Activation of Spores of *Bacillus cereus* by γ -Radiation

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SUMMARY

Spores of *Bacillus cereus* strain PX which were exposed to 0.02, 0.06, 0.18, 0.54 and 1.08 Mrad of γ -radiation became progressively more activated, i.e. they germinated more rapidly in the presence of germinants (e.g. L-alanine, inosine, *n*-dodecylamine, calcium dipicolinate) than did unirradiated spores. Heat- and radiation-activation differed in that irradiated spores but not heated spores germinated faster than untreated control spores in *n*-dodecylamine. The two methods of activation were similar in that both sorts of activated spores could be de-activated, and heated and irradiated spores both had increased contents of titratable thiol groups as compared with untreated spores. Germination-like changes still occurred rapidly in spores which had been subjected to doses of γ -radiation sufficient to render them non-viable. Ionizing radiation, heat and other agents which activate spores probably do so by changing the tertiary structure of spore macromolecules and thereby exposing previously masked reactive sites which are important in germination.

INTRODUCTION

Spores of many bacteria only germinate completely and at maximal rate when first activated by some treatment such as: heating at sublethal temperatures (Curran & Evans, 1945); treatment with reducing agents (Keynan, Evenchik, Halvorson & Hastings, 1964) or calcium dipicolinate (Lee & Ordal, 1963); incubation at low or high pH values (Gibbs, 1967) or in the presence of chloral hydrate (Lewis, Snell & Alderton, 1965), dimethylformamide or dimethylsulphoxide (Widdowson, 1967); incubation in solutions of urea (Z. J. Ordal, unpublished results) or incubation of lyophilized spores in air saturated with water vapour (Hyatt, Holmes & Levinson, 1966). These diverse treatments have in common the ability to cause alterations in tertiary structure of macromolecules, and it was postulated by Keynan *et al.* (1964) and Keynan, Issahary-Brand & Evenchik (1965) that activation directly resulted from such alterations.

Ionizing radiation can also cause changes in the tertiary structure of macromolecules, either by direct action or through the agency of reactive free radicals from water, causing rupture of hydrogen bonds, peptide bonds or other covalent linkages (Bacq & Alexander, 1961; see Charlesbury, 1963). Farkas & Kiss (1964, 1965) showed that high lethal doses of X-radiation caused increased permeability and germination-like changes in *Bacillus cereus* spores which might have resulted from large alterations in spore molecular structure; Levinson & Hyatt (1960) described similar changes in spores of *B. megaterium* and *Clostridium sporogenes* when subjected to high lethal doses of radiation with electrons. Roberts & Ingram (1965) and T. A. Roberts

(personal communication) found that the initial parts of dose/survivor curves of spores of several *Clostridium* species showed an increase in viable count with dose, apparently induced by sublethal doses of γ -radiation. These observations strongly suggested that radiation-activation could occur. It was therefore of interest to study the effect of γ -radiation on the subsequent germination of spores of *B. cereus* in defined media containing known germinants.

METHODS

Spores. Organisms were grown at 30° on the surface of agar containing Oxoid potato extract (0.4 %, w/v), yeast extract (0.4 %, w/v) and glucose (0.25 %, w/v). Sporulation and lysis of sporangia was complete in 3–6 days, after which spores were collected in cold distilled water and washed six times by centrifugation. Suspensions of spores in water (about equiv. 20 mg. dry wt/ml.) were stored at 4°. When used for germination studies suspensions were diluted to about 5×10^8 spores/ml.; for thiol measurements suspensions of spores of *Bacillus cereus* PX were concentrated to equiv. 50–100 mg. dry wt/ml.

Radiation. Suspensions (1.0 ml. amounts in sealed glass ampoules) were irradiated with γ -radiation from ^{60}Co at a dose rate of about 1 Mrad/hr at ambient temperature (about 20°). Following irradiation suspensions were used immediately or stored on ice for not longer than 24 hr.

Heating. Sealed ampoules containing spores for heat activation were immersed in a 70° bath for 30 min., then stored on ice for as short a time as possible before use.

De-activation. Activated spores were partially de-activated by placing suspensions of them in dialysis sacs which were immersed in stirred distilled water for 16 hr at about 20°; control suspensions were stored in sealed ampoules at 4° during this time.

