

# Inactivation of Dried Bacteria and Bacterial Spores by Means of Gamma Irradiation at High Temperatures

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Dried preparations with *Streptococcus faecium*, strain A<sub>2</sub>1, and spores of *Bacillus sphaericus*, strain C<sub>1</sub>A, normally used for control of the microbiological efficiency of radiation sterilization plants and preparations with spores of *Bacillus subtilis*, normally used for control of sterilization by dry heat, formalin, and ethylene oxide, as well as similar preparations with *Micrococcus radiodurans*, strain R<sub>1</sub>, and spores of *Bacillus globigii* (*B. subtilis*, var. *niger*) were gamma irradiated with dose rates from 16 to 70 krad/h at temperatures from 60 to 100 C. At 80 C the radiation response of the spore preparations was the same as at room temperature, whereas the radiation resistance of the preparations with the two vegetative strains was reduced. At 100 C the radiation response of preparations with spores of *B. subtilis* was unaffected by the high temperature, whereas at 16 and 25 krad/h the radiation resistance of the radiation-resistant sporeformer *B. sphaericus*, strain C<sub>1</sub>A, was reduced to the level of radiation resistance of preparations with spores of *B. subtilis*. It is concluded that combinations of heat and gamma irradiation at the temperatures and dose rates tested may have very few practical applications in sterilization of medical equipment.

It has been suggested that combinations of heat and ionizing radiation might be useful for sterilization of interplanetary vessels and food-stuffs (5, 7). Such combinations may act additively or synergistically in inactivation of microorganisms without correspondingly additive or synergistic effects in material damage. Items which do not tolerate a dry heat sterilization or a radiation sterilization may thus be sterilized by a combination of the two methods.

The present work aims at an evaluation of gamma irradiation procedures combined with heat treatments for sterilization of medical equipment. We have applied the bacteriological standard preparations we normally use for control of radiation sterilization and dry heat sterilization of such equipment supplemented with some experiments on a few other radiation-resistant or heat-resistant strains.

## MATERIALS AND METHODS

The following bacterial strains were used in the experiments: *Streptococcus faecium*, strain A<sub>2</sub>1, (ATCC no. 19581); *Micrococcus radiodurans*, strain R<sub>1</sub>, made available by A. W. Anderson, Oregon State

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University, August 1961; *Bacillus sphaericus*, strain C<sub>1</sub>A (E. A. Christensen, manuscript in preparation); *Bacillus subtilis*, the Statens Serum Institute test strain for sterilization by dry heat, formalin, and ethylene oxide; *Bacillus globigii*, (ATCC no. 9372), *B. subtilis* var. *niger*.

Test pieces with *S. faecium*, strain A<sub>2</sub>1, were taken from batches of reference standards prepared for control of the microbiological efficiency of radiation sterilization plants (3). Briefly, these standard preparations are prepared as follows.

Heavily inoculated 5% blood agar plates are incubated at 30 to 32 C for 3 to 4 days. The cultures are scraped off the plates and suspended in serum broth. Droplets (0.02 ml) of the suspension (containing around 10<sup>8</sup> viable units) are placed on polyethylene foil and dried overnight in a glove box at 30% relative humidity as it has been found that humidity levels above 50% lead to reduced radiation resistance of the test pieces (4). Each dried test piece is sealed into a double envelope of polyethylene foil.

Viable counts are determined by means of classical dilution techniques, by using 5% blood agar plates and by incubation at 30 to 32 C for 3 to 4 days.

Test pieces with *M. radiodurans*, strain R<sub>1</sub>, were prepared by similar procedures. The strain was cultivated on tryptone-glucose-yeast extract (TYG) agar, and the test preparations were dried at ambient humidity in a laminar air flow cabinet.

Test pieces with spores of *B. sphaericus*, strain C<sub>1</sub>A, were taken from batches of spore monitors pre-

pared for control of the microbiological efficiency of radiation sterilization plants (3). These test pieces are produced by procedures similar to those used for preparing test pieces with *S. faecium*, strain A<sub>2</sub>1. They are dried at ambient humidity. For experiments at 100 C the test pieces were removed from the polyethylene envelopes and transferred to small aluminum capsules.

Test pieces with spores of *B. subtilis* were taken from batches prepared for control of sterilization by dry heat, formalin, and ethylene oxide (6). Briefly these standard preparations are prepared as follows.

