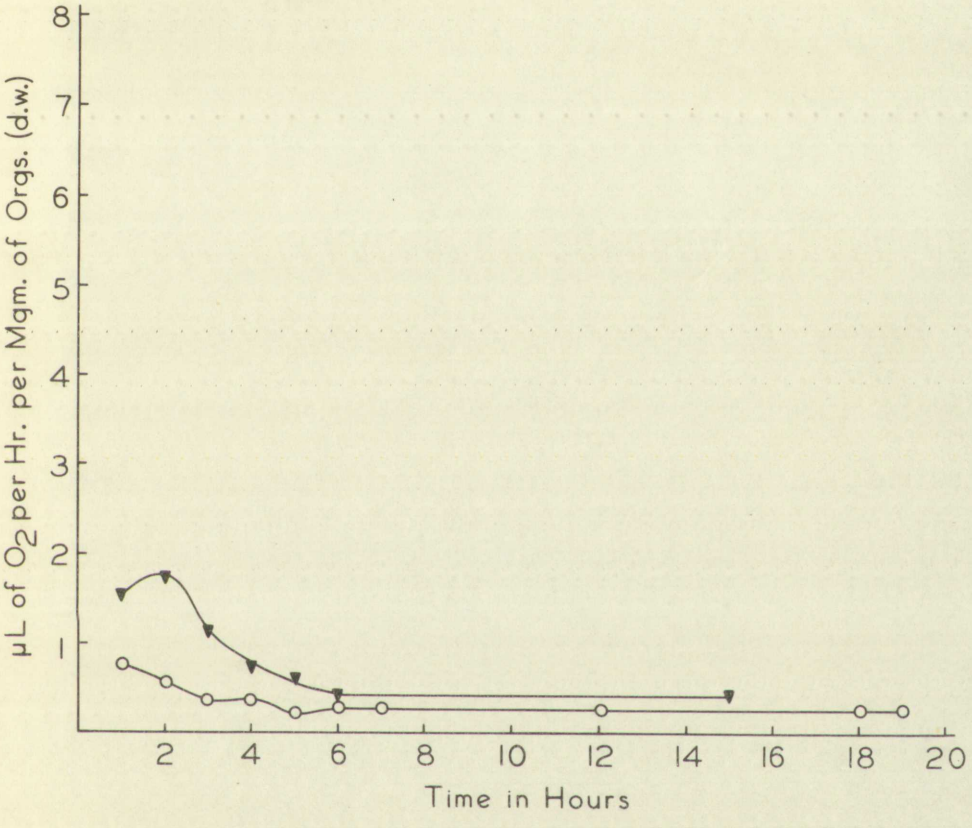
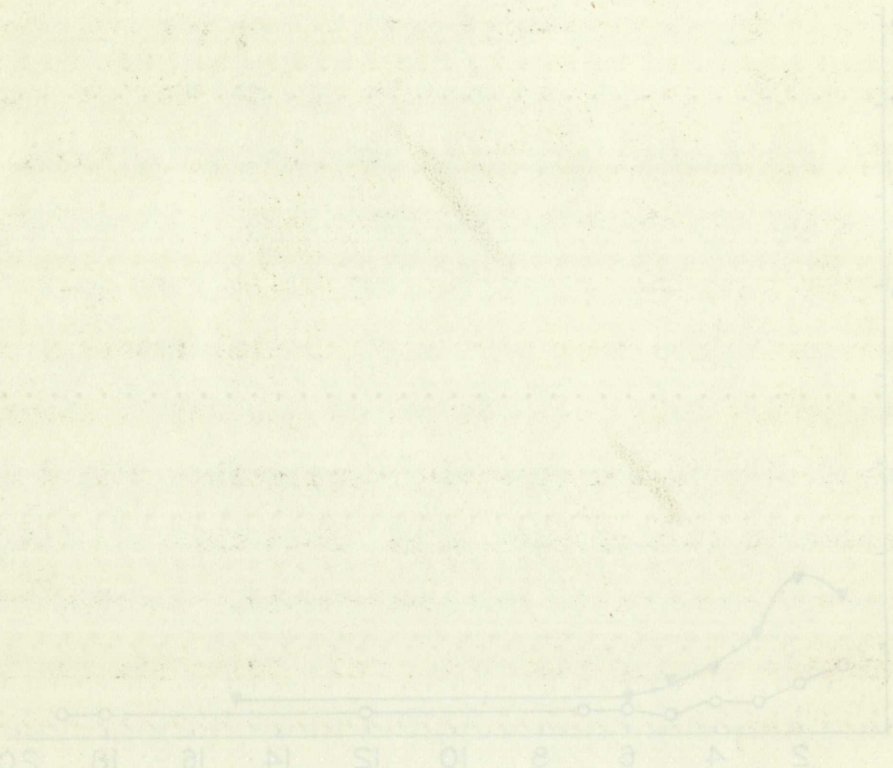


Figure 5. Anthrax that has not been heated.


