NON-INVOLVEMENT OF LYSIS DURING SPORULATION OF
BACILLUS MYCOIDES IN DISTILLED WATER*

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Powell and Hunter (1953) do not concur with the conclusions reached by this laboratory (Hardwick and Foster, 1952) that washed vegetative cells of aerobic bacilli can sporulate in the absence of exogenous nutrition and when growth is precluded. Their view is based essentially on the demonstration that under the conditions maintained in their experiments mass lysis of the cells initially present took place. They believe that the few survivors grow abundantly and sporulate on the lytic products, and imply that sporogenesis is intrinsically connected with increase in cell growth.

In view of the erroneous impression the Powell and Hunter paper creates relative to claims based on our experiments, we present here a considerable amount of additional experimental data which, without exception, support and confirm our original findings, and which appear to preclude the occurrence of mass lysis in our experiments. Differences in results between the two laboratories are discussed later.

Materials and Methods

General procedures, materials, and media were those employed previously (Hardwick and Foster, 1952); their repetition is unnecessary here. Bacillus mycoides was used throughout; the strain was that previously employed. Vegetative cells were always harvested from 12 hour shake cultures of synthetic glucose-glutamic acid-salts medium (GGS) which had been inoculated lightly with free spores from a glucose-yeast-extract slant.

RESULTS

Experiments to Detect Lysis

1.—Twice washed freshly harvested vegetative cells were suspended in sterile distilled water to a final concentration of approximately $10^5$ cells per ml. as judged by plate counts. The suspension was placed on the shaker at

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30°C. and aliquots removed at 12 hour intervals for the measurements (a), (b), and (c) listed below. Microscopic observation showed that about 90 percent of the cells present had sporulated by the 12th hour.

(a) Cell volume: Four ml. were centrifuged in hematocrit tubes for 15 minutes at 2,000 r.p.m. and the volume of packed cells recorded (Fig. 1).

(b) Optical density: Determined in a Klett-Summerson photoelectric colorimeter and recorded as Klett units (Fig. 1).

(c) Soluble nitrogen: This criterion of lysis was studied by Kjeldahl digestion of 10 ml. of the cell-free supernatant liquid, followed by direct nesslerization and reading in the Klett-Summerson photoelectric colorimeter. The values were compared against a standard curve. The data are contained in Fig. 1. Total nitrogen present initially in the form of cells was 12.5 μg. per ml.

None of the parameters in Fig. 1 indicates that significant lysis occurred during the experiment. The gradual slight decline in the Klett readings is not that typical of a mass lysis, as was supposed by Powell and Hunter to have occurred in our experiments, and a standard turbidity curve constructed from various dilutions of the bacterial suspension confirmed the conclusion.

![Graph showing turbidity, cell volume, and supernatant nitrogen during sporulation of a suspension of vegetative cells of B. mycoides in distilled water. The packed cell volumes were obtained with 4 ml. aliquots throughout.](image-url)
that the turbidity change recorded in Fig. 1 does not reflect a significant change in cell concentrations. The small drop observed is attributed to the reduction in size of individual cells (Hardwick and Foster, 1952).

The curve of cell volumes gives no indication of lysis.

There was a gradual low level release of soluble nitrogen from the cells; the increase over the zero hour control was rather insignificant. This curve sharply upward after the 10th hour; sporulation is well under way at the 10th hour and is completed by the 12th hour. Concomitant with sporulation is lysis of the sporangia (Hardwick and Foster, 1952). The cellular debris formed during this lytic period has altered physical characteristics such that it packs with extreme difficulty, which accounts for the apparent increase in cell volume after the 10th hour.

2. Oxygen Uptake.—This experiment was repeated just as it was done in our first paper. The smooth curve obtained was identical with that in Fig. 2 of that paper. The smooth nature of the curve expressing oxygen consumption during the sporulation process is incompatible with the degree of lysis assumed to take place by Powell and Hunter (1953).

