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The Duration of Recovery and DNA Repair in Excision Deficient Derivatives of *Escherichia coli* K-12 after Ultraviolet Irradiation

ANN K. GANESAN and KENDRIC C. SMITH
Radiology Department, Stanford University School of Medicine
Stanford, California 94305

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Summary. Our results indicate that cells of excision deficient (*uvr*) mutants of *Escherichia coli* K-12 which survive exposure to ultraviolet radiation may require several hours to complete their recovery. For example, the duration of the recovery period for cells exposed to 63 ergs mm^{-2} at 254 nm was about 5 hours, the equivalent of slightly more than 4 generations of the unirradiated controls. During the recovery period the rate of cell division was reduced (Figs. 3 and 4), the cells gradually regained resistance to complex medium (Figs. 1 and 3), and they became refractory to photoreactivation (Fig. 1). Over the same period of time their pattern of DNA synthesis changed. More intact molecules, similar to those found in unirradiated controls, and relatively fewer discontinuous molecules were synthesized (Figs. 6 and 7).

Introduction

Derivatives of *Escherichia coli* K-12 which are excision deficient (*uvr*) but recombination proficient (*rec*$^+$) survive better after ultraviolet (UV) irradiation if they are plated on minimal medium than if they are plated on complex medium (Ganesan and Smith, 1968). This response appears to be due to a recovery process which is inhibited by complex medium. Genetic studies have indicated that the response is controlled by the genes *recA*, *recB* and *recC* (Ganesan and Smith, 1970). It can be distinguished from "liquid holding" recovery (Hollaender and Claus, 1937; Hollaender and Curtis, 1935; Roberts and Aldous, 1949; Castellani, Jagger and Setlow, 1964; Harm, 1966), not only because it is controlled by *rec* genes instead of *uvr* genes, but also because it appears to require conditions which permit the cells to grow and synthesize DNA (Ganesan and Smith, 1970; Ganesan and Smith, Biophys. J., **10**, 176a, 1970).

The repair of DNA in *uvr* cells after UV irradiation also requires DNA replication. Immediately following irradiation, DNA containing single strand discontinuities is made (Rupp and Howard-Flanders, 1968; Howard-Flanders *et al.*, 1968). These discontinuities, apparently formed when irradiated dimer-containing DNA provides the template for replication, are repaired in *rec*$^+$ but not in *recA* cells (Smith and Meun, 1970). The repaired molecules may act as templates for succeeding rounds of replication and thus permit the synthesis of intact DNA (Rupp *et al.*, 1971). The recovery of viability in *uvr* cells should reflect this repair process. As one test of this expectation, we wished to compare the time course of recovery with that of DNA repair in UV-irradiated *uvr* cells to determine whether these two processes were compatible in this respect.

In order to determine the time needed for *uvr* cells to complete recovery we examined the ability of UV-irradiated cells to form colonies on complex medium before and after exposure to visible light. Since complex medium appears to inhibit some step in the recovery process, the acquisition of the ability to form colonies on it after UV irradiation should indicate that recovery has progressed beyond this step. Loss of photoreactivability by *uvr* cells, which are defective in excising dimers (Howard-Flanders, Boyce, and Theriot, 1966; Boyle and Setlow, 1970), should indicate when the dimers induced by UV have been modified so that they are no longer susceptible to photoreactivation, or when the irradiated cells have progressed to some stage when dimers, or DNA strands containing them, no longer affect their survival.

We have estimated the time needed by *uvr* cells to complete repair by determining the time after UV irradiation when DNA free of single-strand discontinuities is synthesized. Intact DNA should be synthesized when the irradiated strands have been repaired, or when they no longer provide the only templates for replication.

Materials and Methods

Bacterial Strains. The derivatives of *Escherichia coli* K-12 used are listed in Table 1. All cultures were incubated at 37° C. Except where otherwise indicated, cultures growing exponentially in minimal medium were used for experiments.

Table 1. *Escherichia coli K-12 Derivatives Used*

Designation	Mating type	Relevant genotype	Other markers	Reference
AB1884	F⁻	*uvrC34*	*λ^s thr leu arg his pro ara lac gal mtl xyl str^r $T6^r$ thi*	a
AB1886	F⁻	*uvrA6*	*λ^s thr leu arg his pro ara lac gal mtl xyl str^r $T6^r$ thi*	a
AB2499	F⁻	*uvrB5*	*λ^s thr leu arg his pro thy ara lac gal mtl xyl str^r $T6^r$ thi*	a

[a] Howard-Flanders, Boyce, and Theriot (1966).

