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Mutagenicity Studies of Size-fractionated Oil Fly Ash in the Ames *Salmonella typhimurium* Assay

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Chrisp et al. (1978) have reported that fly ash collected from the stack breeching of a conventional coal-fired power plant contains mutagenic material which can be extracted into horse serum or organic solvents. This result caused concern that similar material might be present in the fly ash released from oil-burning power plants. By using the five tester strains of Salmonella typhimurium, we have demonstrated the presence of mutagens on oil fly ash collected from an oil-burning power plant.

Both an azeotropic mixture of benzene/methanol (60:40 by weight) and horse serum were used to extract mutagens. The azeotropic mixture is commonly used in organic geochemical studies, such as oil shales, sediments, etc., to provide a total solvent-soluble mixture of compounds. It satisfactorily dissolves both nonpolar and polar organic compounds from oil fly ash. Horse serum was chosen as a model for lung alveolar fluid. Both the azeotropic mixture of benzene/methanol and horse serum have been successfully used to extract mutagens from coal fly ash particles (Chrisp et al. 1978; Fisher et al. 1979; Wei et al. 1982).

MATERIALS AND METHODS

Oil fly ash was collected from an oil-burning power plant with a 480 MWe steam cycle electrical generation utility unit burning 260,000 lbs (34,000 gallons) of petroleum oil per hour. During 21 days of sampling, nominal power levels were maintained near 400 MWe. The fuel burned was a residual oil blend consisting primarily of oil derived from Indonesian petroleum, a crude oil of particularly low sulfur and low ash contents. The ash content was less than 0.01%.

The fly ash sampler (Fig. 1) was designed by Dr. Andrew McFarland of the Department of Environmental Engineering of Texas A&M University. The sampler consisted of a cyclone separator designed to collect the coarse, non-respirable particles, followed by a Teflon fabric filter designed to collect fine respirable particles. The filter unit was mounted in a single "bag house" unit designed to be intermittently pulsed with high-pressure air to dislodge collected ash into a collection hopper below the unit. The system was

equipped with a flow meter, jet ejector pump, pressure gauges, and a one-inch diameter sampler inlet probe which

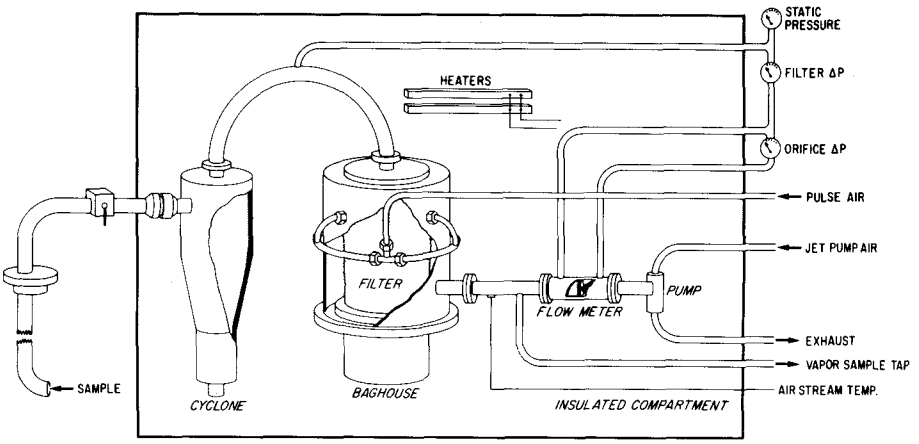

Figure 1. Fly ash sampler used for the collection of size-fractionated oil fly ash.

was inserted into the stack breeching at the power plant. The principal components of the sampler and flow system were enclosed in an insulated metal box equipped with electrically operated thermostatically controlled heaters to maintain the sampler flow gas stream above the dew point of sulfuric acid and water, i.e., approximately 140°C. The sampler flow rate was set at 0.2265 m^3/min (8 ft^3/min) and would provide a sampling linear velocity of about 457 m/min (1,500 ft/min).

