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Report

BIOASSAY OF POLYCYCLIC AROMATIC HYDROCARBONS

by

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A Thesis

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A positive relationship was found between the photodynamic activity of twenty-four polycyclic aromatic hydrocarbons versus published results on the mutagenicity, carcinogenicity, and initiation of unscheduled DNA synthesis. Metabolic activation of benzo(a)pyrene resulted in detection of increased mutagenesis in Paramecium tetraurelia as found also in the Ames Salmonella assay. The utility of Paramecium tetraurelia as a biological detector of hazardous polycyclic aromatic hydrocarbons is discussed.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are a class of organic molecules with two or more fused carbon rings. PAH are now ubiquitous atmospheric pollutants in both vapor and particulate forms due to the use of fossil fuel (Committee on Biological Effects of Atmospheric Pollutants, 1972 and 1976). Waters from fuel conversion processes and smoked or charred foods contain PAH (Guerin et al., 1978, Lo and Sandi, 1978; Mossanda et al., 1979). The major carcinogens in tobacco smokes are known to be polycyclic aromatics (Kier et al., 1974).

Distribution, carcinogenicity, and other biological activities have raised concern about PAH compounds. Problems associated with chemical carcinogenicity testing in animals stimulated interest in a simpler, less expensive method for predicting PAH's carcinogenicity. The relationships between carcinogenicity and reactivity indices, electrochemical properties, molecular characteristics, photodynamic activity, and mutagenicity of PAH have been studied (Cavalieri and Calvin, 1971; Epstein et al., 1965; McCann et al., 1975; Morgan et al., 1976; Morgan and Warshawsky, 1977; Podany et al., 1978; Smith et al., 1978). Mutagenicity is the most thoroughly studied parameter and provides the best relationship with carcinogenicity; however many other properties show good correlations and generate some interesting theories about the mechanisms of both carcinogenicity and mutagenicity.

Most PAH are not mutagenic and presumed not carcinogenic in pure forms and must undergo activation before genetic damage occurs.

Metabolic activation, in which the enzyme system, cytochrome P-450, degrades PAH to produce excretable water soluble molecules is the most thoroughly studied (Pelkonen and Korhonen, 1977). In the process intermediates are formed which are highly reactive and cause genetic damage (Rogan et al., 1976). Recently it was shown that gamma radiation from ^{60}Co can activate several PAH to mutagenic forms (Gibson et al., 1976).

Evidence exists for decomposition and photoactivation of PAH to more reactive forms by near ultraviolet (U.V.) light (approximately 360 nm). Isolation of stable mutagenic photodecomposition products from benzo(a)pyrene (BaP) has been reported (Issaq et al., 1979). With photoactivation, PAH bind covalently to proteins and DNA (Blackburn et al., 1973; Blackburn and Taussig, 1975; Cavalieri and Calvin, 1971; Miller, 1951; Rice, 1964, Ts'o and Lu, 1964). Furthermore, photoactivation of the polycyclics chlorpromazine and methylene blue to mutagenic forms has been demonstrated (Bohme and Wacker, 1963; Gutter et al., 1977; Imray and MacPhee, 1975; Jose, 1979; Kelly-Garvert and Legator, 1973; MacPhee and Imray, 1974 and 1978). Light enhances the induction of skin cancer by anthracene, benzo(a)pyrene, and 20-methylcholanthrene, and the induction of leukemia by 20-methylcholanthrene (Clark, 1964; Heller, 1950; Morton et al., 1940; Morton et al., 1951). These studies indicate possible genetic damage due to synergistic effects of PAH and light.

Photoactivation resulting in killing of cells or destruction of cellular components such as proteins or DNA by certain chemicals in combination with light is defined as photodynamic activity (in the case

of PAH the important wavelength is 360 nm). Santamaria and Prino (1972) have compiled a list of 417 photodynamically active compounds, many of which are PAH. The photodynamic killing by polycyclics has been studied using Escherichia coli (Ellis et al., 1974), Proteus mirabilis (Jacob, 1975), Salmonella (Gutter et al., 1977; Ito, 1978), Paramecium (Epstein and Burroughs, 1962; Epstein et al., 1964), Neurospora (Malling and Chu, 1970), the brine shrimp, Artemia (Morgan and Warshawsky, 1977), and mammalian cells in culture (Morimura et al., 1964).

There is evidence for a wide range of biological activities of PAH in combination with light, but few of these have been discussed in terms of the possible synergistic effects of pollutants and sunlight. In particular, photoinduced carcinogenicity and mutagenicity of PAH have received little experimental attention.

