

Effects of radiation pasteurization on *Salmonella*. II. Influence of repeated radiation-growth cycles on virulence and resistance to radiation and antibiotics

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Populations derived from *Salmonella typhimurium* surviving 1, 5, or 10 exposures to 0.5 Mrad did not show any increase in resistance to antibiotic discs containing 5 µg of tetracycline, 5 µg of chloromycetin, or 50 units of polymyxin. The frequency of resistance to 3 µg of tetracycline, 10 units of ampicillin, 120 µg of chloromycetin, or 30 µg of streptomycin per milliliter of agar, did not increase among progeny derived from either of two strains of *S. typhimurium* surviving 5-10 exposures to 0.5 Mrad. So, too, the virulence and radiation resistance of three strains of *S. typhimurium* exposed to radiation-growth cycling remained essentially unchanged or decreased.

A mixture which originally contained 10 different serotypes of *Salmonella* was cycled at 0.23 Mrad. After 10 exposures the frequency of resistance of the progeny to ampicillin, chloromycetin, and streptomycin increased 87-, 55- and 13-fold respectively. The LD₅₀ was essentially the same, but the radiation resistance (*D* value) increased from 0.053 Mrad in controls to 0.074 Mrad.

The evidence presented seems to indicate that mutants are attained more readily from *Salmonella* cultures recycled at 0.25 Mrad or less than from those recycled at 0.5 Mrad. The pathogenicity of either one was usually essentially unchanged or reduced.

Reduction of the microbial load in poultry by exposure to ionizing radiation has been studied to extend the refrigerated shelf life of the product and reduce the possibility of transmission of *Salmonella* by it (4, 15, 17, 20). Of the microorganisms present, *Salmonella* has been of prime concern because of its relatively high radiation resistance as well as its high incidence. It has been hypothesized that surviving salmonella recycled one or more times through the process in a short or lengthy cyclical fashion may possess a greater radiation resistance, toxigenicity, virulence, and (or) antibiotic resistance (19). Increases in the radiation resistance of *Salmonella* species are not always obtained after irradiation (6). They have been attained (5, 8, 11, 12) only by repeated exposure to radiation at levels that are lower than those usually contemplated for use in a commercial process (15, 20). This report represents an attempt to determine whether repeated exposure of *Salmonella* to doses approximating levels likely to be used for its elimination (0.5 Mrad) selects or induces mutant populations with altered virulence, radiation resistance, or antibiotic resistance.

Materials and Methods

The general methodology used has been described in detail (18). Briefly, the cells were grown for 21 h at 37°C in brain heart infusion (BHI, Difco) or the same media enriched with yeast extract (Baltimore Biological Laboratories) (EBHI). Under the conditions used, enrichment did not influence survival after irradiation (18) nor provide any detectable influence on parameters reported in the data that are to be described. The cultures were centrifuged and resuspended in 1/100 the original volume in saline. A 0.1-ml quantity containing about 10⁹-10¹⁰ cells was inoculated into 2-g pieces of autoclaved chicken and exposed to ⁶⁰Co irradiation at 4°C. *Salmonella typhimurium*, strain RIA, which was to be radiation cycled, was inoculated into germ-free chicken (Charles River Laboratories, Wilmington, Mass.). The type of chicken has been shown to be without influence on radiation survival (18).

Radiation-Growth Cycles

S. typhimurium, strain RIA, and strains resistant to 5 µg discs of chloromycetin (NCDC No. 5110-62) and tetracycline (NCDC No. 5401-62) were inoculated into chicken and exposed to 0.5 Mrad at 4°C.

Fresh broth (1.9 ml) was then added to the chicken-cell mixture and agitated vigorously with a vortex mixer (Labline Instruments, Chicago, Illinois). One milliliter was removed to 75.0 ml of broth and incubated at 37°C for 24 h. Because of a prolonged lag induced by exposure to 0.5 Mrad, less than optimal growth (less than 10⁹ cells/ml) was attained. Therefore, 1.0 ml of the 24-h growth was subcultured in a tube of fresh broth and incubated for 21 h at 37°C. Aliquots (0.1 ml) were then mixed in 1.9 ml glycerol, frozen at -70°C, and stored at -20 ± 2°C for future assay. This method of storage reduced the number of viable cells less than 1 log. The remaining

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aliquot was prepared for inoculation into chicken as described. The cycle of irradiation and growth was repeated 10 times. In each experiment unirradiated control cultures were treated similarly. To simulate the reduction in numbers afforded by exposure to 0.5 Mrad, the control cultures of the tetracycline- and chloromycetin-resistant strains of *S. typhimurium* were diluted to 10^{-6} or 10^{-7} in saline before reincubation in broth.

