

CORRELATION AMONG THE RATES OF DIMER EXCISION, DNA REPAIR REPLICATION, AND RECOVERY OF HUMAN CELLS FROM POTENTIALLY LETHAL DAMAGE INDUCED BY ULTRAVIOLET RADIATION

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ABSTRACT The kinetics of excision repair in confluent cultures of diploid human fibroblasts after ultraviolet irradiation at varying doses was measured by three different methods: (a) removal of thymine-containing dimers, (b) DNA excision repair synthesis, and (c) biological recovery of cells from the potentially lethal effects of the irradiation. Each method gave similar results and indicated that the excision rate was dependent upon the number of thymine-containing dimers induced (substrate concentration). For example, at a dose of 40 J/m^2 (0.2% dimerization), the repair rate was 1.6 J/m^2 per h as determined by a modified method to measure the number of thymine-containing dimers remaining in DNA and 1.65 J/m^2 as measured by excision repair synthesis. At a dose of 7.5 J/m^2 , the repair rate was 0.5 J/m^2 per h as measured by biological recovery, and at a dose of 7 J/m^2 , the repair rate was 0.46 J/m^2 per h as measured by excision repair synthesis.

INTRODUCTION

Irradiation of cells in culture with ultraviolet (UV) (254 nm) light induces pyrimidine dimers (1). Evidence that DNA excision repair of this damage is induced in human and other mammalian cells immediately after irradiation has been obtained by measuring unscheduled DNA synthesis (2–4), repair replication in parental DNA using 5-bromodeoxyuridine (BrdUrd)—CsCl density gradient centrifugation (5, 6), and the disappearance of *Micrococcus luteus*- or bacteriophage T₄ endonuclease-sensitive sites from DNA (7–9). The reported rates of physical removal of pyrimidine dimers from the DNA of UV-irradiated human cells range from 90% in 1 h with a dose of 10 J/m^2 (10) to <50% in 48 h with a dose of 40 J/m^2 (11). Ehmann et al. (11), for example, observed a slow rate of pyrimidine dimer removal in human fibroblasts (GM38) with a period of 6 h occurring before the first excised dimers could be detected. In contrast, they reported that the time required for half-maximal repair

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synthesis (i.e., unscheduled DNA synthesis measured by autoradiography) was 4.5 h. Williams and Cleaver (8) found a similar inconsistency between the slow rate of dimer excision and the rapid rate of loss of endonuclease-sensitive sites from DNA of UV-irradiated African green monkey kidney CV-1 cells and suggested that "dimer excision requires two stages, one detectable by endonuclease-sensitive site removal from high molecular weight DNA and repair replication and the second by thin-layer chromatography (TLC) of dimers excised into acid-fragments."

While these investigations were in progress, we were also carrying out a study with human fibroblasts to compare the rates of removal of DNA lesions induced by several carcinogens, including UV radiation, with both the rate of repair synthesis and the rate of recovery of cells from the potentially lethal effects of the carcinogen (12-14). Because we obtained a good correlation between rate of dimer removal and rate of UV-induced repair replication, we compared our method of determining loss dimers with those used by Ehmann et al. (11) and by Williams and Cleaver (8). The results suggest that the differences observed are related to the methodology. A preliminary report of our studies of rate of dimer excision has already been presented (15).

MATERIALS AND METHODS

Cells and Cell Growth

Cultures of diploid fibroblasts derived from the skin of normal persons were prepared and stored as described (16). The cells were grown in Eagles minimal essential medium (MEM) or Ham's F10, supplemented with 15% fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.) and antibiotics, as described (17).

Irradiation with UV Light

The medium was removed from the dishes and the cells were rinsed twice with phosphate-buffered saline (PBS) at 37°C, drained, and irradiated with the indicated doses of UV at an incidence of 0.1—0.25 J/m² per s. Fresh medium at 37°C was added after the irradiation. The incident radiation at 254 nm was determined by an IL 570 model International Light radiometer (International Light, Inc., Newburyport, Mass.).

