

Characterization and Quantitation of DNA Strand Breaks Requiring *recA*-Dependent Repair in X-Irradiated *Escherichia coli*¹

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The repair of X-ray-induced DNA single-strand breaks was studied after the completion of growth-medium-independent repair in *Escherichia coli* K-12. A comparison of the sedimentation of DNA from bacteriophages T2 and T7 was used to test the accuracy of our alkaline and neutral sucrose gradient procedures for determining the molecular weight of bacterial DNA. The repair of DNA single-strand breaks by cells incubated in buffer occurred by two processes. About 85% of the repairable breaks were resealed rapidly ($t_{1/2} = <6$ min), while the remainder were resealed slowly ($t_{1/2} = \sim 20$ min). After the completion of the repair of DNA single-strand breaks in buffer, about 80% of the single-strand breaks that remained were found to be associated with DNA double-strand breaks. The subsequent resuspension of cells in growth medium allowed the repair of both DNA single- and double-strand breaks in wild-type but not in *recA* cells. Thus the *recA*-dependent, growth-medium-dependent repair of DNA single-strand breaks is essentially the repair of DNA double-strand breaks. © 1986 Academic Press, Inc.

INTRODUCTION

Using alkaline sucrose gradients, one can identify two processes (or groups of processes) in *Escherichia coli* that are involved in the resealing of X-ray-induced DNA single-strand breaks (SSB) (1, 2). One process, called Type II or growth-medium-independent repair, can function in cells held in buffer; it is largely *polA* dependent and is fast ($t_{1/2} = 1-2$ min at room temperature) (1). The other process, called Type III or growth-medium-dependent repair, occurs only when the X-irradiated cells are incubated in growth medium; it is *recA* dependent and is slow (requiring 40-60 min at 37°C for completion) (1).

The processes for the repair of X-ray-induced DNA double-strand breaks (DSB) and for the growth-medium-dependent repair of X-ray-induced SSB share many common characteristics: (i) both are dependent on the *recA* gene (1, 3); (ii) both appear to require postirradiation *de novo* RNA and/or protein synthesis to function (4-6); (iii) both require 40-60 min at 37°C for maximal repair (5); and (iv) the capacity for

¹ This paper is dedicated to the memory of Dr. Henry S. Kaplan (deceased: February 4, 1984), who pioneered the study of the repair of DNA double-strand breaks, and who made major contributions to the study of the repair of DNA single-strand breaks.

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both repair processes is greatly enhanced by growing cells to logarithmic phase in rich medium versus minimal medium (5).

Because of the many common features, we have proposed that for X-irradiated cells, the repair of DSB and the growth-medium-dependent repair of SSB may be the same process (5). That is, all of the SSB that require growth medium for their repair may be involved in DSB, and the repair that occurs in buffer may be completely efficient in the elimination of SSB (and alkali-labile lesions) that are not involved in DSB. If this model is true, then after the completion of the repair of SSB in buffer, the number of SSB per single-strand genome (SSG) ought to equal the number of DSB per double-strand genome (DSG).

In this work we have used alkaline and neutral sucrose gradients to analyze X-irradiated DNA after the completion of growth-medium-independent repair to quantitate the relationship between the growth-medium-dependent repair of SSB and the repair of DSB.

MATERIALS AND METHODS

Bacteria, bacteriophage, and media. *E. coli* K-12 strains used were SR749 (also known as AB1157) and SR248, which have been described (7), and SR894 (*srlA300::Tn10*) and SR895 (*srlA300::Tn10 recA56*), which were derived from SR749 by bacteriophage P1 transduction from a donor strain, SR669, carrying the adjacent *srlA300::Tn10* and *recA56* markers. Bacteriophage T2 and T7 were originally obtained from Miles Laboratories and Biopolymer Inc., respectively. Yeast extract nutrient broth (YENB), minimal medium (SMM), and phosphate buffer have been described (7).