Time in Hours

outline would be the same. Figure 6 shows an experiment in which a partial curve has been determined for B. subtilis. The respiration of this organism is found to increase at a regular rate during the first 6 hours.

In the experiments conducted to determine the respiratory stimulation afforded by glucose, readings were made after the first hour, and again at the end of the sixth or eighth hour. In all cases, the initial respiration was within a range of 0.3 µL. The results of these experiments is shown in table I in which the Q_{O_2} reported is the average for the time interval employed. Respiration in the presence of glucose is about twice that of normal endogenous respiration, with a possible error of plus or minus 20%. This error is introduced primarily by nitrogen determinations carried out on samples smaller than the usual limits set for the method employed.

It must be emphasized that no evidence of germination could be found by careful inspection of spore suspensions at the end of each experiment. The results presented consist of measurements taken on spores whose exine and intine remained intact, and in which no change in refractivity could be observed during the period of investigation.

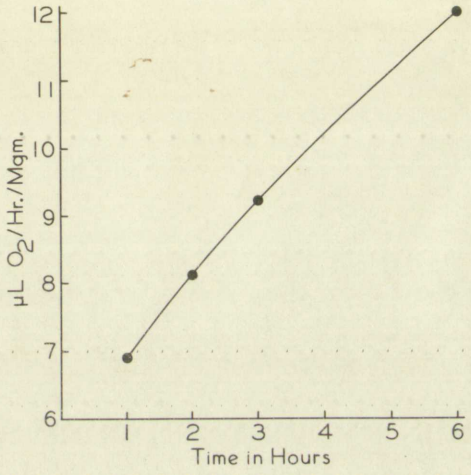


Figure 6. B. subtilis heated to 65° for 30 minutes.

Table I. The effect of 0.5% Glucose in phosphate buffer on the resiration of B. subtilis.

Experiment #	Duration	Substrate	Q_{O_2} x
22-3	6 hr.	Buffer only	5.00
24-3	8 hr.	" "	4.25
25-3	6 hr.	Buffer plus Glucose	10.36
27-3	8 hr.	" " "	9.28

x Average for time interval employed.

Discussion

The primary purpose of this paper was the adaptation of a method to be utilized in metabolic studies of bacterial endospores. The Scholander micro-respirometer has been found to be admirably suited in following the rather low respiratory levels found in this type of investigation. The accuracy of the device, five percent, agrees very well with accuracies obtained by more conventional methods with systems displaying far greater activity. The use of operational periods up to 20 hours has proven practical and there is every indication that even longer periods can be employed without encountering serious difficulties. The theoretical freedom of the apparatus from external variations has been confirmed. When held at 32° C. in a water bath, changes in room temperatures of as much as 5° C. have no effect on the device.

The problems encountered by many earlier investigators with respect to the preparation of pure, stable suspensions of bacterial endospores have largely been solved by using the procedure described in the experimental method. Considerable care must still be employed in the case of B. subtilis, but an ideal test organism has been found in B. cereus var anthraces, from

which spore suspensions of high stability and purity can be obtained with comparative ease.

It should be emphasized that all experimental results came from spores that morphologically appeared intact and in which, by definition, germination did not occur. A more careful cytological investigation is indicated with respect to possible changes initiated by the application of heat. There may also be some changes in the permeability of the spore coat which may not be apparent by ordinary staining methods.

The data obtained from the experiments reported herein call for a revision of previous hypotheses concerning the activity of bacterial endospores and of the permeability of their exine and intine. The latter idea, for instance, is still referred to in most current literature, for example, by Knaysi (1951). The role of these structures has been questioned by Dubos (1947) who mentions a peculiar staining characteristic of spores which would tend to indicate that the refractivity of the spore to staining is a function of the protoplasm itself, and not an inherent property of the spore coats. Physiological support of this view is presented by the work in this and the preceding papers concerning the stimulated respiration caused by glucose which must have penetrated these membranes. The stimulation afforded by transfer of

spores from distilled water to phosphate buffer provides still further evidence of spore coat permeability. A series of future experiments is indicated using a number of metabolites in order to test the range of selective permeability present.

