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Enzymatic Photoreactivation of *Escherichia coli* after Ionizing Irradiation: Chemical Evidence for the Production of Pyrimidine Dimers

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By comparing the results for a *uvrA recA* and a *uvrA uvrB recA phr* strain, we conclude that the photoreactivation (PR) observed after ionizing irradiation involves the same enzyme that is responsible for the PR of ultraviolet (uv) radiation-induced cyclobutadipyrimidines (Py< >Py). A comparison of the photoreactivable sectors in a *uvrA recA* strain after uv irradiation and ^{137}Cs γ irradiation indicates that 10 krad of γ radiation produces about 0.07 Jm^{-2}-equivalents of 254-nm radiation-induced photoreactivable damage. After 400 krad, an acid hydrolysate of the isolated DNA revealed the presence of thymine-containing Py< >Py, as evidenced by their chromatographic properties and their photochemical reversibility. The yield of thymine dimers after 400 krad of γ irradiation, as detected chromatographically, was in close agreement with the yield that was predicted from our PR studies. Thus, the PR observed after ionizing irradiation in strains that are blocked in excision repair and in postreplication repair is due to the production of trace amounts of Py< >Py.

INTRODUCTION

The phenomenon of photoreactivation (PR)[1] has been considered to take place only in cells irradiated with ultraviolet (uv) radiation, but not with ionizing radiation. Recently, however, Myasnik and Morozov (*1*) demonstrated that PR can be observed after the exposure of certain strains of *Escherichia coli* to ionizing radiation, e.g., K-12 (*uvrA recA*) and B_{s-1} (*uvrB lexA*). These authors reported that PR was not produced by preillumination and was mediated by a temperature-dependent process, suggesting that it was acting through photoenzymatic repair rather than by indirect PR (*2*) or by the direct photochemical reversal of the damage (*3*). The only known photoenzymatic repair involves the photoreactivating enzyme (PRE) that specifically monomerizes cyclobutadipyrimidines

[1] Abbreviations used: T, thymine; U, uracil; T< >T, cyclobutane-type thymine dimer; PR, photoreactivation; uv, ultraviolet; Py< >Py, cyclobutadipyrimidines; PRE, photoreactivating enzyme.

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TABLE I

Escherichia coli K-12 Derivatives Used

Strains[a]	*Relevant genotype*	*Other markers*	*Reference or source*
SR18	*uvrA6 recA13*	*pro tsx str thi lac gal*	R. P. Boyce
SR73	*uvrA6 recA13*	*pro tsx str thi lac gal thyA thyR*	This paper
SR362	*uvrA6 uvrB recA56 phr*	*leuB rha lacZ str metE thyR*	D. A. Youngs

[a] SR18 is strain AB2480. SR73 is a thymine-requiring derivative of AB2480, isolated after treatment with trimethoprim (*24*). Strain SR362 was constructed by transducing *uvrA6* (*25*) into KH21 (F⁻ *leuB bio rha lacZ str thyA thyR metE malB*; from R. B. Helling) to obtain DY292; the *phr* and *uvrB* genes of strain HS30 (*26, 27*) were transduced into DY292 to obtain DY314; the *recA56* gene from strain JC5088 (*28*) was transferred into DY314 to obtain strain SR362.

(Py< >Py) in the presence of light (310–480 nm) (*4, 5*). Therefore, two major questions arise: (1) Does the observed PR after ionizing irradiation involve the same PRE as after uv irradiation, and (2) are Py< >Py formed in the DNA of cells by γ irradiation? The present work presents evidence that Py< >Py are produced by ionizing radiation and that the observed PR after ionizing irradiation involves the same PRE that acts after uv irradiation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used are listed in Table I.

The medium used for the growth of strains SR18 and SR362 was YEP broth (*1*) (10 g each of yeast extract, peptone, and NaCl in 1 liter of water). Stationary phase cells were harvested by centrifugation. DTM buffer (6) was used for washing and resuspending the cells at $\sim 1 \times 10^8$/ml for irradiation. A 0.067 *M* $NaKPO_4$ buffer, pH 7.0, was used for diluting the cells for plating. Colony-forming units were determined on YEP agar, which is YEP solidified with 1.6% Difco Noble agar (*7*). Colonies were counted after 24 hr at 37°C.

