THE ENDOGENOUS RESPIRATION OF
BACILLUS CEREUS

I. CHANGES IN THE RATE OF RESPIRATION WITH THE
PASSAGE OF TIME

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I. INTRODUCTION

A starving bacterial cell may continue to respire for some time; Mycobacterium tuberculosis for example, retained an appreciable respiration, even after starving for 15 days, (Loebel, Shorr and Richardson, 1933). Stier and Stannard (1935) found that the respiration of yeast was also resistant to starvation, and proposed the term “endogenous respiration” to describe the respiration of starved cells. Bacillus cereus has a large endogenous respiration which persists for some time. These organisms are all gram-positive. Sevag (1933) has suggested that this character is related to high endogenous respiration, for he found that butyric acid bacilli with a low endogenous respiration failed to retain Gram’s stain. Evidence is given below which shows that, in the case of B. cereus, the gram-positive character and the high endogenous respiration are both associated with a high content of fats in the cell.

II. EXPERIMENTAL METHODS

A strain of B. cereus was used, isolated from a bacon slime by the writer in 1933, and developed from a single cell culture. The strain corresponds closely in physiological characters with that preserved by the Lister Institute for type cultures (no. 2599), but it has a lower optimum temperature, (25 instead of 30°C.) and
forms very long cells in young cultures, especially in nutrient broth.

Standard cultures were produced as follows. A tube culture was made in broth every 24 hours; and when a culture had been incubated at 25°C. for 24 hours 1 ml. was removed for seeding each agar plate on which the cells for an experiment were grown. The broth had the composition:

\[
\begin{align*}
\text{Glucose} & \quad 20 \text{ gm.} \\
\text{Distilled water} & \quad 500 \text{ ml.} \\
\text{NaCl} & \quad 10 \text{ gm.} \\
\text{Pork extract} & \quad 500 \text{ ml.}
\end{align*}
\]

the two solutions being sterilised separately, and the pH of B adjusted to 7.0, while hot, with 40 per cent caustic potash; the solutions were then mixed in equal proportions: the solid medium had the same composition with the addition of 2 per cent Difco Bacto-agar. Plates and tubes were incubated over-night at 25°C. before inoculation, in order to avoid temperature shock. One milliliter of the 24-hour old broth culture was seeded onto each plate, and spread by means of a bent glass rod. The cells were incubated at 25°C. until they reached the age required.

The growth from the plates was suspended in distilled water, shaken, and separated by centrifuging. This process was then repeated twice, in distilled water or buffer solution as the case might require. The thrice-washed and shaken suspension was filtered through a Schott sintered glass filter, porosity G3, into a vessel so arranged as to permit of aeration and dilution of the suspension under aseptic conditions. In this vessel the suspension was diluted with distilled water or buffer solution, to the same turbidity as a standard barium sulphate suspension, so chosen that the bacterial suspension contained about 10 mgm. dry weight of cells per ml. of suspension. A vigorous stream of air was then passed through the diluted suspension for 15 minutes, and after which 1.5 ml. of suspension was measured into each manometer; other solutions were added to these samples in por-
tions of 1.5 ml. Dry weights were determined by drying out a sample of suspension in air at 105°C. for 24 hours.

The oxygen absorption was determined in Barcroft differential manometers, shaken with a traverse of 2.5 cm. at 95 cycles per min. in a water-bath maintained electrically at 25 ± 0.05°C. To absorb carbon dioxide, 0.4 ml. of 40 per cent caustic potash was used, with 2 sq. cm. of Whatman no. 40 filter paper (Dixon 1934); with this absorbent and the rate of shaking given, experiments showed that the manometers would measure rates of oxygen uptake as high as 1,000 c.mm. per hour.

Measurements of size, and percentage of gram-positive cells, were made on samples of aqueous suspensions dried in air at room temperature. The films were stained by Jensen's modification of the Gram stain, and the organisms then measured with an eyepiece micrometer.

