Bull. Environm. Contam. Toxicol. 28, 141–148 (1982)

Relationship Between Fluorescence of Polynuclear Aromatic Hydrocarbons in Complex Environmental Mixtures and Sample Mutagenicity

Simon Litten,[1] John G. Babish,[2] Michael Pastel,[3] Mary B. Werner,[1] and Brian Johnson[2]

[1]Toxic Substances Control Unit, Division of Water, NYS Department of Environmental Conservation, Albany, NY 12233; [2]Department of Preventive Medicine, NYS College of Veterinary Medicine, Cornell University, Ithaca, NY 14853; [3]Department of Atmospheric Science, SUNY Albany, Albany, NY 12222

Many carcinogenic and mutagenic compounds or their metabolites exhibit fluorescence (NIH 1980, McCANN et al. 1975, TONG et al. 1977). Of these compounds, polynuclear aromatic hydrocarbons (PAH) have commanded considerable attention from the perspectives of environmental distribution, metabolism and potential adverse health effects (SELKIRK 1977, GRIMMER 1979). Fluorescence has been used as a screening tool for detecting PAH in atmospheric dust (SAWICKI et al. 1965) and for identifying spilled oils (FRANK 1978) and other hazardous materials (FRANK and GRUENFELD 1978).

Noting these uses of fluorescence, it would be interesting to determine to what extent the fluorescence of a sample is associated with the presence of genotoxic materials. An examination of the intensity of fluorescence relative to quinine (SAWICKI et al. 1960) of 12 PAH and their mutagenicity (McCANN et al. 1975) indicated that compounds exhibiting relative fluorescence intensities of 0.7 or above were 2.6 times as likely to produce a mutagenic response equal to or greater than 0.6 revertants per nmole than compounds with lower relative fluorescence. The figures for relative fluorescence intensity and mutagenic potency were median values for PAH.

The New York State Department of Environmental Conservation is evaluating the effectiveness and interpretability of environmental screens for PAHs and mutagenicity using fluorescence on thin-layer chromatography (TLC) plates and the Salmonella/mammalian-microsome assay. Demonstration of high association between fluorescence and mutagenicity would support the hypothesis that environmentally distributed fluorescent substances contribute to the observed genotoxicity.

EXPERIMENTAL

Collection and Preparation of Samples

Ninety-two samples were collected from sites in New York State. These include 15 sewage treatment plant sludges; 22 industrially affected soils and sediments; 30 non-industrially affected soils

0007-4861/82/0028-0141 $01.60

and sediments; 17 samples from landfills; and 8 biological samples. Samples came from heavily industrialized urban areas, suburban areas, agricultural regions, and places believed remote from direct human influences. The biological samples came from rural, suburban, and industrially affected sites.

Sediment, sludge and soil samples were collected in glass mason jars, sealed with aluminum foil or Teflon and stored at -20 C. Prior to extraction a portion of the sample was thawed and dried at room temperature. Unionid bivalues (Elliptio complanata)were collected from a lake in Rensselaer County, NY. After a preliminary experiment to determine baseline levels of PAH and mutagenic materials in clams from this site, cages containing 12 clams each were held for 8 weeks at 5 study sites in lakes and streams in southeastern NY. Directly after collection, the soft clam tissue was removed from the shells, frozen and stored at -20 C. Before extraction the clam tissue was thawed, homogenized in a Sorvall Omnimix and lyophilized.

Approximately 30 g (4 g for biological samples) of sample were placed in a 45 x 123 mm cellulose extraction thimble which had been washed with the extraction solvent. The sample was covered with a glass wool plug and placed in a Soxhlet extraction apparatus containing 250 mL of a mixture of dichloromethane and acetone (4:1). Samples were extracted for 8-12 h. The range of total dry weight of extracts for the 92 samples was 1.8 mg to 2.8 g, being lowest for biological samples and highest for sludge samples. A portion of the extract (20%) was removed for PAH-fluorescence determination and the remainder dried under nitrogen. This residue was dissolved in dimethylsulfoxide (DMSO) for testing in the bacterial mutagen assay.

PAH Fluorescence

A 10 µL portion of the extract, containing between 0.7 and 1100 µg of material, was spotted on silica gel G1500 plates (20 x 20 cm, 250 µm thickness, activated 1 h at 150 C) and developed with dichloromethane: hexane (2:1) to 10 cm. After development PAH were visualized by exposure to 254 nm and 366 nm light. In order to maintain a permanent record, the plates were photographed during exposure on color transparency film. Scoring of fluorescence was done using a battery-powered, hand-held slide viewer without knowledge of sample mutagenicity results.