Irradiation conditions. γ irradiation was performed using an 8000-Ci ^{137}Cs source (J. L. Shepard and Associates), emitting a γ-photon peak with an energy of 0.66 MeV. X irradiation was performed using the twin-tube 50-kVp X-ray unit described by Loevinger and Huisman (*8*). The X-ray tubes were operated at 48 and 50 mA with 0.25 mm Al added filtration. The dose rates for γ and X irradiation were 4.55 and 6.67 krad/min, respectively, and were determined using ferrous sulfate (*9*). For the analysis of survival, cells ($\sim 1 \times 10^8$/ml in DTM buffer) were irradiated at room temperature without aeration.

Ultraviolet irradiation was performed using an 8-W General Electric germicidal lamp emitting primarily at 254 nm that was situated ~47 cm above a rotating platform. The fluence rate was determined with an International Light germicidal photometer (IL-254) and was corrected for cell-masking effects according to an empirically derived relationship based on the survival of a *uvrB recA56* strain irradiated over a range of cell densities (*10*).

For photoreactivation, cell suspensions ($\sim 1 \times 10^8$/ml) in Pyrex petri dishes were irradiated for 3 min when placed on top of a 0.5-cm glass plate that was 1 mm above three 15-W Daylight fluorescent lamps (General Electric). The lamps were cooled with a small fan. Under these conditions, maximal PR was achieved in less than 1 min. All experiments involving PR were performed under red ambient light.

Assay for Py< >Py. To assay for the production of Py< >Py by γ irradiation, cells of strain SR73 were grown at 37°C in DTM–glucose minimal medium supplemented with thiamine·HCl (0.5 μg/ml), proline (1 m*M*), and thymine (2 μg/ml) until a cell density of $\sim 3 \times 10^8$ cells/ml was reached. The cells were diluted 20-fold into the same medium containing 100 μCi/ml of [*methyl*-^{3}H]-thymidine (Amersham, 48 Ci/mmol) and incubated for 5 to 6 hr at 37°C. Cells were harvested by centrifugation, washed three times, and resuspended in DTM buffer at about 2 to 3×10^9 cells/ml. The cell suspension was γ-irradiated (400 krad) without aeration at 4°C. Irradiated cells ($\sim 2 \times 10^{10}$) were collected by centrifugation at 4°C, and the DNA was isolated by lysing the cells with lysozyme and Sarkosyl treatment followed by phenol extraction (*11*). The extracted DNA was mixed with 50 μg of carrier calf thymus DNA and precipitated with cold trichloroacetic acid (5% final concentration). The DNA precipitate was washed with cold ethanol (95%), dried, and dissolved in trifluoroacetic acid. Hydrolysis of DNA was done in sealed glass tubes at 175°C for 75 min. The acid hydrolysates were spotted on Whatman No. 1 paper strips and fractionated by descending chromatography (15–20 hr; solvent front, 40–43 cm) using: (A) *n*-butanol:acetic acid:H_2O (40:6:15), (B) *n*-butanol:H_2O (43:7), (C) isopropanol:NH_4OH:H_2O (7:1:2), and (D) *sec*-butanol saturated with H_2O.

To quantitate the distribution of radioactivity on the developed chromatograms, the paper strips were cut into 1-cm segments, transferred to scintillation vials, and eluted with 0.5 ml of H_2O. The radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer, using 8 ml of Insta-Gel (Packard) as the counting solution.

^{14}C-labeled T< >T and U< >T were isolated from solvent A-developed paper chromatograms of an acid hydrolysate of [^{14}C]thymine-labeled SR73 that had been uv-irradiated (254 nm) with 1.2×10^3 Jm^{-2}. These samples were used in parallel runs during chromatography to locate the regions corresponding to T< >T and U< >T from hydrolysates of γ-irradiated DNA.

This experiment has been run twice: the first for qualitative results, the second for the quantitative values reported here.