The purpose of the present study was to determine if Paramecium tetraurelia could be used as a detector of mutagenesis and photodynamic activity. Paramecia has been extensively utilized for chemical, ultraviolet, and X-ray mutagenesis studies (for review see Sonneborn, 1970). Photodynamic activity has been studied extensively by Epstein and colleagues using Paramecium caudatum (Epstein and Burroughs, 1962; Epstein et al., 1963, 1963a, 1963b; Epstein et al., 1964; Epstein et al., 1965). For this study Paramecium tetraurelia was used. P. tetraurelia shares all the advantages of P. caudatum for studying photodynamic activity, plus cellular mutagenesis is better studied in P. tetraurelia (Sonneborn, 1970). This species undergoes autogamy while P. caudatum does not. After autogamy all gene loci become homozygous; therefore, induced DNA

damage as both dominant and recessive mutations is expressed in a single generation (Sonneborn, 1970). P. tetraurelia is far more suitable for mutagenesis studies than P. caudatum.

The emphasis of this research was to explore the utility of paramecia: 1) to detect mutagenicity of PAH using metabolic activation, 2) to investigate whether light could activate PAH to mutagenic forms, and 3) to determine photodynamic activity of PAH. The Ames test was also employed to detect mutagenesis and synergistic effects of light and PAH agents.

MATERIALS AND METHODS

1. Culture Conditions

A. Salmonella: Strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538 of Salmonella typhimurium were used. Each of these strains is a histidine requiring mutant, and mutagenesis is detected by the rate of back mutation to wild type. The strains have different types of forward mutations and are susceptible to different types of mutagens to form the back mutants.

Samples of these strains were kindly supplied by Dr. Bruce Ames (Berkeley, Cal.) and cultured according to the methods of Ames et al. (1975). Stock cultures were maintained frozen at -80°C in nutrient broth containing 0.8% DMSO (dimethylsulfoxide). Strains were activated by inoculating 5 ml of nutrient broth with the stock culture and incubating at 37°C overnight.

B. Paramecium: Paramecium tetraurelia stock 51 was used for all experiments. The cells were maintained as daily isolation lines (Sonneborn, 1970). The culture media was a sterile Cerophyl infusion inoculated 24 hours prior to use with Klebsiella aerogenes, incubated at 27°C , then adjusted to pH 6.7 with a sterile solution of 1 N KOH immediately before use.

The fission age was determined as the sum of the $\log_2$ of the number of cells derived from single cells on successive days.

For experimental use, paramecia were grown in mass cultures by inoculating approximately 20 ml of culture media with a single clone of cells from isolation lines. Cultures were used in log phase of growth for all experiments. A mass culture method was also used to obtain large numbers of exponential cells.

2. Preparation of Polycyclic Aromatic Hydrocarbon Solutions

Names, structures, and sources of the PAH used for this study are given in Appendix B. A selected PAH was dissolved in reagent grade DMSO or in acetone if not soluble in DMSO, at a concentration of 1 to 20 mg/ml, and serial dilutions were made in culture media. When the polycyclics were diluted from the organic solvent into the culture media, they immediately precipitated to form a fine suspension. The diluted suspension was incubated with Paramecium. Paramecia were examined using fluorescence microscopy to assure that the precipitate was being taken up by the cells.

In the bacterial assay (sect. 3 below), the PAH were added to top agar without dilution from the organic solvent.

3. Determination of Mutations

A. Salmonella: Mutations were determined by the top agar method of Ames et al. (1975). Bacteria in nutrient broth were rapidly mixed with mutagen, activator, and molten top agar (in a 45°C steam bath), then spread evenly over minimal media plates. Before use, 10 ml of a sterile solution of 0.5 mM L-histidine·HCl-0.5 mM biotin was added per

100 ml of top agar (0.6% Difco agar, 0.5% NaCl). After a 48 hour incubation period at 37°C, the number of colonies per plate were counted. Each colony represents one back mutation to wild type (i.e. not requiring histidine for growth).

B. Paramecium: Postautogamy viability was used to assay mutations in paramecia (Kimball, 1949; Rodermel and Smith-Sonneborn, 1977). Kimball (1969) has shown that paramecia are most sensitive to U.V. induced mutations at 2.5 hours into the interfission cycle. For mutagenesis experiments, synchronized paramecia were always used. Synchronization was accomplished by removing paramecia with the morphology of dividers from a log phase mass culture manually with a micropipet. These cells were incubated at 27°C and used at the appropriate time into the interfission cycle. Following exposure of paramecia to mutagens they were washed free of the mutagen by manually diluting the cells into fresh media six times with a micropipet. These paramecia were then established as single isolates in spot plates with each depression containing 0.3 ml of media and allowed to starve, or in some experiments, maintained in daily isolation lines to provide a source of starved cells. After three to four days incubation at 27°C, the cells were assayed for autogamy by removing at least 20 cells from each depression, staining with acridine orange, then observing the macronucleus with fluorescence microscopy for the morphological changes characteristic of autogamy (Smith-Sonneborn, 1974). When a depression was found to contain paramecia in autogamy (i.e. at least 90% autogamous) 12 or 24 cells were removed from that depression and set up as single isolates in depression plates. These

cells were incubated at 27°C for four days and assayed for viability. Viable progeny were those paramecia which had cleared the media of bacteria.