3. Ability of Supernatant Liquid to Support Growth.—Powell and Hunter (1953) made capital of the fact that their suspensions lysed to such an extent that the soluble organic matter in the supernatant fluid supported excellent growth and sporulation of freshly inoculated cells. They implied that a similar sequence of events could explain our results, and that our interpretation of the events as metamorphosis was erroneous. The following experiment shows that under our conditions the ability of the supernatant fluid to support growth is negligible. Twelve hour GGS twice washed vegetative cells were suspended in distilled water as usual, and distributed equally in 3 flasks which were then shaken for 12 hours till normal sporulation. One flask was removed at zero time, one after 6 hours' shaking, and one after 12 hours' shaking, the cells centrifuged out, and the supernatant liquids sterilized by filtration. Each then received a small inoculum of fresh vegetative cells and was shaken aerobically for 12 hours at which time the cells in each were counted in a Petroff-Hauser chamber. The results were recorded as the average number of cells per smallest ruled square of the chamber and each value represents the average of 200 squares counted. After 12 hours' incubation the inoculated supernatant from the suspension at time of replacement, e.g., zero time supernatant, was 0.28 cell per square; that from the suspension after 6 hours' shaking was 0.33; and that from the suspension after 12 hours' shaking was 0.40. These increases may be taken as additional evidence that under our conditions the amount of soluble nutrients produced as a result of lysis is negligible insofar as it can account for the events under consideration.
SPORULATION OF BACILLUS MYCOIDES

Experiments Showing that a Spore Is Formed for Each Vegetative Cell Initially Present without Significant Change in Cell Numbers during the Entire Process

1. Plate Counts.—Powell and Hunter obtained a yield of only 10 per cent spores based on the cell count at time of replacement, and found that lysis accounted for the other 90 per cent. Spore yield was determined under conditions originally specified by us. An aliquot of the vegetative cell suspension in distilled water was removed at zero time. A portion was diluted in sterile distilled water blanks and 0.10 ml. of the dilutions was spread on the surface of dry GGS agar plates containing 0.1 per cent basamin and 0.1 per cent soluble starch (Foster, Hardwick, and Guirard, 1950). Each dilution was plated in quadruplicate. Plates were counted after 14 hours' incubation at 30°C. Counts were made before overgrowth occurred. A second portion of the original aliquot was promptly heated in a 60°C. water bath for 15 minutes, to kill vegetative cells. Correspondingly, unpasteurized and pasteurized samples of the suspension after it had been shaken for 12 hours (sporulation) were plated out. The data are presented in Table I. Colony counts on the individual plates and for the various dilutions are given to show that the accuracy obtained is suitable for the objective of the experiment. Table I shows that the experiment started with $3.96 \times 10^5$ cells per ml. of suspension, none (<250) of which was a spore (resistant to pasteurization). In 12 hours, when sporulation was observed microscopically to be complete, the suspension contained a total of $7.16 \times 10^5$ viable cells in the unpasteurized sample, and $8.4 \times 10^5$ viable cells in the pasteurized samples. Thus, substantially every cell in the 12 hour sample was a spore, and, most significantly, there was a heat-resistant cell formed for substantially every vegetative cell present initially. The somewhat

### Table I

<table>
<thead>
<tr>
<th>Dilution plated</th>
<th>Suspension at beginning</th>
<th>Suspension after 12 hrs.' shaking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unpasteurized</td>
<td>Pasteurized</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>36, 34, 37, 38 = 36</td>
<td>No colonies on dilutions as low as 43, 37, 40, 33 = 38</td>
</tr>
<tr>
<td>Average</td>
<td>36</td>
<td>Average</td>
</tr>
<tr>
<td>$2 \times 10^{-4}$</td>
<td>24, 22, 22, 21 = 22</td>
<td>0.25 X $10^{-4}$</td>
</tr>
<tr>
<td>Average</td>
<td>22</td>
<td>Average</td>
</tr>
<tr>
<td>$3 \times 10^{-4}$</td>
<td>13, 13, 12, 14 = 13</td>
<td>Average</td>
</tr>
<tr>
<td>Average</td>
<td>13</td>
<td>Average</td>
</tr>
<tr>
<td>Average count per ml. of suspension</td>
<td>$3.96 \times 10^5$</td>
<td>Average count per ml. of suspension</td>
</tr>
</tbody>
</table>

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higher count of cells at the end in relation to the beginning is not due to growth, but to the fact that *B. mycoides* vegetative cells characteristically are in chains of 2, 3, or 4 cells, and the shaking breaks up some of them, this breakage occurring more readily after sporulation. Nothing in these data contradicts the interpretation that substantially every cell initially present metamorphosed directly into a spore. This experiment has been performed several times with results essentially the same as those contained in Table I. Also, the results are in complete agreement with comparable experiments performed by W. A. Hardwick in 1950–51 (personal communication).

**TABLE II**

*Effect of Removal of Original Supernatant Fluid at Hourly Intervals on Subsequent Viable Cell Count during Sporulation*

Figures represent colonies obtained per plate per 0.10 ml. of the particular dilution; and each value is the average of quadruplicate plates. Variation between quadruplicates was of same magnitude as that in Table I.

