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The effect of temperature and of cetrimide on the rate of loss of refractility of spores of *Bacillus megaterium*

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The rate of germination of spores of *Bacillus megaterium* at 30° is not significantly different from the rate at 37° but the onset of germination is delayed; outgrowth is normal. At 45° germination of some spores occurs but the rate is much slower than at 37°, and there is no lag; emergence occurs from only a proportion of the germinated spores and after 3 or 4 vegetative cells have been produced, replication ceases. A single regression equation can represent the germination rate of the spores at 37° in the presence of from 0·0005 to 0·02% w/v of cetrimide and in its absence. In 0·0005% w/v of cetrimide, germ cells emerge from some of the germinated spores but many of them become swollen and disintegrate. Concentrations of 0·00125% w/v or more progressively inhibit swelling and completely inhibit emergence.

Campbell (1957) has described some of the criteria of germination of bacterial spores. These criteria include loss of heat resistance and of refractility, acquisition of stainability and decrease of optical density of spore suspensions. In addition, loss of calcium ion and of dipicolinic acid, and loss of resistance to toxic chemicals have been investigated as criteria of germination.

Campbell regarded loss of heat resistance as a basic criterion of germination and the subsequent stages of swelling of the germinated spore, emergence of the germ cell, and its elongation and division, he termed outgrowth.

Powell (1957) argued the convenience of using loss of refractility as a criterion of germination, since an examination by phase contrast microscopy is easy to perform and there is, he stated, a close association between the phase contrast appearance and the heat resistance of a spore. Levinson & Hyatt (1966) made a comprehensive investigation of the sequence in which the different germination events occur. They found loss of resistance to heat and to mercuric chloride were the first germination events to occur and these were followed by loss of dipicolinic acid, the acquisition of stainability, loss of turbidity of a suspension, and phase darkening of the spores. Levinson & Hyatt stressed the effect on the sequence of the germination events, of species differences, the sporulation and germination media used, the temperature of incubation during germination, the method of stabilizing samples taken for quantitative examination, the definition of the end point for the particular germination event and the technique of measurement of the degree of germinative change.

Gould (1964) has described the effect of a number of preservatives on growth from spores of six *Bacillus* spp. and the stage of development inhibited by each substance.

Gibbs (1964) and Thorley & Wolf (1961) have described the effect of change of incubation temperature on the germination of anaerobic and aerobic spores, and have shown that temperature optima for germination exist for the organisms they investigated.

In the work reported in this paper, loss of refractility has been employed as the criterion of germination and the rate of germination of spores of *B. megaterium* ATCC 8245 at 30°, 37° and 45° has been examined. The effect of cetrimide on the rate of germination at 37° of the spores and on the morphology and viability of germinated spores has been investigated.

MATERIALS AND METHODS

Bacillus megaterium ATCC 8245 *spore suspension.* This was prepared using potato agar as described by Chiori (1964). The washed suspension was stored at 4°. Only an occasional phase dark spore was observed when the suspension was examined by phase contrast microscopy. The total count, estimated by means of a 0·1 mm depth haemocytometer chamber was about $2{\cdot}5 \times 10^{10}$/ml.

MRVP broth (Difco Bacto) and *double strength MRVP broth* were autoclaved in 15 ml volumes at 115° for 15 min, *MRVP agar* was prepared by the inclusion of 1·5% w/v of Ionagar No. 2 (Oxoid) in MRVP broth and *Plain agar* was a 1·5% w/v solution of Ionagar No. 2 clarified and sterilized by filtration whilst molten, through a millipore filter (a.p.d. 0·22 μm). *Phenol agar* was prepared by including 0·5% w/v of phenol B.P. in plain agar; cetrimide B.P. and mercuric chloride (Analar) were used for inhibition experiments and for the stabilization of culture samples respectively.

A Patholux research microscope (Vickers Instruments, Metron Works, Purley Way, Croydon, Surrey) was used for phase contrast examinations of spore samples.

Preparation of agar slides. A layer of molten plain agar or phenol agar, about 0·8 mm thick was poured between two sheets of plate glass. When the agar had set, the upper glass sheet was removed and the layer of agar dried for 10 min at 37°. The peripheral regions of the layer were discarded and the remainder was cut into strips about 1·5 cm × 5·5 cm, each of which was transferred to a microscope slide (0·6 to 0·8 mm thick) on which were etched three sequentially numbered circles.