Abbreviations: (Demerec *et al.*, 1966; Curtiss, 1968; Taylor, 1970). The symbols *arg, his, leu, pro, thi, thr, thy,* denote requirements for arginine, histidine, leucine, proline, thiamine, threonine, thymine, respectively; *ara, gal, lac, mtl,* and *xyl,* the inability to utilize arabinose, galactose, lactose, mannitol, and xylose, respectively; *T6,* λ, and *str,* response to the phages T6 and λ, and to the antibiotic, streptomycin (^{r} indicates resistance; ^{s} sensitivity); *uvr* designates genes affecting host cell reactivation and UV sensitivity.

Media. The media used included a complex medium, YENB (Ganesan and Smith, 1970), and phosphate buffered minimal media (Ganesan and Smith, 1968). When needed, L-amino acids were incorporated at a final concentration of 10^{-3} M; thymine, 10 μg/ml; and thiamine, 0.5 μg/ml.

Irradiation. Conditions used for irradiation have been previously described (Ganesan and Smith, 1970). The dose rate was 750 ergs mm^{-2} min^{-1}, as estimated by the photodecomposition of potassium ferrioxalate (Hatchard and Parker, 1956; Jagger, 1967).

Plating. Three different plating methods were used. (1) Samples were diluted to give approximately 10^3 colony forming units per ml, then 0.1 ml volumes were spread on the surface of agar plates with glass spreaders. (2) Samples were diluted as for (1), but 0.01 ml samples were deposited on the agar surface as drops and allowed to dry without spreading. (3) Samples were diluted as for (1), but 0.01 ml volumes were placed on squares of cellophane

(approximately 5 × 5 cm) on the surface of agar plates (Alper and Gillies, 1958) and spread with small glass spreaders. The cellophane was kindly provided by Dr. A.J. Forage.

Photoreactivation. Cells in liquid, or on agar plates, were exposed to light from two Westinghouse F15T8 BL bulbs, positioned 6 cm above the surface on which Petri dishes containing the cells rested. The tops of the dishes were left in place to act as filters. When cells were photoreactivated in liquid, Pyrex dishes and tops were used; on agar, polystyrene dishes and tops. An exposure time of 40 minutes was used because it gave maximal survival. When cells were photoreactivated in liquid, 10 ml of cells in minimal medium were placed in 10 cm Petri dishes on a wire rack on a platform rotator. The wire rack was used to insulate the cells from the heat of the rotator. The temperature under the lamps during photoreactivation was not more than 37° C, and usually about 35° C. The survival of unirradiated control cells was not significantly affected by these conditions.

DNA Synthesis. The incorporation of ^{3}H-thymine was measured as previously described (Smith and O'Leary, 1968). Radioactive thymine was diluted to give a final concentration of 3 μg/ml (15 μCi/ml).

Alkaline Sucrose Gradients. The procedures used have been described previously (Rupp and Howard-Flanders, 1968; Smith and Meun, 1970), except that ^{3}H-thymine (3.4 μg/ml, 200 μCi/ml final concentration) was used for labeling the cells, and the spheroplast mixture contained 100 μg/ml lysozyme. Each gradient contained 20 μl of spheroplast mixture adjusted before lysozyme treatment to $OD_{650} = 0.51$ (equivalent to about 2×10^8 control cells/ml).

Radioactive Chemicals. Thymine (methyl-^{3}H) (18.2 and 18.5 Ci/mmole) was obtained from New England Nuclear Company.

Determination of Radioactivity. Samples on paper discs were counted in a liquid scintillation spectrometer (Smith and Meun, 1970). The counting efficiency for ^{3}H-thymine on the paper discs under these conditions was 3%.

