Factors Affecting the Germination of Thick Suspensions of *Bacillus subtilis* Spores in L-Alanine Solution

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SUMMARY: The rate of germination (defined as loss of heat-resistance accompanied by change in staining properties under specified conditions, with maintenance of viability) of thick suspensions of *Bacillus subtilis* spores in phosphate-buffered L-alanine solution increased with the time from harvesting. The maximum rate of germination was reached after about 20 days' storage in water at 20°. This effect could be retarded, but not reversed, by storage at low temperatures. The rate of germination may be temporarily accelerated by heat treatment. Germination was considerably retarded after treatment with mercuric chloride, and was completely inhibited by 8-hydroxyquinoline (oxine) and by 2:3-dimercaptopropanol (BAL) at 10 mM concentration. The latter effect was partially reversed by the addition of metals.

Using dilute spore suspensions of *Bacillus subtilis* (10^4 spores/ml.) Hills (1950) found that 80 % loss of heat-resistance occurred during 30 min. incubation at 35° in 0.5 mM L-alanine buffered with 33 mM phosphate at pH 7.3. The present report concerns germination in thicker suspensions, i.e. 5×10^9 spores/ml., with a view to the study of the biochemical changes which accompany loss of heat-resistance in the above simple medium.

EXPERIMENTAL

A laboratory strain of *B. subtilis* was used throughout. Suspensions containing at least 95 % spores were obtained by growth on CCY agar (Gladstone & Fildes, 1940) at 37° for 12 days. The spores were washed five times with distilled water after harvesting. Their age was measured from the day of reaping, and storage was at room temperature (20°) unless otherwise stated. No heat treatment was given after harvesting except that specifically stated in an experiment.

The percentage germination was at first determined by plate counts on peptone agar before and after heating at 60° for 15 min. (Hills, 1950). Later, it was found that when films of the L-alanine-treated spores were made, fixed and stained with hot carbol fuchsin and methylene blue, most of the cells appeared elongated and stained uniformly bluish purple or showed a central blue-staining body (Pl. 1). These were readily distinguishable from the unchanged spores, and their percentage occurrence could be determined by direct counting.

The details of the staining procedure were as follows. Films were treated for 5 min. with hot carbol fuchsin, then washed thoroughly with hot water. Nigrosin (1 %) was then poured on, and left for 2 min. After washing in cold water, the slide was stained with methylene blue for 2 min. It was found important to avoid overstaining with carbol fuchsin since differentiation between the two forms was then very poor. Twenty-two samples of partially germinated suspensions were stained and counted in this way, and the proportion of the blue-staining forms determined. The value obtained agreed very well with that

for non-heat-resisting cells, determined by the plating method in the same samples (Fig. 1). The total number of cells counted in both methods was about 600, and the blue-staining or non-heat-resisting cell count ranged from 300 to 500, so that the standard error of counting was 4–6 %. It thus appears that loss of heat-resistance can be estimated equally well by counting the cells which have undergone the above change in staining properties, which may be connected with a sudden increase of cell permeability. The plating method was therefore abandoned in favour of the less laborious staining technique, except in those cases where it was necessary to determine the viability of the germinated cells.

It was also observed that the turbidity of a spore suspension markedly decreased on incubation with L-alanine. This phenomenon was obviously due to the decrease in refractive index and therefore in the amount of light scattered by the spore during germination. Measurements of percentage transmission of germinating suspensions were made at 610 m μ . in a Coleman spectrophotometer. Using this simple turbimetric method at 37°, many readings may be made at short intervals, and the effect of storing and pre-heating of spores very clearly demonstrated (see below).

RESULTS

The germination rate of thicker spore suspensions, i.e. 10⁹ spores/ml. in 0.5 mM L-alanine at 35° was slower and less complete than that of dilute suspensions. It could be increased by raising the L-alanine concentration to 5 mM and adding 50 mM glucose, the mixture being buffered by 33 mM phosphate at pH 7.8 as before. Under these conditions, 80–90 % germination occurred in 30 min. and plate counts showed that even after 2–3 hr. incubation, all the cells remained viable.