The fat content of the cells was estimated by a modification of the method recommended by Leathes (1925). The cells were dried at 105°C. and the dry weight determined. The residue was treated with 65 per cent caustic potash for 1 hour on the water-bath, an equal volume of alcohol added, and the whole refluxed for 1 hour. The extract was treated with 40 per cent sulphuric acid, and the fatty acids taken up in petrol ether by shaking for 1 hour and estimated by weight after evaporation of the ether and desiccation in vacuo. The fatty fraction determined in this way is larger than that obtained by direct extraction with petrol ether, for it contains the fatty acids from structural lipoids, which are of interest in connection with the staining reaction.

III. EXPERIMENTAL DATA

With this material, three phases of respiration could be distinguished, whatever the age to which the cells had been grown.

1. At first the rate of oxygen uptake decreased. When the period of aeration was omitted from the preparation of the suspensions, this phase was prolonged correspondingly and the initial rate of respiration was much higher. It could also be prolonged by reducing the degree of agitation during washing of the cells,
but it was not shortened by washing a greater number of times. The duration of this phase was greater the older the cells, as is shown in figure 2. It was not appreciably altered by a considerable reduction in temperature (curve f, fig. 2).

2. During the next phase, the changes in respiration depended very markedly on the age of the cells.

![Graph](attachment:image.png)

**FIG. 1. THE ENDOGENOUS RESPIRATION AT 25°C. OF CELLS OF B. CEREUS GROWN TO DIFFERENT AGES:** (a) 12-HOUR CULTURE, (b) 18-HOUR CULTURE, (c) 24-HOUR-CULTURE

(a) With cells from cultures less than 18 hours old at 25°C. (i.e. in the logarithmic phase of growth), the rate of consumption of oxygen rose steadily, to rather high values in the case of cells 12 hours old (curve a, fig. 1). The behavior of 18-hour-old cells
(curve b) was similar but less marked. The 12-hour cultures consisted of long cells (20–50μ) united in chains; in the course of a respiration experiment the total number of cells increased about 10 times, and the length of the cells fell to 3–5μ.

(b) With cells 24 hours old or more (i.e. from the stationary or senescent phases of growth), the total number of cells did not change appreciably, nor did the rate of uptake of oxygen for a

period of several hours. The rate of endogenous respiration during the period of constancy was lower the older the cells (curves c–e, fig. 2). At the end of this phase of respiration, the viable count was always less than 1 per cent of the total number of bacteria.

3. In the final phase, the rate of absorption of oxygen declined following an exponential course with time for at least 10 hours.
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(curve B, fig. 3). Precautions were taken to ensure that contaminating organisms were absent, that oxygen was adequate, and that carbon dioxide did not accumulate in the manometers. This decline of respiration was common to cells of all ages.

The presence of salts did not interfere with the appearance of these phases of respiration; low concentrations caused a slight increase and higher concentrations a decrease in the rates of respiration. It was noted, in particular, that with mature cultures the level of respiration during the second, constant, phase could be changed by sodium chloride, without altering the time at which the respiration began to decay.

To test the possibility that some inhibitor might be produced
during respiration, a sample of cells was aerated in distilled water for 20 hours at 25°C, and the suspending fluid centrifuged off. This might be supposed to contain the inhibitor. Samples of a freshly-prepared suspension were then diluted with an equal volume of distilled water, or the solution centrifuged from the starved cells. It was found that the respiration of the suspension containing "inhibitor" (in half the concentration present in the starved suspension) was about 5 per cent less than that of the normal suspension. However, the pH of the normal suspension was found to be 6.5 at the end of this experiment, and that of the treated suspension 7.0. This difference accounts for the difference between the rates of respiration, so that the "inhibitor" was a substance making the suspensions more alkaline. After 20 hours starvation, the pH of a normal suspension was 7.5, and this was not alkaline enough to account for the low respiration observed.

