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AN INTERLABORATORY COMPARISON OF MUTAGENESIS TESTING OF COAL FLY ASH DERIVED FROM DIFFERENCT COAL CONVERSION TECHNOLOGIES

by

Clarence Chrisp, Charles Hobbs,
Richard Clark and Herbert E. Kubitschek

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AN INTERLABORATORY COMPARISON OF MUTAGENESIS TESTING OF COAL FLY ASH DERIVED FROM DIFFERENT COAL CONVERSION TECHNOLOGIES*

Clarence Chrisp, Laboratory for Energy-Related Health Research, University of California, Davis, CA 95616 - (916) 752-2096

Charles Hobbs and Richard Clark, Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, P. O. Box 5890, Albuquerque, NM 87115

Herbert E. Kubitschek, Argonne National Laboratory, 9700 Cass Avenue, Argonne, IL 60439

A workshop was held (1) to discuss the progress of research on the potential health effects of effluents resulting from coal combustion. Participants were from Argonne National Laboratories (ANL), Argonne, Illinois; the Inhalation Toxicology Research Institute (ITRI), Albuquerque, New Mexico; and the Laboratory for Energy-Related Health Research (LEHR) (formerly the Radiobiology Laboratory) at the University of California, Davis, California. ANL has been studying fly ash from a bench model pressurized fluidized bed combustor (2); ITRI has been studying fly ash from an atmospheric fluidized bed combustor at Morgantown, West Virginia (3); and LEHR has been studying ash from the hopper and from the stack downstream from electrostatic precipitators of a commercial coal-fired power plant (4,5,6).

Results of mutagenicity testing of coal fly ash from these different sources were noted to be quite different. The Ames plate incorporation test, which utilizes mutants of Salmonella typhimurium (7), was used in all three laboratories. Since this test was developed primarily as a qualitative screening test for mutagenicity testing, the question arose as to whether the differences in mutagenicity results in different laboratories might be

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attributed to differences in Ames test methodology. Also, it was noted that solvents used to extract the fly ash were different among the three laboratories, raising the question of whether the methods of extraction might account for the differences in mutagenic activity.

For these reasons, representatives of these laboratories decided to formulate a protocol for exchange and Ames testing of coal fly ashes among the three laboratories. A meeting was held May 17, 1979 with representatives from ITRI and LEHR in order to formulate a protocol for Ames testing of exchanged fly ash. Although a representative from ANL could not be present, that laboratory also participated in the exchange. Dr. William Belser, Professor of Microbiology at the University of California at Riverside, was invited to attend as a consultant. He has done extensive research on the factors contributing to the variation of results in the Ames test. His advice was most helpful in planning the protocol.

I. METHODS

The experimental protocol adopted at the May 17 meeting involved the sending of two fly ashes and one solvent extract of fly ash by each laboratory to the other two participants. The goal of this study was to determine whether the reported differences in mutagenicity of fly ash between the three laboratories were due to (1) differences in Ames testing methodologies in each laboratory, (2) differences in solvent extraction methods, or (3) actual differences in the mutagenicity of fly ash derived from different coal conversion technologies.

A. Experimental Protocol

1. The Ames test methods used in each laboratory were to be described in detail by each laboratory. Descriptions were to include even the smallest

details, including how the tubes were mixed before plating, e.g., whether rolled in the hand, vortexed, etc. Each laboratory was to use their current method of testing unless an exception was stated in this protocol.

2. Only direct mutagenic activity was to be examined. The metabolic activation step was eliminated not only to reduce the workload, but also because little indirect mutagenic activity had been seen in any of the fly ash samples. One hundred microliter amounts were to be tested on each of three replicate plates. A standard amount of histidine, according to Ames (7), was to be used in the top agar.

3. Only Salmonella typhimurium strains TA 98 and TA 100 were to be used.

4. All raw data were to be reported, i.e., number of revertants/plate for each plate in the experiment. The reports were to include all controls and spontaneous revertants.

5. Each laboratory was to submit to the other two laboratories two fly ashes, as well as an extract prepared by their own methods. The amount of extract tested was limited to the amount that could be extracted from 2.0 gms of fly ash. If possible, at least one point was to be determined in the toxic region and three points in the linear portion of the dose-response curve.

6. In order to determine the influence of different solvent extraction methods, each laboratory was to submit solvent extracts of each of three concentrations of one positive fly ash prepared by their usual extraction method. These were to be calculated to represent three concentrations in the linear dose-response portion of the curve where mg of fly ash/ml of solvent is plotted against mean revertants/plate. If solvent extracts were evaporated to dryness, they were to be reconstituted in DMSO just before mailing. Two ml of

each concentration were to be submitted. The solvent and DMSO controls were to be included. This represented a total of five tubes of 2.0 ml each for testing. The laboratories were to ship the tubes one day after extractions were completed. Testing was to be done six days after shipment. The laboratories were to coordinate shipments by telephone for the convenience of the chemists and testing laboratory. Dr. Belser was to mail each laboratory enough 2-nitrofluorene from one lot to use as a positive control. It was to be diluted just before use and tested at 0.25 µg/100µl, 0.5 µgm/100µl, and 1.0 µg/100µl. These concentrations had been calculated to give a linear dose response. With this information any fly ash extracts tested could be compared to a positive control done the same day under the same conditions.

