DNA REPAIR IN *POTOROUS TRIDACTYLUS*

STEVEN N. BUHL, R. B. SETLOW, *and* JAMES D. REGAN

From the University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, and the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. Dr. Buhl's present address is Montreal Children's Hospital, Montreal, Quebec, Canada.

ABSTRACT The DNA synthesized shortly after ultraviolet (UV) irradiation of *Potorous tridactylis* (PtK) cells sediments more slowly in alkali than that made by nonirradiated cells. The size of the single-strand segments is approximately equal to the average distance between 1 or 2 cyclobutyl pyrimidine dimers in the parental DNA. These data support the notion that dimers are the photoproducts which interrupt normal DNA replication. Upon incubation of irradiated cells the small segments are enlarged to form high molecular weight DNA as in nonirradiated cells. DNA synthesized at long times (~24 h) after irradiation is made in segments approximately equal to those synthesized by nonirradiated cells, although only 10–15% of the dimers have been removed by excision repair. These data imply that dimers are not the lesions which initially interrupt normal DNA replication in irradiated cells. In an attempt to resolve these conflicting interpretations, PtK cells were exposed to photoreactivating light after irradiation and before pulse-labeling, since photoreactivation repair is specific for only one type of UV lesion. After 1 h of exposure ~35% of the pyrimidine dimers have been monomerized, and the reduction in the percentage of dimers correlates with an increased size for the DNA synthesized by irradiated cells. Therefore, we conclude that the dimers are the lesions which initially interrupt DNA replication in irradiated PtK cells. The monomerization of pyrimidine dimers correlates with a disappearance of repair endonuclease-sensitive sites, as measured in vivo immediately after 1 h of photoreactivation, indicating that some of the sites sensitive to the repair endonuclease (from *Micrococcus luteus*) are pyrimidine dimers. However, at 24 h after irradiation and 1 h of photoreactivation there are no endonuclease-sensitive sites, even though ~50% of the pyrimidine dimers remain in the DNA. These data indicate that not all pyrimidine dimers are accessible to the repair endonuclease. The observation that at long times after irradiation DNA is made in segments equal to those synthesized by nonirradiated cells although only a small percentage of the dimers have been removed suggests that an additional repair system alters dimers so that they no longer interrupt DNA replication.

INTRODUCTION

UV radiation makes dimers between adjacent pyrimidines in DNA. The dimers may be removed by two known mechanisms: (*1*) enzymic photoreactivation, a process that specifically monomerizes only this photoproduct (J. K. Setlow, 1967, 1972; Cook, 1967; Harm et al., 1971; Patrick Harm, 1973) and that occurs in most species, including marsupial but not placental mammals (Cook and Regan, 1969; Cook, 1970); and (*2*) excision repair (R. B. Setlow, 1968), which has been observed in many cells, in-

cluding those of normal humans (Regan et al., 1968) but is reduced in humans affected with xeroderma pigmentosum (Cleaver, 1968; Setlow et al., 1969) and in hamster and mouse cells (Setlow et al., 1972).

The DNA synthesized shortly after irradiation of *Escherichia coli* (Rupp and Howard-Flanders, 1968), human (Buhl et al., 1972 *a,b*), and other mammalian cell lines (Cleaver and Thomas, 1969; Rupp et al., 1969; Meyn and Humphrey, 1971; Lehmann, 1972; Rauth et al., 1974; Fujiwara and Kondo, 1972) is of lower molecular weight than that from nonirradiated cells. The sizes of these low molecular weight segments in *E. coli* (Rupp and Howard-Flanders, 1968), mouse L5178Y (Lehmann, 1972), and human (Buhl et al., 1972 *a,b*) cells approximate the average distance between pyrimidine dimers in the parental DNA. These data support the notion that dimers interrupt normal DNA replication and that synthesis resumes beyond the dimer. This idea is reinforced by the observation that in *E. coli,* after either photoreactivation or excision of the dimers, the newly synthesized DNA is of nearly normal size (Smith and Meun, 1970). On the other hand, although UV-irradiated Chinese hamster ovary (CHO) and B14 cells (Meyn and Humphrey, 1971) also synthesize DNA in smaller segments, the segments are larger than the distance between dimers (Rauth et al., 1974). Moreover, mouse L cells synthesize DNA in normal-sized segments after low UV doses, and though they synthesize DNA in smaller segments at higher doses, the segments are much larger than the distance between dimers (Chiu and Rauth, 1972). These observations and the facts that in hamster, mouse, and man DNA synthesized at long times after irradiation is approximately the same size as that synthesized by nonirradiated cells, even though a large percentage of dimers remain in the parental DNA (Meyn and Humphrey, 1971; Lehmann and Kirk-Bell, 1972; Buhl et al., 1973), imply that dimers are not responsible for the interruption of normal DNA replication and the formation of gaps in the DNA synthesized from UV-irradiated templates.

The experiments reported here attempt to resolve these differences. We used cells from *Potorous tridactylis* because they have photoreactivation ability (Cook and Regan, 1969; Krishnan and Painter, 1973) and hence a particular lesion—the pyrimidine dimer—can be selectively eliminated by exposure of the cells to photoreactivating conditions. We found that the lesion responsible for interrupting DNA synthesis is photoreactivable and therefore conclude that the photoproducts interrupting DNA replication in UV-irradiated mammalian cells are pyrimidine dimers.

