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# Analysis of Resynthesis Tracts in Repaired *Escherichia coli* Deoxyribonucleic Acid

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Excision repair of ultraviolet radiation-induced damage in a wild-type strain of *Escherichia coli* has been examined, using two methods for characterizing the resynthesis step of the repair process. Comparison of data obtained after both isopycnic analysis of repaired deoxyribonucleic acid and sedimentation velocity analysis of deoxyribonucleic acid after selective photolysis of bromouracil-containing repaired regions has shown that the repaired deoxyribonucleic acid molecules contain a semicontinuous distribution of sizes of repair tracts. Further analysis of our data suggests two major classes of repair patches, one about 20 to 40 nucleotides in length, and the other containing 1,600 to 2,000 nucleotides. Under the conditions employed, approximately 2 to 10% of the fully repaired regions are long repair patches.

Excision repair of DNA damage, one of the primary means by which the cell preserves the integrity of its genetic material (15, 16), is thought to proceed by a coordinated series of steps, including incision at or near the site of the lesion, excision of the damage and adjacent nucleotides, resynthesis of the DNA, and ligation of the repaired region to the parental strand (5, 6). In wild-type *Escherichia coli*, there is evidence for heterogeneity of repair patch size; studies with isopycnic analysis have indicated the presence of extensive regions of new DNA synthesized in response to UV-induced damage (3). However, it could not be determined from those experiments whether these long stretches of DNA synthesis were indeed patches in the sense that they constituted fully ligated regions contiguous to the parental DNA molecule. We report here in vivo measurements of repair resynthesis in wild-type *E. coli*, using two separate techniques. One of these techniques, which relies on selective photolysis of bromouracil-containing DNA by 313-nm radiation, is designed to emphasize repair resynthesis tracts from regions in which rejoining to the preexisting DNA strand has been completed. Both types of measurements show repair patches in excess of 1,000 nucleotides in length which, under the experimental conditions employed, account for repair at about 2 to 10% of the damage sites.

## MATERIALS AND METHODS

**Bacterial strains.** The strains of *E. coli* K-12 used in this study included W3110 (*thy*), KLC124 (*trpA33 rha thy*) (2), and TN207 (*uvrA thy*) (7).

**Media and growth conditions.** Bacteria were grown at 37°C with aeration in minimal (M9) medium (11) supplemented with 2 μg of thymine per ml, 0.2% (wt/vol) vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.), and 40 μg of L-tryptophan per ml; 0.4% (wt/vol) glucose provided the carbon source. M9 salts is unsupplemented M9 medium (without glucose).

**UV irradiation.** A pair of germicidal lamps with an incident dose rate of 1.6 J/m$^2$ per s was the source of 254-nm radiation. Exponentially growing cells, which had been washed and resuspended in ice-cold M9 salts, were irradiated in sterile petri dishes at 0°C on a rotating platform. Subsequent manipulations were performed under dim yellow light.

**313-nm irradiation.** Radiation was provided by illumination of a Hilger quartz prism monochromator (Hilger & Watts, Inc., Morton Grove, Ill.) by a 1,000 W Phillips high-pressure, mercury arc lamp (North American Philips Corp., Hightstown, N.J.). The radiation was passed through a thin Mylar film to eliminate any scattered shorter wavelengths. The average exposure rate at the center of the quartz cuvette containing the sample was 50 J/m$^2$ per s.

**Photolysis by 313-nm radiation.** In a modification of the procedure detailed by Ley and Setlow (10), bacterial cultures (12 ml) were grown in M9 medium containing 8.8 μCi per 2.2 μg of [$^{14}$C]thymine (New England Nuclear Corp., Boston, Mass.) per ml or 16 μCi per 2 μg of [$^3$H]thymine (Schwarz/Mann, Orangeburg, N.Y.) per ml to a density of approximately $10^8$ cells per ml. Label was removed by collecting the cells on membrane filters (Millipore Corp., Bedford, Mass.) and washing with warm M9 salts; cells were suspended in unlabeled M9 medium and incubated for an additional 17 min. The bacteria were then washed with warm M9 salts and resuspended in their original volume of ice-cold M9 salts. After a dose of 15 J of UV radiation per m$^2$, the cells were warmed to 37°C and then incubated for 2 min in medium containing glu-

