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Recombination-deficient Mutant of *Salmonella typhimurium*[1]

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A recombination-deficient (Rec⁻) ultraviolet-sensitive mutant of *Salmonella typhimurium* was isolated. The mutant grows more slowly than the wild-type strain and degrades its deoxyribonucleic acid extensively both during normal growth and after ultraviolet irradiation. Evidence is presented that a growing Rec⁻ population consists of two types of cells, one which can divide and one which cannot.

Recombination-deficient (Rec⁻) mutants of *Escherichia coli* have been isolated (1, 7). Such mutants are also highly sensitive to ultraviolet irradiation and exhibit an abnormally high rate of deoxyribonucleic acid (DNA) degradation, both during normal growth and after ultraviolet irradiation (2). A study of revertants (1) suggested that all of these characteristics are the result of a single mutation. The possibility that the recombination deficiency is due to failure of the male chromosome to enter the female cell during conjugation was eliminated by the demonstration of zygotic induction in a cross of a male lysogenic for phage λ with a sensitive Rec⁻ female. Zygotic induction can be used as a measure of transfer of genetic material since its expression does not depend on recombination but only on the entry of the prophage from the lysogenic donor (8). Although the number of recombinants obtained in the cross was less than in a similar cross with a Rec⁺ female, the number of infective centers resulting from zygotic induction was normal.

In this paper, the isolation and characterization of a similar mutant in *Salmonella typhimurium* is described. Evidence is presented that a growing Rec⁻ culture consists of two populations of cells, one which can divide and one which cannot.

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial strains used were derivatives of *S. typhimurium* LT-2. The male strains were Hfr A *purC7 strA* [SU576 of Sanderson and Demerec (11)], direction of injection *O-metA-leu-pro*; Hfr B2 *metA22* [SU354 of Sanderson and Demerec (11)], direction of injection *O-metG-purC-argB*; and Hfr B3 *hisD23 gal50* [SU436 of Sanderson and Demerec (11)], direction of injection *O-argB-purC-metG*. Two female strains were used: *leu197* and *argB69*. Cells of a *gal*⁻ strain were used as indicator bacteria for plating phage.

The phage strains employed were c_1 or c_2, clear mutants of the temperate phage P22. Wild-type activity of both genes is required for lysogenization of the host (9); infection with either mutant results in lysis of the cell.

Media. L broth, nutrient agar, soft agar for top layers, buffered saline (9), M9, and supplemented M9 (12) have all been previously described. Minimal agar contained 4 g of glucose, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of citric acid, 10 g of KH_2PO_4, 3.5 g of $NaNH_4 \cdot H_2O$, and 20 g of agar (Difco) in 1 liter of distilled water.

Ultraviolet irradiation. Cells were grown at 37 C with aeration in L broth or in supplemented M9 to a concentration of about 10^8 cells/ml. The cells were then centrifuged, resuspended in buffered saline or supplemented M9, and irradiated in a glass petri dish. The cell concentration was no greater than 10^8 cells/ml, and the depth of the suspension in the dish did not exceed 2 mm. Ultraviolet light was administered with a 15-w Westinghouse germicidal lamp at a distance of 50 cm, or as stated. The dose rate at 50 cm was 10 ergs per mm² per sec. All operations subsequent to exposure to ultraviolet light were carried out in the dark or in red glass vessels, to avoid photoreactivation of the irradiated cells.

Bacterial crosses. Hfr and F⁻ cells were grown to log phase in L broth with aeration. Two optical density (OD) units (Gilford 3000, 0.3 OD_{650} unit = 10^8 cells/ml) of the F⁻ culture and 0.4 OD unit of the Hfr strain were concentrated together on a membrane filter (pore size, 0.45 μ; Millipore Corp., Bedford, Mass.). The membrane filter was then placed on a soft-agar layer prewarmed to 37 C and was incubated for 10 min. Finally, the filter was transferred to 15 ml of L broth at 37 C and incubated for an additional 110

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min with gentle aeration. Cells were plated on selective medium. The Millipore filter conjugation technique was adapted from Sanderson and Demerec (11).