Results

To estimate the length of time required for recovery to occur in excision deficient cells, cultures were irradiated with UV, then plated immediately on cellophane carriers (Alper and Gillies, 1958) on agar plates. This procedure permitted transfer of the cells from one medium to another while they were immobilized on the cellophane and ensured that all of the division products of a cell would contribute to the formation of only one colony. The cells were initially plated on minimal medium, then at intervals they were transferred to complex medium (YENB) to prevent further recovery. Immediately after transfer, one set of YENB plates was incubated in the dark, while another was exposed to photoreactivating light. Fig. 1 presents the results of a representative experiment with a *uvrC34* mutant, AB1884. The longer the cells remained on minimal medium before transfer to YENB, the larger the proportion of cells which formed colonies on YENB. Recovery, as estimated by the acquisition of resistance to YENB, appeared to occur over a period of several hours. As resistance to YENB increased, resistance to photoreversal also increased. The proportion of cells which formed colonies on YENB after photoreactivation declined as the length of exposure to minimal medium prior to photoreactivation increased (Fig. 1). By 6 hours after a dose of 125 ergs mm^{-2}, most of the cells had become resistant to YENB and to photoreactivation. If a second dose of UV was given at this time, it could be efficiently photoreactivated (Table 2), indicating that the loss of photoreactivability reflected some change in UV induced lesions rather than a decay in the cells' capacity for photoreactivation.

The same experiment was also performed on a *uvrA6* mutant, AB1886 (data not shown). Although the amount of recovery on minimal medium after the same

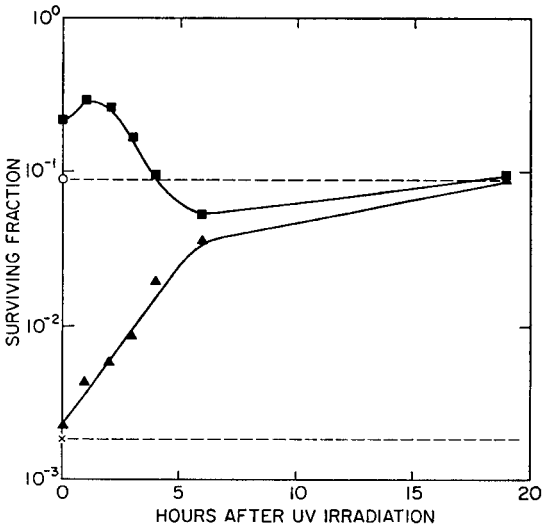

Fig. 1. Photoreactivation of *uvr* cells during recovery on agar minimal medium. AB1884 (*uvrC34*) was starved for amino acids for 2 hours, then irradiated with 125 ergs mm^{-2}, and plated on cellophane carriers: ×---- cells plated on YENB (and left there). ○--- cells plated on minimal medium (and left there). ▲—— cells plated on minimal medium, and transferred to YENB at the time shown on the abscissa. ■—— cells plated on minimal medium, transferred to YENB at the time shown on the abscissa, and then photoreactivated

Table 2. *Efficiency of photoreactivation of a second UV dose given 6 hours after the first dose*

	Time after 1st dose	Survival				
		Relative to unirradiated cells		Corrected for survival of cells given only 1st UV dose		
		without PR	with PR	without PR (a)	with PR (b)	Ratio b/a
1st dose (125 ergs mm^{-2})	0	7.3×10^{-4}	3.3×10^{-1}	1.0	1.0	—
	6	1.1×10^{-1}	7.3×10^{-2}	1.0	1.0	—
2nd dose (38 ergs mm^{-2})	0	1.3×10^{-4}	1.8×10^{-1}	1.8×10^{-1}	5.4×10^{-1}[a]	3.0
	6	5.3×10^{-3}	1.2×10^{-2}	4.8×10^{-2}	1.6×10^{-1}[a]	3.3

Cells of AB2499 (*uvrB5*) were irradiated and plated on YENB. They were then irradiated (on the plates) with a 2nd UV dose either immediately after the 1st dose, or 6 hours later. They were photoreactivated (PR) (on the plates) immediately after the 2nd dose.

[a] Corrected for cells photoreactivated 6 hours after the 1st UV dose.

UV dose was less for this mutant than for AB1884, the time required to attain complete resistance to complex medium and to photoreactivation was the same as for AB1884.