MATERIALS AND METHODS

Cell Lines and Media

The cell lines used were PtK, derived from the kidney of *Potorous tridactylis* (rat kangaroo), and SG-XP4, derived by skin biopsy from an individual with xeroderma pigmentosum. PtK cells have photoreactivating activity but SG-XP4 do not. Cells were normally grown in minimum essential medium (Eagle, 1959) supplemented with 10% calf serum and the nonessential amino acids at 37°C in a water-saturated atmosphere of 2% CO_2 in air. One or two days be-

fore each experiment the cells were trypsinized (0.05% trypsin) and plated on plastic petri dishes (Permanox, Lux Scientific Corp., Thousand Oaks, Calif.) at approximately 30,000 cells per dish. The incubation medium during photoreactivation consisted of the essential and nonessential amino acids and vitamins from minimal essential medium without phenol red (Eagle, 1959) supplemented with 10% calf serum and buffered to pH 7.4 with phosphate-buffered saline (Dulbecco and Vogt, 1954).

UV Irradiation, Photoreactivation, Pulse-Labeling, Incubation, and Harvesting Cells

Each dish of cells was washed with warmed phosphate-buffered saline (Dulbecco and Vogt, 1954) and exposed to UV radiation from a germicidal lamp at a rate of 10 ergs/mm^2 · s. The cells were then washed and incubated or photoreactivated in warmed medium for various times. Photoreactivation was accomplished by exposure to two white fluorescent lamps (GE 15-W daylight) for 0.75–1 h at a distance of 5 cm (longer photoreactivation would reverse more dimers but these cells recover the capacity to make DNA in long pieces several hours after UV, even in the absence of PR; see results). The cells were protected from the shorter wavelengths by 0.25 cm of plate glass placed between the lamps and the petri dishes. They were maintained at 36–39°C during photoreactivation by a hot air curtain. The DNA was pulse-labeled by addition of 12–50 μCi/ml of [methyl-^{3}H]thymidine (sp act 20 Ci/mmol) for 30 min. After pulse-labeling the cells were sometimes further incubated in minimal essential medium plus the nonessential amino acids and 10% calf serum before collection and suspension in an ice-cold isotonic ethylenediaminetetraacetate (EDTA) solution (Setlow et al., 1969).

Measurement of Dimers, Endonuclease-Sensitive Sites, and 313-nm-Sensitive Sites in DNA after Repair Replication in BrdUrd

The DNA was labeled by incubation of cells for 18 h in medium containing either [^{3}H]thymidine (2 μCi/ml, 1.9 Ci/mmol), $^{32}PO_4$ (10–15 μCi/ml), or [^{14}C]thymidine (0.5 μCi/ml, 33 mCi/mmol) (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.). The amount of [^{3}H]thymidine remaining as dimers after UV irradiation and various treatments of the cells was determined chromatographically (Carrier and Setlow, 1971 *a*).

After various treatments DNAs were also analyzed for sites sensitive to repair endonuclease by use of an extract from *M. luteus*, as follows (kindly provided and described by R. J. Wilkins; see Wilkins, 1974). The cells were harvested into an ice-cold EDTA solution. Approximately 2×10^4 ^{3}H-labeled and 2×10^4 ^{14}C- or ^{32}P-labeled cells were mixed, centrifuged, and resuspended in 40 μl of 0.29 M sucrose in 0.05 M Tris buffer (pH 7.6). After 10 min the volume was increased 10-fold with H_2O to swell the cells and make them permeable to the endonuclease. This was followed by refrigeration for 10 min and addition of 10 μl of a solution containing 0.5 M Tris buffer, 0.01 M EDTA, 0.1 M mercaptoethanol, and 6 μg/ml of calf thymus DNA at pH 8.0. The sample was placed in a Vortex mixer for 10 s, and 10 μl of the *M. luteus* extract was added before incubation for 5 min at 37°C, followed by chilling in an ice bath, irradiation with gamma rays, and sedimentation of the DNA in alkaline sucrose.

The amounts of repair replication were determined by the bromouracil photolysis method described by Regan et al. (1971). After radioactive labeling the cells were incubated for 4–6 h, UV-irradiated, and incubated in medium containing 10^{-3} M hydroxyurea and either 10^{-4} M BrdUrd (for ^{3}H-labeled cells) or 10^{-4} M thymidine (for ^{32}P-labeled cells) for 24 h. The cells were then harvested, and 2.0×10^4 ^{3}H-labeled and 2.0×10^4 ^{32}P-labeled cells were mixed, irradiated with 2 krad of gamma radiation, exposed to 313-nm radiation, usually at a rate of 10^5 ergs/min, and sedimented in alkaline sucrose.

Sedimentation in Alkaline Sucrose

After harvesting and various treatments, the cells were prepared for sedimentation as follows. They were irradiated with 2 krad of gamma rays from a ^{60}Co source at a rate of 3.65 krad/min so as to make a few single-strand breaks in the DNA and permit its sedimentation without excess entanglement of strands (Lehmann and Ormerod, 1970). 5–10 × 10^3 cells were then immediately lysed in 0.1 ml of 6.9 mM sodium dodecyl sulfate solution on top of a 3.6-ml gradient of 0.146–0.58 M sucrose, 0.1 M NaCl, and 0.1 M NaOH in a 4-ml polyallomer tube. The gradients were centrifuged at 40,000 rpm for 75 min at 20°C in the SW56 rotor of a Beckman L3-50 centrifuge (Beckman Instruments, Fullerton, Calif.). 0.12-ml aliquots were collected through a hole punched in the bottom of the tube onto Whatman No. 17 paper strips (Carrier and Setlow, 1971 *b*). The strips were washed in 0.31 M trichloroacetic acid, twice in ethanol, and once in acetone (Bollum, 1966), dried, cut, and placed into vials with a toluene-2,5-bis-[2-(5-*tert*-butylbenzoxazolyl)]-thiophene (BBOT) scintillator, and the radioactivity was counted in a liquid scintillation spectrometer. The molecular weights were calculated by computer, using the relationship $M\alpha d^{2.62}$ observed by Abelson and Thomas (1966), with single strands of T4, λ, and φX174 DNAs as standards (Freifelder, 1970).

