

SPORULATION IN DISTILLED WATER

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PLATES 6 AND 7

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INTRODUCTION

Hardwick and Foster (1952) have recently described observations which they have made on the sporulation of vegetative cells washed free of the culture medium and resuspended in distilled water. From these observations, the authors conclude that abundant sporulation can occur "in the absence of nutrients, and consequently, where growth is precluded." When it is considered that (a) the specific gravity of spores is considerably higher than that of vegetative cells (McIntosh and Selbie, 1937) and that (b) spore germination is accompanied by excretion of solid material amounting to 30 per cent of the spore dry weight (Powell and Strange, 1953), it is clear that sporulation involves concentration and possibly *de novo* synthesis of cell material, and must necessarily be a strongly endothermic process. We find it difficult, therefore, to envisage the direct metamorphosis of a vegetative cell to a spore in a non-nutrient medium. If, as Hardwick and Foster (1952) conclude "exogenous nutrition is completed before the metamorphosis commences" the vegetative cell must then contain all the constituents of the spore and, in addition, large reserves of energy needed to concentrate them. This seemed to us unlikely, and we therefore offer an alternative interpretation of these "replacement" experiments which is supported by data we have obtained in this laboratory. The object of our experiments was to determine (1) the amount of cell lysis which occurred when vegetative cells were shaken in distilled water, (2) whether the products of lysis would permit further growth of the surviving organisms, and (3) the number of spores obtained when a known number of vegetative cells was shaken in distilled water.

Materials and Methods

Vegetative cells of *Bacillus subtilis*, *B. cereus* (NCTC 8035), *B. mycoides* (NCTC 926), and a *B. cereus*-*B. megatherium* intermediate were used as a starting material. *B. subtilis* and the *B. cereus* NCTC 8035 were studied in greatest detail. Cultures were grown at 37° in a casein hydrolysate, yeast extract medium (Gladstone and Fildes, 1940) in shaken 1 litre flasks containing 200 ml. of medium. In a typical experi-

ment, five flasks were inoculated with 3×10^4 spores/ml., and growth allowed to proceed until the cultures were within a few hours of sporulation (*vide infra*). At this time, all the flasks were removed, the cells from four flasks were harvested, centrifuged at 4° , and washed three times aseptically with distilled water, the fifth flask being kept at 4° as a control. The washed cells were divided into four equal batches, one of which was taken for the determination of initial cell dry weight. The remaining three batches were each suspended in 200 ml. of distilled water and together with the control flask shaken at 37° for periods up to 44 hours. During this time flasks were removed at intervals, cell counts determined, and stained films made using carbol fuchsin, nigrosine, and methylene blue (Powell, 1950). The cells were then separated from the suspending medium by centrifugation. Cell dry weight was determined by heating continuously at 103° for 48 hours. The amount of soluble material in the suspending medium was determined by freeze drying a measured volume and weighing the residue. The remainder of the medium was sterilized by filtration through sintered glass, reinoculated with 10^5 spores/ml., and examined for visible growth after 18, 24, and 40 hours' incubation at 37° .

RESULTS

Sporulation.—The behaviour of vegetative cells of the four organisms studied was very similar to that described by Hardwick and Foster (1952). After 18 hours' shaking with distilled water, the *B. cereus* suspension consisted almost entirely of free spores (Figs. 1 to 4). The control flask had spored completely in 8 hours. Complete sporulation of the 36 hour *B. subtilis* was slower, even though this culture was already showing definite signs of sporulation when resuspended (Figs. 5 to 8). Here, there was practically complete sporulation in 40 hours. The 24 hour cultures of *B. subtilis* (Figs. 9 to 12), *B. mycoides*, and *B. cereus*—*B. megatherium* all showed signs of sporulation at 24 hours and 80 to 90 per cent free spores after 40 hours' shaking. In the control flasks there was complete sporulation in 24 hours.

Lysis during Incubation in Distilled Water.—During 20 hours' shaking in distilled water the cell dry weight of 22 and 24 hour cultures of *B. cereus* decreased by 90 and 80 per cent respectively. After 40 hours' shaking, a 36 hour culture of *B. subtilis* lost 70 per cent of its cell dry weight. Similar results were obtained with 24 hour cultures of *B. subtilis*, with *B. mycoides*, and with the *B. cereus*—*B. megatherium* intermediate. The weight of soluble material in the suspending medium correspondingly increased. This soluble material, containing 11 to 12 per cent nitrogen and consisting mainly of protein together with small amounts of free amino acids, was obviously derived from cell lysis (Table I). Single dimensional paper chromatograms of hydrolysed and unhydrolysed material from *B. subtilis* were run with collidine as solvent (Consden, Gordon, and Martin, 1944). In the unhydrolysed material, lysine, aspartic and glutamic acids, arginine, and leucine were present together with small amounts of all the common amino acids. After hydrolysis, the amounts of all these acids were greatly increased.

