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Different rate-limiting steps in excision repair of ultraviolet- and *N*-acetoxy-2-acetylaminofluorene-damaged DNA in normal human fibroblasts

(unscheduled DNA synthesis/endonuclease-sensitive sites)

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ABSTRACT **In normal human cells the amount of excision of ultraviolet damage to DNA saturates at high doses. In these cells some chemicals mimic ultraviolet damage as far as their biological and repair characteristics are concerned. One of these chemicals is *N*-acetoxy-2-acetylaminofluorene. We determined whether the limited repair capacity for ultraviolet damage was affected by treatment with *N*-acetoxy-2-acetylaminofluorene. To measure repair we determined unscheduled DNA synthesis and the number of sites sensitive to an ultraviolet endonuclease in an assay using an extract of *Micrococcus luteus*. The nuclease does not act on DNA treated with the chemical. The amount of unscheduled DNA synthesis due to a combined chemical and ultraviolet treatment was the sum of those observed from the separate treatments, even at saturation doses. The combined treatment did not affect the removal of nuclease-sensitive sites. We conclude that there are different rate-limiting steps in excision repair of the ultraviolet and the chemical damage and suggest a model involving a complex of enzymes to explain the data.**

Biological systems possess a number of enzymatic mechanisms to repair physical and chemical damage to their DNA (1–4). One of them—excision repair—involves four (sometimes five) general steps: (*N*-glycosidase action), incision, excision, polymerization, and ligation. This process acts upon a large number of chemically distinct damages induced by agents such as ultraviolet (UV), ionizing radiation, and a variety of chemical mutagens and carcinogens. Although it is reasonable that the versatility in substrate recognition for repair may be provided by a number of specific endonucleases each of which probably recognizes a class of distortion of the damaged site, the number of classes identified so far is small. A crude classification in terms of repair characteristics is into UV and ionizing radiation type (5).

UV-induced pyrimidine dimers and *N*-acetoxy-2-acetylaminofluorene (AAAF) lesions in DNA are substrates for excision repair in human cells, and AAAF damage mimics UV damage in the following ways: (*i*) both are repaired by a long patch mechanism (about 100 nucleotides) (5); (*ii*) xeroderma pigmentosum (XP) cells deficient in repairing UV damage are also deficient in repairing AAAF damage (6, 7); (*iii*) XP cells are more sensitive than normal cells to the cytotoxic and mutagenic activity of both UV and AAAF (8). However, studies based on DNA repair synthesis showed that removal of AAAF and UV lesions differed in the initial rate (9).

The amount of excision repair of pyrimidine dimers saturates at high UV doses (>20 J·m^{-2} of 254 nm) (10–12). Hence, if AAAF truly mimicked UV damage one would expect that the amount of repair from a combined treatment using high doses would be less than the sum of the treatments separately. Therefore, we determined if the limited UV repair was affected by AAAF treatment. For this purpose we employed two techniques to monitor excision repair: unscheduled DNA synthesis (13) and the measurement of sites sensitive to UV-endonuclease (14). The first technique was chosen because it gives information of repair due to both agents, whereas the second, using *Micrococcus luteus* endonuclease, allows us to detect UV-induced pyrimidine dimers in the DNA of cells exposed to a combined treatment of UV and AAAF. We found, contrary to our expectation, that as a result of the combined treatment unscheduled DNA synthesis equaled the sum of those from separate treatments and that AAAF treatment did not inhibit the removal of sites sensitive to exogenous endonuclease.

Abbreviations: UV, ultraviolet; AAAF, *N*-acetoxy-2-acetylaminofluorene; XP, xeroderma pigmentosum; EDTA, ethylenediaminetetraacetate; M_w, weight average molecular weight; M_n, number average molecular weight.

MATERIALS AND METHODS

Cell Line and Tissue Culture. The normal human fibroblasts Rid Mor CRL 1220 were obtained from the American Type Culture Collection. They were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, L-glutamine at 400 μg/ml, penicillin at 140 units/ml, and streptomycin at 140 μg/ml (Grand Island Biological Co.), and kept in humidified 10% CO_2 atmosphere at 37°. When cells reached confluency they were subcultured at a subculture ratio of 1:3. The passages used, 3–24, usually took about 4–5 days to reach confluency.