Determination of DNA degradation. A stationary culture was diluted 1:100 into supplemented M9 medium containing 10 μc of tritiated thymidine per ml (specific activity, 21.5 c/mmole; New England Nuclear Corp., Boston, Mass.) and grown to log phase (about 10^8 cells/ml) in a shaker bath at 37 C. To remove the label, cells were collected on a Millipore filter, washed, and resuspended in supplemented M9. They were then grown with aeration for an additional 45 min in the presence of unlabeled thymidine (100 μg/ml) to exhaust the intracellular pool of label. The cells were irradiated in the same medium and returned to the shaker bath. Samples were removed at appropriate intervals into an equal volume of cold 10% trichloroacetic acid containing 100 μg of unlabeled thymidine per ml and placed in an ice bath for at least 30 min. To determine the amount of radioactivity in the trichloroacetic acid precipitate, the sample was collected on a glass filter (Whatman GF-C) in a Millipore apparatus, washed with cold distilled water, and dried; the filter was then placed in 15 ml of paradioxane scintillation fluid to which 0.5 ml of 5% trichloroacetic acid had been added. The scintillation fluid contained 100 g of naphthalene (Eastman Organic Chemicals, Rochester, N.Y.), 7 g of PPO (2,5-diphenyloxazole; Packard Instrument Co., Downers Grove, Ill.; scintillation grade), and 100 mg of dimethyl POPOP (1,2-bis-2-[4-methyl-5-phenyloxazolyl]-benzene, Packard Instrument Co.; scintillation grade), dissolved in 1 liter of paradioxane (Matheson, Coleman, and Bell, East Rutherford, N.J.). All counting was done in a Packard Tri-Carb liquid scintillation spectrometer.

To determine the amount of label that was simultaneously made acid-soluble, the trichloroacetic acid sample was centrifuged in a Sorvall SS-34 rotor for 15 min at 10,000 rev/min (12,100 $\times$ *g*) to sediment the acid-precipitable fraction. Samples of the supernatant fluids were transferred to scintillation vials, 15 ml of paradioxane scintillation medium was added to each, and the samples were counted.

Sucrose gradient centrifugation to determine cell size. Log-phase cells were sedimented and thoroughly resuspended at a concentration of about 2×10^9 cells/ml in buffered saline containing 0.015% Sarkosyl (sodium sarkosinate, Geigy Chemical Corp., Ardsley, N.Y.). A 1-ml sample of cells was layered on 30 ml of a 5 to 20% sucrose gradient and centrifuged in a swinging-bucket rotor in an International clinical centrifuge (Universal model UV) for 6 to 10 min at 2,000 rev/min (600 $\times$ *g*). The optical density of 1-ml fractions collected from the bottom of the tube was determined, and each fraction was assayed for colony formers. To determine the number of cells capable of supporting phage growth, 0.1 ml of each fraction was added to 1 ml of phage c_1 or c_2 at an estimated multiplicity of 10 to 20. After allowing 5 min for adsorption at 37 C, the mixture was treated with antiphage serum (final $K = 2$) to inactivate unadsorbed phage. The cells were then plated with background bacteria and incubated overnight, after which the number of infective centers was determined.

RESULTS

Isolation of the recombination-deficient (Rec$^-$) mutant. The recombination-deficient mutant used in this study was isolated by a procedure similar to that of Clark and Margulies (1). A 1-ml amount of an overnight culture of *leu197* cells in L broth was concentrated on a Millipore filter, washed twice with 1-ml portions of citrate buffer (0.1 M, *p*H 5.5), and resuspended in 1 ml of citrate buffer containing 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml (Aldrich Chemical Co., Milwaukee, Wis.). Cells were incubated for 1 hr at 37 C, concentrated on a filter, washed once with citrate buffer, and then resuspended in buffered saline. A sample was then spread onto minimal agar plates supplemented with approximately 1 mg of leucine per plate. The plates were incubated at 37 C for 48 hr. Since the original strain required leucine for growth, the addition of leucine to the minimal agar permitted most of the cells to form colonies.

