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- Title of Study:**
1. Soot Mutates Human Cells
 2. Mutagenicity of Soot and Associated Polycyclic Aromatic Hydrocarbons to Salmonella Typhimurium

Name of Chemical: Kerosene soot

CAS#:

Summary: The polycyclic aromatic hydrocarbon fraction of a kerosene soot induces forward mutation in human diploid lymphoblasts when coincubated with rat-liver post mitochondrial supernatant.

Kerosene soot is also positive in the Ames Assay.

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12-09-78

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SOOT MUTATES HUMAN CELLS

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SOOT MUTATES HUMAN CELLS

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ABSTRACT

The polycyclic aromatic hydrocarbon fraction of a kerosene soot induces forward mutation in human diploid lymphoblasts when coincubated with rat-liver post mitochondrial supernatant. Two components of the kerosene soot extract, benzo[a]pyrene and acepyrylene were also tested. Benzo[a]pyrene was not mutagenic at the concentration found in the soot extract, although it was active at higher concentrations. The amount of acepyrylene present could account for approximately 8% of the total mutation observed with the soot. The results are compared to data obtained previously (Kaden et al., 1978, submitted for publication) in a similar mutation assay in Salmonella typhi-
murium.

The protocol described permits the facile assay of mutation at the hprt locus in human lymphoblasts induced by compounds or complex mixtures requiring mixed-function oxygenase activity for metabolism to genetically active derivatives.

ABBREVIATIONS

- PAH - polycyclic aromatic hydrocarbons
- PMS - post-mitochondrial supernatant
- PBS - phosphate buffered saline
- FCS - fetal calf serum
- 6GT - 6-thioguanine

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) and their corresponding heterocyclic compounds are widely distributed in our environment (1,2,3) and are formed by incomplete combustion of organic material. The carcinogenic potency of several PAHs (3) and the association of fossil fuel combustion with PAH production (4) has attracted the attention of the public to the possible hazards of uncontrolled emissions from motor vehicle operation, home heating, and stationary power plant operation. It should be noted that PAHs are also formed from natural sources (5). However, analysis of air samples from large cities has generally reflected the predominant spectrum of PAHs derived from local use of fossil fuels (6).

The full extent of the biological impact of human exposure to combustion products is not known. However, soot was the first recognized human carcinogen; Sir Percival Pott observed a high incidence of scrotal cancer in chimney sweeps in England in 1776 (3). More recently it has been noted that cancers of the lung and larynx occurred at higher than expected frequencies in gas stokers, coke oven chargers, and chimney sweeps (7). Seelig and Benignus (8) reported that death from lung cancer was two to five times more frequent in urban than rural areas and suggested that this was linked to a higher urban PAH concentration.

In vivo studies with soots have produced conflicting results. In inhalation experiments, some studies have been positive for carcinogenicity (8,9,10) and some negative (11,12). Many PAHs have been investigated for their carcinogenic potential as pure compounds. While many are highly active (e.g., benzo[a]pyrene, dibenz[a,h]anthracene, 7,12-dimethylbenz[a]anthracene), many others (e.g., pyrene, anthracene) have been found inactive (?).

Organic solvent extracts of particulate air pollutants collected from industrial and residential urban locations have been found mutagenic to S. typhimurium (13,14,15,16). Polar and non-polar solvent extracts of respirable coal fly ash collected downstream from an electric power plant also exhibited mutagenic activity to bacteria (17).

Individual PAHs have been studied in bacteria (18; Kaden et al., 1978, submitted for publication) and rodent cells in culture (19,20). Maher et al. (21) and Heflich et al. (22) have reported that the oxidized derivatives expected from the action of mixed-function oxygenases and epoxide hydase on benzo[a]pyrene, dibenz[a,h]anthracene, and 7,12-dimethylbenz[a]anthracene were mutagenic to diploid human fibroblasts.

By modifying the method of Krahn and Heidelberger (19) and combining it with the human diploid lymphoblast mutation assay developed previously in our laboratory (23,24), we now are able to perform assays for the mutagenic potency of individual PAH and complex mixtures, such as soot extracts, directly on human cells. We report here the details of our human-lymphoblast mutation-assay-protocol and our observations of the mutagenic activity of benzo[a]pyrene, acetyrylene, and the methylene chloride extract of a kerosene soot. For comparison, we also include results obtained with our newly developed forward mutation assay in Salmonella typhimurium.

MATERIALS AND METHODS

Chemicals

Benzo[a]pyrene, nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, cytidine, hypoxanthine, aminopterin, thymidine, 6-thioguanine, 8-azaguanine and penicillin (potassium salt) were obtained from Sigma Chemical Co., St. Louis, MO. Aroclor 1254 was obtained from Analabs, North Haven, CT. Agarose (ME) was purchased from Miles Laboratories, Elkhart, IN. Acetyrylene (>95% pure, also known as cyclopenta[cd]pyrene) was a gift from Dr. L. Wallcave, Univ. of Nebraska Medical Center, Omaha, NE. Kerosene soot was obtained through the burning of kerosene fuel in a continuous flow combustor and subsequently collected with a water-cooled probe (25). Soot samples were extracted with methylene chloride in a soxhlet extractor for 18 hours, evaporated by rotary evaporation under vacuum, and redissolved in dimethylsulfoxide. The composition of the kerosene soot extract was determined and

has been reported previously (25).

Metabolizing Element

Because human lymphoblastoid lines lack the ability to oxidatively metabolize xenobiotics including polycyclic aromatic hydrocarbons, a rat-liver post mitochondrial supernatant (PMS) was included during treatment. Male Sprague-Dawley rats (Charles River Laboratories, Cambridge, MA), maintained on a diet of standard rat chow and tap water, received a single intraperitoneal injection of 400 mg/kg aroclor 1254 to raise the level of liver mixed-function oxygenases associated with the endoplasmic reticulum. Four days after the injection, the rats were sacrificed by decapitation. The livers were perfused in situ with ice-cold phosphate-buffered saline (PBS) containing 3.0 mg/ml NaCl, 0.2 mg/ml KCl, 1.15 mg/ml Na₂HPO₄, and 0.2 mg/ml KH₂PO₄. The livers were removed, minced with scissors in three volumes of ice-cold PBS, and homogenized in a Dounce homogenizer with six strokes of a loose-fitting pestle. The homogenate was then centrifuged at 9000xg for 30 minutes at 0°C. The supernatant was decanted and filter-sterilized by passing it through a 0.3µ Millipore filter. PMS aliquots were stored at -80°C prior to use. No decrease in the ability to metabolize benzo[a]pyrene to mutagenic derivatives has been observed during freezing and storage for 3 months at -80°C (G. Kurzba: et al., unpublished observations).

Hyman Lymphoblast Line

Cell line HH-4, a subclone of the human near-diploid lymphoblastoid line WI-L2 (a gift of Dr. Arthur Bloom, University of Columbia College Physicians and Surgeons, New York, NY) was used in all experiments. HH-4 demonstrates the remarkable ability to form macroscopic colonies in soft agar in the presence of a feeder layer of human fibroblasts. Also, the frequency of induced mutation in HH-4 is similar to that observed in other human lymphoblast lines (26; H.L. Liber and T.R. Skopek, unpublished results).

Cell line HH-4 was cultured in RPMI 1640 medium supplemented with 15% fetal calf serum (FCS) (Flow Laboratories, Rockville, MD). Cells were maintained in spinner culture at 37°C with daily dilutions to 3.0×10^5 /ml. Doubling time was 12-13 hours. Cell concentration was determined by particle count using a Coulter Model B Counter (Coulter Electronics, Hialeah, FL).

To reduce the spontaneous 6-thioguanine resistant fraction, cultures were grown for two days in RPMI 1640 (15% FCS) containing 2×10^{-7} M aminopterin, 2×10^{-4} M hypoxanthine, 10^{-5} M cytidine, and 1.74×10^{-5} M thymidine. To reverse the inhibitory effects of the aminopterin, cells were then grown for one additional day in RPMI 1640 (15% FCS) supplemented with only hypoxanthine, thymidine, and cytidine. Cultures were diluted with regular medium for three days prior to use in a mutation assay.

Lymphoblast Mutation Assay

Cultures were diluted to 5.7×10^5 /ml in RPMI 1640 (15% FCS, 100 units/ml penicillin) and divided into 16 ml aliquots, which were placed in 25 cm² plastic tissue culture flasks (Corning Glassworks, Corning, NY). Each flask received 18 mg nicotinamide adenine dinucleotide phosphate, 18 mg glucose-6-phosphate, 12 mg MgCl₂, 7.2 units glucose-6-phosphate dehydrogenase (delivered in 0.92 ml RPMI), and 0.9 ml PMS. The compound was delivered to each flask in 0.18 ml dimethylsulfoxide. Cultures were incubated for 3 hours in a 37°C humidified incubator (5% CO₂).

After treatment, the cells were centrifuged, decanted, and resuspended in fresh medium at a concentration of 3×10^5 /ml. To determine toxicity of treatment, duplicate aliquots of 4000 cells were plated in 5 ml of RPMI 1640 with 0.25% agarose, 20% FCS and 100 units/ml penicillin (plating agar) over a 5 ml prejelled layer of the same agarose medium in a 100 mm petri dish. The plates were fed the next day with 3 ml of RPMI 1640 (20% FCS, 100 units/ml penicillin) and counted one week later.

To allow for phenotypic expression, the treated cultures were allowed

to grow for at least five generations. Five generations are sufficient for expression of mutation to 6-thioguanine resistance in HH-4 cells, in contradistinction to the requirement of 10-14 generations, in another human lymphoblastoid line, MIT-2 (23,24). Duplicate aliquots of 4×10^6 cells were centrifuged and resuspended in 10 ml of liquid plating agar. A 10 μ l sample containing 4000 cells was plated for plating efficiency as described above. A 10 μ l aliquot of a 6-thioguanine solution (10 mg/ml in dimethylsulfoxide) was added to the 10 ml suspension, which was then plated in two 5 ml aliquots onto 5 ml layers of solidified plating agar. Thus, the final 6-thioguanine concentration is 5 μ g/ml. The plates were fed the next day with 3 ml of medium.

