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# Survival of *Clostridium botulinum* Spores

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## ABSTRACT

ANELLIS, A. (U.S. Army Natick Laboratories, Natick, Mass.), NICHOLAS GRECZ, AND D. BERKOWITZ. Survival of *Clostridium botulinum* spores. Appl. Microbiol. 13:397-401, 1965.—Radiation survival curves of spores of *Clostridium botulinum* strain 33A exhibited an exponential reduction which accounted for most of the population, followed by a "tail" comprising a very small residual number [7 to 0.7 spore(s) per ml] which resisted death in the range between 3.0 and 9.0 Mrad dose levels. The "tail" was not caused by protective spore substances released into the suspensions during irradiation, by the presence of accumulated radiation "inactivated" spores, or by heat shock of pre-irradiated spores. The theoretical number of spore targets which must be inactivated by irradiation was estimated both by a graphical and by a computation method to be about 80, and the *D* value was calculated to be 0.295 and 0.396 Mrad, respectively, in buffer and in pork pea broth.

Radiation survival curves, obtained by Wheaton and Pratt (1962) for *Clostridium botulinum* spores, consisted of three distinct portions: (i) a shoulder, (ii) an exponentially declining portion consistent with classical hit theory at doses up to approximately 2.0 Mrad, and (iii) a so-called "tail" portion at 2.0 to 5.0 Mrad. The "tail" of the curve did not follow classical hit theory, and was present regardless of the kind of suspending medium or the initial spore concentration used. Similar "tailing" of radiation survivors was apparently encountered by Brown, Vinton, and Gross (1960) with spores of the putrefactive anaerobe PA 3679 in cured ham and raw pork, and by Gunter and Kohn (1956) with nonsporeformers.

The possibility that the "tail" of the radiation survival curves may be due to occasional radiation-tolerant mutants in a bacterial population was entertained both by Wheaton and Pratt (1962) and by Gunter and Kohn (1956). Only the latter workers examined "tail" survivors, but found no exceptional resistance as compared with the parent cultures.

Several investigators have attempted to produce resistant mutants by ionizing radiation. Pepper, Buffa, and Chandler (1956), using spores of *Clostridium sporogenes* and *Bacillus pumilus*, and Koh, Morehouse, and Chandler (1956), using *Escherichia coli*, were unsuccessful in their attempts, whereas Gaden and Henley (1953) and Erdman, Thatcher, and MacQueen (1961) suc-

ceeded in increasing the resistance of *E. coli*. The latter also reported a slight increase (1.3-fold) in the resistance of vegetative cells of *C. botulinum* type A by exposing the cells to 10 consecutive daily irradiations of 0.186 Mrad  $\gamma$  rays. Spores produced by the mutant showed a slight but consistently higher resistance than the original spores. The greater radiotolerance of these spores was transmitted back to the homologous vegetative population.

An alternative possibility may be suggested to explain the "tailing" phenomenon. Spores release certain components in increasing quantity during irradiation which may compete for active radicals or repair cellular damage of the remaining viable organisms, and thus modify the shape of the survival curve to produce the "tail" portion. For example, substances such as protein(s) and dipicolinic acid are released from spores into the menstruum in increasing amounts with increasing radiation levels (Levinson and Hyatt, 1960; Stewart and Slack, 1961). An accumulation of spore exudates could conceivably raise the *C. botulinum* resistance and thus create a "tail."

The present report is concerned primarily with (i) a study of the effect of irradiated spore exudates on the shape of the radiation survival curves, and, incidentally, with (ii) the effect of spore heat shock, (iii) the presence of radiation "inactivated" spores, and (iv) the number of sensitive targets, on the radioresistance of *C. botulinum* strain 33A.

## MATERIALS AND METHODS

*Test organism.* Spores of *C. botulinum* 33A were prepared as described by Anellis and Koch (1962).

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Viable spore counts were made on heat-shocked (80 C for 10 min) samples of the stock suspension by preparing serial decimal dilutions in 0.067 M phosphate buffer (pH 7.0) and subculturing 1.0-ml samples in triplicate tubes of Wynne's agar (Wynne, Schmieding, and Daye, 1955). The solidified medium was stratified with about 2 cm of Wynne's agar and was incubated at 30 C up to 5 days. Maximal colony counts were obtained in 3 to 4 days.