Rec$^-$ mutants selected from the leucine-supplemented plates were identified by a plate mating. Colonies were replica-plated onto minimal agar plates spread with approximately 2×10^9 male (Hfr) cells concentrated from an aerated early stationary-phase culture. After 18 to 24 hr of incubation, the replica was compared with the master plate. Any colony which did not produce prototrophic recombinants on the replica plate was a possible Rec$^-$. One such colony was obtained, and it was further tested for ultraviolet sensitivity by streaking across a nutrient agar plate and exposing half of the plate to ultraviolet light for 10 sec at 50 cm. Streaks from wild-type colonies are unaffected by this treatment. No growth was obtained in the part of the Rec$^-$ streak exposed to ultraviolet light. A stock was made from this Rec$^-$ Leu$^-$ UVS mutant after two successive single-colony isolations.

The original mutant grew very poorly. To separate the Rec$^-$ mutation from other possible mutations induced by nitrosoguanidine, the Rec$^-$ character was transferred into other strains by means of bacterial crosses. This was possible because the ability of the Rec$^-$ strain to recombine is not entirely lacking, although it is greatly reduced. Rare recombinants may be obtained, some of which are still Rec$^-$. HfrA *purC7 strA*, which is streptomycin-resistant (Sm^R) and which inserts the streptomycin locus as a terminal marker, was crossed with the Rec$^-$ strain. The cells were plated on minimal agar containing leucine and streptomycin [1 drop of a 5% (w/v) solution, Chas. Pfizer & Co., Inc., Brooklyn,

N.Y.]. The presence of streptomycin assured that only those cells which had received the entire Hfr chromosome (including the marker for streptomycin resistance) would survive. The rare Sm^R, Pur^+ recombinant colonies were tested for ultraviolet sensitivity, recombination ability, and leucine auxotrophy. A Leu^- Rec^- UV^S Sm^R recombinant was selected and used for the majority of experiments to be described. This strain grew faster than the original Rec^- mutant, probably because a large portion of its chromosome was derived from the male parent.

Some of the Leu^- Rec^- Sm^R recombinants were Hfr. These male cells were still ultraviolet-sensitive, but were able to yield normal numbers of recombinants when mated with a Rec^+ female. By crossing the male Rec^- strain with female strains carrying various markers, it was possible to produce female Rec^- strains containing any desired allele. A Rec^- Arg^- strain was isolated for use in the recombination experiments.

Recombination ability. Matings were carried out between Rec^- strains and two Hfr strains (Table 1). The frequencies of recombination were compared with those in crosses in which Rec^+ females were used. Hfr A showed 10^6 times fewer recombinants in crosses with Rec^- than in similar crosses with Rec^+ females. This was not due to failure of the male chromosome to enter the Rec^- cell. The finding of normal zygotic induction of Rec^- females mated with a male strain lysogenic for phage P22 (Wing, *unpublished data*) showed that the failure to find recombinant progeny must be due to a block at one of the recombination steps.

When Hfr B2 was used, only a 2-log difference in recombination frequency is observed between Rec^+ and Rec^- matings (Table 1). Hertman and Luria (4) have demonstrated that an *E. coli* rec^+ gene begins to function as soon as it enters a rec^- cell. The relatively high frequency of recombination observed with Hfr B2 therefore suggests that the *rec* locus maps near the point of origin of this male so that the chances of insertion of the rec^+ allele into a recipient cell are good. Preliminary interrupted mating experiments between Hfr B3 and the Rec^- strain suggest that the *rec* locus is inserted early by this male too. Therefore, the *rec* locus probably lies between the points of origin of Hfr B2 and Hfr B3 on the *S. typhimurium* map (11). On the other hand, the *rec* gene probably maps far from the origin of Hfr A. When this male was used, the rec^+ allele had a very small probability of entering the Rec^- female cell.