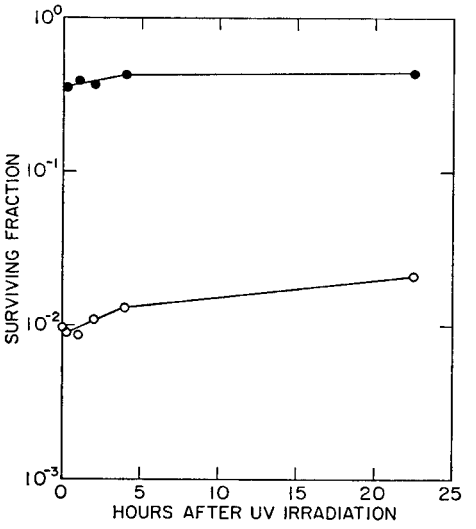

Fig. 2. Photoreactivation of *uvr* cells during amino acid starvation. AB2499 (*uvrB5*) was incubated in minimal medium without amino acids for 2 hours, irradiated with 97 ergs mm^{-2}, and again incubated in minimal medium without amino acids. Samples were plated at the times indicated on the abscissa on YENB, before (○), and after photoreactivation (●)

Both the acquisition of resistance to complex medium and the loss of photoreactivability appeared to require metabolic process(es) inhibited by starvation for required amino acids, since irradiated *uvr* cells starved for amino acids did not lose their sensitivity to YENB (Fig. 2; Ganesan and Smith, 1970) or their photoreactivability (Fig. 2). When incubated in YENB, UV-irradiated *uvr* cells became refractory to photoreactivation even more rapidly than when they were incubated in minimal medium (data not shown).

To try to relate the duration of recovery to generation time, irradiated *uvr* cells were allowed to recover in liquid minimal medium instead of on agar. Samples were plated at intervals on YENB and on agar minimal medium. Fig. 3 illustrates the results obtained with a *uvrB5* mutant, AB2499. During the first 7 hours after irradiation with 125 ergs mm^{-2}, the cells which could form colonies on agar minimal medium increased only about 4-fold while survival on YENB rose about 300-fold until it was equal to that on the minimal medium. Since the number of colonies formed by the irradiated cells on agar minimal medium remained relatively constant compared to the unirradiated controls, it appeared that the rate of cell division was reduced in the irradiated population and that most of the increase in survival on YENB could be attributed to recovery. The time required to recover in liquid minimal medium after a dose of 125 ergs mm^{-2} (6 hours) was comparable to that required for recovery on agar minimal medium after the same UV dose (Fig. 1). The response after 63 ergs mm^{-2} was similar, except that 5 instead of 6 hours was required for recovery, indicating that the dose of UV influenced the duration of the recovery period.

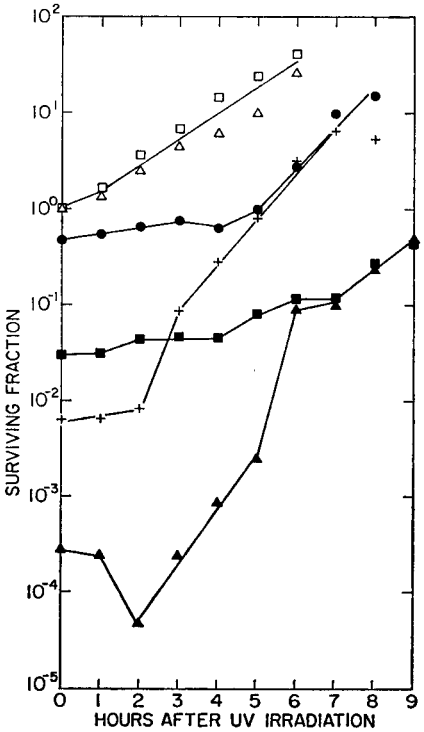

Fig. 3. Recovery of UV-irradiated *uvr* cells in liquid minimal medium. AB2499 (*uvrB5*) was irradiated, incubated in liquid minimal medium, then plated at intervals on YENB and agar minimal medium (*MM*). △ unirradiated, YENB; □ unirradiated, MM; + 63 ergs mm^{-2}, YENB; ● 63 ergs mm^{-2}, MM; ▲ 125 ergs mm^{-2}, YENB; ■ 125 ergs mm^{-2}, MM

The idea that the rate of cell division was reduced by UV irradiation was supported by counting total cells (Fig. 4; Rupp *et al.*, 1971). Even a dose of 31 ergs mm^{-2}, from which nearly 90% of the cells recovered on minimal medium, significantly reduced the rate of cell division (Fig. 4). Although long filaments were not observed, the optical density of the culture (Fig. 4) and the size of individual cells increased during the division delay. The delay appeared to result from the effects of UV on DNA since 1) photoreactivation shortened the delay; 2) the delay occurred in *uvr* mutants, but not in related *uvr*$^+$ cells; and 3) visible light emanating from the UV source did not account for the delay (data not shown).

