Resistance of *Bacillus subtilis* Spores to Inactivation by Gamma Irradiation and Heating in the Presence of a Bactericide

II. Factors Affecting Rates of Inactivation by Phenolic Bactericides

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Aqueous suspensions of *Bacillus subtilis* NCTC 8236 spores, surviving 150,000 or 300,000 rad of gamma irradiation under air from a cesium-137 source, exhibited an enhanced rate of inactivation, compared to nonirradiated spores, when heated with different phenolic bactericides. The apparent magnitude of the enhanced inactivation rate, observed from survival curves, increased progressively with the irradiation dose applied and diminished progressively as the severity of heat treatment with 0.2% chlororesol was increased either by raising the temperature from 70 to 90°C or reducing the pH from 8 to 6. The enhanced inactivation rate was unaffected when the concentration of sodium chloride added to 0.2% chlororesol was altered from 0.4 to 0.8%. The enhancement effect was also observed when the heat treatment was carried out with 0.5% phenol and 0.3% m-cresol.

It has been reported (4–7) that bacterial spores surviving gamma irradiation are more readily inactivated by moist heat treatment than spores which have not been irradiated. Pershina and Vasil’eva (8) observed that vegetative bacteria show enhanced sensitivity to inactivation by treatment with bactericides at normal temperatures after surviving sublethal doses of gamma irradiation.

*Bacillus subtilis* spores surviving gamma irradiation have been previously noted by us (2) to exhibit an enhanced rate of inactivation by moist heat treatment in the presence of a bactericide, compared to nonirradiated spores. This communication describes an appraisal of those factors which influence the magnitude of this enhancement effect when phenolic bactericides are used.

**MATERIALS AND METHODS**

A detailed description and statistical evaluation of the experimental procedures utilized for obtaining survival curve data for *B. subtilis* NCTC 8236 spores have been previously described (2, 3). Spores of equivalent total count, obtained from a stock suspension, were used in experiments for determining comparable survival curves. Gamma irradiation was obtained from a cesium-137 source. Heating-up periods of 4, 5, and 5.5 min, determined with a thermostir, were allowed for 1.5-ml volumes of spore suspensions sealed in 2-ml ampoules to reach 70, 80, and 90°C, respectively, after immersion into an oil bath.

**Chemicals.** Glass-distilled water was used to prepare the medium and chemical solutions. Chlororesol and m-cresol, laboratory grade reagents, and phenol, dextrose, sodium phosphate, and sodium acid phosphate, “analar” grade reagents, were obtained from British Drug Houses, Ltd.

**Treatment of data.** Each survival curve, representing the rate of inactivation of *B. subtilis* spores, was prepared by plotting the log surviving fraction, derived from the mean colony counts of quintuplicate platings, against the duration of bactericidal treatment at the stated temperature. The mean colony count determined after the elapse of the heating-up period was taken to be surviving fraction 1 at zero time, and all other mean colony counts determined for the survival curve were compared against this mean colony count to derive their surviving fractions. A correlation coefficient (1), obtained by correlating the increasing divergence of corresponding log surviving fraction points against increasing duration of bactericidal treatment, was used to determine whether the divergence of each pair of comparable survival curves was significant at the probability level of $P = 0.05$.

**RESULTS**

The divergence of survival curves determined for replicate experiments was insignificant, whereas the divergence of all of the comparable
survival curves reported in this communication was significant, unless otherwise indicated.

Effect of dose of gamma irradiation on spore inactivation. Aqueous spore suspensions were nonirradiated, irradiated with 150,000 rad under air, or irradiated with 300,000 rad under air, and to each suspension was added 0.2% chlorocresol in phosphate buffer (pH 7) just before the heat treatment at 80 C. Figure 1 shows that spores surviving irradiation were more readily inactivated than nonirradiated spores and that the enhancement of the rate of inactivation by heating in the presence of the bactericide was increased as the dose of prior gamma irradiation applied to the spores was increased.