Ultraviolet sensitivity. Figure 1 presents the survival curves for Rec^- and Rec^+ cells irradiated in buffered saline at a distance of 160 cm from the lamp. The Rec^+ strain showed typical wild-type

TABLE 1. *Recombination ability of Rec⁻ strains*

Cross	Mating	Recombinants per Hfr cell mated	Rec⁺/ Rec⁻
I	Hfr A *purC7 strA*		
	× Rec^+ Leu^-	1.3×10^{-1}	10^6
	× Rec^- Leu^-	$<1.5 \times 10^{-7}$	
II	Hfr B2 *metA*		
	× Rec^+ Arg^-	1.6×10^{-2}	71
	× Rec^- Arg^-	2.3×10^{-4}	

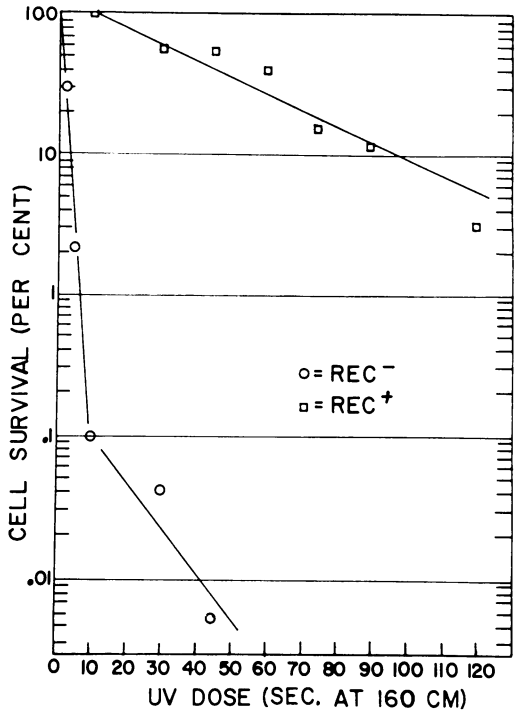

FIG. 1. *Survival curves of Rec^+ and Rec^- cells exposed to ultraviolet irradiation.*

sensitivity, with a 37% survival dose (equivalent to an average of one lethal hit per cell) of about 48 sec. The Rec^- strain showed a diphasic survival curve, with a greater sensitivity at low doses (37% survival dose = 2 sec) than at higher doses. The Rec^- strain was, therefore, initially about 25 times as sensitive to ultraviolet light as the Rec^+ strain.

To determine whether a small fraction of ultraviolet-resistant cells was responsible for the change in the slope of the Rec^- killing curve, cells surviving high doses were picked into broth, incubated to log phase, and retested for ultraviolet sensitivity. All isolates were as sensitive as the original cells. The increased resistance to

ultraviolet killing was apparently due to nonheritable factors.

The Rec⁻ strain was also extremely sensitive to killing by other agents which mimic the action of ultraviolet light, such as streptonigrin and mitomycin C.

DNA degradation. The *E. coli* Rec⁻ strain isolated by Clark and Margulies (1) displays an abnormal amount of degradation of its DNA during growth and even more after ultraviolet irradiation (2). It was therefore of interest to investigate this property in the *S. typhimurium* Rec⁻ strain. Figure 2 shows the results of an experiment in which Rec⁻ and Rec⁺ cells were prelabeled with tritiated thymidine, and solubilization of label was followed with or without prior exposure to ultraviolet light. In the absence of ultraviolet irradiation, the Rec⁻ cells solubilized 30% of the initial label in 2 hr of growth, whereas the control Rec⁺ had lost only 5%. After ultraviolet irradiation (30 sec at 50 cm), the Rec⁻ strain lost label at a greater rate, losing 60% of its DNA in 2 hr. The Rec⁺ control lost only 12% during the same period. The *S. typhimurium* Rec⁻ strain, like the *E. coli* strain, thus showed excessive degradation of DNA.