RESULTS

Photoreactivating Enzyme is Required for Photoreactivation after Ionizing Irradiation

In *E. coli* the PRE is controlled by the *phr* locus, which is located at ~15.9 min on the 100-min *E. coli* chromosome map (*12*, *13*). A mutation in the *phr* locus results in the loss of a cell's ability to be photoreactivated after uv irradiation (Fig. 1). To test whether or not the PRE may be involved in the PR observed after γ irradiation, we compared strains SR18 (*uvrA recA*) and SR362 (*uvrA*

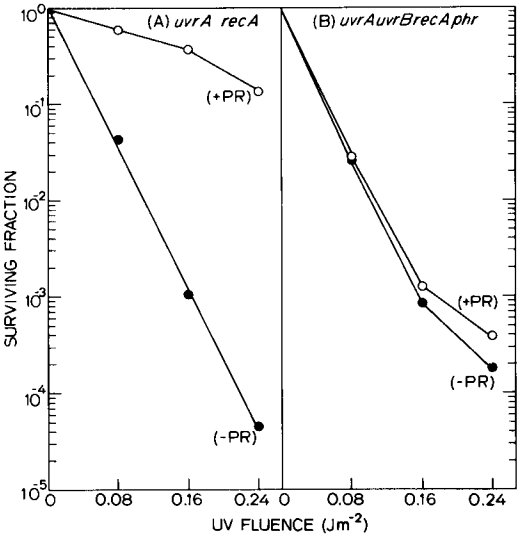

FIG. 1. The effect of postillumination with near-uv radiation on the survival of SR18 (AB2480) (*uvrA recA*) and SR362 (*uvrA uvrB recA phr*) after far-uv (254-nm) irradiation. Stationary phase cells grown in YEP broth were suspended in DTM buffer at 1×10^8 cells/ml and uv-irradiated (254 nm) at room temperature. Samples were either kept in the dark (−PR) or postilluminated (+PR) with 15-W Daylight fluorescent lamps for 3 min, as described under Materials and Methods.

uvrB recA phr) for their ability to be photoreactivated after γ irradiation. As shown in Fig. 2, strain SR18 (AB2480) showed PR after γ irradiation, as reported by others (*1*, *14*, *15*). In contrast, cells of strain SR362 failed to be photoreactivated after γ irradiation. A similar result was obtained when X rays (50 kVp) were used as the ionizing radiation, except that, for similar doses, X rays produced less photoreactivable damage (see below). These results indicate that the same PRE is responsible for the observed PR after ionizing irradiation as after uv irradiation.

A comparison of the photoreactivable sectors in strain SR18 after uv irradiation (Fig. 1) and after γ irradiation (Fig. 2) or X irradiation (data not shown) reveals that 10 krad of ^{137}Cs-γ radiation and 50-kVp X radiation produced about 0.07 and 0.04 Jm^{-2}-equivalents of 254-nm radiation-induced photoreactivable damage, respectively. These values are somewhat less than what we estimate from the published work of Myasnik and Morozov (*1*) and Vinicombe *et al.* (*15*) for ^{60}Co-γ radiation, namely, that 10 krad of ^{60}Co-γ radiation produces $\sim$0.2 Jm^{-2}-equivalents of 254-nm radiation-induced photoreactivable damage.

Production of Py< >Py by γ Irradiation

Since the only substrate known for the PRE is a Py< >Py (*5*), and material has been observed after ionizing irradiation that chromatographs similarly to pyrimidine dimers (*16*, *17*), we examined the possibility of Py< >Py production by γ irradiation. Strain SR73 was labeled in DTM-glucose minimal medium in

