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Chemical and Toxicological Characterization of Residential Oil Burner Emissions: II. Mutagenic, Tumorigenic, and Potential Teratogenic Activity

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Extracts of effluents from a modern residential oil burner have been evaluated in several toxicological assay systems. Bacterial mutagens were detected in extracts from both the particulate and vapor phase emissions. Effluents from continuous operation were an order of magnitude less mutagenic than those from cyclic (5 min on, 10 min off) operations. No difference in the yield of bacterial mutagens per gram of fuel burned was found between cyclic operation under low and moderate sooting conditions. On the basis of elution behavior from alumina it appeared that the bacterial mutagens collected from high sooting effluents were more polar than those from low sooting effluent. An extract that was mutagenic in bacteria did not induce a significant increase in mutation frequency to human lymphoblasts. No evidence of tumorigenicity was observed in a limited number of newborn mice after IP injection of effluent extract when compared to historical control data. Putative nonmutagenic teratogens were detected in effluent using an attachment inhibition assay. The level of these agents was reduced in effluents from continuous oil burner operation.

Introduction

More than 5% of the petroleum burned in the United States is consumed in residential oil fired furnaces (*1,2*). Current operation standards are concerned with visible soot emissions and burner efficiency rather than with the evolution of mutagenic, carcinogenic, or teratogenic substances. Effluents from these devices may therefore conform to rigorous environmental standards and yet emit unacceptable levels of toxic materials to the atmosphere. There is evidence that domestic oil burner effluents contain mutagenic and carcinogenic compounds (*3,4*). Residential oil burner emission extracts have been shown to be inactive or only weakly tumorigenic as initiators in SENCAR mouse skin painting assays when followed by continuous phorbol ester promoting treatment (*4,5*).

In the studies reported here we examined the mutagenic activity of effluent collected from a residential oil burner fitted with a flame retention head. We were interested in how operating parameters affect effluent toxicity. Because of the inherent flame instability of a commercial residential furnace, we felt that a large number of samples would be required to reach valid conclusions.

In lieu of *in vivo* testing, which was precluded by the large number and small size of the samples, a set of *in vitro* tests was used to compare samples and to monitor the purification and chemical characterization of toxic chemical constituents. The methods used were bacterial and human lymphoblast forward mutation assays and a cell attachment assay which is sensitive to nonmutagenic teratogens. In addition, a newborn mouse lung adenoma assay was used to determine if samples were tumorigenic *in vivo*.

Methods

Effluent Samples

Effluent from a residential oil burner fitted with a flame retention head and fired with No. 2 fuel oil at 0.92 gallons/min was sampled through a quartz probe and collected using a sampling train consisting of a teflon-coated glass fiber filter followed by a bed of Amberlite XAD-2 sorbent resin. A total of 62 samples were collected, extracted, and concentrated according to the methods presented in the accompanying paper (*6*). Twenty-seven samples were taken during cyclic (5 min

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on, 10 min off) operation at low sooting Bacharach Smoke No. 1; 6 during continuous operation at Bacharach Smoke No. 1; and 29 during cyclic, moderate sooting Smoke No. 5 firing. Extracts from several runs were often combined.

Bacterial Mutagenesis

Effluent extracts were tested for their ability to induce mutations in *Salmonella typhimurium* with the forward mutation assay of Skopek et al. (*7,8*). Briefly, exponentially growing bacterial cells were incubated with several test material concentrations for 2 hr at 37°C in 0.1 to 1 mL of a medium consisting of minimal E salts and 2% Brain Heart Infusion (Difco). The cells were diluted in buffered saline and plated at appropriate concentrations on nonselective plates to determine cell survival and on selective plates supplemented with 50 μg/mL 8-azaguanine (Sigma) to detect induced drug resistant mutants. Following a 45 hr, 37°C incubation, the plates were counted with an automatic colony counter and the mutation frequency calculated. To simulate *in vivo* metabolic activity, separate trials were conducted in the presence of 5% post-mitochondrial supernatant (PMS) from Aroclor 1254-induced rat liver homogenate (Litton).

Solvent and positive controls were an integral part of each experiment. Solvent control values fell in a narrow distribution about a mean characteristic of each large frozen bacterial stock. This distribution was used to define the statistical basis for significant mutagenic activity. Samples inducing a mutant frequency larger than the 99% upper confidence limit (the 99% UCL) of the solvent control distribution were judged to contain significant mutagenic activity. The sample concentration yielding a mutant frequency equal to the 99% UCL is a useful measure of the sample's mutagenic activity. This unit, the minimum detectable mutagen concentration (MDMC), is small for samples containing high mutagenic activity and is large (or infinite) for agents having little or no activity. Figure 1 shows the dose-response relationship between concentration of a cyclic Smoke No. 1 extract and induced mutant frequency. The graphical method for determining the MDMC is also shown in this figure.

Effluent samples were tested up to a final concentration of 300 μg/mL. Those samples not exhibiting detectable activity at this concentration are listed as having an MDMC of >300 μg/mL.

Human Cell Mutagenesis Assay

Mutagenic activity toward human lymphoblasts was measured according to the methods described in Furth et al. (*9*) and Crespi and Thilly (*10*). Two lines of human lymphoblasts were used, AHH-1 and TK6. AHH-1 cells contain an inducible cytochrome P-450 mixed-function oxidase system and thus are capable of activating some mutagens, such as benzo[a]pyrene, without the addition of exogenous metabolizing elements. Mutants resistant