cose, Casamino Acids, and tryptophan before addition of 20 μg of 5-bromouracil per ml in place of thymine. After 30 min of incubation, reactions were terminated by addition of ice-cold 50 mM Tris-hydrochloric acid (pH 8.0)–5 mM EDTA, and cells were collected by filtration, washed with Tris-EDTA, and resuspended in that buffer. After adjustment of the absorbance at 313 nm to 1.0, a 0.7-ml sample of bacteria was placed in an ice-cooled quartz cuvette of 1-cm path length and exposed to increasing doses of 313-nm radiation as described above. Portions (0.050 ml) were removed and lysed by incubation for 35 min on 5 to 20% alkaline sucrose gradients with a 0.2-ml top layer (0.5 N NaOH, 1% Sarkosyl [Geigy], 10 mM EDTA). Centrifugation in the SW56 rotor was at 20°C and 22,500 rpm for 3, 4, or 5 h in a Beckman L3 centrifuge; $^{14}$C-labeled T4 phage or $^{3}$H-labeled T7 DNA sedimented under identical conditions served as a marker. Sedimentation conditions for each experiment were chosen to minimize the uncertainty in the molecular weight determinations for the sizes of DNA expected. Gradients were collected and radioactivity was determined as previously described (8).

To measure nonspecific DNA breakage caused by exposure to 313-nm radiation, cells from strain KLC124 were grown under identical conditions, except that 20 μg of thymine per ml was substituted for 5-bromouracil in the repair incubation. Breakage efficiency of fully substituted DNA was measured by growing strain KLC124 for several generations in M9 medium in which thymine was replaced by 20 μCi per 10 μg of 5-[6-$^{3}$H]bromouracil per ml; growth was continued for 90 min in the absence of label, and cells were rinsed and resuspended in Tris-EDTA. Without prior UV irradiation, the cells were exposed to 313-nm radiation at an average exposure rate of 20 J/m$^2$ per s; centrifugation was at 30,000 rpm for 3 h.

**Repair resynthesis.** Determinations of repair replication after 15 J of UV irradiation per m$^2$ and 30 min of post-irradiation incubation were made as previously described (8), except that 5-[6-$^{3}$H]bromouracil (Schwarz/Mann) was included at a final concentration of 16 μCi per 14 μg/ml. Samples were harvested by collection and washing on a 0.45-μm Millipore filter rather than by centrifugation.

**Molecular weight determinations.** Number-average molecular weights ($M_n$) were calculated by the equation of Charlesby (1). $^{3}$H-labeled phage T7 DNA, provided by L. A. Dodson, and $^{14}$C-labeled bacteriophage T4 were used as markers in the photolysis experiments; a $^{32}$P-end-labeled, 2,070-base pair restriction fragment from *Gecarcinus lateralis* satellite DNA, the gift of D. M. Skinner, was employed as a marker in the determination of the size of sonic DNA fragments analyzed in the CsCl density gradients.

## RESULTS

**Photolysis of bromouracil-substituted DNA.** The sensitivity of DNA containing 5-bromouracil to breakage by 313-nm radiation provides a valuable and convenient probe for determining the nature and extent of repair replication subsequent to UV-induced damage (10, 12). To facilitate interpretation of this type of experiment, conditions should be chosen such that repair of pyrimidine dimers is nearly complete but semiconservative DNA replication is minimal; thus, the number of repair patches may be safely assumed to equal the number of pyrimidine dimers introduced, and the molecular weight of the repaired DNA molecules is high enough to permit accurate determination of breaks introduced by photolysis. Furthermore, conditions that minimize the resumption of semiconservative replication avoid sensitizing the parental strand complementary to the newly synthesized bromouracil-substituted DNA to 313-nm radiation (9) and assure that photolysis-induced breakage is that of bromouracil-containing repair tracts rather than that of semiconservatively replicating DNA which has incorporated bromouracil. Our previous work (8) suggested that a dose of 15 J of UV irradiation per m$^2$ followed by 30 min of post-irradiation incubation would be suitable for these experiments. These conditions yield 90% survival, cause very little UV-stimulated DNA degradation, and allow essentially complete DNA repair. In an additional control experiment, cesium chloride density gradient analysis showed no signficant amount of semiconservative DNA replication under these conditions (data not shown). Thus, in these experiments, the induction of single-strand breaks in 5-bromouracil-substituted DNA by 313-nm radiation is a function of the number and length of repair tracts and is characterized by a decrease in number-average molecular weight as determined from alkaline sucrose gradient analysis.

