NEAR-ULTRAVIOLET LIGHT-INDUCED STRAND BREAKS IN DNA PRETREATED WITH THE CARCINOGEN, *N*-ACETOXY-2-ACETYLAMINOFLUORENE

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ABSTRACT Neutral sucrose gradients of supercoiled DNA (ΦX-174 RF I) were used to measure the in vitro production of strand breaks by the carcinogen, *N*-acetoxy-2-acetylaminofluorene (AcO-AAF). Treatment with AcO-AAF in 10% dimethyl sulfoxide did not directly yield strand breaks. Breaks in relatively low yield appeared after alkali treatment (pH 13, 60 min) of the RF I previously reacted with AcO-AAF. The DNA treated with AcO-AAF was sensitive to single-strand breakage by 303 nm near-ultraviolet light under neutral conditions. The greater the prior AcO-AAF treatment, the greater the sensitivity to 303 nm light. Post-irradiation alkali treatment greatly enhanced the light-induced strand breakage.

INTRODUCTION

The carcinogenic aromatic amine, 2-acetylaminofluorene (AAF), acts at specific tissue sites. This specificity is due to a requirement for enzymatic activation, believed to proceed via a "proximate" carcinogen, *N*-hydroxy-2-acetylaminofluorene, and one or more ester derivatives, sometimes termed "ultimate" carcinogens. A synthetic ester, *N*-acetoxy-2-acetylaminofluorene (AcO-AAF) has been widely used as a model for the ultimate carcinogenic derivative of AAF (1, 2). This acetoxy-derivative is (*a*) carcinogenic at the site of application; (*b*) chemically reactive with DNA, RNA, and protein in vivo and in vitro (1, 2); and (*c*) mutagenic to transforming DNA (3), to phage (4), and to bacteria, in which it can specifically produce frameshift mutations (5). Under neutral conditions in vitro, reaction with DNA occurs primarily at the 8-carbon of guanine, yielding a nucleic acid containing *N*-(guanine-8-yl)-AAF. Unlike alkylation at N-7, this product is stable and does not lead to spontaneous depurination (6). Reaction with adenine also occurs to a lesser degree, the extent depending on the conformation of the DNA (7, 8). The addition of the -AAF residue, which displays ultraviolet light (UV) absorption above 300 nm, yields an absorption shoulder at 300–310 nm for the DNA-AAF complex (6, 8). This complex can be photochemically modified by irradiation at 310 nm, as determined by changes in absorption and fluorescence (8, 9). The nature of the modification is not known. Physical studies on DNA treated

with AcO-AAF show some cross-links (3, 8, 10) but extensive strand breakage is not observed (10, 11). However, when mammalian cells are treated with AcO-AAF, DNA strand breaks are observed, as measured by alkaline sucrose gradients (12–14). It is not clear whether the breaks produced in vivo occur (*a*) spontaneously at AcO-AAF reaction sites, (*b*) from enzymatic attack at reaction sites, or (*c*) as a result of alkali treatment of the AcO-AAF-altered DNA.

In the experiments reported here, we have used neutral sucrose gradients of supercoiled DNA (ΦX-174 RF I) to analyze for the in vitro production of strand breaks by (*a*) AcO-AAF, (*b*) AcO-AAF followed by alkali treatment, (*c*) 303-nm UV exposure of DNA pretreated with AcO-AAF, and (*d*) alkali treatment of the UV-irradiated AAF-DNA complex.

METHODS

Chemicals

The AcO-AAF was generously provided by Dr. E. Miller. A stock solution of 3–4 mg/ml in dimethyl sulfoxide (DMSO; J. T. Baker Chemical Co., Phillipsburg, N.J.) was stored at −52°C. Absorption spectra indicated that this stock was stable under these conditions for several weeks.

Preparation of RF I

[^{3}H]thymidine-labeled ΦX-174 replicative form I (RF I; closed circular, double-strand, supercoiled) was prepared according to published procedures (15, 16). The purified RF I was suspended in 0.01 M Tris, 0.001 M EDTA, pH 8 (Tris-EDTA Buffer), at 6 μg/ml and stored at −52°C. This preparation was 93% RF I and 7% RF II.

