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EXPLORATORY RESEARCH ON MUTAGENIC ACTIVITY OF COAL-RELATED MATERIALS

Final Report, March 1—June 1, 1980

By
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Work Performed Under Contract No. AS22-78ET00222

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Abstract

The following samples were found to be mutagenic for strains TA1538, TA98 and TA100 Salmonella typhimurium: ETTM-10, ETTM-11, ETTM-15, ETTM-16, and ETTM-17. ETTM-13 was marginally mutagenic for TA1537. ETTM-14 was slightly mutagenic for TA1537, TA1538, and TA98. Mutagenicity by all samples was demonstrated only in the presence of hepatic enzyme extracts (S9) which provided metabolic activation. ETTM-11 was shown to be the most mutagenic sample assayed thus far; specific activity was 2.79×10^4 TA98 revertants/mg sample. Fractionation by serial extractions with increasingly polar organic solvents was done at least 2 x with ETTM-10, ETTM-11, ETTM-15, ETTM-16 and ETTM-17. For some samples highly mutagenic fractions were observed.

Objective

The objectives of this research have been to investigate the mutagenic effects of materials produced from coal gasification/liquefaction processes using a sensitive and rapid *in vivo* biological test system, the *Salmonella typhimurium*/microsome assay.

Background

In the process of making our country energy self-sufficient, technology has turned to coal, our most abundant source of energy. Since the combustion of coal may emit toxic contaminants into the environment, governmental agencies have regulated its use. Various technologies are now being developed to utilize this source of energy and reduce the side effects upon the environment. It is highly probable that during the processing of coal, materials are produced that may be toxic to man. A toxicological investigation of these materials appears to be a prudent endeavor to undertake as we have only to examine the history of the workers in the carbonization of coal to be alerted to the potential hazards.

Workmen employed in the carbonization of coal, whether for coke or for the generation of producer gas, have an unusually high risk of cancer of various sites (1). It has long been recognized that during the combustion or destructive distillation of coal some agent (or agents) is produced that is carcinogenic for the skin of man.

The history of cancers associated with coal begins with the observation of scrotal cancers in London chimney sweeps by Percivall Pott in 1775(2). He noted that this disease was peculiar to persons employed as chimney sweeps and originated from what was described in the trade as a sootwart. For almost a hundred years, Pott's observations were looked upon as a medical curiosity and no attempt was made further to relate cancer experience to occupational exposures.

Volkman, in 1873, reported three cases of scrotal cancer in men handling tar and paraffin recovered from the carbonization of lignite, a "young coal" (3). His report agreed to the last detail with the so-called chimney sweep cancer of the British. Other reports of unusual skin cancer experience among coal carbonization workers and handlers of various by-products soon appeared (4-6). Experimental studies on cancer induction further demonstrated the carcinogenicity of materials produced during the destructive distillation of coal and eventually led to the isolation of the first pure chemical carcinogen. In 1915, Yamigawa and Ichikawa showed that coal tar was carcinogenic for the skin of the rabbit (7). Passey in 1922 induced cancer with an ether extract of chimney soot (8). After many years of research on the constituents of the coal tar distillates, benzo(a)pyrene, a potent carcinogen was isolated (9).

As early as 1892, it was suggested that exposure to coal tar products might be responsible for cancer of internal organs (4). Kennaway observed that a high proportion of noncutaneous cancers in chimney sweeps were situated in the respiratory tract and the alimentary canal above the stomach (10).

The first report of unusual lung cancer experience for men engaged in coal carbonization concerned the Japanese producer gas workers (11). Of the malignant neoplasms observed in men working at these gas generators, 80 percent were lung

cancers. When this was reported in 1936, lung cancer was a relatively rare disease in Japan and accounted for only 3.1 percent of all malignant neoplasms (12). The extremely high lung cancer rate for gas generator workers was even more striking in contrast with the experience of other employees at the same plant. Not a single lung cancer was noted among the 46 malignant neoplasms observed for other employees.

