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Effect of Repeated Irradiation on Various Characteristics of *Salmonella*[1]

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Four *Salmonella* serotypes which had been subjected to either 15 or 20 cycles of irradiation and subculture were examined for possible changes induced by the treatment. With three serotypes virulence remained unchanged, and with one serotype a reduction had occurred. A few minor changes in the biochemical characteristics occurred in some instances, but with all cultures the serological property was affected to some extent. Lag times and growth rates of the treated cultures were not altered, but the maximal viable population was usually less than that for untreated cultures.

In a previous paper (4*a*). it was reported that repeated irradiation and subculturing of *Salmonella* cultures resulted in a culture with increased radioresistance. This observation stimulated interest in determining whether this cyclic irradiation treatment might also produce *Salmonella* cultures with some other altered trait, such as an increase in virulence or a change in metabolism, which might affect taxonomic identification based on biochemical tests. Mutations of this nature could make radio-pasteurized foods potentially hazardous to the public health (6).

The results of an investigation concerned with these and related objectives are reported here.

MATERIALS AND METHODS

Cultures. The *Salmonella* serotypes used throughout this investigation were *S. typhimurium* ATCC 7823, *S. newport* ATCC 6962, *S. thompson* ATCC 8391, and *S. heidelberg* ATCC 8326. Radio-resistant cultures were obtained by subjecting a broth culture to repeated cycles of irradiation and subculture to stationary phase (20 such cycles for *S. typhimurium* and *S. newport* and 15 cycles for *S. thompson* and *S. heidelberg*), whereas the original parent culture represented the radio-sensitive culture (4*a*).

Unless indicated otherwise, cultures were grown in Trypticase soy-yeast extract (TSYE) broth at 37 C, and plate counts were made by surface streaking 0.1-ml portions of serial dilutions on the surface of TSYE agar plates and incubating for 48 to 72 hr.

Virulence of radio-resistant and radio-sensitive cultures. Cultures of the radio-resistant and radio-sensitive strains were grown under aerobic (shake-flask) conditions for 8 hr at 37 C, at which time the cells were in the logarithmic phase of growth, containing a minimum of dead cells. The cultures were centrifuged and then suspended in chilled, phosphate-buffered peptone water (1 g of peptone/liter, *p*H 7). The volume of peptone water was adjusted so that the final cell concentration would be of the order of 10^{10} per ml.

Swiss white male mice, weighing approximately 18 to 25 g, served as one species of test animal. Portions (0.5 ml) of the serially diluted cell suspensions were injected intraperitoneally into groups of 10 mice. The mice were observed for deaths over a 3-day period.

In some tests, 8-day-old embryonated hen's eggs were used as the host species. The eggs were previously candled to eliminate those with dead embryos. Portions (0.5 ml) of the serially diluted cell suspensions were inoculated into the yolk sac in groups of six eggs, and the eggs were observed by candling for mortality of the embryo during a 3-day incubation period at 37 C. A control group of six eggs was also inoculated with 0.5 ml of peptone water and observed during the same period. No embryos in this group succumbed.

A plate count was made either on the primary dilution or on several of the dilutions to determine the number of viable cells being injected.

The LD_{50} was estimated by plotting the percentage of mortality after 3 days as a function of the log of the number of viable cells injected. The number of cells required to cause 50% mortality was determined by graphical interpolation.

Biochemical characteristics of radio-resistant and radio-sensitive cultures. Fifty isolated colonies of each radio-resistant and radio-sensitive serotype on TSYE agar plates were inoculated into each of the following media to test for either growth or biochemical reaction: Triple Sugar Iron Agar, lysine iron

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agar, Phenylalanine Agar, Simmons Citrate Agar, Malonate Broth, urea broth, tryptone broth, and KCN broth. All inoculated media were incubated at 37 C and observed after 24 hr. Negative tubes were incubated up to 72 hr. Strains of *Proteus, Aerobacter,* and *Escherichia coli* were also inoculated into the various media to serve as positive controls for validating the test procedure. Characteristic reactions on these media were described by Edwards and Ewing (1).

