Efficacy of the Inactivation of Bacterial Spores in White Petrolatum and a Hydrophilic Ointment by Gamma Irradiation

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To evaluate the possibilities of using gamma irradiation for the sterilization of ointments, the effect of irradiation on spores of Bacillus pumilus and Bacillus sphaericus in dry material and in two different kinds of ointments was studied. The results indicate that for sterilization purposes irradiation was less effective in white petrolatum as compared to irradiation in the dry state. No such protective effect was found in a hydrophilic ointment. Accordingly, the sterilization dose needed for the sterilization of an ointment can be decided upon only after inactivation experiments with suitable test organisms in the actual preparation.

Irradiation sterilization of pharmaceutical preparations has recently been introduced in Norway. In 1970, the gamma irradiation plant at the Norwegian atomic energy establishment near Oslo was permitted to sterilize disposable medical equipment, and in 1972 this permission was extended to pharmaceutical preparations for topical use.

The irradiation plant has also been used for scientific work. The purpose of the present research program was to investigate the possibilities of radiation sterilization of certain pharmaceutical preparations for which heat sterilization is not possible, e.g., eye ointments and ointments containing steroids. The program also aimed at creating a scientific background for irradiation sterilization in Norway as a service for Norwegian pharmaceutical industry.

The collaborative project has three different parts: chemical and physical tests on irradiated ointment bases, chemical tests on irradiated drugs, and investigation of the microbiological efficiency of irradiation of ointments.

The purpose of the microbiological part of the work has been to compare the effect of irradiation on microorganisms in dry material with the effect of the same dose in two different kinds of ointments.

In 1967, Haraszti et al. (10) studied the effect of irradiation on Bacillus pumilus in hydrophilic and hydrophobic ointments and concluded: "A marked increase in the resistance of the spores due to the ointment medium could be observed. The experiments show that guaranteed sterility is not achieved even with doses as high as 5.0 Mrad if massive contamination is present." Affolter and Speiser (2) discuss different factors that influence the radiation resistance of microorganisms. The present theoretical knowledge is, however, not sufficient to predict whether an ointment will alter the resistance or not. Affolter et al. (1) studied the influence of two ointments and their components on the radiation resistance of spores of Bacillus subtilis. Their results suggested, for example, that in ground-nut oil the spores had an increased sensitivity to irradiation, whereas an o/w ointment containing 20% ground-nut oil seemed to protect the spores. Jacob and Leupin (12) irradiated two different ointments and found that 2.5 Mrad was not a sufficient sterilization dose when the ointments were inoculated with 10⁶ spores of Bacillus sphaericus per g, whereas 4.5 Mrad was considered sufficient.

The health authorities of Norway have established 3.2 Mrad as the minimum required dose for gamma sterilization of disposable medical equipment. The choice of dose was made basically from the work of Christensen et al. (7, 8). The manufacturing of the product and the irradiation plant shall meet the requirements of the International Atomic Energy Agency recommended code of practice for radiosterilization of medical products (11). As the minimum dose of 3.2 Mrad is related to the effect of irradiation of microorganisms in dry material, and considering the partly conflicting and incomplete experience reported in the literature on irradiation of microorganisms in ointments, the present study was carried out.

MATERIALS AND METHODS
The gamma irradiation plant at IFA has a cobalt
The quality of the white petrolatum meets the requirements of the Nordic Pharmacopoeia (14). Chemical analysis of this material irradiated at 1, 2.5, 3.5, and 6 Mrad showed the main compounds formed during the irradiation to be hydrogen and methane. No significant changes in the ultraviolet spectrum were observed (13).

Samples (1 g) of white petrolatum inoculated with spore sand, each containing approximately $10^8$ viable units of B. pumilus or $10^9$ viable units of B. sphaericus, were irradiated in 100-ml bottles. Doses from 0.5 to 3.5 Mrad for B. pumilus and 0.5 to 6.5 Mrad for B. sphaericus were used. Each experiment included at least three dose levels (experiments A and B). Two days after irradiation the white petrolatum was solved in 20-ml sterile isopropyl myristate (membrane filtered; Millipore Corp., GSWP 142-50, pore diameter 0.22 μm), and the solution was filtered through Sartorius membrane filters SM 11406, pore diameter 0.45 μm. The filters were washed according to the procedure in USP XVIII (15) and incubated on solid tryptone-glucose-yeast agar at 32 C. Contact time with isopropyl myristate was made as short as possible, approximately 15 min.

