RADIATION STERILIZATION

I. The Effect of High Energy Gamma Radiation from Kilocurie Radioactive Sources on Bacteria

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One of the main advantages of sterilizing heat-sensitive pharmaceutical preparations by X and gamma radiation lies in the ability of these ionizing radiations to penetrate thick walled packaging materials without inducing radioactivity in the material which is being irradiated (Stanford Research Institute, 1951). This phenomenon is in contrast to the relatively low penetrating power of cathode rays (electrons) and of ultraviolet light (Hink and Johnson, 1951). As the absorption of cathode, X, and gamma radiation is accompanied by a negligible temperature rise (Brasch and Huber, 1948), it may be possible to substitute this technique for the more expensive aseptic procedures now required (Brewer, 1948). A number of investigators have studied the effect of radiation on various types of microorganisms. In the main, these irradiations were carried out with radioactive sources of low intensity, with x-ray machines, or with electron accelerators (Sparrow and Rubin, 1951; Fram *et al.*, 1950; Trump and Van de Graaff, 1948; Brasch and Huber, 1947; Kirsch and Huber, *to be published*. The recent availability of intense sources (1,000 curie) of radioactive isotopes (Manowitz, 1951) and the potential availability of even more intense radioactive by-products from the atomic energy program make investigation of these new radiation sources of interest.

MATERIALS AND METHODS

The early irradiations were carried out with 1.1 to 1.3 million electron volt gamma rays in the tubular radioactive cobalt (Co^{60}) and tantalum (Ta^{182}) sources developed by the Brookhaven National Laboratory (Manowitz, 1951). It has been found by the Brookhaven group that a very uniform radiation field exists within the central volume of these 1,000 curie sources which are contained in lead shields. After having previously measured the radiation dose rate in this volume, bundles of samples are inserted by remote control and allowed to remain in the gamma field for sufficient time to receive the desired total dose.

In order to investigate the effect of lower intensity radiation from these same sources, an arrangement of aluminum tubes at various distances around an underwater kilocurie source was constructed (Tarpley *et al.*, *in press*). With this apparatus it was possible to introduce samples into portions of the radiation field at various intensities for the desired time interval. Bundles of sample tubes were inserted and removed by remote control, and the total radiation dose was controlled by time of exposure.

It was found that the ferrous sulfate to ferric sulfate conversion (Miller, 1948) produced by irradiation was a satisfactory measurement of intensity. All such dosimetry measurements were made in the same positions and same type of container as were used to irradiate the microbial suspensions. As an additional control, the radiation produced ceric sulfate to cerous sulfate conversion was employed (Clark and Coe, 1937; Weiss, 1952). This dosimetry system, in contrast to the ferrous-ferric system, permits measurement at very high total radiation doses. One ceric sulfate dosimeter vial was incorporated in the center of each bundle of vials and was used to monitor the total radiation dose received by each bundle. Comparison of the total dose, computed from the ferrous-ferric intensity measurement and the time of exposure, with that actually measured by the ceric sulfate monitor shows that within the precision of the experiment (± 15 per cent of total radiation dose) the radiation received within the vials was very close to that desired.

A problem involving sterilization of a heat-sensitive suspension of a steroid (cortisone acetate) dictated the medium in which sterilization

was to be studied. Twenty-five mg per ml suspensions of steroid in a modified aqueous phosphate suspending medium (pH 6.9; M/15; isotonic with NaCl) (Hind and Goyan, 1947) were prepared without the addition of chemical preservative. Microorganisms were added to portions of the suspension as indicated. In order that a relatively large number of bacteriological measurements could be made, aliquots of 0.4 ml of uniform suspension were placed under aerobic conditions in rubber capped 0.6 ml vials (40 by 8 by 1 mm thick), and bundles of vials were irradiated at the Brookhaven National Laboratory within 24 hours after inoculation. The extent of contamination was checked by culturing unirradiated controls. After irradiation each vial was cultured in thioglycolate liquid medium (Difco). In some instances one part of the sample was cultivated in thioglycolate medium, another in anaerobic agar (Difco). All tubes were incubated for fourteen days at 37 C. In certain experiments the number of viable organisms was counted by growth in serial dilutions. Any organisms surviving irradiation were subcultured and examined microscopically and for appearance of the colonies on agar.