Preparation of ¹⁴C-bacteriophage. Bacteriophage were propagated on strain SR248 grown in SMM containing casamino acids at 2 mg/ml, thymine at 2 μg/ml, and [¹⁴C]thymidine (51 mCi/mmol, ICN) at 1 μCi/ml. After low and high-speed centrifugations, the bacteriophage were resuspended in 1 ml of phage buffer [Tris(50 mM)-EDTA (1 mM), pH 7.6], layered onto a 3.9-ml CsCl step-gradient (1.3 ml each of 1.3, 1.5, and 1.7 g/cc H₂O), and centrifuged in an SW50.1 Beckman rotor (40,000 rpm, 60 min, 20°C). The phage band was collected and [¹⁴C]DNA was prepared by a modification of the method of Freifelder (8). A 4.5-ml vol of ¹⁴C-bacteriophage was layered onto 0.5 ml of 7.5 M NaClO₄ (pH 7.0-7.5) in a tube and centrifuged in a SW50.1 rotor (35,000 rpm, 30 min, 20°C). After removing the upper layer, the perchlorate layer was carefully removed with a wide-bore (2 mm) pipet and placed in a dialysis bag that had been boiled (15 min in 5% NaHCO₃) and then rinsed thoroughly with water. After dialyzing three times against phage buffer, the ¹⁴C-bacteriophage DNA was stored at 4°C for use.

Preparation and irradiation of cells. Procedures were as before (9), with exceptions included below. Logarithmic-phase SR749 cells were grown to an OD₆₅₀ of 0.4 (8×10^7 CFU/ml) in YENB containing [*methyl*-³H]thymidine (New England Nuclear, 80.2 Ci/mmol) at 20 μCi/ml, before being resuspended in phosphate buffer at an OD₆₅₀ of 1.0 and X-irradiated (50 kVp, single X-ray tube operated at 50 mA) with aeration. [For comparison, note that 50 kVp X rays produce 1.93 more DSB/krad/genome and 1.57 more SSB/krad/genome than ¹³⁷Cs γ rays (10).] Cells were then diluted 20-fold in phosphate buffer and incubated at 24°C in the dark.

Assays for DNA strand breaks. After repair incubation in phosphate buffer, 0.2-ml cell samples were each added to 0.2 ml of ice-cold Tris (0.07 M)-EDTA (0.017 M), pH 7.6, containing lysozyme (Worthington Biochemical Corp.) at 400 μg/ml. After 10 min on ice, 0.1-ml samples of spheroplasted cells were layered onto linear, 4.8-ml alkaline sucrose gradients (sucrose at 5-20%, NaOH at 0.1 N, Triton X-100 at 0.1%) capped with 0.1 ml of 0.5 N NaOH, or they were layered onto linear, 4.8-ml neutral 5-20% sucrose gradients (11), but containing Triton X-100 at 0.1% and NaCl at 72 mM. The NaCl concentration was adjusted so that the ionic strength would be 0.1 M for both the alkaline and neutral gradients. After holding at room temperature for at least 60 (alkaline) or 90 (neutral) min, the gradients were centrifuged in Beckman SW50.1 rotors for 16 h at 10,000-13,100 rpm (alkaline) or for 40 h at 3250-6500 rpm (neutral). The centrifugation conditions were selected to position the center of the DNA profile at a relative distance sedimented of 0.6, so that number-average molecular weight calculations could be confidently used for every profile. The first

moment value [i.e., the distance sedimented by the center of mass of the DNA (12)] for [2-¹⁴C]thymidine-labeled bacteriophage T2 was used as a molecular-weight marker. Methods for gradient fractionation and the determination of trichloroacetic acid-insoluble radioactivity have been described (9). The calculations for the number-average molecular weight and the number of strand breaks per genome have been described (12, 13), except that 2.8×10^9 Da was used here as the molecular weight for a single *E. coli* chromosome (14).

RESULTS AND DISCUSSION

To test the notion that the growth-medium-dependent repair of SSB is the repair of DSB, conditions were selected to insure that the repair of SSB in buffer had gone to completion. Cells were X-irradiated and held in buffer at 24°C for graded times of repair incubation before being assayed for SSB/SSG (Fig. 1). Previous data, obtained in the presence of inhibitors of growth-medium-independent repair, indicate that one should expect an initial yield of 150 SSB/SSG after 25 krad (5). In the data shown in Fig. 1, where inhibitors of this repair were not present, only 68 SSB/SSG were detected. We presume that this difference in initial yield of SSB/SSG can be accounted for by repair occurring in buffer during the 10-min irradiation step. Also, the repair of SSB in buffer appears to be biphasic. Repair during the first 20 min is dominated by a fast process ($t_{1/2} = <6$ min), which is consistent with the original description of the growth-medium-independent process (1). After the first 20 min, however, repair shows a $t_{1/2}$ of about 20 min. We calculate that these lesions that are repaired slowly in buffer should constitute about 15% of the initial yield of SSB that can be repaired in buffer, a fraction that is similar to that reported for alkali-labile lesions [reviewed in (15)]. In similar experiments with neutral gradients, we were not able to detect the repair of DSB in buffer (data not shown).