RESULTS

Size of Pulse-Labeled DNA

Pulse-labeled PtK DNA synthesized during the first hour after UV irradiation sediments more slowly in alkali than that from nonirradiated cells, as shown by the typical data in Fig. 1 *a,b*. The lengths of the pulse label were adjusted to give approximately

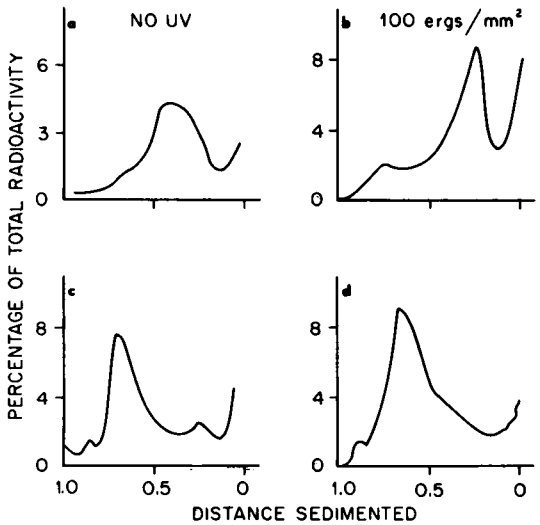

FIGURE 1 Alkaline sucrose gradient profiles of DNA from PtK cells labeled with [^{3}H]thymidine for 0.33 h (*a, c*) or 0.5 h (*b, d*), beginning 0.5 h after irradiation (100 ergs/mm^2). (*a, b*) Sedimentation immediately after the pulse label. (*c, d*) Incubation after pulse labeling for 12 additional h before sedimentation. Total counts per minute for each profile shown were (*a*) 6,090, (*b*) 4,070, (*c*) 3,300, and (*d*) 2,750. The weight-average molecular weights plus or minus one standard deviation from at least four determinations are (*a*) 40 ± 4 × 10^6, (*b*) 10.5 ± 0.5 × 10^6, and (*c, d*) 110 ± 6 × 10^6.

the same amounts of ^3H incorporation per cell in the irradiated and nonirradiated cells, in order to correct for the reduction in the amount of DNA synthesized after UV irradiation.

The size of the newly replicated DNA in the irradiated cells can be correlated with the distance between the dimers in the following manner. UV irradiation with 150 ergs/mm^2 results in 0.068% of the ^3H activity appearing as pyrimidine dimers. This is an average of 4 dimers per 10^7 daltons (Setlow et al., 1969). Irradiation with 100 and 150 ergs/mm^2 results in 1.4 and 1.9 gaps or discontinuities per 10^7 daltons, respectively (see Appendix for a typical calculation) or one gap for every 2.1 dimers in the parental DNA. Due to the relatively long pulse-labeling time, the estimation of the number of gaps per 10^7 daltons is probably too low.

If the irradiated, pulse-labeled cells are incubated an additional 8–12 h before sedimentation, only high molecular weight DNA is observed (Fig. 1 *d*), indicating that the short segments of DNA are elongated and joined as in nonirradiated ones (Fig. 1 *c*).

RESTORATION OF DNA SYNTHESIS IN SEGMENTS OF NORMAL SIZE AFTER IRRADIATION

The ability of irradiated cells to synthesize DNA in segments of normal size can be restored either by dark recovery or by photoreactivation. The first entails incubation of the cells in the dark for long times before pulse-labeling; the second, exposing the irradiated cells to photoreactivating light. Dark recovery is slow, requiring 6–24 h for completion (Fig. 2). The mechanism involved in this recovery is not known; but, as will be shown below, it does not involve excision of dimers.

Exposure of PtK cells to photoreactivating light results in an increase in the size of the DNA synthesized by irradiated cells but does not affect nonirradiated ones. (Fig. 3). The number of discontinuities in the DNA decreases from 2.2 to 1.5 per 10^7 daltons, indicating that ~30% of the interrupting lesions have been removed. On the other hand, the size of the DNA synthesized by human cells is not affected by exposure to photoreactivating light (Fig. 4). Since human cells do not contain photoreactivating enzyme in significant amounts but PtK cells do, and since the enzyme reacts only with and monomerizes pyrimidine dimers (Cook, 1967; Harm et al., 1971; Setlow, 1967, 1972), it is a reasonable inference that pyrimidine dimers are responsible, at least in part, for the small size of the DNA made after UV irradiation. We have used three different experimental techniques in an attempt to make a quantitative correlation between changes in the number of dimers and changes in the size of newly synthesized DNA. These techniques are direct analysis of dimers by chromatography, photolysis of BrdUrd incorporated during repair replication, and treatment of cellular DNA with a repair endonuclease.