The plates were counted after 7-10 days' incubation (37°C, 5% CO₂, humidified). Mutant fraction was calculated by the following formula:

$$\frac{\text{Colonies in the presence of 6-thioguanine}}{(\text{Plating efficiency of culture}) (4 \times 10^6)}$$

RESULTS AND DISCUSSION

Figure 1 illustrates the induction of 6TG-resistant mutants in human lymphoblasts. Kerosene soot at a concentration of 26 μ g/ml induced a mutant fraction of 12×10^{-5} . This dose level contains about 0.52 μ g/ml benzo[a]pyrene, at which concentration benzo[a]pyrene is not significantly mutagenic (no significant increase in mutation at 1 μ g/ml was observed). We tested acepyrylene because it constitutes 15% (wt/wt) of the kerosene soot extract (25) and because it is highly mutagenic to *S. typhimurium* (29; Kaden et al., 1978, submitted for publication, Figure 2). The 26 μ g/ml dose of kerosene soot extract contains about 4 μ g/ml acepyrylene, which can only account for approximately 8% of the mutagenic activity and, thus, cannot explain the high potency of the kerosene soot. Clearly, other factors must be at work; either an undiscovered compound(s) of high mutagenic potency to human cells exists, or, perhaps, synergistic action among compounds is occurring. Much work remains to identify the active and inactive components of the soot polycyclic aromatic fraction in

terms of human cell mutagenesis.

A very important point about the choice of target cells is emphasized when we compare the data obtained with human cells to that obtained with bacterial cells (Figure 2). When the same kerosene soot extract was used to mutate S. typhimurium to 8-azaguanine resistance (Kaden et al., 1978, submitted for publication), a concentration of 100 $\mu\text{g/ml}$ produced a mutant fraction of 100×10^{-5} . This dose level would contain about 2 $\mu\text{g/ml}$ benzo[a]pyrene and 15 $\mu\text{g/ml}$ acepyrylene. Benzo[a]pyrene at 2 $\mu\text{g/ml}$ would not account for the mutagenicity observed; however, 15 $\mu\text{g/ml}$ acepyrylene is considerably more than enough to account for all of the mutagenicity induced by kerosene soot (10 $\mu\text{g/ml}$ produced a mutant fraction of 120×10^{-5}). If one had chosen to characterize the mutagenic potential of kerosene soot components in a bacterial assay, one might have concluded that acepyrylene was wholly responsible for the mutagenicity. While this is conceivably true in bacteria, we have shown that in human cells it accounts for only 8% of total activity.

The difference between the human lymphoblasts and Salmonella can be due to specie-specific differences in the way they respond to a mutagenic stimulus, or to differences in the reactivity of the target DNA in each system. With respect to the latter point, our group has recently isolated a thymidine kinase (tk) heterozygote from the HH-4 line, which enabled us to observe mutation frequency at the tk locus in human cells (30). Preliminary results indicate that the mutation frequency at the tk locus is similar to that at the hgpRT locus (30). Also, we note that the sensitivity of forward mutation to 8-azaguanine resistance in S. typhimurium is similar to that observed in other forward selection systems, including fluorouracil and azetidine-carboxylic acid (T.R. Skopek, 1978, unpublished results). Therefore, we feel that the observed differences in mutagenic response in the human and bacterial systems can, indeed, reflect specie specificity.

The benzo[a]pyrene results compare well with data for other mammalian cells

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in the literature. We note a significant increase in mutation at 3 µg/ml. Krahn and Heideberger (19) used rat-liver cells with V-79 as the target cell and, also, observed an increase in the 8-thioguanine resistant mutant fraction at approximately 3 µg/ml. Huberman (20) obtained an increase in 8-azaguanine-resistant mutant fraction in V-79 cells at 1 µg/ml benzo[a]pyrene in the presence of hamster embryo cells, but the incubation time was two days as opposed to several hours. S. typhimurium also responded to benzo[a]pyrene in the 1-5 µg/ml range. Thus, it would appear that the human lymphoblast system is as sensitive to benzo[a]pyrene as others reported in the literature.

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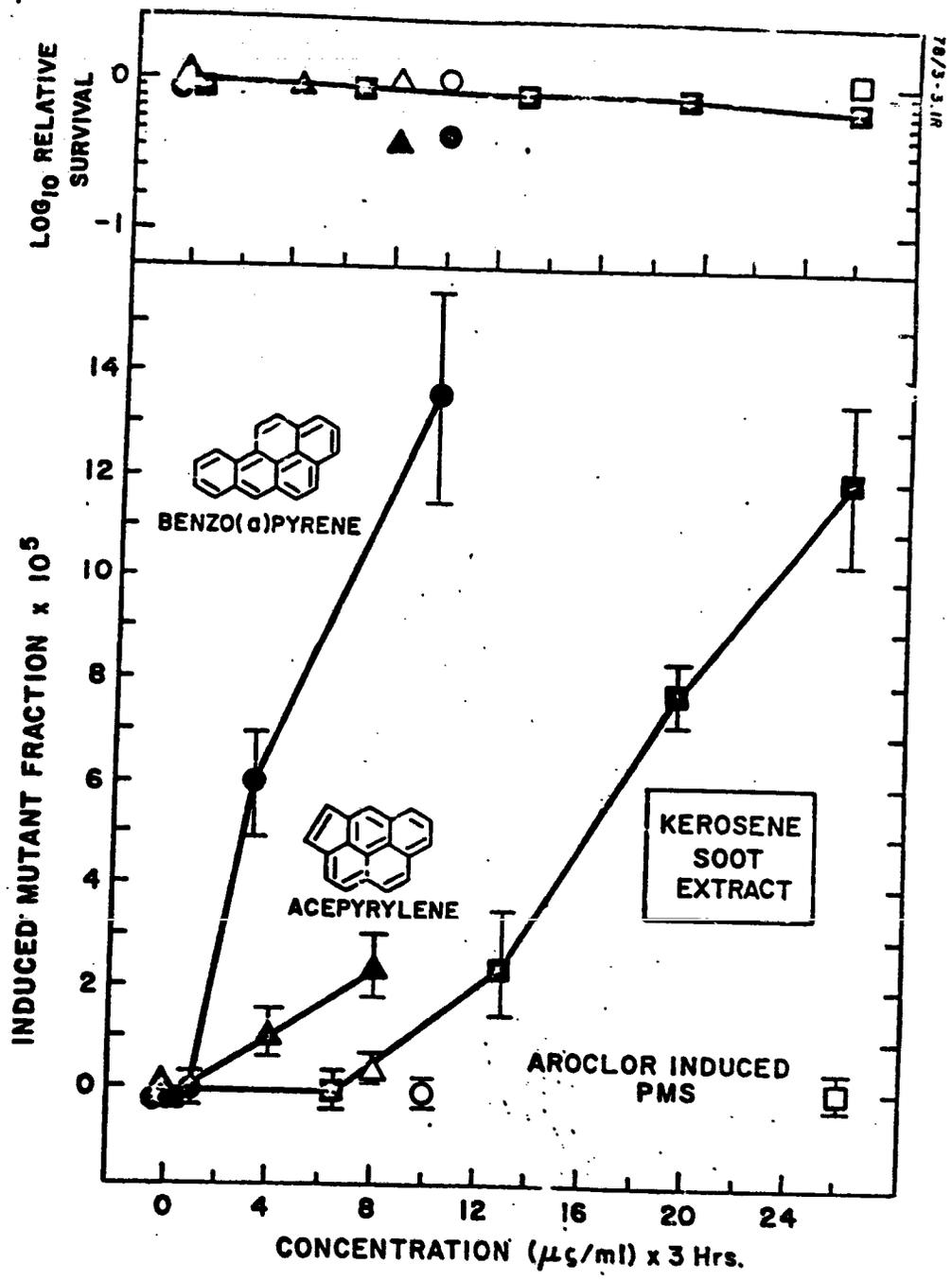
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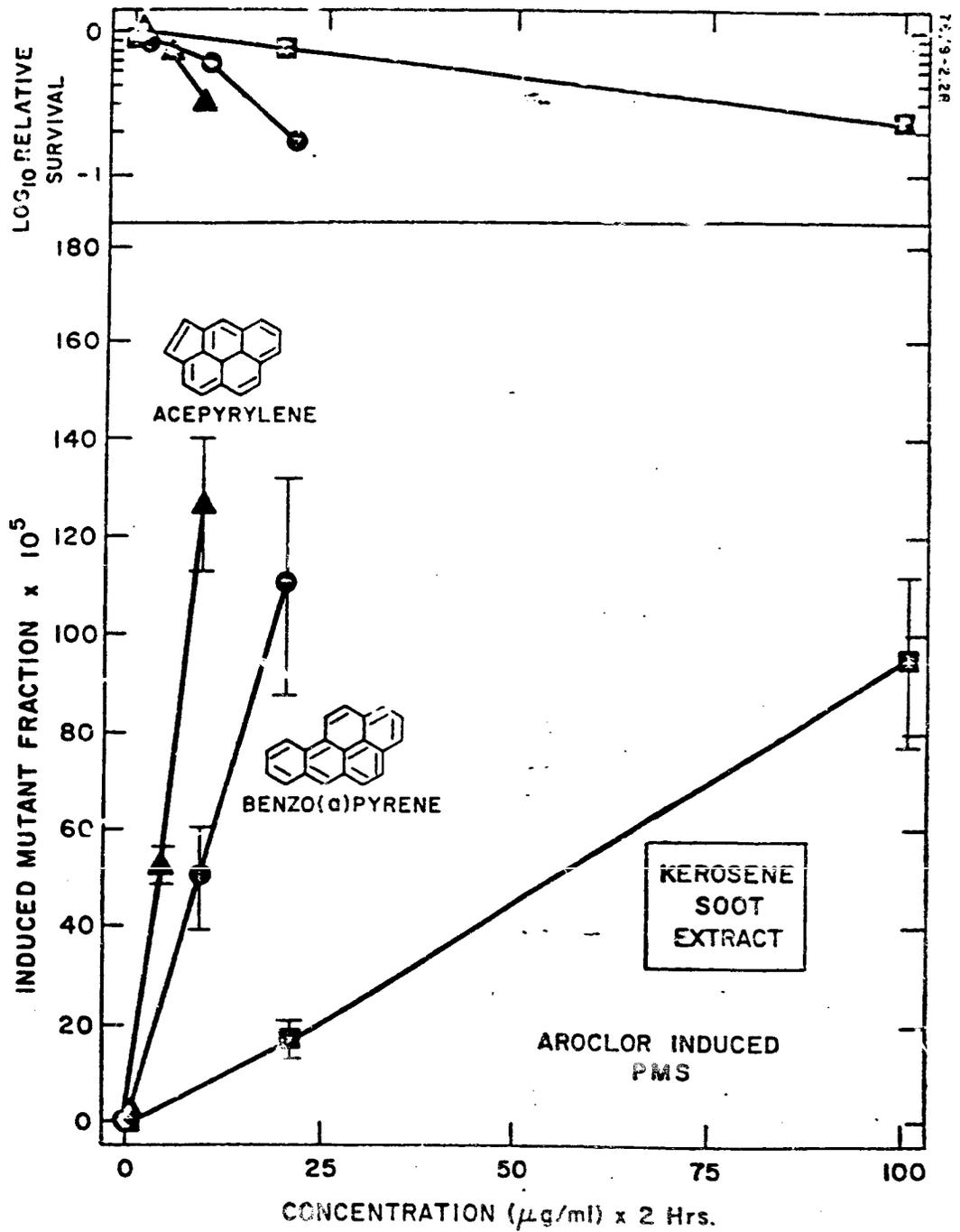
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FIGURE LEGENDS