Recovery of Cells from Potentially Lethal DNA Damage

A series of cultures was grown to confluence, maintained in that state for at least 3 d without refeeding, and irradiated with the indicated doses of UV. One set of cells was immediately released from density inhibition of cell replication and assayed for percent survival of cloning ability by plating at low density. At various times postirradiation, another set of cells was released from confluence and plated at cloning densities. Unirradiated control cultures were treated similarly. During the postirradiation period, confluent cultures were refed with "spent medium," i.e., mitogen-free medium collected from rapidly growing stock cultures. Feeding confluent cultures with this medium does not result in stimulation of DNA replication (18). Following release from confluence, cells received fresh culture medium, which was renewed twice weekly until visible clones developed (\approx 14 d) and were stained as described (19). The cytotoxic effect of UV-irradiation was determined from the percent survival of the colony-forming ability of the irradiated cells compared with that of the unirradiated controls. The cloning efficiency of the unirradiated control cells ranged between 30 and 50%.

Preparation of Cultures for Measurement of Thymine Dimer Excision Rate

Approximately 1×10^6 cells from early passage (5-15th) (generation time, 24h) were plated into 150-mm diameter culture dishes, allowed to grow for 3 d, and then labeled for 24 h with $1 \mu\text{Ci/ml}$ [^3H]-thymidine ([^3H]dThd) (specific activity 50 Ci/mmol, Schwarz/Mann, Div. Becton, Dickinson & Co., Orangeburg, N.Y.). After that period, fresh medium not containing the radioactive label was added and the cells were allowed to grow to confluence (one and a half to two more population doublings). After 3 d in confluence without refeeding, the cells were washed, irradiated as described, and allowed to carry out excision for various periods of time.

DNA Isolation and Hydrolysis

At various times postirradiation, cells were harvested and the DNA was isolated as follows. Cells lysed in 0.1% sodium lauryl sarcosine (Sigma Chemical Co., St. Louis, Mo.) were incubated with 20 U/ml of *Aspergillus* T₁ RNase (Sigma Chemical Co.) and 20 $\mu\text{g/ml}$ of pancreatic RNase (Worthington Biochemical Corp., Freehold, N.J.) for 30 min at 37°C and then for 1 h at 37°C with 0.1 mg/ml of pronase (Calbiochem-Behring Corp., San Diego, Calif.). The DNA was then extracted twice with a 24:23:1 mixture of Kirby phenol, chloroform (Mallinckrodt Inc., St. Louis, Mo), and butanol. Kirby phenol contained 14.3 ml of cresol (Matheson, Coleman and Bell, Northwood, Ohio), 85.7 ml of liquid phenol (Fisher Scientific Co., Pittsburgh, Pa.), and 0.2 g of 8-hydroxyquinoline. After extraction the DNA was dialyzed extensively against standard saline citrate (SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.4) at 4°C and precipitated with 2% ice-cold trichloroacetic acid. A sample of the acid-soluble and acid-insoluble fraction was measured for radioactivity in 15 ml of tritosol scintillation fluid (20) using a Packard Tri Carb Liquid Scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The remaining acid-precipitable fraction was hydrolyzed in 97% formic acid for 60 min at 178°C. The hydrolysate was dried under a stream of N₂ gas, redissolved in water, and analyzed for its thymine-containing dimer content by TLC using the procedure of Cook and Friedberg (21). The average standard deviation of the percent radioactivity remaining in thymine-containing dimers at several times after irradiation was 9.2%. As a standard, thymine dimers chemically prepared from thymine according to the method of Günther and Prusoff (22) were used. In certain indicated experiments, the kinetics of thymine dimer excision was measured by the method described by Ehmann et al. (11) or Williams and Cleaver (8) for the purpose of comparing our method with theirs.