The effect of substituting acetate and bicarbonate buffer for phosphate is shown in Table 1. The buffer-capacity of 33 mM acetate at neutrality is low, so that here the L-alanine concentration was decreased to 0.5 mM. The germination rate in this mixture without glucose was at least equal to, or possibly greater than, that in phosphate buffer of the same pH, and all the cells were viable at the end of the experiment. Germination proceeded as well in bicarbonate as in phosphate buffer at pH 8.1. In this case, glucose was present and the L-alanine concentration was 5 mM, so that germination was more rapid and extensive than in the acetate experiment at pH 6.8. The difference in percentage germination between the phosphate-bicarbonate and the phosphate-acetate comparison experiments cannot be ascribed to the difference in pH, for the germination of a 10⁴ spores/ml. suspension in 0.5 mM L-alanine measured at 1.5 hr. in phosphate buffer of pH ranging between 6.0 and 8.0, did not vary appreciably between pH 6.5 and 8.0.

The germination of freshly harvested spores was very slow and incomplete even in 5 mM L-alanine and glucose. On storing the aqueous spore-suspension at room temperature the germination rate gradually increased to a maximum at about 20 days (Fig. 2). For example, a 3-day-old suspension showed only

Table 1. Comparison of germination of spores of *Bacillus subtilis* on incubation in acetate-, bicarbonate- or phosphate-buffered L-alanine solutions

(Temperature of incubation 35°; samples removed at intervals.)

Composition of incubation solution	pH	Period of incubation (min.)	Film counts total/germinated	Germination (%)
33 mM phosphate 0.5 mM L-alanine	6.86	10	689/260	38
		20	716/387	59
		40	665/384	58
		80	738/446	60
33 mM acetate 0.5 mM L-alanine	6.81	10	627/262	42
		20	620/402	65
		40	585/433	73
		80	598/429	72
33 mM phosphate 5 mM L-alanine 50 mM glucose	8.10	18	701/566	81
		30	706/635	90
		60	678/629	93
		100	657/615	94
33 mM bicarbonate 5 mM L-alanine 50 mM glucose	8.10	18	750/559	75
		30	657/506	77
		60	624/493	78
		100	636/529	83

Fig. 1

Fig. 2

Fig. 1. Comparison of percentage germination in twenty-two samples measured by the filming and plating techniques.

Fig. 2. Effect of increasing periods of storage (3 to 25 days at 20°) on the germination of *B. subtilis* spores in 33 mM phosphate, 50 mM glucose and 5 mM L-alanine at pH 7.3 and 35°.

10 % germination after 15 min. incubation, but after 25 days' storage at 20°, 75 % germination occurred in 15 min.

This spontaneous increase in germination rate during storage, conveniently referred to as ageing, was slower at low temperatures. Table 2 shows the germination rate of two samples of the same suspension, one of which had been stored at 20°, the other at 4° for 31 days, compared with the germination rate

of the same suspension when freshly reaped. Storage of aged cells at low temperature did not, however, result in a decreased germination rate. Neither was it affected by a 3-fold washing of an aged suspension (25 days at 20°). Ageing was therefore not due to breakdown of some spores with loss of diffusible constituents which stimulated the germination of the remainder.

Table 2. *The effect of storage of Bacillus subtilis spores in water at 20° and 4°*

(Germination was examined by incubation at 35° in phosphate buffer (33 mm; pH 7·3) containing L-alanine (5 mm) and glucose (50 mm). Samples were examined after different periods of incubation.)

Treatment of spore suspension	Period of incubation (min.)	Film counts total/germinated	Germination (%)
Control before storage	15	—	10
	30	519/87	15
	60	752/373	50
	120	742/341	46
Stored for 31 days in water at 20°	15	690/497	72
	30	626/515	82
	60	656/564	86
	120	703/604	86
Stored for 31 days in water at 4°	15	633/114	18
	30	739/283	38
	60	543/321	61
	120	596/406	68

In order to determine whether spores would age in the above sense, on prolonged contact with the CCY agar on which they had been grown, the germination of a 3-day-old suspension, reaped after 30 days' incubation, was measured by the staining method; 5, 30 and 72 % germination occurred at 15, 30 and 60 min. respectively, which is a performance characteristic of a still 'young' suspension.

The effect of ageing on germination was also demonstrated by turbidity measurements during incubation with L-alanine. Fig. 3 shows that the rate of increase of light transmission was very much greater in the case of aged than of freshly reaped spores.