Growth-Promoting Effect of Products of Lysis.—36 hour vegetative cultures of *B. subtilis* were shaken for 40 hours in distilled water, the medium centrifuged off, sterilized by filtration, and inoculated with 10^5 spores of *B. subtilis*/ml. Visible growth occurred in 18 hours indicating that the cell concentration was at least 10^7 /ml.; *i.e.*, that the cell concentration had increased 100 times. The

TABLE I
Lysis and Sporulation of Vegetative Cultures in Distilled Water

Organism	Age of culture	Time of shaking in distilled water	Cells/ml. $\times 10^{-4}$	Cell dry weight	Soluble material in medium
	<i>hrs.</i>	<i>hrs.</i>		<i>mg./ml.</i>	<i>mg./ml.</i>
<i>B. cereus</i> NCTC 8035	24	0	0.85	1.45	0
		18	0.17	0.28	0.88
<i>B. cereus</i> NCTC 8035	22	0	1.20	1.70	
		5		1.28	
		10		1.03	
		21	0.22	0.27	
<i>B. subtilis</i>	36	0	3.3	1.53	0
		4	3.3	1.28	0.20
		20	1.2	0.73	0.60
		44	0.37	0.30	0.91
<i>B. subtilis</i>	24	0	3.8	1.13	0
		4	2.7	0.97	0.09
		20	1.6	0.61	0.44
		44	0.19	0.14	0.73
<i>B. mycoides</i> NCTC 926	24	0	2.3	1.05	
		42	0.10	0.20	
<i>B. cereus</i> - <i>B. megatherium</i> intermediate	24	0	2.7	0.86	
		42	0.25	0.20	

suspending medium after shaking for 18 hours was rather less nutritious, giving visible growth in 40 hours. These 40 hour cultures consisted mainly of free spores.

Similar results were obtained with *B. cereus*. On reinoculating the sterilized medium after 20 hours' shaking, visible growth occurred in 18 hours, and complete sporulation in 24 hours.

Quantitative Yield of Spores.—Although sporulation was practically complete after 18 to 40 hours' shaking with distilled water, the final total cell count was considerably reduced. The highest yield of spores was obtained from *B. cereus*,

the final cell count in three experiments was 10, 10, and 20 per cent that of the time of replacement. The other three organisms gave spore yields corresponding to 5 to 10 per cent of the cell count at the time of replacement.

DISCUSSION

While it is true that sporulation occurs when washed vegetative cells of sporing organisms are shaken in distilled water at 37°, it seems unlikely that the process is a straightforward conversion of vegetative cells to spores. We have shown that during shaking with distilled water, a massive cell lysis occurred; after which the suspending medium could promote growth and division of the surviving organisms. Sporulation did not, in fact, take place in distilled water, but in a dilute nutrient medium. It was not until lysis was well advanced that the first signs of sporulation appeared, and this delay was not due simply to interrupted shaking and incubation during replacement: sporulation still occurred very much more rapidly in the control cultures which were cooled and static for 3 to 4 hours during the period of washing and resuspending. It may be significant that a laboratory strain of *B. megatherium* which we examined produced vegetative cells which were very resistant to lysis. These cells showed no signs of sporulation after 48 hours' shaking in distilled water at 37°.

We have not investigated systematically the effect of age of the washed culture on its tendency to sporulate in distilled water. It might be expected, however, that older cultures with greater tendency to lysis will, on the whole; produce spores more readily. This has been reported by Knaysi (1945) who also showed that sporulation was more rapid when distilled water suspensions of vegetative cells were allowed to stand for several hours before incubation and aeration began. We have found that lysis of 24 hour *B. subtilis* vegetative cultures proceeded at about the same rate and to the same extent as that of 36 hour cultures. After 20 hours' resuspension, sporulation was, however, more advanced in the 36 hour culture, and there were some free spores. Approximately 50 per cent lysis had occurred in both cases. The 36 hour cultures were already showing signs of sporulation when resuspended and it is interesting to note that the average dry weight of a single cell was 1.5 times that in the 24 hour cultures, indicating that some concentration of cell material had already taken place. In this connection, it is significant that the dry weight of single spores, which can be roughly calculated from Table I, is generally considerably greater than that of vegetative cells of the same organism. It therefore seems very unlikely that the transformation of vegetative cell to spore can occur without extracellular supplies of nutrients, and it follows that the utilization of these will be susceptible to the presence of growth inhibitors as Hardwick and Foster (1952) have found. It is also to be expected, as these authors describe, that addition of buffered glucose to the distilled water suspension at an early stage will discourage sporulation, but will have no effect if added after 9 to 10 hours'

