porated into RNA is increased by raising energy intake, in one case by an increase in the total RNA per liver without a change in the percentage of phosphorus atoms incorporated in a given time, in the other case by an increase in percentage incorporation rate accompanied by a smaller change in total RNA per liver.

We are most grateful to Dr R. M. S. Smellie for instructing us in the ionophoretic method of separating ribonucleotides. We also wish to thank Dr Robb of the Statistics Department of Glasgow University for helpful discussions on statistical treatment. One of us (H. N. M.) wishes to acknowledge gratefully receipt of a grant for expenses from the Medical Research Council.

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Biochemical Changes Occurring During the Germination of Bacterial Spores

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(*Received* 24 *October* 1952)

Spores of laboratory strains of *Bacillus subtilis* and *Bacillus megatherium*, incubated with L-alanine and glucose respectively, remain viable, but rapidly lose their resistance to heat (Hills, 1950; Powell, 1951). They also become less refractile and more permeable to stains (Powell, 1951). These changes indicate the first stages of spore germination and are accompanied by a decrease in cell dry weight and an increase in total and amino nitrogen of the suspending medium. It appears that excretion is one of the first changes associated with the germination process, whether this occurs in a medium containing only the specific germination stimulant or in a nutrient tryptic digest broth (T.M.B.), or spontaneously in water suspension as a result of prolonged heat activation. This excretion process and the identification of the excreted solids will be described in detail.

ORGANISMS AND METHODS

Most of the work was done with spores of the laboratory strain of *B. megatherium*. These were obtained by growth on meat extract peptone agar containing 0·2% Lab Lemco, 1% peptone, 0·5% NaCl, and 0·2% glucose (Tarr, 1933). They were reaped after 2 days' growth at 37° and washed at least five times with water. During reaping and the first three washings, suspensions were cooled to 0°, since it had been previously found that some incompletely washed suspensions germinated spontaneously at room temperature. Spores of this organism need glucose specifically for germination and, in some cases, a short preliminary heat treatment, i.e. 30 min. at 60° (Powell, 1951). It has been recently found that if a prolonged heat treatment is given, i.e. 2 hr. at 60°, and the suspension is then centrifuged and resuspended in water, spontaneous germination takes place. Within an hour, more than 90% of the spores lose their heat resistance and stain densely, but are still viable. These changes occur in suspensions as thick as $1{\cdot}5 \times 10^{10}$ spores/ml. Germination exudates were obtained in this way, and also by incubating resting spores with 5 mM-glucose and 30 mM-phosphate buffer, pH 7·3. After 30–45 min. incubation the suspension was centrifuged, and the clear and colourless centrifugate removed for analysis.

A less complete investigation was made with spores of the laboratory *B. subtilis*. These were obtained after 3–4 days' growth at 37° on casein-yeast (CCY) agar (Gladstone & Fildes, 1940). They were washed five times with water, and had no tendency to germinate spontaneously during reaping or after heat treatment. In 30 mM-phosphate with 2 mM-L-alanine and 5 mM-glucose, germination was generally not more than 30–50% in suspensions of 5×10^9 spores/ml., and could only be improved by the addition of disproportionally high concentrations of L-alanine which then obscured subsequent analyses and amino-acid chromatograms. Germination was almost complete in suspensions

of 2×10^9 spores/ml. after 30–45 min. incubation with 2 mM-L-alanine and 5 mM-glucose in 30 mM-phosphate at pH 7·3. Analyses were made on centrifugates from these suspensions.

Total nitrogen. This was determined by the Dumas and the micro-Kjeldahl methods. In the latter method, the catalyst suggested by Chibnall, Rees & Williams (1943) was used.

Amino nitrogen. This was determined by the nitrous acid reaction (Peters & Van Slyke, 1932) and α-amino nitrogen by the method of Pope & Stevens (1939).