The size distribution of coarse ash was determined to range from "very small" particles under 5 μm to "huge" particles up to 265 μm in diameter, with a volume median diameter (VMD) of 21.6 μm. The average density of these particles was gravimetrically determined to be 2.69 ± 0.01 g/cm^3. The fine oil ash (3.1 μm VMD) had a uniform granular appearance. The average density was determined to be 2.52 ± 0.01 g/cm^3.

An aliquot of coarse oil fly ash (306 mg) was weighed into a centrifuge tube (30 ml) fitted with a Teflon-lined screw cap. Extraction was carried out with 3 x 10 ml of azeotropic benzene/methanol mixture (60:40 by weight; Mallinckrodt, Nanograde) using an ultrasonic probe for agitation. To avoid any overheating effects, the probe was switched on for 15 seconds every minute for 10 minutes for each extraction. The samples were centrifuged at 2,000 rpm for 15 minutes, the supernatants pipetted and filtered through a pure Teflon filter (Millipore, 0.2 μm pore size, special order, type FG,

lot no. COD 291A9A). Filtraton was accomplished by forcing the solution through the filter with approximately 25 psi of nitrogen (Matheson, Zero gas) purified by passing through a filter of molecular sieve and Drierite. The 3 filtrates were combined, concentrated, and blown down to dryness with a stream of dry, purified nitrogen gas. An aliquot of 6 ml of DMSO (Schwarz/Mann, spectrophotometric grade) was added, and the samples were refrigerated until tested. A control sample was similarly prepared and processed except that no fly ash was placed in the centrifuge tube.

Because of the low concentration of fine fly ash in the effluent stream, the ash collected on the baghouse fabric filter was trapped and could not be removed by pulsing with air as planned. The fine oil ash was therefore extracted together with the Teflon filter. A suitable Teflon filter control was included for extraction procedure.

In view of the small increase in weight of the filter due to ash (2.42 g) and the large weight of the filters themselves (78.08 g and 86.56 g), the weight of fine oil fly ash could only be estimated. Approximately 1/8 of the total filters were cut and weighed. This procedure gave samples of 12% and 13% of the total ash + filter and control filter, respectively. The amount of fine oil fly ash was calculated to be about 290 mg.

The 1/8 filter pieces were carefully cut into small pieces and placed in a centrifuge bottle for extraction. The procedure used was identical to that described above except for the amount of solvent used. The first extraction was carried out using 60 ml of the solvent. However, after centrifugation only 35 ml could be moved due to the absorption of the solvent by the filter. As a consequence, for the second and third extractions, only an additional 35 ml of solvent was added each time. The pooled benzene/methanol extracts were concentrated and evaporated to dryness, prior to the addition of 6 ml DMSO and the samples were refrigerated until analysis.

A 20.15 g portion of the fabric filter impregnated with fine oil fly ash was placed in a Teflon bottle with 300 ml of dichloromethane (Mallinckrodt, Nanograde) and agitated for 2 min using an ultrasonic probe. The ash suspension was filtered through a solvent-washed Millipore Teflon filter and the filtered solvent was reused to remove more ash particles from the fabric filter. This removal procedure was repeated until as much ash was removed as possible, a total of 639 mg.

The CH_2Cl_2 solution was concentrated, transferred to serum bottle and evaporated to dryness. The fine oil fly ash (639 mg) was also transferred to the same bottle for incubation with horse serum. A procedural control using another unused Teflon filter and another aliquot of solvent was also prepared.

To prevent coagulation of serum protein due to the acidity of the fly ash, the ash samples were first neutralized with 1.56M NaOH solution before autoclaving; 1.2 ml and 0.95 ml of NaOH solutions were added to 634 mg of the cyclon coarse ash and to 639 mg of the fine filter ash, respectively. After incubation in a dark oven at 37°C for 7 days at a concentration of 30 mg ash per ml of horse serum (Microbiological Associates, Walkersfield, MD, lot no. 92743), the samples were centrifuged at 35,000 g for 30 min. The supernatants were filtered through a 0.45 μm Millipore membrane filter to remove particulate matter, and the resulting filtrates were refrigerated until tested. The filter control, the solvent control, and a horse serum control were prepared following the same procedure.