7. The density of bacteria in the original overnight culture used was to be recorded.

B. Fly Ash Extraction Methods

1. ANL

Fly ash was extracted with ~~DMSO~~ dimethyl sulfoxide / DMSO / J.T. Baker, spectrophotometric grade for 45 minutes at 37°C on a rotator (Rototorque). The ash was centrifuged down and the supernatant diluted as needed for testing.

2. ITRI

ITRI Sample: Bag filter fly ash BG(3A)-6-3-3 (130 g) was split into two equal portions and placed in one liter amber bottles. Glass distilled methylene chloride (300 ml) was added to each bottle and the samples were sonicated for 2 hours in a water cooled ultrasonic bath. The methylene chloride was filtered to remove the ash and cautiously reduced in volume (using a rotary evaporator) to about 2 ml. This was taken to dryness with a stream of purified N_2. The remaining residue was dissolved in DMSO and

serially diluted so that 0.1 ml of the extract represented 200, 400, and 800 mg of extracted fly ash, i.e., 2 ml samples of 3 different concentrations were sent to LEHR and ANL, representing 2000, 4000, and 8000 mg of extracted fly ash per ml DMSO. ~~These were labeled Dose 1, Dose 2, and Dose 3, respectively.~~ A sample of ITRI's DMSO was also sent (~~Dose 0~~), as well as a solvent blank which was prepared by evaporating 400 ml methylene chloride to dryness and resuspending any residue present in 12 ml DMSO.

LEHR Samples A and B: Three grams of both samples were added to 50 ml of methylene chloride and extracted ultrasonically as described above. The residues were resuspended in DMSO to concentrations representing 1000, 500, 250, 100, and 10 mg extracted fly ash per ml or 100, 50, 25, 10, and 1 mg extracted fly ash per plate, since 0.1 ml of extract was used in the test.

ANL Samples A and B: Three hundred mg of ~~both samples~~ were added to 10 ml methylene chloride and extracted ultrasonically as described above. The residues were resuspended in DMSO to concentrations representing 25, 10, and 5 mg/ml, or 2.5, 1.0, and 0.5 mg extracted fly ash per plate, since 0.1 ml of extract was used in the test.

ITRI Samples Exchanged: Forty-gram samples each of bag filter fly ash BG(6)-6-1-1 and BG(3A)-6-3-3 were sent to LEHR and ANL for extraction and testing.

3. LEHR

In addition to normal trace organic analytical precautions, samples and extracts were protected from light at all stages. Fly ashes from each laboratory (Table 1) were extracted as follows:

Method A. Each sample was extracted three times with an azeotropic benzene/methanol mixture (60:40; Mallinckrodt Nanograde) in a screw-capped

centrifuge tube using an ultrasonic probe (15 sec/min for 10 min). After centrifuging at 2,000 rpm for 15 minutes, the supernatants were filtered through a plug of solvent-extracted cottonwool, then combined and evaporated to dryness under a stream of dry purified nitrogen. After addition of dimethyl sulfoxide (Schwarz-Mann, Spectrophotometric grade), the solutions were sealed in glass ampuls under vacuum using liquid nitrogen.

Method B. Each sample was extracted as in Method A in a Teflon-stoppered centrifuge tube. After centrifuging at 1,800 rpm for 15 minutes, the supernatants were pooled, concentrated on a rotary evaporator, and then filtered through a plug of solvent-extracted cottonwool before evaporation to dryness.

C. Ames Test Procedures

The following are each laboratory's detailed procedures for performing the Ames test.

1. ANL

a. Media Preparation and Strain Maintenance

(1) Top agar is made in 1500 ml quantities. The agar and salt are stirred while heated until completely dissolved. The solution is dispensed in 100 ml quantities, sterilized, and stored at room temperature.

(2) Histidine-Biotin solution is made as per Ames et al. (7) and is stored at room temperature.

(3) Vogel-Bonner plates (VB) containing 25.0 ml of agar are made as per Ames et al. (7). Plates are allowed to dry at least two days at room temperature or overnight at 37°C before use. Plates are stored in plastic bags at room temperature.

(4) An isolated colony is picked from nutrient agar stock plates of strains TA 98 and TA 100 kept at 4°C and used as an inoculum for a 10 ml nutrient broth culture. New stock plates are made monthly. This culture is grown overnight at 37°C on a shaker. The next day this culture is tested with 2-nitrofluorene for strain TA 98 or with benzo(a)pyrene for strain TA 100. The remainder of the culture is stored at 4°C until the plates are read. If the strain is mutagenized by the 2-nitrofluorene, or for TA 100 by benzo(a)pyrene, the stored culture is used as an inoculum for a 300 ml nutrient broth culture. This culture is grown overnight at 37°C on the shaker and tested the next morning against 2-nitrofluorene. If the results are satisfactory, the culture is collected by centrifugation, resuspended in a glycerol (10%)-casamino acids (1.5%) storage medium (10% GCA) and kept at 4°C as the stock culture for doing Ames tests.