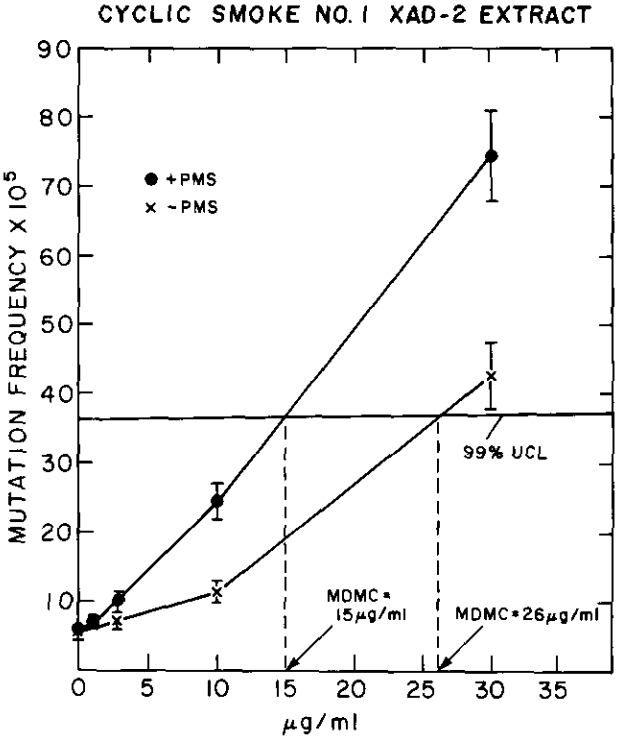

FIGURE 1. Typical dose-response curves. The test mixture consisted of several exhaust samples each recovered from XAD-2 by Soxhlet extraction with methylene chloride. The eluates were combined, dried, redissolved in DMSO, and assayed for mutagenic activity. The oil burner was operated cyclically (5 min on, 10 min off) at a Bacharach Smoke No. 1. Induced mutant frequency in the presence of 5% PMS and cofactors (●); in the absence of metabolizing elements (X). Historical 99% UCL is indicated by horizontal line at 37×10^{-5}. The MDMC are indicated.

to the purine analogue 6-thioguanine (Sigma) were scored. TK6 cells do not have endogenous cytochrome P-450, and thus mutagen activation required the addition of Aroclor 1254-induced rat liver postmitochondrial supernatant (PMS) and cofactors as in the bacterial assay. In addition, effluent extract was incubated with TK6 cells without added PMS to determine whether there was PMS-independent mutagenic activity. Mutants resistant to trifluorothymidine (Sigma) were scored in assays using TK6 cells.

To assay for mutagenic activity, 10^7 TK6 or AHH-1 lymphoblasts in a 30-mL suspension culture were incubated with effluent extract for 2 or 24 hr, respectively, and grown for five (TK6) or eight (AHH-1) generations to permit mutant expression. Appropriate dilutions in media containing selective agent were made and distributed to wells in 96-well microtiter plates. Cells were also plated at low concentration to determine plating efficiency. Following a 2-week incubation, wells with colonies were counted and the mutant fraction determined according to the methods of Furth et al. (*9*).

Putative Teratogen Assay

Potential teratogenicity was measured using the attachment inhibition assay system of Braun et al. (*11*–

13). Samples of effluent extract in DMSO were incubated with tritium-labeled murine ascites tumor cells for 2 hr at 37°C. The cells were poured into 35 mm plastic Petri dishes containing three 1.25 cm diameter polyethylene disks coated with concanavalin A. The disks had been previously derivatized in a solution of 15 μg/mL concanavalin A, 85 μg/mL bovine serum albumin, 2.5% glutaraldehyde for 18 hr and stored in an 0.1 M glycine solution. Treated cells were allowed to sediment onto the disks for 20 min. The disks were removed with fine forceps, washed in PBS, and counted in a scintillation counter. Disks coated with BSA alone were used as nonattachment controls. Extracts were considered inhibitory if they inhibited attachment by more than 50% and this inhibition was not accompanied by an increase in trypan blue permeability. Anti-attachment activity is expressed in terms of the extract concentration required to inhibit attachment by 50% (the ID_{50}). The DMSO vehicle did not inhibit attachment at the concentrations used.

Tumor Induction

A newborn mouse lung adenoma bioassay was performed according to the procedure described by Busby et al. (*14*). Newborn Swiss-Webster BLU:Ha mice (Blue Spruce Farms, Altamont, NY) were injected IP with DMSO vehicle or oil burner extract exchanged into DMSO in three doses containing 1/7, 2/7, and 4/7 of the total dose on days 1, 8, and 15, respectively. The animals were killed at 26 weeks of age, and the separated lobes of the fixed lungs were examined for visible tumors. Lung sections were taken and stained with hematoxylin-eosin for histopathologic examination. Student's *t*-test was employed in the analysis of lung tumor numbers, and the method of Peto et al. (*15*) was used to analyze lung tumor incidence.

Results

Preliminary Experiments

A single large lot of No. 2 fuel oil was used in all the experiments described here. Neither the fuel nor the fractions eluted from alumina were mutagenic to *S. typhimurium*. Some material toxic to bacteria was eluted from alumina with chloroform. Details of the alumina column fractionation procedure are found in an accompanying paper (*6*).

In our procedure, effluent collected on glass fiber filter and XAD-2 resin was extracted successively with methylene chloride and methanol. The organic extracts were usually taken to dryness under a nitrogen stream and dissolved in DMSO for bioassay; see (*6*). It was possible that volatile compounds lost on drying might be mutagenic and hence lead to an underestimate of actual effluent activity. To examine this possibility several replicate methylene chloride extract samples were mixed with an appropriate volume of dimethyl sulfoxide (DMSO), the methylene chloride removed under a stream of nitrogen, and the residual DMSO solution assayed for mutagenic activity. By exchanging the sample into DMSO it was thought that a larger fraction of volatile constituents would be retained. The results (Table 1) show no statistically significant difference between the specific activity of the exchanged and the fully dried samples. Thus, it appeared that exchange into DMSO had no advantage over the complete drying procedure. All further samples were therefore concentrated by drying under a stream of nitrogen.

Bacterial Mutagens in Effluent Extracts

Table 2 lists the specific bacterial mutagenic activity detected in residential oil burner extracts. Specific mutagenic activity was calculated as the extract concentration required to yield a mutant frequency equal to the historical 99% upper confidence limit for solvent controls, the minimum detectable mutagenic concentration, or MDMC. For comparison, the specific activity of a representative group of complex mixtures and pure compounds is listed in Table 3.

Specific bacterial mutagenic activity detected in the absence of PMS was least for continuous Smoke No. 1 (low sooting) extracts and somewhat higher in extracts from cyclic Smoke No. 1 and No. 5 (moderate sooting) operation. This is reflected in the relatively high MDMC of continuous Smoke No. 1 extracts (> 300 μg/mL, -PMS) and the somewhat lower MDMC under cyclic Smoke No. 1 and No. 5 operation (45 μg/mL and 197 μg/mL, respectively). The PMS-dependent specific activity was similar under all firing conditions.

Material collected on filters and XAD-2 resin was first extracted with methylene chloride and then with methanol. With the exception of a single Smoke No. 5 run, no significant mutagenic activity was detected in the methanol extracts.

Table 1. Minimum detectable mutagenic concentration of dried and DMSO-exchanged oil burner effluent extracts.