The lost respiration could not be restored by feeding with glucose. With starving 24-hour-old cells in the phase of constant respiration, the Q_{O_2} (\text{c.mm. oxygen per hour per mgm. dry weight}) was raised roughly from 20 to 40 by the addition of glucose. When the Q_{O_2} of endogenous respiration had fallen to 5, addition of glucose raised the Q_{O_2} only to 7.5. Thus the increase in respiration due to glucose was only \frac{1}{4} of that attained earlier, a fall greater in proportion than that of endogenous respiration. The increase in rate of respiration was independent of glucose concentration within the range 0.005–0.5 M; a similar state of affairs has been reported to occur with Sarcina lutea (Gerard and Falk, 1931).

It was found that the respiratory quotient \left( \text{R.Q.} = \frac{\text{vols. CO}_2}{\text{vols. O}_2} \right) remained low throughout. With cells 24 hours old, respiring at 25°C in phosphate buffer at pH 6, it had an initial value of 0.5; during the second phase of respiration it remained roughly 0.6, and rose gradually to 0.7 after 15 hours. Analysis of cells of \textit{B. cereus} showed that the low R.Q. was accompanied by a disappearance of fat from the cells. Starvation for 23 hours at 25°C.
reduced the fat-content from 4.2 per cent to 1.0 per cent of the dry weight. The loss of fat was accompanied by a diminution in the volume of the cells, and by a loss of the ability to retain Gram's stain. These changes are shown in table 1.

*B. cereus* is normally gram-positive, and even in old cultures all the cells retain stain. Starving of 24-hour-old cells for only 5 hours was sufficient to make half of the cells gram-negative, and even those which continued to retain the stain held it only in small granules, apparently on the margin of the cells. The microscopic appearance of the cells at different stages of starvation was in keeping with the supposition that the diminished volume of the cells resulted from a loss of their outer layers.

### TABLE 1

Changes in fat content, cell-volume, and Gram reaction, during starvation of 24-hour-old cells of *B. cereus* at 25°C.

<table>
<thead>
<tr>
<th>HOURS OF ENDOGENOUS RESPIRATION AT 25°C</th>
<th>FATTY ACIDS AS PERCENT-AGE OF DRY WEIGHT</th>
<th>AVERAGE VOLUME OF A CELL (µm²)</th>
<th>PERCENTAGE OF CELLS RETAINING ANY STAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.2</td>
<td>5.0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>2.6</td>
<td>2.6</td>
<td>62</td>
</tr>
<tr>
<td>4</td>
<td>2.4</td>
<td>4.6</td>
<td>58</td>
</tr>
<tr>
<td>6</td>
<td>2.4</td>
<td>1.7</td>
<td>43</td>
</tr>
<tr>
<td>8</td>
<td>1.8</td>
<td>1.7</td>
<td>25</td>
</tr>
<tr>
<td>12</td>
<td>1.8</td>
<td>1.7</td>
<td>25</td>
</tr>
<tr>
<td>13</td>
<td>1.0</td>
<td>1.7</td>
<td>34</td>
</tr>
<tr>
<td>15</td>
<td>1.0</td>
<td>1.0</td>
<td>16</td>
</tr>
<tr>
<td>23</td>
<td>1.0</td>
<td>1.4</td>
<td>8</td>
</tr>
</tbody>
</table>

The average volume and percentage of gram-positive cells were determined for 100 cells by two independent observers.

IV. DISCUSSION

An initial phase of falling respiration has been observed to occur with *Sarcina lutea* by Gerard and Falk (1931). They attributed it to the accumulation of an oxygen debt from asphyxia during the washing of the cells, the debt being met gradually as the cells became saturated with oxygen. Shoup (1929) has shown clearly that such an oxygen debt is accumulated during anaerobiosis. The relations to washing and aeration support the view
that this took place with *B. cereus*. The unduly large uptake of oxygen would account for the low R.Q. If the process were controlled by the diffusion of oxygen into the cells, one would expect it to be largely independent of temperature, as was the case. Moreover, the increased duration when older cells were used may be an expression of their lower permeability.