Radiation-cycled cultures of a mixture of salmonella species were kindly supplied by Dr. Richard A. Pledger of Hazelton Laboratories, Falls Church, Virginia. They had been prepared by the following procedure. Equal volumes of trypticase soy broth cultures of 10 species of *Salmonella* (*typhimurium*, *infantis* 165, *montivideo*, *newport*, *panama*, *javiana*, *poona*, *paratyphi* B, *enteritidis*, *senftenberg*) were pooled. A total of 10^7 cells was inoculated per square inch into a piece of raw preirradiated chicken measuring $\frac{1}{2}$ in. thick and 2 in. in diameter. The latter had been "sterilized" by exposure to 1.95 ± 0.1 Mrad at a dose rate of 1.03 ± 0.05 Mrad/h. The mixture of cells and chicken was exposed to 0.23 ± 0.02 Mrad at 1°C and then incubated for 2-4 days at 37°C . The outgrowth was washed off the chicken by agitation with 20-25 ml of saline and 0.25 ml inoculated onto another piece of preirradiated chicken for recycling. After each successive growth period an aliquot of the saline wash was incubated for 24 h on a tryptic soy agar slant. A loop was then drawn along the entire length of the slant to provide an extremely heavy inoculum. The latter was incubated for 21 h in BHI at 37°C and replicates were frozen in glycerol broth as described above.

Antibiotic Sensitivity of S. typhimurium, RIA, after One Radiation Exposure

Dilutions were made to provide about 1000 viable cells from control and irradiated samples after exposure to 0.5 Mrad at 4°C on autoclaved chicken. The numbers were estimates derived from previous survival studies (18). The bacteria were grown in EBHI for 24 h at 37°C and subcultured again (0.1 ml per 8.0 ml EBHI) for another 24 h. After centrifugation, resuspension, and dilution in saline, 0.1-ml aliquots containing 4000 ± 1000 cells were streaked on five to seven replicate plates of EBHIA. The number of cells plated was estimated by turbidimetric analysis at 660 m μ on a Coleman model 14 spectrophotometer (Coleman Instruments, Maywood, Illinois) and confirmed by plate counts. Discs of chloromycetin (5 μg), tetracycline (5 μg), polymyxin B (50 units), and streptomycin (10 μg) (Difco) were placed on each plate. The zone of inhibition was measured to the nearest millimeter after incubation for 24 h at 37°C . The data represent an average for three experiments analyzed statistically by the rank order test (21).

Antibiotic Sensitivity of S. typhimurium, RIA, after Repeated Radiation-Growth Cycling

The assay used was essentially the same as the one just described with three exceptions. (1) The cells were derived from cultures stored at -20°C and described above under the section on radiation-growth cycles. (2) The inoculum was incubated for 21 h (rather than 24 h) before it was streaked on EBHIA. (3) The antibiotic discs were positioned on the streaked plates and stored at 4°C for 2-3 h to allow the antibiotic time to diffuse through the media

before incubation at 37°C (7, 22). The data represent averages for two experiments in which 5550 ± 1550 cells were spread per plate and four or five replicate plates were made for each variable in each experiment.

Mutation Frequency

Three micrograms of tetracycline hydrochloride (Panmycin hydrochloride, Upjohn, Kalamazoo, Mich.), 30 μg of streptomycin sulfate (Charles Pfizer, New York, N.Y.), 120 μg of chloromycetin (Parke Davis and Co., Detroit, Mich.), or 10 units of ampicillin (Omnipen, Wyeth Laboratories, Radnor, Pa.) were incorporated per milliliter of BHIA at $45-50^\circ\text{C}$ before the plates were poured. Stock cultures of radiation-cycled bacteria were rapidly thawed and 1 ml inoculated into 75 ml BHI. After incubation at 37°C for 21 h they were centrifuged at $4080 \times g$ at 4°C for 15 min and resuspended in 7.5 ml saline. Tenfold dilutions were spread in triplicate in 0.1-ml volumes on a series of plates with and without antibiotics. The number of colonies were recorded after 48 h at 37°C on all plates excepting those containing streptomycin. The latter were recorded after 24 h. The mutation frequency was derived from the number of colonies appearing on plates with and without antibiotic. Up to three replicates of each unirradiated and radiation-cycled culture were assayed in this manner.