Serological reaction. Isolated colonies of the radio-resistant or radio-sensitive strains on TSYE agar plates were inoculated into Triple Sugar Iron Agar, and these cultures were used for agglutination tests. Tests for O (somatic) antigens were made by the glass-slide method with either Difco *Salmonella* O Antiserum Poly A-I or the appropriate group-specific *Salmonella* O antiserum. Tests for H (flagellar) antigens were made by the tube test method with Difco *Salmonella* Poly H Antiserum. These tests are described in the *Difco Supplementary Literature* (Difco Laboratories, Detroit, Mich.).

Antibiotic sensitivity. Approximately 5,000,000 cells from 16-hr TSYE broth cultures were spread on the surface of pre-poured TSYE agar plates (20 ml of agar per 100-mm diameter plate). Sensitivity discs impregnated with the following antibiotics were placed on each plate: chloramphenicol, 5 μg (BBL); tetracycline, 5 μg (BBL); ampicillin, 2, 5, or 10 μg (BBL). The plates were incubated for 24 hr at 37 C, and the diameter of the zone of inhibition was measured with a millimeter rule under the magnifying lens of a Quebec colony counter. Twenty-nine cultures of *S. typhimurium* and 20 cultures each of *S. newport, S. thompson,* and *S. heidelberg* were tested.

A significant difference at the 95% level in sensitivity to a particular antibiotic between radio-sensitive and radio-resistant cultures was determined by the Student *t* test.

Measurement of growth rates. For anaerobic growth, approximately 100 cells from a 15-hr TSYE broth culture of the radio-resistant or radio-sensitive culture were inoculated into 30 ml of TSYE broth in culture tubes (20 by 175 mm) and incubated in a thermostatically controlled water bath.

For aerobic growth, the cells were inoculated into 100 ml of broth contained in a 500-ml Erlenmeyer flask and pretempered to the desired temperature. The flasks were shaken on a mechanical shaker within a walk-in incubator. Samples were extracted periodically and analyzed for total viable count by surface streaking suitable dilutions on TSYE-agar plates. A growth curve was constructed by plotting log concentration of cells as a function of time. The generation time was determined from the regression slope characterizing the logarithmic growth phase.

RESULTS

Virulence of radio-resistant and radio-sensitive cultures. By using the LD_{50} value for the number of viable cells required to cause death in mice as the criterion, it is considered that there was no difference in virulence between radio-resistant and radio-sensitive cultures of *S. newport, S. thompson,* and *S. heidelberg* (Table 1). With these serotypes, the magnitude of the difference between the LD_{50} values of resistant and sensitive cultures was probably within the realm of experimental error. However, with *S. typhimurium* the number of radio-sensitive cells required to cause 50% mortality in mice was approximately 10 times less than the number needed with radio-resistant cells. A difference of this order of magnitude might be large enough to indicate that the radio-resistant cultures were less virulent than the radio-sensitive culture. Leshkovich (4) noted a marked reduction in the virulence of *Pasteurella pestis* after the culture had been subjected to three X-ray treatments.

When chick embryos were used as host, the results seemed to confirm those obtained with mice, that is, the radio-resistant cultures were not more virulent than the radio-sensitive cultures (Table 1). However, the chick embryo was considerably more sensitive as a host than the mouse, since the introduction of only a few viable cells was sufficient to eventually result in death. In the case of the chick embryo, death was most likely due to an infection, whereas with the mice, death may have been caused by intoxication, since a tremendously large number of cells were involved.

Another criterion of virulence is the rapidity with which death occurs once the host has been challenged. There did not appear to be any major difference in this respect between the resistant and sensitive cultures.

Passage of microorganisms through animals has been known to effect an increase in virulence. Therefore, one experiment was directed to ascertain whether the radio-resistant culture might become more virulent after passage through a live host as compared with the radio-sensitive culture. For this experiment, resistant or sensitive

TABLE 1. *Approximate number of viable cells of radio-sensitive or radio-resistant Salmonella required to cause 50% deaths in mice or chick embryos*

Serotype	Host	Radio-sensitive culture	Radio-resistant culture
S. newport	Mouse	8×10^7	6×10^7
S. typhimurium	Mouse	3×10^6	2×10^7
S. thompson	Mouse	1.5×10^8	3.5×10^8
S. heidelberg	Mouse	7×10^7	2.5×10^7
S. newport	Chick embryo	0.9	0.7
S. thompson	Chick embryo	<5	3.5