(5) Two to three hours before the test, the necessary amount of top agar is melted by placing bottles in boiling water. Melted agar is kept at 45°C. VB plates are labeled and prewarmed by placing them in a 37°C incubator.

(6) Sterile disposable 13 x 100 mm tubes are placed in the heating blocks of a Fisher Isotemp Dry Bath® (Model 145) at 45°C.

b. Ames Test Procedure

(1) Histidine-Biotin (10 ml) is pipetted into 100 ml of melted top agar and is dispensed in 2.0 ml aliquots into tubes in dry bath with a 10.0 ml sterile disposable serological pipette.

(2) The amount of bacterial inoculum needed to give 0.1 ml per plate is placed in tubes and kept on ice.

(3) Plating is carried out in quadruplicate. Plates are taken from the incubator, four at a time.

(4) Bacterial culture (0.1 ml amounts) is dispensed into four top agar tubes with a 1.0 ml graduated glass pipette.

(5) The actual plating takes about 80 seconds for four plates. A 100 λ Eppendorf pipette is used to dispense the sample. The top agar tube containing the cells is removed and the sample is added and vortexed (Vortex-Genie) for 2-3 seconds to mix the sample and the cells. The sample is poured onto the VB plate. The tube is tapped gently on a dry area of the plate three times to get the last drops of liquid. The plate is swirled to evenly distribute agar on its surface. The culture is checked during each test for strain markers and ampicillin resistance and is titered for cell survival.

(6) Plates are allowed to set at room temperature for at least 20 minutes to allow the agar to solidify; then they are inverted and incubated for two days at 37°C. Colonies are counted manually.

2. ITRI (7). The sources of the materials are shown in Table 1.

a. Media Preparation and Strain Maintenance

(1) Agar and NaCl for the top agar (7) are weighed out in 100 ml bottles. Water is added and the agar is sterilized on the morning of the test. The histidine biotin solution (7) is added after the top agar cools to 45°C.

(2) Glucose minimal agar plates are prepared as described by Ames et al. (7) and refrigerated. Plates are taken out of the refrigerator the night before the test.

(3) Tester strains are stored at -70°C in 1 ml aliquots (7). When a nutrient broth culture is prepared, the vials are taken out of the freezer and allowed to melt at room temperature. Approximately 20 ml of nutrient broth is inoculated with 0.1 ml cells and grown overnight in a 37°C shaking water bath.

b. Ames Assay Procedure

(1) Top agar is autoclaved and histidine biotin solution is added.

(2) Top agar is dispensed (2 ml) into sterile disposable 13 x 100 mm tubes with sterile Cornwall® pipette. Tubes are kept in a 43-45°C water bath.

(3) Tester strains are transferred to sterile vials and 0.1 ml is taken for serial dilution to determine the population (diluted 1 x 10^6 and 0.1 ml of this dilution is added to 2 ml top agar and plated on nutrient agar).

(4) Tester strain (0.1 ml), test compound (0.1 ml), and sterile saline (0.5 ml) are added to top agar in that order (triplicate plates). Top agar is mixed by semivigorous rolling of tube between hands, and the mixture is poured onto agar plates. This process requires no longer than 60 seconds per 3 plates.

(5) Sodium azide (TA 100) and 2-nitrofluorene (TA-98) are used as positive control mutagens.

(6) Plates are stored in a 37°C incubator for 60 hours.

(7) Revertant colonies are counted with an Artek® Automatic Colony Counter. Those samples out of the linear range of the counter are recounted manually.

(8) All genetic and biochemical markers for each strain are checked on the day of the test.

(9) The sources of materials used for Ames tests are shown in Table 2.

3. LEHR

a. Media Preparation and Strain Maintenance

(1) Histidine, biotin and ampicillin solutions for master plates and histidine-biotin solution for top agar are made as described by Ames et al. (7). Ampicillin solutions are made monthly.

(2) Top agar (0.6% agar, 0.5% NaCl) is made in 1 liter quantities, autoclaved, and dispensed in 75 ml aliquots in sterile 100 ml bottles.

(3) Minimal medium plates with 1.5% agar are made using Vogel-Bonner Medium E 25X stock solution and adding 40 ml of 50% sterile glucose solution per liter after autoclaving. Each poured plate contains approximately 17 ml minimal glucose agar medium. Poured plates are stacked only 5 high to avoid uneven plate surfaces. Plates are kept inverted in metal cans in a refrigerator no longer than 2 weeks.

(4) Oxoid® nutrient broth is used for overnight growth of test inoculum as suggested by Ames in supplemental literature accompanying shipment of stock cultures from his laboratory. Difco nutrient broth with 0.8% NaCl is used to make nutrient agar plates for controls and inoculum density determinations.