As a comparison samples of spore sand containing the same number of viable units as the samples with white petrolatum were also irradiated in 100-ml bottles. Two days after irradiation, the sand was mixed with peptone water, membrane filtered, and incubated in the same way as the samples containing white petrolatum.

In experiment C the irradiated samples were incubated in liquid tryptone-glucose-yeast medium containing Tween 80. The incubation temperature was 40 C and the bottles were shaken during incubation. No isopropyl myristate was used in this experiment.

The test organism, B. pumilus, was also irradiated in a hydrophilic ointment containing cetomacrogol, propylene glycol, glycerol, and macrogol 400 (experiment D). Two days after irradiation the samples were mixed with peptone water, membrane filtered, and incubated as described above. The ointment has been tested by the chemical group, and their results indicate that the chemical effects induced by irradiation doses in the range required are tolerable.

**RESULTS**

Experiment A: effect of gamma irradiation on bacterial spores in dry state and mixed with white petrolatum studied by the membrane filter technique. In experiment A the effect of gamma irradiation on spores of B. pumilus and B. sphaericus in dry state and mixed with white petrolatum was studied. The inactivation curves (Fig. 1 and 2) for both microorganisms were steeper when irradiated in sand compared to irradiation in white petrolatum. This indicates that the irradiation was less effective for the inactivation of spores irradi-
radiated in white petrolatum. The difference in starting point of the inactivation curves for spores in sand and spores in white petrolatum (Fig. 1 and 2) may be due to toxic effects of isopropyl myristate, and perhaps to difficulties of viable microorganisms to multiply because of a film of white petrolatum on microorganisms and filters.

Experiment B: effect of isopropyl myristate on the results obtained in experiment A. In experiment A different laboratory techniques were used when counting viable units in sand (mixed with peptone water) and in white petrolatum (mixed with isopropyl myristate). To confirm that the results were not due to this difference in laboratory technique the following experiment was carried out.

After irradiating the spores in sand and in white petrolatum as in experiment A, the spore sand was mixed with white petrolatum and both sets of samples were treated with isopropyl myristate in the same way.

As can be seen from Fig. 3, both curves in this experiment have the same starting point. In this experiment, like in experiment A, the irradiation curve for spores irradiated in sand is steeper than the corresponding curve for spores irradiated in white petrolatum. Thus, the difference between the inclination of the two inactivation curves does not seem to be due to differences in laboratory technique.

Experiment C: effect of gamma irradiation on bacterial spores in dry state and mixed with white petrolatum using culture in liquid media. The difference between the two inactivation curves is also confirmed by the results in experiment C (Table 1). In this experiment 12 bottles containing spore sand and 12 bottles containing spore sand in white petrolatum were irradiated and later cultured in liquid medium. Based on the inactivation curves (Fig. 1 and 2) and considering the sensitivity of the method, two dose levels for each microorganism were chosen to show the difference in survival (i.e., for B. pumilus in white petrolatum, 3.1 Mrad, and in sand, 2.5 Mrad; and for B. sphaericus, 6.1 and 5.5 Mrad, respectively). For both microorganisms a higher number of bottles with survivors were found when the spores had been irradiated in white petrolatum.

Experiment D: effect of gamma irradiation on bacterial spores in dry state and mixed with a hydrophilic ointment. In experiment D spores of B. pumilus were irradiated in the hydrophilic ointment. As can be seen (Fig. 4) there is no difference between the inactivation curves for spores irradiated in hydrophilic ointment and in dry state. Each 1-g sample was placed in a 100-ml bottle and exposed to a given dose of gamma irradiation.