Mutagenicity Assays

Salmonella typhimurium strains TA98 and TA100 (AMES et al. 1975) were obtained from Dr. Bruce Ames (Biochemistry Dept., University of California, Berkeley, California). They were stored as permanent cultures at -80 C in nutrient broth with DMSO. The master plates made from the frozen permanent cultures and stored at 4 C were used as the source of inoculum for overnight cultures used in the mutagenesis assays.

The overnight cultures were grown in Oxoid nutrient broth No.

2 at 37 C for 16 h with agitation in a water bath shaker. Cultures contained 1 to 2 x 10^9 viable cells per mL. For each assay cultures were checked for crystal violet sensitivity and ampicillin resistance. In addition, the responses to 7, 12-dimethylbenzanthracence, benzo(a) pyrene (B(a)P), 2-nitrofluorene (TA98 only) and sodium azide (TA100 only) were monitored. Sterility of the S-9 was also monitored with each test. Quality control limits for spontaneous reversion rates for TA98 and TA100 were 20-60 and 110-200, respectively.

The procedure reported by Ames et al. (1975) for preparation of Aroclor 1254 induced S-9 was used with no modifications. Each mL of S-9 mix contained 100 µL of S-9.

The standard plate incorporation assay as described by Ames et al. (1975) was performed with no modifications. A minimum of 5 geometrically spaced doses were used. Only extracts from biological samples were not tested to toxic levels; this was due to a limit in amount of extract available. All other samples were tested to levels demonstrating toxicity to the microorganisms. The two extreme dose ranges tested were 0.07 to 3.50 µg extract per plate and 111.9 µg to 5.6 mg extract per plate. Triplicate determinations with and without S-9 were made at each dose level. Plates were incubated for 48 h at 37 C. Colony counts were made using an automatic colony counter (NBS Biotran II, New Brunswick Scientific).

A positive mutagenic response was defined as a two-fold increase in revertants compared with the appropriate control (i.e. mutagenic index (MI)$\geq$2). This interpretation was originally recommended by AMES et al. (1975).

Statistical Methods

To study the association of fluorescence to MI a four-fold table was constructed. The two characteristics being studied for each environmental sample were fluorescence and mutagenicity. Based on results of testing, a sample may be classified as (i) having fluorescence and MI$\geq$2, (ii) having fluorescence and MI<2, (iii) not having fluorescence and MI$\geq$2, and (iv) not having fluorescence and MI<2. Each of these four outcomes corresponds to a cell in the four-fold table.

The odds ratio (OR) was chosen as the measure of degree of association between the two characteristics, i.e. fluorescence and mutagenicity (EDWARDS 1963). The use of the OR does not assume a linear relationship underlying the two variables for which a measure of association is desired. This characteristic makes the OR a valuable statistic in the field of epidemiology where it is used in survey studies such as described here. An OR equal to 1 indicates no association between the two characteristics; an OR greater than 1 indicates a positive association (increased probability of mutagenicity with fluorescence); an OR less than 1 indicates a negative association (decreased probability of

mutagenicity with fluorescence). Chi-square values were computed to test the null hypothesis OR=1 (SNEDECOR and COCHRAN 1971). The probability of rejecting the null hypothesis when true was set at 5%. The continuity correction was applied to the computation of OR and chi-square (YATES 1934).

RESULTS AND DISCUSSION

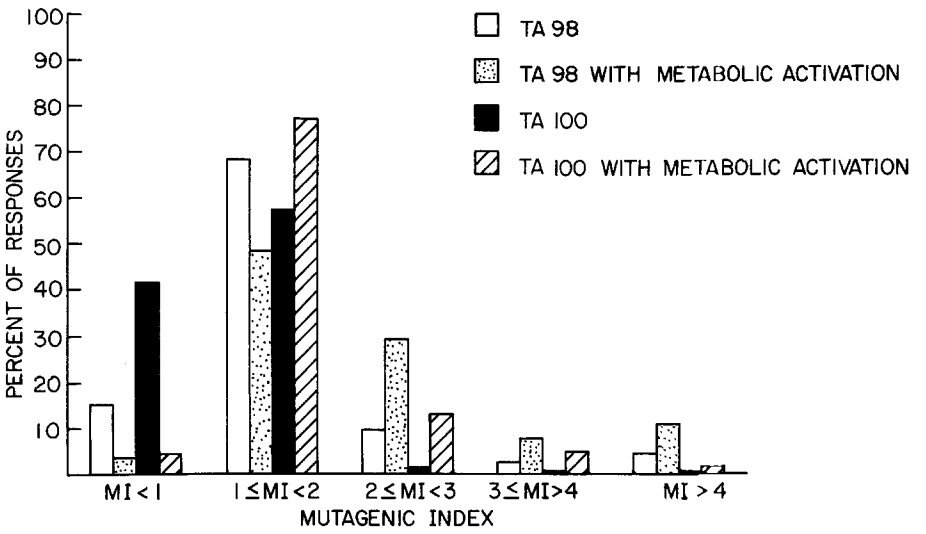

Figure 1. Frequency Distribution of Mutagenic Index (MI) by Test System.