C. S-9 Activation: The S-9 fraction is a microsomal fraction prepared from rat liver which contains activated cytochrome P-450 enzymes. The S-9 was prepared and used according to the methods of Ames et al. (1975).

The enzyme system was activated by giving each rat a single interperitoneal injection of Aroclor 1254 (polychlorinated biphenyl) at a dosage of 500 mg/kg. The Aroclor 1254 was dissolved in corn oil at a concentration of 200 mg/ml. After five days the rats were sacrificed and the livers removed, homogenized, centrifuged at 9000 xg for 10 minutes at 4°C, and the supernatant collected. This supernatant is the S-9 fraction. To preserve the fraction it was divided into 2 ml portions, stored frozen at -80°C, and thawed immediately before use.

An S-9 mix was prepared which contained 20 µl of 0.4 M $MgCl_2$ -1.65 M KCl; 50 µl of 0.1 M Glucose-6-Phosphate; 40 µl of 0.1 M NADP; and 500 µl of 0.2 M Na_2HPO_4 (pH 7.0). Either 50 µl or 100 µl of S-9 was added and the volume brought to 1 ml with cerophyl media in paramecia experiments and sterile distilled water in bacterial experiments. The designation 100-S-9 mix was used to indicate an S-9 mix containing 100 µl of S-9 and 50-S-9 mix to indicate those with 50 µl of S-9.

D. Photoactivation of PAH to possible mutagenic forms: Cells (both Salmonella and Paramecium) preincubated in the PAH were given a dose of near U.V. light resulting in less than 100% survival. The sur-

viving cells were assayed for mutations as described previously. Both BaP and anthracene were tested in the paramecia system and only BaP in Salmonella. Salmonella were exposed to light both while in a mix of BaP in nutrient broth and with cells and BaP in top agar on minimal media plates. Paramecia were exposed to light while in cerophyl media containing PAH in the manner described for determining photodynamic activity. All manipulations of cells (both Salmonella and Paramecium) after mixing with PAH were done under minimal light. A 25W incandescent bulb provided the only light used.

4. Determination of Photodynamic Activity

A. Salmonella: Survival was determined with strains TA 1535, TA 98, and TA 100. Cells in a mix of BaP and nutrient broth were irradiated in sterile depression plates for the various times with a Sylvania F40 BLB light at a distance of 4.4 cm. Immediately after exposure, serial dilutions were prepared in sterile distilled water and 0.1 ml of the final dilution spread on a nutrient agar plate. Several dilutions were done for each treatment. These plates were then incubated at 37°C for 48 hours before counting the colonies.

B. Paramecium: Photodynamic activity in paramecia was determined by placing 25 cells into 0.3 ml of cerophyl with an appropriate dilution of the polycyclic aromatic hydrocarbon. The cells were then exposed to a Sylvania F40 BLB light at a distance of 4.4 cm for various times. Four experimental repetitions, or a total of 100 cells, were used for each treatment. The cells were preincubated for two hours in PAH before exposure. After exposure, cells were covered, placed in the dark and

assayed for survival one hour later. This post exposure incubation allowed severely damaged cells to die before determination of percent survival.

The effect of temperature on photodynamic activity was determined by incubating the cells for the preincubation period at various temperatures (i.e. 15°C, 23°C, 28°C, and 34°C) and removing the paramecia from these temperatures immediately before exposure.

C. Relationship of photodynamic activity in Paramecium to other biological effects of PAH: A literature search was conducted to determine certain biological properties of the polycyclics used in this study. These properties include: photodynamic activity, mutagenicity, carcinogenicity, and initiation of unscheduled DNA synthesis. The present photodynamic activity data was compared to literature reports on the biological effects of the agents used.

RESULTS

1. Photodynamic Activity

A. Salmonella: Salmonella was found to be susceptible to photodynamic killing by the polycyclic aromatic hydrocarbon, benzo(a)pyrene (BaP) (Table 1). It can be observed from this data that the susceptibility of the Salmonella to killing by either near ultraviolet light alone or near ultraviolet light plus BaP varies for different strains. The sensitivity to light in decreasing order was TA 100, TA 98, and TA 1535 respectively.

B. Paramecium: Results can be obtained in a few hours using Paramecium, whereas two days are required with Salmonella. The paramecia assay is much less expensive, and far simpler than the bacterial system. Several parameters that can influence the photodynamic activity of BaP and presumably other PAH are shown in Figure 1. Higher culture temperatures (Fig. 1A) and older fission ages (Fig. 1B) both increase the sensitivity of paramecia to photodynamic killing. Photodynamic activity is dependent on both the amount of light (Fig. 1C) and the concentration of agent (Fig. 1D). Increases in either or both of these parameters leads to decreased survival of paramecia after exposure. The paramecia system was used to assess the photodynamic activity of 24 PAH in this study (Table 2). It is shown here that different compounds have differing photodynamic activities. This difference in photodynamic activity was compared with other properties of these PAH. Compounds exhibiting