Unscheduled DNA Synthesis. Twenty-four hours before treatment cells were seeded at a density of 10^3 cells per cm^2 onto 11 × 22 mm cover slips (Arthur H. Thomas Co.), contained in 60 mm diameter glass petri dishes, and covered with 5 ml of medium. Three hours before treatment, hydroxyurea (Sigma Chemical Co.) was added to a final concentration of 2 mM to inhibit unscheduled DNA synthesis (13). At zero time (time of treatment) the cells in medium at 37° were exposed for 20 min to various concentrations of AAAF (a gift from J. A. Miller) dissolved in fresh $(CH_3)_2SO$ (Fisher Chemical Co.), or the medium was removed and the cells were exposed to various doses of 254 nm radiation at a dose rate of 0.36 W·m^{-2}, or to a combined treatment of UV followed by AAAF. AAAF does not have to be further metabolized in order to bind to DNA and other macromolecules. It is a highly reactive compound with half life of about 7 min in water (15). Therefore, in our experiments it was left in contact with cells for 20 min before being washed out. After treatment fresh medium containing 2 mM hydroxyurea and 2 μCi/ml of [^{3}H]thymidine (6.7 Ci/mmol, New England Nuclear) was added and left for 3 hr. The cover slips were then removed, fixed in Carnoy's solution, hydrated in a descending series of alcohols, and immersed in distilled water. The cells were stained by the Feulgen procedure (16) and the cover slips were mounted back to slides, dipped in 2-

fold diluted Kodak NTB photographic emulsion, and kept in dark, light-tight boxes for 4 days at 4°. Slides were developed in D-19 Kodak developer and fixed in Kodak fixer. Fifty cells were randomly selected and the number of silver grains per nucleus was counted. The appropriate background—hydroxyurea without UV irradiation, or hydroxyurea with $(CH_3)_2SO$ equivalent to that used in AAAF treatment (2.5–10 μl)—was subtracted as a small correction (<1 grain per nucleus).

Endonuclease-Sensitive Sites. The general procedure for the *in vitro* assay has been described before (14, 17). DNA containing pyrimidine dimers is exposed to an endonuclease specific for such dimers. The number of endonuclease-induced breaks equals the number of dimers (18). We wanted to minimize experimental fluctuations that might arise from variations in the molecular weight of extracted DNA or in centrifugation procedures. Therefore, we compared, in a single assay, untreated with treated cells, or unincubated-treated with incubated-treated cells, or UV-irradiated with UV-plus-AAAF-treated cells. To do this we used, per assay, two plates of cells labeled with different radioactive precursors and receiving different treatments. Approximately 100,000 cells were plated in 5 ml of medium in 60 mm plastic dishes. One plate was labeled for 36 hr with [^{3}H]thymidine at 0.5 μCi/ml and the second plate was labeled for 36 hr with [^{14}C]thymidine at 0.1 μCi/ml (50 Ci/mol, New England Nuclear). After the end of the treatment or incubation period the medium was discarded and cells in each plate were washed twice with 2 ml of an ice-cold EDTA-containing NaCl solution (10) before they were rubbed off plates with a rubber policeman into 2 ml of the same solution and mixed together. The mixture was centrifuged, the supernatant was discarded, and the pellet of cells was washed with 2 ml of phosphate-buffered saline and the buffer was removed. The tube was vortexed to disperse the cells. One-half milliliter of lysing solution containing 0.02 M Tris·HCl (pH 8)/0.04 M NaCl/0.002 M EDTA/10% Sarkosyl (Geigy) was added. Lysis occurred almost immediately. Pronase (Calbiochem) was added to give 15 μg/ml and the proteins in the lysate were digested for 60 min at 37°. One milliliter of phenol equilibrated with endonuclease buffer [0.02 M Tris·HCl (pH 8)/0.04 M NaCl/0.02 M EDTA] was added to the lysate and the samples were rotated for 1 hr at room temperature. The phenol was separated from the DNA solution by centrifugation at room temperature. The upper phase was collected and extracted twice with an equal volume of ether saturated with endonuclease buffer to remove most of the phenol and the DNA solution was dialyzed overnight against two changes of the buffer at 4°. The endonuclease used was as a crude extract, equivalent to fraction III of Carrier and Setlow (19). Ten microliters of that extract (2 mg of protein per ml) was added to 100 μl of DNA solution and incubated at 37° for 20 min. Under these conditions the reaction went to completion. The reaction was stopped with 50 μl of 1 M NaOH, the reaction mixture was layered on top of 5–20% alkaline sucrose gradients containing 0.5 M NaCl, and the DNA was sedimented at 20° in an SW 60 rotor of a Beckman L5-50 ultracentrifuge at 50,000 rpm for 90 min. Fractions were collected from the bottom of the gradient and the acid-insoluble radioactive material was counted and analyzed by a computer program as described elsewhere (20). Each gradient had between 3,000 and 10,000 cpm of ^{3}H and 1,000 and 2,000 cpm of ^{14}C.