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cells of *S. thompson* were recovered from chick embryos which had died after being initially infected with about three to five cells. The virulence of cultures propagated from these cells was then determined by using mice as the test species. The LD_{50} value for the resistant and sensitive cultures was about 1.1×10^8 cells and 3.5×10^7 cells, respectively. Prior to passage through chick embryo, the corresponding LD_{50} was 3.5×10^8 cells for the resistant and 1.5×10^8 cells for the sensitive culture. With both cultures there was a comparable decrease in the number of cells required for 50% mortality in mice, but since the standard error was not known, it could only be concluded that the radio-resistant culture was not more prone to an increase in virulence compared to the radio-sensitive culture after one animal passage.

Virulence in pathogenic bacteria has been associated with "smooth" colonies, the "rough" colonies usually giving rise to avirulent cells. Smooth colonies often revert to the rough form, but the opposite reversion has not usually been observed. Irradiation of a "rough" culture of *S. newport* on the surface of TSYE-agar plates did not induce the conversion of rough to smooth colonies.

Biochemical characteristics of radio-resistant and radio-sensitive cultures. The radio-sensitive cultures of the four serotypes all gave typical reactions on the various test media, with one exception. A few radio-sensitive and radio-resistant cultures of *S. newport* showed weak growth in KCN medium, therefore, this particular test was discontinued.

The radio-resistant cultures of *S. heidelberg* or *S. typhimurium* did not show a change in any of the biochemical reactions tested. However, 11 of the 50 radio-resistant cultures of *S. newport* examined did not utilize citrate. Among 50 radio-resistant cultures of *S. thompson*, 5 did not utilize citrate and 2 neither produced hydrogen sulfide nor assimilated citrate.

There are normally some *Salmonella* serotypes which do not grow on Simmons citrate and some which do not produce hydrogen sulfide on Triple Sugar Iron agar. Therefore, whereas the cyclic irradiation treatment did not cause a drastic change in the overall biochemical pattern, in some instances a minor change may have been induced. Erdman et al. (2) found no change in biochemical reactions of a culture of *S. gallinarum* which had been subjected to 14 cycles of irradiation at 200,000 rep and outgrowth, whereas a culture of *E. coli* failed to produce indol after 12 irradiation cycles. It is not surprising to have induced some metabolic block after cyclic irradiation treatment, since ionizing and nonionizing (ultraviolet) radiations have been used for years to produce bacterial mutants with some particular metabolic trait (5). Nevertheless, it was considered that in spite of these minor alterations, generic identification of these radio-resistant cultures as *Salmonella* was still feasible. To verify this assumption, a radio-resistant culture of *S. newport* was submitted to the Communicable Disease Center at Atlanta, Ga., for identification, and it was properly classified and serotyped.

The appearance and reactions of *Salmonella* on certain differential media is often employed as an aid in identification. However, there was no observable difference in colony appearance or reaction between the radio-resistant and radio-sensitive cultures of the four *Salmonella* serotypes on Brilliant Green agar (Difco), Bismuth Sulfite agar (Difco), Salmonella-Shigella agar (Difco),

TABLE 2. *Diameter (mm) of zone of inhibition and standard deviation (in brackets) for radio-sensitive and radio-resistant cultures of Salmonella with antibiotic sensitivity discs*

Serotype	Radio-sensitive culture					Radio-resistant culture				
	Chloramphenicol (5 µg)	Tetracycline (5 µg)	Ampicillin			Chloramphenicol (5 µg)	Tetracycline (5 µg)	Ampicillin		
			2 µg	5 µg	10 µg			2 µg	5 µg	10 µg
S. typhimurium	16.6[a] (0.91)	11.2[a] (0.62)	8.8 (1.5)			15.0 (0.97)	10.6 (0.68)	8.1 (0.96)		
S. newport	18.2[a] (1.0)	11.4 (0.60)	12.9[a] (0.89)	19.7[a] (0.91)	21.7[a] (1.6)	15.6 (1.1)	11.6 (0.68)	11.1 (1.8)	23.4 (1.4)	26.5 (1.5)
S. heidelberg	17.2 (1.1)	(10.0)[a] (0.46)	10.7 (1.2)	18.3[a] (1.1)	21.2[a] (1.1)	16.9 (1.2)	12.1 (0.45)	10.3 (1.1)	19.7 (0.66)	22.8 (0.91)
S. thompson	15.5 (1.2)	10.4 (0.49)	10.1 (0.78)	16.8[a] (1.1)	19.4[a] (1.2)	15.1 (1.1)	10.6 (0.84)	10.4 (1.3)	18.3 (1.2)	20.7 (1.1)

[a] Significant difference with radio-resistant counterpart.