To measure the amount of 313-nm-induced photolysis, radionuclide-labeled cultures of strain KLC124 (wild type) were exposed to UV radiation and then incubated in medium in which thymine was replaced by 5-bromouracil. In a parallel experiment, the UV-irradiated cells were incubated in thymine-containing medium. Cells were exposed to 313-nm radiation (or retained as controls) and lysed, and their DNA was sedimented through alkaline sucrose. Photolysis of 5-bromouracil-substituted DNA by 313-nm radiation was manifested by a decrease in single-strand molecular weight (Fig. 1). These data also indicate that breakage occurs to a much lesser extent in the thymine-containing repaired DNA and, additionally, that the presence of 5-bromouracil does not by itself lead to significant molecular weight reduction without exposure to 313-nm radiation. Typical values for $M_{n_0}$ were about $1.2 \times 10^8$. (The $M_{n_0}$ for bromouracil-substituted DNA in Fig. 1 is $1.4 \times 10^8$, slightly larger than what was obtained in most experiments.)

Data such as those shown in Fig. 1 were used

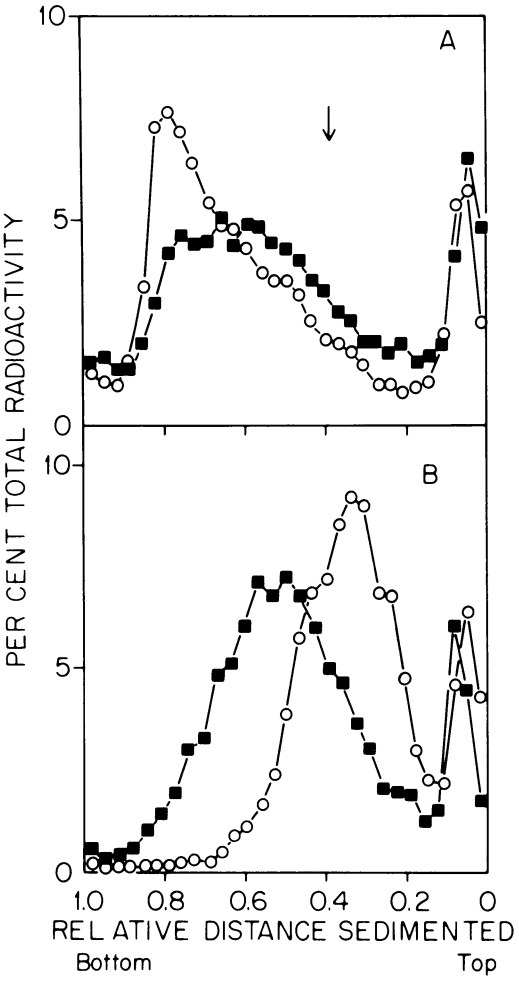


FIG. 1. *Size distribution of DNA after 313-nm photolysis. Isotopically labeled cells from strain KLC124 (wild type) were irradiated with 15 J/m² at 254 nm and incubated for 30 min in the presence of 5-bromouracil or thymine. After suspension in 50 mM Tris (pH 8.0)–0.5 mM EDTA, cells were subjected to photolysis by 313-nm light, and samples were lysed on alkaline sucrose gradients and centrifuged for 3 h as described in the text. Fractions were collected on paper strips and acid precipitated, and their radioactivity was determined. Profiles are shown; arrow denotes the position of T4 DNA sedimented under identical conditions. Direction of sedimentation is from right to left. (A) No 313-nm radiation; (B) 2 × 10⁴ J of 313-nm radiation per m². Symbols: ○, incubated with 5-bromouracil; ■, incubated with thymine.*

to calculate the $M_n$ of DNA from cells exposed to various doses of 313-nm radiation. The difference between the reciprocals ($1/M_n$) of $M_n$ of DNA exposed to 313-nm radiation and that of DNA not subjected to photolysis ($1/M_{n_0}$) yields a value for the number of single-strand breaks introduced by exposure to any given dose of 313-nm radiation. This value is plotted as a function of radiation dose in Fig. 2. These data show that contributions from nonspecific thymine breakage are small in comparison to those resulting from UV-induced repair processes. An important control with an incision-deficient strain (*uvrA*) established that processes independent of the excision-repair pathway, such as postreplicational repair, do not result in significant levels of photolysis under these experimental conditions. In contrast, data obtained from photolysis experiments in which the wild-type strain was repaired with 5-bromouracil show an initial rapid, nonlinear increase in the number of breaks introduced by increasing exposures to 313-nm radiation. Nearly identical results were obtained with wild-type strain W3110 (data not shown).