	MDMC, μg/mL	
Sample number	+PMS	−PMS
Dried samples[a]		
1	12	18
2	14	25
3	13	37
Average (SD)	13 (1)	27 (10)
Exchanged samples[b]		
1	11	15
2	14	23
3	11	24
Average (SD)	12 (2)	21 (5)
Average of all samples	13 (1)	23 (8)

Abbreviations: MDMC: minimum detectable mutagenic concentration; +PMS: assayed in the presence of 5% Aroclor-induced rat liver postmitochondrial supernatant; −PMS: no postmitochondrial supernatant added; SD: standard deviation.

[a]Dried: methylene chloride extracts were taken to dryness under nitrogen and dissolved in DMSO.

[b]Exchanged: an appropriate volume of DMSO was added to methylene chloride extract and the methylene chloride blown off with nitrogen.

Table 2. Specific bacterial mutagenic activity of residential oil burner exhaust extracts.

	Minimum detectable mutagenic concentratrion, μg/mL[a]											
	− PMS ($MeCl_2$)			+ PMS ($MeCl_2$)			− PMS (MeOH)			+ PMS (MeOH)		
Run	Filter	XAD-2	Total[b]	Filter	XAD-2	Total[b]	Filter	XAD-2	Total[b]	Filter	XAD-2	Total[b]
Continuous Smoke 1 (6 runs)												
Average[c,d]		> 300[e]			160			> 300			> 300	
Range		104–> 300			44–> 300			> 300			> 300	
Cyclic Smoke 1 (27 runs)												
Average[c]	58	47	45	220	43	39	120	> 300	> 300	> 300	> 300	> 300
Range	5–> 300	12–> 300	12–> 300	17–146	13–> 300	14–> 300	31–> 300	53–> 300	65–> 300	> 300	173–> 300	187–> 300
Cyclic Smoke 5 (26 runs)												
Average[c]	67	212	197	199	120	152	> 300	> 300	> 300	> 300	> 300	> 300
Range	16–> 300	32–> 300	31–> 300	37–> 300	54–> 300	53–> 300	> 300	287–> 300	> 300	> 300	> 300	> 300
Ambient air	> 300	> 300	> 300	> 300								

[a]Concentration of extract required to yield an induced mutant fraction equal to the historical 99% upper confidence limit of solvent controls.

[b]Calculated according to the formula: $S = (W_f + W_x)/W_f/F + W_x/X)$; where S = summed specific mutagenic activity; F = MDMC of material extracted from particulates retained on glass fiber filter; x = MDMC of material extracted from XAD-2 resin; W_f = weight of material extracted from particulates retained on glass fiber filter, and W_x = weight of material extracted from XAD-2 resin.

[c]Average calculated according to the formulae: $F_a = n/\sum_{i=1}^{n}\frac{1}{F_i}$; $X_a = n/\sum_{i=1}^{n}\frac{1}{X_i}$; $S_a = n/\sum_{i=1}^{n}\frac{1}{S_i}$. $\sum$ represents summation from $i = 1$ to $i = n$. F_a, X_a, S_a = calculated average MDMC of filter, XAD-2, and calculated sum, respectively. F_i X_i, S_i = MDMC of filter, XAD-2, and calculated summed extracts, respectively, from run i. n = number of runs averaged. For the purposes of average calculations, values listed as > 300 were arbitrarily set at 1000 μg/mL.

[d]No material was extracted from the filter.

[e]> 300 indicates that no significant mutagenic activity was detected at 300 μg/mL.

Table 3. Specific bacterial mutagenic activity of representative mixtures and compounds.

	Minimum detectable mutagenic concentration, μg/mL		
Mixture/compound	Test cells[a]	− PMS	+ PMS
St. Louis urban dust extract (*21*)	TM677 (bacteria)	13 μg/mL	> 100 μg/mL[b]
	TK6 (human cells)	> 100	75
Diesel soot (dilution tunnel) extract (*22,23*)	TM677	5	75
	TK6	> 200	55
Diesel soot (hot tube) extract (*22,23*)	TM677	3	13
Washington urban dust extract	TM677	> 300	115
Benzo[a]pyrene	TM677	> 20	0.6
Benzo[a]pyrene, *trans* 7,8-dihydrodiol, 9,10-epoxy (anti)	TM677	0.06	Not tested
Cyclopenteno[c,d]pyrene	TM677	> 10	1
1,8-Dinitropyrene (Sanders, Tencharoen, and Thilly, in preparation)	TM677	0.00017	> 0.15
	TK6	0.05	Not tested
Fluoranthene	TM677	> 100	0.52

[a]TM677: tested in *Salmonella typhimurium* forward mutation assay. TK6: tested in human lymphoblastoid cell forward mutation assay.

[b]The symbol (>) indicates that no significant mutagenic activity was detected at the concentration shown. This was the highest concentration tested.

Ambient air from the room containing the oil burner was passed through the sampling train for 16 hr at a sampling rate of 8 standard cubic feet/min. Three hundred twenty milligrams methylene chloride-extractable material was collected. No significant bacterial mutagenic activity was detected in this material (Table 2).

Perhaps the most striking feature of the data presented in Table 2 is the wide range of specific activity among the extracts from cyclic operations. In nearly all cases there was an order of magnitude difference between the highest and lowest MDMC measurements. It is unlikely that this variation is totally due to variability in the bacterial measurements since replicate measurements rarely differ by more than 50% (see Table 1).

From the perspective of potential public health impact, the total amount of mutagens emitted per gram of fuel burned is of greater interest than the specific mutagenic activity. One may calculate the emitted mutagenic activity per unit weight of fuel by dividing the average MDMC in Table 2 by the calculated weight of extractable material emitted per unit weight of fuel burned. Methods for calculating total emission yield are given in the accompanying paper (*6*). The resulting parameter has derived units of grams fuel fired per milliliter test solution inducing a mutant fraction equal to the 99% UCL. This quantity represents the fuel consumption required to emit a quantity of mutagenic activity sufficient to produce a statistically significant response. The results of this computation are presented in Table 4. As far less extractable material was produced during continuous Smoke No. 1 operation, the total yield of mutagenic material was considerably less than that during cyclic operations. There was no difference in the mutagen yield between the cyclic Smoke No. 1 and cyclic Smoke No. 5 operations.