Paper chromatography. For the detection of amino-acids, this was carried out as described by Consden, Gordon & Martin (1944) and for purines and pyrimidines as described by Markham & Smith (1949).

Hexosamine. This was determined by the method of Elson & Morgan (1933) with the modification suggested by Immers & Vasseur (1950).

Calcium. This was determined by titration with permanganate after precipitation as oxalate (Kramer & Tisdall, 1921).

The Unicam spectrophotometer was used to measure ultraviolet absorption spectra.

RESULTS

Decrease in dry weight during germination

Excretion during germination of *B. megatherium* was assessed both by measuring decrease in cell dry weight and increase in dry weight of the suspending medium. This was centrifuged off and a sample heated in an oven at 103° for 24 hr. Cell dry weight was similarly determined before and after germination. In the latter case, the cells were washed with 0·9% sodium chloride before drying. Weight changes corresponding with complete germination were calculated after determining percentage germination from stained films. The results obtained are shown in Table 1.

The figures given in the last two columns are derived from the means of duplicate dry weights. The values for gain in weight of the medium are consistently higher than those for loss in weight of the cells. This can partly be accounted for by inadequate washing and retention of saline in the centrifuged cell mass. It is also possible that germination may involve depolymerization, i.e. hydrolytic, processes which might be expected to increase the cell dry weight to a small extent. It was interesting to find that, owing to this weight loss during germination, it was possible to separate germinated from resting spores by centrifugation. During germination, spores of *B. megatherium* became also appreciably darker in colour, so that when a partly germinated suspension was centrifuged, the separation of the cells into two layers could be distinctly seen.

Since germination of spore suspensions thicker than 2×10^9/ml. of *B. subtilis* was poor, even in T.M.B., it was not practicable to determine increase in dry weight of the medium, since this increase, with spore suspensions of 2×10^9/ml., is relatively low. Decrease in cell dry weight at spore concentrations of 2×10^9/ml. was determined both in T.M.B. and the alanine-glucose phosphate medium. It was found to be 26% in the meat digest and 35% in the chemically defined medium.

Excretion of nitrogen-containing compounds during germination

When a suspension of *B. megatherium* spores containing 5×10^9 spores/ml. was incubated for 45 min. with 5 mM-glucose in 30 mM-phosphate, 80% germination occurred. The total N (Kjeldahl) of the medium rose to 9·7 and the amino N to 4·5 mg./100 ml. In a similar experiment with spores of *B. subtilis* and the addition of 2 mM-L-alanine to the above medium, there was 38% germination in 60 min. The total N of the medium increased by 3·8 and the amino N by 2·0 mg./100 ml. Slightly greater nitrogen excretion was found with spores of *B. megatherium* when T.M.B. was the germination medium.

Excretion of ultraviolet-absorbing compounds during germination

Resting spores of *B. subtilis* and *B. megatherium* were suspended at 10^9 spores/ml. in 30 mM-phosphate buffer, pH 7·3, and broken up mechanically at 0° in a tissue disintegrator (Mickle, 1948) with Ballotini beads, size 12. After 45 min. shaking, very few intact cells could be found in a stained film. The disintegrated suspension was centrifuged at high

Table 1. *Decrease in dry weight of* Bacillus megatherium *spores during germination*

Medium (10 ml.)	No. of spores	Spore dry wt. (mg.)	Germination (%)	Change in dry weight for complete germination (mg./100 mg.)	
				Cells	Medium
T.M.B.	$1{\cdot}08 \times 10^{11}$	105	90	−30	+33
	$1{\cdot}15 \times 10^{11}$	108	73	−27	+36
5 mM-Glucose, 30 mM-phosphate, pH 7·3	$1{\cdot}08 \times 10^{11}$	105	69	−29	+33
Distilled water (heat-activated germination)	$1{\cdot}15 \times 10^{11}$	108	95	−26 −25	+33

speed, the clear supernatant solution removed, and its ultraviolet absorption measured throughout the range 2000–3000 A. at 25 A. intervals. A characteristic absorption spectrum appeared with maxima at 2625, 2700 and 2775 A. Essentially the same spectrum was obtained when the media in which germination had occurred were similarly examined (Figs. 1 and 2). It seemed, therefore, that the ultraviolet-absorbing material present in resting spores had been excreted during germination. The identification of this material, thought at first to be a mixture of purines and pyrimidines, as dipicolinic acid, will be described in the following paper (Powell, 1953).