(5) Tester strains for routine use are kept at 4°C on minimal glucose agar plates with 0.1 ml of 0.1 M L-histidine, 0.1 ml of 0.5 mM biotin and 0.1 ml of 8 mg/ml ampicillin (for TA 98 and TA 100) spread onto the plates. Strain stocks are kept as frozen permanents at -70°C (7). Master plates are prepared in triplicate by growing overnight 5 ml of nutrient broth inoculated with a sample of the frozen permanent transferred with a sterile stick. Frozen permanents are never allowed to thaw. Master plates are

streaked from the growth inoculum and incubated 48 hours at 37°C. Overnight cultures are tested for spontaneous reversion, mutagenesis and for characteristic genetic mutations (7). Master plates of those cultures showing results consistent with previous tests are sealed with paraffin and used as strain sources for no longer than 50 days.

b. Ames Assay Procedures

(1) Sixteen to twenty hours before the test, a single colony from the masterplate is inoculated into 5.0 ml of nutrient broth in a sterile 18 mm x 150 mm plastic capped tube. The tube is incubated at 37°C in a shaking water bath at a shaker speed sufficient to produce greater than 1 x 10^9 cells/ml in 16 hr. Enough tubes are inoculated to give the proper amount of bacterial suspension when pooled.

(2) On the morning of the test the overnight cultures are removed from the water bath and stored on ice in a covered ice bucket. The cultures are pooled and O.D. 450 is determined with appropriate dilutions using a Gilford® 2450 Spectrophotometer. Cells/ml of the pooled inoculum is determined using an O.D. 450 versus cells/ml standard curve. The pooled inoculum is then diluted with nutrient broth to give ~1x10^9 cells/ml and kept on ice until used.

(3) Top agar is melted in a microwave oven, then kept at 48° in a water bath. Histidine-biotin solution is added with a 20 ml plastic disposable syringe.

(4) Two ml of top agar with histidine and biotin is added with a plastic disposable 20 ml syringe to sterile 13 x 100 mm plastic capped tubes kept in a 48°C waterbath.

(5) Plates containing approximately 17 ml of minimal medium are removed from refrigerator and left at room temperature to warm up. If the room is cold, the plates are put in an incubator for 15-30 min.

(6) Serial ten-fold dilutions of the inoculum to 1 x 10^{-7} are made (~2 ml of each dilution). These are then used to determine cells/ml, either by plating 0.1 ml of 1 x 10^{-5} to 1 x 10^{-7} dilutions onto nutrient broth plates, or by plating 0.1 ml of the same dilutions with top agar. Predetermined correction factors are used to correct for the type of pipet tip used, e.g., glass or plastic.

(7) Tubes containing top agar are transferred to a Sybron© Thermolyne Dri-Bath kept at 47°-48° in a Labgard© Laminar Flow Hood.

(8) Test samples are thawed and diluted, if necessary. The test samples and bacteria are dispensed and plates are poured in a laminar flow hood. Samples are dispensed in 100 µl amounts with an Oxford© adjustable 50-200 µl micropipet using sterile, individually wrapped, polypropylene tips. Bacteria are dispensed in 3-drop aliquots (~0.1 ml) with sterile 9" Pasteur pipet. The order of addition is sample, then cells. Tubes are vortexed for 4 seconds on a Van Waters and Rogers© Vari-Whirl vortexer at a "5" setting. After the contents of the tube are poured onto the center of the plate, the tube is shaken over the plate to remove any sample left in the tube. Plates are then tilted to distribute the top agar. Total time from addition of cells to the completion of pouring is approximately 25 seconds per plate.

(9) Plates containing only bacteria are prepared to test the spontaneous and mutagen induced reversion properties of the tester strains. Plates with the mutagens 2 nitrofluorene (TA 98) and methylmethane sulfonate (TA 100) are added to paper discs placed in the center of poured and cooled

plates to confirm the response of the strains to known mutagens. Plates containing bacteria and various doses (0-15 µg/plate) of the 2-nitrofluorene incorporated into the top agar are also prepared to determine mutagenic activity of a known mutagen for both TA 98 and TA 100.

(10) Plates are kept at room temperature for at least 20 minutes to set the top agar. Plates are then inverted and placed in a Forma Scientific ®Model 3314 incubator in equal stacks evenly spaced to ensure maximum uniform air flow. Plates are incubated 63-65 hrs at 37° and counted on a New Brunswick © Model CIII Biotran II colony counter which is regularly calibrated by comparing machine and hand counts over the range of colonies per plate. If very small colonies are present, it is often necessary to do hand counts because machine counts are not accurate.

(11) The lawns of plates with a representative range of colonies and with a range of the dose levels tested are examined under a dissection microscope to check for proper lawn appearance (small, barely visible, crowded colonies in healthy lawn) and for toxicity effects (widely spaced large colonies).

(12) A minimum of 100 colonies from plates representing several concentrations of each test sample are selected with a flamed loop and transferred to minimal medium plates with 0.5 mM biotin to form a grid for replicate plate testing. Colonies are replica plated onto minimal-biotin plates and nutrient agar plates 2-3 times to test for false revertants, which may appear as a result of toxicity.

(13) All bacterial strains are tested at the end of the test for strain markers by streaking on appropriate plates and checking growth.

(14) The source of materials for Ames testing is shown in Table 3.