shaking. In the first case, cell lysis is reduced by the addition, while after 9 to 10 hours lysis has already proceeded far enough to supply the required nutrients for sporulation.

SUMMARY

Spores are formed when vegetative cells of sporing aerobes are shaken with distilled water at 37°. These spores are derived from the small number of cells which survive lysis. The sporulation process involves increase and concentration of solid material in the cell, and is achieved at the expense of the products of lysis of 80 to 90 per cent of the resuspended cells.

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EXPLANATION OF PLATES

The magnification of all the figures is 2300.

PLATE 6

FIG. 1. 22 hour culture of *B. cereus* NCTC 8035 at time of resuspension in distilled water. The clear areas in these cells do not develop directly into spores.

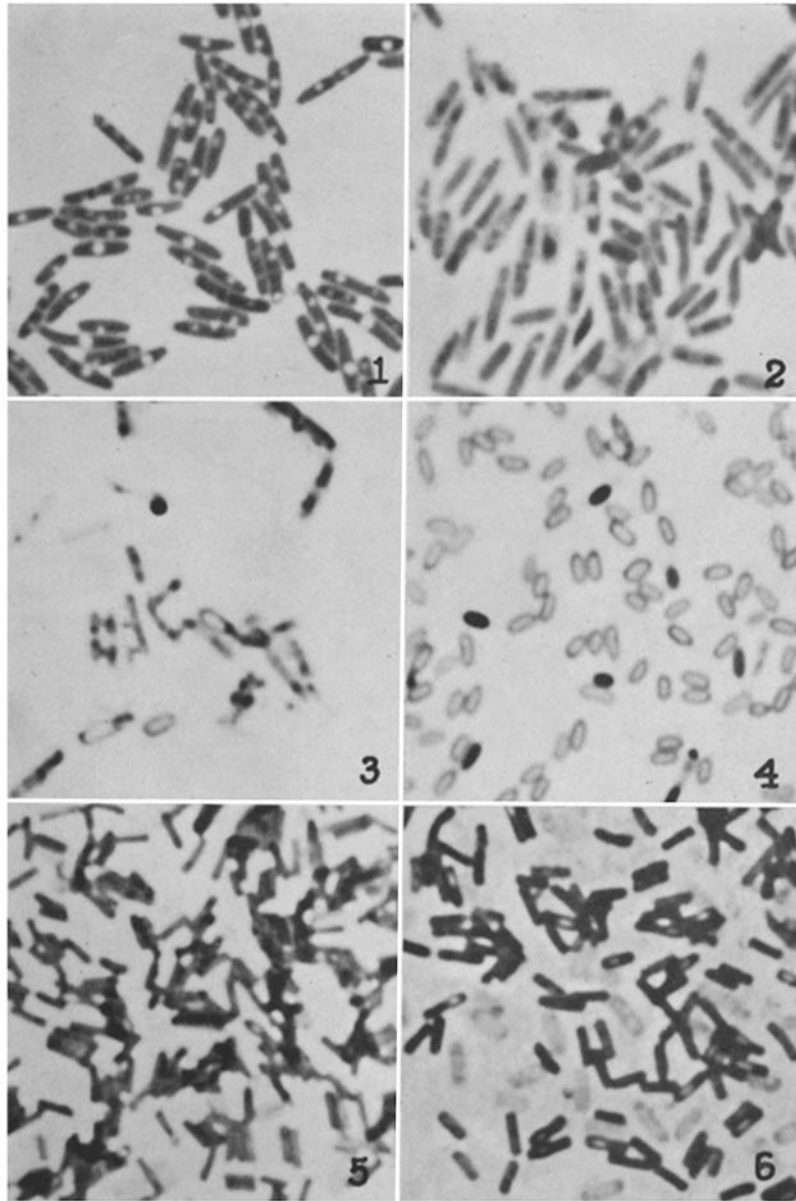
FIG. 2. *B. cereus* culture after 5 hours' shaking with distilled water. Cells staining diffusely, and swelling slightly prior to lysis.