A temporary increase in the rate of germination was produced by heating an aqueous spore suspension at 60° before its addition to the buffered alanine. Table 3 compares the effect of pre-heating a freshly reaped, i.e. a 3-day-old spore suspension for 15 min., and for 3·5 hr. In Fig. 4, the result of a similar experiment with a 7-day-old spore suspension is shown, the turbidity of the suspension being measured at short intervals during germination. Here, the times of pre-heating were 30 min. and 3·5 hr. The effect of heat treatment was almost completely lost in about 48 hr. (Table 3), so that the heat-activation process seems not to involve the same mechanisms as the ageing process.

In order to test for germination stimulants produced in spores during heating, a 10⁹ spores/ml. suspension, heated for 2 hr. at 60°, was broken up with glass beads in a Mickle tissue disintegrator (Mickle, 1948). After centrifuging and Seitz filtration, 1 ml. of the extract was added to 9 ml. of a 10⁴/ml. spore sus-

Table 3. *Heat activation of germination of Bacillus subtilis spores*

(The spore suspensions were treated as shown. The degree of germination after different periods of incubation as 35° in phosphate buffer (33 mm; pH 7.3) containing L-alanine (5 mm) and glucose (50 mm) was then measured.)

Treatment of spore suspension	Period of incubation (min.)	Film counts total/germinated	Germination (%)
Control (age 3 days)	15	667/70	11
	30	623/179	29
	63	643/266	41
	100	603/252	42
15 min. pre-heated at 60°	15	592/67	13
	30	622/339	38
	63	612/330	54
	100	625/333	53
3.5 hr. pre-heated	15	623/317	49
	30	662/440	66
	63	695/498	72
	100	650/474	73
48 hr. after 3.5 hr. pre-heating	15	525/89	17
	30	577/222	38
	63	590/352	60
	100	570/312	55

Fig. 3

Fig. 4

Fig. 3. Comparison of the germination rates of a 7-day-old ($\times$ — $\times$) and a 38-day-old ($\bullet$ — $\bullet$) spore suspension of *B. subtilis* (storage at 20°) in 33 mm phosphate, 50 mm glucose and 5 mm L-alanine at pH 7.3 and 35°. Measurements of percentage transmission made at 610 m μ .

Fig. 4. The effect of pre-heating for 30 min. ($\circ$ — $\circ$) and for 3.5 hr. ($\times$ — $\times$) on the germination of *B. subtilis* spores in 33 mm phosphate, 50 mm glucose and 5 mm L-alanine at pH 7.3 and 35°. Control unheated spores, $\bullet$ — $\bullet$. Measurements of percentage transmission made at 610 m μ .

pension in phosphate buffer. Thus there was available to each spore an amount of extract equivalent to 10^4 heated spores. Even after incubation for 2 hr. at 35°, there was no evidence of germination, as measured by the plating technique. Similarly, the germination of a 10^4 /ml. spore suspension after 1 hr. incubation in 0.5 mm L-alanine was not stimulated by the addition of pre-heated spore

extract. In the latter experiment there was available to each spore an amount of extract equivalent to 8×10^4 heated spores.

Effect of certain enzyme inhibitors

A few experiments were done to determine the effects of certain enzyme inhibitors on the germination process in L-alanine. Keilin & Hartree (1947) showed that the germination of *B. subtilis* spores in glucose yeast extract medium was completely inhibited by a $20 \mu\text{M}$ concentration of 8-hydroxy-quinoline (oxine). The inhibition was removed by washing and re-suspending the spores in fresh medium. These authors generally used the sudden increase in rate of oxygen uptake accompanying proliferation as an index of germination; in the oxine experiment, however, the inhibition was 'confirmed by microscopic examination'. The resting metabolism of both the spore and the vegetative cell was found to be considerably less sensitive to oxine, a concentration of 1 mM being required to decrease the rate of oxygen uptake by 80 %. The germination of *B. subtilis* spores in the present investigation, as measured by both the staining and the plating techniques, was unaffected by 1 mM oxine and all the cells remained viable. In the presence of 10 mM oxine germination was completely inhibited. In these experiments the spores in $10^9/\text{ml}$. suspension were first incubated with glucose, buffer and oxine for 20 min.; the L-alanine was then added, and germination measured after a further 30 and 60 min. incubation.