In the same year that the Japanese reported lung cancer in gas generator workers, Kennaway and Kennaway noted an excess in British gas producermen (13). Their survey of death certificates for England and Wales, 1921 to 1932, showed that other coal carbonization and by-product workers had experienced higher than expected lung cancer mortality. In this and a subsequent report for 1921 to 1938, the Kennaways reported excess lung cancer mortality for gas producermen, chimney sweeps and several categories of gas workers employees (14). Other investigators have shown that coal carbonization workers have excess risk of lung cancer when compared to the general population (1,15-17).

The processing of coal to produce liquid fuels has been associated with an increased risk of cancer, and it has been demonstrated experimentally that certain of these coal-derived liquids are carcinogenic (18-22). Heavy exposure to coal hydrogenation materials produced both benign and malignant skin tumors (21). The incidence of skin cancer in workmen exposed to the coal hydrogenation process was between 16 and 37 times greater than that of West Virginia or the United States as a whole. Any operations performed on the hot, tarry residue left after all of the more volatile components have been removed are potential sources of exposure to carcinogens (20).

Several process stream oils and products were painted on the skin of mice. Middle stream oil (b.p. 260° - 320°C), light oil stream residue (b.p. 260° - 380°C), pasting oil (b.p. 320° - 450°C) and pitch product (boiling above 450°C) were all carcinogenic (19). Tumor incidence in the test animals increased and the length of induction decreased as boiling points of various fractions rose. Any stage in the process of high-boiling coal-derived liquids or their vapors should be considered a potential hazard (23). The pasting oil, which was found to be highly carcinogenic, has significant implications for liquefaction processes which will use recycled oil for slurry formation.

Bioassay studies (24) on various fractions of Bergius and Fischer-Tropsch oils obtained from coal hydrogenation/liquefaction operations were found to be carcinogenic. The data further suggested that the carcinogenic effects may not be restricted to tissues in which these materials are deposited but may extend to remote organs. Lastly bioassays (25) of three coal liquefaction materials using three samples of extraction solvent and refinery feedstock indicated all three coal conversion materials were carcinogenic for the skin of mice. The average latent period range from 24 weeks for 0.2% benzo(a)pyrene in N,N-dimethylformamide to 65 weeks for 50% solution of extracted coal in toluene. A further result from this work indicated that although the presence of a small quantity of benzo(a)pyrene (BaP) imparts carcinogenic properties, the lack of any detectable quantity of BaP does not ensure the fraction is noncarcinogenic.

Synthetic natural gas is not expected to pose a carcinogenic risk. Any trace element, organic carcinogens or cocarcinogens present in the raw product should be removed during subsequent clean up and scrubbing operations. In coal gasification, it is the conversion process itself, rather than the fuel produced which should be the primary concern in the future with regard to carcinogens (26).

The major processes under development for coal liquefaction are pyrolysis, hydrogenation, solvent refining and Fisher-Tropsch synthesis. Composition data (28-32) suggest that polycyclic substances of considerable carcinogenic potential are likely to be produced by various liquefaction processes. These compounds are most likely to be found in the highly boiling aromatic fractions of product liquids (33). The total products of hydrogenation, the high-boiling distillates, the centrifuged oils, char, residues, recycled solvent oil, recycled solvent and liquid coal are all potentially hazardous materials (23).

The greatest hazard potential in coal gasification exists in the early stages of the processes in which coal goes through a series of structural degradations of complex organic compounds. In these early stages, leaks and spills will contain hazardous material (23). Potentially carcinogenic polycyclic organic material found during gasification of coal is likely to concentrate in the tars, oils and char (26,33). In addition if the crude gas contains tars of high boiling oils, the composition must be considered to represent a potential hazard.

In summary products, intermediate streams and wastes associated with coal conversion processes contain known or suspected carcinogenic substances. Most of the carcinogens are associated with tars and oils. It is apparent then that the opportunity for synthesis of hazardous chemicals exists whenever coal is subjected to severe conditions such as those which exist during pyrolysis, hydrogenation, or gasification. As use of synthetic fuels will be likely to increase, sensitive and rapid in vitro mutagenic studies must be carried out on products, intermediate streams and wastes of coal conversion processes.