FIG. 3. *B. cereus* culture after 10 hours' shaking with distilled water, showing some free spores and partly lysed cells.

FIG. 4. Complete sporulation of *B. cereus* after 21 hours' shaking with distilled water.

FIG. 5. 36 hour *B. subtilis* culture at time of resuspension in distilled water, already showing signs of sporulation.

FIG. 6. 36 hour *B. subtilis* culture after 4 hours' shaking with distilled water. Partly lysed cells can be seen in the background.



(Powell and Hunter: Sporulation in distilled water)

PLATE 7

FIG. 7. 36 hour *B. subtilis* culture sporulating after 20 hours' shaking with distilled water, and showing massive cell lysis.

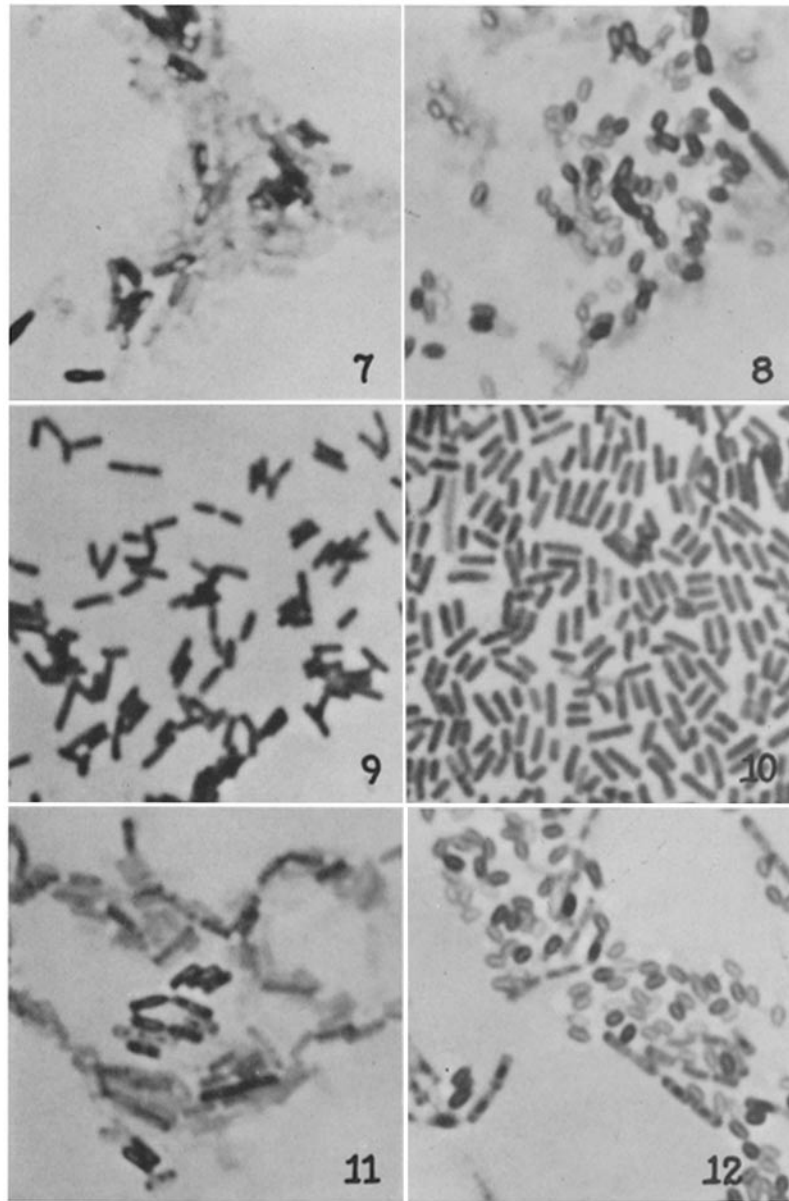
FIG. 8. Practically complete sporulation of 36 hour *B. subtilis* culture after 44 hours' shaking with distilled water.

FIG. 9. 24 hour *B. subtilis* culture at time of resuspension in distilled water.

FIG. 10. 24 hour *B. subtilis* culture after 4 hours' shaking with distilled water showing sporulation and lysed cells.

FIG. 11. 24 hour *B. subtilis* culture after 20 hours' shaking with distilled water showing sporulation and lysed cells.

FIG. 12. Practically complete sporulation of 24 hour *B. subtilis* culture after 44 hours' shaking with distilled water.



(Powell and Hunter: Sporulation in distilled water)