Growth characteristics. Under normal conditions of culture (L broth at 37 C), Rec⁻ cells grew more slowly than Rec⁺ cells. In one determination, the doubling time for Rec⁺ cells, as measured by colony count, was 23 min and for the Rec⁻, 35 min. The ratio of Rec⁻ to Rec⁺ doubling time was 1.5, a value consistently obtained. Another finding was that the ratio of OD to number of colony formers was greater for the Rec⁻ strain than for the Rec⁺ strain. Thus, 0.3 OD units corresponded to approximately 10^8 Rec⁺ colony-forming units, but only 4×10^7 Rec⁻ colony-forming units. This observation could be explained if (i) the Rec⁻ cells are larger than Rec⁺ cells or (ii) the Rec⁻ culture contains a substantial proportion of cells which contribute to optical density but are not colony formers. Both possibilities may be operating simultaneously.

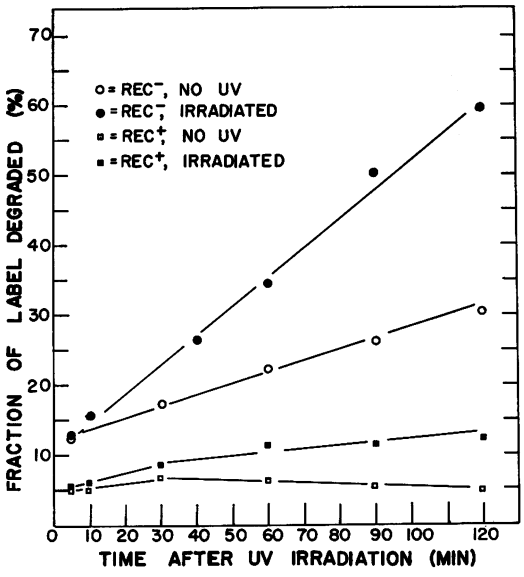

FIG. 2. *Sucrose gradient sedimentation of Rec⁻ and Rec⁺ cells. Each fraction was assayed for colony formers and optical density.*

Cells which are incapable of dividing (e.g., ultraviolet-inactivated cells) can often support phage growth and yield infective centers. If a culture contains a mixture of colony-forming and nondividing cells, all of which can support phage growth, then the number of infective centers produced by that culture will exceed the number of colonies. To determine whether the Rec⁻ strain contains two such types of cells, log-phase cells were plated for colony production; another sample of cells was infected with phage c_2 at an estimated multiplicity of 20 phage/cell, to insure that every cell, whether or not it was a colony former, would be infected. Unadsorbed phage were inactivated with antiphage serum, and the number of infective centers assayed. The results of three independent experiments are shown in Table 2.

The Rec⁺ control showed approximately equal numbers of infective centers and colony formers. In contrast, the Rec⁻ cultures contained about 1.5 times as many cells capable of supporting phage growth as capable of forming colonies. Thus, about one-third of the cells in a Rec⁻ culture are nondividers. Similar results were obtained with very early or very late log-phase cells. Therefore, the nondividing cells appear to arise from the colony formers. A growing Rec⁻ culture is thus heterogeneous; some cells can divide but others cannot.