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TABLE 3. *Growth characteristics under aerobic or anaerobic conditions for radio-resistant or radio-sensitive cultures of various Salmonella serotypes*

Serotype	Growth temp	Generation time		Lag time		Maximal concn log cells/ml	
		Sensitive culture	Resistant culture	Sensitive culture	Resistant culture	Sensitive culture	Resistant culture
	C	*min*	*min*	*min*	*min*		
S. typhimurium							
Anaerobic growth	10	690	720			8.92	8.76
	20	91	86.5	180	210	8.25	7.90
	30	36.8	34.7	90	80	8.44	8.38
	34	26.0	26.3	85	95	8.78	8.61
	38	29.0	27.8	84	60	8.80	8.41
	43	24.0	24.5	35	40	8.43	8.29
	45	30.0	32.2	110	110	8.18	8.24
Aerobic growth	20	85.8	79	190	200	10.30	10.15
	37	20.5	23	120	85	10.0	9.90
S. newport							
Anaerobic growth	10	590	780			8.86	8.18
	20	96.5	141	270	660	8.90	8.35
	37	22	25.4	85	75	9.20	8.90
	45	24	24	96	90	8.90	8.70
Aerobic growth	20	87	128	330	345	10.20	9.65
	37	19.3	21.6	140	150	9.80	9.63
S. heidelberg							
Anaerobic growth	37	22.8	24.2	70	70	9.15	8.90
Aerobic growth	20	79	79	350	380		
	37	22.5	25.2	60	150	9.95	9.80
S. thompson							
Anaerobic growth	10	620	610			8.70	8.83
	20	98.5	103	375	375	8.75	8.65
	30	30	26.4	84	108	8.85	8.82
	34	24.3	24.3	60	60	8.85	8.75
	37	24.8	25.1	96	84	8.95	8.81
	43	26	26	96	60	8.64	8.63
	45	38	37.5	48	72	7.70	7.58
Aerobic growth	20	81.5	83	252	260		
	37	17.9	20.3	180	135	9.85	9.75

and MacConkey agar (Difco). On each specific diagnostic medium, all four serotypes formed similar colonies except on Bismuth Sulfite Agar, in which case there were differences among serotypes and even within a serotype of the sensitive cultures.

Serological reaction. One hundred colonies of *S. typhimurium* isolated from a radio-sensitive culture and 100 colonies from a radio-resistant culture all gave positive agglutination with *Salmonella* O poly A-I antiserum, although many of the radio-resistant cultures agglutinated weakly. Twenty-five colonies from each culture were then tested with the *Salmonella* O antiserum specific for group B, and in every instance a positive reaction was obtained. These same cultures, both sensitive and resistant, also were agglutinated with *Salmonella* H antiserum specific for group i.

With *S. newport*, 70 cultures of either the radio-sensitive or radio-resistant strains were reacted with polyvalent *Salmonella* O antiserum, and again a weak agglutination was observed with the radio-resistant cultures as compared with the parent radio-sensitive cultures. The radio-sensitive cultures agglutinated strongly with group C_2-specific O antiserum, but with the radio-resistant cultures there was either no agglutination or a very weak reaction. Some difficulty was also experienced with the resistant cultures in serological reaction for flagellar antigens with *Salmonella* H antiserum group e, h.

Similarly, radio-resistant cultures of *S. thompson* or *S. heidelberg* did not agglutinate as strongly

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as the parent radio-sensitive cultures with polyvalent somatic or flagellar antisera, but typing was still possible.

Thus, it is evident that cyclic irradiation ininduced a change in the serological properties of *Salmonella*.