II. RESULTS

The range in numbers of bacterial cells plated in each laboratory is shown in Table 4. The spontaneous reversion rates (number of His^+ revertants per plate) are shown in Table 5. Reversion rates on solvent control plates were similar to spontaneous reversion rates. Data from three replicate plates were used to summarize results as stated in the experimental protocol. In some cases data from four replicate plates were submitted, and the counts/plate for one plate were chosen at random and eliminated from the analysis.

The specific mutagenic activity (His^+ revertants per mg of fly ash extracted) for each fly ash extract was determined by fitting the number of revertants by linear regression, i.e. $y = ax + b$ where the slope, a, is in terms of revertants per mg of fly ash extracted. The correlation coefficient, r, was also calculated. Tables 6, 7, and 8 each illustrate Ames test results of one solvent extract in all three laboratories using one bacterial strain, Salmonella typhimurium TA 98.

The specific activity of an ANL fly ash extracted with DMSO and tested with Salmonella typhimurium strain TA 98 was about threefold greater as determined at ANL than at ITRI or LEHR (Table 6). Also, the specific activity of 2-nitrofluorene, contributed by Dr. Belser to all three laboratories, was approximately fivefold greater at ANL than at the other two laboratories. It is clear that the mutagenicity of both the fly ash extract and 2-nitrofluorene was significantly greater at ANL. The degree of difference was rather unexpected, and the reasons are not obvious. Differences between laboratories in either Ames test methodology or specific substrain sensitivities could account for the variations in test results. The ratios of the extract slope to the

2-nitrofluorene slopes were compared for each laboratory in order to see if results would be more similar between laboratories when normalized to results with the positive control mutagen. The interlaboratory differences in this ratio were no greater than threefold.

The specific activity of the ITRI fly ash extracted with methylene chloride was quite low in all three laboratories (Table 7). Although ANL specific activity was slightly higher, no significant differences were apparent between laboratories. The difference in slope ratios is probably meaningless when the mutagenic activity is this low.

The specific activity of a LEHR fly ash extracted with benzene/methanol was also greater at ANL than at the other two laboratories (Table 8). Again, the maximum difference was no greater than three- to fourfold, and the difference in ratios of extract slope to the 2-nitrofluorene slope was no greater than threefold. When the Ames test data are examined, it should be realized that Ames test mutagenicity data for the same complex mixture within all three laboratories can vary as much as twofold between test days. Therefore, this degree of variation could be expected between laboratories.

During the second part of the experiment, two fly ashes from each laboratory were exchanged and extracted using a different extraction method in each laboratory, and each was tested with both TA 98 and TA 100. The results are shown in Tables 9-15.

Both fly ashes from the bench model fluidized bed combustor submitted by ANL were quite mutagenic for TA 98 in all three laboratories, regardless of the extraction method (Table 9). ANL-A fly ash was the same one submitted by ANL as part of the extract exchange discussed previously (Table 6). The DMSO specific activity was identical to that previously determined by ANL. Again,

the specific activity was higher at ANL than at ITRI or LEHR. Because of the dissimilarities between Ames test results when common extracts or control mutagens were tested, the determination of effect of different extraction methods on mutagenicity testing was somewhat confounded. However, because of the much lower specific activity of the benzene/methanol extract of ANL-A as determined at LEHR compared to that seen by LEHR with a DMSO extract of the same fly ash (Table 6), we suspect that benzene/methanol may be less efficient than DMSO for extraction of mutagens from this particular complex mixture. In contrast, the pattern of specific activities of ANL-B extracts was quite different. We can only assume that DMSO was a very inefficient solvent for this particular fly ash, or else the ANL Ames test was much less sensitive for this particular complex mixture. On the other hand, the specific mutagenic activities of both ANL-A and ANL-B fly ash extracts were quite comparable in all three laboratories when tested with strain TA 100 (Table 10). It should be noted that strain TA 100 is generally regarded as being less sensitive than strain TA 98 for the determination of mutagenic activity due to frameshift mutagens.

A very low amount of specific activity for strain TA 98 was found in ITRI-A fly ash by all three laboratories (Tables 11 and 15). ITRI-B fly ash extracts were positive in all three laboratories; however, the results suggest that benzene/methanol may be a superior solvent for this particular fly ash. A methylene chloride extract of this same fly ash was submitted during the extract exchange which was previously discussed (Table 7). ITRI-A extracts were slightly positive for TA 100 at ANL and ITRI, but not at LEHR (Tables 12 and 16).

LEHR-A fly ash proved to be quite mutagenic for TA 98 in all three laboratories (Table 13 and 15). However, results suggest that methylene chloride may be an inferior solvent for extraction of mutagens from this particular fly ash. This impression was further confirmed by the results with TA 100 (Tables 14 and 16), which show a rather low specific activity for the methylene chloride extract when compared to the DMSO or benzene/methanol extracts. LEHR-B fly ash proved to be only marginally positive with strain TA 98 at LEHR and negative at ANL and ITRI (Table 13 and 14). It should be noted that a much larger amount of fly ash was extracted at LEHR than at the other two laboratories. LEHR-B was not mutagenic for strain TA 100 in any of the three laboratories (Tables 14 and 16).