The two positive coal fly ash control samples, fraction 2 and fraction 3 samples of the previously collected coal fly ash (McFarland et al. 1977) were used as positive controls. These samples were previously reported to be mutagenic in the Ames mutagenicity test (Chrisp et al. 1978; Fisher et al. 1979). The fraction 2 ash sample was reported to have a volume median diameter of 6.3 μm and the fraction 3 ash sample, 3.2 μm. They both had geometric standard deviations of approximately 1.8.

Sterile samples of fraction 2 and fraction 3 ashes were mixed with sterile horse serum at 18 mg/ml and 40 mg/ml, respectively, and stored in a dark oven at 37°C for 7 days. The incubated samples were processed following same procedures described above.

The standard plate incorporation assay (Ames et al. 1975) was followed and triplicate plates were tested for each dose of each sample. Ash filtrates were tested at 0.1 ml per plate either with or without metabolic activation. To metabolically activate the sample, an aliquot of 0.1 ml of a 10% liver S-9 mix was added to each plate. The samples were tested with Salmonella typhimurium strains TA1535, TA1537, TA1538, TA98 and TA100.

In each experiment, microscopic examination of the background bacterial lawn was performed to check the bacterial toxicity of the compound. The number of spontaneous revertants of each tester strain fell within the range reported by de Serres & Shelby (1979). Concurrent positive controls were also included in all experiments; 2-nitrofluorene was used for strains TA 1538 and TA 98, methanesulfonate for TA 1535 and TA 100, and 9-aminoacridine for TA 1537 in the absence of S-9; 2-aminoanthracene was used for all the five strains in the presence of S-9.

The groups of three replicates of observed numbers of revertants were compared to each other and filter controls using the one-tailed Mann-Whitney rank-sum non-parametric test (Snedecor & Cochran 1967). Significant differences were assumed to exist for $p<0.1$ using this test. The student t-test was not used since the distributions of individual values had widely different observed variances and may not have been normally distributed. The

non-parametric test avoided the presumption of normality and did not involve use of the variances.

RESULTS AND DISCUSSION

The results of the mutagenicity studies with five tester strains are shown in Tables 1 and 2. The benzene/methanol extracts or the horse serum filtrates of oil fly ash did not show statistically significant levels of mutagenic activity at the 10% significance level (one-tailed) in any of the test systems except in two separate cases of a benzene/methanol extract from fine ash. One case was at the highest concentration (4.83 mg/0.1 ml DMSO/plate) with TA 100 with S-9 and the other was 0.483 mg/0.1 ml DMSO/plate with TA 1538 without S-9 (Table 1).

Although it would be more convincing to have shown a positive dose-related increase in the number of histidine-independent colonies before declaring that the sample was mutagenic, our limited supply of ash samples has prohibited us from further testing of the dose-related mutagenicity. We, therefore, had to rely on statistical analyses for the results. These results indicated that the fine fly ash might contain promutagens to cause mutation to strain TA 100 upon metabolic activation. Fine fly ash might also contain some weak and direct-acting mutagens that would cause frameshift mutation to strain TA 1538 thus, indicating the possible potential of oil fly ash to cause genetic disorders.

The benzene/methanol extract of fine fly ash at 4.83 mg per plate was toxic to tester bacteria if liver S-9 was not added to the test system. The toxicity could be attributed to the high content of vanadium and nickel in the fine ash, which has been demonstrated in our laboratory (personal communication). These metals are extracted by methanol because of its extreme polarity. When S-9 mix was incorporated, the toxicity of the benzene/methanol extract of fine fly ash was reduced, possibly due to the neutralization or binding action of liver proteins to the metals.

The results showing significant increases ($p<0.1$) in mutagenic activity suggest that there may exist some mutagenic, and possibly carcinogenic, organic compounds on fine, respirable oil fly ash. Because this fine ash also contained high levels of vanadium and nickel that are already known to be toxic to pulmonary alveolar macrophages (Waters et al. 1974 & 1975), additional studies on the health hazard, especially to the respiratory system, of the fine oil fly ash are needed.