Since sensitivity to irradiation is different among different bacterial species and genera (Dunn *et al.*, 1948), a variety was studied. The organisms used were obtained from the following sources and were cultured as indicated:

(a) *Escherichia coli*, strain "Texas". The strain was received from Dr. D. Billen, Department of Bacteriology, University of Tennessee. A 24 hour culture on agar slants.

(b) *Micrococcus pyogenes* var. *aureus*, strain P-209 (ATCC 6528-P). A 24 hour culture on agar slants.

(c) *Pseudomonas aeruginosa*. The strain was received from Dr. Cowles, Yale University. A 24 hour culture on agar slants.

(d) *Bacillus subtilis* (strain ATCC 6633). A 4 day culture (37 C) on agar slants.

(e) *Bacillus coagulans*, received from the National Canners Association under the name of *Bacillus thermoacidurans*, strain 43-P. According to instructions *B. coagulans* was cultivated on the acid proteose peptone agar at 37 C for 14 days.

(f) *Clostridium sporogenes* (strain ATCC 10000). A 4 day old culture in fluid thioglycolate medium.

(g) *Candida albicans* (from Dr. E. L. Keenney, Baltimore). A 24 hour culture on Sabouraud agar.

(h) *Aspergillus niveus* (strain NRRL no. 1955), *Penicillin citrinum* (strain ATCC 8506), and *Mucor racemosus* (Schering strain) were cultivated separately for 7 days on Sabouraud agar at room temperature.

Culture mixtures were prepared for irradiation as follows: The culture of *C. sporogenes*, grown for 4 days in thioglycolate medium, was recovered by centrifugation and after being washed, was resuspended in physiological saline. All other cultures were washed from agar slants with physiological saline. They were mixed and after being shaken for 15 minutes in a sterile flask with glass beads were centrifuged. The packed cells were resuspended in saline, transferred to large test tubes, and were left at room temperature for 2 hours to allow gross particles to settle. After this time the turbid supernatant fluid was withdrawn, well shaken, and added to the steroid suspension for irradiation.

The measurement of the radiation dose necessary to accomplish sterilization of a variety of microorganisms and the effect on the sterilization dose of intensity of the radiation field were investigated, but no attempt was made to study the mechanism of inactivation. Since pharmaceutical preparations, in contrast to foodstuffs, are seldom heavily contaminated with microorganisms, no measurements were made concerning the extent of destruction of the bacterial enzyme systems (Proctor *et al.*, 1952).

RESULTS AND DISCUSSION

The results of this investigation are embodied in tables 1 and 2. Table 1 compares the effective sterilization dose of radiation with the intensity with which it was applied. In grouping the results, the average intensities of radiation are shown for the various positions since these did not vary by more than ±10 per cent and similar radiation doses were grouped together.

From an examination of table 1, it may be seen that at the very heavy microorganisms contamination levels used a total dose greater than 2.6, but less than 3.2 megarep was required to accomplish sterilization. All vials irradiated with from 1.32 to 2.0 megarep were not sterile although greater than 99.99 per cent of the organisms were killed. This is in accord with the findings of other workers (Dunn *et al.*, 1948) who reported

that from 2 to 5 times as much radiation are necessary to sterilize as to kill 94 to 99.99 per cent of the organisms. The vials irradiated with doses from 2.73 to 5.16 megarep were found sterile.

