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Relative Resistances of Microorganisms to Cathode Rays

III. Bacterial Spores

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Previous communications from this laboratory have described the resistance to cathode rays of vegetative bacterial cells and various yeasts and fungi.

The present report concerns studies on the resistance to cathode rays of spores from numerous identified and unidentified species of bacilli. The lethality response as affected by number of spores in an exposure and their physical state at the time of exposure was studied.

TABLE 1. Fortified brain-heart infusion agar

Components	Amount
Bacto peptone.....	10 g
Dextrose.....	8 g
Yeast extract.....	10 g
Sodium thioglycollate.....	1 g
Agar.....	15 g
Brain-heart infusion (Difco).....	50 g
Distilled water.....	1000 ml

pH 7.2.

The response of cathode ray resistant isolates to subsequent irradiation was also studied.

During these investigations the question arose as to whether the presence of a large quantity of bacterial cells in a suspension would have a sparing effect on some of the species of spores studied. Experiments were designed to explore this possibility.

MATERIALS AND METHODS

Aerobic spores. The majority of aerobic spores were produced on trypticase soy agar (BBL); nutrient agar

(BBL) was employed for sporulation of unidentified bacilli, E-594 A, B and C. Cultures were incubated for 6 days at 37 C, harvested and washed twice with distilled water.

Anaerobic spores. For all but two *Clostridium* species, spores were produced on fortified brain-heart infusion agar (table 1). Sporulation of *Clostridium novyi* and *Clostridium aereofoetidum* was accomplished in cooked meat phytone (BBL). Anaerobic cultures were incubated for 6 days at 37 C in an atmosphere of helium (Bridges *et al.*, 1952).

Spores produced on agar were harvested by simple rinsing from the surfaces. Spores in cooked meat phytone were separated by filtration through cheese-cloth followed by centrifugation at 300 rpm for 5 min to sediment gross particles of the medium. Subsequent centrifugation at higher speeds and washing twice in distilled water provided suspensions for storage at 4 C. Numbers of viable spores were determined by plate counts prepared with aliquots which had been heated to 80 C for 15 min and incubated at 37 C, 1 to 2 days for aerobes and 3 to 5 days for anaerobes.

Radiation. The relative resistances of bacterial spores to cathode rays were determined with suspensions containing 5×10^5 viable spores per ml. One-tenth-ml aliquots of a suspension were transferred to filter paper discs¹ for each irradiation dose investigated. The 4 replicate discs were inserted in double sterile

¹ Schleicher & Schuell Co., Keene, New Hampshire. No. 740-E, diameter 12.7 mm.

polyethylene² envelopes, hermetically sealed and exposed to the cathode beam. After irradiation, the packets were chemically disinfected for 18 hr. Discs impregnated with aerobic spores were transferred to trypticase soy broth and those impregnated with anaerobic spores to fluid thioglycollate medium I (U.S.P.) containing 0.1 per cent soluble starch.

To study the relationship of numbers of spores and the radiation dosage necessary to sterilize³ such an inoculum, suspensions of 2 unidentified *Bacillus* species (E-588 and E-601) were logarithmically diluted. One-tenth-ml aliquots were transferred to discs which were packaged, irradiated and subcultured as described above.

Studies of the effect of physical state on radiation resistance were made with *Bacillus pumilus*. Discs were impregnated with spores and then treated so that the spores were in either a moist, frozen or dried condition. For determination of resistance of moist spores, discs were packaged in double envelopes which were immediately sealed and irradiated. To obtain irradiated frozen spores, impregnated discs, sealed in envelopes, were stored for 1.5 hr in dry ice and irradiated while frozen. Controls, treated similarly, were thawed just prior to irradiation. The resistance of dried spores was determined by drying impregnated discs at 37 C for 18 to 24 hr prior to packaging and irradiation. Control samples, similarly dried, were hydrated immediately prior to packaging and irradiation.