Using the same conditions as in the above experiment, similar results were obtained with 2:3-dimercaptopropanol (BAL). This compound inhibits certain metal-activated enzyme systems at concentrations of 1–10 mM (Webb & van Heyningen, 1947; Barron, Miller & Meyer, 1947). Concentrations of BAL up to 2 mM did not appreciably diminish spore germination as indicated by staining. On increasing the BAL concentration, inhibition became noticeable at 4 mM, and complete at 10 mM. A partial reversal of the inhibition was obtained by the addition of soluble salts of zinc, magnesium, copper and iron. The germination thus observed after 30 and 60 min. incubation varied between 10 and 30 %. It was not possible to make satisfactory counts with these suspensions since even after treating the slide with dilute acid to remove precipitated BAL-metal complex, the cells remained in clumps.

To test whether the viability of spores was affected by concentrations of BAL which inhibited germination, a 10^9 spores/ml. suspension was incubated with 10 mM BAL in L-alanine glucose buffer mixture for 30 min. The suspension was also treated with 10 mM BAL alone for 30 min. Both samples were then diluted $1/10^6$ and plated. In the former case the viable count had fallen by 20 %, but remained unaltered in the latter.

The effect of pre-treatment of spores with mercuric chloride before incubation with L-alanine was also studied. A 10^9 spores/ml. suspension in 5 % mercuric chloride was allowed to stand at room temperature for 40 min. after which the mercuric chloride solution was removed, and the spores washed four times with water. No visible colonies were produced by plating the treated suspension on peptone agar. When, however, the 'non-viable' suspension was

incubated with L-alanine and glucose, within 60 min. 80 % of the spores showed the change in staining properties associated with germination. The process was, however, markedly slower than that in a control suspension (Table 4).

With regard to the viability of mercuric chloride treated spores, 80 % of the original viable count was reached when the spores were plated on peptone agar to which 0.1 % sodium thiolacetate had been added. The reviving effect of 0.1 % BAL was also tested, but found to be less than that of thiolacetate. This may be due to the decomposition of BAL during the drying of the plates; the

Table 4. *Germination of mercury-treated Bacillus subtilis spores*

(Spores (10^9 /ml.) in 5 % mercuric chloride for 40 min. at room temperature. Spores then centrifuged out and washed four times with 20 ml. lots of water. Effect on germination examined by incubation at 35° in phosphate buffer (33 mM; pH 7.3) containing L-alanine (5 mM) and glucose (50 mM), samples being examined after the periods shown.)

Treatment of spore suspension	Period of incubation in buffer (min.)	Film counts total/germinated	Germination (%)
Control	15	735/461	63
	30	579/463	80
	60	664/545	82
Mercury treated	15	—	10
	30	671/339	50
	60	747/594	80

medium became heavily clouded with sulphur. A mercury-treated suspension which had been incubated with L-alanine for 1 hr., and showed 80 % change in staining properties, was also tested for viability on thiolacetate agar. Here, only 15 % revival was obtained. This was slightly increased, however, by including 0.1 % thiolacetate in the final dilution, which was allowed to stand for 1 hr. before plating. When BAL was substituted for thiolacetate in the plated dilution, 50 % revival was obtained.

DISCUSSION

Curran & Evans (1945) showed that pre-heating stimulated the germination of certain thermo-tolerant bacteria, e.g. *Bacillus coagulans* and *B. calidolactis*. A similar effect was observed by Goddard (1939) in the germination of ascospores of *Neurospora tetrasperma*. Here it was found that the heated spores became deactivated on storage for a few hours under anaerobic conditions, or in the presence of cyanide, but could be reactivated by further heat-treatment. With regard to the mechanism of heat-activation, it is reasonable to apply the suggestion made by Goddard (1939), namely, that the rate of spore germination may be at least partly determined by the concentration of a stimulatory compound in the spore, and that the supply of this compound is limited by a heat-activated reaction.

The irreversible ageing process is more difficult to explain. Spores which remain in contact with the solid medium for long periods germinate far less

readily in L-alanine solution than do those which have been stored in watery suspensions for the same period. It is possible that the newly formed spore contains substances which antagonize its germination and that these substances are lost when the spore is harvested, but not when it remains in contact with the medium in which it has grown and which may now contain an unfavourable balance of nutrients as well as products of metabolism. Since the ageing effect can be retarded by storage at low temperatures, it may be suggested that the inhibitory substance is lost by a heat-activated adsorption process (Taylor, 1932).

