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

III. Factors Affecting Rates of Inactivation by Phenylmercuric Nitrate

P. B. DEASY, E. KÜSTER,1 AND R. F. TIMONEY

College of Pharmacy, Pharmaceutical Society of Ireland, Shrewsbury Road, Dublin, Ireland

Received for publication 6 July 1971

Aqueous suspensions of Bacillus subtilis NCTC 8236 spores, surviving gamma irradiation from a cesium-137 source, exhibited an enhanced rate of inactivation compared to nonirradiated spores when heated with 0.04% phenylmercuric nitrate. The enhanced rate of inactivation, observable from survival curves, was noted when spores were irradiated with 150,000 rad under air in either the presence or absence of the bactericide. The magnitude of the enhanced inactivation rate increased as the irradiation dose under air increased from 150,000 to 300,000 rad. The inactivation rates of spores surviving irradiation with 150,000 rad under either oxic or anoxic conditions did not exhibit a simple quantitative relationship. The enhancement effect was observed when the severity of the heat treatment was increased by either reducing the pH from 8 to 6 or raising the temperature from 70 to 90 C.

Phenylmercuric nitrate is a commonly used mercurial bactericide. Mitchell (5) noted the suitability of 0.004% phenylmercuric nitrate for inclusion in ophthalmic vehicles to facilitate sterilization by heating at 100 C. In the British Pharmacopoeia 1968 (The Pharmaceutical Press, London) sterilization process of "heating with a bactericide," 0.002% phenylmercuric nitrate is added to thermo-labile injections which are then heated at 98 to 100 C for 30 min to effect sterility.

It has been reported by us (1, 3) that prior treatment of Bacillus subtilis spores with low doses of gamma irradiation enhances their rate of inactivation, compared to nonirradiated spores, by various heat treatments with phenolic bactericides. Accordingly, it was decided to attempt to establish a similar enhancement effect in the inactivation of the spores when phenylmercuric nitrate was used as bactericide. Such studies would indicate the usefulness of a sterilization process based on combined minimal dose of gamma irradiation and thermal treatment with a bactericide.

MATERIALS AND METHODS

The preparation and storage of the stock spore suspension of B. subtilis NCTC 8236, the viable count procedure, the dosimetry of the cesium-137 gamma irradiation facility, and the utilization of the heat source have been described (1-3). The count of viable spores in the stock suspension used was ca. 2 x 109 organisms per ml and did not diminish significantly (P = 0.05) in viability during storage at 5 C throughout the 9 months of usage in the experiments described. Spores of equivalent total count, obtained from the stock suspension, were used for determining comparable survival curves. The method of construction and statistical analysis of divergence between survival curves has been described (3).

Medium. Preliminary experiments indicated the suitability of nutrient agar (Oxoid) containing 1% dextrose, sterilized by autoclaving at 10 psi for 15 min, to which was added aseptically at 55 C 0.3% sodium thioglycolate previously sterilized by filtration. The sodium thioglycolate was prepared and assayed just prior to incorporation into the medium as described by Steel (6).

Chemicals. Glass-distilled water was used to prepare the medium and chemical solutions. Laboratory grade phenylmercuric nitrate and thioglycolic acid and analar grade dextrose, sodium phosphate, sodium acid phosphate, and sodium hydroxide reagents were obtained from The British Drug Houses, Ltd.

Phenylmercuric nitrate carryover. Serial dilutions of disinfection mixtures containing phenylmercuric nitrate were made in sterile water. The appropriate serial dilutions used for the enumeration of surviving spores were adjusted to contain 0.00004% phenylmercuric nitrate, as preliminary experiments showed that this procedure minimized the carryover effect of the bactericide on viable counts.
RESULTS

It was considered desirable to cause inactivation of spores through several log cycles of surviving fraction for the realistic evaluation of the sterilization procedure. Preliminary experiments indicated that a suitable concentration of phenylmercuric nitrate for such a study was 0.04% when proposed experimental conditions were varied. The divergence of survival curves determined for replicate experiments was insignificant, whereas the divergence of all the comparable survival curves reported in this communication was significant, unless otherwise indicated.

Effect of presence or absence of phenylmercuric nitrate during irradiation on spore inactivation. Volumes of spore suspension in 0.04% phenylmercuric nitrate, phosphate buffer (pH 7), were either nonirradiated or irradiated with 150,000 rad under air. Figure 1 shows that spores surviving irradiation were more readily inactivated than nonirradiated spores by subsequent heat treatment at 80°C. There was no observable alteration in the pH of the suspensions after treatment, but the phenylmercuric nitrate was noted to have undergone less than 5% radiolytic decomposition, when estimated by the method recommended by Eldridge and Sweet (4). This alteration in the effective concentration of the bactericide could affect the magnitude of the divergence between the survival curves discussed.