With actively-dividing cells from young cultures division appears to have been continued on suspension in distilled water, without any corresponding increase in cell material. The high rate of respiration associated with this process presents analogies to the high rates of metabolism observed when bacterial cultures emerge from the lag phase of growth by the cleavage of large cells into smaller cells of normal size. (Martin, 1932; Bayne-Jones and Adolf, 1932; Walker et al. 1934).

With mature cells there seemed to be a period of equilibrium with the menstruum, for no division occurred and the respiration remained constant. This has been observed in *B. subtilis* (Callow, 1924) and in yeast (Stier and Stannard, 1935). The equilibrium was disturbed by some cause which brings about an exponential decline with time in the rate of respiration. This might be attributed to (i) death of the cells, (ii) the production of some inhibitor during respiration at the end of the period of equilibrium, (iii) the exhaustion of a food-supply once it is reduced below a certain critical level or (iv) the decay of the enzyme systems of the cells according to a monomolecular law.

(i) It has been shown, e.g. by Rahn (1929), that under certain conditions the viable fraction of a population of cells may remain constant for some time and then decline, the decline being roughly exponential with time only in its later stages. Although the general behaviour of endogenous respiration was similar, the decline was exponential throughout. Moreover, the interpretation in the case of viability depends on the fact that this is an "all or none" property: there is no evidence that the respiration of a single cell ceases abruptly in the same way. The changes in endogenous respiration were not directly related to the viability of the cells, since it was reduced to one-hundredth without any corresponding change in respiration. This probably took place
principally during the first phase of respiration, for Winslow and Brooke (1926) found the viability of washed cells of *B. cereus* to be reduced by 99 per cent after exposure to distilled water for 1 hour. Dieckman (1934) quotes many instances in which, during a period of constant respiration, the number of viable cells fell to an insignificant proportion of the whole.

(ii) An experiment has already been described which showed that specifically inhibiting substances were not responsible for the low respiration of starved suspensions. Their alkalinity was probably due to the production of ammonia and carbon dioxide (compare Shaughnessy and Winslow, 1927), the latter being removed by the potash in the manometers while the ammonia accumulated in the suspensions.

(iii) Working with yeast cells more than 2 days old, Stier and Stannard (1935) observed phases of constant respiration and of exponential decay with time. They believed that this represented the exhaustion of the glycogen reserve, and that decay set in directly its concentration fell below a critical value. This conclusion was based on 3 observations. The first two of these were that glycogen disappeared and that the R.Q. remained 1 throughout endogenous respiration; these facts proved that a carbohydrate was the substrate of respiration. The third was that sugar fed to starved cells caused a large increase in respiration, the R.Q. remaining 1; this showed that the enzyme system attacking carbohydrate was intact after starving. This hypothesis does not apply to *B. cereus*, for two reasons. Firstly, when the rate of utilisation of the food reserve was diminished by salt, the period of constant respiration was not prolonged (curve C, fig. 3). Secondly sugar-feeding indicated that the respiratory enzymes were destroyed during starvation, those oxidising glucose even more than those responsible for endogenous respiration.

(iv) The data of Martin (1932) demonstrated that there is a close relation between the surface area of a bacterial cell and the rate at which it utilises oxygen; this would occur if the amount of the respiratory enzymes were related to the area of the superficial layers of the cell. The simultaneous diminution in the size of cells of *B. cereus* and in their ability to retain Gram’s stain sug-
suggests that it was these outer layers that were respired away; two observations indicated that this was fatty material. The low R.Q. was associated with a fall in the total fatty acids of the cell; Stephenson and Whetham (1922, 1923) found that low values of the R.Q. were associated in *Mycobacterium phlei* with the oxidation of fat in the cell. Secondly, Churchman (1929) and Burke and Barnes (1929) have shown that the power of retaining Gram's stain lies in the outer sheath of bacteria, which agrees with the microscopic appearance of starved cells of *B. cereus*, while Jobling and Peterson (1914) and Tamura (1914) have shown that the gram-positive character is associated with fat in the cell, an association confirmed for *B. cereus*.