**Isopycnic analysis of repaired regions.** Although we have implied a correlation between the long-patch repair detected in isopycnic analysis (3, 4) and the data shown in Fig. 2, it was of value to make a direct comparison by examining

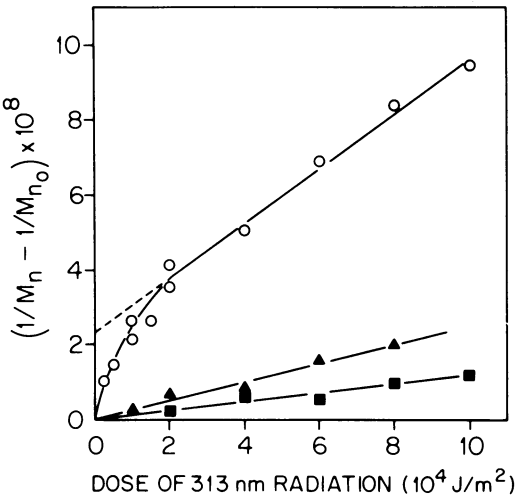


FIG. 2. *Induction of breaks by photolysis with 313-nm radiation. Isotopically labeled cultures were irradiated with 15 J/m² at 254 nm and then incubated with either thymine or 5-bromouracil and irradiated with the indicated doses of 313-nm radiation. Sedimentation profiles recovered from alkaline sucrose gradients as described in the legend to Fig. 1 were used to calculate the number-average molecular weights ($M_n$) of the DNA. The difference between the reciprocal of $M_n$ for DNA subjected to photolysis and the reciprocal of $M_n$ for the unphotolysed control ($M_{n_0}$) is taken to equal the number of breaks introduced by 313-nm radiation and is plotted as function of 313-nm radiation dose. Symbols: ○, strain KLC124 (wild type) incubated with 5-bromouracil; ■, strain KLC124 incubated with thymine; ▲, strain TN207 (uvrA) incubated with 5-bromouracil.*

the buoyant density of DNA repaired in strain KLC124 under conditions identical to those used in the experiments of Fig. 1 and 2. Cells from strain KLC124 labeled with [$^{14}$C]thymine were irradiated with 15 J of UV per m$^2$ or retained as an unirradiated control and then incubated with 5-[6-$^3$H]bromouracil as the repair label, as previously described (8). After lysis of the bacteria, the DNA was sedimented in a CsCl gradient. Fractions containing the peaks of $^{14}$C-labeled parental (unreplicated) DNA (Fig. 3A and B) were isolated and subjected to a second isopycnic centrifugation to yield the profiles of radioactivity shown in Fig. 3. Figure 3C shows little incorporation of [$^3$H]bromouracil by the unirradiated sample; peak fractions of the irradiated sample (Fig. 3D) show a 2.5-fold stimulation of incorporation of the repair label. After subtraction of the background incorporation observed in the unirradiated control, calculation from the specific activity of the [$^3$H]bromouracil yielded an estimate of an average value of 60 nucleotides inserted per pyrimidine dimer.

A portion of those fractions of the gradient shown in Fig. 3D containing the peak values of $^{14}$C-labeled DNA was subjected to further analysis in experiments similar to those reported by Cooper and Hanawalt (3). Rebanding in alkaline CsCl revealed a slight skewing of the $^3$H-labeled DNA toward greater density (Fig. 3E), suggesting that this label had been incorporated in tracts long enough to shift the density of the parental DNA into which it had been integrated. This interpretation was confirmed by the experiment of Fig. 3F, in which a portion of the same DNA was subjected to sonication before sedimentation in alkaline CsCl. The DNA fragments analyzed in this experiment were approximately 1,000 nucleotides long, as determined by sedimentation analysis in alkaline sucrose with a $^{32}$P-end-labeled restriction fragment of defined length as a marker. As seen in Fig. 3F, the long repair tracts, which would comprise a larger portion of the smaller DNA pieces, appreciably altered the density of those fragments which contained the long patches, whereas short patches did not affect the density of repaired DNA molecules. Repair tracts having the same density as the fully substituted marker must then be at least 1,000 nucleotides in length.

