

# THE KINETICS OF THYMINE DIMER EXCISION IN ULTRAVIOLET-IRRADIATED HUMAN CELLS

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**ABSTRACT** We have investigated the kinetics of the loss of thymine dimers from the acid-insoluble fraction of several ultraviolet (UV)-irradiated cultured human cell lines. Our results show that UV fluences between 10 and 40 J/m<sup>2</sup> produce an average of  $21\text{--}85 \times 10^5$  thymine dimers per cell and an eventual maximal loss per cell of  $12\text{--}20 \times 10^5$  thymine dimers. The time for half-maximal loss of dimers ranged from 12–22 h after UV irradiation. In contrast, the time for half-maximal repair synthesis of DNA measured by autoradiography was 4.5 h. This figure agrees well with reported half-maximal repair synthesis times, which range from 0.5 to 3.6 h based on our analysis. The discrepancy in the kinetics of the loss of thymine dimers from DNA and repair synthesis is discussed in terms of possible molecular mechanisms of thymine dimer excision *in vivo* and in terms of possible experimental artifacts.

## INTRODUCTION

Ultraviolet (UV) light exposure causes damage to the DNA of living cells by producing cyclobutane dipyrimidine products (pyrimidine dimers) between adjacent intrastrand pyrimidines in their DNA (1). Many species, including many mammalian cells, can repair this form of DNA damage by enzymatic excision of a sequence of nucleotides that includes the dimers (nucleotide excision repair) (1–11). The molecular mechanism of nucleotide excision repair is best understood in bacterial systems (1–8). These have served as useful general models for mammalian cell systems; however, there are indications that the details of the mechanism of excision of nucleotides from chromatin may be more complex (12–14).

Endonucleolytic incision of a DNA strand adjacent to the site of a pyrimidine dimer is believed to be an early, if not initial, event in nucleotide excision repair (1–10,15). This is followed by excision of a dimer-containing oligonucleotide sequence as well as by repair synthesis, though the relative order of these events is not known (10). These latter two reactions can be monitored by measuring DNA repair synthesis and by measuring the loss of thymine dimers from DNA. Measurement of DNA repair synthesis involves the use of a variety of techniques that measure the incorporation of nucleotides (usually radioactively labeled) into DNA after irradiation (16,17), and it is generally assumed that this incorporation occurs at sites where pyrimidine dimers were excised. Loss of thymine dimers from DNA is most conveniently measured by determining the ratio of thymine-containing pyrimidine dimers to thymine monomers

in the acid-insoluble fraction of the cellular DNA (18,19). The kinetics of DNA repair synthesis have been carefully studied in a number of laboratories using a variety of techniques (20–31). In general, these studies suggest that nucleotide excision repair of DNA after UV-irradiation of mammalian (including human) cells in culture is relatively rapid. There have been few reports on the detailed kinetics of the loss of thymine dimers from acid-precipitated cells, and most of these suggest that dimer loss continues well beyond the time in which repair synthesis is completed (32–34).

In the present study we have investigated in detail the kinetics of dimer excision in human fibroblast cells and compared the results with both our data and the data of others on repair synthesis. A preliminary account of these findings was reported earlier (35).

## METHODS

### *Cells and Cell Growth*

The following cell types were used: GM38, a normal human diploid skin fibroblast line obtained from the Medical Research Institute, Camden, N. J.; WI-38, a normal diploid human fibroblast line from fetal lung, originally obtained from Dr. L. I. Hayflick; VA13, an SV-40 transformed WI-38 cell line also obtained from Dr. L. I. Hayflick; human KB cells (a tumor cell line) obtained from the American Type Culture Collection; xeroderma pigmentosum skin fibroblast lines CRL1223 (JayTim, XP12BE, complementation group A), and CRL1160 (BeWen, XP5BE, complementation group D); two xeroderma pigmentosum variant skin fibroblast lines, CRL1162 (WoMec, XP4BE) and CRL1258 (PeHay, XP13BE); and the Fanconi's anemia skin fibroblast line, CCL122 (HG261). Experiments were carried out with fibroblasts at passage levels between 10–30.

All cells were cultured in Eagle's modified essential medium (Gibco autoclavable, Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 15 mM HEPES buffer, 12 mM sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 µg/ml), and Fungizone (0.25 µg/ml, G. R. Squibb & Sons, Princeton, N.J.).

### *Measurement of Kinetics of Thymine Dimer Excision in vivo*

Cells were plated in plastic tissue culture dishes (60 or 100 mm diameter) and allowed to grow for 3 days, at which time they were approaching confluence. The DNA was then labeled with [<sup>3</sup>H]thymidine prepared at 2–10 µCi/ml (40–60 Ci/mmol), in fresh medium 20–24 h before irradiation. The medium was removed just before irradiation and the cells were rinsed with phosphate-buffered saline. The cells were then irradiated with an 8 W low-pressure mercury vapor germicidal lamp (General Electric G8T5, General Electric Co., Cleveland, Ohio), which emits light principally at 254 nm, at dose rates from 0.45 to 0.85 J/m<sup>2</sup> s<sup>-1</sup>. In most cases irradiation was performed at 37°C. After irradiation, prewarmed medium was added to the cell cultures, which were then incubated at 37°C. At various times between 0 and 72 hr after irradiation, medium was removed from the dishes, the cells were rinsed with phosphate-buffered saline, and 10% cold trichloroacetic acid was added. In some experiments with WoMec cells, caffeine (2.0 mM) was present during the entire post-UV incubation. The acid-treated cells were harvested by scraping the dishes with a rubber policeman and centrifuged at 3,000 g for 10–15 min at 4°C. The acid-insoluble fractions (precipitates) were hydrolyzed at 176°C in 95–97% formic acid for 45 min, as described by Goldmann and Friedberg (36). The hydrolysates were dried by evaporation and resuspended in 5–10 µl distilled water. Labeled thymine dimers in the hydrolysates were separated from thymine monomers by one-dimensional

thin-layer chromatography and quantitated by the procedure of Cook and Friedberg (37). Since this technique gives a 15–20% variation in thymine dimer contents, we have obtained duplicate or triplicate values for each point. These either represent duplicate or triplicate dishes of cells and/or duplicate chromatographic measurements from a single hydrolysate. In addition most experiments were repeated on at least three occasions with comparable results.