Determination of DNA Repair Synthesis

Excision repair of lesions formed in DNA by UV irradiation was determined by measuring the incorporation of [^3H]-dThd into parental strands using a modification of the alkaline BrdUrd-CsCl gradient centrifugation technique of Painter and Cleaver (23). Approximately 1×10^6 cells were seeded into 150-mm diameter plastic culture dishes (Falcon Labware, Oxnard, Calif.), allowed to reach confluence, and maintained at confluence without refeeding for about 3 d. 2 h before irradiation the cells were preincubated with culture medium supplemented with 25 μM BrdUrd, 0.1 μM aminopterin (Sigma Chemical Co.), and 25 μM hypoxanthine (Calbiochem). Following irradiation they were allowed to carry out excision repair for the indicated periods of time in the presence of the same medium further supplemented with 15 $\mu\text{Ci/ml}$ [^3H]-dThd. The DNA was extracted as described above. Density-labeled daughter DNA was separated from DNA of normal buoyant density (parental DNA) by isopycnic centrifugation for 60 h in alkaline CsCl at 20°C at 40,000 rpm using a 50 Ti rotor in a Beckman L5-50 centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). Each centrifuge tube contained 2.5 ml of the DNA sample in SSC, 2.0 ml of 0.0375 M sodium phosphate (pH 12.4), and 6.27 g solid CsCl. The parental DNA was pooled, dialyzed exhaustively against SSC, and rebanded under the same conditions. After the second centrifugation, the gradients were fractionated using a Densiflow (Buchler Instruments, Div. Searle Diagnostics, Inc., Fort Lee, N.J.), and the optical density profile was

determined at 260 nm with a Beckman model 25 spectrophotometer fitted with a flow cell. 1 ml of water and 15 ml of tritosol were added and the radioactivity was determined. Results were expressed as counts per minute per microgram of DNA recovered from the gradient. The total amount of parental DNA recovered was determined from the optical density profile by comparing the area under the curve with the area obtained with known amounts of DNA under similar conditions, using a molar absorbance of 8.13×10^3 at 260 nm per base of DNA dialyzed against SSC.

RESULTS

Rate of Excision of Thymine-Containing Dimers by Normal Fibroblasts

The rate of excision of dimers induced in confluent cultures of human skin fibroblasts by 40 J/m² of UV as measured by the method described in Materials and Methods is presented in Fig. 1A. The values reported are the mean and standard deviation of four to six independent experiments for times greater than zero and of 11 independent experiments for the initial level of dimerization. Irradiation of these cells with 40 J/m² resulted in an average dimerization of 0.2% of thymine. The values ranged from 0.18 to 0.22. These values are similar to those reported by Ehmann et al. (11) and by Williams and Cleaver (8). We prelabeled the cells with comparatively low concentrations of [³H]-dThd (1 μ Ci/ml) to avoid a high incidence of