Preparation of RF II

The RF II used in some experiments was formed by irradiating RF I with 60 krads of ^{60}Co gamma rays, yielding greater than 98% conversion of RF I to RF II (16).[1]

Reaction of AcO-AAF with RF-DNA

All reactions were performed at 37°C under dim light in a mixture of 1 vol AcO-AAF in DMSO and 9 vol of RF I stock. Under these conditions, absorption spectrum changes indicated an AcO-AAF half-life of about 45 min. For reactions with the AcO-AAF above about 200 μg/ml, a blue-white turbidity gradually appeared. This did not affect the recovery of the labeled RF-DNA in the subsequent Sephadex G-25 column separation. We believe that this turbidity represents an insoluble breakdown product(s) of AcO-AAF.

Removal of Unbound AcO-AAF

Immediately after the reaction, the AcO-AAF and DNA mixture (50–200 μl) was passed through a small Sephadex G-25 column (0.5 × 5 cm) to remove unbound AcO-AAF. Tris-EDTA Buffer containing no DMSO was used to elute the column. The ^{3}H-labeled RF appeared in the excluded volume. A second peak of UV-absorbing, unlabeled material appeared in the retarded volume, as would be expected for AcO-AAF and its degradation products. Recovery of RF was virtually 100% in all cases. The low concentration of RF precluded

[1] Burns, L. R., and W. D. Taylor. Unpublished observation.

accurate determinations of its UV absorption spectra before and after reaction. However, such spectra as could be obtained did indicate that little unbound AcO-AAF remained with the eluted RF.

303 nm Irradiation

RF samples of 0.2–0.5 ml from the G-25 columns were exposed in microcuvettes to 303 nm UV from a high-intensity water prism monochromator. As determined by a calibrated photocell, the intensity was 300–500 ergs/mm^2 s. The sample transmission at 303 nm was always greater than 95%. A plastic filter allowing no transmission below 286 nm was inserted in the light beam just before the sample. The exposure to the sample in ergs/mm^2 often called "dose" will be designated "energy fluence" in keeping with more modern terminology (17).

Alkali Treatment

In some experiments, the RF DNA treated only with AcO-AAF or with AcO-AAF followed by 303 nm UV light was adjusted to pH 13 with 0.5 M NaOH. The sample was held at this pH at 23°C for 60 min and then neutralized with 0.5 M HCl before being layered on the neutral sucrose gradients. Experiments utilizing other holding times at pH 13 showed that the alkali effect was maximal by 30 min and that no further change occurred up to at least 90 min.

Sucrose Gradients

Gradients were 5–20% sucrose prepared in the Tris-EDTA buffer at 5°C and layered with 50–200 μl of the RF preparation. Sedimentation was at 5°C in a Beckman SW 50.1 rotor (Beckman Instruments, Spinco Div., Palo Alto, Calif.) for 215 min at 50,000 rpm in an L2-65B centrifuge. Fractions (31–33 per gradient) of six drops each were counted in a Beckman LS-230 counter utilizing a toluene:triton:water emulsion system (18). The counting efficiency did not change significantly along the gradients.

RESULTS

Effect of AcO-AAF on S-Values of RF I and II

At neutral pH and in low salt, untreated ΦX-174 RF I sediments at an $s_{20,w}$ of 23.7 (19, 20). If a single-strand break is introduced, yielding RF II, sedimentation is at 17.3 S (19, 20). After denaturation by alkali at pH 13 and subsequent neutralization, RF I renatures to a double-stranded supercoiled form of 24.6 S (estimated from this study). RF II yields two single-strand forms, one a closed molecule and one a linear molecule, both of 14.4 S (15). A double-strand break with no denaturation would yield RF III of about 12 S (16). Under our sedimentation conditions, RF I and II sedimented to fractions 11 and 17, respectively; the alkali denatured-neutralized forms of RF I and II sedimented to fractions 10 and 20, respectively. RF III would be expected to sediment to fraction 22 before alkali treatment and to fraction 20 after alkali.

