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PHOTOTRANSFORMATION OF POLYCYCLIC AROMATIC HYDROCARBONS INTO STABLE, MUTAGENIC COMPONENTS.

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INTRODUCTION

Many potent photodynamic chemicals induce a variety of biological responses ranging, for example, from the modification of amino acids to the causation of erythemal responses on the skin of mammals (1,2). The polynuclear aromatic hydrocarbons (PAH) have been viewed with particular interest since the discovery of their photodynamic properties in the mid 1930's (3,4). The most significant findings have demonstrated high correlations between the photodynamic action of certain PAH with their ability to induce carcinogenic responses (5,6,7). More recently, other investigators have shown that PAH can be transformed into reactive cytotoxic and mutagenic intermediates following their exposure to natural sunlight and other sources of radiation (8,9, 10,11).

Recent observations in our laboratory (11) have indicated that although benzo[a]pyrene (BaP), 7,12-dimethylbenz[a]anthracene (DMBA), and other related model polycyclics are cytotoxic following activation by near ultraviolet light (UVA), they do not induce a significant photomutagenic response in either bacterial or mammalian culture test systems (Strniste and Chen, unpublished data). These results were perplexing since under similar conditions complex organic mixtures known to contain PAH were considerably photomutagenic (12,13,14). Because these latter studies indicated the presence of heretofore unidentified photodynamic constituents, with potent mutagenic activity, we began to systematically analyze the photomutagenic properties of polycyclic aromatic amines, another class of organics found in these complex mixtures.

In this report we compare the mutagenicity of several PAH, including three aromatic amines following exposure to sunlight or an artificial source of UVA. The most active of these compounds, 2-aminofluorene (2-AF), was further investigated to determine the mechanism of its photoactivation and the chemical identity of the induced and reactive photoproducts.

MATERIALS AND METHODS

Chemicals

BaP, 2-AF, 9,10-dimethylanthracene (DMA), 2-aminoanthracene (2AA), and 2-aminonaphthalene (2-AN) were obtained from Aldrich Chemical Company. These chemicals were routinely dissolved in spectrophotometric grade dimethyl sulfoxide (DMSO) at concentrations of 1 mg/ml. The various derivatives of 2-AF used as standards in high pressure liquid chromatography (HPLC) were also obtained from Aldrich Chemical Company.

Irradiation

Sunlight exposures were conducted on the rooftop of the Health Research Laboratory at the Los Alamos National Laboratory, Los Alamos, NM, latitude 36°, elevation ~7300 ft above sea level. The incident fluence as measured through a Petri dish cover by an Eppley thermopile (Eppley Laboratory, Inc.) was ~950 $J/m^2/sec$.

Two parallel 15-watt blacklights (GE F15T8 BLB) were used as an artificial source of UVA radiation (300-400 nm wavelength). Incident fluence through a Petri dish cover average 6.8 $J/m^2/sec$ as measured by the same thermopile. Irradiations were performed in 60 mm glass Petri dishes with the lids attached. Colored, glass filters (WG 360, GG 420 and GG 495) which eliminate >90% of wavelengths of light below 360, 420 and 495 nm respectively, were obtained through Melles Griot, Irvine, CA.

Ames/Salmonella Bioassay

Standard plate assays as described by Ames *et al.* (15) were performed with *Salmonella typhimurium* tester strain TA98. Except where diagnostic mutagens made it necessary, the typical assay did not utilize rat liver S9 homogenates. BaP and 2-nitrofluorene were employed as diagnostic mutagens to insure proper functioning of the assay system. Linear dose response curves were generated for 0-50 µl of each irradiated sample. Data in this report, however, is presented from single dose points (from the linear dose response curves) to enable condensation of the material.

Historical his^+ revertant background in this laboratory for TA98 (without S9) is 23 ± 4.

High Pressure Liquid Chromatography

2-AF photoproducts were analyzed by reverse phase HPLC using a Beckman Model 334 Gradient Liquid Chromatograph system fitted with a 10 μ Radial-PAK C_{18} cartridge and a radial compression module (RCM-100, Waters and Associates). The samples were usually applied to the column in 20 μl of DMSO and eluted with a linear gradient of triethylammonium carbonate buffer (1 mM, pH 8.3): acetonitrile (95:5 to 0:100 in 45 min) at a changing flow rate of 2 to 3 ml/min. Photoproducts of 2-AF were detected by absorbance at 254 nm. In semi-preparative HPLC analysis, 250 μl samples were applied to the column and the A_{254} peaks from consecutive runs were pooled into fractions, rotary evaporated, redissolved in DMSO and then assayed for mutagenic activity. Retension times (R_t) of various 2-AF photoproducts were compared to R_t of authentic oxidized derivatives of 2-AF.