A variety of techniques are available for assessing the environmental risks and potential health effects of these technologies. These include chemical and physical characterizations, microbial assays, acute toxicity and irritation tests, subchronic toxicity and teratology, chronic toxicity, and carcinogenesis, among others. The conduct of such a myriad of testing on all new developments would not be appropriate, as each change of experimental condition or operation would lead to new health study requirements. This would lead to prohibitive costs, and the resultant data would fill unopened files of defunct processes. A delay in biological assessment until a process has reached the state of production feasibility would be inappropriate. An acceptable position must be found for new fossil energy processes. This compromise consists of chemical characterizations and rapid bioassay studies in small-scale developmental programs followed by detailed characterization and short-term and long-term toxicological testing programs on pilot processes.

For this project material from advanced coal process technologies including gasification and liquefaction, ranging from solid residue to liquid products and waters, have been selected and screened using the Salmonella/microsome mutagenesis assay. This assay developed by B.N. Ames and associates is recognized as one of the most useful short-term assays for mutagenesis, based on the number of compounds detected as mutagens and positive correlation with known carcinogens (34-38). In general, this assay consists of plating histidine requiring organisms in the presence of growth-limiting concentrations of histidine. Only those organisms which are reverted to histidine independence are able to produce colonies under these conditions. The compound to be tested is added to the plate bearing the test bacteria, and mutagenicity is scored as the number of colonies reverted to histidine independence. The tester set of Salmonella strains includes two different frameshift mutants, hisC3076 and hisD3052, and one missense mutant, hisG46 with additional mutations which allow them to respond to a wide variety of chemical mutagens. The set with no capacity for

excision repair (∇ uvrB) and a minimal cell wall (∇ gal, rfa) is the most responsive to the majority of mutagens (39). Two strains, TA98 and TA100, a frameshift and a missense mutant, carry the R plasmid pKM 101. The plasmid confers increased sensitivity to a number of mutagens (40).

The Salmonella themselves, lacking cytochrome P450-associated enzymes, have no capacity to metabolize compounds such as polycyclic aromatic hydrocarbons or other procarcinogens. By addition of mammalian microsomal preparations to the plate assay, activation of compounds and detection as mutagens can occur (41-42).

The Salmonella/microsome system has gained acceptance as a screening assay for potentially hazardous chemicals. It has found application both in the investigation of compounds already in the environment, as well as an indicator of possible health effects of materials under development. It has particular utility in the evaluation of mixtures of substances such as cigarette smoke concentrate (43-44), synthetic crude oil (45) and organic extracts of drinking water (46).

The Salmonella/microsome assay is a reversion assay, measuring the ability of compounds to cause mutations at three specific DNA targets. Agents which interact with DNA sequences not included in the tester set could escape detection as could those causing only deletions or gross chromosomal abnormalities. A small percentage of known organic carcinogens, notably chlorinated hydrocarbons are known to be negative in this assay (37). While a few inorganic carcinogens have been shown to be mutagenic for Salmonella (47), the most widespread application has been in surveying organics.

The greatest difficulty in the evaluation of data from in vitro assays is in the extrapolation to the human situation. Although in two studies (36,38), approximately 90% of known carcinogens were mutagenic for Salmonella, sufficient correlative data do not exist to suggest that a positive response in this assay predicts carcinogenicity or a potential mutagenic hazard for humans. This, however, does not detract from its applicability as a screen for identifying agents for further study. As a screen this assay offers several advantages (34,38).

1. It has been used in more comparative studies than other microbial tests, such as the Saccharomyces cerevisiae Ad-2 assay or the repair assays using Escherichia coli strains.

2. It is less expensive and easier to undertake than the mammalian cell transformation assays which offer a high degree of positive response/known carcinogen correlation (35,38).

3. Results are obtained in 24-48 hours.

4. Results are more simple and less time consuming to score than with chromosome aberration or sister chromatid exchange (48).

The goals underlying the conduct of the mutagenic screenings performed on various coal conversion materials under this project have been as follows:

1. To develop comparative data on the relative hazards as indicated by short-term microbial assays of a wide spectrum of coal conversion materials. Limitations exist in short-term tests regarding interpretation and extrapolation of results, but they do have value

for predicting effects. To be effective in a specific study such as that being performed, the comparative data must be developed within a single laboratory with standardized and uniform procedures.

2. To utilize the developed data base for the identification of priority materials to be tested to higher tier assays.

3. To attempt correlation of results from tests on small-scale process operations to the results obtained from toxicology programs on larger-scale continuous process operations.