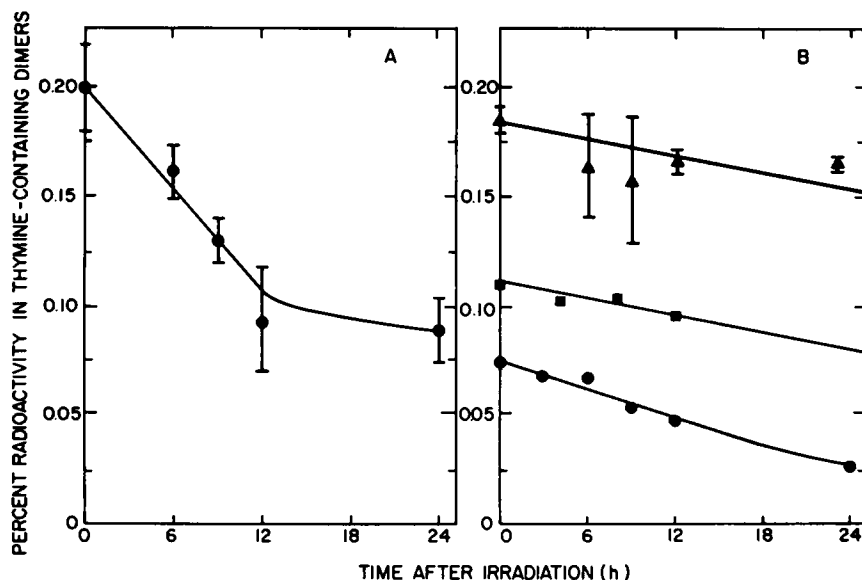


FIGURE 1 Kinetics of excision repair of thymine-containing dimers in human fibroblasts. Curves fitted by eye. (A) ●, cells, prelabeled with 1 μ Ci/ml [³H]-dThd were irradiated after a chase period with a UV dose of 40 J/m². DNA was isolated, hydrolyzed, and assayed on TLC for the amount of thymine-containing dimers. The data are presented as the mean and standard deviation of 11 independent experiments for the initial induction and 4–6 independent experiments for each time-point. (B) ▲, cells, prelabeled with 10 μ Ci/ml [³H]-dThd, were irradiated without a chase period with a UV dose of 40 J/m². DNA was isolated, hydrolyzed, and assayed as described above. The data are presented as the mean and standard deviation of four experiments. ■, cells, prelabeled with 1 μ Ci/ml [³H]-dThd, were irradiated after a chase period, with a UV dose of 20 J/m². Cells were hydrolyzed and assayed as described above. ●, cells prelabeled with 1 μ Ci/ml [³H]-dThd, were irradiated after a chase period with a UV dose of 20 J/m². DNA was isolated, hydrolyzed, and assayed as described above.

single-strand breaks induced by the ^3H disintegration which takes place when high specific activities are used. Further growth in unlabeled medium before irradiation was allowed to deplete the intracellular nucleotide precursor pools of this label and to enable all labeled DNA to attain a high molecular weight. This growth period also puts most of the radioactive label into the parental DNA strands. Thus only thymine-containing dimers induced in the parental DNA will be detected. This method is similar to the method described in the following section which measures repair replication in the parental strands. Before determining the percent of thymine-containing dimers remaining in DNA by TLC, we isolated and hydrolyzed the DNA from the cells rather than nuclei (10) or whole cells (8, 11).

Fig. 1A indicates that from a level of 0.2% dimerization, the number is reduced to 0.1% in ≈ 12 h (i.e., from the dimer equivalent of 40 J/m^2 to that of 20 J/m^2) to give an average rate of $\approx 1.6 \text{ J/m}^2$ equivalents per h. This contrasts with the observations of Ehmann et al. (11), who report that excision of dimers by GM38 cells cannot be detected during the first 6 h after irradiation with 40 J/m^2 (initial level, 0.21% thymine as dimers). For purposes of comparison, we also measured the excision of dimers in cells irradiated with a lower dose (20 J/m^2) in which the percent thymine as dimers was 0.075%. The results are shown in Fig. 1B, lowest curve. It can be seen that although the rate of excision is slower than that expected from repair synthesis experiments (see next section), there is still a significant reduction in the number of dimers in the first 9 h (35%). However, if we labeled with $10 \mu\text{Ci/ml}$ [^3H]-dThd, omitted the chase period, and irradiated cells immediately after the labeling period, we observed that even at an initial dimerization level of 0.18% (40 J/m^2), the kinetics of dimer removal was slow, and after 24 h of incubation, only 14% of the dimers appear to have been rendered acid-soluble (see Fig. 1B, highest curve).

If we measured the rate of dimers excised from hydrolyzed whole cells rather than from hydrolyzed DNA isolated from irradiated cells, we obtained the results shown in Fig. 1B, middle curve. In this experiment, cells were labeled with $1 \mu\text{Ci/ml}$ [^3H]-dThd and allowed a period of growth in unlabeled medium as described in reference 8. The initial level of dimerization was 0.11%. Using this method, a half-maximal excision time of ≈ 24 h was determined. Considering experimental variability in the initial number of dimers and the variability in measuring the number of dimers remaining after different periods of time, we concluded that this method gives results comparable to those of Fig. 1B, lowest curve.