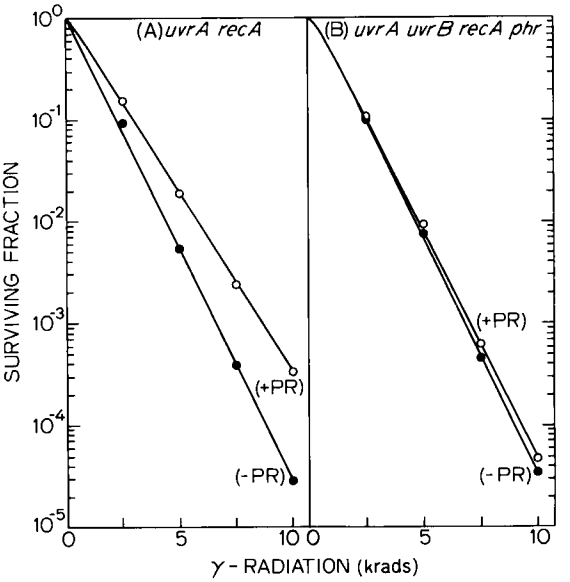

FIG. 2. The effect of postillumination with near-uv radiation on the survival of SR18 and SR362 after ^{137}Cs-γ irradiation. Otherwise, the experimental conditions were as described in the legend to Fig. 1. The results are the average of three experiments.

the presence of 100 μCi/ml of [^{3}H]thymidine and was irradiated in DTM buffer at 4°C with 400 krad of γ radiation. The DNA was extracted, hydrolyzed with trifluoroacetic acid, and chromatographed on Whatman No. 1 paper, as described under Materials and Methods. In assays for T< >T, radioactivity eluted from the T< >T region in solvent A was rechromatographed in the same solvent to eliminate the streaking of thymine in the T< >T region. Of the isolated material, only about 25% could be reversed to thymine by irradiation in aqueous solution at 254 nm (1.3×10^5 Jm^{-2}). In the subsequent development with solvent B, material having an R_f of 0.2 was separated from the T< >T region. Using solvent C, two other contaminating materials (R_f of 0.31 and 0.42, respectively), which cochromatographed with T< >T in both solvents A and B, were separated from T< >T. The isolated material that chromatographed identically with T< >T in solvents A, B, and C also chromatographed identically with T< >T in solvent D (Fig. 3A) and was completely reversed to thymine upon exposure to 254-nm uv radiation (Fig. 3B). This purified material, therefore, can be confidently identified as T< >T.

In assays for U < > T, material corresponding to the U < > T region from solvent A was chromatographed in solvents B and C. There was radioactive material that chromatographed as U < > T in solvents B and C, but the separation of U< >T from other contaminating materials was not satisfactory, and its yield could not be quantitated with confidence.

The purified T< >T isolated from cells after irradiation with 400 krad of γ radiation represents 7.4×10^{-5} of the total [^{3}H]thymine radioactivity. Therefore, 400 krad of ^{137}Cs-γ radiation is equivalent to 2.6 to 3.7 Jm^{-2} of 254-nm

radiation in producing Py< >Py, if we assume the initial rate of production of T< >T in the DNA of uv-irradiated cells is 2.0 to 2.75 × 10^{-5} radioactivity in T< >T per total radioactivity as thymine per Jm^{-2} (*18, 19*). This result is in good agreement with our *in vivo* estimate based upon survival curves (i.e., since 10 krad of ^{137}Cs-γ radiation = 0.07 Jm^{-2}-equivalents of 254-nm radiation, the value calculated for 400 krad is 2.8 Jm^{-2}-equivalents).

DISCUSSION

The phenomenon of PR observed in a *uvrA recA* strain of *E. coli* after ionizing irradiation [Fig. 2, and Refs. (*1, 14, 15*)] requires the same enzyme that is responsible for PR after uv irradiation. No PR was observed in a *phr* derivative of a *uvrA uvrB recA* strain after uv or γ irradiation (Figs. 1 and 2).

The known specificity of the PRE to catalyze the monomerization of Py< >Py via a light-dependent process (*5*) suggests that Py< >Py are produced in cells by γ irradiation. We have tested this hypothesis and have identified T< >T from acid hydrolysates of DNA purified from γ-irradiated cells. The identification of T< >T after γ irradiation was based upon its having chromatographic properties in several solvents that were identical with those of authentic T< >T from uv-irradiated cells, and also on its property of total photochemical (254-nm) conversion in aqueous solution to thymine (Fig. 3). Therefore, the most logical explanation for the phenomenon of PR after ionizing irradiation in a *uvrA recA* strain is the photoenzymatic monomerization of γ-radiation-produced Py< >Py.