Because the division delay made relating recovery to cell generation time of dubious significance, an attempt was made to relate recovery to DNA replication. The incorporation of radioactive thymine by the *uvrB5* mutant, AB2499, was measured at various times after irradiation. As Fig. 5 indicates, the rate of incorporation by irradiated cultures was less than in the unirradiated controls, but greater than could be accounted for by assuming that only survivors replicated DNA and did so at the same rate as unirradiated cells. Since it appeared that

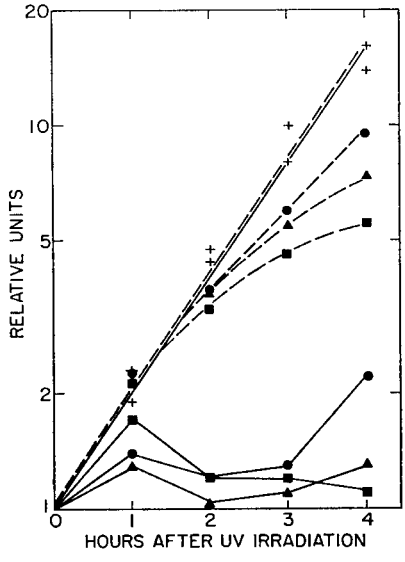

Fig. 4. Division delay in irradiated *uvr* cells. AB2499 (*uvrB5*) was irradiated and incubated in minimal medium. The optical density at 650 nm (----) and total cell number (——)were followed. Total cell number was determined using a Petroff-Hausser counting chamber. UV dose: None (+); 31 ergs mm^{-2} (●) (survival on minimal medium = 87%); 63 ergs mm^{-2} (▲) (survival on minimal medium = 65%); 125 ergs mm^{-2} (■) (survival on minimal medium = 13%)

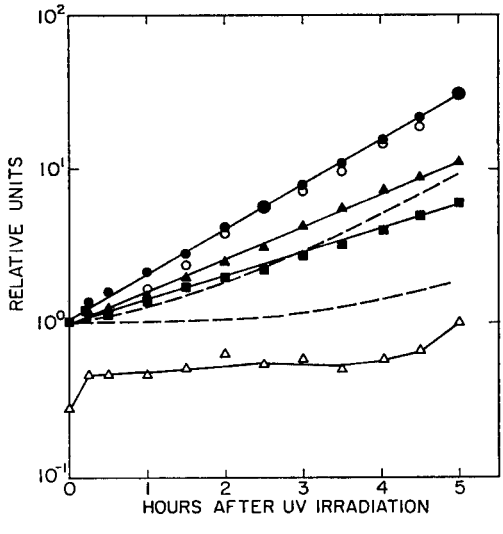

Fig. 5. DNA synthesis and colony formation by irradiated *uvr* cells. AB2499 (*uvrB5*) was irradiated with 63 or 95 ergs mm^{-2}, and incubated in minimal medium containing ^{3}H-thymine. At intervals the cultures were sampled for radioactivity and for survival on agar minimal medium. Unirradiated control: radioactivity (●), survival (○). Irradiated cultures: 63 ergs mm^{-2} — radioactivity (▲), survival (△); 95 ergs mm^{-2} — radioactivity (■), survival (not shown) = 2.8×10^{-2} at 0 hours. The dashed lines represent the radioactivity expected if only the surviving cells synthesized DNA and if they incorporated ^{3}H-thymine at the same rate as unirradiated control cells: upper curve, 63 ergs mm^{-2}; lower curve, 95 ergs mm^{-2}