"Tail" colonies. Initial populations of  $7 \times 10^8$  to  $9 \times 10^8$  spores per milliliter of 0.067 M phosphate buffer (pH 7.0) were irradiated from 1.0 to 9.0 Mrad at 1.0 Mrad intervals, with and without prior heat shock (80 C for 10 min). Colony counts were made of the survivors. Triplicate recovery tubes, subcultured from the 9.0 Mrad-exposed undiluted spore samples, developed a total of four colonies. Each colony was subcultured to individual tubes of Wynne's broth and was examined for growth, *C. botulinum* type A toxin, and spore production. Growth of the four individual cultures was somewhat delayed in the primary recovery tubes, but was normal upon subsequent transfer. Characteristic type A toxigenicity was produced by all four isolates. The ability to form spores appeared to be comparable with that of the parent unirradiated culture.

Preparation of suspending substrates. The effect of substances released from spores during irradiation on the radioresistance of the remaining viable spores was studied in two basic suspending menstrua: phosphate buffer (pH 7.0), and pork pea infusion broth (pH 7.0; Andersen, 1951). Each of these two substrates was varied as indicated in Table 1. Phosphate buffer had three modifications as described under A, B, and C, and pork pea infusion had four modifications: A, B, C, and D.

Irradiation. Heat-shocked or unheated viable spores were added in known concentrations to each of the various menstrua. The suspensions were distributed in 1.0-ml quantities in sets of 10 replicate cotton-plugged Pyrex glass tubes (10 × 75 mm). The tubes were inserted in polystyrene holders contained in metal cans (307 × 409). The cans were evacuated, flushed with nitrogen, sealed, and irradiated as previously described (Anellis, Cichon, and Rayman, 1960), with 0 to 2.4 Mrad in increments of 0.3 Mrad at the Argonne High Level Gamma Irradiation Facility.

The contents of each replicate set of 10 irradiated tubes were pooled, and triplicate colony counts of the surviving spores were made as above. Thus, the counts represented averages of 10 individual samples per radiation dose level.

Multitarget computation of survival curves. Since the resulting radiation-survival curves appeared to follow the multitarget model (Hutchinson and Pollard, 1961), the following equation was used to calculate the number of targets needed to inactivate the organisms:

$$\frac{n}{n_0} = 1 - (1 - e^{-VD})^N$$

TABLE 1. Substrates\* for suspending spores of *Clostridium botulinum* during irradiation

Substrate modification	Addition of spores	Treatment of substrate
A	None	None
B	None	Irradiated to 4.5 Mrad
C	Added $10^{10}$ spores per 100 ml of initial substrate	Irradiated to 4.5 Mrad; spores removed after irradiation by centrifugation
D	Added $10^{10}$ spores per 100 ml of initial substrate	Irradiated to 4.5 Mrad; spores not removed

\* Two initial substrates, sterilized by autoclaving for 20 min at 15 psi, were used: (i) phosphate buffer (pH 7.0; modification A, B, and C), and (ii) pork pea broth (modification A, B, C, and D). All spores were heat-shocked 10 min at 80 C immediately before use.

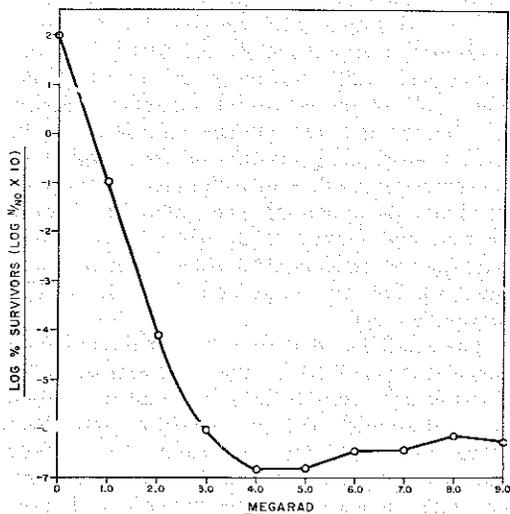


FIG. 1. Survival of spores of *Clostridium botulinum* 33A exposed to  $\gamma$  radiation. Initial spore load  $9 \times 10^8$  per milliliter, heated for 10 min at 80 C prior to irradiation, suspended in phosphate buffer (pH 7.0), irradiated up to 9.0 Mrad at 0 C.

where  $n$  = number of surviving spores;  $n_0$  = initial number of unirradiated spores;  $e = 2.7183$ ;  $V$  = constant indicating volume of each target;  $D$  = radiation dose, and  $N$  = number of targets per spore.