If AcO-AAF directly produced single- or double-strand breaks in RF I, the amount of material at 23.7 S would decrease, along with a concomitant increase in material at 17.3 or 12 S. No quantum shifts of S-value of this sort were observed after treatments with AcO-AAF, with no alkali. Thus, AcO-AAF did not by itself lead to single- or

TABLE I

S-VALUES OF RF I AFTER VARIOUS REACTIONS WITH AcO-AAF

Reaction time	$s_{20,w}$	
	150 μg/ml	300 μg/ml
min		
0	23.7	23.7
2.5	22.0	19.3
5	20.3	19.0
10	18.0	20.4
20	19.4	23.0
40	21.0	24.4

double-strand breaks under neutral conditions, up to at least our treatment with 300 μg/ml for 40 min.

Instead, with increasing AcO-AAF concentrations and reaction times, the sedimentation constant of the RF I continuously shifted to lesser and lesser values until it approached that of RF II; then it increased again for the still more extensive treatments, ultimately becoming slightly greater than for the original untreated RF I. Table I shows the S-values for the AcO-AAF-treated RF I for increasing reaction times at two concentrations. Fig. 1 shows the $s_{20,w}$ for RF I and II as a function of AcO-AAF concentration for a 10-min reaction period. Because the treatment was used in

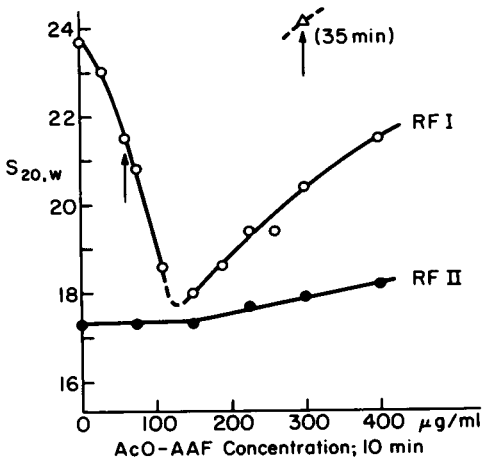

FIGURE 1 The sedimentation constants ($s_{20,w}$) of RF I and RF II as a function of AcO-AAF concentration for 10 min reaction time. The RF I values (-○-) were obtained as described in the text, with additional untreated RF II as a marker; the RF II values (-●-) were obtained after reaction of AcO-AAF with RF II under the same conditions as for RF I, with additional untreated RF I as a sedimentation marker. The --△-- point was obtained at 300 μg/ml for 35 min. The arrows indicate the two reaction conditions used to treat the RF I before the exposure to light. Unbound AcO-AAF was always removed by Sephadex G-25 column immediately after the end of the reaction period.

experiments to be presented later, the result for 35-min reaction with 300 μg/ml AcO-AAF is also shown.

The observed S-value changes for RF I and II are consistent with the interpretation that intercalation is occurring and that the degree of intercalation increases with reaction time and AcO-AAF concentration. It is known from other work (21) that the intercalation of increasing numbers of hydrophobic ring structures, such as ethidium bromide, proflavine, and certain other drugs and antibiotics, between the bases of RF I can at first unwind the supercoil to yield an untwisted closed molecule with about the same S-value as RF II; upon further intercalation, the RF I rewinds to give a supercoil of opposite twist, thus increasing the S-value again. RF II, already a relaxed, nicked circle, is little affected in S-value by such intercalation (21). The actual amounts of total and intercalative binding have not been measured in our experiments. Even though our results are qualitatively consistent with intercalation, we have not yet attempted to quantitatively correlate the degree of binding with the amount of untwisting, as has been done in other work (21).

Action of 303-nm Light on AcO-AAF-Treated RF I

To test for light-induced alterations of AcO-AAF-treated RF I, 303-nm light was chosen because it is absorbed much more strongly by the -AAF residue than by the normal bases of DNA (6, 8–10). Fig. 2 presents typical results. As noted earlier, no strand breakage was produced directly by the AcO-AAF (top panels), although the S-values were shifted by the amounts shown in Fig. 1. Single-strand breaks were induced in the treated DNA by the light, as indicated by the reduction in the amount of material in the RF I peak and the concomitant increase in the amount of RF II. The greater the energy fluence, the greater the conversion. Less light was required to produce single-strand breaks in the more extensively treated RF I.