Endonuclease-Sensitive Sites: Calculations. The weight-average molecular weight M_w was calculated from the distribution of radioactivity. We used the weight-average (M_w) rather than the number average (M_n) because the latter is very sensitive to fluctuations in the amount of DNA near the top of the gradient, and took $M_n = M_w/2$ on the assumption that the breaks were distributed randomly. The reciprocal of M_n gives the number of breaks per unit molecular weight. In the absence of endonuclease treatment there was less than a 5% change in molecular weight after a 24 hr incubation following exposure to the highest doses used (20 J·m^{-2} ± 20 μM AAAF). Hence there is equivalence among the three following methods used to calculate the number of endonuclease-sensitive sites removed during incubation. (*i*) If cells in one plate were treated and those in the other were not treated but both plates were incubated, the difference between the numbers of breaks in the DNA of treated and untreated cells gives the numbers of sites remaining after incubation. Subtracting the number of sites remaining after incubation from the number at zero time gives the number of sites removed during incubation. (*ii*) Cells on both plates were treated but one plate was incubated and the other was not before the cells were mixed and the DNA was assayed. The difference between the numbers of breaks in the two DNAs is the number of endonuclease-sensitive sites removed during the incubation period. (*iii*) Cells on one plate were irradiated with UV and those on the other were irradiated and treated with AAAF. Both plates were incubated before the cells were mixed and the DNA was assayed. The difference between the numbers of breaks in the two samples is the inhibitory effect of AAAF on the removal of UV-endonuclease-sensitive sites. Methods *ii* and *iii* are suitable for measuring small changes.

RESULTS

Unscheduled DNA Synthesis. Control slides showed that $(CH_3)_2SO$ alone caused little increase in grain count above background and that the average number of grains due to UV did not change when it was added to the medium. The results of the autoradiographic studies on the effects of various doses of UV, AAAF, and a combined treatment of both agents are shown in Fig. 1. A dose of 5 J·m^{-2} results in the same number of grains as does 5 μM AAAF and 10 J·m^{-2} is equivalent to 10 μM AAAF. Moreover, the numbers of grains above cells given a combined treatment are close to those expected from an additive effect of the individual treatments. Another way of presenting these data (Fig. 2) shows that a combined treatment of UV plus AAAF results in much more unscheduled DNA synthesis than the saturation level observed with UV alone at doses of 20 J·m^{-2}.

Endonuclease-Sensitive Sites. *M. luteus* endonuclease provides a sensitive way to detect pyrimidine dimers in DNA and we have employed it to analyze the DNA in cells treated with both UV and AAAF. The enzyme preparation is particularly useful because, as we show below, it does not break phosphodiester bonds in the DNA of AAAF-treated cells and hence permits us to measure pyrimidine dimers in cells subjected to combined treatments of UV and AAAF.

The DNA extracted immediately from cells exposed to 10 μM AAAF and treated with the endonuclease showed no difference in sedimentation profile from the DNA of cells without AAAF treatment (Fig. 3*A*). This effect was not due to the inhibition of the endonuclease by the AAAF-treated DNA because when we treated one set of cells with a combination of UV plus AAAF and another set with only UV and extracted the DNA immediately the sedimentation profiles (Fig. 3*B*) showed that the endonuclease worked equally well on the UV lesions in both sets of cells.

Fig. 4 shows typical sedimentation data for cells treated with UV alone and incubated for different times after irradiation. The dose used, 20 J·m^{-2}, is near the saturation level for unscheduled synthesis and removal of endonuclease-sensitive sites

