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Gamma-irradiation of spores of *Bacillus subtilis*

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Freeze-drying from a 5% aqueous solution of glucose produced a marked protection of spores of *Bacillus subtilis* subjected to spent fuel gamma-irradiation over the range $2{\cdot}5$–$12{\cdot}5 \times 10^5$ rads. Freeze-drying from aqueous suspension produced no protection. Irradiation in 5% aqueous glucose and in aqueous suspension gave log % survivor/dose regressions with similar slopes.

BACTERIAL spores have been used in studies of radiation resistance, but only recently has evidence been forthcoming that conditions during (Tallentire, 1958; Powers & Kaleta, 1960) and after (Powers, Webb & Ehret, 1960; Tallentire & Davies, 1961) irradiation have any marked effect on the recovery after irradiation.

We have examined the effect of irradiation on spores freeze-dried from water and glucose and lactose solutions and compared their viability with that of suspensions in water and glucose after irradiation.

Experimental

MATERIALS AND METHODS

Spore suspension. *Bacillus subtilis* NCTC 8236 was grown on a Lemco agar containing 0·0001% manganous sulphate at 37°. After 14 days spores washed from the surface were washed five times with sterile water, heated at 78–80° for 20 min to kill vegetative cells, and stored at 0–4°. Suspensions were made in water or 5% glucose or lactose as required.

Counting. Decimal dilutions were made in sterile distilled water. Ten replicates of 0·5 ml were spread on overdried peptone agar (Oxoid) plates, and incubated at 37° for at least 36 hr. Colonies were counted at 18 and at 36–40 hr.

Freeze-drying. 0·1 ml samples of spore suspension in water or 5% glucose or lactose solutions were snap-frozen in ampoules and dried over phosphorus pentoxide for 5–6 hr (Model L.T.5, Edwards and Co. Ltd.). The phosphorus pentoxide was then replenished and drying continued overnight, maintaining a pressure of 0·01 mm Hg by continuous pumping. Ampoules were sealed under air. Freeze-dried spores were recovered by adding 1 ml sterile water to the ampoule, transferring the reconstituted suspension to 9 ml sterile water, and rinsing the ampoule at least 5 times with the bulk dilution.

Storage of aqueous spore suspension. No change in microscopical, colonial or biochemical characteristics was detected in 2 years of storage in water or 5% glucose solution, and the resistance of the spores to wet heat (100°), dry heat (100°) and phenol, chlorocresol, phenylmercuric nitrate, chloramine-T, crystal violet, cetrimide, aminacrine HCl, chlorhexidine diacetate, benzylpenicillin and streptomycin remained the same.

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Irradiation. Irradiation at the Spent Fuel Gamma Irradiation Unit of the Atomic Energy Research Establishment, Harwell, was at approximately 20° and over the dose range of 2·5–12·5 × 10^5 rads. Storage before and after irradiation was at 16–17°. At least 3 ampoules were irradiated at each dose level. Spores were irradiated in the presence of air.

Storage of irradiated spores in aqueous suspension. At 0–4° the viable count of the stock spore suspension fell from 6 × 10^8 spores/ml to 3 × 10^8 spores/ml after 1 year. Serial dilutions of irradiated spore samples were stored at 0–4°, and plated at intervals up to 215 days. Although a fall in count occurred in irradiated and unirradiated samples, the slope of the regression of log % survivors against dose did not change significantly over this period.