Figure 1 Toxicity and mutagenicity of benzo[a]pyrene (o), acepyrylene (z) and kerosene soot extract (□) to diploid human lymphoblasts. Treatment was for 3 hours in the presence (closed symbols) or absence (open symbols) of 5% (vol/vol) rat-liver post-mitochondrial supernatant. The control 6-thioguanine resistant mutant fraction ($2-4 \times 10^{-5}$) was subtracted from experimental points to yield the parameter "induced mutant fraction." Each point is the average value of two independently treated cultures. Error bar equals one standard deviation.

Figure 2 Toxicity and mutagenicity of benzo[a]pyrene (o), acepyrylene (Δ), and kerosene soot extract (□) to *Salmonella typhimurium* in 2 hour exposures. Treatment was carried out in liquid culture in a procedure similar to that used for the lymphoblasts. The control 8-azaguanine resistant mutant fraction ($2-5 \times 10^{-5}$) was subtracted from experimental points to yield the parameter "induced mutant fraction." Each point is the average of two independently treated cultures. Error bar equals one standard deviation. [Data is from Kadlubar et al., 1977, submitted for publication.] Assay procedure used in this study is described in references (27) and (28).





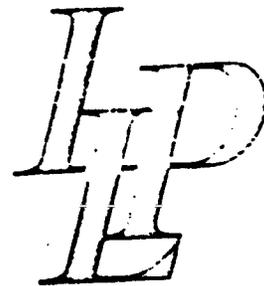
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Mutagenicity of Soot and Associated Polycyclic
Aromatic Hydrocarbons to Salmonella typhimurium¹

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³The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; PMS, postmitochondrial supernatant.

Key Words: genetic toxicology
 mutagenesis
 polycyclic aromatic hydrocarbons
 soot

SUMMARY

The mutagenic activity of the polycyclic-aromatic-hydrocarbon-containing fraction of several soot samples was measured in Salmonella typhimurium using resistance to the purine analog 8-azaguanine as a genetic marker. A postmitochondrial supernatant derived from livers of phenobarbital- and/or aroclor-pretreated male Sprague-Dawley rats was incorporated into all assays to allow metabolism of pro-mutagens to their active forms.

The mutagenic activity of the soot extracts ranged from ten to twenty times higher than could be accounted for by the amount of benzo(a)pyrene present. The possibility of synergism occurring between benzo(a)pyrene and some component in the soot extracts was discounted by the simple additive relationship of the mutagenicity of a soot extract and added benzo(a)pyrene.

To examine the alternative explanation that other components of soot may have undiscovered mutagenic activity, seventy polycyclic aromatic hydrocarbons were quantitatively assayed for their mutagenic potential. Thirty-four of these compounds induced a significant increase in the mutant fraction resistant to 8-azaguanine. Of particular interest is the extreme mutagenicity of perylene, acerylene, and fluoranthene, all of which exhibit greater mutagenicity than benzo(a)pyrene at equimolar concentrations.

Using the measured activities of each polycyclic aromatic hydrocarbon constituent in a kerosene soot, we are able to account for the mutagenic activity of the whole polycyclic aromatic hydrocarbon fraction in terms of additive mutagenicity of its individual components.

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH)³ are found throughout the environment (1,2,3). They are formed by the incomplete combustion of organic material. Sources of PAH include the decomposition of organic matter in soil and sediments (4), heat and power generation, refuse burning, coke production, and motor vehicles (5).

PAH from fuel combustion found in the atmosphere are generally bound to particulate matter such as soot or fly ash. Two to fifteen percent of the fine particle mass in a typical urban atmosphere is soot (6).

Numerous experiments have demonstrated that soot is carcinogenic to experimental animals (7-14) and epidemiological observations have suggested similar activity in man (15). Extracts of particulate matter have induced transformation in rat and hamster embryo cells in culture (16), as well as mutation in bacterial cultures (17-21).

Benzo(a)pyrene, a known mutagen and carcinogen, has been identified as one of the active constituents of soot, fly ash, and particulate samples (1,2,22). Several other mutagenic and carcinogenic constituents have also been identified (2,22-27). However, the mutagenic and carcinogenic potency of soot or its total PAH fraction seems greater than could be accounted for on the basis of the amounts of constituents with known activity (14,18).

We have begun an analysis of this problem with knowledge of the compound distributions in soots (23-25) and a new quantitative bacterial assay for laboratory generated mutation which is particularly useful in the analysis of complex mixtures (28).

MATERIALS AND METHODS

Sources of Soot

|| Nitrogen- and sulfur-containing soots were generated from mixtures

containing equal parts of pyridine, decalin and o-xylene, and from thiophene, decalin and o-xylene, respectively. Mixtures were burned in an alcohol burner, and soot was collected on the bottom of a water-cooled flask placed directly over the flame. The soot was washed from the collection flask with glass wool and methylene chloride (26). Kerosene soot was obtained through the burning of a kerosene fuel in a turbulent, continuous-flow combustor and subsequent collection with a water-cooled probe (23). Furnace black was obtained from a commercial source.

Soot samples were extracted in methylene chloride in a Soxhlet extractor for 18 hours, evaporated by rotary evaporation under vacuum, and redissolved in dimethylsulfoxide.

Sources of Chemicals

Chemicals were obtained from the following sources: acepyrene (cyclopenta(cd)pyrene) was a generous gift of Dr. Lawrence Wallace, University of Nebraska Medical Center, Omaha, NE. 1,2-Benzodibenzo-(b,d)thiophene was generously supplied by Dr. LeRoy H. Klann, University of Oregon, Eugene, OR. 1H-Benz(g)indole and 1H-benz(e)-indol-2-acid were donated by Dr. Stewart W. Schneller, University of South Florida, Tampa, FL. Dibenz(a,e)fluoranthene was supplied by Dr. R.C. Lao, Environmental Health Centre, Ottawa, Ontario. Acenaphthylene, 4-azafluorene, benzene, 7H-benz(d,e)anthracen-7-one, benzo(b)fluorene, benzo(ghi)perylene, benzo(e)pyrene, 5,6-benzoquinoline, 7,8-benzoquinoline, 4H-cyclopenta(def)phenanthrene, 2,6-dimethylquinoline, 2,6-dimethylnaphthalene, isoquinoline, 3-methylisoquinoline, 2-methylquinoline, 4-methylquinoline, perylene, 2-phenylnaphthalene, 2-phenylpyridine, 4-phenylpyridine, pyrene, pyridine, triphenylene, 2,7-dimethylquinoline, corone, and fluoranthene were purchased from Aldrich Chemical Co., Milwaukee, WI. Anthanthrene, aroclor 1254, 1,1'-binaphthyl, 9-phenyl-

anthracene, picene, 6-terphenyl and 2-terphenyl were purchased from Analabs, Inc., North Haven, CT. 2,3,6-Trimethylindole was obtained from Chemical Samples Co., Columbus, OH. 3,4-Benzoquinoline was purchased from Eastern Chemicals, Hauppauge, N.Y. Benz(a)anthracene, chrysene, fluorene, 1-methylnaphthalene, naphthalene, and phenanthracene were obtained from Eastman Chemical Co., Rochester, N.Y. Anthraquinone, anthrone, and indole were purchased from Fisher Scientific Co., Medford, MA. Dibenz(b,d)thiophene was obtained from Fluka A.G., Switzerland. Acenaphthalene, 1-cyanonaphthalene, 2-cyanonaphthalene, 1-methylpyrene, 1-methylphenanthrene, and 2-methylphenanthrene were obtained from I.C.N./K&K Life Sciences Group, Plainview, N.Y. 2-Methylanthracene and 9-methylanthracene were obtained from I.C.N. Pharmaceuticals, Inc. Plainview, N.Y. Sodium phenobarbital and methylene chloride were obtained from Mallinckrodt Inc., St. Louis, MO. Quinoline and dimethylsulfoxide, reagent grade, were obtained from Matheson, Coleman and Bell, Norwood, OH. 1-Methylisoquinoline was obtained from Pflatz & Bauer, Inc., Stamford, CT. Anthracene, benzo(a)pyrene, dibenz(a,c)anthracene, dibenz(a,h)anthracene, 7,12-dimethylbenz(a)anthracene, 2,3-dimethylquinoxaline, m-dinitrobenzene, 3-methylcholanthrene, and 2-methylindole were obtained from Sigma Chemical Co., St. Louis, MO.

Bacterial Mutation Assay

Mutation assays were carried out as specified by Skopek *et al.* (28, 29). Exponentially growing cultures of *Salmonella typhimurium* strain TM677 were exposed to several concentrations of the test agent for two hours in the presence of 10% (v/v) of a postmitochondrial supernatant (PMS), prepared as a 25% (w/v) liver homogenate of phenobarbital- or aroclor-pretreated male Sprague-Dawley rats (Charles River Breeding Laboratories, Cambridge, MA). Details of

PMS preparation are reported elsewhere (28). One mg/ml glucose-6-phosphate, 1 mg/ml NADP⁺, 670 μg/ml MgCl and 0.4 units/ml glucose-6-phosphate dehydrogenase were included as cofactors for the drug-metabolizing system. Following the two-hour incubation at 37 C, bacteria were centrifuged (2000 rpm x 15 minutes), resuspended in a phosphate-buffered saline, and plated under selective (50 μg/ml 8-azaguanine) and non-selective conditions. Colonies were counted after two days' growth at 37 C.