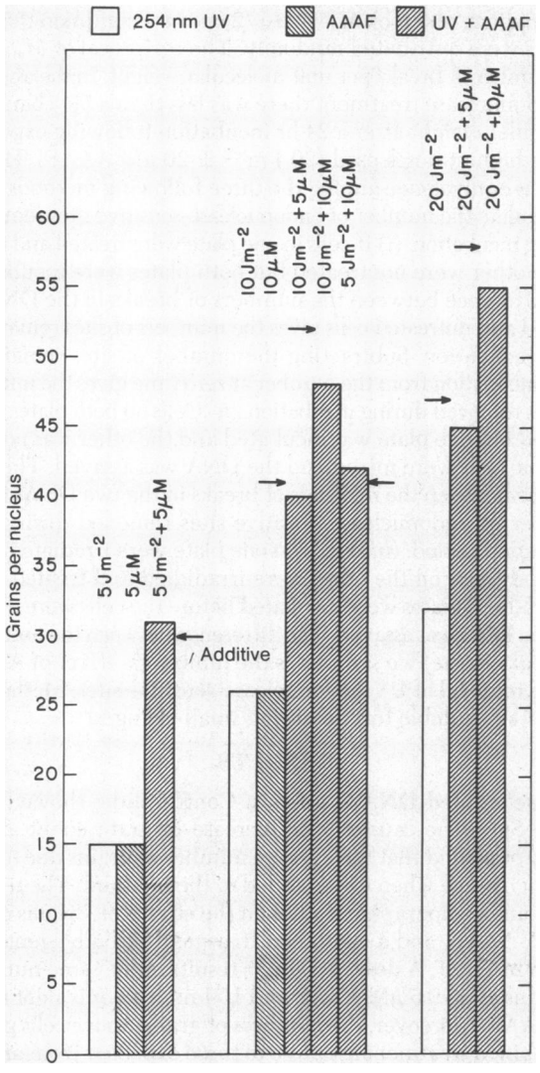

FIG. 1. The average number of grains per nucleus as a function of various doses of UV, AAAF, and combined treatments. Cells were seeded on coverslips, hydroxyurea was added 3 hr before treatment, and after the indicated treatment new medium was added containing [^{3}H]thymidine and hydroxyurea. The cells were left to do unscheduled DNA synthesis for 3 hr. Radioautographs were made (see *Materials and Methods*). An average of 50 nuclei was used. Background has been subtracted.

(see below). We calculate that out of an initial number of 36 sites per 10^8 daltons, 14 were removed in 6 hr and 28 were removed in 24 hr. To see if AAAF treatment affected the removal of sites *in vivo* we gave one set of cells a combined treatment of 20 J·m^{-2} plus 20 μM AAAF while the other set received only 20 J·m^{-2}. Both sets were incubated for 6 hr before DNA extraction and assay. The sedimentation profiles of the two sets of cells (Fig. 5) were what we would expect due to removal of some sites after 6 hr of incubation and were similar for both treatments, indicating that AAAF treatment does not significantly affect the removal of sites *in vivo*. we did a number of experiments to determine the number of UV-endonuclease-sensitive sites removed after a combined treatment with both agents as compared to UV alone. Fig. 6 shows the results of such experiments. It can be seen that the number of sites removed at 6 hr was less than at 24 hr. The 6 hr incubation gives an approximation to the initial rate of removal while the 24 hr gives approximately the extent of site removal. We note that at 6 hr the removal of sites is saturated at a dose of 20 J·m^{-2}. The combined

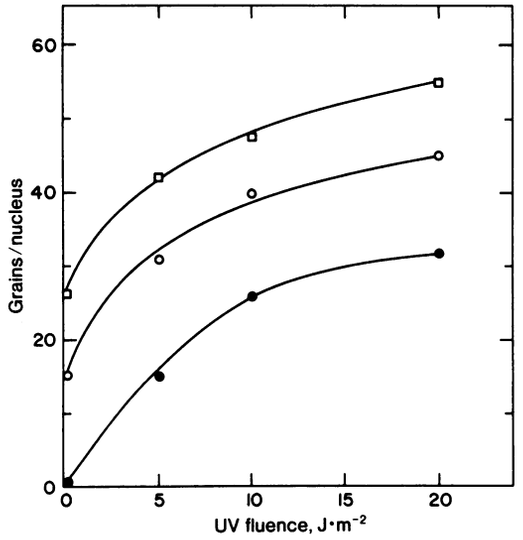

FIG. 2. Relationship between unscheduled DNA synthesis, expressed as number of grains per nucleus, and various doses of UV. For UV alone (●); UV + 5 μM AAAF (○); UV + 10 μM AAAF (□).

treatment of UV plus AAAF gives results similar to those due to UV alone, indicating that AAAF treatment does not inhibit the removal of sites sensitive to exogenous UV endonuclease, even though the concentrations of AAAF used were in some cases equivalent to UV doses of 20 J·m^{-2} in terms of unscheduled DNA synthesis (see Fig. 1).