Germination studies. Samples of spores for germination studies were washed out of ampoules into 24 ml. distilled water. One ml. of this dilution was then pipetted into absorptiometer tubes containing 4 ml. medium at 37° to initiate germination. The extinction (which was always initially about 0.3) was immediately read with an absorptiometer with 580 m μ peak transmission filter. Further readings were taken at intervals to follow the decrease in extinction which is characteristic of germination. Germination media used were: (1) L-alanine (10 mM); (2) L-alanine (1 mM) + adenosine (200 μM); (3) inosine (100 μM); (4) *n*-dodecylamine ($\frac{1}{2}$ saturated solution in water); (5) calcium dipicolinate (40 mM- CaCl_2 + 40 mM-sodium dipicolinate mixed immediately before adding spores). Germination media nos. 1–4 contained sodium phosphate buffer (100 mM; pH 8.0), and no. 5 contained tris HCl buffer (100 mM; pH 8.0).

Thiol titrations. Spore thiol groups were titrated by using silver nitrate in the back-titration method of Hamm & Hofmann (1965). Samples of spores equiv. 50–100 mg. dry wt suspended in 8 M-urea were used for each estimation.

Colony counts of viable spores. Duplicate samples were decimally diluted in water and poured plates made with yeast glucose agar. Colonies were counted after incubation of plates at 37° for 24 hr.

RESULTS

Viability of γ -irradiated and heated spores

γ -Irradiated spores lost viability as measured by colony counts, the shape of the survivor curve being identical with that reported by Hitchins, King & Gould (1966).

The percentage survivals at the dose values used are shown in Table 1. Spores which are not viable in that they are unable to give rise to colonies may still have their germination capacity unimpaired, or even stimulated (see below). Thus, of the doses of γ -irradiation used later, 0.06 Mrad left more than 75 % of the irradiated spores still viable (i.e. able to grow into colonies), whereas spores irradiated with 0.54 Mrad

Fig. 1

Fig. 2

Fig. 1. Inosine germination of γ -irradiated and heat-activated spores of *Bacillus cereus* strain PX. Spores were incubated at 37° in sodium phosphate buffer (0.1 M; pH 8.0) containing inosine (100 μ M). Germination was accompanied by a decrease in extinction of suspensions. Treatment of spores was as follows: $\circ$, unirradiated and unheated control; $\bullet$, irradiated with 0.06 Mrad; $\triangle$, 0.18 Mrad; $\blacksquare$, 0.54 Mrad; $\square$, heated at 70° for 30 min.

Fig. 2. Calcium dipicolinate germination of γ -irradiated and heated spores of *Bacillus cereus* strain PX. Spores were incubated at 37° in Tris HCl buffer (0.1 M; pH 8.0) containing equimolar calcium chloride and sodium dipicolinate (40 mM). Treatment of spores: $\circ$, unirradiated and unheated control; $\bullet$, 0.06 Mrad; $\triangle$, 0.18 Mrad; $\blacksquare$, 0.54 Mrad; $\blacktriangle$, 1.08 Mrad; $\square$, heated at 70° for 30 min. The line for 0.06 Mrad has been omitted for clarity.

Table 1. Viability of γ -irradiated *Bacillus cereus* spores

Survivors were estimated by colony counts as described in Methods.

γ -radiation dose(Mrad)	Survivors (%)
0.02	98
0.06	78
0.18	27
0.54	3.4
1.08	0.011

were more than 95 % non-viable (Table 1), although still capable of showing typical germination changes in appropriate media. The heat activation treatment used (70° for 30 min.) did not measurably decrease the viability of *B. cereus* PX spore suspensions.

Activation of spores with γ -radiation and with heat

Spores of *Bacillus cereus* PX which were γ -irradiated, subsequently germinated more rapidly than did unirradiated spores and, over the range studied, the greater

the γ -dose the greater was the degree of activation. Activation was particularly obvious when inosine was used as germinant (Fig. 1). When L-alanine was used as germinant, radiation-activation was detectable but was less marked. With different batches of spores, activation was always clearly seen when inosine was used as germinant, but was variable in extent with L-alanine, sometimes being slight and sometimes more marked. The reasons for these differences are not understood and not investigated further. Activation was always slight when tested with the mixed germinants adenosine + L-alanine. Whichever germinant was used, heat caused greater activation than did the highest radiation dose used (Fig. 1). The degree of radiation-activation of spores of different species varied markedly. For instance, of those tested at the 0.18 Mrad level only spores of strains of *B. cereus* with inosine as germinant were significantly activated (Table 2).

Table 2. *Germination of γ -irradiated spores of different organisms*

Unheated spores were left unirradiated for controls or exposed to 0.18 Mrad of γ -radiation. Subsequent germination was measured as described in Methods. L-Alanine was used at 10 mM and inosine at 1 mM.