III. SUMMARY AND CONCLUSIONS

This experiment showed that mutagenicity of fly ash derived from different coal conversion technologies as determined by the Ames plate incorporation test was similar in all three laboratories. The differences in mutagenic activity of each fly ash between laboratories with different solvent extraction methods were no greater than one order of magnitude. In most cases the differences between laboratories were much less.

In addition, there were much smaller, but still significant differences in mutagenic activity between laboratories when the same solvent extract of a particular fly ash was tested in each laboratory. There were also significant differences in mutagenicity of the positive control mutagen (maximum of fivefold) between laboratories. Because of this difference in Ames test sensitivity between laboratories, the influence of the solvent extraction methods on differences in mutagenicity was not clear. However, the data suggested that either there were significant differences in the degree of sensitivity of Ames tests for different complex mixtures within each laboratory, or else there were differences in mutagen extraction efficiency between different solvent extraction methods. Both Ames test sensitivity and solvent extraction may be important. Further work would be necessary to separate the contribution of these two factors.

An important aspect of further work would be to separate the contribution of the innate sensitivity of substrains of Ames tester strains in each laboratory from the possible effects of differences in Ames testing methodology. This could be done by testing the same extracts of fly ash and positive control mutagens with substrains of tester strains exchanged between laboratories. This work also implies that caution should be exercised in assuming that the same solvent would have the same efficiency for extraction of mutagens from different fly ashes even within the same laboratory.

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Table 1. Methods for Fly Ash Solvent Extraction at LEHR

Fly Ash Source		Weight (gm)	Method	Extraction Volume		DMSO Volume (ml)	Final Concentration (gm/ml)
ANL	A	0.53	A	3 x 10 ml		0.53	1.0
	B	0.52	A	3 x 10 ml		0.52	1.0
ITRI	A	30.43	B	3 x 125 ml		1.5	20.3
	B	30.31	B	3 x 125 ml		1.5	20.2
LEHR	A	30.04	B	3 x 125 ml		1.5	20.0
	B1	4.00	A	3 x 20 ml	a	4.0	2.0
	B2	4.01	A	3 x 20 ml			
Procedural Control	1	-	A	3 x 20 ml	a	6.0	-
	2	-	A	3 x 20 ml			
DMSO Control		-	-	--		6.0	-

[a]Supernatants combined

Table 2. Source of Materials Used for Ames Test at ANL

Bacto Nutrient Broth	Difco
Bacto Agar	Difco
Bacto Nutrient Agar	Difco
Glucose	American Drug and Chemical Company
$NaNH_4HPO_4 \cdot {}_4H_2O$	Fisher Scientific
Citric Acid $\cdot H_2O$	Fisher Scientific
$MgSO_4 \cdot 7H_2O$	Mallinckrodt
KCl	Allied Chemical
Na_2HPO_4	Mallinckrodt
$NaH_2PO_4 \cdot H_2O$	J. T. Baker
NaCl	Fisher Scientific
K_2HPO_4	Fisher Scientific
NADP	Sigma
Histidine	Sigma
Biotin	Sigma
Ampicillin	Sigma
Glucose-6-Phosphate	Sigma
2-Nitrofluorene	Aldrich
Aroclor 1254	Litton Bionetics

Table 2_A Source of Materials Used for Ames Test at ITRI

Bacto Nutrient Broth	Difco
Agar (50:50 mixture)	Difco:Sigma
Glucose	Mallinckrodt
$NaNH_4HPO_4 \cdot 4H_2O$	Mallinckrodt
Citric Acid $\cdot H_2O$	Mallinckrodt
$MgSO_4 \cdot 7H_2O$	Mallinckrodt
$MgCl_2$	Mallinckrodt
KCl	Mallinckrodt
$Na_2HPO_4 \cdot 7H_2O$	Mallinckrodt
$NaH_2PO_4 \cdot H_2O$	Mallinckrodt
NaCL	Mallinckrodt
K_2HPO_4	Sigma
NADP	Sigma
Histidine $\cdot$ HCl	Sigma
Biotin	Sigma
Sodium azide	Sigma
Glucose-6-phosphate	Sigma
2-nitrofluorene	Aldrich
Aroclor 1254	Analabs, Inc.
Distilled water	

Table 3. Source of Materials Used for Ames Test at LEHR

Material	Source
Citric Acid (anhydrous)	Sigma
$K_2HPO_4 \cdot 3H_2O$	Sigma
$MgSO_4$ (anhydrous)	Sigma
$NaNH_4HPO_4 \cdot 4H_2O$	MCB
Biotin	Calbiochem
Histidine	Calbiochem
Ampicillin	Bristol
Ampicillin discs 10 µg/disc	Difco
Agar	Difco
NaCl	Allied Chemical
50% Glucose (sterile)	Abbott
Nutrient broth for plates	Difco
Nutrient broth for inoculum growth	Oxoid
Methyl methene sulfonate (MMS)	Aldrich
2 nitrofluorene (2NF)	Aldrich