Fractionation

As a first step in identifying the principal bacterial mutagens emitted from the residential oil burner, large

Table 4. Total emitted bacterial mutagenic activity of exhaust emissions from cyclic and continuous oil burner operation.

	Total mutagenic activity in g fuel fired/mL (MDMC)[a]											
	Methylene chloride extract						Methanol extract					
	− PMS			+ PMS			− PMS			+ PMS		
Run	Filter	XAD-2	Total[b]	Filter	XAD-2	Total[b]	Filter	XAD-2	Total[b]	Filter	XAD-2	Total[b]
Continuous Smoke 1												
Average		> 14[b]			7.5			> 13			> 13	
Range					4–> 14							
Cyclic Smoke 1												
Average	20	1.6	1.3	72	1.9	1.4	15	> 10	> 8	> 27	> 10	> 8
Range	8–> 320	0.4–> 9	0.7–> 9	21–> 320	0.4–> 9	0.4–> 9	2–> 27				5–> 10	
Cyclic Smoke 5												
Average	11	3.3	2.6	24	2.6	2.7	> 35	17	> 17	> 35	3.7	> 17
Range	3.5–43	1.2–> 6	1.3–> 5	8.0–> 51	1.2–> 6	1.9–> 5		3.9–> 34			0.6–> 34	

[a]Total mutagen yields were calculated for individual runs as described in the text. Then average values were calculated according to the formulae of Table 2.

[b]The symbol (>) indicates that no significant mutagenic activity was detected at the concentration shown. This was the highest concentration tested.

samples of methylene chloride extractables were fractionated on alumina columns. The details of this procedure are described in an accompanying paper (*6*). Neither the original No. 2 fuel oil nor alumina fractions of the fuel were mutagenic.

The results of several fractionations are presented in Table 5. Mass recovery was greater than 70%. Mutagenic activity in the cyclic Smoke No. 1 samples eluted predominantly in the benzene fraction (> 86%), while activity in cyclic Smoke No. 5 samples was found primarily in the chloroform fraction (82% of recovered activity). All the activity detected in the presence of PMS was recovered, although there was only about a 20% recovery of PMS-independent mutagenic activity.

Subfractionation

A 750-μg aliquot of the mutagenic alumina fraction from cyclic Smoke No. 1 operation (fraction 2, Table 5) was applied to a cyanopropyl HPLC column and eluted with a programmed sequence of 98%:2% hexene:methylene for 10 min at 2 mL/min followed by 100% methylene chloride for 10 min. Details of this subfractionation procedure are described in an accompanying paper (*6*). Roughly 95% of the applied extract weight was eluted in the first subfraction (X2-A) and was composed of aromatic hydrocarbons. The second subfraction (X2-B) was no more than 5% of the total mass injected and contained a variety of nitro-aromatic compounds (*6*).

The two subfractions were dried under nitrogen, dissolved in 0.2 mL DMSO, and tested for bacterial mutagenic activity. The results of these tests are shown in Table 6. While less than 55% of the applied PMS-dependent mutagenic activity was recovered, all of this activity was confined to fraction X2-B, the subfraction known to contain nitro-aromatic compounds.

Human Cell Mutagen Assay

The preceding results demonstrated the presence of bacterial mutagenic activity in residential oil burner effluent extracts and, for effluent from cyclic Smoke No. 1 operation, indicated an association of mutagenic activity with a subfraction containing nitro-aromatic compounds. It was of interest to determine whether this material was mutagenic in human cell systems. Bacterial and human cells responses can differ by orders of magnitude for certain mutagens. For example, 1,8 dinitropyrene is mutagenic to *S. typhimurium* at 0.1 ng/mL (1 hr dosage) but became significantly mutagenic to TK6, human lymphoblasts, only at doses of more than 50 ng/mL (24 hr dosage) (Sanders, Tencharoen, and Thilly, in preparation).

A cyclic Smoke No. 1 XAD-2 extract found to be a relatively strong bacterial mutagen (MDMC of 25 μg/mL without PMS and 13 μg/mL with PMS) was tested in two different human lymphoblast mutagenesis assays. The results, shown in Figures 2–4, show that this extract did *not* increase the mutant frequency in either system at concentrations up to 100 μg/mL.

Potentially Teratogenic Activity

Selected effluent samples were tested for their ability to inhibit tumor cell attachment to concanavalin A coated surfaces. A large class of nonmutagenic teratogens inhibits attachment at concentrations related to those causing congenital defects *in vivo* (*12*). In addition, tumor promoters inhibit attachment (*16*). Thus, the attachment inhibition assay tests for biologically significant activity that is not detected in standard genotoxicity systems.

The results of these tests are summarized in Table 7. In the first section of Table 7, the specific activity is expressed in terms of the extract concentration required to reduce attachment by 50% (the ID_{50}). As more active extracts inhibit attachment at low concentrations, low ID_{50} values indicate active material while high ID_{50} values indicate relatively inactive material. The general trend exhibited in the data of Table 7 corresponds to the trend of bacterial mutagenic activity in

Table 5. Bacterial mutagenic activity yield from alumina column fractions of domestic oil burner effluent extract.[a]

	Applied[c]	Fraction number[b] 1	2	3	4	Calculated sum[d]
Cyclic Smoke No. 1, 1st fractionation						
− PMS						
MDMC	23	> 300[e]	30	35	> 300	174
Potency	10.9	(< 0.13)	0.55	0.85	(< 0.02)	> 1.55
% Applied potency[f]		(< 1.2)	5.1	7.8	(< 0.2)	> 13
+ PMS						
MDMC	13	238	1	13	124	12
Potency	19.2	0.53	16.6	2.3	0.14	19.6
% Applied potency		2.8	86	12	1	102
Weight (mg)	250	125	17	30	18	190
% of Applied	100	50	7	12	7	76
Cyclic Smoke No. 1, repeat fractionation						
− PMS						
MDMC	23	> 300	30	28	138	123
Potency	10.9	(< 0.11)	0.67	1.2	0.13	> 2.1
% Applied potency		(< 1)	6.2	11	1.2	> 18
+ PMS						
MDMC	13	> 300	1	16	> 300	11
Potency	19.2	(< 0.11)	20	2.1	0.02	> 22
% Applied potency		(< 1)	104	11	0	> 115
Weights (mg)	250	109	20	34	18	171
% of Applied	100	44	8	14	7	72
Cyclic Smoke No. 5						
− PMS						
MDMC	147	> 300	> 300	88	> 300	> 300
Potency	0.80	(< 0.2)	(< 0.04)	0.16	(< 0.01)	> 0.16
% Applied potency						20
+ PMS						
MDMC	80	> 300	20	12	> 300	> 65
Potency	1.46	(> 0.2)	0.6	1.2	(< 0.1)	> 1.8
% Applied potency			41	82		> 123
Weight (mg)	117	59	12	14	3	88
% of Applied	100	50	10	12	3	75

[a]The methylene chloride extracts from the XAD-2 resin from 25 combined cyclic Smoke No. 1 or 5 combined cyclic Smoke No. 5 runs were applied to an alumina column and eluted with solvents of increasing polarity. The eluates were dried, weighed, dissolved in DMSO and tested for mutagenic activity. Sufficient smoke No. 1 sample was available to permit duplicate fractionations.