Rate of DNA Excision Repair Replication Induced by UV-Irradiation in Confluent Cultures of Human Cells

As an independent biochemical measurement of the rates of excision repair by these human fibroblasts, we determined the rates of incorporation of [^3H]-dThd into parental DNA after irradiation with various levels of UV light (Fig. 2). For these studies, as for the dimer removal experiments, the cells were grown to confluence and irradiated in the confluent state. In measuring the kinetics of repair replication, the deoxynucleotide precursor pool for DNA polymerization was controlled by the composition of the medium (see Material and Methods) to insure that the specific activity of the [^3H]-dThd used to measure repair replication during the course of the experiment remained constant. Detailed studies validating the use of this medium to quantitate DNA repair synthesis has been submitted for publication elsewhere.¹

The data in Fig. 2 can be analyzed in terms of dimer equivalents excised per hour if one assumes that following exposure to 7 J/m^2 essentially all the dimers have been removed when

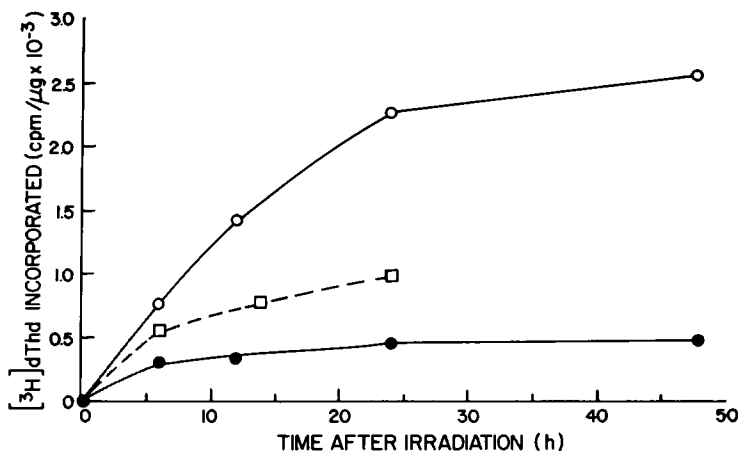


FIGURE 2 Rate of incorporation of [^3H]-dThd into parental DNA by repair replication. Cells were plated into dishes, grown to confluence, irradiated with 40 J/m² (O), 15 J/m² (□), or 7 J/m² (●) of UV radiation, and incubated for the indicated time periods in labeling medium. The DNA was extracted, and incorporation of [^3H]-dThd into parental DNA was determined as described in Materials and Methods.

repair synthesis is complete. The cells excised 50% of the DNA damage (3.5 J/m² worth) in 7.5 h to give a rate of about 0.46 J/m² equivalents per h. Assuming that the same number of counts per minute per microgram correspond to repair of the same number of dimers at the other doses as it does for 7 J/m² (490 cpm/μg for 7 J/m² or 70 cpm/μg per J/m²), then the rate of repair synthesis induced by 15 J/m² is 1.25 J/m² per h and that induced by 40 J/m² is 1.65 J/m² per h. For cells irradiated with 40 J/m², the rate of excision as determined by repair synthesis is in close agreement with the initial rate of dimer excision shown in Fig. 1A. The rate at 15 J/m², however, is faster than that calculated from the dimer excision data shown in Fig. 1B, lowest curve.

Rate of Biological Recovery of UV-Irradiated Density Inhibited Noncycling Human Cells

Recent reports by Simons (24) and Maher et al. (18) describe a method of measuring recovery of human cells from potentially lethal DNA damage (liquid holding recovery [24]) after UV irradiation. When cells were held in a replication-inhibited, confluent state, a time-dependent gradual increase in their survival up to 100% was observed. The increase in survival appears to be the result of excision repair of thymine-containing dimers during the time held in confluence because XP cells with virtually no dimer excision repair capability (from complementation group A) exhibited no (18) or only a very small amount (24) of biological recovery under these conditions. These results indicate that it is possible to determine the number of potentially cytotoxic lesions originally present in a UV-irradiated population of confluent cells and the number remaining at various times postirradiation by assaying the population for percent survival. The survival values of such populations can then

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be related to "dimer equivalents of DNA damage." Use of this method can, therefore, yield information on the rate of removal of potentially lethal DNA damage by cells during the period in which they are being held confluent. The rate of recovery can then be compared with the rate of repair replication (Fig. 2) and of dimer excision (Fig. 1) following irradiation with similar doses.