DISCUSSION

A number of techniques have been used to show that both the rate and the extent of excision repair of pyrimidine dimers saturate at high doses (>20 J·m^{-2}) (10–13, 21). The amount of repair, or the number of dimers excised, becomes approximately independent of dose at high doses. Unscheduled DNA synthesis following AAAF treatment also saturated after treatment with high concentrations (21), and by this repair measurement and others there is a reasonable equivalence between UV and AAAF treatments (5, 21, 22). Five J·m^{-2} is equivalent to 5 μM in our experiments (Fig. 1). Moreover, as outlined in the introduction, AAAF damage mimics UV dam-

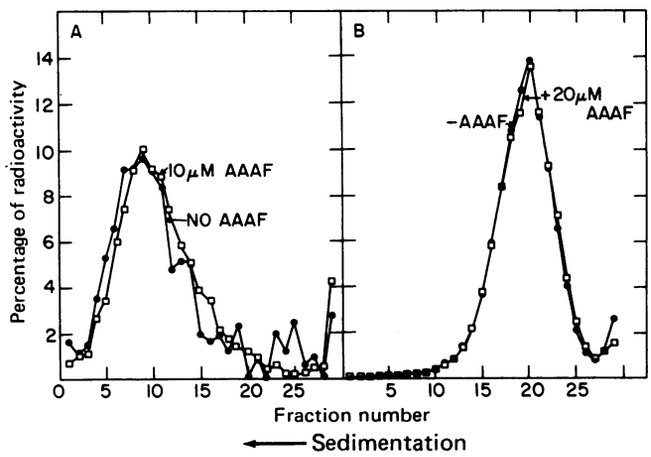

FIG. 3. Sedimentation profiles of extracted DNA, after treatment of cells with UV, AAAF, or AAAF plus UV. DNA was extracted immediately after treatment and samples were incubated with endonuclease after extraction. (*A*) Untreated cells (●); cells treated with 10 μM AAAF (□). (*B*) Cells exposed to 20 J·m^{-2} of UV (●); cells treated with UV plus 20 μM AAAF (□).

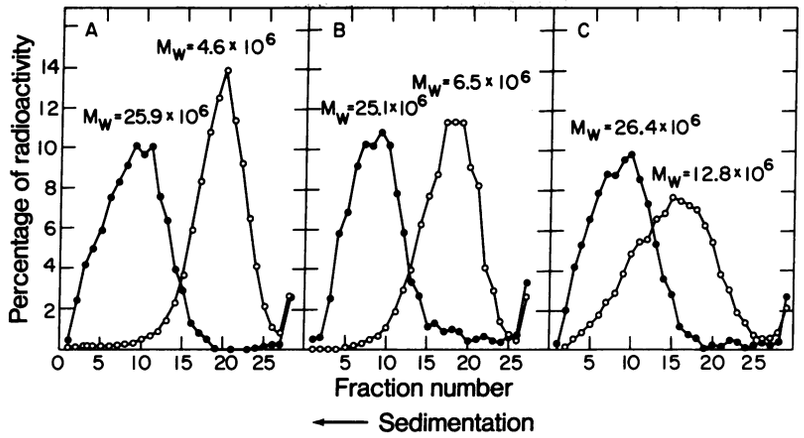

FIG. 4. Sedimentation profiles of extracted DNA, after treatment with UV endonuclease. Cells were exposed to no UV (●) or 20 J·m^{-2} (○). (*A*) Extraction immediately after irradiation. (*B*) Extraction 6 hr after irradiation. (*C*) Extraction 24 hr after irradiation. The weight average molecular weights, M_w, of the molecules giving the profiles are indicated.

age to human cells in other and more convincing ways. Hence, we expected that the pathway for repair of UV damage and for AAAF damage would be similar in normal human cells and that the amount of unscheduled synthesis resulting from a combined treatment at high doses would saturate at the same level as for separate treatments. The expectation was not observed. The amount of unscheduled synthesis resulting from a combined treatment was the sum of those observed with separate treatments (Fig. 2).

We used a completely independent technique to verify the surprising result observed using unscheduled synthesis measurements. An endonuclease preparation from *M. luteus* makes single-strand breaks in DNA containing pyrimidine dimers and the number of breaks equals the numbers of dimers, but does not do so in DNA from cells treated with AAAF. Therefore, the nuclease may be used to measure the numbers of dimers remaining after repair has taken place in UV-irradiated cells or in cells given a combined treatment of UV plus AAAF. Our original naive expectation led us to predict that at saturating UV doses AAAF treatment would give rise to more lesions in DNA and compete at the level of the rate-limiting step for excision repair and inhibit the loss of UV-endonuclease-sensitive sites. As in the case of unscheduled synthesis, the prediction was wrong. AAAF had no significant effect on the disappearance of nuclease-sensitive sites (Fig. 6). Therefore, we conclude that the enzymatic steps involved in repair of the two types of damage are not identical, although they may have many steps in common.