[b]Fractions: (1) eluted with hexane; (2) eluted with benzene; (3) eluted with chloroform; (4) eluted with methanol.

[c]Applied: MDMC of material applied to column.

[d]Calculated: MDMC calculated from individual MDMC values, using the equations in the footnotes of Table 2 and weight fractions obtained by normalizing the weight of material eluted in the corresponding fraction by the total.

[e]The symbol (>) indicates that no significant mutagenic activity was detected at the concentration shown. This was the highest concentration tested.

[f]Potency: weight of material divided by MDMC. Figures in parentheses are upper potency estimates, as no mutagenic activity was observed in these samples.

these samples. Samples taken from cyclic Smoke No. 1 and cyclic Smoke No. 5 operation have similar activity. Samples taken from continuous operations have considerably less activity. In the lower portion of Table 7, the inhibitory yield per kilogram fuel fired has been calculated, taking into consideration sampling fraction and extract yield in terms of kilogram fuel fired, as described earlier (see Table 4). The general picture remains the same, with highest inhibitory yield in the extract effluents and lowest in the continuous effluents.

Tumorigenicity

A newborn mouse lung adenoma bioassay was performed on the same pooled cyclic Smoke No. 1 sample tested in the human lymphoblast assays. The results are displayed in Table 8. Because of the limited amount of material available, only 20 mice were injected. Lung tumors were not observed in 29 mice concurrently injected with DMSO vehicle, but were noted in 3 of the 20 animals treated with a total of 1.05 mg oil burner extract.

The increase in both total lung tumor incidence (15%) and in the numbers of lung tumors per mouse (0.15) in the treated animals was statistically insignificant ($p > 0.05$) in comparison with historic control data obtained over the past several years in this laboratory. However, there was an indication of a marginally significant response ($0.025 < p < 0.05$) when compared with concurrent controls. Simple probability calculations based

Table 6. Bacterial mutagenic activity in cyanopropyl HPLC subfractions.[a]

Fraction	Weight[b]	MDMC	Potency[c]	% Recovery
Material applied to column	0.740 mg	1.0 μg/mL	0.74	
Fraction X2-A	0.577	> 35[d]	> 0.02	> 3%
Fraction X2-B	0.163	0.4	0.408	55%
Combined X2-AW and X2-B	0.740 mg	2.2 μg/mL	0.34	45%

[a]The pooled XAD-2 extract from 25 cyclic Smoke No. 1 runs described in Table 5 was applied to alumina, and the fraction eluted with benzene was subfractionated on a cyanopropyl HPLC column. As described in the text, two subfractions were taken and tested for mutagenic activity with postmitochondrial supernatant.

[b]These values assume the following: all the 740 μg of X2 extract applied to the column was recovered; 78% of the applied weight, 577 μg, was eluted in fraction X2-A; and 22% of the applied weight, 163 μg, was eluted in fraction X2-B.

[c]Potency and percent recovery were calculated as described in the footnotes of Table 5.

[d]The symbol (>) indicates that no significant activity was detected at the concentration shown. This was the highest concentration tested.

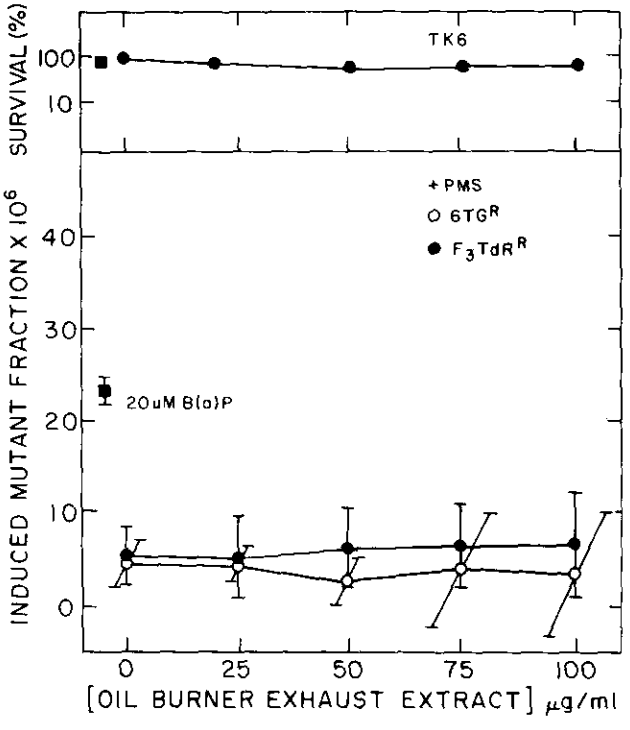

FIGURE 2. Lack of mutagenic activity directed toward TK6 human lymphoblasts by domestic oil burner effluent extract. TK6 cells were treated for 2 hr with a pooled extract Smoke No. 1 operation in the presence of 2.5% Aroclor 1254-induced PMS and cofactors (NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) as described under "Methods." Symbols: Lower panel: (○) 6-thioguanine resistant mutant yield; (●) trifluorothymidine resistant mutant yield; (■) 20 μM benzo[a]pyrene positive control (trifluorothymidine resistant mutants). 95% confidence limits based on counting statistics are shown. Upper panel: Survival following extract (●) and benzo[a]pyrene (■) treatment.