Plate cultures from agar plates incubated for 5 days at 37 C are scraped off the plates and suspended in physiological saline and then mixed with sterilized quartz sand. The mixture is immediately vacuum-dried at a pressure of 2 to 4 mm Hg for 24 h, and finally homogenized in a mortar. Each test piece, which consists of 120 mg of this spore sand and contains  $2 \times 10^6$  viable units, is wrapped in two layers of paper.

Spores of *B. globigii* were used in two kinds of preparations: dried serum broth preparations prepared in the same way as test pieces with spores of *B. sphaericus*, strain C<sub>1</sub>A, and sand preparations prepared in the same way as test pieces with spores of *B. subtilis*.

Most of the gamma irradiations were carried out in <sup>60</sup>Co-facility II, a gamma cell, which at the time of the experiments was loaded with 600 curies. During the time period, where experiments were undertaken, the dose rate in the center of the irradiation chamber decreased from 75 krad/h to 64 krad/h. Irradiations under this condition will be referred to as 70 krad/h. The dose rate could be reduced by lead shieldings. Two shielding configurations were used. In one of these configurations the dose rate was reduced by a factor of 2.8, and irradiations under this condition will be referred to as 25 krad/h. In another configuration the reduction factor was 4.45 or 16 krad/h. For simultaneous heating and irradiation test, pieces were placed in the gamma cell in a 2-cm-wide brass cylinder, which was surrounded by a heat jacket. The temperature in the center of the cylinder was measured by an electrical thermocouple. The temperature varied around the setting with  $\pm 2$  C. In experiments where heating was applied before or after irradiations, irradiations were carried out in <sup>60</sup>Co-facility III, loaded with 4,500 curies. The dose rate in the center of this gamma cell was 400 krad/h.

## RESULTS

Radiation inactivation curves for dried serum broth preparations with *S. faecium*, strain A<sub>2</sub>1, *M. radiodurans*, strain R<sub>1</sub>, spores of *B. sphaericus*, strain C<sub>1</sub>A, and spores of *B. globigii* irradiated at room temperature are seen in Fig. 1. Figure 2 presents radiation inactivation curves for the same preparations at 80 C and 25 krad/h. It will be seen that the inactivation curves for the two spore preparations were unchanged, but that the LD 99.99 value for the preparations with the two vegetative strains was

reduced by about a factor four. (LD 99.99 is the radiation dose, or heating time, that reduces the surviving fraction to  $10^{-4}$ , in Fig. 2: 0.8 Mrad for *S. faecium*, strain A<sub>2</sub>1.) When the standard preparations with the vegetative bacteria were kept at 80 C for 48 h the surviving fraction for both strains was higher than 10%, so the simultaneous application of dry heat at 80 C and gamma irradiation acted clearly synergistically in the experiments with these two vegetative strains. At 80 C and 70 krad/h, the LD 99.99 was 1.4 Mrad for *S. faecium*, strain A<sub>2</sub>1, and 1.6 Mrad for *M. radiodurans*, strain R<sub>1</sub>. At 60 C and 70 krad/h, the LD 99.99 for strain A<sub>2</sub>1 was 2.8 Mrad and for strain R<sub>1</sub> it was 3.4 Mrad.

Table 1 presents LD 99.99 values for irradiation of the four kinds of spore preparations at room temperature and at 100 C. It can be seen that the radiation resistance of the test pieces with spores of *B. subtilis*, normally used for control of sterilization by dry heat, was the same at 100 C and room temperature at the three dose rates tested. The radiation resistance of test pieces with spores of *B. sphaericus*, strain C<sub>1</sub>A, normally used for control of radiation sterilization plants was lower at 100 C than at room temperature. At 70 krad/h the reduction was small; at 16 and 25 krad/h the resistance was reduced by about a factor of four to the level of resistance observed with the subtilis preparations. The radiation resistance of the two kinds