### *Isolation of Nuclei*

In some experiments the thymine dimer content was measured in hydrolysates of acid-precipitated nuclei rather than whole cells. Isolated nuclei were prepared by two different procedures, one using the lysing solution described by Lieberman and Poirier (38), consisting of 320 mM sucrose, 1 mM potassium phosphate buffer (pH 7.5), 1.5 mM  $\text{CaCl}_2$ , and 1% Triton X-100, and the other consisting of 10 mM Tris buffer (pH 8.0), 50 mM sucrose, 1 mM  $\text{MgCl}_2$ , and 0.5% Triton X-100 (Rohm and Haas Co., Philadelphia, Penn.). In both cases the dishes of cells to be prepared for dimer analysis were rinsed with phosphate-buffered saline, 1.5 ml of the lysis solution was applied to the monolayer, and the cells were dislodged from the Petri dishes with a rubber policeman. The cells were allowed to remain in the lysis solution for ~5 min. before lysis by 10–25 strokes with the tight-fitting pestle of a Dounce homogenizer. The lysates were made iso-osmolar by dilution with sucrose and the nuclei pelleted by gentle centrifugation at 200 g for 5 min. The entire nuclear separation procedure was performed at 4°C. After the supernatants were discarded, the pellets were resuspended in 10% cold trichloroacetic acid; after 10 min, precipitates were pelleted by centrifugation and analyzed for thymine dimers as described above.

### *Unscheduled DNA Synthesis*

Cells were plated on 22-mm-square cover glasses, previously laid into individual  $35 \times 10$  mm units of a multidish (Linbro Scientific, Hamden, Conn.). The cells were placed at either  $1.5 \times 10^5$  or  $2.5 \times 10^5$  cells per dish 3 days before irradiation. Immediately after irradiation and at specifically designated intervals thereafter, [ $^3\text{H}$ ]thymidine (10  $\mu\text{Ci}/\text{ml}$ ) was added to appropriate wells of a multidish and the cells were allowed to incubate for 1 h. The labeled medium was removed and the cells were fixed for autoradiography by placing the cover slips in cold 10% trichloroacetic acid solution overnight. The cover slips were then mounted on slides and dipped into NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y.) at 41°C, dried at room temperature for 1 h, and stored for 6–7 days in the dark at 4°C. Slides were developed in Kodak D-19 developer for 4 min at 20°C, rinsed in water, and fixed with Kodak fixer for 20 min (Eastman Kodak Co., Rochester, N.Y.). Cells were stained with Gill-2 hematoxylin (Lerner Laboratories, Stamford, Conn.) through the NTB-2 emulsion for 20 min, before gradual dehydration in a series of ethanol concentrations beginning with 25% and gradually increasing up to 100% and finally clearing in xylene. For each time point the number of grains in at least 75 cells was counted microscopically and averaged.

### *Isolation of High Molecular Weight DNA on Alkaline Sucrose Gradients*

[ $^3\text{H}$ ]thymidine-labeled cells in Petri dishes were exposed to UV radiation and incubated for varying times as described above. Medium was aspirated from the dishes and the cultures were rinsed once with phosphate-buffered saline. 1 ml of 0.15 M NaCl was pipetted onto each Petri dish and the dishes were placed on dry ice-acetone for quick freezing. Petri dishes were stored at  $-20^\circ\text{C}$  for periods between 1 and 6 days in different experiments. Thawed cells were harvested from the dishes with a rubber policeman and added to 1.0 ml of a lysis solution consisting of 0.5 N NaOH and 0.05 M EDTA (39). The mixtures were aspirated five times with a Pasteur pipette and incubated at room temperature for about 20 min. Each lysate was then layered onto a 5–20% sucrose gradient (36.0 ml) at pH 12.0 and centrifuged in a SW27 rotor

(Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at speeds between 16,000 and 24,000 rpm in different experiments for varying times at 20–25°C. In each experiment the specific speed and time of centrifugation was calculated to provide  $\omega^2 t$  value =  $3.2 - 4.4 \times 10^{11}$ . Fractions ( $\sim 2.0$  ml) were collected from the bottom of each gradient and the radioactivity in a small sample was measured by liquid scintillation spectroscopy. After determination of the sedimentation profile of the DNA in each gradient, fractions containing DNA of molecular weight greater than  $\sim 5 \times 10^6$  daltons were pooled. The pooling was based on extrapolation from previous unpublished experiments in which the identical sedimentation conditions including a phage T7 DNA molecular weight marker were used. Unlabeled calf thymus DNA was added as carrier to a final concentration of 10  $\mu\text{g}/\text{ml}$  and the DNA was precipitated with cold trichloroacetic acid (5% final concentration). After standing on ice for 1–2 h, the precipitates were harvested by centrifugation in a Sorvall fixed-angle head rotor (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.) at 48,200 g for 2 h. The resulting pellets were washed with cold 10% trichloroacetic acid and recentrifuged for 1–5 h. The thymine dimer content of these samples was measured as described above.