Sampling and stabilization of samples. To estimate the proportion of germinated spores in samples from an incubating suspension of spores in nutrient medium, it is necessary to ensure that germinative processes are stopped immediately after removal of the sample. Three methods were compared during the course of the work (*a*) a loopful of incubating suspension was transferred to a numbered site on a plain agar slide maintained at 4°, and refrigeration continued until microscopic examination (see below) could be made, (*b*) a loopful of incubating suspension was transferred to a phenol-agar slide at room temperature, (*c*) 0·1 ml of incubating suspension was transferred to an ignition tube containing 0·1 ml of 4 mM $HgCl_2$; after mixing, a loopful was transferred to a plain agar slide at room temperature.

When dry (approximately 20 s), each agar slide was covered with a thin cover slip. All slides were stored in large Petri dishes containing moist cotton wool to prevent drying out.

Differential counts of about 1000 spores in a partially germinated suspension were made by each of the three methods; there was no significant difference between the % of phase bright spores stabilized by each of the three methods. There was no increase in the % of phase dark spores after 8 h storage, nor did phase dark spores outgrow. The phenol-agar preparations were the most convenient, and phenol-agar was used for most of the stabilizations.

Estimation of rate of loss of refractility. To 10 ml of double-strength MRVP broth warmed to 37° ± 0·5° in a water bath were added 5·0 ml of sterile water or

of a solution of cetrimide, and 5·0 ml of a spore suspension containing about 10^8 mature spores/ml, which had been previously heat activated at 80° for 10 min and cooled. The mixture was then incubated with shaking at 37°, samples being withdrawn by sterile platinum loop at zero, and at 2 or 3 min intervals, during the subsequent 1 h, and then at 30 min intervals for 4 h. A final sample was withdrawn after 24 h incubation.

Duplicate slide preparations were made at each sampling time. All prepared slides were stored as previously described.

Differential counts of spore samples. Within 2 h of sampling, the slides were examined by phase contrast microscopy using a ×100 "Fluorite" phase contrast objective and ×10 eye-pieces. Photomicrographs were taken of many suitable fields of each sample, so as to have records of from 500 to 700 spores for each. In general, more photomicrographs were taken of the samples in which 40 to 60% of the spores had become dark. The negatives were projected onto white paper, differential counts were made and the % of phase dark spores at each sampling time calculated. Spores which were completely dark or in which the outer phase dark ring of the dormant spore had thickened, were scored as phase dark.

RESULTS

Rate of loss of refractility at 37°. From the estimates of % phase dark spores at each time interval, regressions of % phase dark spores on time were calculated using data representing from about 25 to about 75% of dark spores; six experiments were made.

A variance ratio test showed that the six regression equations could be fitted with a common line having the equation:

$$y = 2{\cdot}78x - 28{\cdot}25$$

observed variance ratio $F_{55,10} = 1{\cdot}49$

tabulated variance ratio

(Fisher & Yates, 1963) $F_{55,10} = 2{\cdot}63$ ($P' = 0{\cdot}05$)

The regression equation constant (−28·25) which determines the position of the regression line can be given expression by the term GT50, i.e. the time required for 50% of the spores to lose refractility. The variance of y, (Vy), the variance of the regression coefficient b, (Vb), the GT50 and its limits of error for the common regression are shown in Table 1.

The plot of rate of loss of refractility is sigmoidal, arising from the heterogeneity of the spore population with respect to microlag (Vary & Halvorson, 1965). (Micro-

Table 1. *Regression equations for the rate of loss of refractility of* B. megaterium *spores incubated at different temperatures in MRVP broth*

Temperature	Regression equation ($y = bx + c$)	Vy	Vb	t-test of difference of b from b at 37°	GT50 and its limits of error at $P = 0{\cdot}95$ (min)	t-test of difference of c from c at 37°
30°	$y = 2{\cdot}68x - 49{\cdot}2$	5·6	0·017	0·74 (n = 75)	37·0 ± 2·1	6·8 (n = 75)
37°	$y = 2{\cdot}78x - 28{\cdot}25$	3·95	0·0012	—	28·15 ± 1·4	—
45°	$y = 1{\cdot}24x + 1{\cdot}0$	2·86	0·005	19·3 (n = 74)	39·2 ± 3·3	7·7 (n = 74)

n = degrees of freedom.
For n = 74 and 75, $t = 1{\cdot}99$ at $P' = 0{\cdot}05$ (Fisher & Yates, 1963).

lag can be interpreted as the time, from the commencement of incubation, required for any particular spore to commence phase darkening.) The progressively slower rate of phase darkening in the spore population after about 75% had lost refractility which we observed was therefore to be expected, and the small proportion (up to 3%) of spores which failed to become phase dark even after several hours incubation may be regarded as superdormant (Gould, Jones & Wrighton, 1968).