Chromatographic Analysis of Dimers

Table I shows the results of radiochromatographic analysis for thymine-containing dimers in irradiated PtK cells treated in various ways. Exposure to photoreactivating light for 1 h removes 35 and 55% of the dimers formed after 250 and 300 ergs/mm^2 of

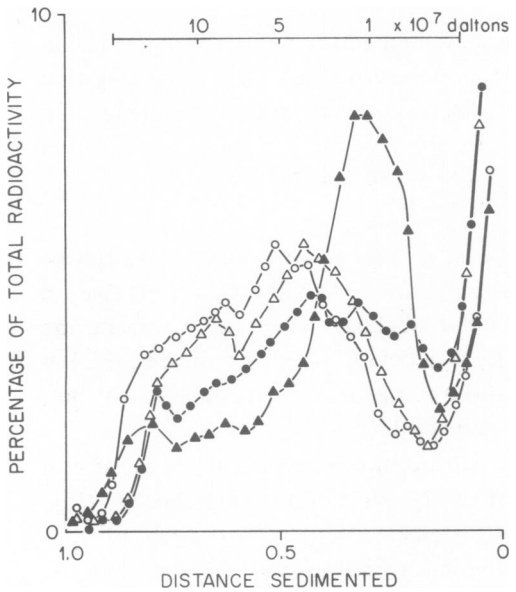

FIGURE 2

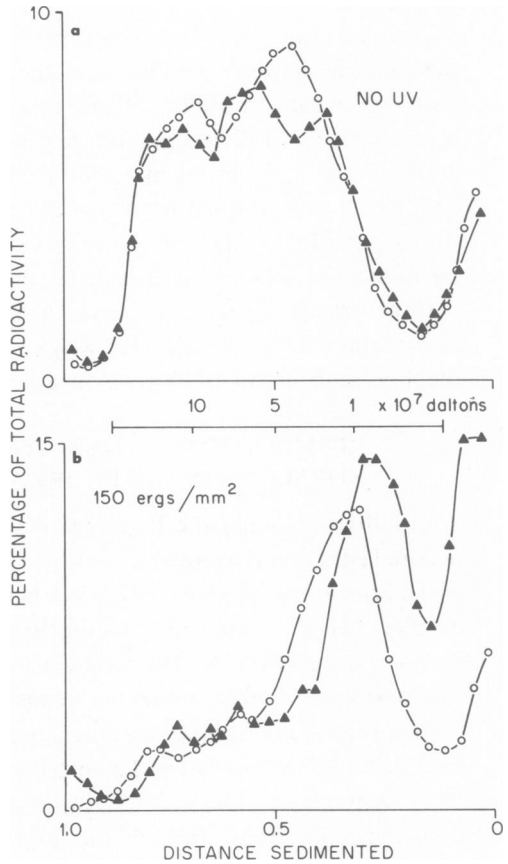

FIGURE 3

FIGURE 2 Alkaline sucrose gradient profiles of DNA from PtK cells that were pulse-labeled for 0.5 h and not irradiated (○) or UV-irradiated (100 ergs/mm^2) and pulse-labeled 0.5 (▲), 6 (●), or 24 (△) h after irradiation. Pulse-labeling was with [^{3}H]thymidine for 0.5 h, and the DNA was sedimented immediately after pulse-labeling. Total counts per minute for each profile shown were (●) 2,670, (▲) 2,550, (○) 4,530, and (△) 3,070. The weight-average molecular weights are (▲) 10.8 × 10^6, (●) 27.0 × 10^6, (○) 54 × 10^6, and (△) 43 × 10^6.

FIGURE 3 Alkaline sucrose gradient profiles of pulse-labeled DNA from PtK cells. Control and irradiated (150 ergs/mm^2) cells were incubated in the dark (▲) or under photoreactivating conditions (○) for 1 h before pulse-labeling with [^{3}H]thymidine for 0.5 h and sedimentation. Total counts per minute for each profile shown were (*b*, ▲) 3,410, (*a*, ▲) 5,350, (*a*, ○) 8,370, and (*b*, ○) 5,030. The weight-average molecular weights plus or minus one standard deviation for at least three experimental determinations of each profile are (*a*, ▲, ○) 56 ± 2 × 10^6, (*b*, ○) 10.8 ± 0.3 × 10^6, and (*b*, ▲) 7.6 ± 0.3 × 10^6.

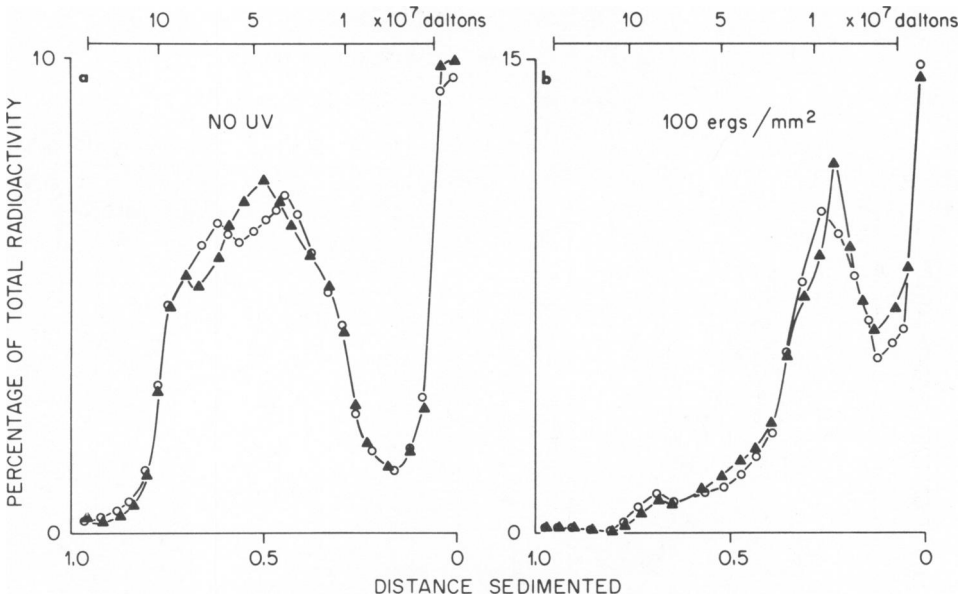

FIGURE 4 Alkaline sucrose gradient profiles of pulse-labeled DNA from SG-XP4 cells. Control and irradiated (100 ergs/mm^2) cells were incubated in the dark (▲) or under photoreactivating conditions (○) for 1 h before pulse-labeling with [^{3}H]thymidine for 0.5 h and sedimentation. Total counts per minute for each profile shown were (*b*, ○) 3,890, (*b*, ▲) 2,720, (*a*, ▲) 6,250, and (*a*, ○) 8,590. The weight-average molecular weights of the profiles are (*a*, ▲) 54 × 10^6, (*a*, ○) 53 × 10^6, (*b*, ○) 10.3 × 10^6, (*b*, ▲) 9.8 × 10^6.