TABLE 1
SURVIVAL OF SALMONELLA FOLLOWING
PHOTODYNAMIC TREATMENT WITH BaP.^a

Treatment	TA 98	Survival ^b TA 100	TA 1535
Control	100.0	100.0	100.0
BaP alone	26.4	1.6×10^{-3}	41.0
5 min. light	0.11	1.5×10^{-4}	3.3
5 min. light plus BaP	3.6×10^{-3}	1.6×10^{-5}	0.44
15 min. light			1.6
15 min. light plus BaP			2.1×10^{-3}
30 min. light			1.0
30 min. light plus BaP			7.2×10^{-3}
60 min. light			1.2
60 min. light plus BaP			2.8×10^{-4}
90 min. light			1.8
90 min. light plus BaP			*

^aBenzo(a)pyrene (BaP) was combined with a nutrient broth culture to a concentration of 5 µg/ml one hour prior to irradiation. Exposures were done in depression plates with a volume of 0.3 ml while covered with Saran Wrap. All exposures were done in nutrient broth at a distance of 4.4 cm from a Sylvania F40 BLB light.

TABLE 1
(CONTINUED)

^bSurvival is in percent of control and was determined by serially diluting 0.1 ml of each treatment into sterile distilled water and spreading 0.1 ml of the final dilution onto a nutrient agar plate. These plates were incubated for 48 hours at 37°C and the colonies counted.

*The smallest dilution done was 10^{-4} and no plates in this group contained any colonies.

Figure 1. Parameters Influencing the Photodynamic Response of Paramecium to BaP.*

- A. Temperature: The paramecia were incubated in the treatment mixture for two hours, removed from the incubator, and immediately exposed to light. The cells used were 50 fissions old and irradiated for 165 seconds with a BaP concentration of 20 $\mu\text{g/ml}$.
- B. Cellular Age: Cells of the same lineage, but different fission ages, were exposed to light for 105 seconds with a BaP concentration of 20 $\mu\text{g/ml}$.
- C. Light Exposure: Young cells were preincubated for two hours in BaP at a concentration of 20 $\mu\text{g/ml}$, then exposed to light for varying times.
- D. Concentration of BaP: 125 fission old cells were irradiated for 210 seconds at the various concentrations of BaP.

*A Sylvania F40 BLB light at a distance of 4.4 cm was the light source.

—: BaP plus light

x—x: Light only

Fig. 1A

Fig. 1B

Fig. 1C

Fig. 1D

TABLE 2
BIOLOGICAL EFFECTS OF POLYCYCLIC AROMATIC HYDROCARBONS.^a

Compound	mut ^b	car ^b	uds ^b	Photo-dynamic ^c
1. Anthracene	-	+	+/-	++
2. Anthracene, 2-methyl-		?		++
3. Anthracene, 9-methyl-		?		+
4. Benz(j)aceanthrylene, 1,2-dihydro-3-methyl-	+	+	+	++
5. Benz(a)anthracene	+	+	+	++
6. Benz(a)anthracene, 9,10-dimethyl-	+	+	+	++
7. 9,10(1',2')-Benzenoanthracene, 9,10-dihydro-		?		-
8. Benzo(rst)pentaphene	+	+		-
9. Benzo(ghi)perylene	+	-		++
10. Benzo(a)pyrene	+	+	+	++
11. Benzo(b)triphenylene	+	+	+	-
12. Chrysene	+	+		w+
13. Dibenz(a,h)anthracene	+	+	+	-
14. Dibenzo(b,def)chrysene		+		-
15. Dibenzo(def,mno)chrysene	+	+		+
16. Naphthacene		-		w+
17. Naphthalene	-	-		-
18. Naptho(1,2,3,4-def)chrysene		+		-
19. Pentacene		-		++
20. Phenanthrene	-	-	-	w+
21. Phenanthrene, 2-methyl-		?		w+
22. Picene		-		-
23. Pyrene, 1-methyl-		-		+
24. Triphenylene	+	-		-

TABLE 2, Cont.

^aThe mutagenicity (mut), carcinogenicity (car), and initiation of unscheduled DNA synthesis (uds) of the compounds were determined from a literature survey and ultimately compiled from the following references:

Andrews et al., 1978; Brown et al., 1979; Cavalieri et al., 1978; Epstein et al., 1964; Epstein et al., 1964a; Lake et al., 1978; Lo and Sandi, 1978; Malling and Chu, 1970; Martin et al., 1978; McCann et al., 1975; Morgan et al., 1977; Mossanda et al., 1979; NIOSH, 1976; Podany et al., 1978; Poirier and Weisburger, 1979; Poirier and de Serres, 1979; Sirianni and Huang, 1978; Smith et al., 1978; Zytkevicz, 1978.

^bTable designations are defined as follows:

- (+): those compounds demonstrating these properties
- (-): those compounds not causing these effects
- (+/-): those compounds tested with approximately equal evidence for + and -.
- (?): those compounds untested for carcinogenicity or tested with indecisive results.