We applied this method and obtained the results shown in Fig. 3. Cultures of cells after 3 d in confluence were irradiated with 5, 7.5, 18, and 24 J/m² and assayed for survival immediately and at later times. Their initial survivals were 40, 20, 5, and 2%, respectively, but if the cultures were held in confluence, i.e., kept from replicating, they exhibited increasingly higher levels of survival with time. These results can be analyzed for rate of dimer removal as follows: cultures irradiated with 7.5 J/m² show an initial survival of 20%, but if allowed 6 h at confluence before being assayed, they exhibit a survival of 40%, the same as that shown by cultures initially irradiated with only 5 J/m² and released immediately. Thus, these confluent cells removed the dimer equivalent of 2.5 J/m² in 6 h which is a rate of ≈ 0.42 J/m² per h. This corresponds closely to the initial rate of excision exhibited by cells irradiated with 7 J/m², viz., 0.46 J/m² dimer equivalents per h, Fig. 2. Similarly, cells irradiated with 18 J/m² exhibit 5% survival if assayed immediately, but show a survival of $\approx 18\%$ if allowed 12 h for excision repair. A survival of 18% corresponds to a UV dose of ≈ 9 J/m². Thus, the cells removed the dimer equivalent of 9 J/m² in 12 h, a rate of 0.75 J/m² per h.

The increase in survival of the irradiated cultures cannot be attributed to cell turnover resulting from loss of the density inhibition because autoradiographic studies indicated that

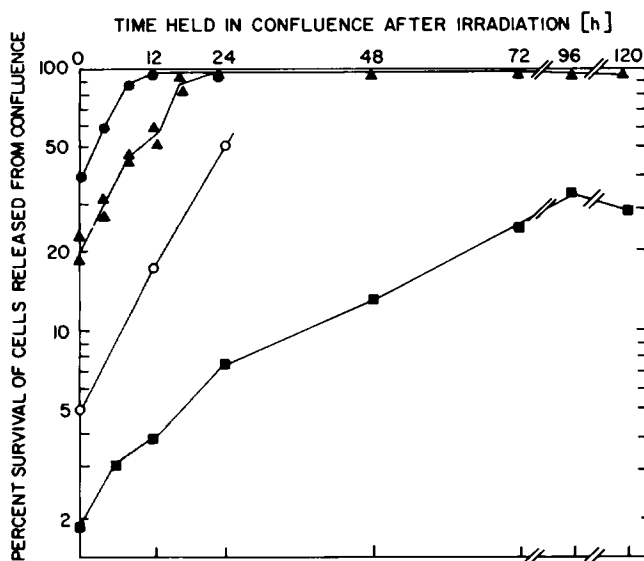


FIGURE 3 Recovery from potentially lethal DNA damage in human fibroblasts after irradiation with UV. Cells in confluence were irradiated and allowed the designated times to repair before they were released and assayed for survival of cloning ability. The cells were irradiated with 5 J/m² (●), 7 J/m² (▲), 18 J/m² (○), and 24 J/m² (■), respectively. Each point was accompanied by an unirradiated, but identically treated control to correct for the cloning ability. Each experimental point represents the mean value among 12 identical dishes. See Materials and Methods for experimental details.

there was no increase in scheduled DNA synthesis as a result of UV irradiation (18). Fig. 3 also indicates that cells that have received sufficient damage to reduce the initial survival to 5% did not reach 33% survival even if held in the nonreplicating state for up to 120 h. A similar inability to reach 100% survival after exposure to 12 J/m² of UV was reported by Weichselbaum et al. (25). That this inability to reach 100% survival was not caused by maintaining cells in confluence for 7 d was shown by the observation that the cloning efficiency of the unirradiated control cultures (45%) did not vary significantly over this period of time.

A comparison of the rates of excision repair in human fibroblasts after irradiation with different UV doses as measured by the described methods is presented in Table I. The rate of excision is seen to be dose dependent; a faster rate was observed in cells exposed to high doses, a slower rate in those irradiated with low doses.