UV, respectively. The difference between the numbers for the two UV doses is due mainly to different cell responses on different days and only slightly to low errors (±10%) in the assays themselves. Incubation for 23 h in the dark results in the loss of only 10–15% of the dimers from the acid-insoluble fractions of both photoreactivated and nonphotoreactivated cells. Such a small change (presumably the result of a low

TABLE I

PERCENTAGE OF ^{3}H RADIOACTIVITY IN THYMINE-CONTAINING DIMERS*

UV dose	No photoreactivation		Photoreactivation	
	1 h after UV irradiation	24 h after UV irradiation	1 h after UV irradiation	24 h after UV irradiation
ergs/mm^2				
250	0.118	0.099	0.078	0.066
300	0.131	0.117	0.057	0.052

*Cells labeled with [^{3}H]thymidine were UV-irradiated and either incubated in the dark for 1 h or exposed to photoreactivating light for 1 h and, immediately or after 23 h of further incubation, analyzed by acid hydrolysis and chromatography for ^{3}H-containing dimers in the acid-insoluble fraction of cells. The percentage of ^{3}H radioactivity in the dimer region of the chromatogram in nonirradiated cells and cells exposed to photoreactivating light only was less than 0.001% in each experiment, and the total counts per minute of [^{3}H]thymidine was between 2.5 and 3.2 × 10^5 for each observation.

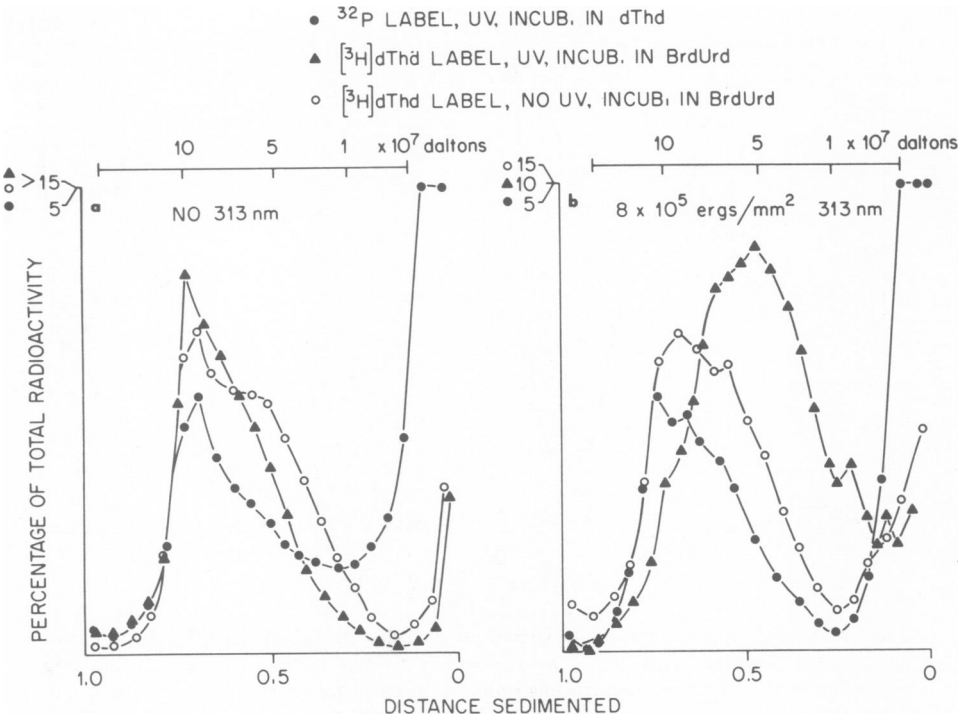

FIGURE 5 Alkaline sucrose gradient profiles of DNA from PtK cells that were treated as indicated for 24 h and then exposed to 313-nm radiation. The UV 254-nm dose was 150 ergs/mm^2. Total counts per minute for each profile shown were (*a*, ○) 7,640, (*b*, ○) 7,400, (*a*, ▲) 18,100, (*b*, ▲) 11,350, (*a*, ●) 22,940, (*b*, ●) 20,210. The weight-average molecular weights plus or minus one standard deviation for at least four experimental determinations of each profile are (*a*, ▲, ○, ●) 110 ± 10^6, (*b*, ●, ○) 97 ± 5 × 10^6, (*b*, ▲) 60 ± 7 × 10^6.

level of excision repair), although difficult to measure quantitatively by direct analysis of dimers, indicates that recovery of the ability to make DNA of normal size (Fig. 2) after UV irradiation is not the result of dimer excision.

Photolysis of BrdUrd-Containing DNA

The low level of excision repair was confirmed by the use of the more sensitive BrdUrd photolysis technique (Regan et al., 1971). Fig. 5 shows DNA profiles from these experiments. The parental DNA of UV-irradiated cells incubated in BrdUrd is more sensitive to 313 nm than the other DNAs. Evaluation of these profiles indicates that 0.12 breaks per 10^7 daltons are introduced into the repaired DNA by photolysis. (Excision proficient human cells show fivefold more breaks after photolysis [Regan et al., 1971].)