Effect of temperature change. Estimations of the rate of loss of refractility at 30° and at 45° were made and the data are shown in Table 1.

Rate of loss of refractility in the presence of cetrimide. This was investigated by replacing the 5 ml of sterile water in the normal germination procedure, by 5 ml of a sterile solution of cetrimide. The cetrimide concentration in the medium in each of the seven experiments made was (% w/v), 0·0005, 0·00125, 0·0025, 0·005, 0·02, 0·1 and 0·25 respectively.

A regression equation was calculated for each experiment and the data are shown in Table 2. The 13 equations (6 for normal germination and 7 for cetrimide germination) could be fitted with a common regression coefficient:

observed variance ratio $F_{12,105} = 1{\cdot}58$
tabulated variance ratio $F_{12,105} = 1{\cdot}89$ ($P' = 0{\cdot}05$)

Table 2. *Regression equations for rate of loss of refractility of* B. megaterium *spores incubated at* 37° *in MRVP broth containing cetrimide*

Cetrimide % w/w	Regression equation ($y = bx + c$)	Vy	Vb	GT50 and its limits of error at $P = 0{\cdot}95$ (min)
0·0005	$y = 2{\cdot}91x - 28{\cdot}5$	9·9	0·024	27·0 ± 2·70
0·00125	$y = 2{\cdot}90x - 33{\cdot}8$	8·7	0·023	28·9 ± 2·47
0·0025	$y = 2{\cdot}94x - 35{\cdot}6$	13·7	0·032	29·1 ± 3·40
0·005	$y = 3{\cdot}14x - 39{\cdot}6$	13·9	0·043	28·5 ± 2·96
0·02	$y = 3{\cdot}08x - 38{\cdot}6$	5·1	0·021	28·8 ± 1·73
0·1	$y = 2{\cdot}78x - 20{\cdot}3$	12·7	0·034	25·3 ± 3·22
0·25	$y = 2{\cdot}91x - 15{\cdot}9$	11·6	0·045	22·6 ± 3·01
Nil (Common regression for 6 experiments)	$y = 2{\cdot}78x - 28{\cdot}25$	3·95	0·001	28·15 ± 1·40

The variance of the regression constants was too great for a common equation to be fitted:

observed variance ratio (with respect to constants) $F_{12,105} = 23{\cdot}3$

After omission of the regressions for 0·25% and 0·1% w/v cetrimide germination, it was found that the remaining 5 cetrimide and 6 normal regressions could be represented by the single equation $y = 2{\cdot}84x - 29{\cdot}6$

observed variance ratio $F_{91,20} = 1{\cdot}67$
tabulated variance ratio $F_{91,20} = 1{\cdot}69$ ($P' = 0{\cdot}05$)

Outgrowth of vegetative cells. Outgrowth is regarded as embracing pre-emergent swelling of the germinated spore, emergence and elongation of the new cell and cell division (Hitchins, Gould & Hurst, 1963). Within 30 min incubation at 37°, about half of the *B. megaterium* spores had become phase dark and many of the dark spores had begun to swell (Fig. 1A); the degree of swelling corresponded to

germination swelling as reported by Hitchins & others (1963). After 1 h virtually all the spores were phase dark and most of them exhibited considerable one-sided swelling (Fig. 1B) corresponding to the pre-emergent swelling described by Hitchins & others (1963). Within 3 h, almost all dark spores had produced a germ cell and about half of the germ cells had divided (Fig. 1C).

At 45° most of the emerged germ cells were slightly swollen and after 24 h incubation, most had replicated but had produced only 3 or 4 vegetative cells, many of which were distorted or had disintegrated (Fig. 1D). At 30° each of the stages of outgrowth occurred later than at 37° but the outgrown cells appeared normal.