Alkali Treatment

Since alkaline conditions frequently have been used to assay for strand breaks in DNA after various treatments (for example, ref. 12), it was of interest to determine what additional effects alkali might have on the AcO-AAF and light-treated RF DNA. The top panels of Fig. 3 show the effect of alkali on the AcO-AAF-treated DNA that was not exposed to 303 nm light. The RF I renatured and returned to about the same S-value as before the alkali treatment. The remainder of the RF, having undergone at least one strand break, renatured to forms which sedimented more slowly. Some sedimented in a peak at about 14 S (fractions 20–21). This is the expected value for the neutralized single-strand circles and linear molecules that result from the alkali treatment of RF II. In addition, for the 300-μg/ml treatment, another peak was observed at about fraction 17 (about 17 S). This corresponds to the expected S-value of renatured RF II. Our present interpretation is that this material is RF II that contains cross-links, thus allowing it to readily renature to a double strand upon pH neutralization. The lower panels of Fig. 3 indicate that the amount of this 17 S material increased after exposure to 303-nm light after both the 60- and 300-μg/ml treatments,

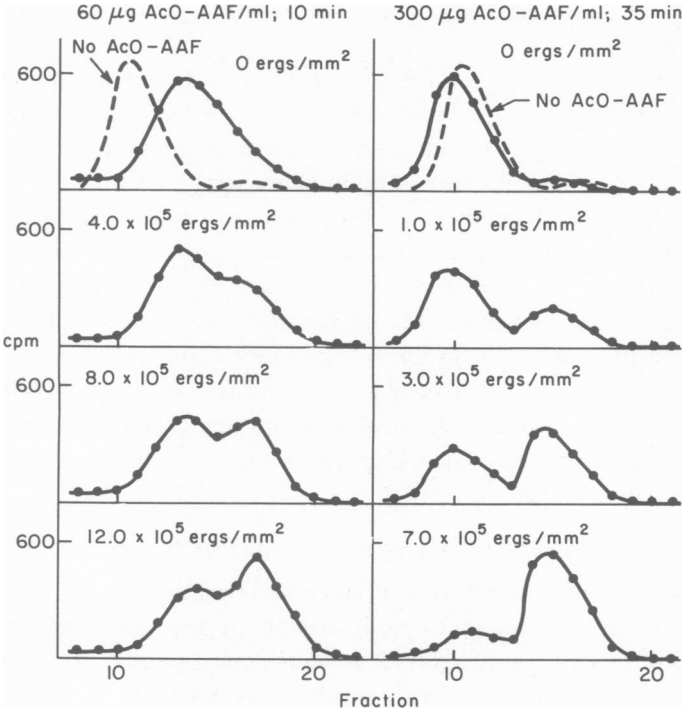

FIGURE 2 Neutral sucrose gradients showing the effect of 303-nm UV light on AcO-AAF-treated RF I. Only that region of the gradient containing counts is plotted. (Left) Pretreatment with 60 μg AcO-AAF/ml for 10 min. The AcO-AAF-treated RF I peaks at fractions 13–14 and RF II at fraction 17. (Right) Pretreatment with 300 μg AcO-AAF/ml for 35 min. The AcO-AAF-treated RF I peaks at fraction 10 and RF II at fraction 15. ———, treated RF I; – – – – – –, untreated RF I control. Note the 7% RF II present in the initial RF stock. Sedimentation was from right to left.

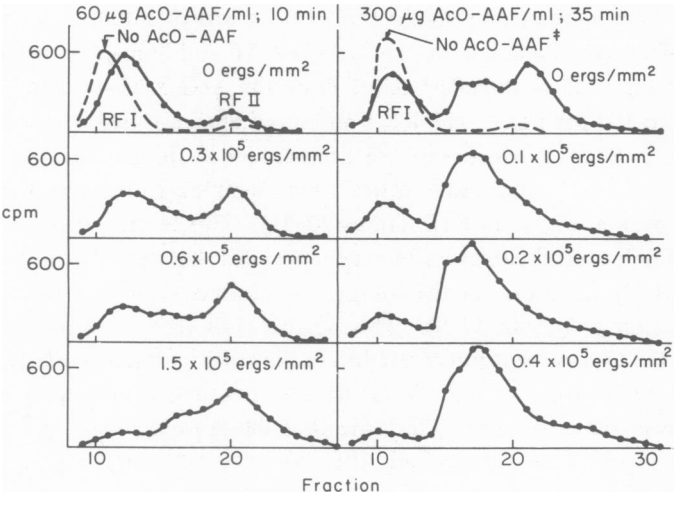

FIGURE 3 Neutral sucrose sedimentation patterns obtained after postirradiation (303 nm) alkali denaturation and neutralization of AcO-AAF-treated RF I. Notation is as in Fig. 2. (‡, this "No AcO-AAF" curve was obtained with half the amount of RF used for the treated samples.)