Material and Methods

Assay Materials

Table 1 describes the samples selected for assessment of mutagenicity. These materials were selected for study due to their availability and to the desirability of testing materials of wide property differences in order to identify comparative effects. The samples are not considered process discharges and may not be identical to materials generated from advanced coal processes eventually developed to commericialization. Although the samples are not representative of all coal-derived materials from advanced processes, their immediate availability dictated their use. ETTM-10 was a brownish black liquid. ETTM-11 consisted of large lumps of a blackish material which was easily reduced to a powder. ETTM-13 and ETTM-14 were clear yellow and of translucent brown liquids, respectively. ETTM-15 through ETTM-17 were brownish black liquids. The test materials were stored at 5°C in the dark.

Preparation of Samples for Assay

ETTM-10, ETTM-11, ETTM-15 through ETTM-17 were prepared for assay by weighing a small amount, 20-70 mg, and adding dimethyl sulfoxide (DMSO) so as to obtain a presumptive concentration of 10 mg/ml. At no time did all the material dissolve. The amount of this insoluble sample was subtracted from the total to give the adjusted concentrations used in calculating the mutagenic rates. On one occasion the insoluble portion of ETTM-11 apparently picked up a great deal of water or retained solvent after evaporation to apparent dryness. It was impossible to calculate an accurate concentration for this sample. For this reason data generated from the assay of this preparation has not been included in the report.

ETTM-13 and ETTM-14 were prepared for assay by either weighing a volume and diluting with dimethylsulfoxide (DMSO) or by mixing equal portions of sample and DMSO. Both liquids were mixable with the solvent. Upon addition of solvent, both ETTM-13 and ETTM-14 underwent exothermic reactions with a change of color to blue-green. It is likely that this indicated oxidation of the sample or formation of complexes of metals and DMSO. The diluted ETTM-14 was observed to separate into two layers upon standing for several hours. Dilutions of this material were vortexed vigorously just prior to assay. Sample solutions were routinely filter sterilized and applied as 0.1 ml aliquots. Dilutions were made in dimethylsulfoxide (DMSO) so that these percentages of the original solution were tested: 100%, 50%, 10%, 5%, 1%.

Preparation of Organic Extracts

Organic extracts of ETTM-10, 11, 15-17 were prepared in the following manner. Approximately five grams or less of each sample was weighed and a volume of solvent equal to 5 x the weight was added. This mixture was agitated vigrously in the dark at room temperature for two hours. After centrifugation to settle particulates the solvent was removed. An equal amount of fresh solvent then was added to the insoluble residue and the mixture was agitated for another two hours followed by another centrifugation. These two extracts were pooled and evaporated under N_2. This procedure was carried out sequentially with hexane, toluene, methylene chloride and acetonitrile. There was generally a quantity of unextracted material remaining, which is referred to in the text as the residue. Organic extract fractions were prepared for assay in the same manner as the whole sample. All were tested as DMSO solutions. This simple organic extraction regimen was chosen for its ease and appropriateness to the type of samples being assayed. Moreover, the type of organic extracts produced are suitable for analysis by high performance liquid chromatography.

Mutagenicity Assays

Salmonella/microsomal assays were carried out according to the methods described by Ames (42). Microsomal extracts (S9) were made from livers of male Sprague-Dawley rats (150-250 g) which had been administered 500 mg/kg Aroclor 1254 on day one and killed on day six. S9s from four animals were pooled on the day of assay. Plates were scored using an automatic colony counter. Only those counts 2 x the spontaneous values were considered indicative of mutagenicity. Specifics of the procedures are addressed in Appendix A.

Procedures for quantitative toxicity assay which can be directly correlated with the plate incorporation assay have not been established. Toxicity, however, could be scored on the mutagenicity assay plates. Clearing of the background bacterial lawn, reduction of colony counts below the range observed for spontaneous reversion and by the appearance of pinpoint his⁻ "feeder" colonies.

Results and Discussion

As our previous work (final report DOE #EW-78-S-22-0222) indicated that spot tests were of limited usefulness in the evaluation of coal materials, they were not done on these samples. All data reported herein were generated in quantitative agar incorporation assays. Whenever possible five-point dose responses were performed in duplicate for each sample.