Endonuclease Treatment

The removal of dimers by photoreactivation can also be correlated with the loss of sites sensitive to *M. luteus* repair endonuclease (Wilkins, 1974). Fig. 6 shows typical

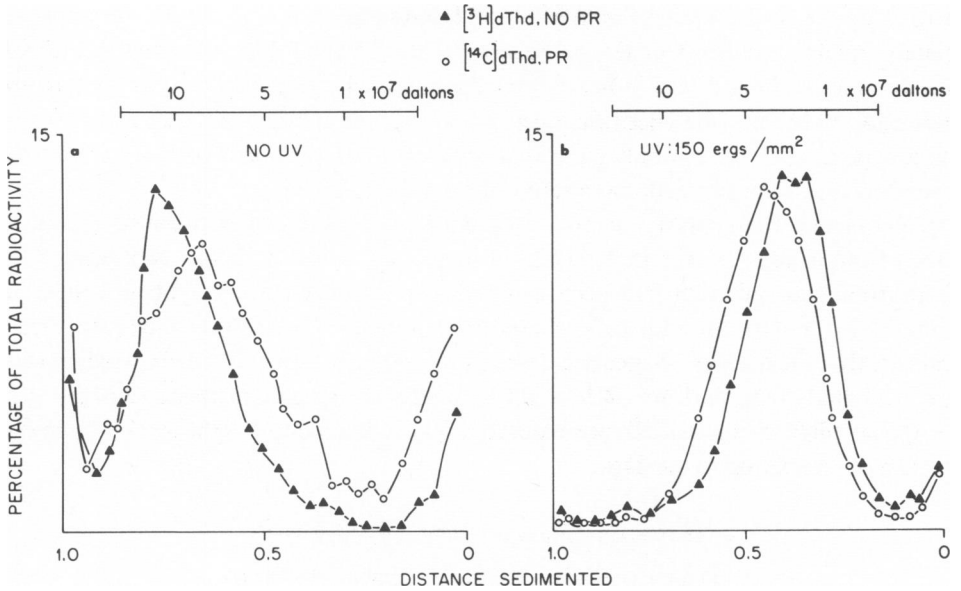

FIGURE 6 Alkaline sucrose gradient profiles of DNA from PtK cells that were prelabeled with [^{3}H]thymidine or [^{14}C]thymidine and photoreactivated or kept in the dark for 1 h after irradiation before incubation with repair endonuclease. The weight-average molecular weights plus or minus one standard deviation for at least four experimental determinations are (*a*, ▲) 110 ± 6 × 10^6, (*a*, ○) 95 ± 4 × 10^6, (*b*, ○) 44 ± 2 × 10^6, and (*b*, ▲) 31 ± 4 × 10^6.

data. Exposure of unirradiated cells to photoreactivating illumination for 1 h results in a small decrease in sedimentation distance, which represents a slight increase in the number of endonuclease-sensitive sites, whereas photoreactivating illumination of UV-irradiated cells results in a significant decrease in endonuclease-sensitive sites. The numbers of sites that disappear as a result of such treatment are calculated in Table II. 48% of the endonuclease-sensitive sites are lost as a result of photoreactivation. Note that the number of sites is only about 1/10 of the number of dimers, indicating that most dimers are not accessible to the endonuclease when this technique

TABLE II

CALCULATION OF ENDONUCLEASE-SENSITIVE SITES PER 10^7 DALTONS OF DNA REMAINING 1 h AFTER IRRADIATION*

Treatment	$(1/M_n) \times 10^7$	
	No photoreactivation	Photoreactivation
(1) UV or no UV, no enzyme	0.18	0.18
(2) No UV, enzyme	0.21	0.21
(3) UV, enzyme	0.64	0.45
Endonuclease-sensitive sites per 10^7 daltons (treatment 3 – treatment 2)	0.43	0.24

*For experimental details see the legend to Fig. 6.

is used in PtK cells, whereas with isolated DNAs the sites and dimers are approximately equal in number (Carrier and Setlow, 1970). The fact that the curves in Fig. 6 *b* are reasonably symmetrical indicates that there is no large group of cells that is impermeable to the enzyme. Because most dimers are not endonuclease-sensitive sites in these experiments, we can only surmise a correlation between loss of dimers and loss of endonuclease-sensitive sites as a result of photoreactivation.

To complete the analysis of repair endonuclease-sensitive sites, we also examined DNA from irradiated cells that had been incubated in the dark for 24 h and DNA from irradiated cells that had been exposed to photoreactivating light and then incubated in the dark for 23 h before enzyme treatment. The results shown in Fig. 7 indicate that the number of sites decreases from 0.30 to 0.18 per 10^7 daltons when cells are incubated in the dark for 24 h, and that nearly all the endonuclease-sensitive sites disappear when irradiated cells are exposed to photoreactivating light for 1 h followed by 23 h of incubation in the dark.