Evaluation of possible protection afforded by contamination with a large quantity of bacterial cells was accomplished by adding aliquots of a spore preparation of *Bacillus pumilus* containing 1×10^6 per ml to an equal quantity of a concentrated aqueous suspension of viable *Escherichia coli*. Discs were impregnated with the mixture, packaged and irradiated in the moist state. Controls, similarly prepared, consisted of equal numbers of spores suspended in distilled water.

Progeny of irradiated spores were obtained from *B. pumilus* and *Clostridium sporogenes* spore populations which had survived maximum sublethal irradiation doses. Spores of the progeny were produced and 2-ml aliquots, containing 1×10^6 spores per ml, were placed into 100- by 11-mm glass tubes and sealed for irradiation with various doses. Suspensions of spores from the parent strain were similarly prepared. Following irradiation, aliquots were transferred to agar plates and incubated at 37C. Plates inoculated with *B. pumilus* were examined after 1 to 2 days' incubation; those with *C. sporogenes* after 4 to 5 days. Survival curves of parent and derived (R) strains were plotted. By irradiating R strains, other derivative (RR) strains were obtained.

² Thickness: 5 mils.

³ Sterility here denotes the inability of the microorganisms to reproduce under these conditions of test.

TABLE 2. Resistances of bacterial spores

Spores of		Highest Dose Showing All Positive Cultures	Lowest Dose Showing All Negative Cultures
		megareps*	megareps
E-3	<i>Clostridium tetani</i>	1.5	2.1
E-95	<i>Bacillus pumilus</i>	1.3	2.1
E-601	<i>Bacillus species</i>	1.2	1.9
E-594B	<i>Bacillus species</i>	1.2	1.9
E-594C	<i>Bacillus species</i>	1.0	1.9
E-40	<i>Bacillus mesentericus</i>	1.3	1.8
E-58	<i>Clostridium sporogenes</i>	1.3	1.7
E-93	<i>Bacillus subtilis</i>	1.1	1.6
E-53	<i>Clostridium aereofoetidum</i>	1.0	1.6
E-587	<i>Clostridium species</i>	1.0	1.5
E-2	<i>Bacillus subtilis</i>	1.3	1.5
E-594A	<i>Bacillus species</i>	1.1	1.5
E-44	<i>Clostridium sporogenes</i>	1.0	1.4
E-86	<i>Clostridium tetani</i>	1.0	1.4
E-42	<i>Clostridium novyi</i>	1.1	1.4
E-603	<i>Bacillus species</i>	1.2	1.4
E-599	<i>Bacillus species</i>	1.0	1.4
E-608	<i>Bacillus species</i>	1.1	1.4
E-606	<i>Bacillus species</i>	1.0	1.3
E-588	<i>Bacillus species</i>	<1.0	1.2
E-85	<i>Bacillus coagulans</i>	<1.0	1.2
E-92	<i>Bacillus subtilis</i>	<1.0	1.2
E-627	<i>Bacillus species</i>	0.8	1.2
E-533A	<i>Bacillus coagulans</i>	0.5	1.0
E-626	<i>Bacillus species</i>	0.4	0.6

* 1 Megarep = 1 million reps.

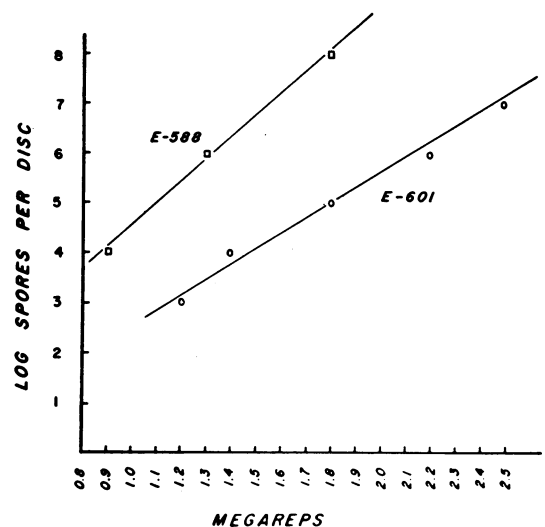


FIG. 1. Radiation dosages required to sterilize are a function of numbers of spores exposed.