^c60 minute irradiations at a distance of 4.4 cm from a Sylvania F40 BLB light were used in determining all photodynamic activities. Photodynamic activity responses are defined as follows:

- (++): Strong: 0% survival at a concentration of 1 µg/ml
- (+): Moderate: 0% survival at 10 µg/ml but greater than 0% survival at 1 µg/ml
- (w+): Weak: greater than 0% survival but less than 90% survival at 10 µg/ml
- (-): Nonphotodynamic: 90% or greater survival at 10 µg/ml

For recent review see Brown et al., 1979.

strong photodynamic activity are very likely to also cause genetic damage. Of the strongly photodynamic compounds, 5/7 (71.4%) are known carcinogens, 5/6 (83.3%) are mutagens, and 5/5 (100%) are capable of initiating unscheduled DNA synthesis. Note that not all of the 24 PAH have been tested for all parameters to detect DNA damage, restricting complete analysis of possible correlations.

2. Mutagenesis by Metabolic Activation of BaP

A. Salmonella: Both Salmonella and paramecia were used to detect mutagenesis. Table 3 shows the induction of mutations in Salmonella with metabolically activated BaP. The Ames mutagenesis test was done to determine the reliability of the activated S-9 fraction before use with paramecia.

B. Paramecium: S-9 activated BaP was found to cause increased mutations in paramecia, detected as reduced viability after autogamy (Table 4). A 50-S-9 mix was found to give decreasing survivals after autogamy with higher doses of BaP; however the 100-S-9 mix gave somewhat skewed results. The dose of 30 $\mu\text{g/ml}$ BaP plus 100-S-9 mix gave a very high survival when compared to doses of 15 $\mu\text{g/ml}$ and 60 $\mu\text{g/ml}$ plus 100-S-9 mix. This could be due to the small sample size of the 30 $\mu\text{g/ml}$ group.

3. Photoactivated PAH Mutagenesis

A. Salmonella: Mutagenesis due to light activated BaP. Salmonella were irradiated while in nutrient broth (Table 5) then put in top agar on minimal media plates. When the number of revertants per 10^{10}

TABLE 3
INDUCTION OF MUTATIONS IN SALMONELLA WITH S-9 ACTIVATED BaP.^a

Treatment	TA 98	TA 100	Strain TA 1535	TA 1537	TA 1538
Control	7	61	4	0	0
BaP. alone	4	66	7	0	0
S-9 mix	16	125	0	3	13
S-9 mix plus BaP	39	280	2	23	29

^aAll table values are the average number of revertants (colonies) counted per plate.

The top agar plate method was used. 0.1 ml of nutrient broth culture of the bacterial strain, 0.5 ml of a 50-S-9 mix (where applies), and 0.1 ml of a 5 mg/ml solution of BaP in pure DMSO (where applies) were added to 2.0 ml of molten top agar. After the top agar hardened the plates were incubated at 37°C for 48 hours before assaying revertants.

TABLE 4
 POST AUTOGAMY VIABILITY^a IN PARAMECIUM FOLLOWING
 TREATMENT WITH S-9 ACTIVATED BaP.^b

S-9 mix	BaP ($\mu\text{g/ml}$)			
	15	30	60	none
none				90.3 (12)
50	87.5 (10)	67.5* (10)	62.0* (9)	91.0 (12)
100	60.4* (4)	86.1 (3)	61.1* (6)	90.7 (9)

^aThe upper numbers in the table indicated the percent viability and numbers in parentheses indicate the number of depressions (clones) from which autogamous paramecia were taken for the viability assay.

^bDividers were taken from a mass culture of 80 fission old cells, immediately placed into a depression containing 0.3 ml of the treatment mixture, and incubated at 34°C for 2 hours. Paramecia were washed free of the treatment mixture, set up as single isolates, and incubated for five days at 27°C. Autogamy was assayed in these, and from each depression found to contain autogamous cells, 12 were set up as single isolates for viability determination, and incubated at 27°C for 4 days. Viable progeny were those paramecia which had cleared the media of bacteria.

The numbers in the S-9 mix column of the table indicate the amount (in μl) of S-9 in the S-9 mix.

*These values are significantly different from the control value of 90.3 at 95% confidence. Significance was determined by placing confidence intervals on the difference of the two proportions using the normal approximation (Mendenhall, 1975).

TABLE 5
MUTAGENESIS IN SALMONELLA FOLLOWING TREATMENT
WITH LIGHT ACTIVATED BaP.^a

Treatment	Revertants ^b		Revertants per 10 ¹⁰ Bacteria Plated ^c	
	TA 98	TA 1535	TA 98	TA 1535
Control	20.3	15.7	0	8.3
BaP alone	23.0	12.3	0	15.8
1 min. light	18.7	8.7		
1 min. light plus BaP	15.3	13.7		
5 min. light	12.0	11.0	6.6	177.4
5 min. light plus BaP	14.3	14.0	230.0	1686.4

^a0.3 ml of a 5 µg/ml suspension of BaP in nutrient broth cultures of strains TA 98 and TA 1535 were placed into sterile depression plates, covered with Saran Wrap, and incubated for 2 hours at 37°C. Four repetitions were done for each experimental treatment. After incubation, irradiations were done for the appropriate times with a Sylvania F40 BLB light at a distance of 4.4 cm with the Saran Wrap cover in place.