In one experiment a 165 nucleotide marker was added to the harvested cells before mixing with lysis buffer. This oligonucleotide was obtained as a product of digestion of  $^{32}\text{P}$ -labeled mitochondrial DNA with  $\text{EcoR}_I$  restriction endonuclease and was generously provided by Mr. James F. Battey, Department of Pathology, Stanford University.

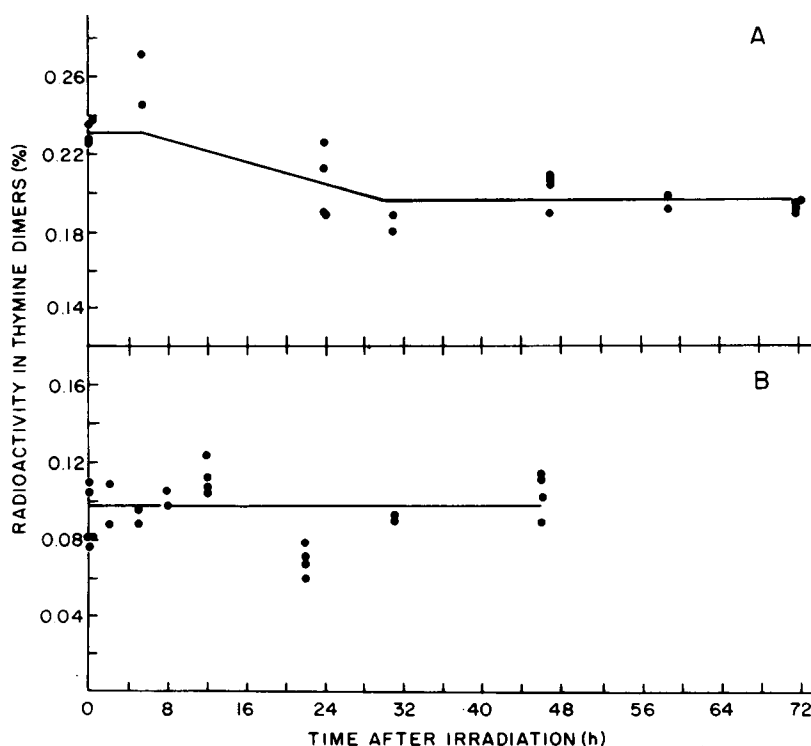


FIGURE 1 Thymine dimer content of two human fibroblast lines after UV irradiation. A, GM38 cells (normal) irradiated with 40  $\text{J}/\text{m}^2$ ; B, JayTim cells (XP complementation group A) irradiated with 15  $\text{J}/\text{m}^2$ . Each data point represents results from a single chromatogram.

## RESULTS

The thymine dimer content of the DNA of acid-precipitated cells was measured as a function of the time of postirradiation incubation. As indicated in Figure 1 A, with normal (GM38) cells, little if any dimer loss occurred within the first few hours after irradiation. After 6–8 h, the fraction of dimers in the acid-insoluble DNA began to decrease and continued to decrease until 24–30 h after irradiation. There was little subsequent loss even until 72 h after irradiation. In contrast, with XP cells from com-

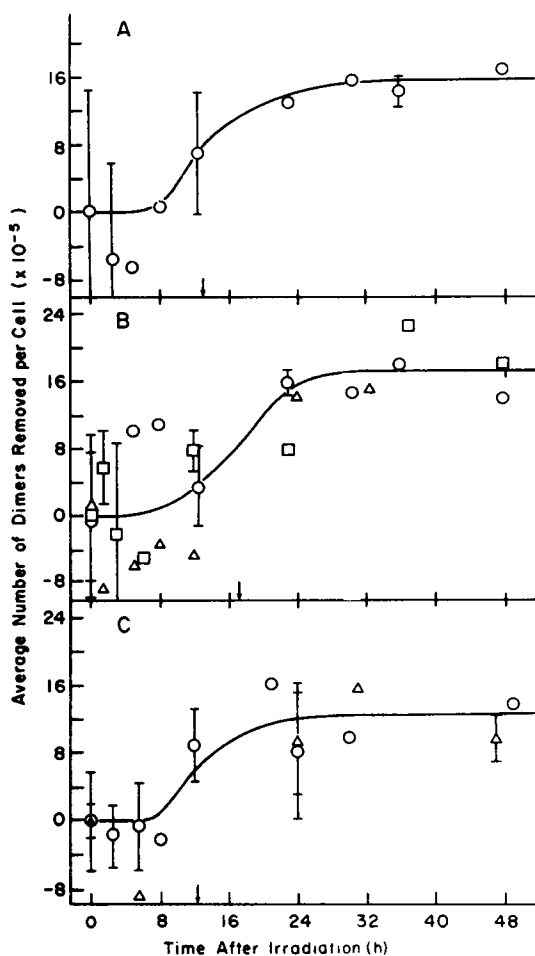


FIGURE 2 The average number of dimers removed per cell for GM38 cells irradiated with (A) 10 J/m<sup>2</sup>, (B) 20 J/m<sup>2</sup>, or (C) 40 J/m<sup>2</sup>. The number of dimers per cell was calculated under the assumption that each cell has  $6 \times 10^{-12}$  g DNA (58). This value was selected as one that would reasonably approximate the average DNA content of a population of cells in various phases of the cell cycle. Where results from three or more chromatograms are averaged, the standard deviation is shown. Most other points are averages of results from two chromatograms. Each symbol represents results from a different experiment.