although at lower exposures after 300 μg/ml. For this latter treatment, little material remained as separated single strands (14 S) after 2×10^4 ergs/mm^2.

Since the interpretation of some aspects of the rather complex gradients observed after AcO-AAF, light, and alkali treatment is uncertain, we have so far focused our attention primarily on the production of strand breaks, that is, on the loss of material from the RF I peak. The alkali treatment by itself induced strand breaks in 16% of the initial RF I reacted with 60 μg AcO-AAF/ml for 10 min and in 70% of that reacted with 300 μg/ml for 35 min. Less than 3% was converted by alkali treatment of the RF I not treated with AcO-AAF. The alkali treatment also greatly enhanced the appearance of strand breaks in the light-treated RF I-AAF complex. Since uncrosslinked RF II and RF III would sediment in the same place after alkali treatment and neutralization, we cannot positively identify all of the material lost from the RF I peak as resulting from single-strand breaks. Possibly some double-strand breaks have also occurred. However, since much of the material initially found at about 14 S (fractions 20–21) after the 300 μg/ml treatment goes to the position of renatured RF II after light exposure, rather than to the position of RF III, we tentatively conclude that a single-strand break has occurred in much of the material lost from the RF I peak.

Strand Breaks as a Function of Energy Fluence

The fraction of the total gradient counts (after background subtraction) remaining under the RF I peak is a measure of the relative amount of RF I that has undergone no strand breakage. Fig. 4 shows plots of the log of this fraction as a function of the

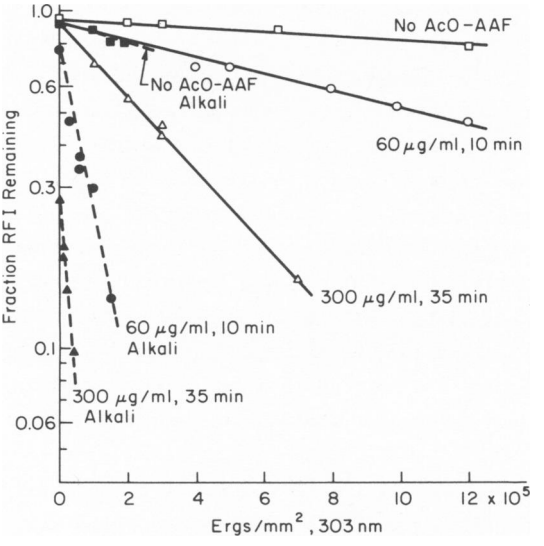

FIGURE 4 Log of the fraction of RF I remaining as a function of the energy fluence (ergs/mm^2) of 303-nm light, for untreated RF I DNA (—□—, no alkali; - -■- -, alkali-denatured), 60 μg, AcO-AAF/ml for 10 min (—○—, no alkali; - -●- -, alkali-denatured), and 300 μg AcO-AAF/ml for 35 min (—△—, no alkali; - -▲- -, alkali-denatured).

TABLE II

PARAMETERS FOR STRAND-BREAK PRODUCTION BY 303 nm LIGHT

Treatment		D_{37}	σ
		$ergs/mm^2 \times 10^{-5}$	$cm^2 \times 10^{19}$
No alkali:	no AcO-AAF	82	0.08
	60 μg/ml; 10 min	18	0.36
	300 μg/ml; 35 min	4.0	1.6
Alkali:	No AcO-AAF	15	0.44
	60 μg/ml; 10 min	0.9	7.3
	300 μg/ml; 35 min	0.3	22

energy fluence in ergs/per square millimeter. The curves show first-order ("single-hit") kinetics. These "survival" curves for unbroken RF I can be analyzed in terms of the energy fluence yielding 37% of the initial amount of RF I (D_{37}). The inverse of this parameter is a measure of the effectiveness ("action cross section") for the production of strand breaks by the light. Table II gives the D_{37} ($ergs/mm^2$) and action cross section, σ (inverse of D_{37} in photons per square centimeter), for the various AcO-AAF and alkali treatments used here, along with the control results for no AcO-AAF treatment.