The inactivation experiments were repeated with the exception that the 0.04% phenylmercuric nitrate, phosphate buffer (pH 7), was added to the nonirradiated and irradiated spores just prior to the commencement of the heat treatment. The survival curves (Fig. 1) indicated that the spores surviving irradiation were also more readily inactivated than nonirradiated spores by heating with the bactericide in the absence of its radiolytic products.

Effect of dose of irradiation on spore inactivation. Aqueous spore suspensions were either nonirradiated, irradiated with 150,000 rad under air at 80°C, or irradiated with 300,000 rad under air. These suspensions were then divided into replicates, each of which was exposed to 150,000 rad, and the surviving fraction was determined. The suspensions irradiated with 300,000 rad under air had a significantly lower surviving fraction than those irradiated with 150,000 rad under air, and those nonirradiated had the lowest surviving fraction. Therefore, the dose of 150,000 rad under air was chosen for further experimentation.
Effect of temperature on spore inactivation. Figure 5 shows survival curves for aqueous suspensions of spores, either nonirradiated or irradiated with 150,000 rad under air, before heating at either 70 or 90°C in the presence of 0.04% phenylmercuric nitrate, phosphate buffer (pH 7). The severity of the bactericidal treatment was increased by raising the temperature, and it was observed that spores surviving irradiation showed an enhanced rate of inactivation compared to nonirradiated spores at both temperatures.

DISCUSSION

When aqueous suspensions of spores were irradiated, damage induced in the spores must be attributable to both direct and indirect action of radiation. It is probable that one or several
radiochemical reactions in the spore initiated a sequence of events, which resulted in either spore inactivation or a predisposition of surviving spores to inactivation during storage and heat treatment with the bactericide.

The enhanced rate of inactivation of spores surviving irradiation, compared to nonirradiated spores, caused by heating with phenylmercuric nitrate, phosphate buffer (pH 7), was observable in the presence or absence of the radiolytic products of the bactericide. The magnitude of the enhancement effect was observed to increase as the dose of prior gamma irradiation applied to the spores was increased. However, the enhanced inactivation rate, caused by moist heat treatment at 80°C with 0.04% phenylmercuric nitrate of the spores surviving irradiation under either oxic or anoxic conditions, did not exhibit a simple quantitative relationship. The enhancement effect was also noted when the severity of the moist heat treatment with the bactericide was increased by either raising the temperature from 70 to 90°C or reducing the pH from 8 to 6.

These results are qualitatively similar to those reported previously (3) when using 0.2% chlorocresol as a bactericide. Such findings indicate that the enhancement effect, induced by prior irradiation of the spores, may be exhibited in other systems by using heat treatment with different bactericides.

The enhanced bactericidal efficiency of a combined process, based on minimal gamma irradiation and heating with a bactericide treatment, may be of use for the sterilization of substances which undergo significant degradation with conventional sterilization techniques. The combined process should facilitate the ready inactivation of those organisms in the mixed flora of the substances which are particularly sensitive to inactivation by any of the individual bactericidal agents comprising the process. Findings reported in this and previous communications (1, 3) suggest that such a combined process has potential and is worthy of further investigation. The results of these investigations, presently in progress, will be published later.

ACKNOWLEDGMENT

The cesium-137 gamma irradiation unit used is part of a generous gift of nuclear equipment to the Irish Government from the United States Government.

LITERATURE CITED

1. Deasy, P. B., E. Küster, and R. F. Timoney. 1968. Influence of γ-irradiation and heating in the presence of a bactericide on the inactivation of Bacillus subtilis spores. Appl. Microbiol. 16:810-811.
2. Deasy, P. B., E. Küster, and R. F. Timoney. 1970. Resistance of Bacillus subtilis spores to inactivation by gamma irradiation and heating in the presence of a bactericide. I. Suitability of viable count procedures. Appl. Microbiol. 20:455-460.
3. Deasy, P. B., E. Küster, and R. F. Timoney. 1970. Resistance of Bacillus subtilis 50 spores to inactivation by gamma irradiation and heating in the presence of a bactericide. II. Factors affecting rates of inactivation by phenolic bactericides. Appl. Microbiol. 20:461-464.
4. Eldridge, A., and T. Sweet. 1956. Spectrophotometric determination of phenylmercuric acetate. Anal. Chem. 28:1268-1271.
5. Mitchell, J. A. 1962. The bactericidal activity at 100 degrees C of some ophthalmic vehicles. Aust. J. Pharm. 43:1139-1143.
6. Steel, K. J. 1958. A note on the assay of some sulphhydril compounds. J. Pharm. Pharmacol. 10:574-576.