In the second series of experiments, cells were X-irradiated with 0, 10, 20, and 30 krad and incubated in buffer for 180 min before assaying for the numbers of SSB/SSG and DSB/DSG (Fig. 2a). From these data we calculate that the rates of accumulation of nonrepaired strand breaks are 1.0 SSB/SSG/krad and 0.77 DSB/DSG/krad, i.e., 77% of the SSB that were not repaired in buffer were involved in DSB.

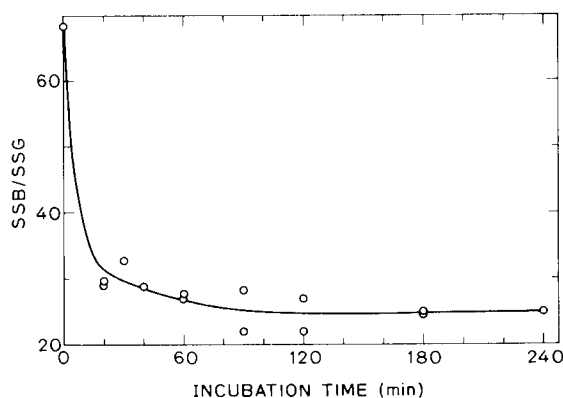


FIG. 1. Repair of X-ray-induced DNA single-strand breaks (SSB) in *E. coli* held in buffer. Cells were grown in yeast extract nutrient broth, resuspended in buffer, and X-irradiated (25 krad) before incubation in buffer at 24°C. Data for SSB per single-strand genome (SSG) were pooled from three experiments.

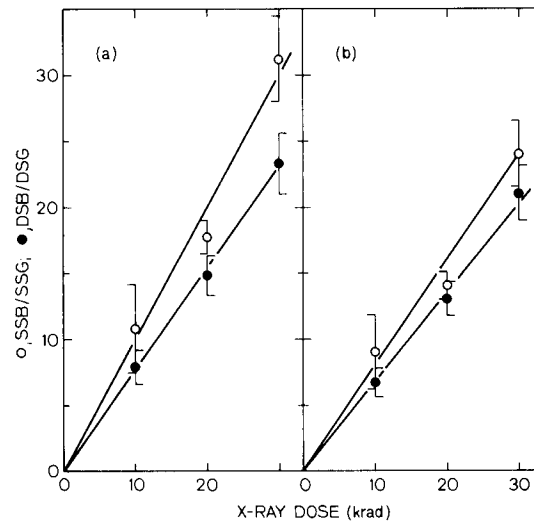


FIG. 2. Yield in *E. coli* of X-ray-induced DNA single-strand breaks (SSB) per single-strand genome (SSG) and double-strand breaks (DSB) per double-strand genome (DSG) after the completion of strand-break repair in buffer. Cells grown in yeast extract nutrient broth were X-irradiated and incubated in buffer for 180 min at 24°C in the dark before conversion to spheroplasts and layering onto alkaline (○) or neutral (●) sucrose gradients. Points are the means of data from triplicate or more experiments. Error bars indicate ± 1 SD. (a) Uncorrected data; (b) data were corrected based upon the relative sedimentation of bacteriophage T2 and T7 DNA (see text). Calculated molecular weights of DNA from nonirradiated cells were 1.0×10^8 (○) and 2.1×10^9 (●) for (a), and 1.3×10^8 (○) and 2.4×10^9 (●) for (b).

To test the validity of measuring SSB with alkaline gradients and DSB with neutral gradients, and quantitatively comparing the strand-break yields, we compared the sedimentation of DNA from bacteriophages T2 and T7 on the two kinds of gradients. The alkaline gradients were centrifuged at 13,100 rpm for 16 h at 20°C, and the neutral gradients were centrifuged at 7100 rpm for 40 h at 20°C. The centrifugation conditions were selected to sediment the T2 DNA through a relative distance of 0.6. The first moment (see Materials and Methods) was calculated for each bacteriophage DNA on each kind of gradient, and their ratio was determined (four experiments, each with duplicate gradients for T2 and for T7). The first moment ratio (T2/T7) was 4.05 ± 0.31 using neutral gradients and 4.13 ± 0.36 using alkaline gradients, which indicates that this sedimentation relationship between the two species of DNA was not significantly different on the two kinds of gradients.