To determine whether the two types of cells differ in size, a log phase Rec⁻ culture was subjected to sucrose gradient centrifugation. For each fraction, the optical density and the number of colony formers was determined. In the Rec⁺ control (Fig. 3, left), the peaks for OD and colony formers overlap, as expected if each cell contributes to both OD and colony formation. In the Rec⁻ gradient, however (Fig. 3, right), the peak of the OD curve was displaced to the left of the peak of colony formers by several fractions. The extent of displacement varied in different experiments, but was usually noticeable. The Rec⁻ culture thus contained some cells which con-

tributed to the OD but could not form colonies. The increased sedimentation rate of the nondividers showed that they are larger than the dividing cells. The Rec$^-$ optical density curve was also much broader than the Rec$^+$, indicating a greater heterogeneity in cell size.

Visual corroboration of the sucrose gradient results came from microscopic examination of fractions from the gradients. The heavier fractions of the Rec$^-$ gradient contained cells which were two to five times as long as normal cells. No abnormally large cells were observed in Rec$^+$ fractions.

The nondividing, larger Rec$^-$ cells may be the fraction previously shown to support phage growth but not to form colonies. If so, then phage infection of each fraction should yield a curve of infective centers resembling the optical density curve. Figure 4 (right) shows such an analysis of a Rec$^-$ gradient. The infective centers curve overlaps the OD curve, verifying the hypothesis that the heavy cells are the nondividing fraction which can support phage growth. As expected, the peaks of all the curves of the Rec$^+$ control gradient (Fig. 4, left) coincide.

TABLE 2. *Comparison of infective centers and colony formers in Rec$^-$ and Rec$^+$ cultures*

Expt	Strain	Cell titer	Infective centers	Infective centers per colony former
1	Rec$^-$	0.78×10^8	1.09×10^8	1.4
	Rec$^+$	1.6×10^8	1.66×10^8	1.05
2	Rec$^-$	0.44×10^8	0.67×10^8	1.5
	Rec$^+$	1.24×10^8	0.88×10^8	0.71
3	Rec$^-$	0.24×10^8	0.52×10^8	2.1
	Rec$^+$	1.3×10^8	1.1×10^8	0.95

DISCUSSION

A recombination-deficient mutant has been isolated in *S. typhimurium* which has properties similar to "reckless" Rec$^-$ mutants of *E. coli* (2). It is highly sensitive to killing by ultraviolet irradiation, with a 37% survival dose about 25 times smaller than a wild-type strain. Its ability to form recombinants in genetic crosses is reduced by a factor of 10^2 to 10^6, depending on the male strain used. The Rec$^-$ strain degrades its DNA extensively, both during normal growth and after ultraviolet irradiation.

Evidence has been presented for the existence of a substantial fraction of cells in a growing Rec$^-$ culture—approximately one-third—which can metabolize but cannot divide. This fraction may arise in the following way. During normal growth, lesions in the DNA, such as single-strand nicks, may occur. The Rec$^-$ strain can repair a large fraction of the lesions in its DNA (2, 7). Consequently, most of the single-strand breaks may be repaired, while extensive degradation may occur at the remaining repair sites. Cells in which extensive degradation occurs would be unable to