TABLE I  
KINETICS OF THYMINE DIMER LOSS FROM ACID-PRECIPITATED CELLS

Cell type	Fluence	Number of thymine dimers lost per cell ( $\times 10^{-5}$ )					$t_{1/2}$
		6 h	12 h	18 h	24 h	48 h	
	$J/m^2$						$h$
WI-38	15	0.6	3.8	7.6	13.2	20.0	22.0
	40	1.4	4.4	8.5	12.0	14.0	16.0
GM38	10	0.4	5.8	11.0	13.7	15.0	13.2
	15	0.9	6.2	11.5	15.0	17.7	13.8
	20	0.5	3.5	9.3	15.3	17.0	17.3
WoMec	40	0.2	5.8	10.4	12.2	12.5	12.2
	10	0.2	3.3	7.9	10.7	12.0	15.2
	15	3.9	9.3	12.4	14.5	16.0	10.2
	20	1.6	5.0	7.3	9.0	10.8	13.0
PeHay	50	1.7	4.7	7.7	10.3	17.4	20.0
	20	2.6	5.6	8.3	9.2	12.5	13.6
Mean values		$1.3 \pm 1.2$	$5.2 \pm 1.7$	$9.3 \pm 1.8$	$12.3 \pm 2.3$	$15.3 \pm 2.9$	$15.1 \pm 3.5$

plementation group A (XP12BE) no significant loss of thymine dimers was observed (Fig. 1 B). Similar results were obtained with XP cells from complementation group D (XP5BE) (data not shown). In Fig. 2 (in which the total number of thymine dimers lost per cell is plotted as a function of the post-UV incubation time) a similar pattern was observed in experiments with GM38 cells using fluences between 10–40  $J/m^2$ . Although there was some variation from experiment to experiment, in none did signifi-

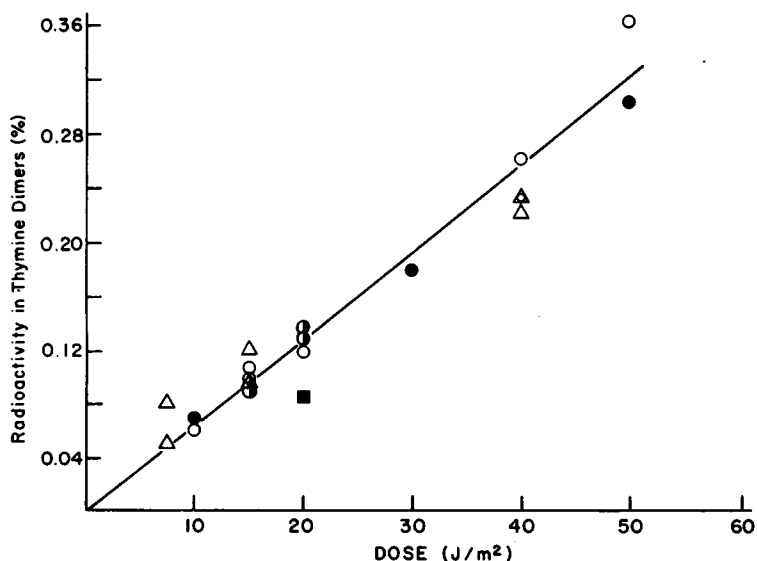


FIGURE 3 Dose response for thymine dimer production in monolayer cultures of mammalian cells.  $\circ$ , GM38 cells;  $\bullet$ , WoMec cells;  $\Delta$ , WI-38 cells;  $\blacktriangle$ , JayTim cells;  $\blacksquare$ , PeHay cells.

cant dimer loss occur within the first few hours, or later than 30 h after irradiation. An analysis of the results from these and other experiments using a number of diploid cell lines showed that on the average less than 10% of the total number of thymine dimers lost at 48 h had been lost by 6 h after irradiation (Table I). Preliminary experiments with KB, VA13, and HG261 (Fanconi's anemia) cells showed similar results; very little if any loss of thymine dimers was observed in the first 6 h after irradiation (40).

The maximum number of thymine dimers lost seemed to be independent of fluence from 10 to 40 J/m<sup>2</sup> and varied from 12 to 20 × 10<sup>5</sup> (Table I). The apparent saturation of dimer loss at some fluence equal to or less than 10 J/m<sup>2</sup> is not a function of dosimetric problems, since in all cases studied the thymine dimer content of irradiated cells bore the expected linear relationship to UV fluence (Fig. 3). Whether or not dimer loss is linear with respect to UV fluence up to 10 J/m<sup>2</sup> is difficult to determine because of the uncertainty in measuring thymine dimer contents of DNA at very low fluences. None-