RESULTS AND DISCUSSION

Sunlight Induced Mutagenicity of PAH

Five commercially available PAH were dissolved in DMSO, exposed to direct sunlight, and bioassayed in the Ames/Salmonella test for direct mutagenic activity. The data presented in Table 1 illustrates that photoactivated 2-AF elicits a highly mutagenic response in *S. typhimurium* TA98 in the absence of exogenously supplied metabolic enzymes (induced rat liver S9 homogenate). This activity remained stable for at least one month when the irradiated 2-AF was stored in the dark at room temperature. Of the PAH listed, only 2-AF showed a significant mutagenic response in the absence of exposure to light (i.e., at 50 μg/plate 150 ± 10 his^+ revertants were induced). The origin of this activity has not yet been determined, but the possibilities include minor contaminants and undetectable levels of oxidized 2-AF intermediates. Under similar irradiation conditions, solutions of BaP, DMA, 2-AA, and 2-AN showed little or no significant mutagenic activity on TA98. Because of structural similarities between 2-AF and the latter three compounds, their lack of photomutagenic activity is an unexpected result. However, photodimerization is known to occur in a variety of substituted anthracenes (16). Conversion to non-

TABLE 1

SUNLIGHT-INDUCED MUTAGENICITY OF PAH IN *S. TYPHIMURIUM* TA98

Compound	Exposure time (min)[i]	Amount tested (µg)	His^+ revertants ± S.D.
2-aminofluorene	60	50	1318±236
9,10-dimethylanthracene	60	50	64±16
benzo(a)pyrene	60	50	45±8
2-aminoanthracene	60	50	53±7
2-aminonaphthalene	30[ii]	50	54±8
DMSO	60	55	26±4

[i] fluence 950 $J/m^2/sec$ [ii] maximum time tested

mutagenic photodimers is a possible explanation for the lack of activity observed with DMA and 2-AA.

Mutagenicity of Irradiated 2-AF

Data from Table 1 as well as other recent observations (17) demonstrate that sunlight irradiation of 2-AF leads to the production of stable mutagen(s) when measured on *Salmonella typhimurium*. The kinetics of this mutagenic response as a function of exposure time to direct sunlight or artificial UVA is depicted in Figure 1. The initial induction of his^+ revertants was rapid and linear between 0 to 0.5 hr (0 to 1.7×10^6 J/m^2) of exposure to sunlight and maximized between 0.5 and 1 hr. Additional irradiation reduced the direct-acting mutagenicity of 2-AF solutions where by ~6 hr exposure (20×10^6 J/m^2) less than 50% of the maximal activity remained. This latter observation suggests that prolonged irradiation of 2-AF solutions eventually results in the photodecomposition of the active component(s).

The dose response for photo-induced, direct-acting mutagenicity of 2-AF by sunlight can be reproduced by substituting natural sunlight with an artificial source of UVA. Irradiation conditions were identical to those for sunlight except that two 15-watt blacklights with a peak output between 300-400 nm were used. The data shown in Figure 1 indicate that a) UVA was approximately 30-40 times more efficient (per unit dose) in inducing mutagenic 2-AF photoproducts than was natural sunlight, and b) continual irradi-

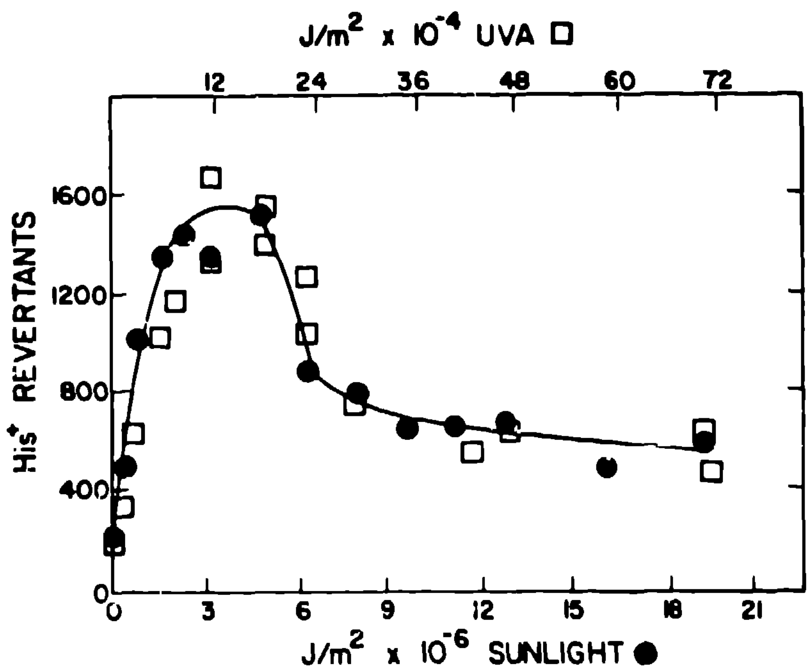

FIGURE 1. Mutagenic activity of 2-AF as a function of increasing exposure to sunlight or artificial UVA. Mutagenicity (histidine reversion) was determined in S. typhimurium TA98. Closed circles : sunlight; open squares : UVA.

ation with UVA also resulted in the photodestruction of induced 2-AF mutagenic components. These results are consistent with spectral output estimates of sunlight reaching the earth's surface which indicate that wavelengths of light in the UVA region represents approximately 3% of total sunlight irradiance power (13).