The rate-limiting step in the excision repair mechanism seems to be the incision step because: (*i*) during excision in normal human cells there are very few single-strand breaks compared to the number of dimers (13, 23); (*ii*) many fewer strand breaks are detected in excision-deficient XP cells (13, 23); and (*iii*) addition of exogenous nuclease restores unscheduled DNA synthesis in UV-irradiated, Sendai-virus-treated XP cells (24). However, our results indicate that there are different rate-limiting steps in excision repair of UV and AAAF damage and they suggest that the rate-limiting step is not a common incision step. Hence, we are led to consider models involving coordinated repair complexes, such as those proposed for *Escherichia coli* and human fibroblasts (25, 26). In such models defects in

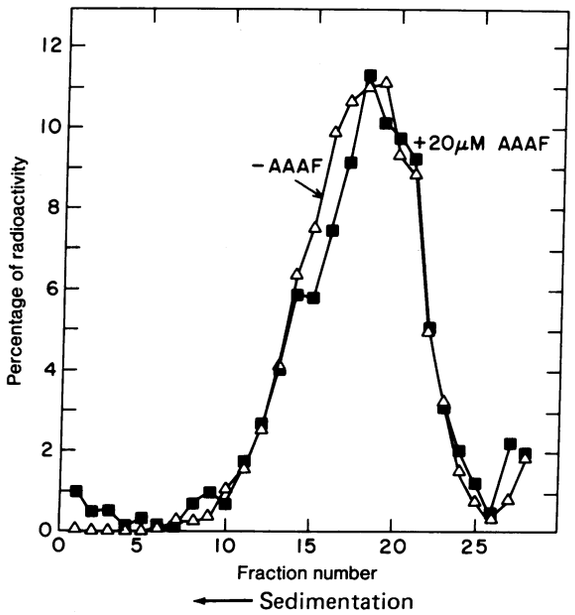

FIG. 5. Sedimentation profiles of DNA after incubation with UV endonuclease. DNA was extracted 6 hr after cells were exposed to 20 J·m^2 (△) or 20 J·m^{-2} plus 20 μM AAAF (■).

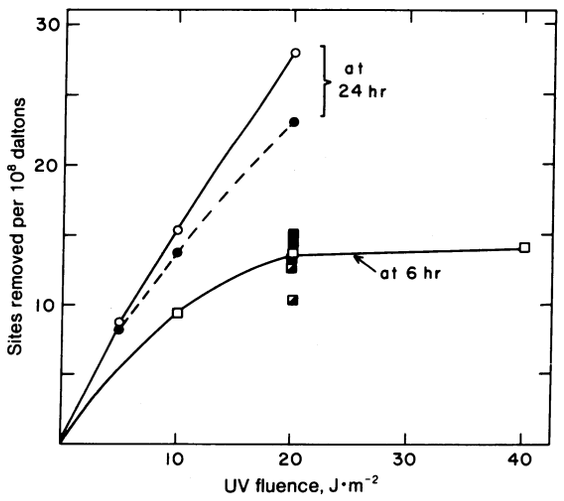

FIG. 6. The number of UV-endonuclease-sensitive sites removed as a function of UV dose for two incubation times (6 hr, squares; 24 hr, circles) and several AAAF treatments given immediately after irradiation (○, □, no AAAF; ◪, 10 μM AAAF; ●, ■, 20 μM AAAF).

one repair enzyme may affect the activity of all others in the complex even though the complex may include endonucleases for several kinds of DNA damage. The defect in XP cells that results in the failure to carry out normal excision repair of UV, chemical, and one kind of ionizing radiation damage (27) may be at the level of formation or stability of this presumptive repair complex. The existence of complementation groups among XP cells (28) is explicable in terms of the exchange of subunits between inactive complexes so as to give rise to active ones and an association of photoreactivating enzyme (29) with such a complex could also explain the observation that XP cells are defective in activity of that enzyme.

An accompanying paper by Amacher *et al.* (30) shows by direct measurement of excision of dimers and loss of AAAF products from DNA in cells of several species that the two types of damage are removed at very different rates and hence presumably by different enzymatic pathways.

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