It has been reported that some strains of the *Pseudomonas aeruginosa* are completely inactivated by a relatively low radiation dose while other strains of the *Pseudomonas* group require larger doses (Dunn *et al.*, 1948). *E. coli*, *M. pyogenes* var. *aureus*, and *Serratia marcescens* are more resistant. Furthermore, young cells were

TABLE 1

Effect of gamma ray intensity on total dose of sterilization

Extent of contamination prior to irradiation—viable organisms per ml	Average intensity	Total dose* rec'd, megarep	Ratio sterile†	Total dose rec'd megarep	Ratio sterile	Total dose rec'd, megarep	Ratio sterile	Total dose rec'd, megarep	Ratio sterile
		377 hr‡		308 hr		260 hr		126 hr	
Escherichia coli, 4–5 × 10^9	12.7	5.16	5/5	3.43	5/5	3.22	5/5	1.43	0/5
Micrococcus pyogenes var. *aureus*, 3–4 × 10^9	Kilo	4.68	5/5	3.68	5/5	3.14	4/4	1.55	0/5
Bacillus subtilis, 1–2 × 10^9	rep/hr	4.64	5/5	3.95	5/5			1.66	0/5
Clostridium sporogenes, 0.1–1 × 10^9				3.56	5/5				
Candida albicans, 0.1–1 × 10^9									
		122 hr		100 hr		85 hr		41 hr	
	37.8	4.98	5/5	3.18	4/4	3.08	5/5	1.32	0/5
	Kilo	4.77	5/5	3.77	5/5	2.85	5/5	1.37	0/5
	rep/hr	4.66	5/5			2.73	5/5	1.43	0/5
		4.06	5/5						
		97 hr		80 hr		67 hr		33 hr	
	47.5	4.18	4/4	4.02	5/5	3.36	5/5	1.38	0/5
	Kilo	4.87	5/5	3.44	5/5	2.89	5/5	1.42	0/5
	rep/hr			3.54	5/5			1.66	0/5
				4.04	5/5				
		41 hr		33 hr		28 hr		14 hr	
	116.5	4.83	5/5	3.66	5/5	3.33	5/5	1.55	0/5
	Kilo	4.75	5/5	3.73	5/5	3.30	5/5	1.62	0/5
	rep/hr	4.59	5/5	3.96	5/5	3.14	5/5	1.68	0/5
		From 27–40 hr						From 3–16 hr	
Pseudomonas aeruginosa, *Aspergillus niveus*	150	4.1	6/6					0.5	0/4
Escherichia coli, *Penicillin citrinum*	Kilo	4.57	6/6					0.97	0/4
Micrococcus pyogenes var. *aureus*, *Mucor racemosus*	rep/hr	5.22	6/6					1.62	0/4
Bacillus subtilis, *Candida albicans*		5.6	6/6					2.0	0/4
Clostridium sporogenes, *Bacillus coagulans*									
Total 0.1 × 10^9									

* The radiation administered to the bacteria suspensions is expressed in roentgen-equivalent-physical units, rep; intensities of the radiation field in kilorep per hour = 1,000 rep per hr; total dose in megarep = 10^6 rep.

† The number of sterile vials appears in the numerator, while the number irradiated appears in the denominator.

‡ Duration of exposure of samples to gamma radiation field.

found to be more sensitive to irradiation than old cells; vegetative forms were more sensitive than bacterial spores; and dried bacteria more resistant than bacteria in aqueous suspension.

Dunn *et al.* (1948) have reported the dose required for complete destruction of various microorganisms with x-rays produced by a 3 million electron volt cathode beam of a Van de Graaff accelerator impinging on a gold target. Their findings show that to destroy the most resistant sporeforming organisms from 1 to 2 megarep are required when the initial count of microorganisms was of the order of 0.4 × 10⁹ organisms per ml. An apparently higher radiation dose was found to be necessary in the present investigation when using cobalt 60 gamma rays. This may be due either to greater radiation resistance of *C. sporogenes* or to the higher initial microorganism concentration (Sparrow and Rubin, 1951). A third possible explanation for the increase (approximately 30 per cent) may lie in the different dosimetry technique used by Dunn *et al.* (ionization chamber). The response of bacteria to radiation may be related to the response of a chemical dosimeter (ferrous-ferric or ceric-cerous) in some different fashion from that of an ionization chamber. It has been suggested (Brasch and Huber, 1948) that very high intensities of radiation (electron beams) released in ultrashort times (1 microsecond) may lead to lower inactivation doses. However, over the time period of the present investigation (28 to 377 hours), no difference in the dose requisite for sterilization was observed. Over a twelvefold difference in intensity, the total dose of radiation determines the extent of bacteria inactivation. It may be concluded then that if low intensity radiation is used for a longer period of time or if higher intensity radiation is used for a shorter period of time no difference in the sterilization dose will be detected.