A digital computer was programmed to fit the

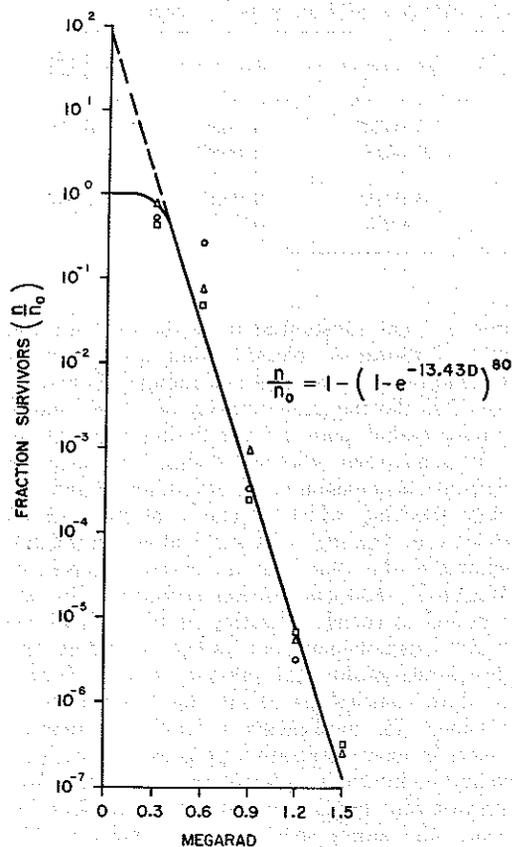


FIG. 2. Effect of phosphate buffer substrates on radiation survival of spores of *Clostridium botulinum* 33A. Initial spore load  $3.3 \times 10^8$  to  $7.5 \times 10^6$  per milliliter. Data represent averages of triplicate counts on a pooled suspension of 10 irradiated tubes with 1 ml of spore suspension per tube. Symbols: unirradiated buffer,  $\circ$ ; buffer irradiated to 4.5 Mrad,  $\square$ ; irradiated spore exudate ( $3.2 \times 10^{10}$  spores irradiated in buffer to 4.5 Mrad and removed),  $\triangle$ .

above formula to the experimental data by the method of Pratt (1960).

#### RESULTS

*Behavior of the survival curve.* Two unheated and one of two heat-shocked spore suspensions yielded survival curves with "tails" at dose levels above 3.0 Mrad. "Tailing" could not be detected in the duplicate heated spore suspension. No explanation can be offered at this time for this anomaly. The most striking survival curve, produced by a heat-shocked spore suspension is reproduced in Fig. 1. The exponential portion of the curve indicates that  $9 \times 10^8$  spores per milliliter should have been reduced to less than one survivor by a dose of 3.0 to 3.5 Mrad; but

the "tail" portion survivors fluctuated between about 7 to 0.7 spore(s) per ml up to 9.0 Mrad, the highest dose employed.

*Effect of substrate modification.* The series of studies with various modifications of substrates were conducted specifically in the radiation range which gave exponential death. It was assumed that any change in the shape of the survival curves would be reflected chiefly within this "sensitive" region, and, hence, may be related to changes in radioresistance and "tailing."