Mutant fraction was calculated by dividing the number of colonies observed under selective conditions by the number of colonies observed under permissive conditions, and multiplying by appropriate dilution factors.

RESULTS AND DISCUSSION

All experiments were performed utilizing one of two frozen batches of bacterial strain TH677. Analysis of the variation among assays shows a normal distribution of the background mutant fraction (Chart 1). The mean background mutant fraction for experiments performed from the first frozen batch (all experiments between June 26, 1977 and September 28, 1977) was 7.1×10^{-5} . The mean background mutant fraction for all experiments performed from the second frozen batch (all experiments between October 1, 1977 and December 1, 1978) was 5.6×10^{-5} . Standard deviations were 4.0×10^{-5} (n=157) and 2.2×10^{-5} (n=146), respectively.

The 99.9% confidence limit on the mean background fraction (mean + 3 standard deviations) is used as our criterion of minimum significance .

Using this criterion, the methylene chloride extracts of nitrogen-containing, sulfur-containing, furnace black and kerosene

soots were all found to be mutagenic at 20-80 $\mu\text{g/ml}$ culture medium in a two hour exposure (aroclor preinduced rat liver PMS). Initial slopes of the concentration dependence of induced mutation (Chart 2) show the sulfur-containing soot to have 10% of the activity of pure benzo(a)pyrene; the nitrogen-containing soot, 10%; furnace black, 13%; and kerosene soot, 17%. Mutation induced utilizing phenobarbital-induced PMS was not observed until higher concentrations were attained for all soot extracts tested, as well as for benzo(a)pyrene (Data not presented).

Benzo(a)pyrene, often considered the highest contributor to soot's mutagenicity, constitutes less than one percent of the kerosene soot extract (28), and accounts for less than three percent of the observed mutagenicity of that soot extract. Thus, the observed mutagenicity could not be explained by a simple additive contribution of all known mutagenic components of the soot extract.

Two possibilities could logically account for this phenomenon: mutagenic components could act synergistically among themselves and non-mutagenic components with regard to their mutagenic activity; alternatively, other components of the soot extract could have yet undiscovered significant mutagenic activity which could cumulatively account for the soot extract's mutagenic activity.

To test the hypothesis of synergism, the mutagenic activity of benzo(a)pyrene was measured in the presence of PMS from aroclor-pretreated rats over a wide range of concentrations in the presence and absence of 80 $\mu\text{g/ml}$ nitrogen-containing soot extract. We observed a strictly additive relationship when benzo(a)pyrene was added to a set amount of nitrogen-containing soot extract and the mixture was assayed for mutagenic activity (Chart 3). This observation is, of course, inconsistent with a significant contribution of

synergism of soot components with benzo(a)pyrene.

In order to test the second hypothesis, seventy PAM components of various soots were quantitatively assayed for mutagenic activity in the presence of PMS from aroclor-pretreated rats. When mutagenicity was not observed with PMS from aroclor-pretreated rats, the compounds were retested in the presence of PMS from phenobarbital-pretreated rats.

Thirty-four of the components induced a significant increase in mutant fraction as measured by 8-azaguanine resistance (Table 1). Data for three of the thirty-seven remaining components (4-azafluorene, anthracene, and 1,2-benzodibenzo(b,d)thiophene) suggests possible low-level mutagenicity. Solubility problems encountered with several of the compounds prevented testing at higher concentrations (Table 1). The lowest concentration yielding significant mutation was calculated by interpolation for each compound from the concentration response curve and is recorded in Table 1 (see Skopek *et al.* (29) for method of calculation). In addition, the mutagenic potency relative to benzo(a)pyrene was calculated by dividing the initial slope of the concentration response curve (mutant fraction in two hours/concentration) by the "slope" of the simultaneously performed (80 μ M) benzo(a)pyrene standard, which lies on the linear portion of the benzo(a)pyrene concentration response curve.

For those interested in comparing the mutagenicity to the carcinogenicity of these compounds, we have included animal carcinogenicity data, where available, in Table 1 (32).

Although the mutagenicity of some of the compounds (quinoline, acetyrylene, benz(a)anthracene, chrysene, benzo(a)pyrene, benzo(e)pyrene, 7,12-dimethylbenz(a)anthracene, 3-methylcholanthrene, dibenz(a,c)-anthracene, benzo(ghi)perylene, and dibenz(a,h)anthracene) has been

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previously recognized (e.g., 30,31), this is the first report of mutagenic activity in bacteria of twenty-three of the polycyclic aromatic hydrocarbons associated with soot. Of particular interest is the extreme mutagenic activity of perylene, acropylene, and fluoranthene, all of which exhibit greater mutagenicity than benzo(a)pyrene at equimolar concentrations (Chart 4). Although the mutagenic response of perylene reaches a stable maximum at concentrations greater than $12 \mu\text{M}$, it induces significant mutation at concentrations as low as $1.1 \mu\text{M}$, as compared to $4.0 \mu\text{M}$ for benzo(a)pyrene when a 10% (v/v) PMS from aroclor-pretreated rats is used.

The compounds acenaphthalene, acenaphthylene, 4-phenylpyridine, 5,6-benzoquinoline, and 1-methylnaphthalene (see Charts 4-6) illustrate the absolute necessity of simultaneous measurement of the toxicity incurred as a result of treatment when examining chemicals of unknown biological activity. All are mutagenic only at concentrations which are also toxic to the bacteria. Failure to account for this toxicity in calculations of mutagenic potency leads one to the erroneous conclusion that none of these compounds are mutagenic to S.typhimurium, since the actual number of mutants is not increased by treatment.

It is necessary to further emphasize that the potency calculations of Table 1 are all based on 10% PMS (v/v) determination. The dependence of apparent mutagenicity of many polycyclics on PMS concentration is not a simple monotonic increasing or saturation relationship, but often demonstrates a maximum in the range of 1 to 20% (v/v). Thus, the activity relative to benzo(a)pyrene will certainly vary for many compounds if different amounts of PMS are used or if PMS concentration is optimized for each compound.

Looking at the facts in mind, it is however worthwhile to note some interesting relationships between structure and activity in the data of Table 1. For instance, the mutagenic activity of a given PAH often seems to be lower than the corresponding aza-compound (Charts 6-8). This may be important in terms of analyzing the soots of fuels such as coal, which contain significant amounts of nitrogen. Present limitations in chemical analytical technique prevent a quantitative analysis of all the aza-aromatics in nitrogen-containing soot. Fortunately, a complete chemical analysis of the polycyclic aromatics in our kerosene soot sample has been performed (23) and is reported in Table 2. Using this information, we can attempt to compare the mutagenic activity of the crude methylene chloride extract to the sum of the expected activities of each of its polycyclic aromatic components.

Our analysis is summarized in Table 2, in which the fraction of each polycyclic aromatic component present in the methylene chloride extract of kerosene soot is listed together with its potential contribution to the mutagenicity of the soot extract. This contribution is calculated by examining the mutant fraction induced by the concentration of a given compound present in the kerosene soot extract, and dividing by the mutant fraction induced by the kerosene soot extract.

As can be seen in Table 2, the individual PAH constituents can more than account for the activity of the kerosene soot methylene chloride extract. Examination at 20 and 100 μ g/ml kerosene soot extract show that compounds may contribute to the extract's mutagenicity to different extents, depending on the concentration present. However, at all levels examined there is sufficient activity present

in the individual components to account for the extract's high activity.

The fact that the sum of the components' mutagenicity is greater than that of the kerosene soot extract may be due to either the imprecision of our estimates, or a partial competitive inhibition of metabolizing reactions. When the kerosene soot extract was reconstituted from the available PAH constituents, mutagenic activity similar to that of the kerosene soot extract itself was observed (Chart 9).

Thus, the mutagenic activity of the PAH fraction of the kerosene soot extract appears to be due to a simple additive contribution of its mutagenic components. Whether this is true of soots prepared from other fuels, using other combustion conditions remains to be determined.

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TABLE 1 (Continued)

Compound	M.W.	PMS ^a	Significant Induced Mutation ^b	Concentration ^c	Relative Mutagenic Activity ^d	Carcinogenesis in Animals ^e
Carbazole	167	A, PB	-	3 mM*		NA
Dibenzo(b,d)thiophene	168	A, PB	-	300 μ M*		NA
m-Dinitrobenzene	168	A	+	2 mM	<0.01	NA
2,3,6-Trimethylnaphthalene	170	A, PB	-	600 μ M*		NA
Anthracene	178	A, PB	-	225 μ M*		-
Phenanthrene	178	A, PB	-	300 μ M*		-
3,4-Benzoquinoline	179	A	+	140 μ M	0.05	NA
5,6-Benzoquinoline	179	A, PB	+	84 μ M	0.11	+
7,8-Benzoquinoline	179	A	+	56 μ M	0.13	NA
4H-Cyclopenta(def)phenanthrene	190	A, PB	-	1 mM*		NA
2-Methylantracene	192	A	+	80 μ M	0.15	NA
9-Methylantracene	192	A, PB	+	75 μ M	0.08	NA
1-Methylphenanthrene	192	A, PB	+	90 μ M	0.50	NA
2-Methylphenanthrene	192	A, PB	+	40 mM	0.30	NA
2-Phenylnaphthalene	192	A, PB	-	4 mM		NA
Anthrone	194	A, PB	-	520 μ M*		-
Pyrene	202	A	+	140 μ M	0.07	-
Benzo(b)fluorene	204	A	+	25 μ M	0.08	NA
Anthraquinone	208	A, PB	-	100 μ M*		-
1H-Benz(e)indole-2 acid	210	A, PB	-	2 mM*		NA
1-Methylpyrene	216	A	+	180 μ M	0.15	+
1,2-Benzodibenzo(b,d)thiophene	228	A, PB	-	500 μ M*		NA
Acetypyrene	228	A	+	7.3 μ M	1.51	NA
Benz(a)anthracene	228	A, PB	+	65 μ M	0.14	+
Chrysene	228	A, PB	+	45 μ M	0.20	+/-

Table 1 Continued . . .