No emergence of germ cells occured in MRVP broth containing 0·00125% w/v or more of cetrimide, and little or no swelling of the spores occurred. Even in

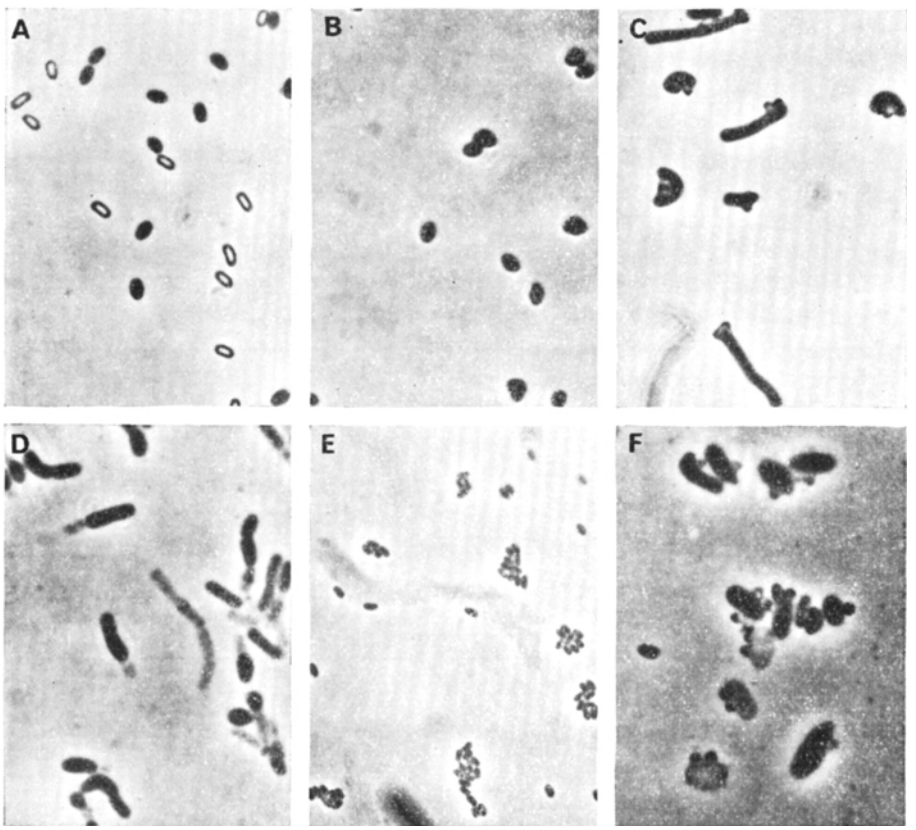

FIG. 1. A. Spores incubated in MRVP broth at 37° for 28 min, showing phase bright and phase dark spores exhibiting varying degrees of enlargement (×1750).

B. Spores incubated in MRVP broth at 37° for 50 min, showing pre-emergent swelling (×1750.)

C. Spores incubated in MRVP broth at 37° for 170 min, showing emerged germ cells and vegetative replication (×1750).

D. Spores incubated in MRVP broth at 45° for 24 h, showing distorted germ cells and vegetative cells, and disintegrated cells (×1750).

E. Spores incubated at 37° for 24 h in MRVP broth containing 0·00125% w/v of cetrimide, showing clumping and only partial enlargement (×1100).

F. Spores incubated at 37° for 4 h in MRVP broth containing 0·0005% w/v of cetrimide, showing disintegration of germ cells (×1750).

0·00125% w/v of cetrimide, no pre-emergent swelling was seen (Fig. 1E). At the higher cetrimide concentrations (0·1% w/v and 0·25% w/v) the microgermination time, i.e. the period commencing at the inception of phase darkening of each spore and ending when the spore is completely phase dark (Vary & Halvorson, 1965), was prolonged, being 4–5 min compared with about 1 min in the absence of cetrimide. A proportion of spores never became completely phase dark in these concentrations of cetrimide.

In 0·0005% w/v of cetrimide, germination swelling and pre-emergent swelling appeared to be normal and to occur at a normal rate. Some germ cells emerged and replicated in an apparently normal manner, whilst others became swollen and then disintegrated, often before they had completely emerged from the germinated spore (Fig. 1F).