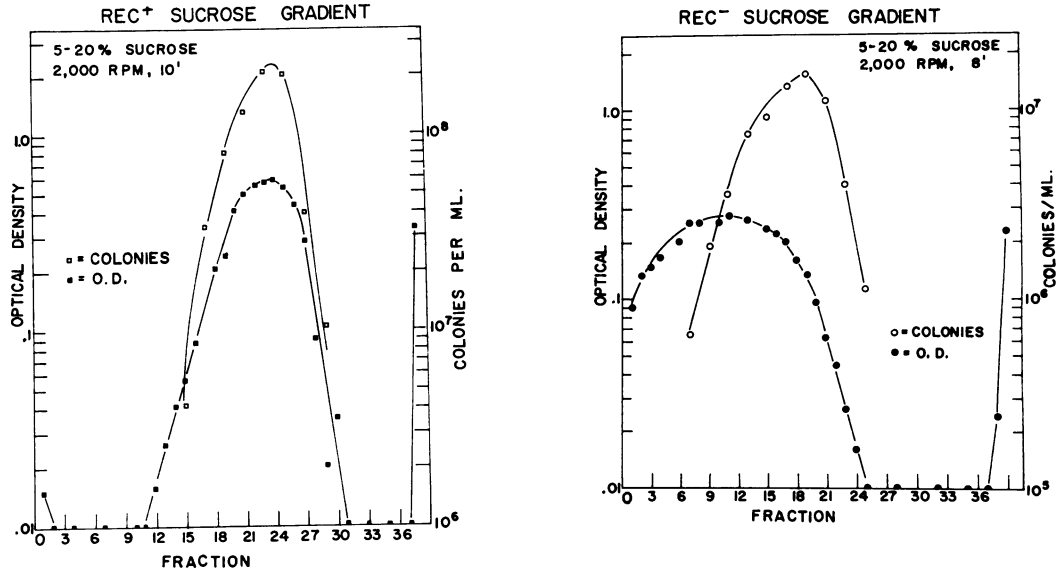

FIG. 3. *Sucrose gradient sedimentation of Rec$^-$ and Rec$^+$ cells. In addition to optical density and colony formers, each fraction was assayed for the number of cells capable of supporting phage growth.*

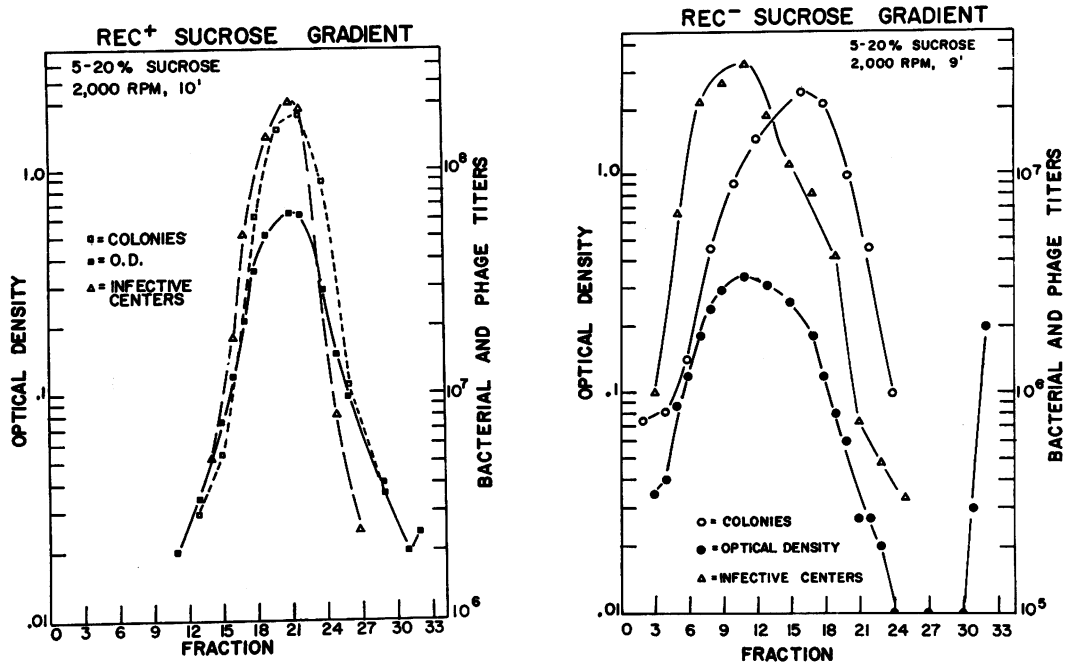

FIG. 4. *DNA degradation in Rec⁻ and Rec⁺ cells.*

divide and would form the nondividing fraction of the Rec⁻ population. Such cells would then be responsible for the extensive solubilization of DNA observed in growing Rec⁻ cultures. These cells might continue to metabolize for a short time (perhaps one to two generation times, as indicated by their sizes under microscopic examination), increasing in size but not dividing. The formation of this fraction could account for the apparent longer generation time of the entire culture. The growing nondividing cells would account for the heterogeneity in size of the Rec⁻ culture.