## DISCUSSION

Using data such as those presented in Fig. 2, the average length of the repair patches, $n$, can be calculated by assuming a Poisson distribution:

$$P = 1 - B/B_{max} \simeq e^{-knD} \quad (1)$$

where $P$ is the probability of no breakage event, $B$ equals the number of breaks per $10^8$ daltons caused by photolysis of repair patches, $B_{max}$ represents the maximum number of breaks possible, $D$ is the dose of 313-nm radiation in J/m$^2$, and $k$ is the sensitivity of 5-bromouracil-substituted DNA to 313-nm radiation. In a separate experiment with fully bromouracil-substituted DNA (data not shown), $k$ was measured to be $2 \times 10^{-10}$ breaks per dalton per J/m$^2$, a value in good agreement with earlier determinations (10, 13). Under conditions in which $n$ is small compared to the interdimer distance, $B_{max}$ will be nearly equal to the number of lesions, about 32.4 pyrimidine dimers per $10^8$ daltons in these studies (14). Values for $B$ as a function of $D$ can be obtained from Fig. 2 by subtracting the curve defined by the closed squares, thereby eliminating the contribution due to nonspecific breakage of thymine-containing DNA. To estimate the value of the patch size $n$, it is useful to rewrite equation (1) as:

$$\ln (1 - B/B_{max}) = -knD \quad (2)$$

and to plot $-\ln (1 - B/B_{max})$ as a function of $D$, using corrected values of $B$ obtained from the data in Fig. 2.

The departure from linearity seen in Fig. 4A indicates that the observed functional dependence of breakage ($B$) on dose of photolytic radiation ($D$) cannot be adequately described by using a Poisson distribution with a single average value of $n$. For low values of $D$, the slope of the solid line in Fig. 4A is better described with a much larger value of $n$, suggesting a significant heterogeneity in patch size, with some repair sites having very long regions of repair resynthesis. It is instructive to dissect the function describing the variation of $B$ with $D$ into two components, one representing short patches of about 30 nucleotides (the linear portion of Fig. 4A) and the other representing longer, more heterogeneous tracts of repair resynthesis. For small values of $n$, equation (1) can be approximated as:

$$B \simeq B_{max} nkD. \quad (3)$$

This expression, with $n = 30$ nucleotides, describes the linear region of Fig. 2 and, indirectly, Fig. 4A. By subtracting equation (3) (shown as a dotted line in Fig. 4B) from the curve generated by the circles in Fig. 2, we arrive at a function (shown as a solid line in Fig. 4B) which we interpret as describing the long-patch component of excision repair first described by Cooper and Hanawalt (4). The curve so generated indicates saturation at approximately 2.4 breaks per $10^8$ daltons. A dose of $7.2 \times 10^3$ J of