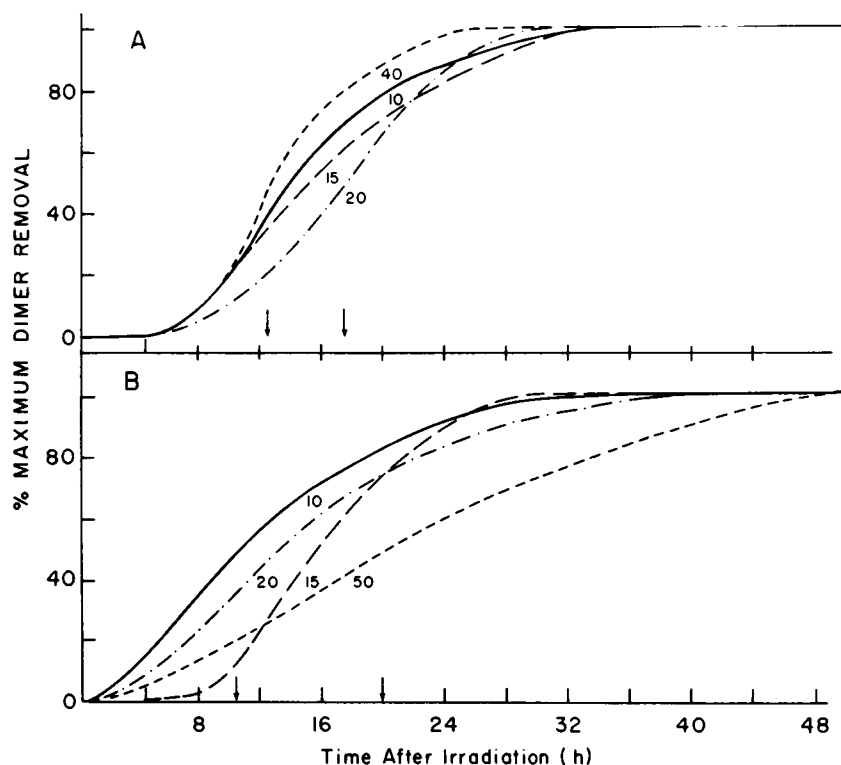


FIGURE 4 Normalized loss of thymine dimers where the total number of dimers lost at a given fluence represents 100%. Each line represents the kinetics of dimer loss at a particular fluence (in joules per square meter) indicated by the accompanying number. (Data points were omitted for clarity. See Figs. 1 and 2 for typical experiments). A. normal human fibroblasts (GM38) B. Xeroderma pigmentosum variant fibroblasts (WoMec). Arrows indicate the earliest and latest times at which 50% of the dimer removal occurred ( $t_{1/2}$ ).

theless, a single experiment at  $7.5 \text{ J/m}^2$  indicated that all thymine dimers were lost by 36 h after irradiation (data not shown).

We have normalized the maximal extent of thymine dimer loss to 100% to allow for a direct comparison between different experiments as well as a comparison with the kinetics of other parameters of excision repair (Fig. 4). In our view no significant differences exist between the normalized kinetics of dimer loss measured in normal (GM38) and xeroderma pigmentosum variant cells (CRL1162 - WoMec) (Fig. 4). In the former, the time at which one half the maximal number of dimers was lost ( $t_{1/2}$ ) ranged from about 12 to 17 h, while in the latter the range was from about 10–20 h (Fig. 4). Caffeine at 2.0 mM had no observable effect on the kinetics of thymine dimer loss in these cells (data not shown). The mean  $t_{1/2}$  value for data presented in Table I for all cells studied was  $15.1 \pm 3.5 \text{ h}$ .

While these experiments were in progress, a report by Amacher et al. (41) indicated a much more rapid loss of thymine dimers from prelabeled human WI-38 cells. The technique used by these authors did not differ substantively from ours except that the thymine dimer content was measured in acid-precipitated nuclei rather than whole cells. In two experiments in which a direct comparison was made between the results

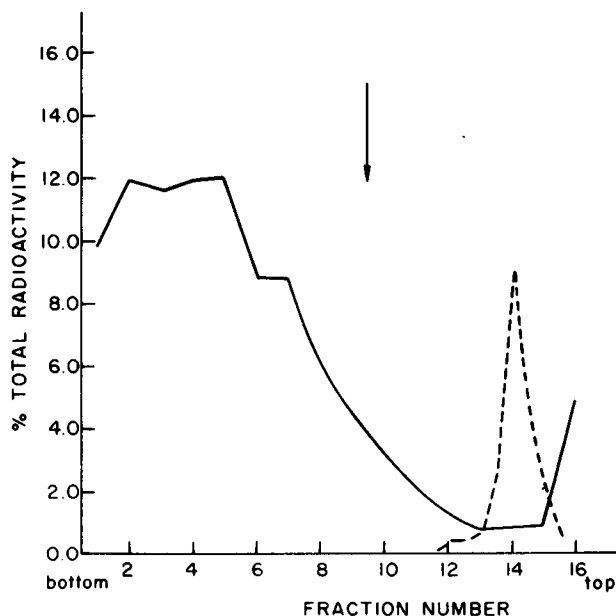


FIGURE 5 A typical sedimentation profile of cellular DNA on an alkaline sucrose density gradient. The DNA was sedimented at 17,900 rpm for 16 h in an SW27 Beckman rotor. All DNA which sedimented to the left of the arrow [ $>5 \times 10^6$  daltons (39)] was pooled for measurement of thymine dimer content. The dashed peak indicates the sedimentation profile of a 16S-nucleotide marker, which was centrifuged with a whole cell lysate in a separate gradient. See text for further details.



from whole cells and nuclei isolated by two different techniques, no significant differences were observed in our hands (data not shown).

We considered the possibility that cells rapidly excised thymine-containing pyrimidine dimers in acid-insoluble oligonucleotides and these were slowly degraded to an acid-soluble form. This possibility was tested by measuring the thymine dimer content of high molecular weight DNA isolated from the cell by alkaline sucrose gradient sedimentation. All fractions of the gradients that contained DNA of molecular weight  $>5 \times 10^6$  daltons were pooled and the DNA was precipitated and assayed for thymine dimers (Fig. 5). If dimer-containing oligonucleotides of up to  $\sim 150$  bases had been present in the cells, they should have been separated from the larger DNA molecules on the sucrose gradient, since when we included a marker of 165 nucleotides on a gradient with cells, it remained near the top, well separated from the high molecular weight DNA. The kinetics of dimer loss measured by acid precipitation of high molecular weight DNA was similar to the kinetics measured by precipitation of whole cells (Fig. 6). We therefore conclude that the cells do not excise dimers as acid-insoluble oligonucleotides.