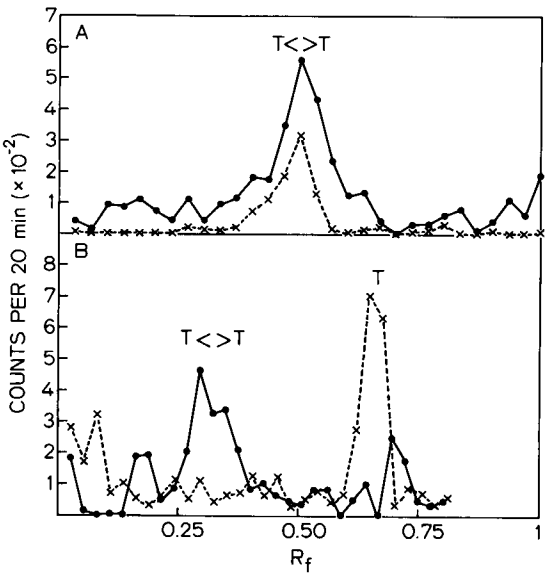

FIG. 3. (A) Cochromatography in solvent D of purified [^{3}H]T< >T-like material (·—·) from γ-irradiated cells with authentic [^{14}C]T< >T(× – – – ×) from uv-irradiated cells. (B) Chromatography in solvent A of a sample of the [^{3}H]T< >T material shown in Fig. 3A prior to (·—·) and after (× – – – ×) exposure in aqueous solution to 1.3 × 10^5 Jm^{-2} of 254-nm radiation.

Based upon a comparison of the PR sectors of the *uvrA recA* strain after exposure to uv radiation and to ^{137}Cs-γ radiation, we estimate that 10 krad of ^{137}Cs-γ radiation produces about 0.07 Jm^{-2}-equivalents of 254-nm radiation-induced Py< >Py, or about 4 dimers per *E. coli* genome [assuming 0.1 Jm^{-2} at 254 nm produces ~6 Py< >Py per genome (*19*)].

In a *uvrA recA* strain of *E. coli*, which is defective in the two major dark-repair system for counteracting Py< >Py, the average lethal hit is 1 to 2 Py< >Py per genome (*20*). Therefore, the formation of only one or two Py< >Py per genome by γ irradiation would be expected to have a significant effect on the survival of *uvrA recA* cells following γ irradiation. The fact that *uvrA recA* cells show PR after γ irradiation confirms that Py< >Py do play a significant role in the killing of *uvrA recA* cells by γ irradiation.

The absence of detection of PR in *uvrA*, *uvrB*, or wild-type cells after γ irradiation (*1*) may be explained on the basis that the few Py < > Py that are formed are rapidly repaired by other repair processes, since the γ radiation doses used for the PR studies have an equivalent uv effect that corresponds to the shoulder regions of the uv inactivation curves for these strains (data not shown). A very small amount of PR may have been observed for a *recA* strain after ^{60}Co-γ irradiation (*15*); a strain that is proficient in the excision repair of Py< >Py, but not in postreplication repair.

It is of interest that similar strain differences have been observed for PR after exposure to 365-nm radiation; a type of radiation that produces relatively few Py< >Py (for a review, see *21*). Thus, a *uvrA recA* strain shows a considerable amount of PR, a *recA* strain only a very slight amount of PR, and the *uvrA* and wild-type strains no PR (*21*, *22*).

The radiation-chemical reaction leading to the formation of Py< >Py by ionizing radiation is of considerable interest. The PR sector remains the same whether the cells are γ-irradiated under aerobic or anaerobic conditions [data not shown; and Refs. (*14*, *15*)]. This result suggests that the formation of Py< >Py by γ irradiation is not affected by the presence or absence of oxygen during irradiation. Since the triplet states of both thymine and guanine are produced in X-irradiated DNA (*23*), the formation of Py< >Py by ionizing radiation may occur by excitations rather than ionizations.

Because different ionizing radiations produce varying amounts (^{60}Co-γ > ^{137}Cs-γ > 50-kVp X rays) of photoreactivable damage [^{60}Co-γ data from Refs. (*1*, *15*)], it would be of interest to determine if these radiations do produce different amounts of excitations in the nucleic acids.

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