TABLE 1 (Continued)

Compound	M.W.	PMS ^a	Significant Induced Mutation	Concentration ^c	Relative Mutagenic Activity ^d	Carcinogenesis in Animals ^e
Triphenylene	228	A	+	44 μ M	0.07	-
7H-Benzo(de)anthracen-7-one	230	A, PB	+	100 μ M	0.20	NA
o-Terphenyl	230	A, PB	-	900 μ M*		NA
m-Terphenyl	230	A, PB	-	900 μ M*		NA
Fluoranthene	235	A	+	5 μ M	1.00	-
3,3'-5,5'-Tetramethylbenzidine	240	A, PB	-	420 μ M*		NA
Benzo(a)pyrene	252	A	+	4 μ M	1.00	+
Benzo(e)pyrene	252	A, PB	+	90 μ M	0.11	+/-
Perylene	252	A	+	1.1 μ M	6.00	-
1,1'-Binaphthyl	254	A, PB	-	800 μ M*		NA
9-Phenylanthracene	254	A, PB	-	400 μ M*		-
7,12-Dimethylbenz(a)anthracene	256	A, PB	+	25 μ M	0.44	+
3-Methylcholanthrene	268	A, PB	+	190 μ M	0.06	+
Benzo(ghi)perylene	276	A, PB	+	72 μ M	0.08	+/-
Anthanthrene	276	A, PB	+	40 μ M	0.08	+/-
Dibenz(a,c)anthracene	278	A, PB	+	13 μ M	0.77	+
Dibenz(a,h)anthracene	278	A	+	75 μ M	0.09	+
Picene	278	A, PB	-	36 μ M*		-
Coronene	300	A, PB	-	170 μ M*		-
Dibenz(b,e)fluoranthene	302	A	+	26 μ M	0.88	NA

^a Where there is more than one pretreatment listed, calculations refer to underlined pretreatment.

^b - No significant induced mutation.

^c + Significant induced mutation.

^d Number listed is concentration of significant induced mutation for positive responses, highest concentration tested for negative responses.

^e Mutagenic activity relative to that of the B(a)P

positive control performed simultaneously with test compound.

^f Reference (32).

* Denotes solubility limits in this assay.

TABLE 2

Compound	Wt. (%)	At 20 µg/ml:		At 100 µg/ml:	
		Amt. present (µg/ml)	Mutation con-tribution	Amt. present (µg/ml)	Mutation con-tribution
Acenaphthylene	23	4.6	0	23	0
Acetylene	15	3.0	1.5	15	1.1
Pyrene	8	1.6	0	8	0.01
Benzo(ghi)perylene + anthanthrene	8	1.6	.13	8	0.02
Coronene	5	1.0	0	5	0
Fluoranthene	4	0.8	0	4	0.7
Naphthalene	3	0.6	0	3	0
Benzo(ghi)fluoranthene	3	0.6	---	3	---
Phenanthrene + Anthracene	2	0.4	0	2	0
Benzacenaphthalene	2	0.4	---	2	---
Benzofluoranthene	2	0.4	---	2	---
Perylene	2	0.4	.07	2	0.2
Acenaphthalene	1	0.2	0	1	0
Indeno(1,2,3-cd)pyrene	1	0.2	---	1	---
Benzo(a)pyrene + benzo(e)pyrene	1	0.2	.03	1	0.02
4H-Cyclopenta(def)phenanthrene	1	0.2	---	1	---
Benzo(a)fluorene	0.4	.008	0	0.4	0
Fluorene	0.3	.006	0	0.3	0
Uncharacterized Material	18.3	3.7	---	18.3	---
Σ Components			1.7		2.1
MeCl ₂ Extract	100	20	1.0	100	1.0

CHARGE NUM	TITLE	QUOTED	ADJUSTED AUTHORIZED SPEND PLAN AMOUNT	STOP DATE	SPENT LAST WEEK	SPENT TO DATE	AUTHORIZED AMT REMAIN	TOTAL REMAIN
1-12	VACATION	17.0	17.0	9/30/96			XXXXX	17.0
F0144 -206	HAPS - \$6000 for DC	24.5	24.5	9/30/95#		24.5		
F0144 -217	TSCATS DATABASE SUPPORT	154.5	154.5	9/29/95#		154.5		
F0144 -401	TSCATS DATABASE MAINTENANCE	300.0	300.0	7/31/96	30.0	30.0	270.0	270.0
F0187 -105 -003	WORK AUTHORIZATION #3	114.0	114.0	9/30/95#	10.0	114.0		
F0187 -107	COMPARATIVE RISK ANALYSIS	51.0	51.0	9/30/95#		1.0	50.0	50.0
F0187 -109	SUPPORT FOR ABSTRACTING SELECTED TSCA	125.0	125.0	9/30/95#		1.0	124.0	124.0

NOTE: * DENOTES AUTHORIZATION EXCEEDED; # DENOTE AUTHORIZATION HAS EXPIRED; XXX DENOTES AUTHORIZATION OPTION IS NOT IN EFFECT
 16 WEEK LINEAR PROJECTION BREAKDOWN

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	REMAIN
F0144 -401	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	169.2
TOTAL	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	169.2

TABLE 1

Compound	Mutagenicity of Soot Components to <i>S. typhimurium</i>		Significant Induced Mutation ^b	Concentration ^c	Relative Mutagenic Activity ^d	Carcinogenesis in Animals ^e
	M.W.	PMS ^a				
Benzene	78	A, PB	-	13 mM	<0.01	-
Pyridine	79	A, PB	+	6 mM		-
Indole	117	A, PB	-	4 mM		-
Naphthalene	128	A, PB	-	2 mM	0.10	+
Quinoline	129	A	+	80 μM		NA
Isoquinoline	129	A, PB	-	8 mM		NA
2-Methyl indole	131	A, PB	-	2 mM	0.01	NA
1-Methylnaphthalene	142	A	+	6 mM		NA
2-Methylquinoline	143	A, PB	-	7 mM	0.05	NA
4-Methylquinoline	143	A	+	90 μM		NA
1-Methylisoquinoline	143	A, PB	-	7 mM		NA
3-Methylisoquinoline	143	A, PB	+	4 mM*	0.07	NA
Acenaphthylene	152	A, PB	+	1 mM		NA
1-Cyanonaphthalene	153	A, PB	-	1 mM		NA
2-Cyanonaphthalene	153	A, PB	-	1 mM		NA
Acenaphthalene	154	A, PB	+	490 μM	0.01	-
2-Phenylpyridine	155	A, PB	-	6 mM		NA
4-Phenylpyridine	155	A, PB	+	650 μM	0.10	NA
2,6-Dimethylaphthalene	156	A, PB	-	160 μM*		NA
2,6-Dimethylquinoline	157	A, PB	-	2 mM		NA
2,7-Dimethylquinoline	157	A, PB	-	2 mM		NA
2,3-Dimethylquinoline	158	A, PB	-	3 mM		NA
Fluorene	166	A, PB	-	300 μM*		-
4-Azafluorene	167	A, PB	-	1 mM*		NA
1H-Benz(g)indole	167	A, PB	-	5 mM*		NA

Table 1 Continued . . .

CHART LEGENDS

- Chart 1: Distribution of background mutant fraction.
Each event represents a single determination of the background mutant fraction. A. All experiments utilizing bacterial batch frozen June 24, 1977. B. All experiments utilizing bacterial batch frozen September 26, 1977.
 n =total number of determinations, \bar{x} =mean, s_x =standard deviation.
- Chart 2: Dose-dependent mutagenicity (open symbols) and toxicity (closed symbols) of the methylene chloride extracts of xerosene (o,●), furnace black (⊗), nitrogen-containing (□,⊠) and sulfur-containing (Δ,▲) soots to S.typhimurium in the presence of aroclor-induced PMS.
Each point represents the average of two independent determinations.
- Chart 3: Dose-dependent mutagenicity of benzo(a)pyrene in the presence and absence of 80 μg/ml of a methylene chloride nitrogen-containing soot extract.
All points were assayed in the presence of aroclor-induced PMS.
Each point represents the average of two independent determinations.
- Chart 4: Dose-dependent mutagenicity (open symbols) and toxicity (closed symbols) of acepyrylene (□,⊠), fluoranthene (Δ,▲), benzo(a)pyrene (o,●) and perylene (o,●) to S.typhimurium.
All points were assayed in the presence of aroclor-induced PMS. Each point represents the average of two independent

determinations.

Chart 5: Dose-dependent mutagenicity (open symbols) and toxicity (closed symbols) of acenaphthylene (□,■), acenaphthalene (○,●), and 4-phenylpyridine (Δ,▲) to S.typhimurium. Acenaphthalene was assayed in the presence of aroclor-induced PMS. 4-Phenylpyridine and acenaphthylene were assayed in the presence of phenobarbital-induced PMS. Each point represents the average of two independent determinations.