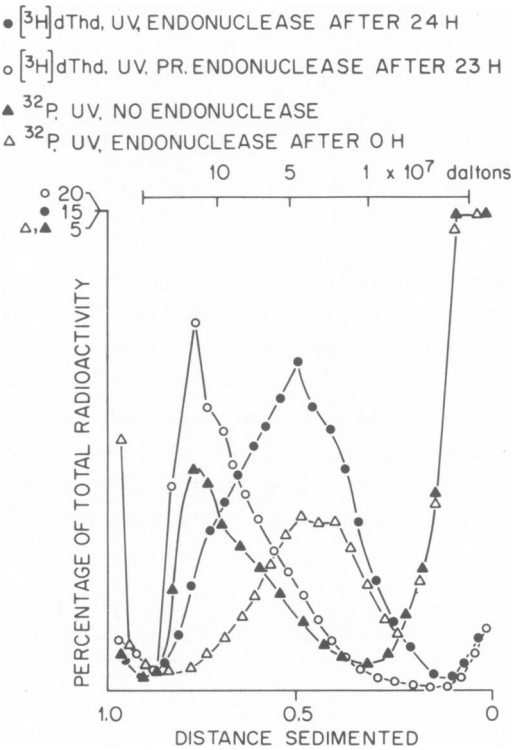

FIGURE 7 Alkaline sucrose gradient profiles of DNA from PtK cells that were prelabeled with either [^{3}H]thymidine or ^{32}P; UV-irradiated (150 ergs/mm^2); and incubated with repair endonuclease at 0 or 24 h, or at 23 h after 1 h of photoreactivation. (▲) Note treated with repair endonuclease, sedimented 24 h after UV. (●, △) Cells combined before treatment with the endonuclease, treated and cosedimented. (▲, ○) Cells mixed after their respective treatments and cosedimented. The weight-average molecular weights for each profile shown are (○) 115 × 10^6, (▲) 116 × 10^6, (△) 42 × 10^6, and (●) 57 × 10^6.

DISCUSSION

DNA synthesized by irradiated PtK cells is made in segments equal to the average distance between 1 or 2 dimers in the parental DNA. These data are consistent with suggestions, made on the basis of similar data in other mammalian cells, that pyrimidine dimers interrupt normal DNA replication and result in gaps of $\sim 10^3$ nucleotides in the newly synthesized DNA (Lehmann, 1972; Buhl et al., 1972 *a,b*). Upon incubation these short segments become larger and form high molecular weight DNA as in nonirradiated cells, but the mechanism of that growth has not been investigated to determine whether it is similar to that in *E. coli*—involving DNA exchanges between parental and newly synthesized DNA (Rupp and Howard-Flanders, 1968; Iyer and Rupp, 1971)—or that in other mammalian cells—involving insertion of exogenous nucleotides into the gaps in the DNA (Lehmann, 1972; Buhl et al., 1972 *b*). The observation that photoreactivating light specific for monomerizing pyrimidine dimers results in an increase in the size of the newly synthesized DNA commensurate with the percentage of dimers monomerized is direct evidence for the conclusion that dimers are responsible for the interruptions in DNA synthesized shortly after UV irradiation in mammalian cells.

Monomerization of dimers is not the only mechanism by which irradiated PtK cells can recover the ability to synthesize DNA in segments of nearly normal size. Cells incubated in the dark for 24 h after irradiation synthesize DNA in normal sizes, even though over 85% of the dimers remain in the DNA. Similar results have been obtained with other mammalian cells (Meyn and Humphrey, 1971; Lehmann and Kirk-Bell, 1972; Buhl et al., 1973). The recovery mechanism is not known, but we have suggested that dimers may be altered in some manner so that normal DNA replication can proceed without an interruption (Buhl et al., 1973).

The three methods (chromatographic detection, BrdUrd photolysis, and endonuclease treatment) used to determine the numbers of dimers and the changes that follow treatment of the cells yield similar results. They all indicate, for example, that excision repair removes only a small percentage of dimers in PtK cells (10–20% in 24 h).

Photoreactivating illumination results in a loss of dimers (chromatographically) and a loss of repair endonuclease-sensitive sites. The data indicate that the *M. luteus* endonuclease recognizes (at least some) dimers as lesions in UV-irradiated mammalian DNA, although only one break is introduced for every 8–10 dimers in the DNA. On the other hand, the endonuclease does not introduce measurable numbers of breaks into the DNA of irradiated cells that have been exposed to photoreactivating light and then incubated in the dark for an additional 23 h, even though $\sim 50\%$ of the dimers remain in the DNA after such treatment. These data show that there is not a unique correlation between dimers and endonuclease-sensitive sites in PtK cells. Perhaps not all dimers are susceptible to endonuclease (although they seem to be for DNA irradiated in vitro), or perhaps only a small, possibly discrete, fraction is accessible to the repair endonuclease in vivo.

The interrupting effects on DNA replication caused by dimers can be ameliorated

in three ways: monomerization of the dimers by photoreactivation, removal of the dimers by excision repair, and modification of the dimer-containing regions by a mechanism that is as yet unknown. Our observations suggest that any dimer can be acted on by any of these repair systems. The repair system that acts on a particular dimer is probably determined by the dimer's immediate environment, which changes with its position in the cell cycle and/or the extent of aggregation or coiling of the chromatin at the particular time after irradiation when the repair systems operate.

APPENDIX

Calculations of Breaks

For convenience of interpretation the data have been reduced to the form of discontinuities or breaks per 10^7 daltons of DNA. The reciprocal of the number-average molecular weight (M_n) gives the number of discontinuities per dalton. However, calculation of M_n directly from the gradient data is subject to large errors for DNAs that sediment near the top of the gradient. Hence, we have calculated the weight-average molecular weight (M_w) from the gradient data and assumed that $M_n = M_w/2$. The assumption is a poor one for the obviously nonrandom distributions in Figs. 1 *a, b,* 2, and 3 *b,* so for those gradients the material in the high molecular weight shoulders was not used in the calculation. Since calculated values of M_w are very dependent on the larger molecular weights, this procedure results in a largely (~50%) underestimated M_w, which is designated M'_w. However, the value of M_n ($=M'_w/2$) is only 10–20% lower than the value calculated directly from the entire gradient. We prefer the smaller value, since it does not include the contributions of the few segments rejoined during the radioactive pulse.