DISCUSSION

Recent work (22, 23) has introduced new factors related to the nature of the binding of AcO-AAF to DNA. After treatment of DNA with AcO-AAF in certain solvents (ethanol; ethylene glycol), Sephadex G-25 columns do not remove all noncovalently bound carcinogen (22). This result may depend upon the reaction conditions. In our experiments it is not clear how much noncovalently bound AcO-AAF remains after passage through G-25 after reaction with RF in 10% dimethylsulfoxide. However, on the basis of the method of analysis used by Waring (21), and our S-value shifts with increasing AcO-AAF treatment, the extents of intercalation would be expected to be on the order of 200 and 800 AAF residues per RF molecule (5,000 base pairs) for the 60-μg/ml, 10-min, and the 300-μg/ml, 35-min treatments, respectively. This assumes that the untwisting produced per intercalated residue is about the same as for typical intercalators (21). At least some of the intercalation probably involves covalent linkage at the C-8 of guanine. Covalent binding and intercalation in vivo has been suggested as the initial step in the production of frameshift mutations in *S. typhimurium* by AcO-AAF (5).

Our AcO-AAF treatment of RF I in vitro does not by itself yield single- or double-strand breaks under neutral conditions. This supports the previous views that no breaks would be expected from the predominant reaction at the C-8 of guanine (6). In addition, there appears to be no other minor reaction products that directly yield breaks at neutral pH.

Alkali-labile alterations leading to strand breaks result from the AcO-AAF treat-

ment. It appears probable that only a very small fraction of the bound -AAF residues serve as alkali-sensitive sites. This conclusion is based on the observation that 30% of the RF I molecules undergo no alkali-induced breakage even after 35 min treatment at 300 μg/ml, a treatment that may be yielding intercalation of on the order of 800 AAF residues per RF molecule. This suggests that the alkali-labile lesion may be a minor reaction product, or one occurring at a specific site in the RF DNA.

For the 300 μg/ml, 35-min treatment, after alkali almost one-half of the RF II appears to contain cross-links. Such cross-links would not be detectable in the undenatured RF I or II of the neutral pH experiments nor in the renatured RF I after alkali treatment. Hence it is not clear whether they result from the AcO-AAF alone or from the effects of the alkali on the AcO-AAF adducts. The yield of cross-links per bound AAF residue appears to be low. As indicated earlier, cross-linking of DNA by AcO-AAF has been previously reported (3, 8, 10).

Our results indicate that it is unlikely that the in vivo breaks observed by Laishes and Stich (12) and Sarma et al. (14) in mammalian cell DNA after AcO-AAF treatment are directly produced by the AcO-AAF or the alkali gradients.

AcO-AAF treatment of RF I DNA as performed here sensitizes this DNA to strand breakage by 303 nm UV. Subsequent alkali treatment of the RF I exposed to light induces many more breaks. Since the identity and exact number per RF molecule of light-sensitive AcO-AAF reaction products are not known, it is not possible at present to determine reliable quantum yields for the photochemical process(es) leading to strand breakage. However, some order-of-magnitude estimates may be valuable. If the absorbing species leading to breakage is assumed to be the AAF adduct to the C-8 of guanine, then the absorption cross-section at 303 nm per residue can be calculated (10). In addition, if the number of these per RF is reliably indicated by the S-value shifts, then quantum yields in terms of breaks per absorbed photon per RF molecule can be estimated. This method of analysis gives quantum yields of about 3×10^{-6} for the nonalkali-treated RF I and about 5×10^{-5} for the alkali-treated. The quantum yield for cross-linking would be about 1×10^{-4} in the alkali experiments.