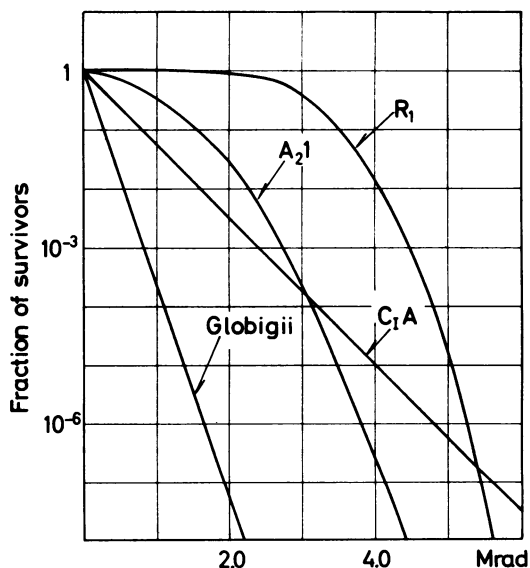


FIG. 1. Radiation inactivation curves for dried serum broth preparations with *S. faecium*, strain A<sub>2</sub>1, *M. radiodurans*, strain R<sub>1</sub>, spores of *B. sphaericus*, strain C<sub>1</sub>A, and spores of *B. globigii* irradiated at room temperature.

of preparations with spores of *B. globigii* at 100 C was lower than the resistance of the preparations with spores of *B. subtilis* and *B. sphaericus* at the same irradiation conditions. The radiation resistance of spores of *B. subtilis* and *B. globigii* in sand preparations was close to the resistance reported for clean spores of these strains (2, 7). The radiation resistance of the *B. globigii* spores in serum broth preparations at room temperature was within the range reported for similar preparations with spores of *B. subtilis* and *B. globigii* (1, 2).

A few experiments with application of heat (100 C) and irradiation (400 krad/h) separately were carried out with preparations of spores of *B. sphaericus*, C<sub>1</sub>A. The LD 99.99 for heat alone was 100 h. When spores were pre-irradiated with 0.6 or 1.0 Mrad the heat LD 99.99 value for the spores which survived the irradiation was reduced to 70 h. The radiation LD 99.99 value for spores which had survived 48 h at 100 C was 3.0 Mrad.

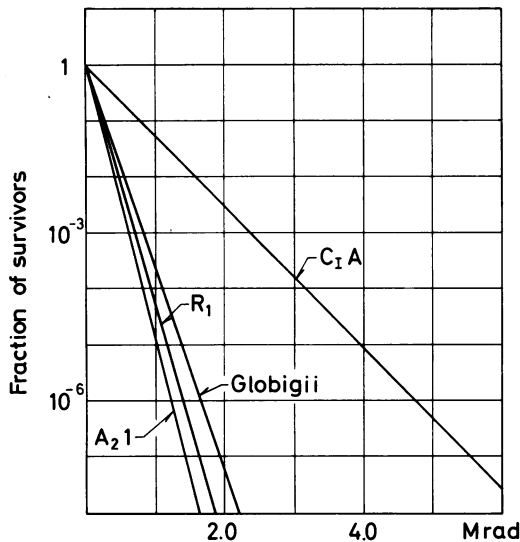


FIG. 2. Radiation inactivation curves for the same preparations as in Fig. 1 irradiated at 80 C with 25 krad/h.

## DISCUSSION

In the experiments with irradiation at 80 C, the two vegetative strains had a reduced radiation resistance, whereas the spores of *B. sphaericus* and *B. globigii* in serum broth preparations had an unchanged response. There were no indications of the so-called paradoxical effect, which has been reported with spores of *B. megaterium* and *Clostridium botulinum* (5, 8). In these reports the spores had a higher radiation resistance at 80 to 90 C than at room temperature. This paradoxical effect disappeared at 100 C where the radiation resistance dropped to below the level observed at room temperature. In cases where it is known that sporeformers are totally absent, it may be beneficial to increase the irradiation temperature to 80 C in order to reduce radiation doses, but if the occurrence of sporeformers cannot be excluded, there may be a limitation of any benefits by irradiation at this temperature.

At 100 C the spore monitor for radiation sterilization *B. sphaericus*, strain C<sub>1</sub>A, was the most resistant at all conditions tested. This, however, does not indicate per se that these preparations can be regarded as ideal monitors for evaluation of any combination of radiation and heat for sterilization, as more suitable strains may be isolated from the relevant environments. However, the present data may be applied in a preliminary analysis aiming at an identification of combinations of radiation and heat which may warrant further consideration, excluding combinations where benefits are too small to warrant investments in microbiological research and technical development. Figure 3 represents an attempt to do such an analysis based on the data obtained with *B. sphaericus*, strain C<sub>1</sub>A, at 100 C. In this analysis we arbitrarily have demanded that any combination of heat and radiation, which may warrant further consideration, at least should reduce both radiation LD 99.99 values and heat LD 99.99 values with a factor of two.