None of the materials tested, ETTM-10,11,13-17 was mutagenic for any Salmonella strain when tested without rat hepatic extracts (S9). All of these samples exhibited some degree of mutagenicity in the presence of S9. These results are summarized in Table 2, which presents the numbers revertant/µg calculated from the linear portions of the dose response curves.

ETTM-10 was generally toxic for Salmonella strains TA1535, TA1538, TA98 and TA100, occassionally producing evidence of bacterial killing at low concentration. When metabolized by S9 ETTM-10 was mutagenic for all but TA1535 (Table 3). While there were instances of colony counts in excess of 2 x the average number of spontaneous

revertants no ETTM-10 dose-related mutagenesis of TA1535 was observed. The frameshift mutants TA1538 and TA98 were more sensitive to ETTM-10 than was TA100, and an enhancement of activity due to misrepair (encoded on pKM101) was noted.

ETTM-11 was more selectively toxic. TA1535 and TA1538 were unaffected while their R factor bearing cognates were killed by concentrations of 5% or higher. This proved to be the most active of this set, reverting strains TA1538, TA98 and TA100 (Table 4). The frameshift mutant strains were somewhat more than twice as sensitive as TA100. The frameshift mutagens in these materials apparently are not greatly dependent upon the type of misrepair promoted by pKM101. This is indictated by the similar mutagenesis responses by TA1538 and TA98 (TA1538/pKM101).

ETTM-13 and ETTM-14 both were toxic for the test organisms at the higher doses tested. ETTM-14 also showed evidence of bacetrial killing at 100 µg/plate. The toxicity of both samples was diminished in the presence of S9. Tables 5 and 6 present activation assay data for samples ETTM-13 and ETTM-14, as well as positive and negative controls. ETTM-13 is apparently not mutagenic for strains TA1535, TA1538, TA98 and TA100 when assayed in the presence of 50 µl/plate Aroclor 1254-induced S9. Only marginal activity was observed with TA1537, which was not strictly dose dependent. ETTM-14 was slightly dose dependent. ETTM-14 was slightly mutagenic for strains TA1535, TA1537, TA1538, and TA98 when tested in the activation assay. Concentrations above 1000 µg/plate were toxic for TA1537 and TA1538, above 5000 µg/plate for TA98 and TA1535. TA100 was apparently killed at concentrations above 500 µg/plate ETTM-14. Data in the Summary Table 2 highlights the very weak but detectable mutagenicity of ETTM-14, no greater than 100 revertant colonies/mg. The -1 frameshift lesion in TA1538 and TA98 was more susceptible to the components of ETTM-14 than either the missense mutation (TA1535) or the +1 frameshift (TA1537). TA100 was killed at the concentrations of sample producing a mutagenic response in the other four strains. Organic solvent extracts were not prepared from samples 13 and 14. ETTM-14 was totally soluble in hexane, so that no separation could be affected.

ETTM-15, 16, and 17 were similar to each other in their mutagenic activity (Tables 7-9). All were reverted strains TA1538, TA98 and TA100 to approximately the same extent, 1-2 x 10^3 colonies/mg material. The responses were higher with the frameshift mutants TA1538 and TA98 than for the missense mutant TA100 for ETTM-15. TA98 and TA100, both containing the reversion inducing R factor pKM101, were more responsive to samples ETTM-16 and ETTM-17 than was the non-R factor carrying strain TA1538. TA1535, bearing a missense mutation, but not the reversion-inducing R factor, showed no dose-dependent response to either ETTM-15, ETTM-16 or ETTM-17.

Sequential organic solvent fractions were prepared two times from samples 10, 11, 15-17 by the procedure described in the Methods section. Whenever possible five-dose points were done on two Salmonella strains for each extract. For purposes of comparison a dose response assay of the unfractionated, whole sample was run in tandem with these assays. Results of these assays are presented in Tables 10-16. None of the extracted materials or the nonextractable residue was mutagenic for TA98 and TA100 when tested without S9.