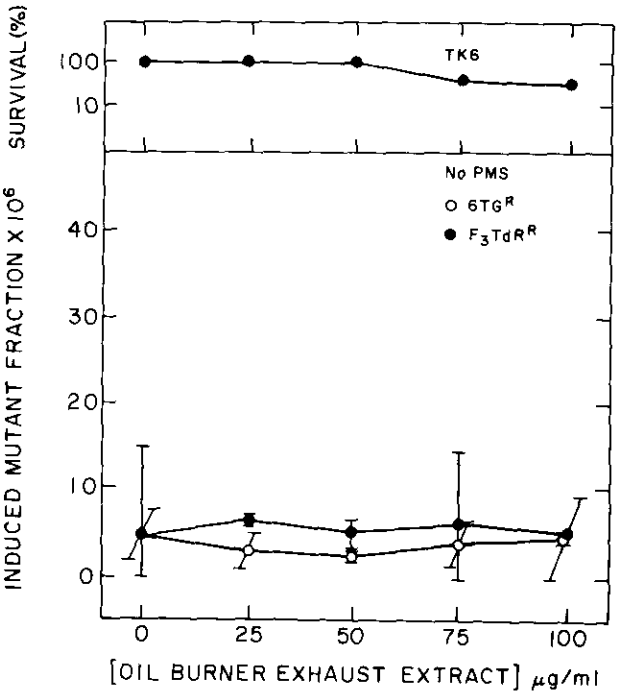

FIGURE 3. Lack of mutagenic activity directed toward TK6 human lymphoblasts by domestic oil burner effluent extract in the absence of exogenous metabolizing elements. Symbols: Lower panel: (○) 6-thioguanine resistant mutant yield; (●) trifluorothymidine resistant mutant yield; (■) 20 μM benzo[a]pyrene positive control (trifluorothymidine resistant mutants). 95% confidence limits based on counting statistics are shown. Upper panel: Survival following extract (●) and benzo[a]pyrene (■) treatment.

on a random distribution of tumors in historic control mice indicated that there was a 7% chance of the same size group of 29 untreated mice not developing any tumors. Accordingly, no basis for significance of these results can be justified.

Discussion

Bacterial Mutagenesis

Bacterial mutagens were routinely detected in the effluent of a modern residential oil burner under operating conditions similar to those of normal residential use. Despite far larger quantities of particulates emitted during cyclic operation at Smoke No. 5 compared to operation at Smoke No. 1 (*6*), the mutagenic activity emitted per gram of fuel burned during cyclic operation was comparable under high and low sooting conditions (Table 4). Indeed, for the burner used in this study, increased smoke production was not accompanied by increased emission of extractable material (*6*) and did not lead to production of extracts with higher bacterial mutagenic potency (Table 2). Thus, an important conclusion of this study is that residential oil burner soot or smoke yields cannot be used to estimate possible genotoxic emissions.

It is of interest to compare the present results with those of an earlier, exploratory study conducted by

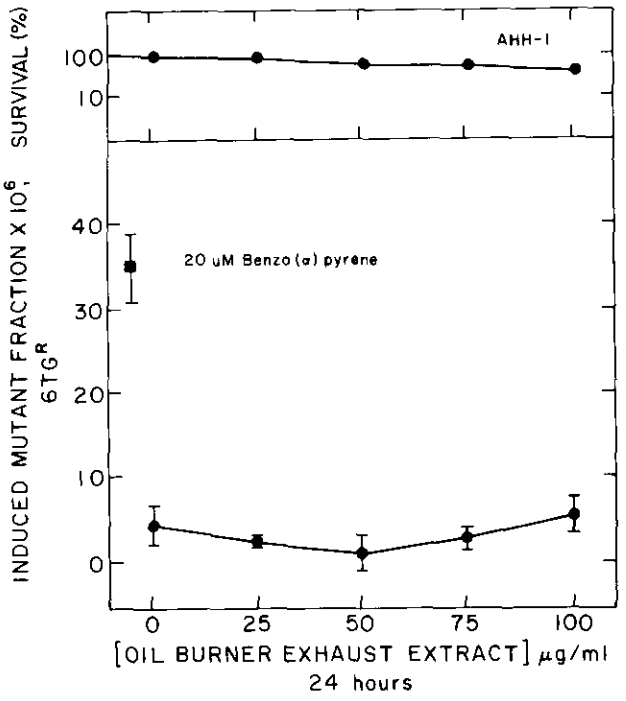

FIGURE 4. Lack of mutagenic activity directed toward AHH-1 human lymphoblasts by domestic oil burner effluent extract. AHH-1 cells were treated with the indicated concentration of oil burner effluent extract for 24 hr. The methods used are described under "Methods." 6-Thioguanine resistant mutants were scored. Mutant yield is shown in the lower panel and cell survival in the upper panel.

Table 7. Anti-attachment activity in oil burner effluents.

Runs	Methylene chloride extract		Methanol extract	
	Filter	XAD-2	Filter	XAD-2
Specific activity: ID_{50} in μg extract/mL assay mixture				
Continuous Smoke 1 average	NC	> 300 (3)[a,b]	NC	> 300 (2)
Cyclic Smoke 1 average	94 (2)	89 (7)	> 300 (4)	NT
Cyclic Smoke 5 average	NT	120 (2)	> 300 (1)	210 (1)
Calculated total activity: ID_{50} in kg fuel burned/mL assay mixture				
Continuous Smoke 1 average	NC	> 0.5 (3)	NC	> 3.0 (2)
Cyclic Smoke 1 average	3.6 (2)	0.05 (7)	> 0.4 (4)[c]	NT
Cyclic Smoke 5 average	NT	0.04 (2)	> 0.9 (1)	0.06 (1)

Abbreviations: NC = none collected; no material was extracted from filters with methylene chloride. NT = not tested.

[a]Average of (*n*) samples tested.

[b]The symbol (>) indicates that no significant inhibitory activity was detected at the concentration shown. This was the highest concentration tested.

[c]For samples with specific activities > 300 μg/mL, the calculations assumed an ID_{50} of 500 μg/mL.

Prado et al. (*17*). The results of the work reported here and those of Prado et al. are summarized in Table 9. The exploratory study differed from the current work primarily in the design of the oil fired boiler used in the Prado study and in the details of the sampling train. While the mass of extractable materials was similar in both studies (*6*), the specific and total mutagen yields differed greatly. Under comparable operation conditions the specific activity of the effluent extracts from the burner used in the Prado study were 10-fold higher than those observed here. Both studies agree that a major reduction in mutagen yield occurs when the burner was shifted from cyclic to continuous operation.