![Fig. 3. Comparison of inactivation of B. pumilus spores irradiated in white petrolatum and in dry state. (I) Irradiated in white petrolatum, mixed with isopropyl myristate, membrane filtered. (II) Irradiated in sand, mixed with white petrolatum, mixed with isopropyl myristate, membrane filtered. Each curve is based on the results of two irradiation experiments with three dose levels.](http://aem.asm.org/)

![Fig. 4. Comparison of inactivation of B. pumilus spores irradiated in hydrophilic ointment and in dry state. (I) Irradiated in hydrophilic ointment, mixed with peptone water, membrane filtered. (II) Irradiated in sand, mixed with peptone water, membrane filtered. Each curve is based on the results of one irradiation experiment with three dose levels.](http://aem.asm.org/)
tion curves of spores in sand and spores in this hydrophilic ointment. The results conform with curve II (Fig. 1). Thus, this hydrophilic ointment does not seem to alter the effect of gamma irradiation on spores.

DISCUSSION

The degree of safety for sterilization of drugs is defined in the Nordic Pharmacopoeia (14) as follows. "Sterile drugs must be prepared and sterilized under conditions which aim at such a result that in one million units there will be no more than one living microorganism." This definition is used irrespective of the sterilization method.

When a certain degree of safety is desired in irradiation sterilization, the minimum sterilization dose depends on the initial number of microorganisms in the product. In Norway, for instance, the dose 3.2 Mrad for disposable medical equipment refers to products with an initial count of less than 50 microorganisms per unit. When it exceeds this limit the dose must be increased to maintain the inactivation required. If the type and sensitivity of the most resistant organisms contained in the product is not known, reference should be made to the inactivation factors obtained for the most resistant organisms under environmental conditions which maximize their resistance (such as the biological reference standard) (11).

When the minimum dose for radiation sterilization was decided, it was based on studies of the microbial flora on such equipment and on the fact that the microorganisms were irradiated in dry state. In ointments, however, the environment is totally different, as it may contain water as well as various chemicals, which might influence the effect of irradiation. The information we have on radiation resistance of microorganisms on medical equipment therefore may not be relevant for ointments.

The microbial flora present in ointments may also be different from that of dry medical equipment. It is known that ointments manufactured without special considerations regarding hygiene may hold high numbers of microorganisms, since raw materials may contain a high number of microorganisms, and many ointments allow microbial growth.

Little information, however, is available on the kind and number of microorganisms present in ophthalmic ointments and topical ointments containing steroids manufactured under the hygienic conditions required today.

In 1972, Bowman et al. (3) tested 82 different ophthalmic ointments and found that 17% of the non-antibiotic and 22% of the antibiotic ointments were contaminated. The contamination was 1 to 10 microorganisms per g and consisted of mold, yeast, and Bacillus sp.

Wargo (16) reported in 1973 about contamination in different ointments containing antibiotics and steroids. The contamination consisted mainly of gram-positive cocci and diplococci, but also gram-negative rods were found.

In their work Haraszti et al. (10) and Affolter et al. (1) studied some of the factors of importance for radiation sterilization of ointments. Their results, however, do indicate that no generally applicable sterilization dose can be settled.

This information is confirmed by our results. Our experiments show that with the same degree of contamination the hydrophilic ointment used in our studies can be sterilized with approximately the same irradiation dose as dry materials, such as syringes. The white petrolatum, on the other hand, would need a considerably higher dose for sterilization. The final sterilization dose, however, cannot be established without further experiments with sporeformers and viruses.

The reason for this difference of inactivation found in our experiments is not known. Differences in effect of irradiation on microorganisms in ointments might be caused by protecting or sensibilizing substances present in the ointment, or by a combination of such effects. Oxygen, for example, is known to enhance the inactivation, whereas glycerol has been suggested as a protective agent (2). In our experiments, however, the presence of glycerol in the hydrophilic ointment did not seem to have any protective effect.

The fact that the effect of irradiation on microorganisms depends to such a large extent on their environment makes it necessary in each case to determine the inactivation curves for various test organisms in the pharmaceutical, and should also be kept in mind when the formulation of an ointment intended for irradiation sterilization is decided.

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LITERATURE CITED