Figure 1 shows the frequency distribution of MI by test system for all 92 samples. Samples with MI less than 2 were considered nonmutagenic; equal to or greater than 2 but less than 3($2 \leq MI < 3$), weakly mutagenic; equal to or greater than 3 but less than 4($3 \leq MI < 4$), moderately mutagenic; and equal to or greater than 4($MI \geq 4$), strongly mutagenic. The test system demonstrating the greatest number of positive responses was TA98 with metabolic activation. Strain TA100 with no activation produced the fewest positive responses and was most sensitive to toxicity.

Strain TA98, which detects frameshift mutagens, gave a positive response to 16% of the samples. With activation the

figure was increased to 48% of the samples. Within the categories of mutagenic response, 60, 13, and 27% of the positive responses without activation were classified as weakly, moderately, and strongly mutagenic, respectively. The distribution of positive responses with activation was nearly identical -61, 16, and 23% respectively.

The only positive response seen with strain TA100, which detects primarily base pair substitutions, was weakly mutagenic. TA100 with activation gave a positive response to 18% of the samples; of the positive responses 70% were weakly mutagenic, 24% were moderately mutagenic, and one response was strongly mutagenic.

The distance fluorescent spots traveled on the TLC plate was measured relative to the distance traveled by a control sample of B(a)P placed on each plate (R_S value). Of the 45 samples exhibiting fluorescence, 7 samples gave rise to streaks on which distinct spots could not be seen. Figure 2 shows the frequency distribution by R_S value for 109 spots from these 38 samples. Also shown is the frequency distribution of R_S values from samples which were mutagenic (MI≥2) in TA98 with activation. Chi-square analysis indicated that the distribution of spots from mutagenic samples was not different from the distribution of spots from fluorescent samples ($\chi^2 = 3.13$; df=6).

As seen in Figure 2, spots with migration distances equal to or greater than that of B(a)P ($R_S \geq 0.9$) showed a greater relationship between fluorescence and mutagenicity than those with migration distances less than B(a)P. PAH which would be expected to migrate further than B(a)P in this system include benz[a]anthracene, benzo(e)pyrene, fluoranthene, and chrysene.

The proportion of samples exhibiting fluorescence or mutagenicity is listed in TABLE 1 by sample type and overall. Notice the high proportion of fluorescence in sludge samples and mutagenicity in industrially affected samples. Overall, the proportion of samples fluorescent and mutagenic was very similar.

TABLE 1

Proportion of Samples Exhibiting Fluorescence or Mutagenicity

Sample	N	Percent Fluorescent (n)	Percent Mutagenic (n)
Industrially Affected	22	73 (16)	91 (20)
Non-Industrial	30	33 (10)	33 (10)
Sewage Sludges	15	87 (13)	53 (8)
Landfill Affected	17	35 (6)	41 (7)
Biological Samples	8	0 (0)	25 (2)
Overall	92	49 (45)	51 (47)

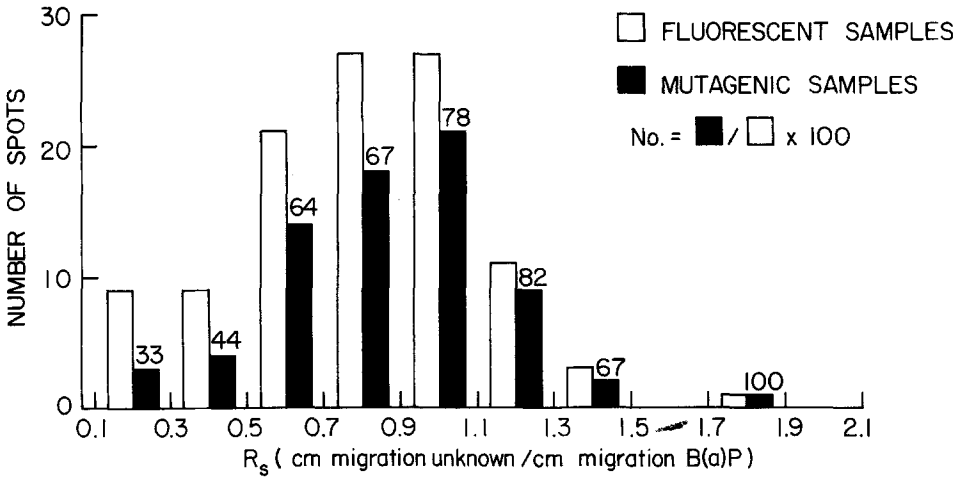

Figure 2. Frequency Distribution of Spots on TLC Plates and Their Migration Distance Relative to the Migration Distance of B(a)P for Fluorescent and Mutagenic Samples.