Organisms	Decrease in extinction (%) after incubation of spore suspension for 20 min. at 37° in the presence of			
	L-Alanine		Inosine	
	Control spores	Irradiated spores	Control spores	Irradiated spores
<i>B. brevis</i> NCTC 7577	3.4	4.6	1.6	2.6
<i>B. subtilis</i> ATCC 6051	38.3	39.4	0	0
<i>B. coagulans</i> NCTC 3991	41.9	42.4	0	0
<i>B. polymyxa</i> strain MI	43.4	45.4	0	0
<i>B. cereus</i> strain PX	4.8	6.7	18.3	39.5
<i>B. cereus</i> NCTC 945	6.4	9.1	22.2	47.4

Calcium dipicolinate (CaDPA), which is a spore constituent, will germinate spores of most bacteria, probably by de-stabilizing some spore-Ca-DPA structure (Riemann & Ordal, 1961). The long chain primary alkane *n*-dodecylamine (laurylamine) will also germinate many spores, probably by virtue of its surface-active properties, and has been called a 'chemical germinant' to distinguish it from germinants like amino acids and ribosides (Rode & Foster, 1961). Figure 2 shows the effect of radiation and of heat on germination of spores of *Bacillus cereus* PX in CaDPA. Radiation-activation was recognizable with CaDPA or with *n*-dodecylamine as germinant, and therefore differed from heat activation, because heat did not activate spores for germination in *n*-dodecylamine.

Effect of γ -radiation on heat-activated spores

When spores of *Bacillus cereus* PX were activated by heat and subsequently γ -irradiated, the overall effect differed according to the germinant used to assess activation. Spores which had been heated and then γ -irradiated germinated in inosine about as rapidly as when only heated. In L-alanine, however, the germination rate of heated spores was decreased by the additional dose of γ -radiation (Fig. 3). Irradiation appeared to de-activate the heat-activated spores.

Titrateable thiol content of activated spores

During heat-activation of *Bacillus cereus* PX spores the titrateable thiol content of the spores increased, probably because thiol groups became unmasked by heat-induced tertiary-structure changes in some molecules of the spores. γ -Irradiated spores were found to have a higher content of titrateable thiol groups than had unirradiated spores, but not as high as heat-activated spores (Table 3).

Fig. 3

Fig. 4

Fig. 3. Effect of γ -irradiation on germination of heated spores of strain *Bacillus cereus* strain PX. Experimental conditions as defined in Fig. 1, but with L-alanine (10 mM) as germinant in place of inosine. Heating was at 70° for 30 min., radiation dose was 0.18 Mrad. Treatment of spores as follows: ○, unirradiated, unheated control spores; ●, irradiated spores; △, heated spores; ■, spores heated and then irradiated.

Fig. 4. Reversal of heat-activation and γ -radiation-activation of spores of *Bacillus cereus* strain PX. Experimental conditions as in Fig. 1 with inosine (500 μ M) as germinant. Treatment of spores: ○ and ●, spores heated 70° for 30 min.; □ and ■, spores irradiated with 0.54 Mrad. Open symbols, before partial deactivation; closed symbols, after partial deactivation. Spores were partially de-activated by storage for 16 hr at 20° in dialysis sacs in water as described in Methods.

De-activation of heat-activated and radiation-activated spores

Heat activation of *Bacillus cereus* spores is reversible in the sense that heat-activated spores become partially de-activated on storage (Keynan *et al.* 1964). γ -Radiation-activated and heat-activated spores of *B. cereus* PX were stored for 16 h in aerated water and then tested for changes in germinability. Figure 4 shows that both heated and γ -irradiated spores germinated less rapidly after such storage, and thus both showed a degree of de-activation.

Radiation activation of Bacillus cereus strain T

In contrast to *Bacillus cereus* strain PX, spores of *B. cereus* strain T were not activated by doses of γ -radiation below about 0.54 Mrad. Doses of 1.08 and 2.16 Mrad were

necessary to cause consistently measurable increase in rate of germination of spores of strain T, at which dose values more than 99.9 % kills occurred, as measured by colony count.

Table 3. *Titrateable thiol groups in γ -irradiated and heated spores of *Bacillus cereus**

Time for 30 % decrease in extinction is an approximate measure of germination rate, and therefore of the extent of activation.		
Treatment of spores	Thiol titre (μ mole SH equiv./ 100 mg. dry weight of spores)	Time for 30 % decrease in extinction of spore suspension in inosine medium (min.)
Untreated control	0.4	> 180
Irradiated, 0.18 Mrad	1.4	58
Irradiated, 0.54 Mrad	1.8	28
Heated to 70° for 30 min.	2.7	11.5