DISCUSSION

Loss of refractility by a spore has been used as a criterion of germination to determine the percentage of spores that have germinated after a measured period of incubation. From the data, regressions representing the rate of germination of the spore population have been calculated. A comparison of the regression equation for rate of germination at 37° (regarded as standard), with those for germination at 30 and 45°, shows that at 45° a proportion of the spores germinated soon after incubation commenced and that the rate of germination, 1·24 spores/min, was significantly lower than at 37° (2·78 spores/min) ($t = 19·3$). The GT50 value (39·2 min) was significantly greater than at 37° (28·15 min). Emergence occurred at 45° from only a proportion of the germinated spores; many of the germ cells were swollen and replication ceased after only 3 or 4 cells had been produced.

At 30°, over the range 25 to 75% of germinated spores, the rate of germination was not significantly lower than at 37° ($t = 0·74$). The GT50 value was significantly greater than at 37° ($t = 6·8$), due to lag in the onset of germination; the theoretical lag (estimated by solving the regression equation for x at $y = 0$) was 18·3 min, compared with 10·2 min at 37°. Outgrowth was apparently normal, although delayed.

Inclusion of from 0·0005% w/v to 0·02% w/v of cetrimide in the medium did not produce a significant change in the rate of germination of spores at 37°, or in the GT50 values; a single regression equation can represent the rate of germination in MRVP broth at 37° in either the absence or the presence of cetrimide over this range of concentration.

In the presence of 0·25% w/v of cetrimide, germination commenced earlier (theoretical lag 5·5 min) than in all other cetrimide experiments (mean lag 11·0 min) but the rate of germination was normal; the theoretical lag in the case of 0·1% w/v cetrimide (7·3 min) was also less than normal. In these two experiments, the effect of the prolonged microgermination time was to produce only very gradual phase darkening of each individual spore so that a decision to count a spore as phase bright or phase dark was very subjective.

Cetrimide, 0·0005% w/v, did not inhibit swelling of incubated spores but inhibited the emergence of germ cells from many of the spores. Most of those cells which did emerge were sensitive to this low level of cetrimide and became swollen and then burst. Concentrations of 0·00125% w/v or more, progressively inhibited swelling, completely inhibited emergence, and induced clumping of the spores (Fig. 1E).

These results show that the germinative processes of *B. megaterium* spores are not sensitive to concentrations of cetrimide of up to 0·02% w/v. The outgrowth processes

of the germinated spore on the other hand, are sensitive to 0·00125% w/v of cetrimide or more, and indeed work which we are at present carrying out, suggests that the germinated spore is in fact killed by low concentrations of cetrimide. Levinson & Hyatt (1966) have reported the sensitivity of germinated but not of ungerminated spores of *B. megaterium* to 2 mM $HgCl_2$ and Vinter (1970), has reviewed the inhibition both of germination and of outgrowth. Our results using 0·0005% w/v of cetrimide indicate that emerged germ cells are even more sensitive than are germinated spores.

In short, mature spores of *B. megaterium*, germinated spores and vegetative cells are progessively more sensitive to cetrimide.

Parker, Barnes & Bradley (1966), using a Coulter counter to detect spore swelling, reported swelling of an order that must be interpreted as emergence or as pre-emergent swelling in *B. subtilis* NCTC 3610 spores incubated in broth containing 0·02% w/v of cetrimide. Parker (1969) reported finding up to 5% non-proliferating, swollen and distorted forms when he repeated the work. We detected some swelling, but not outgrowth of *B. subtilis* spores supplied by Parker when they were incubated in similar conditions and examined by the method we have described; the spores behaved similarly to our *B. megaterium* spores, i.e. semi-abortive emergence occurred only when the cetrimide concentration was reduced to 0·001% and 0·0005%. It may be that the swollen cells which Parker detected were already present in his suspension. At any rate, Parker reported no extensive emergence of germ cells and, indeed, only 27% increase in volume of his spores during germination in 0·02% w/v of cetrimide, and in these respects, his results are similar to ours. The use of thin strips of plain agar or agar containing phenol to mount either stabilized or unstabilized spore samples, we found preferable to other methods of mounting samples, because it preserved the phase properties of the spores throughout each experiment and also prevented Brownian movement of the spores; in addition it was not necessary to re-hydrate the spores for phase microscopy, as is necessary when spore samples are allowed to dry on slides or coverslips.

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