RESULTS

Relative resistances. The radiation dose required to sterilize discs impregnated with the bacterial spores tested varied from 0.6 megareps to 2.1 megareps (table 2). It was not possible to demonstrate that the clostridia were more resistant than the bacilli.

Effect of numbers of spores. As the concentration of spores increased, larger doses of cathode rays were

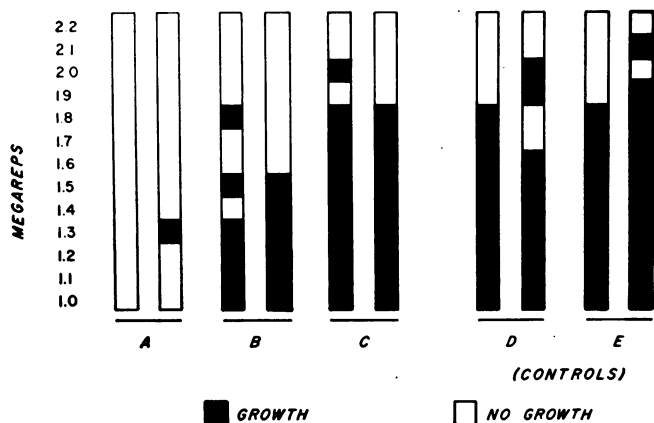


FIG. 2. Cathode ray sterilization of bacterial spores exposed in dried, frozen and moist states. A, dried; B, frozen; C, moist; D, frozen and thawed; E, dried and rehydrated (duplicate experiments).

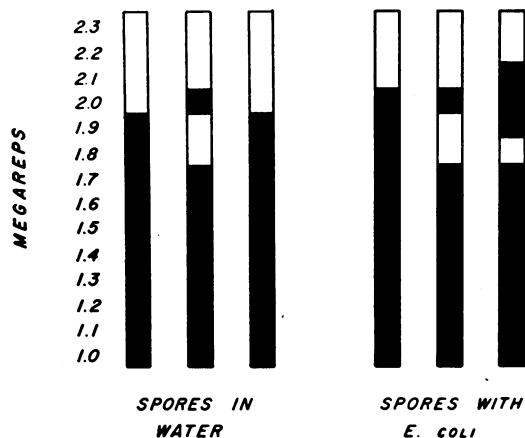


FIG. 3. Cathode ray sterilization of bacterial spores contaminated with a large quantity of *Escherichia coli*.

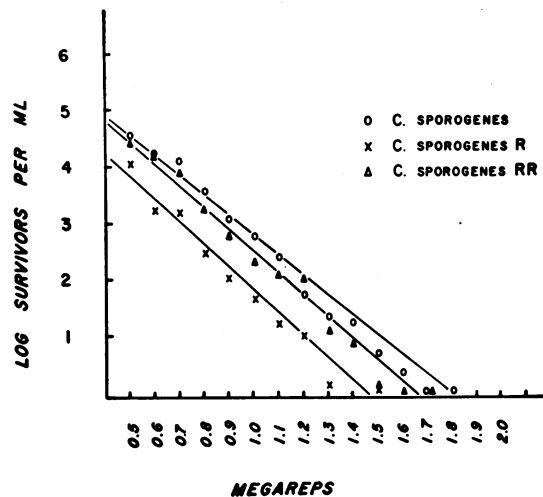


FIG. 4. Survival curves of parent and irradiated strains of *Clostridium sporogenes*.

required to sterilize. This relationship between radiation sterilizing dose and numbers of spores is shown in figure 1.