Wavelength Dependence in the Photoactivation of 2-AF

The importance of sunlight wavelengths in or below the UVA region in the induction of mutagenic properties of 2-AF is more clearly illustrated in the experiment depicted in Table 2. Glass cut-off filters that eliminate >90% of wavelengths of light below designated wavelengths were utilized in irradiation of solutions of 2-AF with sunlight. The results indicate that only 32%, 15%, and 1%, respectively, of the total mutagenic activity remained when 360, 420, and 495 nm cut-offs were used to filter out sunlight.

These results are also consistent with the absorption spectrum of 2-AF, which shows negligible absorptivity of light above 400 nm.

TABLE 2

WAVELENGTH DEPENDENCE FOR 2-AF PHOTOACTIVATION

Filter	Exposure Time (min)	Amount Tested (μg)	His^+ Revertants[i] ± S.D.	% Control
None	30	50	1349±32	100
WG 360	30	50	426±33	32
GG 420	30	50	209±27	15
GG 495	30	50	15±15	1

[i]Data represents his^+ revertants minus reversion frequency resulting from 50 μg/plate of 2-AF prior to irradiation.

High Pressure Liquid Chromatography of Irradiated 2-AF

In an attempt to identify the biologically important 2-AF photoproduct(s), UVA irradiated 2-AF solutions were subjected to reverse phase HPLC. The profiles in Figure 2 were obtained following the HPLC analysis of 2-AF solutions through a C_{18} μ-Radial PAK column eluted with a linear water:acetonitrile gradient (see Materials and Methods). Profiles A, B, and C, respectively, represent 2-AF solutions exposed to 1) no irradiation, 2) 2 hr of UVA treatment, and 3) 4 hr of UVA treatment. The compounds and arrows illustrated in profile B indicate R_t of commercially available derivatives of 2-AF under the conditions employed in these experiments. The numbers and arrows in profile C indicate A_{254} peaks which were collected and pooled into fractions following consecutive semi-preparative runs (see Materials and Methods).

The irradiation of 2-AF solutions with UVA light clearly induces the conversion of 2-AF into several new components. Two of these components co-chromatograph with <u>2-aminofluorenone</u> (fraction 1) and <u>2-nitrofluorene</u> (fraction 6). Pooled samples of each A_{254} peak were dried, redissolved, and reassayed for direct acting mutagenicity in the Ames test.