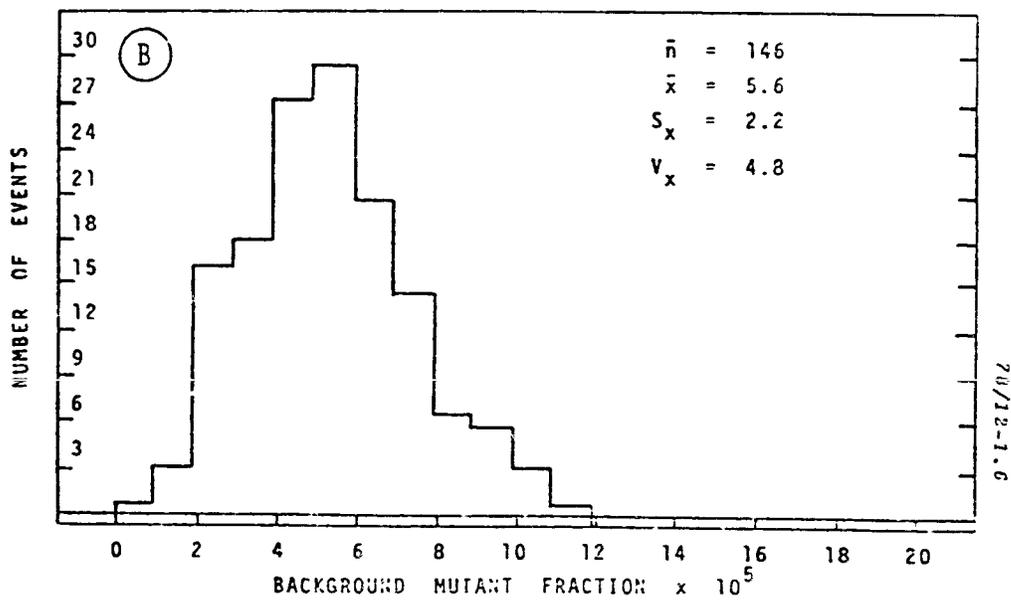
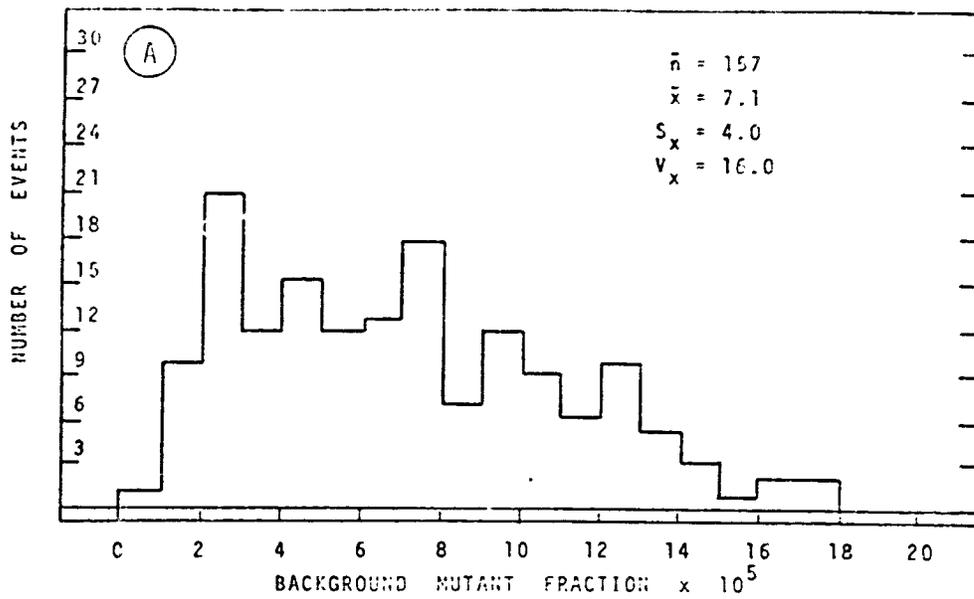
Chart 6: Dose-dependent mutagenicity (open symbols) and toxicity (closed symbols) of 4-methylquinoline and 1-methylnaphthalene to S.typhimurium in the presence of aroclor-induced PMS. Each point represents the average of two independent determinations.

Chart 7: Dose-dependent mutagenicity (open symbols) and toxicity (closed symbols) of 5,6-benzoquinoline (Δ,▲), 7,8-benzoquinoline (□,■), 3,4-benzoquinoline (○,●), and phenanthrene (⊙,⊙) to S.typhimurium. 3,4-Benzoquinoline, 5,6-benzoquinoline and 7,8-benzoquinoline were assayed in the presence of aroclor-induced PMS. Phenanthrene was assayed separately in the presence of either aroclor- or phenobarbital-induced PMS. Each point represents the average of two independent determinations.

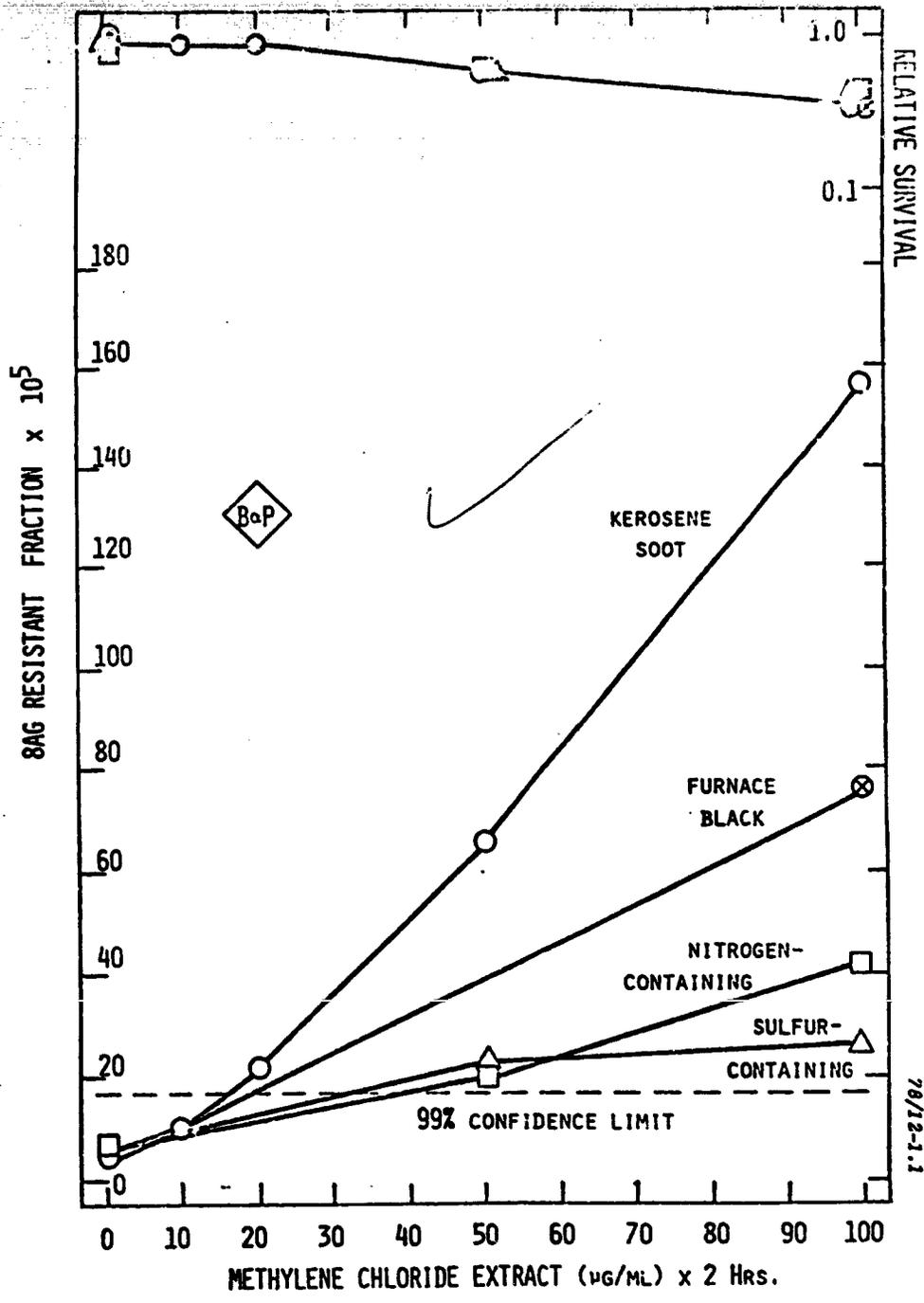
Chart 8: Dose-dependent mutagenicity (open symbols) and toxicity (closed symbols) of quinoline (○,●), isoquinoline (□,■), and naphthalene (Δ,▲) to S.typhimurium. Quinoline and isoquinoline were assayed in the presence of aroclor-induced PMS. Naphthalene was assayed separately in the

presence of aroclor- or phenobarbital- induced PMS. Each point represents the average of two independent determinations.

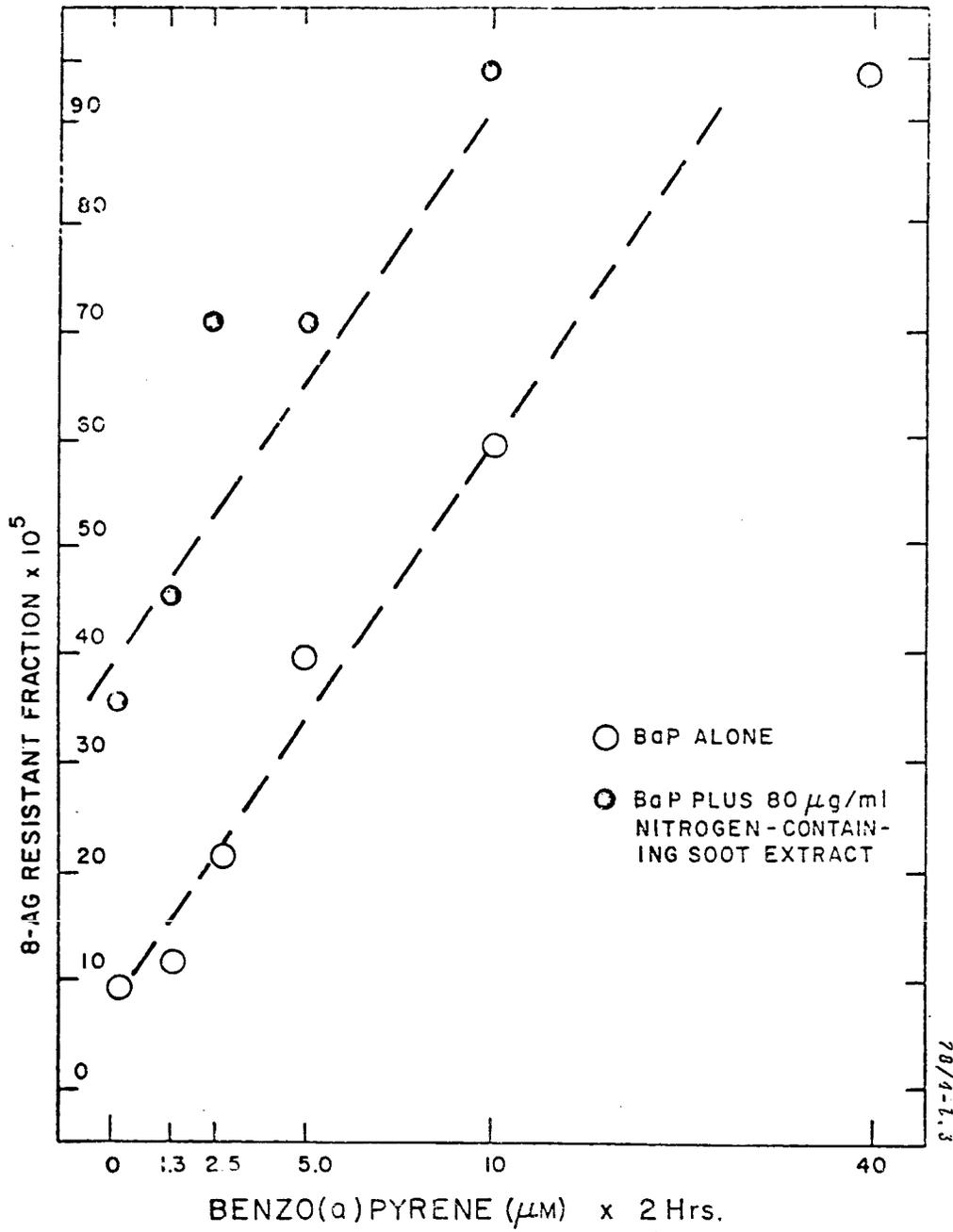
Chart 9: Dose-dependent mutagenicity (open symbols) and toxicity (closed symbols) of a methylene chloride kerosene soot extract (o,●) and a reconstituted kerosene soot extract (Δ,▲) to S.typhimurium in the presence of aroclor-induced PMS. Each point represents the average of two independent determinations.