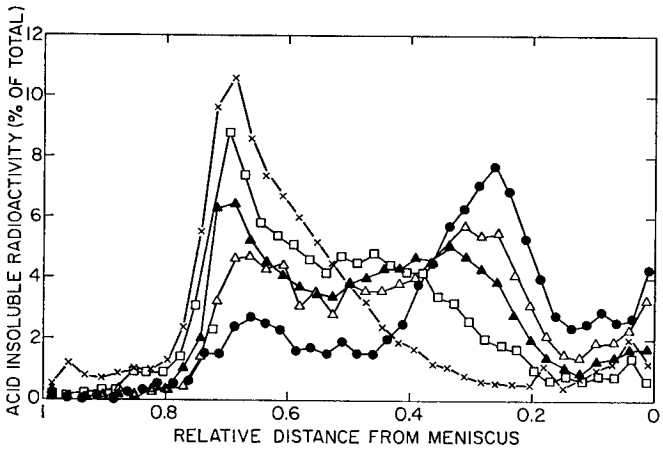

Fig. 6. Sedimentation on alkaline sucrose gradients of DNA synthesized at various times after UV irradiation. Cells of AB2499 (*uvrB5*) were irradiated with 63 ergs mm^{-2}, then reincubated in minimal growth medium. At intervals, samples were removed and labeled for 10 minutes with ^{3}H-thymine and analysed on alkaline sucrose gradients as described in Materials and Methods. Unirradiated controls (×); cells labeled immediately after irradiation (●); cells incubated for 60 minutes (△), 120 minutes (▲), or 240 minutes (□) after irradiation before being labeled

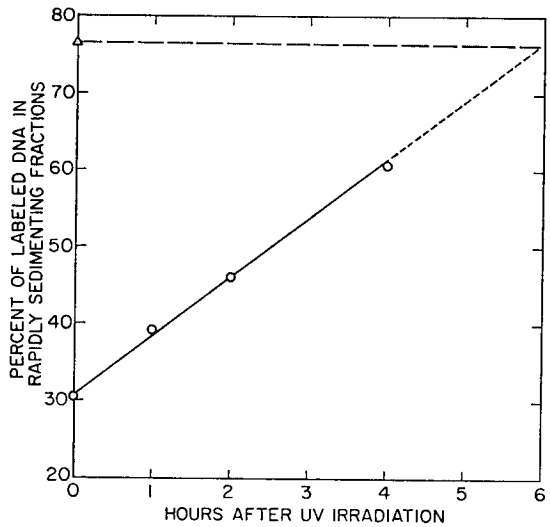

Fig. 7. Changes in the relative amount of rapidly sedimenting DNA synthesized at different times after UV irradiation. Data from Fig. 6. A point was arbitrarily chosen halfway between the peak of DNA from unirradiated control cells and the peak of DNA from cells labeled immediately after irradiation (Fig. 6). The rapidly sedimenting fractions were those between this point and the bottom of the gradient. The amounts of radioactivity represented by the points are: control = 8150 cpm; irradiated cells: 0 hour = 930 cpm, 1 hour = 1560 cpm, 2 hours = 2620, 4 hours = 5600. Unirradiated control (△); irradiated cells (○)

UV-irradiated cells which could not form colonies continued to make DNA for some time after irradiation (Fig. 5; Smith, 1969), it was not possible to determine by this type of experiment how many complements of DNA were synthesized by the survivors during the recovery period.

The DNA synthesized by the irradiated *uvr* cultures appeared to contain discontinuities, presumably resulting from replication of radiation damaged template DNA (Rupp and Howard-Flanders, 1968; Howard-Flanders *et al.*, 1968; Smith and Meun, 1970). In order to determine how much time elapsed after irradiation before DNA was synthesized without these discontinuities, the following experiment was performed. An exponentially growing culture of AB2499 (*uvrB5*) was irradiated and reincubated in minimal growth medium. At intervals, samples were removed and incubated with ^{3}H-thymine for 10 minutes. They were then washed and analysed on alkaline sucrose gradients. Fig. 6 shows the results of a representative experiment. Consistent with previous results (Smith and Meun, 1970; Rupp *et al.*, 1971), most of the DNA made immediately after irradiation appeared to be discontinuous, and sedimented more slowly than DNA from unirradiated controls. By 2 hours, nearly half of the newly synthesized DNA sedimented at a rate similar to that of the control, while the rest still appeared to contain discontinuities (Figs. 6 and 7). By 4 hours after irradiation, most of the DNA synthesized behaved like that from control cells; relatively fewer discontinuous molecules were observed, and these appeared to be larger than the ones made immediately after irradiation (Figs. 6 and 7). Extrapolation from these data indicated that only intact molecules should be detected by about 6 hours after irradiation (Fig. 7).