DISCUSSION

Ionizing radiation will cause tertiary-structure changes in macromolecules (Bacq & Alexander, 1961) by breaking covalent linkages including peptide bonds, and even by destroying basic amino acids in proteins (Robinson, Weiss & Wheeler, 1966). Destruction of the integrity of spore deoxyribonucleic acid (DNA) by γ -radiation may well cause death of bacterial spores (i.e. destroy their colony-forming ability), but although functional DNA is essential for cell growth it is probably not essential for spore germination and early outgrowth (Steinberg, Halvorson, Keynan & Weinberg, 1965). Indeed, bacterial spores irradiated with 0.54 Mrad during the present work became more than 95 % non-viable (not forming colonies) and not only germinated rapidly but also outgrew and shed their coats when incubated on slide cultures of yeast glucose agar. However, growing and dividing forms were never observed. The relative resistance of the germination and outgrowth processes of irradiated spores compared with later vegetative growth has been reported for a number of organisms, e.g. *Clostridium botulinum* (Costilow, 1962), *Bacillus megaterium* (Levinson & Hyatt, 1960) and *B. subtilis* (Hermier, 1963). Clearly the enzymes and cofactors involved in germination must be more resistant to γ -radiation than the sites of damage caused by radiation which lead to loss of colony-forming ability.

From the hypothesis that the various methods known to activate bacterial spores do so by altering tertiary structures (Keynan *et al.* 1964) it was possible to predict that ionizing radiation should cause activation. The efficiency of radiation as an activating agent, however, was (like heat) not the same for all spores. It was more effective for *Bacillus cereus* strains PX and NCTC 945 than for *B. cereus* strain T, and γ -radiation was not an obvious activator for spores of four of the organisms shown in Table 2 or of *B. subtilis* strain SJ2 (Hermier, 1963). Spores of *Clostridium botulinum* suspended in phosphate buffer and irradiated with electrons subsequently germinated at similar rates to un-irradiated spores (Costilow, 1962), but since these spores were heat-activated any radiation-activation may have been masked. Spores of *C. botulinum* irradiated with sublethal doses of γ -radiation in cured ham did become activated (Anellis, Berkowitz, Jarboe & El-Bisi, 1967). Activation of *B. cereus* spores by heat and γ -radiation were similar qualitatively except when *n*-dodecylamine was used as germinant; then

radiation caused activation but heat did not, as noted by Rode & Foster (1961). The mode of action of *n*-dodecylamine as a germinant is not understood, so the different activation responses cannot yet be explained. A further similarity was that heat-activated and radiation-activated spores both became de-activated on storage. De-activation is known to occur with *B. cereus*, but not with all bacterial spores (e.g. *B. subtilis* 5230; Busta & Ordal, 1964), and probably reflects reformation of original tertiary structures (Keynan *et al.* 1964).

Although activation treatments probably alter tertiary structure of some spore macromolecules, the sites of those molecules within the spore are not known. It may be that the structural changes which lead to activation are general and occur to molecules in a variety of spore sites. Evidence supporting this view was obtained when activated spores of *Bacillus cereus* PX were found to have a higher content of titratable thiol groups than control spores, for tertiary-structure changes in proteins containing cysteine residues are known often to result in unmasking or masking of thiol groups. The increased thiol titres measured in radiation-activated spores might be the result of a general loosening of structure and unmasking of groups in a variety of spore molecules. The same loosening may be caused by alkanes, for Rode & Foster (1965) discovered that spores of *B. megaterium* QMB 1551 could be germinated by *n*-alkanes, in particular by butane and pentane, and proposed that the alkanes acted by disrupting hydrophobic bonds in spores. On the other hand, it has been suggested that a major effect of ionizing radiation is disruption of cell membranes (Bacq & Alexander, 1961), so that alterations in membrane permeability might be a factor leading to activation of spores. The observations of Levinson & Hyatt (1960) and Farkas & Kiss (1964, 1965) that electron-irradiated and X-irradiated spores showed changes (which Farkas & Kiss called 'pseudogermination') which were consistent with loss of permeability would support this view. However, the radiation doses necessary to cause pseudogermination were high lethal doses, whereas activation can be caused by low non-lethal doses of γ -radiation. This suggests that activation is due to smaller, more subtle changes than the gross changes in permeability measured by Farkas & Kiss, though probably of the same type. A similar pattern is shown by the effect of heating in that whereas low sublethal heating may activate spores, high sublethal or lethal heating causes increase in spore permeability (Black & Gerhardt, 1962) and leakage of some spore constituents (El-Bisi, Lechowich, Amaha & Ordal, 1962). The observation that ionizing radiation can activate spores is thus compatible with the idea that activation is due either to changes in tertiary structure of spore macromolecules or to an increase in permeability of a spore membrane. In either case the result would be to unmask previously masked reactive sites within the spore and allow more rapid germination.

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