DISCUSSION

The results reported in this study are consistent with the hypothesis that the excision of pyrimidine dimers followed by the resynthesis of the excised region leads to the biological recovery of human cells from potentially lethal DNA damage. Similar dose-dependent kinetics of excision repair were observed using three different experimental approaches (Table I). The rate of repair of UV-induced damage we observed using repair replication agrees with previously published rates of repair as measured by repair replication (5) (incorporation of [³H]dThd into parental DNA), by unscheduled DNA synthesis (11) (autoradiography), or by the removal of *Micrococcus luteus* endonuclease-specific sites (7). The rate of removal of thymine-containing dimers in human diploid cells falls within the range reported in the literature. Amacher et al. (10) observed a much faster rate of removal of pyrimidine dimers than we observed, whereas Regan et al. (26), Setlow et al. (27), and Ehmann et al. (11) observed rates that were either comparable or slower. For example, the kinetics described by Regan et al. (26) for initial dimerization of 0.079% do not differ significantly from those of Fig. 1B (lowest curve), which shows an initial dimerization of about 0.075%. However, the

TABLE I
DOSE-DEPENDENT INITIAL EXCISION REPAIR RATES DETERMINED BY VARIOUS
METHODS IN HUMAN FIBROBLASTS

| Dose | Rate of repair (dimer equivalents removed per h) | Method used |
|------------------|---|---------------------|
| J/m ² | (J/m ² /h) | |
| 40 | 1.6 | Dimer removal |
| 40 | 1.65 (1.9)* | Repair replication |
| 18 | 0.75 | Biological recovery |
| 15 | 1.25 | Repair replication |
| 7.5 | 0.5 | Biological recovery |
| 7 | 0.46 | Repair replication |
| 5 | 0.25 | Biological recovery |

*A value of 1.65 J/m² per h was obtained using the experimental result that 490 cpm/μg corresponds to complete repair of damage induced by 7 J/m² (70 cpm/μg per J/m², Fig. 2 lowest curve.) A value at 1.9 was obtained assuming that repair was complete at 48 h for 40 J/m².

latter rate is less than one would predict from repair replication data (Fig. 2). This may reflect the difficulty in obtaining accurate measurements of the number of dimers remaining in DNA after low doses of radiation. There was good agreement between the two methods at the higher dose (40 J/m²).

A difference between dimer excision and repair replication experiments is that the former require high levels of [³H]-dTd to be incorporated into cellular DNA before irradiation. Disintegration of ³H in the DNA induces single-strand breaks (28), and the accumulation of breaks might interfere with excision repair of dimers. In a very careful study, however, Williams and Cleaver (8) preirradiated cells with a dose of x rays. This introduces as many single-strand breaks into the DNA as does prelabeling with [³H]-dTd at 5 μ Ci/ml and measured repair replication induced by UV light. No significant difference between the control cultures and the x-irradiated cultures could be demonstrated, suggesting that induction of strand breaks by ³H does not account for the relatively slow excision of dimers observed (8). It could be that the single-strand breaks only interfere with the excision-step, but not with the polymerization step. This seems unlikely, however.

The observed differences might be attributed to significantly different abilities of cells to perform excision repair, depending upon their physiological states. In contrast to the methods used by others, our method of determining the loss of dimers included a period of time after labeling and before irradiation to allow the cells to reach confluence. This period was also introduced to make these results more comparable to those obtained in the biological recovery experiments in which confluence is used to assure a nonreplicating state. However, when we measured the rate of dimer excision in exponentially growing cells, no difference could be found between growing and confluent cells (15).

The objective of the biological recovery experiments performed in this study was to compare the rate of recovery with the excision rate at various doses measured by the two biochemical methods. The results (Table I) indicate that the initial rate of excision repair of UV-induced damage is dose dependent. The higher the relative dimer load on the DNA, the faster the rate of removal, suggesting that the overall reaction is dependent on the concentration of the substrate, a result that agrees with observations reported by Williams and Cleaver (8) and Ahmed and Setlow (29).

The data in the present study suggest that measurement of the removal of thymine dimers and determination of DNA repair synthesis induced by UV irradiation are two different methods of assaying the same molecular process, a process that is able to eliminate potentially lethal damage from DNA.

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