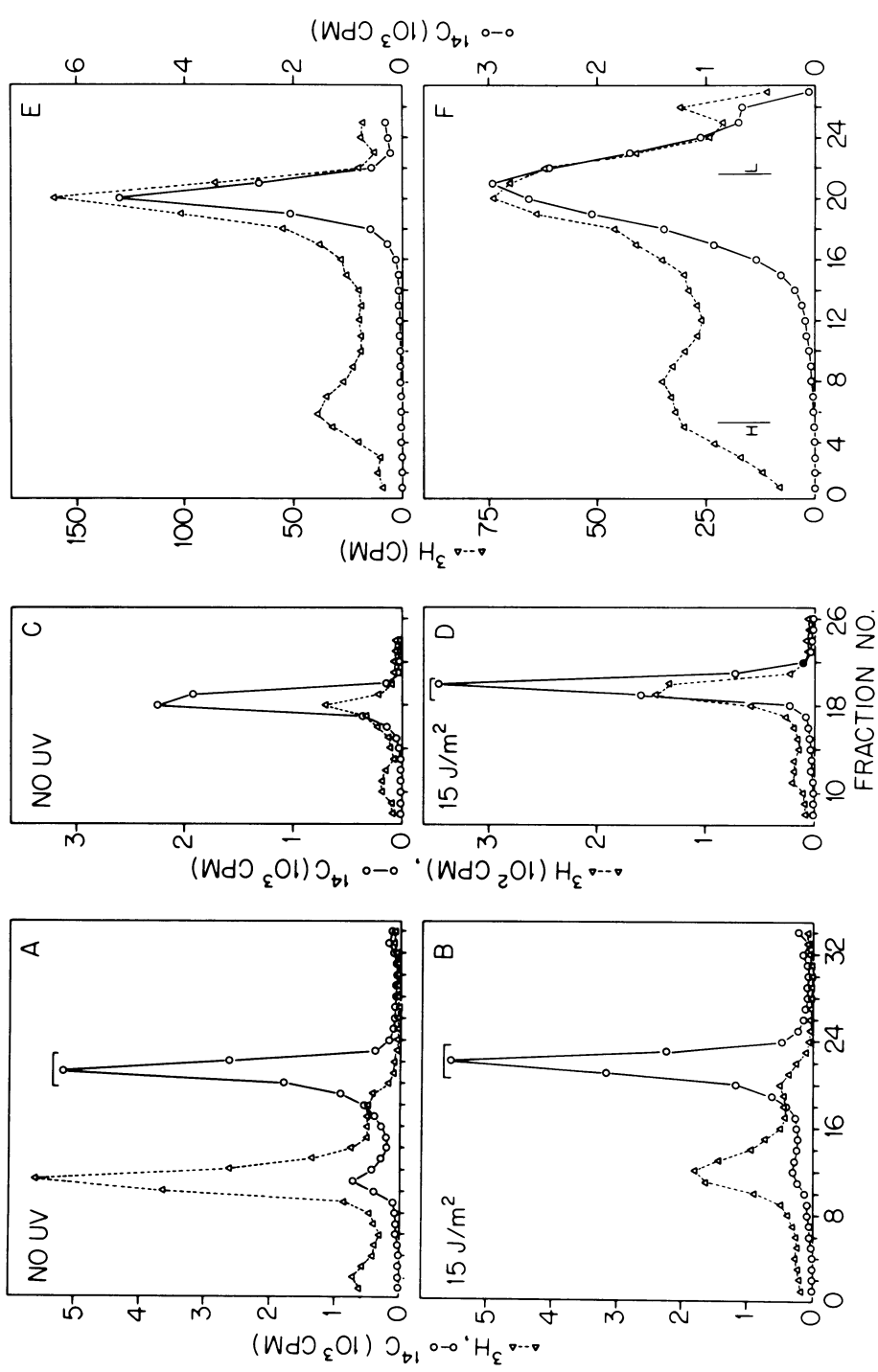


FIG. 3. *Repair resynthesis in unirradiated and irradiated KLC124. Strain KLC124 was grown in [$^{14}$C]thymine-containing M9 medium, resuspended in M9 salts, and irradiated with 0 or 15 J of UV per m$^2$. After 30 min of incubation in the presence of nutrients and [$^3$H]bromouracil, cells were lysed and subjected to isopycnic analysis as described previously (8). Profiles of radioactivity recovered from these gradients are shown. The fractions indicated by brackets in frames A and B were pooled, and after adjustment of CsCl concentration they were rebanded in neutral CsCl and centrifuged to equilibrium. Profiles of radioactivity recovered from these gradients are shown. The fractions indicated by brackets in frame D were pooled and diluted in NET buffer. One portion was denatured and centrifuged in alkaline CsCl for 60 h at 20°C. The other portion was fragmented by sonication and denatured; one part of this sonicated sample was retained for alkaline sucrose gradient analysis, and the remainder was centrifuged in alkaline CsCl for 80 h at 37,000 rpm. Profiles of radioactivity recovered from these gradients are shown. (E) Unsonicated; (F) sonicated. Symbols: ○, [$^{14}$C]- thymine prelabel; △, $^3$H repair label. Direction of sedimentation is from right to left; bars indicate the positions of fully bromouracil-substituted and unsubstituted markers.*