The kinetics of unscheduled DNA synthesis in GM38 cells were clearly distinct from those of thymine dimer loss. When the total amount of [ $^3\text{H}$ ]thymidine incorporated by nonreplicating GM38 cells in the first 24 h after irradiation was considered to represent maximal unscheduled DNA synthesis, the time at which half-maximal synthesis occurred was approximately 4.5 h after irradiation (Fig. 7). This result was obtained with

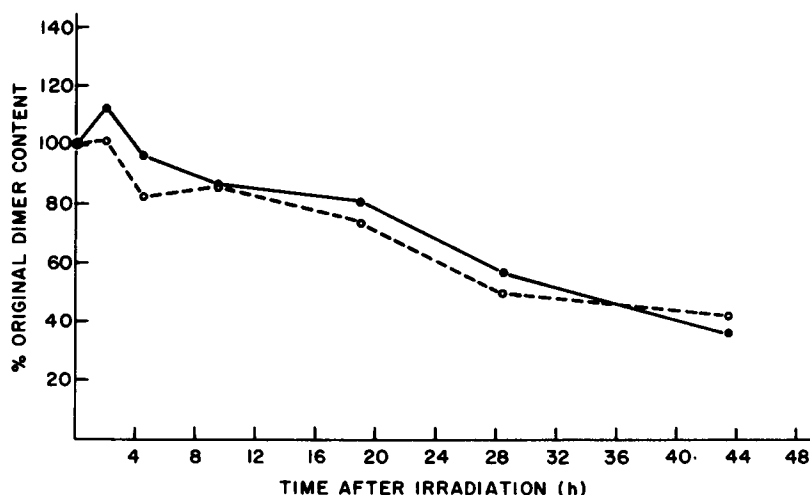


FIGURE 6 Comparison of the kinetics of loss of thymine dimers from the acid-insoluble fraction of whole GM38 cells (●) and from high molecular weight DNA harvested from an alkaline sucrose gradient as indicated in Fig. 7 (○). The thymine dimer content of the whole cell fraction immediately after irradiation was 0.084% and of the high molecular weight DNA was 0.103%. Each data point is the mean of triplicate or duplicate chromatography. The experiment was performed on five separate occasions with similar results. See text for further details.

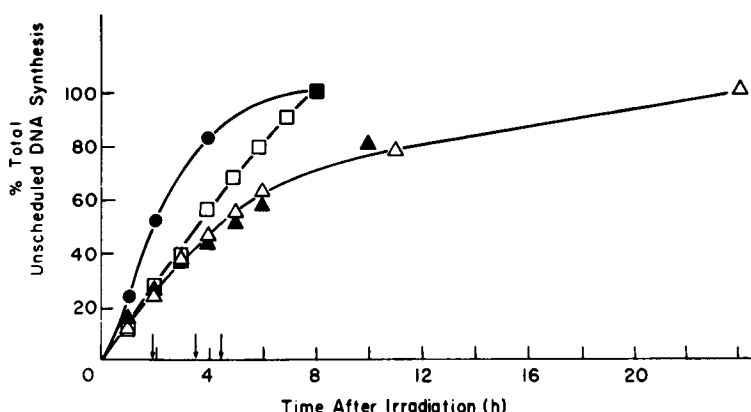


FIGURE 7 Kinetics of unscheduled DNA synthesis as measured by autoradiography. The total amount of unscheduled DNA synthesis within the entire course of the experiment is designated as 100%.  $\Delta$ , GM38 cells irradiated with 15 J/m<sup>2</sup>;  $\blacktriangle$ , GM38 cells irradiated with 40 J/m<sup>2</sup>;  $\bullet$ , normal human fibroblasts from a healthy donor irradiated with 10 J/m<sup>2</sup> (data from Rasmussen [29]);  $\square$ , WI-38 cells irradiated with 15.7 J/m<sup>2</sup> (data from Bowman et al. [28]). Arrows on abscissa indicate times at which 50% of unscheduled DNA synthesis occurred.

fluences of both 15 and 40 J/m<sup>2</sup> (Fig. 7). Our measurements of unscheduled DNA synthesis compared well with those of others who have reported this technique for measuring the kinetics of DNA repair, i.e., about half the maximal repair occurred within the first few hours after irradiation (Fig. 7). Similarly, we have compared by normalization the relative kinetics of repair synthesis measured by others either by radioactivity incorporation in the presence of hydroxyurea (22–31) (Fig. 8 A) or by isopycnic sedimentation using bromodeoxyuridine as a repair synthesis probe (20, 21,42) (Fig. 8 B). The data provide estimates of the half-maximal repair time between 0.5 and 3.2 h.

## DISCUSSION

The present studies indicate that the kinetics of thymine dimer loss from the acid-insoluble fraction of cells is complex. There is an initial phase of undetectable loss for about 6 h after irradiation; this is followed by a loss of up to, but not more than approximately  $15 \times 10^5$  dimers per cell during the next 20–30 h. Apparently, after 30 h, there is no further loss of dimers from the acid-insoluble fraction. The same kinetics of dimer loss were found at all doses between 10 – 40 J/m<sup>2</sup> and for all cell lines studied (except the excision defective xeroderma pigmentosum cells from complementation groups A and D). The results of our studies on the rate of loss of thymine dimers from whole cells, nuclei, or high molecular weight DNA precipitates indicate that this event occurs with a mean half-maximal time of about 15 h after irradiation.