Table 4. Range in Numbers of Bacterial Cells Plated in Each Laboratory

Laboratory	Strain	
	TA 98	TA 100
ANL	$3.5 - 8.1 \times 10^8$[a]	1.2×10^9
ITRI	$1.2 - 2.0 \times 10^9$	$1.0 - 1.3 \times 10^9$
LEHR	$9.0 \times 10^8 - 2.1 \times 10^9$	$6.5 \times 10^8 - 1.9 \times 10^9$

[a]Bacterial cells per milliliter

Table 5. Average Spontaneous Reversion Values for *Salmonella Typhimurium* Strains TA 98 and TA 100

Laboratory	TA 98	TA 100
ANL	38 ± 11[a]	179 ± 23
ITRI	32 ± 8	144 ± 32
LEHR	25 ± 7	107 ± 16

[a]His^{+} revertants/plate ± one standard deviation

Table 6. Extract Exchange: ANL Fly Ash Extracted with DMSO[a] and Tested with TA 98

Lab Testing	Slope of Extract (Rev/mg)	r[b] of Extract	Slope of 2-NF (Rev/µg)	r of 2-NF[c]	Extract Slope/ 2-NF Slope
ANL	419	0.99	1117	0.98	0.38×10^{-3}
ITRI	121	0.99	182	0.99	0.67×10^{-3}
LEHR	184	0.99	195	0.99	0.95×10^{-3}

[a]Dimethyl sulfoxide

[b]Correlation coefficient

[c]2-Nitrofluorene

Table 7. Extract Exchange: ITRI Fly Ash Extracted with $MeCl_2$[a] and Tested with TA 98

Lab Testing	Slope of Extract (Rev/mg)	r[b] of Extract	Slope of 2-NF[c] (Rev/µg)	r of 2-NF	Extract Slope/ 2-NF Slope
ANL	1.1	0.97	1028	0.96	0.001×10^{-3}
ITRI	0.9	0.99	188	0.99	0.005×10^{-3}
LEHR	0.8	0.98	212	0.99	0.004×10^{-3}

[a]Methylene chloride

[b]Correlation coefficient

[c]2-Nitrofluorene

Table 8. Extract Exchange: LEHR Fly Ash Extracted with Be/Me[a] and Tested with TA 98

Lab Testing	Slope of Extract (Rev/mg)	r[b] of Extract	Slope of 2-NF[c] (Rev/µg)	r of 2-NF	Extract Slope/ 2-NF Slope
ANL	118	0.94	1028	0.96	0.11×10^{-3}
ITRI	31	0.99	188	0.99	0.16×10^{-3}
LEHR	63	0.99	212	0.99	0.30×10^{-3}

[a]Benzene/Methanol

[b]Correlation coefficient

[c]2-Nitrofluorene

Table 9. Fly Ash Exchange: ANL Fly Ash A and B Extracted amd Tested with TA 98

Lab/ Solvent	Slope (Rev/mg)	r[a]	Linear Dose Range (mg/pl)	Maximum Dose (mg/pl)	Toxic Dose or Range (mg/pl)
ANL A					
ANL (DMSO)[b]	335	0.99	0 - 0.8	1.0	_
ITRI ($MeCl_2$)[c]	131	0.99	0 - 2.0	2.0	_
LEHR (Be/Me)[d]	54	0.99	0 - 10.0	10.0	_
ANL B					
ANL (DMSO)	76	0.99	0 - 5.0	5.0	_
ITRI ($MeCl_2$)	123	0.99	0 - 1.0	1.0	_
LEHR (Be/Me)	100	0.99	0 - 10.0	10.0	_

[a]Correlation coefficient

[b]Dimethyl sulfoxide

[c]Methylene chloride

[d]Benzene/Methanol

Table 10. Fly Ash Exchange: ANL Fly Ash A and B Extracted and Tested with TA 100

Lab/ Solvent	Slope (Rev/mg)	r[a]	Linear Dose Range (mg/pl)	Maximum Dose (mg/pl)	Toxic Dose or Range (mg/pl)
ANL A					
ANL (DMSO)[b]	61	0.70	0 - 1.0	1.0	—
ITRI ($MeCl_2$)[c]	69	0.98	0 - 2.5	2.5	—
LEHR (Be/Me)[d]	56	0.99	0 - 10.0	10.0	—
ANL B					
ANL (DMSO)	57	0.97	0 - 8.0	10.0	—
ITRI ($MeCl_2$)	93	0.98	0 - 2.5	2.5	—
LEHR (Be/Me)	55	0.98	0 - 10.0	10.0	—

[a]Correlation coefficient

[b]Dimethyl sulfoxide

[c]Methylene chloride

[d]Benzene/Methanol

Table 11. Fly Ash Exchange: ITRI Fly Ash A and B Extracted and Tested with TA 98

Lab/ Solvent	Slope (Rev/mg)	r[a]	Linear Dose Range (mg/pl)	Maximum Dose (mg/pl)	Toxic Dose or Range (mg/pl)
ITRI A					
ANL (DMSO)[b]	0.340	0.70	0 - 50	50	–
ITRI ($MeCl_2$)[c]	0.025	0.88	0 - 2000	2000	–
LEHR (Be/Me)[d]	0.071	0.97	0 - 1000	1000	–
ITRI B					
ANL (DMSO)	0.9	0.88	0 - 50	50	–
ITRI ($MeCl_2$)	0.9	0.99	0 - 800	800	–
LEHR (Be/Me)	2.8	0.99	0 - 200	1000	400 - 1000