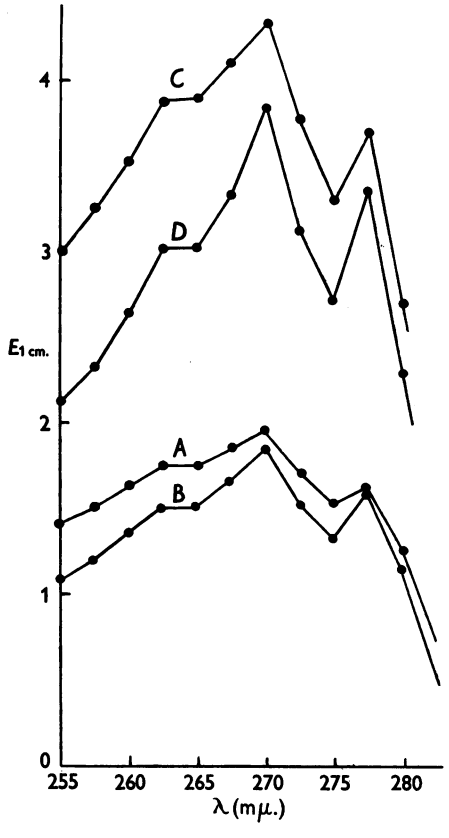

Fig. 1. Curve *A*, ultraviolet absorption of an extract from a disintegrated resting suspension of *B. subtilis* containing 10^9 spores/ml. Curve *B*, absorption of L-alanine phosphate medium after complete germination of *B. subtilis* has occurred at 10^9 spores/ml. Curve *C*, ultraviolet absorption of an extract from a disintegrated resting suspension of *B. megatherium* containing 10^9 spores/ml. Curve *D*, absorption of glucose phosphate medium after complete germination has occurred at 10^9 spores/ml.

Analysis of exudate from Bacillus megatherium *spores during heat-activated germination*

From the above results, it appears very likely that the excretory process associated with germination in both the organisms examined is essentially the same, whatever the medium in which it occurs.

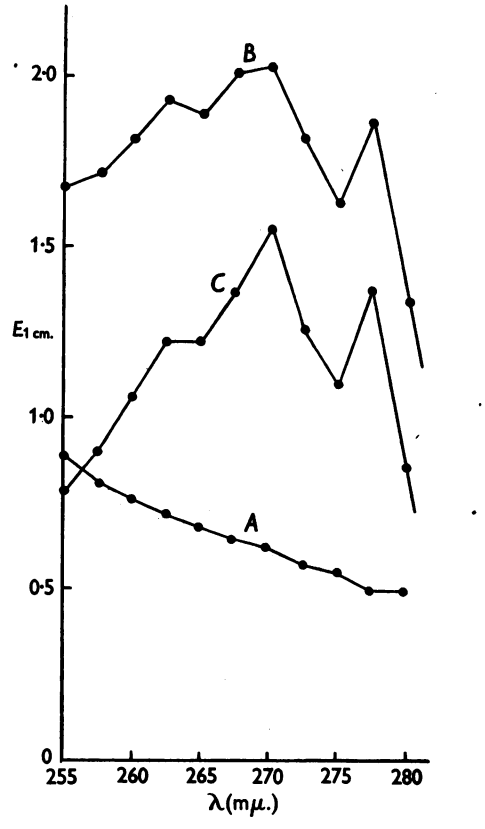

Fig. 2. Curve *A*, ultraviolet absorption of diluted meat digest medium. Curve *B*, absorption of meat digest medium after complete germination of *B. megatherium* has occurred at 4×10^8 spores/ml. Curve *C*, absorption of germination exudate derived by subtracting Curve *A* from curve *C*.