It is not known whether the reduced ability of the radio-resistant cultures to agglutinate with polyvalent O antiserum was due to a genetic defect or to a physical phenomenon related to the pleomorphism of the cells. Nevertheless, in the hands of experts, serological typing of these cultures was still possible, since the culture causing us the most difficulty, *S. newport*, was serotyped at the Communicable Disease Center, Atlanta, Ga.

Antibiotic sensitivity. The relative sensitivity of radio-resistant (radiation cycled) and radiosensitive (noncycled) cultures of the four test *Salmonella* serotypes to various antibiotics, as measured by the sensitivity disc method, can be evaluated from the data presented in Table 2. The differences in sensitivity to a given antibiotic between radio-resistant and radio-sensitive cultures were not large enough to be of any practical significance. In accordance with the manufacturers guide sheet, both radio-resistant and radiosensitive cultures were classified as sensitive to the antibiotic on the basis of their diameter zones of inhibition. Therefore, it would not appear that irradiation treatment would generally produce mutant strains of *Salmonella* that would be resistant to antibiotics that are ordinarily effective against these species. However, it was considered that a statistical comparison of these differences, however small they may be, might reveal a general trend of the radio-resistant cultures toward increasing or decreasing antibiotic sensitivity, but none was found except possibly with ampicillin, in which case the radio-resistant cultures were slightly more sensitive.

Growth rate of radio-resistant and radiosensitive cultures. Growth characteristics of radiosensitive and radio-resistant cultures of the four test *Salmonella* serotypes under aerobic or anaerobic conditions are presented in Table 3. In general, there was no difference in growth rates between resistant or sensitive cultures either under aerobic or anaerobic conditions and over a temperature range of 10 to 45 C, with the exception of *S. newport* at 10 or 20 C, in which instance the radio-resistant cultures grew at a slower rate. Witkin (7) reported that there was no difference in growth rates of *E. coli* B, and the radioresistant mutant B/r, but that the lag period was shorted for the resistant mutants. Lag times were defined as the time required for the initial inoculum to double in numbers. In the present study, lag times based on this definition were determined by graphical interpolation of the growth curves; these values are included in Table 3. These lag times are at best only approximations of the true values. Still, one can see that there is no particular trend for the resistant cultures to have a shorter or longer lag than the radio-sensitive parent cultures. Idziak and Thatcher (3) also found no difference in lag times between a radioresistant and a radio-sensitive strain of *E. coli* when grown at 37 C under shake flask or stationary conditions.

It has been observed that the final viable concentration of radio-resistant cultures is usually slightly less than that of the parent radio-sensitive cultures (2, 7). This phenomenon was also confirmed in this present study with radio-resistant cultures of *Salmonella*.

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LITERATURE CITED

1. Edwards, P. R., and W. H. Ewing. 1955. Identification of *Enterobacteriaceae*. Burgess Publishing Co., Minneapolis.
2. Erdman, I. E., F. S. Thatcher, and K. F. MacQueen. 1961. Studies on the irradiation of microorganisms in relation to food. II. Irradiation resistant mutants. Can. J. Microbiol. 7:207–215.
3. Idziak, E. S., and F. S. Thatcher. 1964. Some physiological aspects of mutants of *Escherichia coli* resistant to gamma irradiation. Can. J. Microbiol. 10:683–697.
4. Leshkovich, L. I. 1958. The action of x-rays on the virulence and antigenic properties of *Pasteurella pestis*. J. Microbiol. Epidemiol. Immunobiol. 29:199–204.
4a. Licciardello, J. J., J. T. R. Nickerson, S. A. Goldblith, C. A. Shannon, and W. W. Bishop. 1969. Development of radiation resistance in *Salmonella* cultures. Appl. Microbiol. 18: 24–30.
5. Tatum, E. L. 1946. Induced biochemical mutations in bacteria. Cold Spring Harbor Symp. Quant. Biol. 11:278–283.
6. Thatcher, F. S. 1963. Some public health aspects of the microbiology of irradiated foods. Int. J. Appl. Rad. Isotopes. 14:51–58.
7. Witkin, E. M. 1946. Inherited differences in sensitivity to irradiation in *Escherichia coli*. Genetics 32:221–248.

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