The ordinate in Fig. 3 is LD 99.99 in hours,

TABLE 1. LD 99.99 in Mrads for radiation inactivation of four kinds of spore preparations at three different dose rates at room temperature and 100 C

| Strain               | Preparation technique | Dose rate (krad/h) | LD 99.99 (Mrad) |           |
|----------------------|-----------------------|--------------------|-----------------|-----------|
|                      |                       |                    | Room temp       | 100 C     |
| <i>B. subtilis</i>   | Sand                  | 16, 25, 70         | 0.7 ± 0.2       | 0.7 ± 0.2 |
| <i>B. sphaericus</i> | Serum broth           | 16, 25             | 3.2 ± 0.2       | 0.8 ± 0.2 |
| <i>B. sphaericus</i> | Serum broth           | 70                 | 3.2 ± 0.2       | 2.6 ± 0.3 |
| <i>B. globigii</i>   | Sand                  | 25                 | 0.4 ± 0.1       | 0.3 ± 0.1 |
| <i>B. globigii</i>   | Serum broth           | 25                 | 1.1 ± 0.1       | 0.5 ± 0.1 |

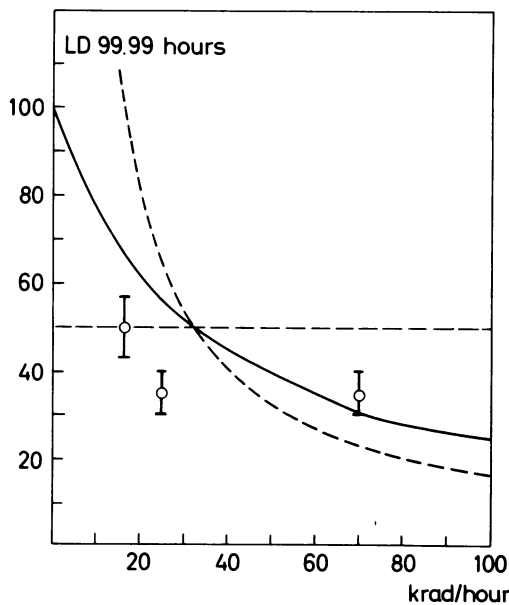


FIG. 3. Radiation inactivation of dried serum broth preparations with spores of *B. sphaericus*, strain C<sub>1</sub>A, at 100 C and three different dose rates. For explanation see text.

the abscissa is the dose rate in krad per hour. In this scheme we have drawn a full line, which represents the LD 99.99 values one would obtain if radiation and heat acted additively. The experimental results obtained at the three dose rates tested are plotted for comparison. It will be seen that at 16 and 25 krad/h the combination of heat and radiation acted more effectively than a pure additive effect, and that the results at 70 krad/h correspond to an additive effect. The dotted lines are auxiliary lines for use in the analysis. At all points below the horizontal line the heat LD 99.99 values are below half the LD 99.99 for heat treatment of *B. sphaericus*, strain C<sub>1</sub>A; standard preparations, all points below (or to the left of) the bowed dotted line, are below half the radiation LD 99.99 value for *B. sphaericus*, strain C<sub>1</sub>A, standard preparations.

It can be seen that the only set of experimental data, which was below both "half" lines, were the data obtained at 25 krad/h. At this point the LD 99.99 values for both heat and radiation were reduced with about a factor of three. At 16 krad/h the reduction in radiation LD 99.99 value was the same as at 25 krad/h, whereas the heat value was only reduced by a factor of two. At 70 krad/h the data were below the "half" line for heat, but above the line for radiation. It appears from this analysis that combinations of heat and irradiation, where halving of both sterilization time and dose may

be expected, are concentrated to a narrow dose-rate interval, approximately 20 to 40 krad/h. It appears unlikely that sterilization times and doses may be reduced by more than a factor of three at any combinations of 100 C heat and irradiation. Technological and economic considerations must decide whether the possibility of introducing this combination method in medical sterilization can justify the amount of microbiological work that will be necessary in order to ensure the general microbiological safety of the process.

In view of the data presented here the sterilization times and doses suggested by Reynolds and Garst (7) for sterilization of spacecraft appear to be about one order of magnitude too low for sterilization of equipment for medical use. Although they use a slightly higher temperature (105 C), their suggestions of 132 krad at 12 krad/h or 252 krad at 36 krad/h, for example, as possible sterilization process parameters appear to be very optimistic. With any of the four kinds of spore preparations we have tested at 100 C, irradiation with 250 krad gave inactivation factors smaller than 10<sup>4</sup>.

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