For more detailed investigation of the process, analyses were made of exudates from *B. megatherium* spores germinating spontaneously in distilled water after heat treatment. These exudates, prepared as described above, were filtered through sintered glass and freeze-dried. The resulting white feathery material had the composition C, 37·9; H, 4·9; N, 8·6 (Dumas); Ca, 9·5%. It contained no phosphorus. There was good agreement between values obtained for nitrogen content of the crude exudate by the Dumas and the Kjeldahl techniques. This was surprising in view of a later finding that analysis

Table 2. *Analysis of freeze-dried exudate from heat-activated* Bacillus megatherium *spores*

	Nitrogen (g./100 g.)				
	Total	Ammonia N	Amino N	α-Amino N	Hexosamine (g./100 g.)	Sulphated ash (g./100 g.)
Unhydrolysed	8·48 (Kjeldahl) 8·60 (Dumas)	0·52	3·15	2·48	0	35·2
Hydrolysed	—	—	4·50	2·92	5·70	—

of the pure ultraviolet-absorbing material by the Kjeldahl method gave a value rather less than half of that obtained by the Dumas method. A summary of the analyses made on the crude exudate is given in Table 2.

The results indicated that amino-acids were present (see below), together with small amounts of peptide. The presence of hexosamine was first detected on amino-acid chromatograms of hydrolysed exudate. These compounds and ammonia nitrogen account for about 60 % of the nitrogen present. A further 30 % is supplied by the ultraviolet-absorbing compound, which constituted 50 % of the total excreted solids. Purines and pyrimidines may be present, but their characteristic absorption would be masked by the other ultraviolet-absorbing material. The ash content of the exudate was remarkably high, and could pratically all be accounted for as calcium.

Paper chromatograms of unhydrolysed and hydrolysed exudate were run with phenol and collidine. In the unhydrolysed material, glutamic acid and glycine were present in the largest amounts, together with smaller quantities of alanine, leucine, isoleucine, serine and valine. Arginine, aspartic acid, histidine, lysine, methionine, and tyrosine were also detected. After hydrolysis, the amount of alanine and aspartic acid present had increased markedly, the threonine, αε-diaminopimelic acid and glucosamine appeared. Both glucosamine and αε-diaminopimelic acid appeared to be associated with a non-dialysable peptide fraction, which constituted approximately 20 % of the total solid exudate (see below).

In an attempt to separate and identify the ultraviolet absorbing fraction, paper chromatograms were run in *tert.* butanol/hydrochloric acid and photographed using ultraviolet light (Markham & Smith, 1949). The absorbing material travelled as a single spot, and faster than any of the components of hydrolysed yeast nucleic acid.

Separation of the glucosamine-containing peptide from exudate of germinating Bacillus megatherium

A specimen of the crude freeze-dried exudate was dialysed in a cellophan sac at 0° against distilled water. The sac contents and the diffusate were freeze-dried. The diffusate contained only a trace of hexosamine. The freeze-dried dialysate was dissolved in water and precipitated with 2 vol. of ethanol. The precipitate was centrifuged, washed with 70 % ethanol, dissolved in water, dialysed again and finally freeze-dried. The resulting material gave a positive biuret, and a weakly positive Molisch reaction. It contained 6·9 % N (Kjeldahl). The glucosamine content after 20 hr. hydrolysis at 100° with 3N-hydrochloric acid in a sealed tube was 28 %. This agreed with the value of 26 % reducing sugar found by the Hagedorn & Jensen (1923) method, after 20 hr. hydrolysis at 100° with 0·5N-HCl. Paper chromatography on hydrolysates demonstrated the presence of glucosamine, aspartic and glutamic acids, serine, glycine, αε-diaminopimelic acid, alanine, valine and arginine.