H. Reiter (Bacteriol. Proc., p. 48, 1967) has shown that DNA degradation observed after ultraviolet irradiation of a wild-type *Bacillus subtilis* culture is the property of a limited number of cells in the population, cells which can no longer divide. This finding provides some support for the idea that the nondividing fraction of the *S. typhimurium* Rec⁻ culture may be responsible for the DNA degradation observed in the absence of ultraviolet treatment. Since exposure to ultraviolet light for only 10 sec at 160 cm allows only 0.1% survival, it is likely that following such a dose, *every* Rec⁻ cell degrades its DNA extensively.

A prediction of the above hypothesis is that the heavier, nondividing, sucrose gradient fractions of Rec⁻ cells should show more DNA degradation than the lighter fractions. This may be tested by uniformly labeling a Rec⁻ culture with tritiated thymidine and then subjecting it to sucrose gradient centrifugation. Fractions from different parts of the gradient can be pooled, and the solubilization of label in each pooled fraction can be followed. Technical problems have thus far prevented a conclusive result.

ACKNOWLEDGMENTS

The suggestion of G. S. Stent to subject a Rec⁻ culture to sucrose gradient centrifugation is hereby gratefully acknowledged.

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LITERATURE CITED

1. CLARK, A. J., AND A. D. MARGULIES. 1965. Isolation and characterization of a recombination-deficient mutant in *E. coli*. Proc. Natl. Acad. Sci. U.S. **51**:451–459.
2. CLARK, A. J., M. CHAMBERLIN, R. BOYCE, AND P. HOWARD-FLANDERS. 1966. The abnormal metabolic response to ultraviolet light of a recombination-deficient mutant of *Escherichia coli* K-12. J. Mol. Biol. **19**:442–454.
3. GELLERT, M., 1967. Formation of covalent circles of lambda DNA by *E. coli* extracts. Proc. Natl. Acad. Sci. U.S. **57**:148–155.
4. HERTMAN, I., AND S. E. LURIA. 1967. Transduc-

tion studies on the role of the *rec*+ gene in the ultraviolet induction of prophage lambda. J. Mol. Biol. **23**:117–133.

5. HOWARD-FLANDERS, P., AND R. BOYCE. 1964. A biochemical pathway in the repair of DNA after UV irradiation that may have steps in common with those of genetic recombination by breakage-reunion. Genetics **50**:256–257.
6. HOWARD-FLANDERS, P., AND R. P. BOYCE. 1966. DNA repair and genetic recombination: Studies on mutants of *Escherichia coli* defective in these processes. Radiation Res. Suppl. **6**:156–184.
7. HOWARD-FLANDERS, P., AND L. THERIOT, 1966. Mutants of *E. coli* K-12 defective in DNA repair and genetic recombination. Genetics **53**:1137–1150.
8. JACOB, F., AND E. L. WOLLMAN. 1953. Spontaneous induction of the development of bacteriophage lambda in genetic recombination of *Escherichia coli* K-12. Compt. Rend. **239**:317–319.
9. LEVINE, M., 1957. Mutations in the temperate phage P22 and lysogeny in *Salmonella*. Virology **3**:22–41.
10. LEVINE, M., AND M. BORTHWICK. 1963. The action of streptonigrin on bacterial DNA metabolism and on induction of phage production in lysogenic bacteria. Virology **21**:568–574.
11. SANDERSON, K. E., AND M. DEMEREC. 1965. The linkage map of *Salmonella typhimurium*. Genetics **51**:897–913.
12. SMITH, H. O., AND M. LEVINE. 1964. Two sequential repressions of DNA synthesis in the establishment of lysogeny by phage P22 and its mutants. Proc. Natl. Acad. Sci. **52**:356–363.