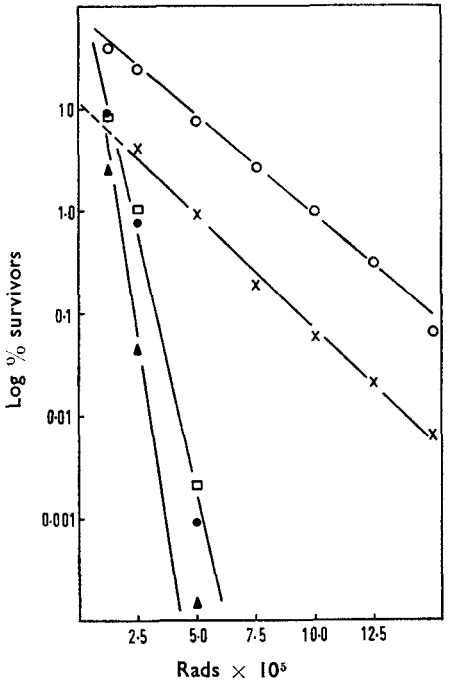

FIG. 1. Gamma irradiation of *Bacillus subtilis* spores. Regressions of log% survivors against radiation dose for: □ aqueous suspension of spores. ● suspension of spores in 5% aqueous glucose. ▲ spores freeze-dried from aqueous suspension. ○ spores freeze-dried from 5% aqueous solution of glucose. × spores freeze-dried from 5% aqueous solution of lactose.

Statistical analysis. Results from irradiation experiments were expressed as % survivors, using counts from unirradiated ampoules as representative of 100%. Log % survivor/dose regressions were calculated, including the 100% value except in the case of freeze-dried lactose. Correlation coefficients indicated that the regressions could be considered linear.

Groups of regression coefficients were compared for parallelism using an analysis of variance (Yousef, 1954). If a significant difference between regression coefficients was thus established, the slopes of pairs of lines were compared by a modified '*t*' test (Bailey, 1959). In some cases a total regression was calculated assuming that all the points were scattered along one regression line and a common slope established.

Results

Exponential survivor/dose curves were obtained upon gamma-irradiation of *B. subtilis* spores, whether in aqueous suspension or freeze-dried and also in the presence of glucose but not lactose.

No significant difference was established in slopes of the curves of spores in aqueous suspension, spores suspended in 5% aqueous glucose, or spores freeze-dried from water (slope (b) = $-0{\cdot}9331$, $-1{\cdot}0112$, and $-1{\cdot}1663 \times 10^{-5}$ rads respectively).

Freeze-drying from 5% aqueous glucose resulted in a marked protective effect (b = $-0{\cdot}1949$ rads $\times 10^{-5}$) (Fig. 1).

Addition of glucose to spores freeze-dried from water was without effect on the radiation resistance.

Freeze-drying from 5% aqueous lactose produced an initial rapid fall in viability to 5–10% of the original, whereupon the slope of the regression changed to almost that of freeze-dried glucose ($-0{\cdot}2226$ rads $\times 10^{-5}$).

Discussion

Linear log survivor/dose curves have previously been reported for the radiation inactivation of *B. subtilis* spores by Donnellan & Morowitz (1957) and Woese (1958).

Moos (1952) interpreted a statistically significant protective effect on freeze-drying *Pasteurella pestis* and *Escherichia coli* from distilled water as a reduction of the water content of the cells preventing formation of certain toxic radicals on subsequent irradiation. Experiments with rigidly controlled water content show sensitisation to X- and gamma-radiation of spores irradiated in the presence of, and stored in the presence of, oxygen. Sensitivity is a function of water content, decreasing sensitivity occurring with increasing water content (Tallentire & Davies, 1961; Tallentire & Powers, 1963). Intracellular dehydration is therefore unlikely to be the reason for the increased radiation resistance demonstrated.

Freeze-drying from 5% aqueous glucose under the conditions described produced a glass. This may be regarded as a supercooled syrup, amorphous in nature, and containing some water. A true glass is impermeable to gases, but if crystallisation occurs, pores form and permit passage of gases. Since the glass forms during freeze-drying, and is impermeable, spores encased in it may exhibit typical anoxic radiation resistance, which is greater than resistance in the presence of air (Proctor, Goldblith, Oberle & Miller, 1955). If the glass is incompletely formed, or unstable, as with lactose, only some of the spores will remain protected by the residual entire glass.

Alternative explanations should also be considered. As the bacterial spore is permeated by glucose (Black & Gerhardt, 1961) a glass may form within the spore. Stabilisation of toxic free-radicals similar to that reported (Cloutier, 1961) in a boric acid glass may then occur.

There is also evidence that the state of molecular aggregation has a marked effect on the radiation decomposition of α-D-glucose (Phillips & Baugh, 1963). It may be possible for the most resistant aggregation to protect spores encased in it against radiation damage.

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