In order to assess the association of fluorescence with mutagenicity, it is necessary to examine the joint occurrence of both variables. This was done for each test system and the results are presented in TABLE 2. Significant associations between fluorescence and mutagenicity were observed for TA98 with and without activation and for TA100 with activation.

For TA98 fluorescent samples were 3.2 and 4.6 times as likely to be mutagenic (without activation and with activation, respectively) as nonfluorescent samples. Strain TA100 with metabolic activation was 4.0 times as likely to be mutagenic when fluorescence was observed.

The overall association of fluorescence and mutagenicity is also presented in TABLE 2. An OR of 5.05 indicates that overall, samples demonstrating fluorescence were over 5 times as likely to be mutagenic as nonfluorescent samples.

The observed OR suggest a strong association (MONSON 1980) between fluorescence of environmental samples and a genotoxic effect. Most of the association was due to compounds requiring metabolic activation for mutagenicity. These findings are consistent with published reports indicating the need for metabolic activation of most environmental carcinogens and mutagens (WEISBURGER 1978).

TABLE 2

Joint Occurrence of Fluorescence and Mutagenicity by Test

Test	Fluorescence	MI≥2	MI<2	Totals	Odds Ratio
TA98	+	11	34	45	
without activation	-	4	43	47	
Totals		15	77	92	3.22*
TA98	+	30	15	45	
with activation	-	14	33	47	
Totals		44	48	92	4.55*
TA100	+	1	44	45	
without activation	-	0	47	47	
Totals		1	91	92	3.20
TA100	+	13	32	45	
with activation	-	4	43	47	
Totals		17	75	92	4.02*
Overall	+	32	13	45	
	-	15	32	47	
Totals		47	45	92	5.05*

*OR≠1.0 with $p<0.05$

A stronger association between fluorescence of PAH and mutagenicity could be obtained primarily be reducing the number of fluorescent samples declared nonmutagenic (MI<2). In light of potential competing toxicity from other components of the extract, the suggested MI cut-off of 2 may be too high for environmental samples. Using a positive dose-response, regardless of MI, as evidence of mutagenicity might achieve a reduction in the number of false negatives.

Additionally, a detailed study of the excitation and emission spectra of fluorescent environmental extracts may strengthen the relationship discovered, as well as indicate what structural factors are important in relating fluorescence and mutagenicity.

ACKNOWLEDGEMENT

This work was supported by BR56 Grant 507 RR05462 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health and TSCA Cooperative Agreement Grant C5806861 - 01.

REFERENCES

AMES, B.N., J. McCANN and E. YAMASAKI: Mutat. Res. 31, 347(1975).

EDWARDS, A.W.F.: J. Roy. Statist. Soc. 126, 109(1963).

FRANK, U.: Toxicol. and Env. Chem. Rev. 2, 163(1978).

FRANK, U. and M. GRUNDFELD: In: National Conference on Control of Hazardous Materials Spills, Miami Beach, Fl., April 11-13, pp. 119-123,1978.

GRIMMER, G.: In: Monitoring Environmental Material and Speciman Banking. Ed. N.P. Lupke pp. 298-312 Proceedings of International Workshop, Berlin, Germany 1978.

McCANN, J., E. CHOI, E. YAMASAKI, and B. AMES: Proc. Nat. Acad. Sci. 72, 5135(1975).

MONSON, R.R.: In: Occupational Epidemiology. Boca Raton: CRC Press, 1980.

NIH publication; No. 80-453 Bethesda: Nat. Inst. of Health, 1980.

SAWICKI, E., T.R. HAUSER and T.W. STANLEY: Int. J. Air Poll. 2, 253(1960).

SAWICKI, E., S.P. McPHERSON, T.W. STANLEY, J. MEEKER and W.C. ELBERT: Int. J. Air Wat. Poll. 9, 515(1965).

SELKIRK, J.: In: Advances in Modern Toxicology, vol3, Ed. by H.F. Kraybill and M.A. Mehlman, pp. 1-25, John Wiley & Sons: New York, 1977.

SNEDECOR, G.W. and W.G. COCHRAN: In: Statistical Methods, 6th ed. Amers: Iowa State Univ. Press, 1971.

TONG, S., C. IOANNIDES and D.V. PARKE: Biochem. Soc. Trans. 4, 1372(1977).

WEISBURGER, E.K.: Ann. Rev. Pharmacol. Toxicol. 18, 395(1978).

YATES, F.: J. Roy Statis. Soc. Supplement 1, 217(1934).