Inspection of table 2 indicates agreement with other published data (Dunn *et al.*, 1948) that nonsporulating bacteria and molds are killed more

TABLE 2

Effect of less than sterilization dose on mixed bacteria suspensions

EXTENT OF CONTAMINATION PRIOR TO IRRADIATION—VIABLE ORGANISMS PER ML	NO. VIALS	TOTAL DOSE RECEIVED, MEGAREP	EXTENT OF CONTAMINATION AFTER IRRADIATION—VIABLE ORGANISMS PER ML	REMARKS
Pseudomonas aeruginosa, Aspergillus niveus *Escherichia coli, Penicillin citrinum*	4	0.5	Mixed bacterial growth but no molds	Bacteria alive atypical growth*
Micrococcus pyogenes var. *aureus, Mucor racemosus*	4	0.97	Only sporeforming bacteria	Little growth on transfer
Bacillus subtilis, Candida albicans *Clostridium sporogenes, Bacillus coagulans*	4	1.6	Less than 1,000 (*C. sporogenes*)	
Total 0.1 × 10⁹	4	2.0	Less than 100 greater than 10 (*C. sporogenes*)	Atypical growth on transfer*
Escherichia coli, 4–5 × 10⁹ *Micrococcus pyogenes* var. *aureus*, 3–4 × 10⁹	15	1.4–1.7	All samples less than 1,000 (*C. sporogenes*)	Irradiated at 12.7 kilorep/hr
Bacillus subtilis, 1–2 × 10⁹ *Clostridium sporogenes*, 0.1–1 × 10⁹	15	1.3–1.4		Irradiated at 37.8 kilorep/hr
Candida albicans, 0.1–1 × 10⁹	15	1.4–1.7		Irradiated at 47.5 kilorep/hr
	15	1.6		Irradiated at 116.5 kilorep/hr

* For example: There was clearly visible turbidity in the fluid thioglycolate medium. Microscopic examination showed multiple convolutes of long, irregular gram positive and gram negative threads with multiple forms of involution. When subcultured some of the samples failed to grow, and some grew slowly. Colonies of *Bacillus subtilis* and *Clostridium sporogenes* were transparent.

easily than sporeforming bacteria. In the present investigation, *C. sporogenes* appeared to be the organism most difficult to kill, which suggests that the spores of this organism are particularly resistant to ionizing radiation. It is of interest that *B. coagulans* (*B. thermoacidurans*) which is resistant to heating at 100 C for more than 90 minutes (phosphate buffer solution, pH 7.0) (Yesair, 1947) is readily inactivated by radiation. A further observation in agreement with published information is that, while many organisms may not be completely inactivated by the smaller radiation doses, they are significantly altered and show morphological and cultural aberrations when transferred to fresh culture medium.

Data indicating the lack of chemical effect of intense gamma radiation fields on steroid suspensions will be published elsewhere (Tarpley *et al.*, *in press*).

SUMMARY AND CONCLUSIONS

The successful sterilization of heavily contaminated steroid suspensions by gamma rays produced by kilocurie sources of radioactive cobalt (Co^{60}) and tantalum (Ta^{182}) has been accomplished.

Radiation doses of between 2.7 and 3.2 megarep (rep $\times 10^6$) are required for sterilization when 0.1×10^9 organisms per ml are present initially. *Clostridium sporogenes* appears to be the most resistant organism studied in this investigation, whereas molds and nonsporulating bacteria are killed more readily. A large fraction of the contaminating sporulating microorganisms was destroyed by radiation doses of about 1.5 megarep, and those not killed showed morphological and cultural aberrations on subculture.

Within the relatively long irradiation periods of this investigation, irradiation at lower intensity over long periods of time has no important bearing on the efficiency of the sterilization. This leads to the conclusion that many thicknesses of the material to be sterilized may be arranged around a radioactive source. By removing various portions at appropriate time intervals high utilization efficiency of the radiation field may be achieved.

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