Virulence

Each cycled culture was rapidly thawed after storage and 0.1 ml inoculated into 10 ml of BHI and incubated at 37°C for 16-18 h. This storage method has been used successfully in these laboratories to maintain the virulence of several serotypes of *Salmonella* for many years. Ten twofold serial dilutions were made in saline and 0.5 ml injected intraperitoneally into CD-1 male mice weighing 21 ± 2 g (Charles River Labs, Wilmington, Mass.). A total of 50 animals in groups of 5 per dose were used for each titration and survival was followed for 14 days after infection. The LD_{50} was derived by the method of Miller and Tainter (14).

Radiation Resistance

The cycled cultures were rapidly thawed and 1.0 ml incubated in 75 ml of BHI at 37°C for 21-22 h. After centrifugation at $4080 \times g$ for 15 min at 4°C they were

TABLE 1
Antibiotic sensitivity^a of *S. typhimurium*, RIA,
after radiation exposure

Antibiotic	Radiation dose	
	0 Mrad	0.5 Mrad
Chloromycetin (5 μg)	20.1	20.3
Tetracycline (5 μg)	17.7	19.6
(30 μg)	25.2	25.1
Polymyxin (50 units)	14.2	14.6
Streptomycin (10 μg)	11.8	11.3

^aDiameter of zone of inhibition, in millimeters, against discs containing the concentration of antibiotic shown.

resuspended in saline, inoculated into chicken as described, and exposed to doses ranging between 0 and 0.7 Mrad in increments of 0.1 Mrad at 4°C. The chicken was then washed with 1.9 ml saline by vigorous agitation with a Vortex mixer. Tenfold serial dilutions were streaked on EBHIA and incubated for 48 h at 37°C. Resistance values were derived as the radiation dose in Mrad which reduced the initial population by 90% (D). They were read from a straight line drawn to the ordinate of a semi-logarithmic plot of the fraction of cells surviving versus radiation dose as was described earlier (18).

Results

Antibiotic Sensitivity after a Single Exposure to 0.5 Mrad

After a single exposure to 0.5 Mrad, the resistance of *S. typhimurium*, strain RIA, to discs containing 5 µg chloromycetin, 5 µg or 30 µg tetracycline, 50 units polymyxin B, or 10 µg strepto-

mycin was not increased above control levels (Table 1). The greatest change observed was one of increased susceptibility to 5 µg of tetracycline. The zone of inhibition increased from 17.7 to 19.6 mm following growth after exposure to 0.5 Mrad ($P < 0.01$). This was not evident when 30 µg of tetracycline was used.

Antibiotic Sensitivity after Radiation-Growth Cycling

The sensitivity of *S. typhimurium*, RIA, to 5 µg tetracycline, 5 µg chloromycetin, and 50 units of polymyxin B was unaltered after as many as 5 to 10 repeated exposures to irradiation with intervening periods of growth (Table 2). The right-hand column shows clearly that the zones of inhibition for control cultures subcultured 10 times in broth are remarkably close to those which were exposed 10 times to 0.5 Mrad. In fact, the variability among control subcultures transferred through broth 0, 5, and 10 times exceeded that observed between irradiated and unirradiated counterparts.

Frequency of Mutation to Antibiotic Resistance after Radiation-Growth Cycling

The disc method provides information concerning the resistance of a total population of cells but no quantitative information about the frequency with which small numbers of resistant cells appear in a large population of survivors. Therefore, the frequency of mutation to antibiotic resistance was determined for different strains of *S. typhimurium* exposed repeatedly to

TABLE 2

Antibiotic sensitivity^a of *S. typhimurium*, RIA, after repeated growth-radiation cycling

Antibiotic	Rad. dose Mrad	No. cycles		
		0	5	10
Chloromycetin (5 µg)	0	17.1	17.3	17.8
	0.5		17.9	17.4
Tetracycline (5 µg)	0	15.0	15.8	14.9
	0.5		15.5	15.4
Polymyxin B (50 units)	0	14.1	14.2	14.9
	0.5		14.6	14.4

^aDiameter of zone of inhibition, in millimeters, against discs containing the concentration of antibiotic shown.