In a study designed to compare Source Assessment Sampling System (SASS) and dilution tunnel sampling, Merrill et al. (*3*) examined the mutagenic activity of oil burner effluent during cyclic Smoke No. 1 and No. 3 operation. The residential oil burner used in their study was not fitted with a flame retention head and sampling took place after the damper. TA98 *his* reversions were scored as a measure of mutagenicity. Despite these differences the current results are in broad agreement with those of Merrill et al. Comparable mutagenic activity was detected in the presence and absence of PMS under both low and moderate smoke numbers. Total mutagen yield was similar at moderate and low smoke numbers. However, we find that the specific activity at Smoke No. 5 was considerably lower than that at Smoke No. 1. Merrill et al. found little difference in the specific mutagenic activity between Smoke No. 1 and Smoke No. 3.

There was a wide variation in bacterial mutagen yield from run to run in a single burner (Tables 2 and 4) and from burner to burner (Table 9) under similar operating conditions. While a portion of this variation is due to the intrinsic variability of the sampling, extraction, and bioassay techniques, it is likely that the bulk of the variation was due to uncontrolled differences in the operating conditions of the burner.

It must be borne in mind that the mass of the effluent extract which was mutagenic was extremely small, particularly when compared to the mass of fuel consumed. For example, under cyclic conditions at Smoke No. 1, 20 g of fuel burned yielded 44 μg of extract (Tables 2 and 4). Taking the sampling fraction into consideration, only 0.0008% of the fuel burned appears as particulate and XAD-2 extract. Furthermore, from the fractionation data in Tables 5 and 6 it can be seen that mutagens constitute no more than 0.2% of the extract. Hence, the weight ratio of mutagens produced to fuel consumed under cyclic Smoke No. 1 conditions was less than 2×10^{-7}. Thus, it is unlikely that minute alterations in effluent composition having very large effects on mutagen yield would be reflected in detectable changes of gross burner parameters such as heat output or total hydrocarbon emissions.

The variation in mutagen yield from run to run and burner to burner (Tables 4 and 9) implies that slight differences in operating conditions and burner design may greatly affect toxic product emissions. This sug-

Table 8. Tumorigenicity of exhaust extract from cyclic Smoke 1 firing of residential oil burner in newborn mice.

Compound	Initial dose (total dose)	Sex	No. of mice	No. of mice with lung tumors, %	No. of lung tumors/ mouse[a]
Historical vehicle control					
DMSO	5 μL (35 μL)	M	156	15[b] (10%)	0.10 ± 0.03
		F	167	13 (8%)	0.08 ± 0.02
Total			323	28 (9%)	0.09 ± 0.02
Concurrent vehicle control					
DMSO	5 μL (35 μL)	M	15	0	0
		F	14	0	0
Total			29	0	0
Oil burner extract	150 μg/5 μL (1050 μg)	M	11	1 (9%)	0.09 ± 0.09
		F	9	2[b] (22%)	0.22 ± 0.15
Total			20	3* (15%)	0.15 ± 0.08**

[a]Mean ± SE.
[b]An adenocarcinoma was present in one animal.
*Comparison with total concurrent DMSO controls, $0.03 < p < 0.04$; not significant ($p > 0.05$) compared with total historical controls.
**Comparison with total concurrent DMSO controls, $0.025 < p < 0.05$; not significant ($p > 0.05$) compared with total historical controls.

Table 9. Comparison of oil burner effluent bacterial mutagenic activity with that measured by Prado et al. (17).

	Minimum detectable mutagenic concentration (μg/mL)[a,b]											
	This study						Prado et al. (17)					
	− PMS			+ PMS			− PMS			+ PMS		
Run	Filter	XAD	Total	Filter	XAD	Total	Filter	XAD	Total	Filter	XAD	Total
Specific activity												
Smoke 1 continuous		> 300[c]			98		9	10	3	14	33	9
Smoke 1 cyclic	13	56	84	52	55	44						
Smoke 5 continuous							13	140	71	35	> 300	> 300
Smoke 5 cyclic	37	126	106	94	123	143	3	14	4	16	> 300	83
Total activity[d]												
Smoke 1 continuous		> 14			7.5		9	9	9	1600	1450	1460
Smoke 1 cyclic	20	1.6	1.3	72	1.9	1.4						
Smoke 5 continuous							35	109	78	680	> 2000	> 1500
Smoke 5 cyclic	11	3.3	2.6	24	2.6	2.7	0.7	3	3	70	> 1300	> 700

[a]Mutagenic activity was measured in both studies under identical protocols.
[b]Average methylene chloride extract values taken from Table 2.
[c]The symbol (>) indicates that no significant mutagenic activity was detected at the concentration shown. This was the highest concentration tested.
[d]Average methylene chloride extract values taken from Table 4.

gests that a substantial reduction of mutagen emission from residential oil burners is possible through improved combustor design and control. Future studies should therefore concentrate on the sources of these variations and develop residential oil burner designs in which potentially toxic product emissions are minimized.

The current studies clearly demonstrate that cyclic operation greatly increases the emission of mutagenic species. Cyclic operation is employed to control time averaged heat output. Cycling could be reduced by increasing the length of the on and off periods and by using the minimal size burner for each installation.

Fractionation

Although the XAD-2 extracts from cyclic Smoke No. 1 and No. 5 effluents had similar specific and total bacterial mutagenic activity, their fractionation patterns on alumina differed (Table 5). Thus, it is probable the chemical nature of emitted mutagens varied with smoke number. Comparison of the elution patterns suggests that extract Smoke No. 5 mutagens requiring exogenous metabolic activation were eluted by more polar solvents than those from Smoke No. 1 operation.

Our results indicate that alumina fractionation is not a satisfactory method for the analysis of complex mixtures arising from hydrocarbon combustion. Although there was quantitative recovery of PMS-dependent mu-

tagens following alumina fractionation, 80% of the PMS-independent mutagenic activity was lost (Table 5). It is probable that many of these PMS-independent mutagens are chemically reactive without further modification and may bind irreversibly to alumina.

Recent studies in this Center indicate that fractionation on cyanopropyl columns results in reproducible and quantitative recovery of both PMS-dependent and PMS-independent mutagens from complex mixtures (*18*).

Human Lymphoblast Mutagenic Activity

An extract from cyclic Smoke No. 1 operation having moderate bacterial mutagenic activity was not mutagenic to AHH-1 cells having endogenous cytochrome P-450 enzymes or TK6 human lymphoblasts in the presence or absence of exogenous P-450 at concentrations up to 100 μg/mL (Figs. 2–4). The volumes required for human lymphoblast mutagenesis assays (> 30 mL) and the limited amount of effluent extract available prevented tests at higher concentrations. Nevertheless, it is clear that the emission extracts tested were considerably less mutagenic to human cells than to bacterial cells.