We also determined the number-average molecular weight for T2 DNA on the two kinds of gradients in the fashion that we normally employ for *E. coli* (see Materials and Methods), but in this case T7 DNA was the molecular-weight standard [$2.5 \pm 0.1 \times 10^7$, Ref. 16]. The molecular-weight values calculated for the T2 chromosome were 9.97×10^7 using neutral-gradient data and 8.68×10^7 using alkaline-gradient data. The average reported molecular weight for T2 DNA is $1.12 \times 10^8 \pm 0.16$ (16). Thus the molecular-weight values for T2 DNA determined in this work appear to be slightly low, more so when determined with alkaline gradients than with neutral gradients. Assuming that the average of the reported molecular-weight values for T2 (16) is more

TABLE I
Rates of Induction of X-Ray-Induced Strand Breaks Determined with Different
Sucrose Gradient Sedimentation Techniques^a

Protocol	DSB/DSG per krad (A)	SSB/SSG per krad (B)	$\frac{(A)}{(B)} \times 100$
DNA sedimented at various speeds to a constant point in the gradients (data from Fig. 2a)	0.77	1.00	77%
DNA sedimented at various speeds to a constant point in the gradients and corrected by a T2/T7 factor (data from Fig. 2b)	0.67	0.80	84%
DNA sedimented to various points in the gradients, but at the same speed (data not shown) ^b	0.93	1.04	89%

^a DSB data were derived from DNA sedimentation on neutral sucrose gradients; SSB data were derived using alkaline sucrose gradients.

^b Quadruplicate experiments performed essentially as under Materials and Methods except that the repair incubation was for 20 min at 37°C in phosphate buffer, the gradients for all doses (0, 10, 20, 30 krad) were centrifuged either at 10,000 rpm for 16 h (alkaline) or at 3,700 rpm for 40 h (neutral), and the neutral sucrose gradients were prepared as in (11), but containing Triton X-100 at 0.1%.

correct than our values, we have corrected the data in Fig. 2a by multiplying all of the molecular-weight values that we used in the calculation of strand breaks by 1.12 (neutral gradients) or by 1.29 (alkaline gradients) to obtain the data shown in Fig. 2b. These data suggest that 84% of the SSB are associated with DSB.

The rates of DNA strand-break production derived from Figs. 2a and b, as well as from another set of data in which all of the DNA samples were centrifuged at the same speed rather than to the same point in the gradient, are listed in Table I. From

TABLE II
Effect of Growth Medium and the *recA* Gene on the Repair of X-Ray-Induced DNA Strand Breaks Remaining after Repair Incubation in Buffer^a

Conditions preceding assay of nonrepaired strand breaks	SSB/SSG (20 krad)		DSB/DSG (3 krad)	
	Wild type	<i>recA</i>	Wild type	<i>recA</i>
Buffer incubation for 180 min at 24°C (or for 20 min at 37°C) ^b	17 (17)	22 (18)	1.7 (1.9)	2.7 (2.4)
After buffer incubation (above), cells were resuspended in YENB and further incubated for 120 min at 37°C	8.0 (4.0)	48 (36)	-0.2 (-0.3)	10 (10)

^a X-Ray doses were selected on the basis that SSB and DSB in YENB-incubated cells can only be accurately quantitated in the dose ranges of 10–60 krad and 1–4 krad, respectively (Sargentini and Smith, unpublished results). Data are the means from duplicate experiments.

^b Similar data were obtained for cells incubated for an additional 120 min at 37°C in buffer, and for buffer-incubated cells that were resuspended in YENB and sampled immediately.

this comparison we conclude that about 80% of the X-ray-induced SSB that are left after the completion of repair in buffer (i.e., after 180 min at 24°C or after 20 min at 37°C) are associated with DSB.

To confirm for our assay conditions that the X-ray-induced SSB and DSB that were not repaired in buffer after 180 min at 24°C (or after 20 min at 37°C) were those breaks that require both growth medium and a functional *recA* gene for their repair, we measured the repair of both SSB and DSB after the addition of YENB to buffer-incubated X-irradiated wild-type and *recA* cells. The results indicate that, by returning the buffer-incubated cells to growth medium, the repair of DSB and SSB was facilitated in the wild-type cells, but not in the *recA* cells (Table II). In fact, the *recA* cells showed an increase in strand breaks with incubation in growth medium.

This *recA* and growth-medium dependence for the repair of the SSB and DSB that remain after repair has been completed in buffer, is consistent with earlier work (1, 3, 4-6). However, what is evident from the present work is that the *recA*-dependent, growth-medium-dependent repair (Type III repair) of DNA single-strand breaks detected in rich medium-grown cells (5) is essentially the repair of DNA double-strand breaks.

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