^b0.1 ml of each treatment mixture was mixed with 2.0 ml of molten top agar and spread evenly over a minimal media plate. These plates (3 per treatment) were incubated at 37°C for 48 hours before assaying for revertants. The number of revertants per plate were assayed and listed here.

^cSurvival was determined by serially diluting 0.1 ml of each treated mixture into sterile distilled water and spreading 0.1 ml of the final dilution onto a nutrient agar plate. These plates were incubated at 37°C for 48 hours, colonies counted, survival determined, and revertants per 10¹⁰ bacteria calculated from these survival values.

surviving cells plated is calculated, there is significant increase in revertants above the control levels in the light and light plus benzo(a) pyrene treatments (Table 5).

B. Paramecium: Anthracene (Table 6) and BaP (Table 7) mutagenesis was studied with Paramecium. Although the percent vegetative survival of paramecia declined for a set dose of anthracene or BaP with increased light exposure, the survivors showed no induced DNA damage when their progeny were examined for reduced viability after autogamy. Survivors in the higher dose treatments exhibited difficulty undergoing autogamy resulting in fewer autogamous clones being obtained from these survivors. The number of autogamous clones examined for the higher doses of light were too few to conclude that no mutagenesis had occurred.

TABLE 6
 PERCENT SURVIVAL OF PARAMECIUM FOLLOWING TREATMENT
 WITH LIGHT ACTIVATED ANTHRACENE.^a

Treatment	Vegetative	Autogamy ^b
Control	100	91.0 (12)
Anthracene alone	100	99.3 (12)
16 min. light	100	98.6 (12)
6 min. light plus anthracene	87.5	95.8 (12)
8 min. light plus anthracene	80.0	98.6 (12)
10 min. light plus anthracene	11.2	95.8 (4)
12 min. light plus anthracene	0	
14 min. light plus anthracene	0	
16 min. light plus anthracene	0	

^a Anthracene was diluted from 10 mg/ml DMSO to a concentration of 10 µg/ml in cerophyl media containing Klebsiella aerogenes. The paramecia used were 60 fissions old. Dividers were manually taken from a log phase mass culture, 20 dividers placed in the anthracene mixture in depression plates at a volume of 0.3 ml (four repetitions were done for each treatment), and irradiated at 2 hours into the interfission cycle for the appropriate times with a Sylvania F40 BLB light at a distance of 4.4 cm. The paramecia were washed from the anthracene manually after a one hour post irradiation incubation. At that time the vegetative survival was assayed. The surviving cells were set up as single isolates and assayed for mutations by viability after autogamy.

^b The values in parentheses are the number of autogamous clones used, and the upper numbers are the percent viable after autogamy. Twelve progeny from each autogamous clone were assayed for viability.

TABLE 7
 PERCENT SURVIVAL OF PARAMECIUM FOLLOWING TREATMENT
 WITH LIGHT ACTIVATED BaP.^a

Treatment	Vegetative	Autogamy ^b
Control	100	96.9 (8)
BaP alone	100	90.6 (8)
3 min. 45 sec. light	100	94.8 (8)
6 min. light	100	93.8 (8)
3 min. 30 sec. light plus BaP	82.5	97.9 (4)
3 min. 45 sec. light plus BaP	83.8	92.7 (8)
4 min. light plus BaP	80.0	98.3 (5)
4 min. 15 sec. light plus BaP	63.8	96.4 (7)
4 min. 30 sec. light plus BaP	52.5	100 (3)
4 min. 45 sec. light plus BaP	60.0	95.8 (2)
5 min. light plus BaP	30.0	100 (1)
5 min. 15 sec. light plus BaP	22.5	100 (1)

^aThe procedures here were identical to those used for mutagenesis with light activated anthracene except BaP was diluted from an initial concentration of 20 mg/ml DMSO to 10 µg/ml in cerophyl media containing Klebsiella areogenes, the cells were 80 fissions old, the volume of mixture used for irradiations was 0.15 ml, and the duration of the exposure to light was varied.

^bThe values in parentheses are the number of autogamous clones used, and the upper numbers are the percent viable after autogamy. Twenty-four progeny from each autogamous clone were assayed for viability.