It is interesting to note that Schwann (1924) found that in the case of *Bacillus anthracis*, the percentage germination as directly observed on nutrient agar decreased from 95 % in young spores, i.e. up to 7 days old, to 55 % after storage for a year. In these experiments, however, germination was taken as the appearance of the first cell division, and the ageing process took place under drying conditions, so that these results cannot be directly compared with those reported here.

Spore germination in L-alanine is comparatively insensitive to oxine, a concentration of 10 mM being required for complete inhibition of the change in heat resistance and staining properties. This concentration is high compared with that found by Keilin & Hartree for *B. subtilis* spores in yeast extract medium. These authors, however, included growth and proliferation in their criterion of germination, and it is likely that the two latter processes are more sensitive to oxine than is the initial change involving loss of heat-resistance. The concentration found in the present work to be necessary for inhibition of germination is of the same order as that found by Keilin & Hartree to inhibit the resting metabolism of the spore, i.e. the respiration in glucose and phosphate buffer.

The effect of BAL on spore germination is very similar to that of oxine. Both these compounds readily form complexes with metals and may be expected to inhibit those enzyme systems which are metal-activated. The reversal of BAL inhibition by addition of excess of metal salts has not been satisfactorily assessed.

The behaviour of mercury-treated spores with L-alanine is surprising. Since a high proportion of spores can be revived after mercury treatment, it seems fairly certain that the change in staining properties is a genuine vital process, and not due to damage to the cell by mercury. The results suggest that the change in heat-resistance can occur in cells which are incapable of growth and division; this is also suggested by the experiments with oxine. It may, however, be argued that the mercury ion does not, in fact, enter the spore, but remains attached externally to the spore coat and exerts its bacteriostatic effect during the next stage of development. Germination is appreciably slowed by pre-treatment with mercury salt, but it cannot be proved that the phenomenon is due to entry of mercury and not to a change in permeability of the spore-wall caused by combination with mercury.

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REFERENCES

- BARRON, E. S. G., MILLER, Z. B. & MEYER, J. (1947). The effect of 2:3-dimercapto-propanol on the activity of enzymes and on the metabolism of tissues. *Biochem. J.* **41**, 78.
- CURRAN, H. R. & EVANS, F. R. (1945). Heat activation inducing germination in the spores of thermotolerant and thermophilic aerobic bacteria. *J. Bact.* **49**, 335.
- GLADSTONE, G. P. & FILDES, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.
- GODDARD, D. R. (1939). The reversible heat activation of respiration in *Neurospora*. *Cold Spr. Harb. Sym. quant. Biol.* **7**, 362.
- HILLS, G. M. (1950). Chemical factors in the germination of spore-bearing aerobes: observations on the influence of species, strain, and conditions of growth. *J. gen. Microbiol.* **4**, 88.
- KEILIN, D. & HARTREE, E. F. (1947). A comparative study of spores and vegetative forms of *Bacillus subtilis*. *Antonie v. Leeuwenhoek, J. Microbiol. Serol.* **12**, 115.
- MICKLE, H. (1948). Tissue disintegrator. *J. R. micr. Soc.* **68**, 10.
- SCHWANN, M. B. R. (1924). On the germination period and mortality of the spores of *Bacillus anthracis*. *J. Path. Bact.* **27**, 130.
- TAYLOR, H. S. (1932). The adsorption of gases by solids. *Trans. Faraday Soc.* **28**, 131.
- WEBB, E. C. & VAN HEYNINGEN, R. (1947). The action of British anti-lewisite on enzyme systems. *Biochem. J.* **41**, 74.

EXPLANATION OF PLATE

Bacillus subtilis spores showing densely staining forms after incubation at 35° for 15 min. in 33 mM phosphate, 50 mM glucose and 5 mM L-alanine and stained with carbol fuchsin and methylene blue. Magnification, × 2850.

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J. F. POWELL—FACTORS AFFECTING THE GERMINATION OF THICK SUSPENSIONS OF *BACILLUS SUBTILIS* SPORES IN L-ALANINE SOLUTION. PLATE I