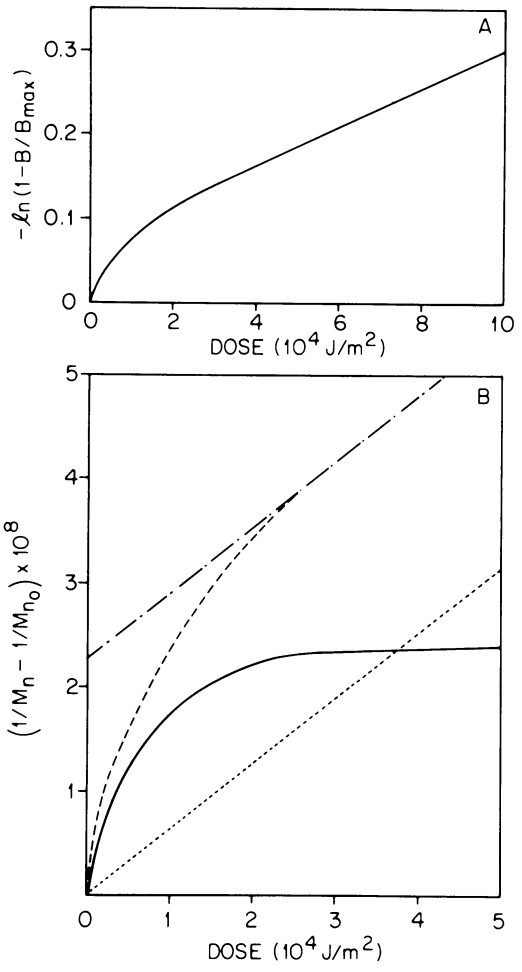


FIG. 4. *Analysis of photolysis data. (A) Total number of breaks ($B_t$) in photolysed DNA was taken to equal ($1/M_n - 1/M_{n_0}$) and was determined as a function of dose (D) of 313-nm radiation from the solid curve defined by the open circles in Fig. 2. This value was then adjusted for nonspecific breakage by subtracting the number of breaks found when 5-bromouracil was replaced by thymine (the closed squares in Fig. 2) to give a corrected value (B) for single-strand breaks caused by bromouracil photolysis. Using $B_{max}$ = 32.4 breaks per $10^8$ daltons as discussed in the text, the function $-ln(1 - B/B_{max})$ was calculated and plotted as a function of D. (B) Value B (corrected for nonspecific breakage) was calculated as described above, plotted as a dashed line, and dissected into two functions by subtracting the equation $B' = B_{max}\, nkD$ from each value of B so as to generate two curves, $B' = B_{max}\, nkD$, shown by the dotted line, and B" (D) (defined as B" = B − B'), shown by the solid line.*

313-nm radiation per m$^2$ produces breakage equivalent to 63% of the saturation value. Using equation (1) and our measured value for $k$, this gives a value of 1,900 nucleotides (nearly 200 helix turns) for the long repair tracts. Since, on the average, 2.4 of the 32.4 available lesions yield long patches, we estimate that about 7% of the pyrimidine dimers are eliminated via this repair mode under our experimental conditions.

The isopycnic gradient analysis in Fig. 3 also allows an estimation of patch size distribution (3). These data show that about 50% of the repair label was incorporated into "short" repair patches that did not significantly alter the buoyant density of the DNA into which they were incorporated. Another 20% of the $^3$H label was found near the position of fully bromouracil-substituted DNA, and the remainder was found at intermediate positions. The degree of bromouracil substitution in each DNA fragment (about 1,000 nucleotides long) determines the $^3$H radioactivity as well as the position in the CsCl gradient at which each fragment comes to equilibrium. Thus, the relative number of DNA fragments at each position in the CsCl gradient can be estimated by correcting the $^3$H radioactivity found at that position for the fraction of [$^3$H] bromouracil contained in each fragment. It is clear from Fig. 3F that most of the DNA fragments contain only small amounts of bromouracil. The molar ratio of 5-bromouracil to thymine in fractions containing the peak of $^{14}$C radioactivity, coupled with knowledge of the number of pyrimidine dimers introduced under these experimental conditions, yields an estimate of about 40 nucleotides inserted per short DNA repair patch. Those repair patches of the order of 1,000 nucleotides long account for only 20% of the total $^3$H radioactivity, suggesting that they are initiated at only 2 to 5% of the damage sites. Thus, although the two techniques employed in this study emphasize different populations of DNA molecules, there is reasonably good accord between values obtained with each method. The results obtained by selective photolysis of bromouracil-containing DNA demonstrate that the long repair resynthesis tracts are rejoined to the preexisting DNA strand and therefore carry the potential for contributing to the damaged organism's survival. Further quantitative analysis of the long patches with both techniques may aid in elucidating the factors that promote these extensive resynthesis tracts, the enzymatic basis of their synthesis, and their influence on survival and mutation in the organism in which they occur.

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