TABLE 3

Mutation frequency ($\times 10^{-7}$) of *Salmonella* after repeated growth-radiation cycling^a (no. cycles, 0 and 10)

Organism	Rad. dose, Mrad	Tetracycline, 3 µg/ml		Ampicillin, 10 units/ml		Chloromycetin, 120 µg/ml		Streptomycin, 30 µg/ml	
		0	10	0	10	0	10	0	10
<i>S. typhimurium</i> RIA	0	12.4 (2)	> 17.8 (2)	14.1	5.4	34.2	32.8	93.1 $\times 10^2$	12.4 $\times 10^2$
	0.5		15.8 (2)		10.4		31.9		7.4 $\times 10^2$
Tetracycline	0			2.1	TNTC ^c	41.3	6.2 $\times 10^3$		
	0.5				> 5.1 $\times 10^6$		4.6		
<i>Salmonella</i> sp. ^b	0	1.1 (2)		0.015		1.5		1.6 $\times 10^2$	
	0.23		1.3 (1)		1.3 (1)		82.1		21.0 $\times 10^2$

^aEach figure represents the average of three separate determinations, except those for which the number is shown in parentheses.

^bTen species of *Salmonella* originally stored on a tryptic soy agar slant.

^cA 10^{-7} dilution provided a plate with colonies too numerous to count (TNTC), indicating far more than 10^7 cells/ml of culture.

0.5 Mrad as well as a mixture of *Salmonella* species cycled at 0.23 Mrad. Neither of two strains of *S. typhimurium* demonstrated an increase in frequency of resistance to 3 µg of tetracycline, 10 units of ampicillin, 120 µg of chloromycetin, or 30 µg of streptomycin per milliliter of agar (Table 3). In fact, the reverse was true. The spontaneous alterations in mutation frequency manifested among controls cycled 10 times at 0 Mrad far exceeded changes observed in cultures exposed to 0.5 Mrad. In all cases the frequency of mutation to antibiotic resistance of

the radiation-cycled cultures was less than that of uncycled controls (0 cycle, 0 Mrad) or less than the corresponding culture after growth and transfer to broth 10 times (10 cycle, 0 Mrad).

The mixture of irradiated *Salmonella* species exposed to 0.23 Mrad did not show a change in mutation frequency to 3 µg tetracycline (Table 3). However, it did manifest increases in frequency of 87-, 55-, and 13-fold in the presence of ampicillin, chloromycetin, and streptomycin respectively. A similar mixture transferred and grown 10 times without radiation exposure was not available for comparative purposes.

TABLE 4

Virulence ($LD_{50} \times 10^{-5}$)^a of viable *Salmonella* after repeated growth-radiation cycling

Organism	Rad. dose, Mrad	No. cycles		
		0	5	10
<i>S. typhimurium</i> RIA	0	5.3 (2.3)	3.6 (1.9)	2.1 (0.8)
	0.5		3.9 (1.7)	3.3 (1.3)
Chloromycetin resistant sp.	0	0.42 (0.14)	0.73 (0.22)	0.86 (0.43)
	0.5		> 8.9 (—)	> 20.0 (—)
Tetracycline resistant sp.	0	0.37 (0.26)	~ 300 (—)	0.35 (0.18)
	0.5		> 16 (—)	9.4 (6.4)
<i>Salmonella</i> sp. ^b	0	400 (110)		
	0.23		110 (76)	150 (87)

^aThe standard error is shown in parentheses.

^bTen species originally stored on a tryptic soy agar slant.

TABLE 5

Radiation resistance^a of *Salmonella* after repeated growth-radiation cycling

Organism	Rad. dose, Mrad	No. cycles		
		0	5	10
<i>S. typhimurium</i> RIA	0	0.080	0.085	0.085
	0.5		0.085	0.092
Chloromycetin resistant sp.	0			0.068
	0.5			0.060
Tetracycline resistant sp.	0	0.060		0.060
	0.5			0.060
<i>Salmonella</i> sp. ^b	0	0.053		
	0.23			0.074

^aD value in Mrad.

^bTen species of *Salmonella* originally stored on a tryptic soy agar slant.

Virulence after Radiation-Growth Cycling

The virulence of different strains of *S. typhimurium* decreased with repeated exposure to 0.5 Mrad (Table 4). There was an increase in the number of cells required for an LD_{50} after 5 or 10 radiation exposures compared to unirradiated cells subcultured 5–10 times in BHI. After five growth cycles the LD_{50} of the tetracycline-resistant strain was about 300×10^5 cells for controls and more than 16×10^5 cells for 0.5 Mrad cycled organisms. However, the latter figure exceeds the LD_{50} requirement of the original cultures, of 0.37×10^5 cells, indicating a loss of virulence after radiation cycling. Spontaneous changes in unirradiated cycled cultures caused greater fluctuations in virulence than was observed after repeated exposure to 0.5 Mrad.