<table>
<thead>
<tr>
<th>Dilution plated</th>
<th>Supernatant removed and replaced by distilled water after shaking for the following hrs.:*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>$3 \times 10^{-4}$</td>
<td>31</td>
</tr>
<tr>
<td>$4 \times 10^{-4}$</td>
<td>23</td>
</tr>
<tr>
<td>$7 \times 10^{-4}$</td>
<td>17</td>
</tr>
<tr>
<td>$8 \times 10^{-4}$</td>
<td>17</td>
</tr>
</tbody>
</table>

| Cells per ml. of undiluted suspension; † ($\times 10^{-4}$) | 1.22 | 1.10 | 1.00 | 1.03 | 1.35 | 2.41 | 2.01 |

* Total elapsed shaking time before and after replacement of supernatant fluid = 12 hours.
† If one eliminates the highest dilution ($8 \times 10^{-4}$) from these averages, owing to the relative fewness of the colonies at this dilution, these values become respectively ($\times 10^{-4}$): 1.13, 1.10, 1.04, 1.11, 1.36, 2.29, 2.06.

2. *Removal of Lysate Nutrients in Supernatant.*—If lysis were at play in our experiments to the extent it was in Powell and Hunter's experiments, removal of the soluble products of lysis supposedly being utilized to support the growth of the majority of the cells present at the end of the experiment, ought to depress markedly the final count. A water suspension of washed *B. mycoides* vegetative cells was allowed to shake at 30°C. in the usual fashion. At hourly intervals aliquots were removed, centrifuged, and the cells made up to volume in distilled water and placed again on the shaker. Plate counts, made as described earlier, were performed on the suspension at zero time, and on each of the washed aliquots after a total shaking time of 12 hours for each aliquot. The results (Table II) demonstrate that removal of the nutrients present in
the supernatant liquid at various times throughout the sporulation experiment did not lead to a reduction in the number of viable cells present at the end of the experiment, within the accuracy of the method. At any one time the amount of soluble nutrients produced as a result of lysis could not, therefore, have served as substrate for the production of a significant proportion of the cells found at the end of 12 hours.

3. Counts by Direct Microscopic Examination.—A washed cellular suspension vigorously shaken by hand for 10 minutes at the time it was prepared was placed on the mechanical shaker and aliquots were removed periodically, shaken vigorously by hand for 10 minutes, and the cells counted microscopically in a Petroff-Hauser counting chamber (Table III). Some uncertainty in counting individual cells was encountered. For comparative purposes, the short chains consisting of 1 to 4 cells were considered as equivalent in the counting. There was no sign that cells initially present as a single, a 2 cell chain, a 3 cell chain, or a 4 cell chain divided to yield longer chains. Average

<table>
<thead>
<tr>
<th>Time, hrs.</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count*</td>
<td>0.61</td>
<td>0.59</td>
<td>0.61</td>
<td>0.63</td>
<td>0.64</td>
<td>0.67</td>
<td>0.73</td>
</tr>
</tbody>
</table>

* Average per smallest square of the Petroff-Hauser chamber. Each value represents the average of 200 squares counted. See text.

chain length was 3 cells. No signs of lysis of individual cells were observed; "ghost" cells were not observed to appear.

It is evident from Table III that no significant reduction in the chain count occurred at any interval during the sporulation process in the suspension in distilled water; presumably lysis could not, therefore, have occurred. The typical sporulation pattern was observed at the 12th hour. The slight increase in count near the end represents breaking up of some of the chains.

4. Continuous Microscopic Observation of the Metamorphosis of Individual Cells.—Minute hanging drops of suitable dilutions of water suspensions of vegetative cells were held under more or less continuous inspection for 2 days. The drop consisted of about 1 to 2 μl. which was discharged from a 10 μl. micropipet and was visible in its entirety under the 8 mm. lens of the microscope (× 430). The drop, surrounded by other drops of water to prevent drying, was placed on a coverslip which was sealed in an inverted position over the concavity of a hanging drop slide. In this way it was possible to study 4 to 12 cells, the identity and status of each individual cell being recognizable throughout the period of observation. At no time were any of the cells observed to lyse, or appear to become "ghost" cells and disappear, nor was
generation of any new cells observed. In one experiment starting with 12 short chains, some of the longer chains broke up to give 17 chains at the time of sporulation, but there was no decrease or increase in the number of cells at any time. Each cell initially present eventually sporulated, although the time required for complete sporulation was about 4 times longer than that generally required in shaken suspensions. Undoubtedly limitations imposed by the difference in physical conditions account for the delay.