If it be supposed that during the third phase of endogenous respiration (1) the outer sheath and respiratory enzymes were destroyed at a rate proportional to the rate of respiration, and (2) that the rate of respiration was proportional to the amount of enzymes remaining, we have—

$$\frac{dE}{dt} = K \cdot E_t$$

(where *E*ₜ is the amount of enzyme remaining at any instant) and thus

$$\ln E_t = C - K \cdot t$$

(*C* and *K* constant) whence

$$\log R_t = \log R_o - K' \cdot t$$

(*t* being measured from the instant at which the decline of respiration begins)

This is the relation represented by the falling straight lines of figure 3, the slopes of the lines representing values of *K'*.

Sevag (1933), from a study of butyric acid bacilli, came to a similar conclusion. These bacilli were gram-positive in young, and gram-negative in old cultures. Data were obtained which are shown in table 2.
The loss in the ability to retain Gram's stain was concomitant with a diminished endogenous respiration, and a corresponding loss of the ability to utilise added sugar. In these cases the changes in respiration were almost certainly due, in the main, to the differing ages of the cells, since there are similar but smaller differences in the respiratory activity of cells of *B. cereus* at different ages without any change in the gram-reaction. However, Sevag also stated that the same changes could be brought about by starving butyric acid bacilli, and that during starvation the cells became smaller. These statements, which are amply confirmed by the data presented above for *B. cereus*, led Sevag to the same conclusion: that the respiratory enzymes, and the loci retaining Gram's stain, are situated in the ectoplasm of the cells, and are respired away during starvation.

### TABLE 2

*Intensity of respiration and Gram reaction in butyric-acid bacilli*

(Calculated from the data of Sevag, 1933)

<table>
<thead>
<tr>
<th>Age of the Parent Culture (hours)</th>
<th>Percentage of Gram-Positive Cells</th>
<th>QO₂, IN: Buffer</th>
<th>Buffer + glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>6</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>24</td>
<td>55</td>
<td>8.5</td>
<td>21.5</td>
</tr>
<tr>
<td>14</td>
<td>92</td>
<td>20.5</td>
<td>62.5</td>
</tr>
</tbody>
</table>

V. SUMMARY

When cells of *Bacillus cereus*, grown on nutrient agar at 25°C, for less than 24 hours, are washed and suspended in distilled water or buffer solution cell-division continues, and gives rise to an unusually high rate of respiration.

If the cells are 24 hours old, or older, no cell division occurs, and the endogenous respiration passes through three phases.

The first phase usually lasts less than two hours. Its duration is greater with older cells, but is not much affected by temperature. The large uptake of oxygen is attributed to the satisfaction of an oxygen debt arising from asphyxia during washing.
The second phase lasts about five hours at 25°C. and during this time the rate of respiration remains constant. The magnitude of this constant rate decreases with increasing age of the cells.

During the third phase, the rate of respiration declines exponentially with time. The time which elapses before this phase of respiration begins is largely independent of the rate of respiration with cells of any given age, which suggests that the decline in respiration is not brought about by the failure of a food supply.

During endogenous respiration the cells diminish in size and lose their fatty materials and their gram-positive reaction. The low R.Q. (0.7) indicates that the fat is probably destroyed by respiration, and this apparently leads to the loss of the outer gram-positive sheath of the cells. The exponential decline of the respiration accords with the view that the respiratory enzymes are destroyed during this phase, and thus associates the respiratory enzymes with the gram-positive sheath of the cell.

The addition of sodium chloride does not destroy the three phases of endogenous respiration. Its effect on respiration may therefore be measured with convenience, by comparing the constant rates of respiration during the second phase, in the presence and absence of salt.

REFERENCES

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