The higher 303 nm action cross sections (σ) for breakage after the 300 μg/ml treatment as compared to the 60 μg/ml treatment (about 4.4 times for the nonalkali and about three times for the alkali-treated) are consistent with the approximately fourfold greater degree of intercalation estimated from the S-value shifts of Fig. 1 and analysis based on the work of Waring (21).

On the order of 1×10^4 ergs/mm^2 of 303-nm light is required to produce a detectable sunburn in undarkened human skin (24, 25). This amount of light in the vicinity of 303 nm could be received from the sun in about 1 h of exposure. The amount of sunlight needed to potentiate DNA damage in exposed cells after AcO-AAF treatment, particularly of the type leading to breaks in alkali ($< 10^5$ ergs/mm^2), would thus be comparable to that yielding a moderate to severe sunburn resulting from a few hours of exposure to sunlight.

The synergistic effects of near UV light on AcO-AAF-treated DNA and cells must be considered when evaluating molecular and cellular responses to this chemical.

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REFERENCES

1. MILLER, J. A. 1970. *Cancer Res.* **30**: 559.
2. MILLER, J. A., and E. C. MILLER. 1969. *Prog. Exp. Tumor Res.* **11**:273.
3. MAHER, V. M., E. C. MILLER, J. A. MILLER, and W. SZYBALSKI. 1968. *Mol. Pharmacol.* **4**: 411.
4. CORBETT, T. H., C. HEIDELBERGER, and W. F. DOVE. 1970. *Mol. Pharmacol.* **6**: 667.
5. AMES, B. N., F. D. LEE, and W. E. DURSTON. 1973. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 782.
6. KRIEK, E., J. A. MILLER, U. JUHL, and E. C. MILLER. 1967. *Biochemistry.* **6**: 177.
7. KRIEK, E., and J. REITSEMA. 1971. *Chem.-Biol. Interactions.* **3**: 397.
8. MICHELSON, A. M., A. M. KAPULER, and F. POCHON. 1972. *Biochim. Biophys. Acta.* **262**: 441.
9. KAPULER, A. M., and A. M. MICHELSON. 1971. *Biochim. Biophys. Acta.* **232**: 436.
10. FUCHS, R., and M. DUANE. 1972. *Biochemistry.* **11**: 2659.
11. TROLL, W., E. RINDE, and P. DAY. 1969. *Biochim. Biophys. Acta.* **174**: 211.
12. LAISHES, B. A., and H. F. STICH. 1973. *Can. J. Biochem.* **51**: 990.
13. FARBER, E. 1973. *Cancer Res.* **33**:2537.
14. SARMA, D. S. R., R. O. MICHAEL, B. W. STEWART, R. COX, and I. DAMJANOV. 1973. *Fed. Proc.* **32**: 833.
15. BURTON, A., and R. L. SINSHEIMER. 1965. *J. Mol. Biol.* **14**: 327.
16. CHRISTENSEN, R. C., C. A. TOBIAS, and W. D. TAYLOR. 1972. *Int. J. Radiat. Biol.* **22**: 457.
17. RUPERT, C. S. 1974. *Photochem. Photobiol.* **20**:203.
18. KHOURY, A. T., and R. A. DEERING. 1973. *J. Mol. Biol.* **79**: 267.
19. KIGER, J. A., E. T. YOUNG II, and R. L. SINSHEIMER. 1968. *J. Mol. Biol.* **33**: 395.
20. COZZARELLI, N. R., R. B. KELLY, and A. KORNBERG. 1968. *Proc. Natl. Acad. Sci. U. S. A.* **60**: 992.
21. WARING, M. 1970. *J. Mol. Biol.* **54**: 247.
22. LEVINE, A. F., L. M. FINK, I. B. WEINSTEIN, and D. GRUNBERGER. 1974. *Cancer Res.* **34**:319.
23. CHANG, C., S. J. MILLER, and J. G. WETMUR. 1974. *Biochemistry.* **13**: 2142.
24. EVERETT, M. A., R. M. SAYRE, and R. L. OLSON. 1969. *In* The Biologic Effects of Ultraviolet Radiation. F. Urbach, editor. Pergamon Press, Inc., Elmsford, N. Y. 181–186.
25. JOHNSON, B. E., F. DANIELS, JR., and I. A. MAGNUS. 1968. *Photophysiology.* **4**:139.