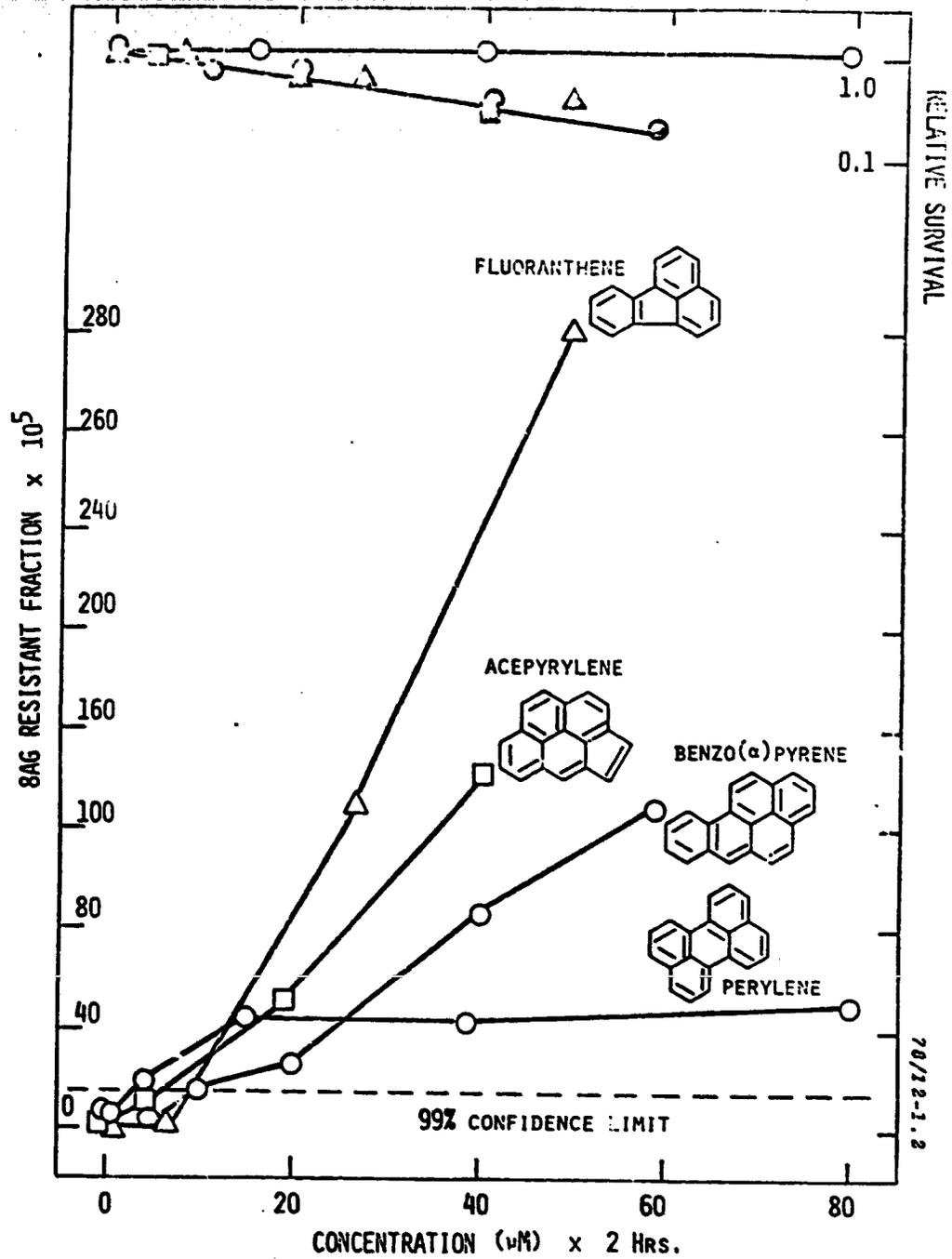


0047



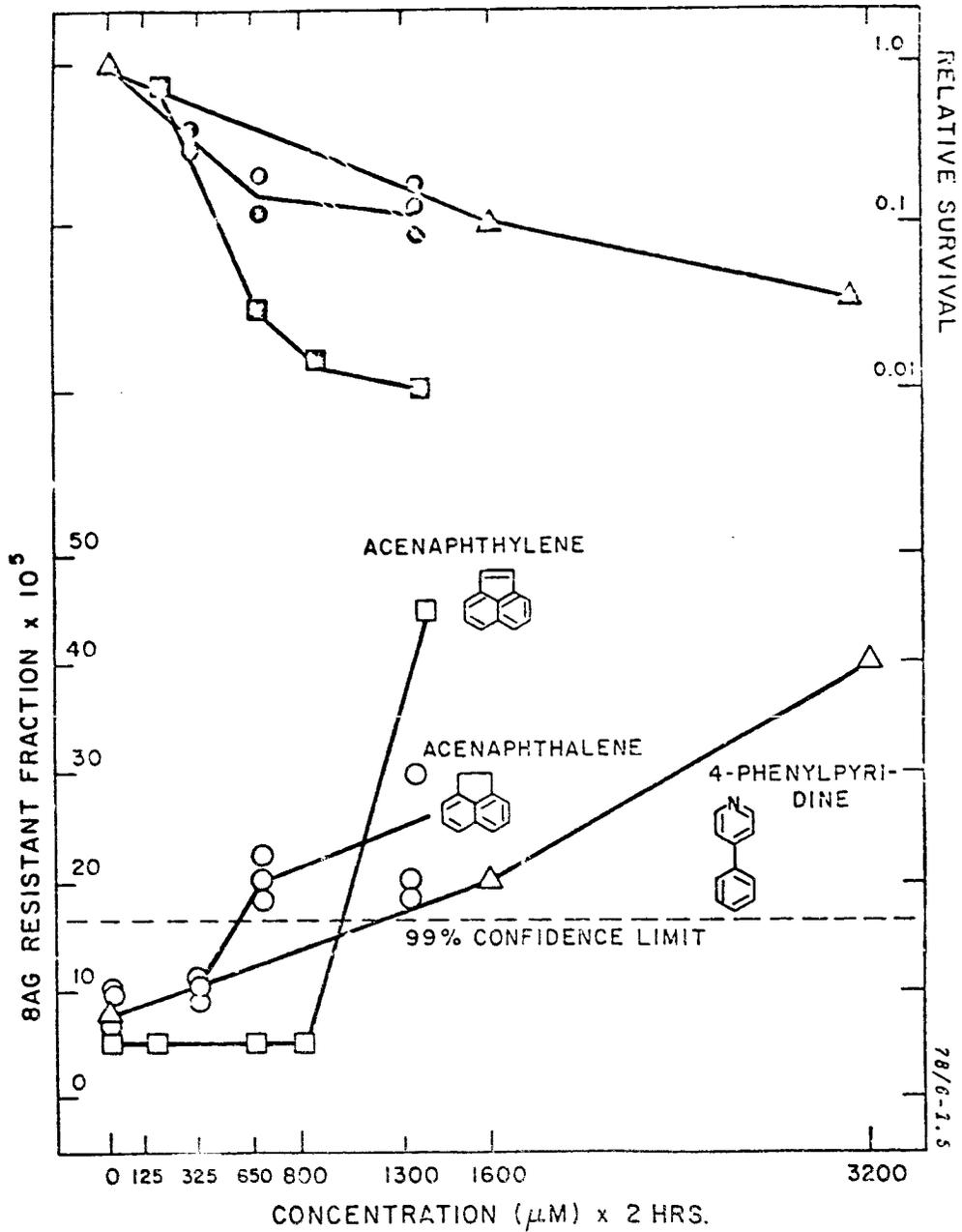
0048





0050

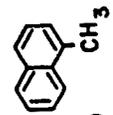
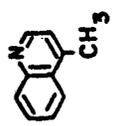
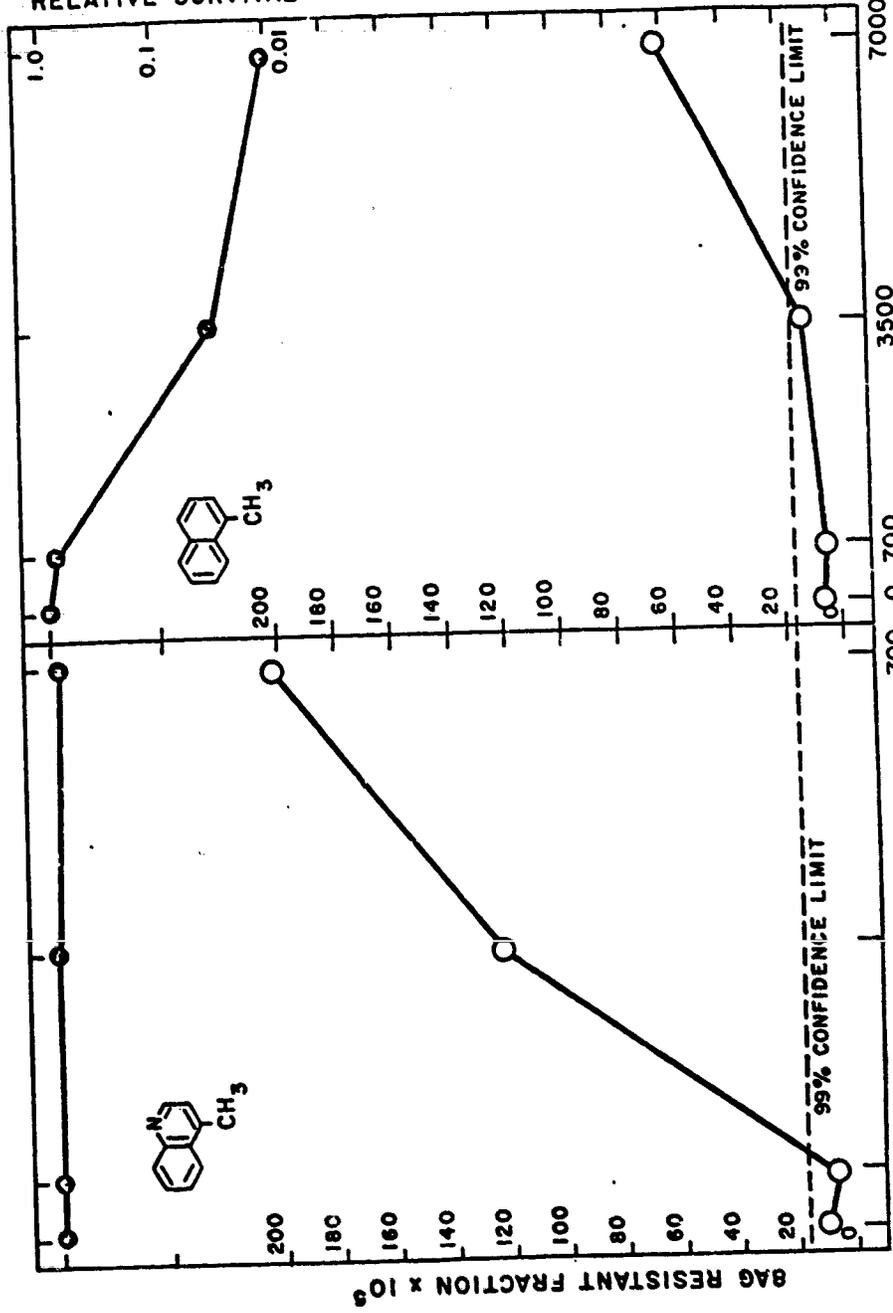
228



78/6-1.5

RELATIVE SURVIVAL