Material Balance during Sporulation in Distilled Water

On this score data obtained under our conditions are not in accord with conclusion of Powell and Hunter (1953) "... that the dry weight of single spores... is generally considerably greater than that of vegetative cells of the same organism." At time of maximum sporulation (12 hours) of a suspension,

<table>
<thead>
<tr>
<th></th>
<th>Dry weight of cells*</th>
<th>Dry weight of supernatant liquid</th>
<th>Total solids in cells and supernatant liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension at time of replacement (0 hrs.)</td>
<td>1.55</td>
<td>0.10</td>
<td>1.65</td>
</tr>
<tr>
<td>Suspension at time of sporulation (12 hrs.)</td>
<td>0.93</td>
<td>0.35</td>
<td>1.28</td>
</tr>
</tbody>
</table>

* Approximately 10⁸ cells per ml.

we have never observed the remarkable reduction in cell dry weight reported by the British workers. To be sure, our weights were obtained on 12 hour freshly spored suspensions in which the spores are contained in sporangia derived from the original vegetative cells. The important point here is that the degree of solubilization (loss of cell weight) in our suspensions (Table IV) is not compatible with a mass lysis. The spored cells have 60 per cent (0.93/1.55) of the weight of the progenitor vegetative cells. Considering that the sporangium and its non-spore contents undoubtedly account for an appreciable portion of the weight of the spored cells, it is obvious that in our experiments a freshly formed spore weighs distinctly less than the original vegetative cell whence it was derived. Also to be noted (Table IV) is that of the 0.62 mg. per ml. reduction in cell weight, only 0.25 mg. can be accounted for as soluble matter (0.35 minus 0.10). There was an over-all loss from the system of 0.37 mg. solids; e.g., 24 per cent of the original dry cell weight. Assuming 10 per cent of the weight of a bacterial cell to be mineral this means a reduction of about 26.4 per cent of the organic matter in the system. In all probability this
loss was the result of oxidation, a portion of the energy derived therefrom driving the endergonic reactions of sporulation. The reactions presumably consume only a portion of the exergonically produced energy.

DISCUSSION

None of the many different experiments that we have performed since presentation of our first results (Hardwick and Foster, 1952), provides evidence implicating massive lysis followed by rapid and abundant growth and sporulation of the few survivors at the expense of nutrients in the lysate. This is true in spite of several attempts designed to detect these events assumed by Powell and Hunter (1953) to account for our observations. The British investigators estimated a final cell recovery of about 10 per cent of the starting cell population in their experiments. At that, no viability or heat resistance measurements were reported. According to their data, the cells present at the end are the result of a 100-fold multiplication at the expense of lysate nutrients derived from the rest of the initial population. Hence, in a typical experiment of theirs 99.9 per cent of the initial population must have undergone lysis. Lysis of this magnitude should have been revealed in our experiments. None of the experimental results we have as yet obtained under our conditions (see also Foster and Perry, 1954) is inconsistent with our earlier work. At the present time the observed results are most satisfactorily explained as a direct conversion of the vegetative cell to a spore. Such results do not, of course, preclude the utilization of exogenous nutrients during sporulation in a complete growth medium, although even here exogenous nutrients may well be equivalent to nutrients of endogenous origin utilized for sporogenesis.

Apart from interpretation of data is the divergency of experimentally obtained results in our laboratory and in Powell and Hunter's laboratory. Obvious deviations from our present experiments noted in Powell and Hunter's experiments relate to growth medium, incubation temperature during growth, temperature during replacement, age of cells at time of replacement, time of analysis of replacement cultures; methods of evaluating numbers of cells, determination of heat resistance, and concentration of cells in replacement suspensions. Whether any or all these factors can explain the differences in results, or whether other or more subtle and elusive forces are at play, remains to be seen.

SUMMARY

Washed vegetative cells of Bacillus mycoides obtained and treated under specified conditions have been found to sporulate when shaken in distilled water under specified conditions. Within limitations of the methods, a heat-resistant cell (spore) is produced for each heat-sensitive vegetative cell present initially. Several different experiments designed to detect massive lysis and
cell growth during sporulation in distilled water yielded uniformly negative results. Evidence is furnished for the conclusion that a freshly formed spore (heat-resistant cell) weighs considerably less than its progenitor vegetative cell. The observed results are most satisfactorily explained as a direct conversion of a vegetative cell to a spore.

BIBLIOGRAPHY