Previous studies from this laboratory led to the suggestion that xeroderma pigmentosum variant cells may be defective in the excision of thymine dimers from DNA and pointed out that direct measurements of this parameter had not been reported (43).

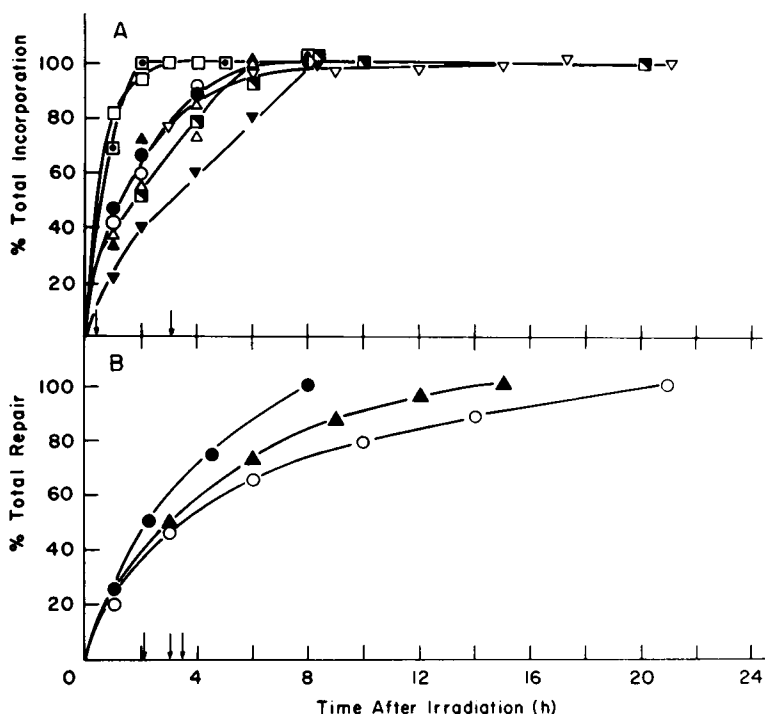


FIGURE 8 The kinetics of repair synthesis in several different cell types. A. Unscheduled DNA synthesis in several cell types as measured by uptake of [ $^3\text{H}$ ] thymidine. The total amount of unscheduled DNA synthesis during the entire course of the experiment is designated as 100%.  $\circ, \bullet$ , human lymphocytes:  $\circ$ , 25 J/m $^2$ ;  $\bullet$ , 50 J/m $^2$  (data from Evans and Norman [26]);  $\Delta, \blacktriangle$ , human fibroblasts:  $\Delta$ , 10 J/m $^2$ ;  $\blacktriangle$ , 50 J/m $^2$  (data from Stefanini et al. [25]);  $\nabla$ , human lymphocytes, 16 J/m $^2$  (from Burk et al. [27]);  $\square, \blacksquare$ , human lymphocytes:  $\square$ , 140 J/m $^2$ ;  $\blacksquare$ , 35 J/m $^2$  (from Clarkson and Evans [23]);  $\nabla$ , polymorphonuclear leucocytes 5 J/m $^2$  (from Conner and Norman [22]);  $\square$ , kidney cells 100 J/m $^2$  (from Smets [24]). Arrows on abscissa indicate times at which 50% of unscheduled DNA synthesis occurred for the fastest (human kidney,  $\square$ ) and slowest (polymorphonuclear lymphocytes,  $\nabla$ ) cells. The calculated points on this figure are not necessarily at the same times as the authors' reported time points, but were derived directly from the authors' curves. B. Repair replication in three different cell types as measured by isopycnic gradient sedimentation. The total amount of repair replication which occurred during the course of the experiment was designated as 100%.  $\bullet$ , WI-38 and VA13 cells irradiated with 10 J/m $^2$  (from Smith and Hanawalt [20]);  $\circ$ , HeLa cells irradiated with doses ranging from 2.4–62.4 J/m $^2$  (from Edenberg and Hanawalt [21]);  $\blacktriangle$ , CV-1 green monkey kidney cells irradiated with 25 J/m $^2$  (from Williams [42]). Arrows on abscissa indicate times at which 50% of repair replication had occurred. The calculated points on this figure are not necessarily at the same times as the authors' reported time points, but were derived directly from their published curves.

The present studies indicate no differences in the rate or extent of loss of thymine dimers from the acid-insoluble fraction of whole cells in normal and xeroderma pigmentosum variant fibroblast lines. In the latter caffeine was without effect.

The results obtained on the kinetics of the loss of thymine dimers from acid-precipitated cells are clearly distinct from those of repair synthesis measured on the same cell types grown under identical conditions, except for the necessary radioactive

prelabeling for dimer loss experiments. Specifically, after UV fluences of either 15 or 40 J/m<sup>2</sup>, repair synthesis measured by autoradiography occurs relatively rapidly, with 50% of the maximal level of [<sup>3</sup>H]thymidine uptake occurring at about 4 h after irradiation.

It is relevant to compare the results of our present studies with similar data reported previously by others. As regards the kinetics of repair synthesis in mammalian cells, our value of 4 h is consistent with the half-maximal repair synthesis values estimated from an analysis of the available literature (0.5–3.6 h), irrespective of the particular technique employed for measuring repair synthesis of DNA. In some of the experiments quoted from the literature we cannot be certain from the data provided that maximal levels of repair were achieved. Thus our estimate for the half-maximal repair times for these data may be artifactually reduced. Nonetheless, in all such experiments, it is clear from the kinetic curves that most repair synthesis took place within the first few hours of irradiation (Figs. 5 and 6).