Discussion

Excision-deficient (*uvr*) derivatives of *E. coli* K-12 are able to recover from UV-induced damage by a process that is controlled by the genes *recA*, *recB* and *recC* (Ganesan and Smith, 1970) and inhibited by complex growth medium (Ganesan and Smith, 1968). This type of recovery is relatively slow since *uvr* cells required several hours after UV irradiation to reach a stage in recovery when they were no longer sensitive to complex medium. This was true whether the irradiated cells were immobilized on solid minimal medium or allowed to recover in liquid minimal medium (Figs. 1 and 3). The duration of the recovery period after a dose of 125 ergs mm^{-2} was about 6 hours (Figs. 1 and 3); after 63 ergs mm^{-2} it was approximately 5 hours (Fig. 3).

During the recovery period the rate of division of the irradiated cells was greatly reduced relative to the unirradiated controls (Figs. 3 and 4). After most of the survivors had attained resistance to complex medium they then began to divide at a more normal rate (Fig. 3). By this time, judging from the incorporation of radioactive thymine, sufficient DNA had accumulated in the irradiated cultures to provide several genetic complements for each cell present (Fig. 5), although it was not clear how this DNA might be distributed among the surviving and moribund cells.

Over approximately the same period of time that the irradiated *uvr* cells were acquiring resistance to complex medium, their pattern of DNA synthesis

changed. More intact molecules, similar to those found in unirradiated controls, and relatively fewer discontinuous molecules were synthesized (Figs. 6 and 7). Shortly after UV irradiation, *uvr* cells make DNA containing discontinuities which appear to be formed when DNA containing pyrimidine dimers acts as a template for replication (Rupp and Howard-Flanders, 1968; Howard-Flanders *et al.*, 1968; Smith and Meun, 1970). Except in *recA* cells (Smith and Meun, 1970) these discontinuities seem to be repaired within about 30 minutes after their formation (Rupp and Howard-Flanders, 1968; Howard-Flanders *et al.*, 1968; Smith and Meun, 1970). The repaired strands may then serve as templates for further replication. Providing that any parental DNA used to repair discontinuities in the daughter strands does not contain dimers, the repaired regions should permit synthesis of intact DNA (Rupp *et al.*, 1971). Some intact molecules appeared to be synthesized in *uvr* cells by 60 minutes after a dose of about 60 ergs mm^{-2} (Figs. 6 and 7; Rupp *et al.*, 1971) indicating that repaired dimer-free regions might be serving as templates by this time. However, discontinuous molecules still represented the predominant species at this time. In contrast, in uvr^+ cells, which can excise dimers from their DNA, only intact molecules were detected after 60 minutes under these same conditions (Smith and Meun, 1970). In *uvr* cells, discontinuous molecules were still frequent after 2 hours, and had not disappeared by 4 hours after irradiation (Figs. 6 and 7). However, increasing amounts of intact molecules were synthesized at these times, and extrapolation from the data of Fig. 6 indicated that by 6 hours after a dose of 63 ergs mm^{-2} the frequency of intact molecules should have returned to the control value (Fig. 7). Thus the time course of the increase in the synthesis of intact molecules was at least roughly comparable to the time during which the irradiated cells became resistant to complex medium.

The loss of photoreactivability observed in UV-irradiated *uvr* cells would be compatible with an increase in the use of dimer-free DNA as a template for replication. Concurrent with their loss of sensitivity to complex medium, cells which initially could be revived by exposure to visible light gradually lost this property (Fig. 1) suggesting that photoreactivable lesions, presumably pyrimidine dimers (Setlow, 1966), were modified so that they were no longer susceptible to photoreactivation, or that the DNA strands containing them no longer contributed to the survival of the irradiated cells.

Evidence from mating experiments indicates that in *E. coli* K-12 dimers are still susceptible to photoreactivation after at least one round of replication of the DNA containing them, even when there are gaps in the newly synthesized complementary strands (Howard-Flanders *et al.*, 1968; Cole, 1971). On this basis we inferred that the progressive loss of photoreactivability observed in irradiated *uvr* cultures was not a direct consequence of the initial round of replication after irradiation, but might reflect the progressive decrease in the use of dimer-containing strands as templates for DNA synthesis which appeared to occur during approximately the same time period.

Our results indicate that in UV-irradiated *uvr* cells, the time required for repair, as judged by the increased synthesis of intact DNA molecules, is not appreciably different from the time required for the cells to recover viability, as judged by the loss of sensitivity to complex medium. Both processes required

several hours incubation in minimal growth medium for completion, e.g. 5–6 hours after 63 ergs mm^{-2} at 254 nm.

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Ann K. Ganesan
Department of Biological Sciences
Stanford University
Stanford, California 94305/USA