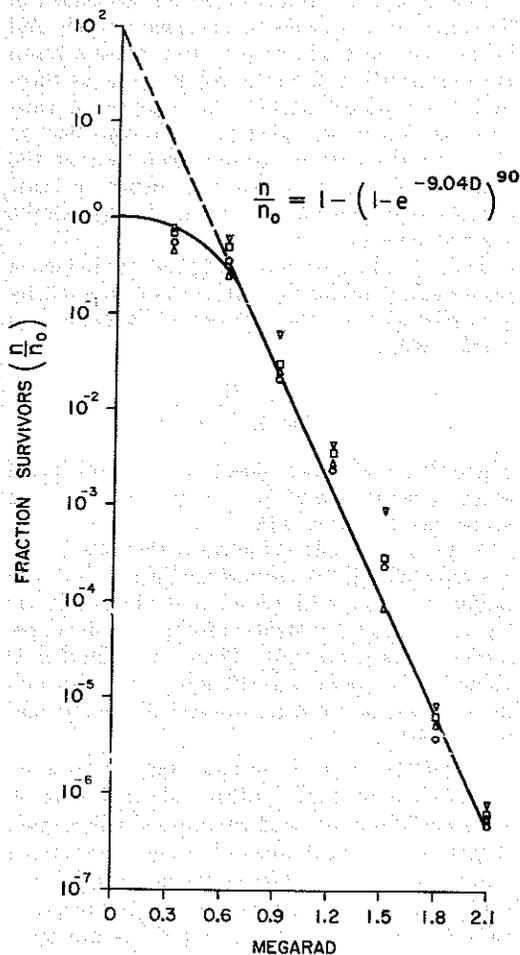


FIG. 3. Effect of pork pea broth substrates on radiation survival of spores of *Clostridium botulinum* 33A. Initial spore load  $1.1 \times 10^8$  to  $2.8 \times 10^8$  per milliliter. Data represent averages of triplicate counts on a pooled suspension of 10 irradiated tubes with 1 ml of spore suspension per tube. Symbols: unirradiated broth,  $\circ$ ; broth irradiated to 4.5 Mrad,  $\square$ ; irradiated spore exudate ( $3.2 \times 10^{10}$  spores irradiated in broth to 4.5 Mrad and removed),  $\triangle$ ; irradiated spores added to broth ( $3.2 \times 10^{10}$  spores treated with 4.5 Mrad),  $\nabla$ .

TABLE 2. Analysis of variance of effect of substrate modification on *D* values

Substrate	Source of variation	Degrees of freedom	Sum of squares	Mean square	F test (1% level)*
Phosphate buffer	Treatments	2	0.00567	0.00283	0.329
	Residuals	6	0.0518	0.00863	
Pork pea broth	Treatments	3	0.00258	0.000859	0.160
	Residuals	15	0.0803	0.00536	

\* Not significant.

The radiation survival of spores suspended in the three modifications of phosphate buffer and in the four variations of pork pea infusion broth are shown in Fig. 2 and 3, respectively, as theoretical multitarget curves. An analysis of variance indicated that substrate variations had no effect on the rate of spore destruction or the shape of the survival curves. Hence, a least squares regression line was fitted through all the survival points.

The *D* value, or dose required to reduce a bacterial population by 90%, was computed by a modification of the formula of Schmidt and Nank (1960):

$$D = \frac{R}{\log N_0 - \log N}$$

where *R* = radiation dose; *N*<sub>0</sub> = total initial spore population; and *N* = number of surviving spores per radiation dose.

A statistical analysis indicated that the *D* values representing the various modifications of the buffer or of the broth were not significant at the 1% level (Table 2). Hence, results were averaged for each basic substrate. Thus, the radiation *D* values for *C. botulinum* 33A spores in buffer and in pork pea broth were, respectively, 0.295 and 0.396 Mrad.

*Multitarget survival curves.* Computations by the method of Pratt (1960) indicated that 80 sensitive sites must be inactivated within a spore to destroy it. This number of targets was verified graphically by extrapolating the exponential portion of the survival curves to the Y-intercept, or zero dose response (Fig. 2 and 3), as predicted by the multitarget equation (Hutchinson and Pollard, 1961).

#### DISCUSSION

It was initially anticipated that substances released during irradiation of spores may protect the remaining viable spores by either competing for active radicals or by assisting in the repair of cytoplasmic or nuclear damage. This assump-

tion was not substantiated by the experimental data. Substances released from spores during irradiation had no apparent radioprotective effect. Nor did the large numbers of "dead" spores protect viable spores from radiation inactivation.

It is not clear whether "tailing" represents a natural phenomenon or an experimental artifact. Heat shocking, which on one occasion appeared to prevent "tailing" of a survival curve, did not eliminate the "tail" in a second experiment. Wheaton (*personal communication*) also attempted to correlate heating of spores with the "tail" phenomenon, but failed to detect any direct relationship. At present, it is premature to draw conclusions about the significance of "tailing" for application to radiation requirements in food sterilization processes. Until more definitive information is obtained, radiation food sterilization standards will have to take into account the entire survival curve including the radioresistant "tail."

An additional factor possibly playing a role in the radioresistance of *C. botulinum* is the number of radiosensitive targets in a particular strain. Wheaton and Pratt (1962) estimated that spores of *C. botulinum* strain 12885A had 13 sensitive targets. One could expect, therefore, that strain 33A, with 80 sensitive targets, should have a higher radiotolerance than strain 12885A. Studies have indicated that, under identical experimental conditions, the comparative *D* values of these two strains were 0.334 and 0.241 Mrad, respectively (Anellis and Koch, 1962). Additional information is needed before it can be suggested that a relationship exists between the number of sensitive targets and the *D* values in strains of *C. botulinum* spores.

The cause of "tailing" appears to be due to factors other than those examined in this study. The investigation of the "tail" phenomenon is being continued.

#### ACKNOWLEDGMENT

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