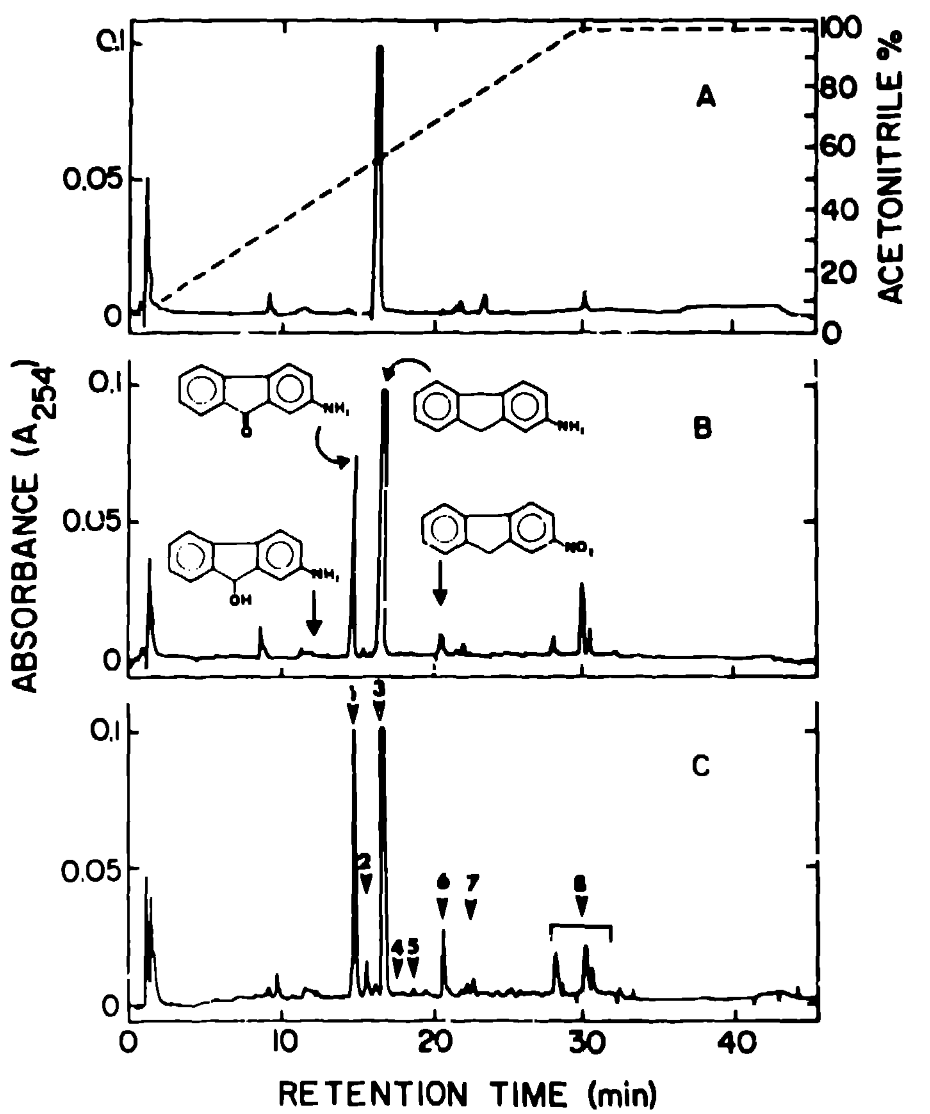

FIGURE 2. HPLC analysis of UVA irradiated 2-AF exposed to 0; 4.9 x 10^4 and 9.8 x 10^4 J/m^2 UVA, respectively, for Panels A to C. Dashed line (Panel A) represents the chromotographic acetonitrile gradient.

These results are illustrated in Table 3. Only two fractions contained significant activity---fraction 3 which is parental 2-AF and fraction 6, which co-eluted with 2-nitrofluorene. Only 10% of the initial activity was recovered from this pooling experiment. The basis for this loss of activity is still undertermined, but could result from activity lost in the chromatographic separation (e.g., by irreversible binding to column resin).

TABLE 3

MUTAGENICITY OF IRRADIATED 2-AF FOLLOWING FRACTIONATION BY REVERSE-PHASE HPLC

Fraction No.	Amount Tested[i] (µl per plate)	His^+ Revertants $\pm$ S.D.
1	50	24±4
2	50	21±3
3	50	71±2
4	50	31±2
5	50	24±3
6	50	230±12
7	50	31±5
8	50	26±6
Unfractionated	50	943±6

[i] 1.5 mg of 2-AF in 1.5 ml DMSO was exposed to UVA for 4 hours and applied to the HPLC column (in six independent 250 µl applications). Each pooled fraction was redissolved in 1.5 ml DMSO. 50 µl of each fraction would therefore be equivalent to 50 µl of the original irradiated 2-AF solution.

Fraction 6 has also been presumptively identified as being a nitrofluorene moiety by infrared spectral comparisons (Okinaka, Nickols, Whaley, and Strniste, in preparation). In addition, 2-nitrofluorene has been found to be a relatively stable compound and is potently mutagenic in nitroreductase proficient Salmonella tester strains (18). These results strongly implicate <u>2-nitrofluorene</u> as a major mutagenic product of 2-AF phototransformation following exposure to UVA light. The precise identity of the other photoproducts is currently being ascertained.

These results suggest that the photodynamic action of 2-AF proceeds via a different mechanism than does the metabolic transformation of 2-AF and its potent sister compound 2-acetylaminofluorene (2AAF). While the eventual mutagenic precursor in both activation systems may be N-hydroxylamine derivatives, these studies would imply that the formation of a nitroarene may be an important intermediate in the photo-activation of 2-AF. The first step in the metabolic activation of 2-AF and 2-AAF is thought to proceed by direct enzymatic oxidation mechanisms to the production of reactive N-hydroxy moieties (19,20).

The notion that phototransformation of 2-AF results in the formation of a nitro-compound gains importance in view of the relatively recent discovery that nitroarenes in general are unexpectedly potent mutagens in microbial systems (21). This unusual activity has been ascribed to nitroreductase activities in bacteria which can readily convert nitroarenes to more reactive arylhydroxylamines (18). The precise relationship between mutagenic potency of nitroarenes in microbes and their carcinogenic activity in mammalian systems, however, is unknown (18).

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Key Words

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Sunlight
2-aminofluorene
2-nitrofluorene
UVA