The number of discontinuities on the newly synthesized DNA as a result of synthesis from irradiated DNA templates is taken as $[1/M_{n(\text{irradiated})} - 1/M_{n(\text{nonirradiated})}] \times 10^7$. For example, in Figs. 1 *a, b* the values of M_n the values of M_n for nonirradiated and irradiated samples are 20×10^6 and 5.2×10^6, respectively. The difference between the reciprocals of these numbers, 0.14×10^{-6}, represents 1.4 discontinuities per 10^7 daltons.

The number of breaks per 10^7 daltons resulting from endonuclease treatment or 313-nm photolysis of the DNA was calculated for Figs. 5, 6, and 7 as $[1/M_{n(\text{treated})} - 1/M_{n(\text{untreated})}] \times 10^7$.

We are grateful to Drs. J. S. Cook and J. K. Setlow for their comments on the manuscript.

At the time of this research Dr. Buhl was a predoctoral trainee supported by grant GM1974 from the National Institute of General Medical Sciences, National Institutes of Health.

Oak Ridge National Laboratory is operated by Union Carbide Corporation for the United States Atomic Energy Commission.

Received for publication 10 June 1974 and in revised form 25 July 1974.

REFERENCES

ABELSON, J., and C. A. THOMAS. 1966. *J. Mol. Biol.* **18**:262.

BOLLUM, F. J. 1966. *In* Procedures in Nucleic Acid Research. G. L. Cantoni and D. R. Davies, editors. Harper and Row, Publishers, New York. 296.

BUHL, S. N., R. B. SETLOW, and J. D. REGAN. 1972 *a. Int. J. Radiat. Biol.* **22**:417.

BUHL, S. N., R. B. SETLOW, and J. D. REGAN. 1973. *Biophys. J.* **13**:1265.

BUHL, S. N., R. M. STILLMAN, R. B. SETLOW, and J. D. REGAN. 1972 *b. Biophys. J.* **12**:1183.

CARRIER, W. L., and R. B. SETLOW. 1970. *J. Bacteriol.* **102**:178.
CARRIER, W. L., and R. B. SETLOW. 1971 *a. Methods Enzymol.* **21**:13.
CARRIER, W. L., and R. B. SETLOW. 1971 *b. Anal. Biochem.* **43**:427.
CHIU, S. F. H., and A. M. RAUTH. 1972. *Biochim. Biophys. Acta.* **259**:164.
CLEAVER, J. E. 1968. *Nature (Lond.).* **218**:652.
CLEAVER, J. E., and G. H. THOMAS. 1969. *Biochem. Biophys. Res. Commun.* **36**:203.
COOK, J. 1967. *Photochem. Photobiol.* **6**:97.
COOK, J. 1970. *Photophysiology.* **5**:191.
COOK, J., and J. D. REGAN. 1969. *Nature (Lond.).* **223**:1066.
DULBECCO, R., and M. VOGT. 1954. *J. Exp. Med.* **99**:167.
EAGLE, H. 1959. *Science (Wash. D.C.).* **130**:432.
FREIFELDER, D. 1970. *J. Mol. Biol.* **54**:567.
FUJIWARA, Y., and T. KONDO. 1972. *Biochem. Biophys. Res. Commun.* **47**:557.
HARM, W., C. S. RUPERT, and H. HARM. 1971. *Photophysiology.* **6**:279.
IYER, V. N., and W. D. RUPP. 1971. *Biochim. Biophys. Acta.* **228**:117.
KRISHNAN, D., and R. B. PAINTER. 1973. *Mutation Res.* **17**:213.
LEHMANN, A. R. 1972. *J. Mol. Biol.* **66**:319.
LEHMANN, A. R., and S. KIRK-BELL. 1972. *Eur. J. Biochem.* **31**:438.
LEHMANN, A. R., and M. G. ORMEROD. 1970. *Biochim. Biophys. Acta.* **204**:128.
MEYN, R. E., and R. M. HUMPHREY. 1971. *Biophys. J.* **11**:295.
PATRICK, M. H., and H. HARM. 1973. *Photochem. Photobiol.* **18**:371.
RAUTH, A. M., M. TAMMEMAGI, and G. HUNTER. 1974. *Biophys. J.* **14**:209.
REGAN, J. D., R. B. SETLOW, and R. D. LEY. 1971. *Proc. Natl. Acad. Sci. U.S.A.* **68**:708.
REGAN, J. D., J. E. TROSKO, and W. L. CARRIER. 1968. *Biophys. J.* **8**:319.
RUPP, W. D., and P. HOWARD-FLANDERS. 1968. *J. Mol. Biol.* **31**:291.
RUPP, W. D., E. ZIPSER, C. VON ESSEN, D. RENO, L. PROSNITZ, and P. HOWARD-FLANDERS. 1969. *Brookhaven Natl. Lab. Publ. BNL 50203 (C-57).* 1.
SETLOW, J. K. 1967. *Comp. Biochem.* **27**:157.
SETLOW, J. K. 1972. *Res. Prog. Org. Biol. Med. Chem.* **3**(1):335.
SETLOW, R. B. 1968. *Prog. Nucleic Acid Res. Mol. Biol.* **8**:257.
SETLOW, R. B., J. D. REGAN, and W. L. CARRIER. 1972. *Biophys. Soc. Annu. Meet. Abstr.* **12**:19 *a.*
SETLOW, R. B., J. D. REGAN, J. GERMAN, and W. L. CARRIER. 1969. *Proc. Natl. Acad. Sci. U.S.A.* **64**:1035.
SMITH, K. C., and O. H. C. MEUN. 1970. *J. Mol. Biol.* **51**:459.
WILKINS, R. J. 1974. *Nature (Lond.).* **274**:35.