It has been observed by Curran and Evans (1945) that germination can be speeded up by a preliminary exposure of spores to heat; the amount of heat necessary being termed the "heat of activation" of the spore being tested. Although no evidence in support of a corresponding metabolic change has previously been shown, such a relationship is clearly demonstrated in the data obtained from those experiments conducted to determine the effect of preliminary heating on endogenous respiration in endospores. Such heating was shown to initiate a burst of metabolic activity followed by a uniform oxygen consumption which was proportional to the amounts of heat employed. Thus, spores held at 20° C. (room temperature) develop a Q_{O_2} of about 0.3 under the conditions of the experiment, while at 65° C., spores respire at a constant rate with a Q_{O_2} of about 1.7, and 80° C. elicits a Q_{O_2} of about 5. The increase appears to be an exponential function of the temperature. It is of interest to note that in the experiment in which spores were pre-treated for 1.5 hrs. at 65° C., the same level of

respiration developed as in the case of spores pre-treated at the same temperature but for only 0.5 hours, despite the fact that only 50% survival was observed in the spores treated for 1.5 hours, whereas essentially all spores survived the 0.5 treatment.

In view of the above noted oxygen consumption, the question arises as to the source of the substrate being utilized. No stored food materials have been reported as occurring in endospores despite the fact that such reserves must be available. Lipoidal substances may be so utilized and ribonucleic acid is also present, but the latter has been shown in at least one case to be unavailable as a source of energy (Knaysi and Baker, 1947). Actually, little is known about the presence and utilization of these or similar substances by bacterial spores. Within the period of time employed in the experiments reported in this paper, there appeared to be a steady level of respiration achieved after the initial spurt of activity. This must surely lead to an ultimate exhaustion of stored material, especially at the rapid rate of activity initiated by 80° C. The problem of what happens upon complete exhaustion of these stored food products remains to be solved. The techniques developed in this work are suited to a delineation of this problem.

It is not inconceivable that the energy necessary

for germination could be burned out as a result of heat stimulation so that the ultimate development into vegetative cells would be impossible even in the presence of easily assimilable nitrogen. On the other hand, experiments on permeability suggest that such energy might be provided by adding glucose to the medium. This could happen only if those irreversable changes that have been observed in starving mammalian cells as a result of catabolism of proteins important to vital function do not occur.

The actual release mechanism that permits heightened levels of metabolic activity is purely speculative at this time. A likely mechanism might involve the liberation of bound water as a result of heat shock. This could be investigated by cryoscopic determinations although such techniques are liable to considerable criticism due to their inherent lack of accuracy (Rahn, 1944).

Summary

The Scholander micro-respirometer has been modified to permit its use in measuring the metabolic activity of bacterial endospores. A method of preparing spores to provide a pure, stable suspension for use in this device has been evolved in which B. cereus var anthraces has been found the organism of choice.

Experiments were conducted to determine the effect of heat of activation on the endogenous respiration of anthrax spores. Following half hour exposures to 65° and 80° C., there was observed a burst of activity which reached a peak and then dropped to a steady rate of oxygen consumption. This level was at a Q_{O_2} of 1.7 following exposure to 65° C., and at a Q_{O_2} of 5.0 following exposure to 80° C., while those suspensions receiving no heat treatment developed a Q_{O_2} of 0.3.

The previously reported respiratory stimulation of B. subtilis spores caused by glucose has been confirmed; the result was a twofold increase over endogenous respiration. This confirms the earlier work of Keilin and Hartree. Stimulation has also been observed upon transfer of spores from distilled water to phosphate buffer.

Literature Cited

Butterfield, L. J., 1951. Personal communication.

Cook, R. P., 1932. "Bacterial spores." Biol. Rev. & Biol. Proc. Cambridge Philos. Soc. 7: 1-23.

Curran, H. R. and F. R. Evans, 1945. "Heat activation inducing germination in the spores of thermotolerant and thermophilic aerobic bacteria." J. Bact. 49: 335-346.

Dubos, R. J., 1947. "The Bacterial Cell." Harvard University Press, Cambridge, Mass.

Keilin, F. and E. F. Hartree, 1947. "Comparative study of spores and vegetative forms of Bacillus subtilis. Antonie von Leeuwenhok Jour. of Microbiol. and Serol., Jubilee Vol. Albert F. Kluyver 12(½): 115-128.

Knaysi, G., 1951. "Elements of Bacterial Cytology." Second ed., Constock Publishing Co., Ithaca, N. Y.

_____ and R. F. Baker, 1947. "Determination, with the electron microscope, of a nucleus in Bacillus mycoides grown in a nitrogen free medium." J. Bact. 53: 539-553.

Rahn, O., 1945. "Physical methods of sterilization of micro-organisms." Bact. Rev. 9: 1-48.

Ruehl, G. L. A., 1923. "The enzymic content of bacterial spores." J. Bact. 8: 487.

Scholander, P. F. and G. A. Edwards, 1942. "Volumetric microrespirometer for aquatic organisms." Rev. Sci. Instruments 13(7): 292-295.

Tarr, H. L. A., 1933. "Some observations on the respiratory catalists present in the spores and vegetative cells of certain aerobic bacillae." Biochem. J. 27:136-143.


DIETER
BINDS
BETTER
DENVER COLO