DISCUSSION

The data of this study show a relationship between photodynamic activity of PAH and genetic damage incurred by these PAH. PAH with strong photodynamic activity have a high probability of being carcinogenic, mutagenic, or UDS initiating. Epstein et al. (1964a) also found a correlation between photodynamic activity and carcinogenicity of PAH in a similar study using P. caudatum. He defined photodynamically active compounds as those PAH for which the reciprocal of the concentration of the PAH (in $\mu\text{g/ml}$) producing 90% lethality in 30 minutes, was 2.00 or less. Using this method he found 54% of carcinogens and 22% of noncarcinogens are photodynamically active. If recent data on carcinogenicity is incorporated, 13.2% (9/68) of the PAH which were then considered noncarcinogenic are now classified as carcinogenic. Changing his definition of photodynamic activity to PAH producing 90% lethality at 10 $\mu\text{g/ml}$ in 50 minutes makes the relationship: 93% (55/59) of the carcinogens and 37% (35/90) of the noncarcinogens are photodynamically active.

In the literature many of the PAH can be found listed as both positive and negative for carcinogenicity, mutagenicity, and initiation of UDS depending on the source. Further studies are necessary to establish the status of these compounds. Many PAH currently classified as noncarcinogenic may upon further study be reclassified as carcinogenic. Problems in classification stem from the carcinogenicity testing system

itself. Currently accepted methods for determining chemical carcinogenicity (Sontag et al., 1977) are inadequate for denoting a substance as noncarcinogenic. The sample sizes used for carcinogenicity testing in animals limit the level of cancer induction which is statistically significant (McCann and Ames, 1976). When the small number of animals (hundreds) used in the test is compared to the size of the human populations at risk (hundreds of millions), it is quite possible for substances classified as noncarcinogens to represent a significant health hazard. The expense and time required to test chemicals for carcinogenicity limits the number of substances and conditions for which carcinogenic status can be determined (currently three years and \$250,000 are required to test one substance under one set of conditions, see Ames, 1979). Many substances might fall into a category similar to anthracene which is only carcinogenic in combination with light, and the hazard will not be realized for many years.

The true health risk of these PAH could be shrouded by the lack of knowledge concerning the real carcinogenic status of the substances.

The induction of mutations in paramecia with metabolically activated BaP demonstrates the potential of paramecia as a possible system for mutagenesis testing of chemicals. There was decreasing postautogamy viability with increasing doses of BaP in those groups receiving the activated 50-S-9 mix. This pattern was not consistent in the 100-S-9 mix groups possibly due to cytotoxic effects manifested as decreased vegetative survival and difficulty of the survivors to enter autogamy. The small sample size of the survivors could account for the observed

variation among groups receiving the 100-S-9 mix. Cytotoxicity due to metabolically activated BaP in tissue culture has been noted (Morimura et al., 1964).

Induction of mutations following photoactivation of PAH could not be detected in Paramecium, though evidence for light activation of PAH was found for Salmonella when the number of revertants per surviving bacteria was calculated. Salmonella is susceptible to mutations caused by photoactivation of the polycyclic methylene blue, but only with the proper dose. The dose which caused mutations most efficiently had a survival of about 10% of the treated bacteria (Imray and MacPhee, 1975). With higher survival rates the dose was not sufficient, while with lower survival rates the photoinduction of mutations was refractory, resulting in reduced mutagenesis. Similar results have also been noted with photoinduction of carcinogenicity. In some cases the carcinogenicity of PAH is enhanced by near U.V. irradiation (Clark, 1939; Clark, 1964; Heller, 1950; Morton et al., 1951), but in others the carcinogenicity is reduced (Morton et al., 1940; Morton et al., 1942; Morton et al., 1951). Clark (1964) has described this as a situation similar to that described previously for mutagenesis in Salmonella where both high and low doses cause lesser effects than the intermediate doses.

Photoactivated PAH produced no measurable mutations in paramecia even though a broad range of vegetative survivals were tested (87% to 11.2%). It is possible that repair of genetic damage could have interfered with mutagenesis. Paramecia has a photoreactivation repair system which requires near U.V. light to function. This system monomerizes

pyrimidine dimers (cyclobutane linkages of pyrimidines, see Setlow, 1966). BaP binds covalently to pyrimidines by a cyclobutane linkage upon irradiation by near U.V. light (Rice, 1964). This cyclobutane linkage of BaP to DNA could be susceptible to photoreactivation repair. Use of repair deficient paramecia in further studies might alleviate possible interference of repair with induction of mutations.

Paramecia is promising as a short-term photodynamic assay for PAH and as a more generally applicable mutagenesis test system. Several characteristics of Paramecium tetraurelia make it especially suitable for this purpose. These include: 1) the size of paramecia makes culture conveniently compact yet manipulation and observation is still easily accomplished using a dissecting microscope, 2) the biology of paramecia is well studied (Sonneborn, 1950, 1970, and 1974), 3) photodynamic activity of PAH has been studied previously using Paramecium caudatum (Epstein and Burroughs, 1962; Epstein et al., 1963, 1963a, 1963b, 1964, and 1965), and requires only 2 to 3 hours providing a more rapid assay than with Salmonella, 4) unicellular eukaryotic organisms fill a phylogenic link between prokaryotic and multicellular organisms, and some DNA damaging agents negative in prokaryotes may be positive in eukaryotes, 5) postautogamy viability of P. aurelia is an established means of determining mutagenesis in paramecia (Kimball, 1949), requiring only 7 to 10 days for the assay, and mutagenesis has been widely studied in paramecia (Sonneborn, 1970), 6) in this study a precipitate was taken into paramecia food vacuoles to cause mutagenesis indicating that this method might be useful for mutagenesis studies of particulate materials.