[a]Correlation coefficient

[b]Dimethyl sulfoxide

[c]Methylene chloride

[d]Benzene/Methanol

Table 12. Fly Ash Exchange: ITRI Fly Ash A and B Extracted and Tested with TA 100

Lab/ Solvent	Slope (Rev/mg)	r[a]	Linear Dose Range (mg/pl)	Maximum Dose (mg/pl)	Toxic Dose or Range (mg/pl)
ITRI A					
ANL (DMSO)[b]	0.755	0.62	0 - 50	50	—
ITRI ($MeCl_2$)[c]	0.035	0.83	0 - 2000	2000	—
LEHR (Be/Me)[d]	0.033	0.50	0 - 1000	1000	—
ITRI B					
ANL (DMSO)	0.74	0.49	0 - 50	50	—
ITRI ($MeCl_2$)	0.15	0.98	0 - 2000	2000	—
LEHR (Be/Me)	0.64	0.95	0 - 200	1000	400 - 1000

[a]Correlation coefficient

[b]Dimethyl sulfoxide

[c]Methylene chloride

[d]Benzene/Methanol

Table 13. Fly Ash Exchange: LEHR Fly Ash A and B Extracted and Tested with TA 98

Lab/ Solvent	Slope (Rev/mg)	r[a]	Linear Dose Range (mg/pl)	Maximum Dose (mg/pl)	Toxic Dose or Range (mg/pl)
LEHR A					
ANL (DMSO)[b]	86	0.96	0 - 20	20	–
ITRI ($MeCl_2$)[c]	18	0.99	1 - 50	100	–
LEHR (Be/Me)[d]	61	0.99	0 - 15	115	–
LEHR B					
ANL (DMSO)	-0.11	-0.12	0 - 20	20	–
ITRI ($MeCl_2$)	0.04	0.26	1 - 100	100	–
LEHR (Be/Me)	0.11	0.95	0 - 800	1000	1000

[a]Correlation coefficient

[b]Dimethyl sulfoxide

[c]Methylene chloride

[d]Benzene/Methanol

Table 14. Fly Ash Exchange: LEHR Fly Ash A and B Extracted and Tested with TA 100

Lab/ Solvent	Slope (Rev/mg)	r[a]	Linear Dose Range (mg/pl)	Maximum Dose (mg/pl)	Toxic Dose or Range (mg/pl)
LEHR A					
ANL (DMSO)[b]	19	0.90	0 - 10	10	—
ITRI ($MeCl_2$)[c]	3	0.99	0 - 100	100	—
LEHR (Be/Me)[d]	35	0.99	0 - 15	115	20 - 115
LEHR B					
ANL (DMSO)	1.4	0.22	0 - 10	10	—
ITRI ($MeCl_2$)	-0.2	-0.47	0 - 100	100	—
LEHR (Be/Me)	0.2	0.66	0 - 200	1000	1000

[a]Correlation coefficient

[b]Dimethyl sulfoxide

[c]Methylene chloride

[d]Benzene/Methanol

Table 15. Comparison of Specific Mutagenic Activity[a] of Coal Fly Ash Tested with Salmonella Typhimurium TA 98

Sample Fly Ash	ANL/DMSO[b]	Lab/Solvent ITRI/$MeCl_2$[c]	LEHR/Be/Me[d]
ANL A	307 - 363[e]	105 - 157	51 - 57
ANL B	71 - 81	108 - 137	97 - 103
ITRI A	0.18 - 0.50	0.015 - 0.034	0.06 - 0.08
ITRI B	0.7 - 1.2	0.8 - 1.0	2.5 - 2.9
LEHR A	74 - 98	16 - 20	59 - 64
LEHR B	-0.53 - 0.30	-0.04 - 0.12	0.09 - 0.13

[a]Revertants/mg fly ash extracted

[b]Dimethyl sulfoxide

[c]Methylene chloride

[d]Benzene/Methanol

[e]95% confidence interval of specific activity

Table 16. Comparison of Specific Mutagenic Activity[a] of Coal Fly Ash Tested with Salmonella Typhimurium TA 100

Sample Fly Ash	ANL/DMSO[b]	Lab/Solvent ITRI/$MeCl_2$[c]	LEHR/Be/Me[d]
ANL A	28 - 94[e]	52 - 86	53 - 59
ANL B	49 - 65	73 - 124	59 - 71
ITRI A	0.25 - 1.26	0.01 - 0.06	-0.007 - 0.073
ITRI B	0.11 - 1.37	0.12 - 0.17	0.48 - 0.80
LEHR A	14 - 23	2.8 - 3.4	32 - 38
LEHR B	-1.87 - 4.69	-0.447 - -0.001	-0.002 - 0.392

[a]Revertants/mg fly ash extracted

[b]Dimethyl sulfoxide

[c]Methylene chloride

[d]Benzene/Methanol

[e]95% confidence interval of specific activity