The lack of human cell mutagenicity found here is in marked contrast to activity detected by Lewtas (*4*) in mouse L5178Y lymphoma cells with extracts taken from a nonretention head residential burner operated at Smoke No. 3. As our assay systems differ, it is impossible to directly compare results. However, two possible reasons for the discrepancy between activity in the two mammalian systems can be suggested. First is the possibility that the extracts tested differed in mutagen content. Both our data (*6*) and that of Merrill et al. (*3*) show that the bulk of the material in the dichloromethane extract from residential oil burner effluent is unburned fuel. Thus, differences in the mutagen content of the original fuel may account for differences in the activity of the effluent extract. To exclude the possibility that the mutagens detected in the effluent were present in the original fuel, the fuel should be tested for activity as well as the effluent. Tests of the fuel used in this study failed to detect any mutagenic activity. Alumina fractions were also inactive. Merrill et al. do not report any tests of the fuel (*3*). Another possible origin for differences in mutagen content between the two studies is the burner itself. Retention head burners restrict the size of the flame and reduce contact with the internal surface of the fire box.

A second possible reason for the difference in mammalian cell response to mutagens in effluent extract is a basic biological difference between mouse lymphoma and human lymphoblast cells. Until comparative response studies between the two systems are made it will be impossible to exclude this possibility.

Putative Teratogens

The reproductive toxicity of environmental agents is generally neglected by workers using short-term assays. With the recent development of simple *in vitro* tests for chemical teratogenicity, the embryotoxicity of complex mixtures can be more fully evaluated. In the current work we have used one of these new assay techniques to evaluate the teratogenic risk associated with oil burner effluents. The assay technique is based on the observation that most nonmutagenic teratogens (such as thalidomide metabolites) inhibit the attachment of cells to plastic surfaces coated with plant lectins (*12*). Attachment is thought to model morphogenic cell-cell or cell-extracellular matrix interactions. Agents interfering with attachment may also interfere with these morphogenic interactions in the developing embryo and hence lead to congenital malformations. There is quantitative and qualitative correlation between inhibitory activity *in vitro* and teratogenic activity *in vivo* (*12*).

The general pattern of results in the attachment assay is quite similar to bacterial mutagenesis tests in several respects (compare Tables 2 and 6). The cyclic Smoke No. 1 and Smoke No. 5 methylene chloride extracts had similar anti-attachment activity, and this activity was considerably larger than the activity from continuous Smoke No. 1 methylene chloride extracts. Little activity was detected in the methanol extracts.

There are a wide variety of chemical teratogenesis mechanisms. Agents interfering with every normal metabolic process, damaging cellular structures, and altering osmolarity and intracellular pH have been implicated in the etiology of birth defects (*19*). Two large subclasses of teratogenic chemicals are those damaging DNA and those altering membrane function. Schreiner and Holden (*20*) have observed that most chemicals damaging DNA and therefore causing mutations are teratogenic to animals at some, generally maternally toxic, dose. Agents affecting cell membrane function are usually teratogenic at somewhat less maternally toxic doses. In the context of the present study, both mutagenic and anti-attachment activity should be considered to be indicators of potential teratogenic activity in the effluent samples.

Tumorigenicity

The bioassay data presented here, with only a limited number of mice, suggest that the single pooled oil burner extract sample tested was not tumorigenic when comparison was made with historic DMSO control data. Larger numbers of animals and/or larger doses of extract will be required to unequivocally establish whether extractables from oil burner emissions are nontumorigenic in mice under these assay conditions.

These results, though limited, appear to be in general agreement with other published data on residential oil burner emissions. Nesnow et al. (*5*), in a comparative study of emission extracts from several combustion sources (mainly diesel engines), concluded that oil burner extract was inactive in a mouse skin painting assay. However, a slight increase in tumor yield was noted at the highest dose tested (10 mg/mouse) in approximately 25% of the animals after 6 months of treat-

ment. A weak tumorigenic response (0.12 papillomas/mouse/mg extract) was reported by Lewtas (4) following treatment with emission extracts from a nonflame-retention head residential oil burner. Considerably greater response was found with extracts from diesel engine (0.24 – 0.59 papillomas/mouse/mg) and coke oven effluents (2.1 – 3.2 papillomas/mouse/mg).

Concordance

Sooting as an Indicator of Biological Activity. Effluents at high sooting conditions and low sooting conditions yielded the same total bacterial mutagen and potential teratogen activity per unit weight of fuel consumed. Smoke number or sooting is therefore not a reliable measure of potential biological potency.

Comparison of Cyclic versus Continuous Operation. In comparison to cyclic operation the effluent during continuous operation contained greatly reduced amounts of extractable organic material, bacterial mutagens, and potential teratogens when expressed on the basis of unit weight of fuel consumed.

Chemical Accounting and Biological Activity. Almost half of the methylene chloride extractable organic material in effluents during cyclic operation can be accounted as unburned fuel. Therefore, measurement of toxic activity in raw fuel must be part of any evaluation of a combustion system.

The principal kinds of chemicals producing bacterial mutations changed as a function of sooting conditions. Bacterial mutagenic activity under low sooting conditions was associated primarily with the slightly polar fractions; mutagenic activity under moderate sooting conditions was associated with more polar fractions.

To date, our studies of oil burner, small diesel engine, and other combustion systems have shown that different systems lead to the primary bacterial mutagenic activity eluting from alumina or cyanopropyl silica chromatographic columns with different eluting solvents.

In the case of diesel engines and certain laboratory flames, benzene eluted a group of potent mutagens which, depending on combustion system, included cyclopenta[c,d]pyrene, fluoranthene, perylene, and the alkyl phenanthrenes. In the case of the oil burner operating under cyclic low sooting conditions, the bacterial mutagenicity was also found with the benzene eluate but was not accountable in terms of polycyclic aromatic hydrocarbons. Instead, further fractionation showed the activity associated with a fraction containing *inter alia* alkylated nitro-aromatic compounds such as alkylnitrobiphenyls.

Finally, for the oil burner operating under cyclic but moderate sooting conditions, the bacterial mutagens were associated with the more polar chloroform eluate which contained oxygenated polycyclic aromatics among which phenalene-1-one was found to contribute appreciably to observed mutagenicity.

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