Paramecium tetraurelia has distinctive advantages, which would make it very useful as part of a battery of tests for predicting the biological hazard of chemical substances.

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APPENDICES

APPENDIX A

COMPOUND NAME CROSS INDEX

1. Anthanthrene (see 37)
2. Anthracene
3. Anthracene, 2-methyl-
4. Anthracene, 9-methyl-
5. Benz(j)aceanthrylene, 1,2-dihydro-3-methyl-
6. Benz(a)anthracene
7. Benz(a)anthracene, 7,12-dimethyl-
8. 1,2-Benzanthracene (see 6)
9. 2,3-Benzanthracene (see 50)
10. 9,10-o-Benzoanthracene, 9,10-dihydro- (see 11)
11. 9,10(1',2')-Benzoanthracene, 9,10-dihydro-
12. 3,4-Benzchrysene (see 57)
13. Benzo(a)chrysene (see 57)
14. Benzo(b)naphthacene (see 54)
15. Benzo(rst)pentaphene
16. Benzo(ghi)perylene
17. 2,3-Benzophenanthrene (see 6)
18. 9,10-Benzophenanthrene (see 62)
19. Benzo(b)phenanthrene (see 6)
20. Benzo(a)pyrene
21. Benzo(b)triphenylene
22. 1,12-Benzperylene (see 16)
23. 1,2-Benzphenanthrene (see 28)
24. Benz(a)phenanthrene (see 28)
25. 3,4-Benzpyrene (see 20)
26. Cholanthrene, 3-methyl- (see 5)
27. Cholanthrene, 20-methyl- (see 5)
28. Chrysene
29. Chrysogen (see 50)
30. Dibenz(a,c)anthracene (see 21)
31. Dibenz(a,h)anthracene
32. 1,2,3,4-Dibenzanthracene (see 21)
33. 1,2,5,6-Dibenzanthracene (see 31)
34. 2,3,6,7-Dibenzanthracene (see 54)
35. 1,2,3,4-Dibenznaphthalene (see 62)
36. Dibenzo(b,def)chrysene
37. Dibenzo(def,mno)chrysene
38. 1,2,5,6-Dibenzonaphthalene (see 28)
39. Dibenzo(a,i)phenanthrene (see 57)
40. Dibenzo(a,e)pyrene (see 53)
41. Dibenzo(a,h)pyrene (see 36)
42. Dibenzo(a,i)pyrene (see 15)
43. 1,2,7,8-Dibenzphenanthrene (see 57)
44. 1,2,4,5-Dibenzpyrene (see 53)
45. 1,2,6,7-Dibenzpyrene (see 36)

46. 1,2,7,8-Dibenzpyrene (see 15)
47. 3,4,8,9-Dibenzpyrene (see 36)
48. 3,4,9,10-Dibenzpyrene (see 15)
49. Isochrysene (see 62)
50. Naphthacene
51. Naphthalene
52. Naphthanthracene (see 6)
53. Naptho(1,2,3,4,-def)chrysene
54. Pentacene
55. Phenanthrene
56. Phenanthrene, 2-methyl-
57. Picene
58. Pyrene, 1-methyl-
59. Rubene (see 50)
60. Tetracene (see 50)
61. Tetraphene (see 6)
62. Triphenylene
63. Triptycene (see 11)

APPENDIX B

COMPOUND NAME AND STRUCTURE

1. Anthracene*
2. Anthracene, 2-methyl-*
3. Anthracene, 9-methyl-*
4. Benz(j)aceanthrylene, 1,2-dihydro-3-methyl-**
5. Benz(a)anthracene*
6. Benz(a)anthracene, 9,10-dimethyl-
7. 9,10(1',2')-Benzenoanthracene, 9,10-dihydro-**
8. Benzo(rst)pentaphene**
9. Benzo(ghi)perylene**
10. Benzo(a)pyrene*
11. Benzo(b)triphenylene**
12. Chrysene*
13. Dibenz(a,h)anthracene**
14. Dibenzo(b,def)chrysene**
15. Dibenzo(def,mno)chrysene**
16. Naphthacene*
17. Naphthalene*
18. Naphtho(1,2,3,4-def)chrysene**
19. Pentacene **
20. Phenanthrene*
21. Phenanthrene, 2-methyl-*
22. Picene**
23. Pyrene, 1-methyl-*
24. Triphenylene**

*Obtained from Sigma Chemical Co.

**Compliments of Dr. John McKay, Laramie Energy Technology Center,
Laramie, Wyoming.