The kinetics of thymine dimer loss as described in the literature show considerable variation (19,32–34,41,44). In general, the comparison with our data is difficult since the studies referred to were, in most instances, not specifically aimed at detailed kinetic analyses and frequently insufficient information is available. Nonetheless, it is, for example, apparent from the data of Setlow et al. (34) that when normal human fibroblasts were irradiated at 7.5 or 15 J/m<sup>2</sup>, loss of thymine dimers from the acid-insoluble fraction of whole cells or purified DNA continued for at least 12–24 h. The data of Regan et al. (32) create a similar impression. On the other hand, data presented by Ben-Hur and Ben-Ishai (44), Isomura et al. (33), Duncan et al. (19), and Amacher et al. (41) indicate an early loss of thymine dimers. It is not evident what experimental variations may account for these discrepancies, but it should be noted that in general, the accurate measurement of very low thymine dimer contents (<0.05%) is difficult.

Several explanations of our results are tenable. First, we have deliberately avoided equating the loss of thymine dimers from acid-insoluble DNA with thymine dimer excision, because it is conceivable that pyrimidine dimers are excised as acid-precipitable oligonucleotides that subsequently become reduced to acid-soluble products by enzymatic degradation. Clearly, if this were the case, measurements on acid-precipitable fractions would reflect the kinetics of enzymatic degradation of oligonucleotides rather than thymine dimer excision. The excision of thymine dimers as acid-insoluble oligonucleotides has been reported by Ben-Ishai and Peleg in primary mouse cultures (45,46). They observed that a reduction in thymine dimer content could be demonstrated in acid-precipitated whole nuclei, but not in whole cells, suggesting that after excision, the dimer-containing oligonucleotides moved to the cytoplasm either *in vivo* or during the experimental manipulations.

The general hypothesis that dimers are excised as acid-insoluble pieces of DNA could explain the variations observed in different laboratories. Different growth conditions and experimental procedures could affect the relative activity of nucleases (or other events) that reduce acid-insoluble oligonucleotides to acid-soluble products.

This hypothesis has previously been tested by Setlow et al. (34), who, after irradiating and incubating cells, measured the thymine dimer content of purified cellular DNA that had been alkali denatured, renatured, and chromatographed on Sepharose 4B columns to separate high molecular weight material from single-stranded pieces smaller than 100 nucleotides. They reported no change in the thymine dimer content compared to whole cell preparations. We have approached this question using alkaline sucrose velocity sedimentation to fractionate cellular DNA. Our results also indicate that the kinetics of loss of thymine-containing pyrimidine dimers from high molecular weight DNA is similar to that observed with precipitated whole cells. These results argue strongly against excision of acid-insoluble oligonucleotides as the explanation for the observed kinetics.

A second possible experimental artifact to consider is the different treatment of the cells inherent in the techniques used for measuring repair synthesis and thymine dimer excision. The latter requires labeling of cellular DNA with radioactive thymidine before irradiation, whereas the former does not. It has been shown that tritium decay in mammalian cells causes strand breaks in DNA (47), mutation (48), inhibition of cell division by a block in the G<sub>2</sub> phase of the cell cycle (48–50), chromosome aberrations (51), and cell death (52,53). All of these effects can be created with [<sup>3</sup>H]thymidine with a specific activity an order of magnitude less than that used in our experiments. It is therefore possible that cells damaged by tritium decay have an altered capacity for nucleotide excision repair. However, evidence has recently been presented to show that prior X-irradiation of cells does not alter the amount of repair replication in response to subsequent UV-irradiation (42).

Two further hypotheses concern possible molecular events associated with nucleotide excision repair *in vivo*. The rapid kinetics of repair synthesis compared to the slower loss of thymine dimers may reflect a “patch and cut” type of mechanism in which repair synthesis occurs before the enzymatic removal of thymine dimers (10). In this event one would expect the kinetics of the loss of UV endonuclease-sensitive sites to be at least as rapid as that of repair synthesis. This has been shown in African green monkey cells (30). Data reported by Paterson et al. (54), are more difficult to analyze. At very low UV fluences (6 J/m<sup>2</sup>) we estimate that the time of half-maximal loss of endonuclease sensitive sites was about 3 h. However, at 25 and 50 J/m<sup>2</sup> this value is about 12 h. These data suggest that contrary to loss of thymine dimers and repair synthesis, where total repair levels apparently saturate at relatively low UV fluences (7.5–20 J/m<sup>2</sup>), the loss of endonuclease sensitive sites does not. This might indicate that with increasing UV fluence, not all sites that undergo endonucleolytic incision necessarily undergo subsequent steps in nucleotide excision repair. Consistent with this idea is the fact that in some studies many breaks in the cellular DNA are still evident as late as 10–20 h after irradiation (34,55).

Finally, it is necessary to consider the possibility that repair synthesis (by whatever technique measured) does not reflect pyrimidine dimer excision in mammalian cells. In a study involving seven Chinese hamster cell lines, Lohman et al. (56), observed no consistent relationship between the amount of repair replication and the extent of

dimer excision in those cell lines that demonstrated both processes. In addition, when embryonic chick cells were exposed to photoreactivating light after UV irradiation, pyrimidine dimers disappeared, yet levels of repair synthesis comparable to those observed in the dark still occurred (57). Further studies are aimed at systematically testing these hypotheses.

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