Effect of variation in physical state of spores. Results obtained when *B. pumilus* spores were irradiated in the

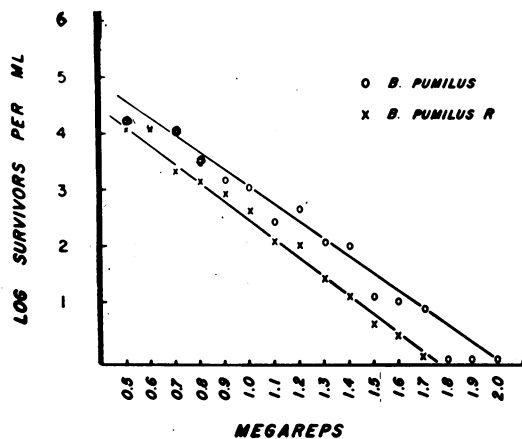


FIG. 5. Survival curves of parent and irradiated strains of *Bacillus pumilus*.

moist, frozen and dried states are shown in figure 2. Freezing or drying *per se* apparently did not affect the sensitivity of the spores to irradiation effects since survival patterns of the 2 control preparations, which were thawed or hydrated just prior to irradiation, were similar to that of the spores irradiated in the moist state. Spores irradiated in the frozen state were somewhat more susceptible to cathode rays; most sensitive were spores irradiated in the dry state. Similar results with dried spores were obtained in other studies when the drying period was extended from 1 to 2 days.

Effect of contamination with a large quantity of bacterial cells. A concentrated suspension of viable *E. coli* cells failed to protect spores of *B. pumilus* from lethal effects of cathode rays as shown in figure 3. *E. coli* survived none of the radiation doses.

Resistances of progeny of irradiated spores. Spores produced by radiation-resistant survivors of *B. pumilus* and *C. sporogenes* were found not to differ from their parents in their radiation resistance. When spores from the progeny of radiation-resistant isolates were subsequently exposed to cathode ray doses identical to those received by the parent, the survival curves were similar to those of the parent (figures 4 and 5).

DISCUSSION

Bacterial spores are more resistant to sterilization by cathode rays than bacterial vegetative cells (Koh *et al.*, 1956), yeasts or fungi (Bridges *et al.*, 1956).

Differences in resistances to radiation sterilization were noted in both the *Bacillus* and *Clostridium* genera (table 2). One megarep or more was required to sterilize 24 of the 25 species studied. Only 2 of the 25 species required more than 2 megareps. The difference in megareps between the highest dose yielding all positive cultures and the lowest dose giving all negative cultures was not consistent for all the spores studied. This difference varied from 0.2 megarep in culture E-626, a *Bacillus* species, to 0.9 megarep in culture E-594-C, also a *Bacillus* species (table 2). Such findings would

suggest that, in addition to the variations in the lethality response, there is an individual sensitivity to lower radiation doses.

Edwards *et al.* (1954) have reported that the radiation dose required to sterilize is related to the number of spores exposed. These workers also found that spores in the frozen state are somewhat more susceptible to cathode rays than are moist spores. The results of the present study are in agreement with their observations.

Survival patterns obtained in the present studies confirm the observation of Dunn (1948) that 95 to 99 per cent of the spores exposed to cathode rays were killed by 0.5 megarep while an additional 1 to 1.5 megareps were required to destroy the remainder.

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SUMMARY

Marked variations in resistances of bacterial spores of various species to cathode rays were observed. Dosages required to sterilize varied from 0.6 to 2.1 megareps. Species of *Clostridium* were found not to be more resistant than species of *Bacillus*. Bacterial spores are more resistant to the lethal effects of irradiation

than are vegetative bacterial cells, yeasts, or molds. When spores in various physical states were irradiated it was found that the resistance of moist spores was greater than frozen spores and greater than dried spores. No protection from irradiation effects were observed when large quantities of *Escherichia coli* were employed with a spore suspension subjected to cathode rays. When spores prepared from cathode ray resistant isolates were irradiated, the survival patterns were similar with those of the parent strain. The radiation resistance of a species of bacterial spore is a function of the numbers exposed.

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