Effect of pH on spore inactivation. Figure 2 shows survival curves for aqueous suspensions of spores, either nonirradiated or irradiated with 150,000 rad under air, before heating at 80 C in the presence of 0.2% chlorocresol in phosphate buffer of pH 8 or pH 6. It was observed that, as the pH decreased, the rate of inactivation of both irradiated and nonirradiated spores by heating in the presence of the bactericide was increased. Spores surviving irradiation were more readily inactivated than nonirradiated spores at both pH values. However, the apparent magnitude of the divergence of comparable survival curves at each pH was observed to diminish progressively as the severity of the bactericidal treatment was enhanced by reducing the pH.

Effect of temperature on spore inactivation. The survival curves shown in Fig. 3 indicated that aqueous suspensions of the spores, surviving 150,000 rad of irradiation under air, were more readily inactivated than nonirradiated spores by subsequent heat treatment at either 70 or 90 C in the presence of 0.2% chlorocresol in phosphate buffer (pH 7). However, it was noted that, as the severity of the bactericidal treatment was increased by raising the temperature, the apparent magnitude of the divergence of the comparable survival curves determined at each temperature decreased.

Effect of sodium chloride on spore inactivation. It may be observed from Fig. 4 that spores surviving 150,000 rad of irradiation under air were more readily inactivated than nonirradiated spores by heat treatment at 80 C with 0.2%
chlorocresol and either 0.4 or 0.8% sodium chloride in phosphate buffer (pH 7). The rates of inactivation of either nonirradiated or irradiated spores were not significantly altered by the change in the sodium chloride concentration and associated toxicity of the system.

**Effect of different phenolic bactericides on spore inactivation.** Phenol (0.5%) and m-cresol (0.3%) are also used in the sterilization process of heating with a bactericide. Aqueous suspensions of the spores were either nonirradiated or irradiated with 150,000 rad under air before separate treatment with each bactericide in phosphate buffer (pH 7) at 80°C. Survival curves shown in Fig. 5 indicate that equivalent treatment with each bactericide caused a faster rate of inactivation of the irradiated spores compared to the nonirradiated spores.

**DISCUSSION**

The various experimental parameters in the bactericidal treatments were chosen to simulate possible environmental conditions present during a sterilization procedure involving heating with a bactericide. Counts of viable spores in suspensions were determined just before the commencement of the heating-up period in such treatments. These counts indicated that, when spore inactivation was appreciable, irradiated spores were more readily inactivated than nonirradiated spores by the same bactericidal treatment during this period.

The rate of inactivation of the most resistant members of spore populations undergoing bactericidal treatments was not followed because of the necessity to ensure, by adequate serial dilution of disinfection mixtures, minimization of the carry-over effect of bactericides on the growth of surviving spores. Accordingly, it must be accepted that survival curve plots shown yield an estimate of sporidical efficiency based upon less than the maximum resistance of the test organism.

The magnitude of the enhanced rate of inactivation of spores surviving irradiation, compared to nonirradiated spores, by heating with a bactericide was observed to increase as the dose of gamma irradiation applied to the spores was increased. This was probably due to greater subinactivation radiation damage produced in the
irradiated spores which increased their sensitivity to subsequent inactivation by heating with a bactericide.

It was observed that the apparent magnitude of the enhanced rate of inactivation of irradiated spores was progressively reduced as the severity of the heat treatment with a bactericide was increased, either by raising the temperature or by reducing the pH. This finding suggested that differences in the sensitivity to inactivation of the nonirradiated spores and the spores surviving relatively low doses of gamma irradiation tended to become insignificant in altering the rate of observed spore inactivation caused by severe heat treatment with a bactericide.

The enhanced rate of inactivation of irradiated spores was noted when the spores were heated with three different phenolic bactericides. This is an indication that the enhancement effect is a general phenomenon exhibited with all phenolic bactericides. The mechanism of action of this effect is at present under investigation and may indicate other methods for increasing its magnitude. The British Pharmacopoeia 1968 (The Pharmacuetic Press, London) states that "sterilisation by heating with a bactericide" involves heating certain thermlabile injections at 98 to 100 °C for 30 min with 0.2% chlorocresol. The results reported indicate that it should be possible to reduce the severity of the heat treatment in this process, although achieving an equivalent probability of sterility, by use of suitable prior irradiation treatment.

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