78/6-1.7



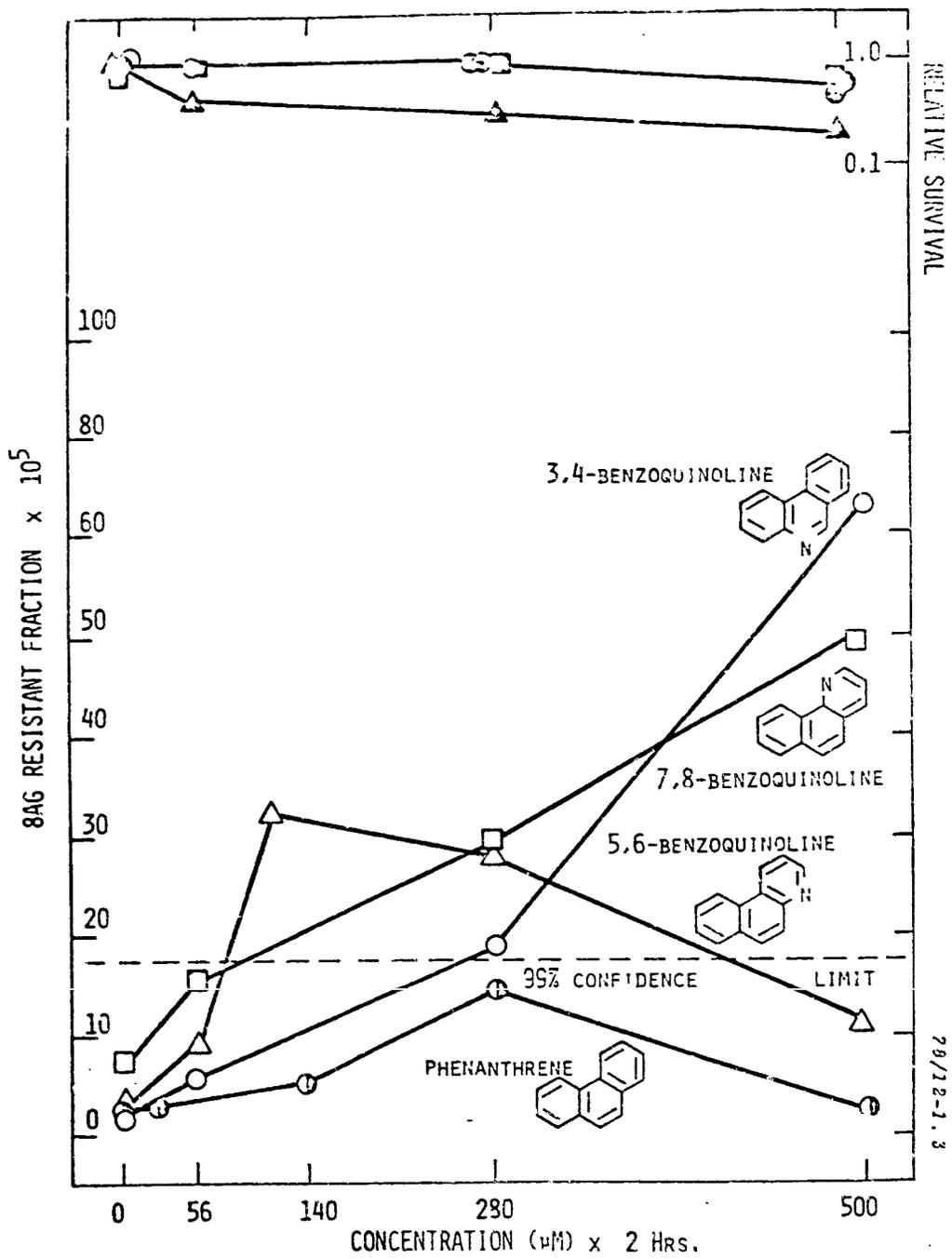
4-METHYLQUINOLINE (μM) x 2 HRS.

1-METHYLNAPHTHALENE (μM) x 2 HRS.

99% CONFIDENCE LIMIT

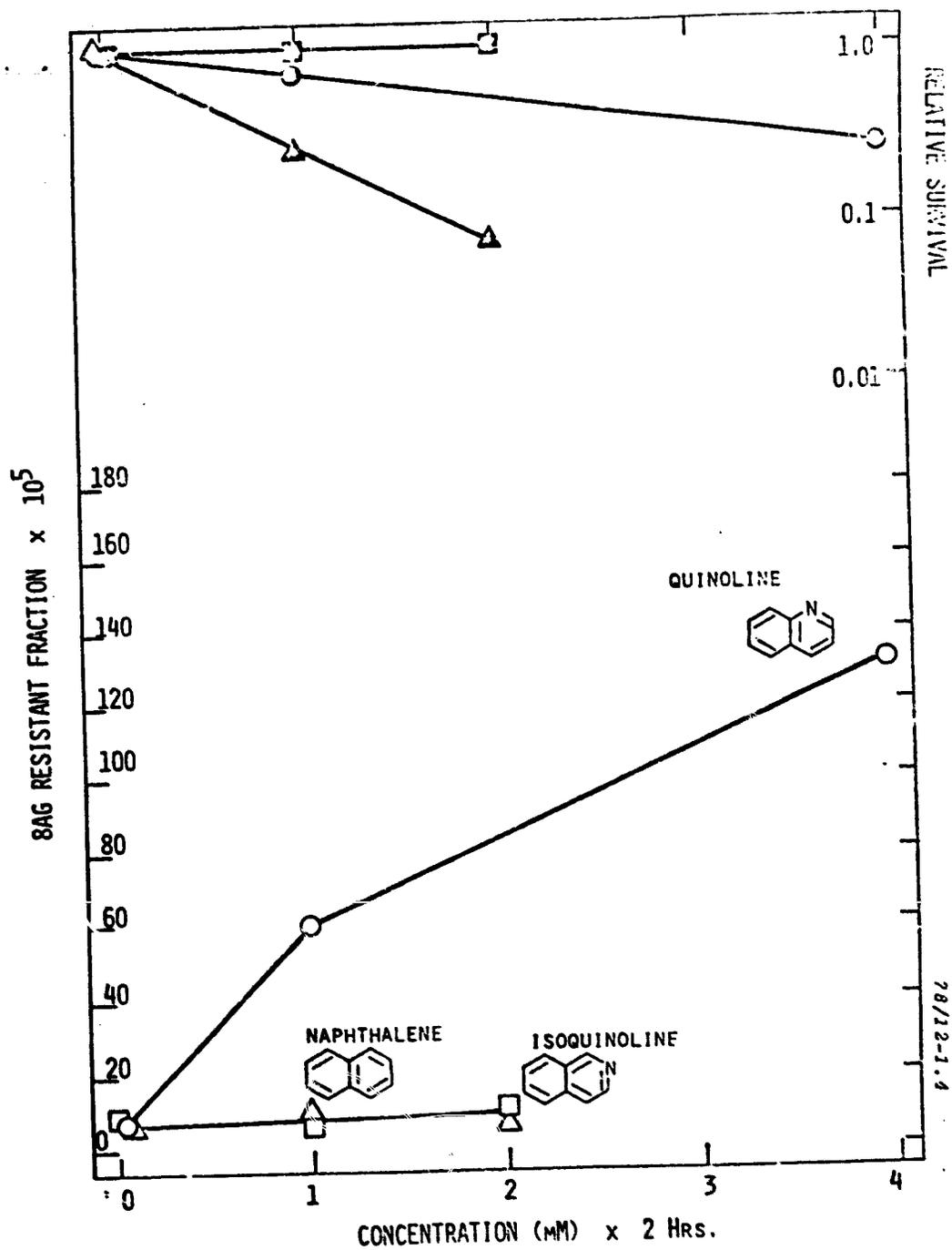
99% CONFIDENCE LIMIT

BAG RESISTANT FRACTION x 10⁹



78/12-1.3

0053



0 0 5 4