There was a slight increase in virulence of the salmonella mixture cycled at 0.23 Mrad. The LD_{50} was 400×10^5 , 110×10^5 , and 150×10^5 cells after 0, 5, and 10 radiation-growth cycles (Table 4).

Radiation Resistance after Radiation-Growth Cycling

The radiation resistance of the cycled cultures followed the same pattern as virulence. That is, for individual strains of *S. typhimurium*, the resistance values after radiation cycling were not significantly higher than those of controls, while the mixture of *Salmonella* species exposed to 0.23 Mrad showed an apparent increase in resistance. In the latter, the D value rose from 0.053 to 0.074 Mrad after 10 exposures to irradiation (Table 5).

Discussion

The prediction that the antibiotic resistance of *Salmonella* surviving irradiation might increase

(19) was not documented among populations of *S. typhimurium*, strain RIA, surviving 0.5 Mrad, even with cultures that were exposed to 10 radiation-growth cycles (Tables 1 and 2). The only significant change observed was towards increased sensitivity to 5 µg of tetracycline after a single exposure to irradiation (Table 1). The results indicate that the total surviving population and progeny did not shift towards greater resistance. Two individual strains of *S. typhimurium* recycled 10 times at 0.5 Mrad also did not show increases in the frequency of mutation to antibiotic resistance (Table 3). The method of subculture used provided latent mutants ample time for expression. Back mutation could possibly account for a lack of increase in the appearance of mutants. However, it seems more likely that the results reflect a phenomenon previously observed, namely, that at a dose above a certain threshold, radiation-induced lethality overshadows its mutagenic effects (3, 13, 16). Others have reported that the phage type and serotype of *S. typhimurium* does not change after exposure to 0.65 Mrad (10). In this same context, failure to observe an increase in virulence (Table 4) or radiation resistance (Table 5) among cultures exposed to 0.5 Mrad might have been anticipated. A decrease in virulence also was recently reported after 12 or more exposures of *S. typhimurium* to 0.18–0.30 Mrad (5). So, too, the toxicity of type A *C. botulinum* did not increase after recycling 10 times at 200 000 roentgens equivalent physical (6). These results support a prediction made nearly two decades ago that biochemical mutants arising from prototrophs would be expected to manifest unaltered virulence or even show a decrease in this characteristic (1).

Data obtained with the mixture of *Salmonella* species recycled 10 times at 0.23 Mrad allow interesting comparisons to be made with that obtained from other laboratories, and inferences to be drawn concerning the differences between the effects of cycling at 0.23 and 0.5 Mrad. The experimental evidence seems to indicate that induction or selection of mutants occurs after 0.23 Mrad exposure. Increases in frequency of resistance of the mixture of salmonellae to three different antibiotics were observed (Table 3). The control of each is probably governed by different genetic loci (2). So, too, the alterations in virulence (Table 4) and radiation resistance (Table 5)

which were noted with the mixture are most likely governed by separate loci. The probability that spontaneous mutation or alterations in the ecology of the mixture could account for all of the changes simultaneously is extremely low. It appears to be less likely in view of reports that cycling of *S. anatum* and *S. enteritidis* at 0.25 Mrad caused a significant increase in radiation resistance but did not change their biochemical attributes (8). Such alterations are dependent on the strain and species (6) as well as the use of a level of irradiation that allows a significant number of cells to survive (3, 13, 16). The origin of radiation-resistant mutants has been related to the dose at which cycling takes place as well as the number of exposures (11, 12). Within limits, doses that cause a lesser degree of killing require a lesser number of cycles to establish a trend towards increased resistance. Failure to obtain radiation resistant populations of *S. typhimurium* when recycled 10 times at 0.5 Mrad (Table 5) probably was due to the relatively high dose used. A single exposure to less irradiation (e.g. 0.05 Mrad) produces populations that manifest significant increases in the frequency of resistance to antibiotics even with the relatively genetically stable *S. typhimurium*, strain RIA (Previte *et al.*, in press).*

The evidence seems to indicate that mutants are attained more readily among cultures recycled at 0.25 Mrad or less (5, 8, 11, 12) than from those recycled at 0.5 Mrad (Tables 3, 4, and 5). However, the pathogenicity of cultures recycled by either method did not increase significantly. Rather, the reverse was generally true (Tables 4, 5).

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strains of *S. typhimurium* and who confirmed the serotypes of individual species used in this series of studies.

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