Glucosamine attached to a non-dialysable peptide has also been found in exudates from spores of *B. subtilis* germinating in the L-alanine medium. A crude preparation of this peptide contained 5·25 % N and 22·6 % hexosamine. It also contained aspartic and αε-diaminopimelic acids, alanine, serine, glycine and small amounts of arginine and lysine.

DISCUSSION

It appears that one of the first processes occurring during spore germination is the excretion of solid material to the extent of approximately 30 % of the spore dry weight. Resting spores have a remarkably high specific gravity of 1·46 (McIntosh & Selbie, 1937) and little or no metabolic activity (Keilin & Hartree, 1947; Spencer & Powell, 1952). This, considered together with their heat-resisting properties, suggests that their water content must be extremely low. So far, only one recorded attempt has been made to determine the water content of resting spores and to compare it with that of vegetative cells (Henry & Friedman, 1937). These authors measured the loss in weight which occurred when 'air-dried' cells were desiccated over calcium chloride and then heated at 105–110° for 4 days. They found that 'air-dried' vegetative cells and spores lost approximately the same amount of water, i.e. 16 %, under these conditions. It is certain, however, and the above authors themselves point out, that 'air-dried' cells do not represent a satisfactory starting material, so that the value of these experiments seems somewhat doubtful.

We are attempting in this laboratory to measure the amount of water in resting spores of *B. subtilis* from a comparison of their wet and dry weights. Wet weight is calculated from measurements of spore density and volume. Dry weight is determined by heating a known number of spores at 104° for 2–3 days, followed by desiccation over phosphorus pentoxide *in vacuo* at 78°. Preliminary experiments strongly support the view that resting spores contain little or no water. It would seem that the germination process involves a sudden change in cell permeability followed by a simultaneous exchange of water from the medium for solids from the spore.

Of the solid materials excreted, the calcium, the ultraviolet-absorbing compound identified as dipicolinic acid (Powell, 1953), and the glucosamine peptide are perhaps the most interesting. It has been suggested that the high calcium content of spores may contribute in some way to their heat resistance, especially since thermophilic organisms contain substantially more calcium than mesophilic vegetative organisms (Curran, Brunstetter & Myers, 1943). The loss of calcium associated with loss of heat resistance supports this view. The role of dipicolinic acid in sporulation and development of heat resistance has still to be investigated. Preliminary experiments with spores of other members of the *Bacillus* group indicate the presence of dipicolinic acid in every case. The compound does not appear to be present in vegetative cells of this group.

The glucosamine-containing peptide appearing in the medium during germination is non-dialysable. It appears at a very early stage in the development of the spore, i.e. long before the emergence of the typical vegetative cell and the shedding of the spore coat (Harris & Powell, 1951). It may be derived from the spore integument when this undergoes the radical change in permeability associated with germination. It would be interesting to compare the peptides 'excreted' by these and other members of the *Bacillus* group with a view to the possibility of their possessing specific antigenic properties (Lamanna, 1940).

SUMMARY

1. The germination process in spores of *Bacillus megatherium* and *Bacillus subtilis* is accompanied by a 30 % loss in cell dry weight.

2. The material excreted consists mainly of amino-acids, peptides, hexosamine attached to a non-dialysable peptide, and a substance absorbing strongly in the ultraviolet, with maxima at 2625, 2700, 2775 A.

3. It is suggested that resting spores contain little or no water, and that during germination there is a simultaneous exchange of water from the medium for solids from the spore.

The authors wish to thank Mr F. Belton for growing large spore crops, Dr R. B. Record for freeze-drying spore exudates, Mr S. Bailey and Mr N. Harkness for technical assistance, and Mr F. E. Charlton for a micro-analysis. A specimen of $\alpha\epsilon$-diaminopimelic acid was very kindly provided by Mrs Elizabeth Work. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

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