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Table 1. Mutagenicity of Benzene/Methanol Extracts of Oil Fly Ash

Sample[b]	Number of Revertants[a]				
	TA 100	TA 1535	TA 98	TA 1538	TA 1537
	+(S-9)/-(S-9)	+(S-9)/-(S-9)	+(S-9)/-(S-9)	+(S-9)/-(S-9)	+(S-9)/-(S-9)
DMSO only					
0.1 ml	73±8/73±4	10±2/9±2	23±3/14±3	5±3/6±2	7±1/8±4
Filter Control					
0.1 ml	68±4/67±13	8±3/7±2	22±2/26±12	11±4/5±2	---
Coarse Oil Ash (per 0.1 ml)					
5.1 mg	85±11/63±2	10±4/11±1	33±3/30±3	16±3/16±4	5±1/8/4
0.51 mg	76±17/69±11	7±4/10±1	21±5/21±3	16±1/9±3	8±3/8±3
0.051 mg	60±10/65±6	9±3/11±4	28±3/24±4	10±1/11±3	4±2/6±2
Fine Oil Ash (per 0.1 ml)					
4.83 mg	*150±33/Toxic	Toxic/Toxic	45±26/Toxic	26±16/Toxic	Toxic/Toxic
0.483 mg	81±15/63±8	5±1/6±4	29±5/28±11	14±3/17*±3	8±2/7±3
0.0483 mg	71±10/65±5	9±4/12±4	18±4/18±5	13±6/11±7	6±2/8±3
Coal Ash (Cut 2)[c]					
1.8 mg	188±18/126±23	15±6/8±6	103±27/127±4	65±13/53±15	12±4/17±2

[a]Mean ± standard deviation from triplicate plates.

[b]Dose per plate.

[c]Known mutagenic coal ash used as positive control.

*Statistically significant $p<0.1$.

Table 2. Mutagenicity of Horse Serum Filtrates of Oil Fly Ash

Sample[b]	Number of Revertants[a]				
	TA 100	TA 1535	TA 98	TA 1538	TA 1537
	+(S-9)/-(S-9)	+(S-9)/-(S-9)	+(S-9)/-(S-9)	+(S-9)/-(S-9)	+(S-9)/-(S-9)
Horse Serum Control					
0.1 ml	130±18/116±12	12±4/15±2	30±2/26±4	21±6/16±4	9±4/5±3
Solvent Control					
0.1 ml	114±5/113±9	9±2/15±1	33±2/28±5	24±4/24±5	5±1/9±6
Filter Control					
0.1 ml	145±6/100±22	9±4/±13±5	31±4/28±5	26±6/27±4	4±0/10±1
Coarse Oil Ash (per 0.1 ml)					
3 mg	152±8/142±3	11±2/8±1	22±2/16±3	16±2/17±2	6±1/11±3
2 mg	117±38/119±4	13±1/7±1	22±2/13±3	16±4/15±2	3±1/6±1
1 mg	114±6/123±9	6±2/7±2	30±4/20±7	17±2/11±2	9±3/11±2
0.5 mg	122±7/109±8	7±1/8±3	27±4/22±1	14±3/16±2	8±3/16±3
0.1 mg	123±18/102±14	12±4/10±3	24±9/21±2	14±4/13±4	11±2/10±2
Fine Oil Ash (per 0.1 ml)					
3 mg	102±24/71±9	Toxic/Toxic	14±2/16±2	31±4/13±1	5±3/3±2
2 mg	91±5/92±11	Toxic/Toxic	17±4/17±2	13±1/23±2	3±3/4±4
1 mg	100±18/111±28	5±3/6±3	18±3/28±4	13±3/15±2	5±3/8±2
0.5 mg	131±11/105±39	8±2/6±3	20±3/24±3	23±3/27±1	6±2/12±3
0.1 mg	108±17/123±9	10±2/11±1	32±4/22±3	27±5/27±1	2±2/12±2
Coal Ash (Cut 3)[c]					
4 mg	261±41/214±16	9±3/12±4	148±4/154±2	204±19/276±25	17±7/23±4

[a]Mean ± standard deviation from triplicate plates.

[b]Dose per plate.

[c]Known mutagenic coal ash used as positive control.

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