The distribution of material into solvents was very similar for repeated extractions of ETTM-10, referred to as ETTM-10_A and ETTM-10_B (Table 10). In both cases, approximately 97% of the sample was hexane soluble. The smallest portion was acetonitrile extractable. Mutagenicity results differed somewhat. In both sets of extractions a small amount of highly mutagenic material was recovered from the toluene extract (Figure 1). The mutagenicity of this material was approximately four times greater with the A extract than with the B. In both sets of extracts, all fractions, including the DMSO-soluble portion of the non-extractable residue was mutagenic for both strains. Normalization of colonies/mg based on contributions of each fraction to the activity of the whole show that somewhat greater mutagenic activity is recovered when components are separated by extraction.

Distribution of ETTM-11 into organic fractions was not well reproduced upon repetition of the extraction procedure. Table 11 compares the percentage of material extracted by the solvents for 5 separate extractions. On the occasion of extraction D, large particles of the sample rather than powder was used extracted for extraction. In this instance was noted a large weight gain probably due to solvent retention or the accumulation of moisture. This set of extracts was not used in mutagenicity testing. In the course of the extraction very fine insoluble particles were generated; these could not be readily separated by centrifugation, nor even by overnight settling. It is probably these particles which account for the variation observed in the amount of acetonitrile and residue fraction collected. There is apparently some carry-over between the toluene and methylene chloride fractions. The amount of material extractable by each solvent may most reasonably be ranked in this manner: hexane=acetonitrile<toluene=methylene chloride<residue. An examination of the component parts by analytical means may indicate similar or the same components present in the toluene and methylene chloride fractions of ETTM-11.

The extract's mutagenic activities were not surprisingly subject to variation tables (12-13). One consistent observation was that of a small amount hexane soluble material which was quite mutagenic for TA98 and much less active for TA100. This was generally true for the acetonitrile fraction as well. The residue, which was the largest fraction, was only slightly mutagenic for TA98 and slightly or non-mutagenic for TA100.

In the first extraction a highly mutagenic component was uncovered in the methylene chloride fraction. Although data from repeat assays of this particular fraction were in good agreement for both strains, this observation was not reproduced in repeated extractions. The absence of this activity from the subsequent extracts does not seem to be entirely due to a spreading of methylene chloride extractables to the other solvents. Previous work with other samples has indicated that methylene chloride (which is itself mutagenic) does not remain after evaporation. The possibility of some sort of solvent effects on mutagenesis promotion cannot, however, be ruled out.

Some variation was also seen in the distribution of ETTM-15 into the hexane and non-extractable fractions upon repeat extractions (Table 14). Generally the majority of the sample was hexane soluble and this fraction accounted for the bulk of the observed mutagenic activity. While the acetonitrile fraction was the most mutagenic, it was present in very small amounts.

ETTM-16 (Table 15) was similar in that most material was hexane soluble, with the remaining 25% split into toluene and methylene chloride extractable components: the methylene chloride fraction was apparently non-mutagenic from the first extraction but the most active upon the repeat extraction. As observed for the majority of the samples, the residue accounts for very little of the total biological

activity.

ETTM-17 (Table 16) was 98% by weight hexane extractable material which was mutagenic in the range of $1X10^3$ colonies/mg. About 1% was toluene coluble material which was approximately 3X as mutagenic for TA98. The very high methylene chloride activity from the A extraction was based on only one set of assay plates with containing a very small amount of material. This resulted in a probable overestimation of its mutagenic potency. Repeated extractions using much greater amounts of sample would be necessary to establish whether or not there is a highly mutagenic,albeit rare, methylene chloride component.

The relative TA98 mutagenicities of the organic fractions are ranked for comparison below:

ETTM 10: T>A>M>H=R	H=hexane soluble
ETTM 11: A>H>T=M>R	T=toluene
ETTM 15: A>M>T>H>R	M=methylene chloride
ETTM 16: A=M>T>H	A=acetonitrile
ETTM 17: T>H=M	R=residue

In Table 17 are presented comparative values for mutagenicity of all coal-related materials found positive to date (ETTM-13 which is only marginally mutagenic for TA1537 alone has been omitted). These have been ranked by their mutagenicity for strain TA98. This ranking is roughly the same for TA1538 and TA100. Only one gasification by-product, a tar, is active in the Salmonella/microsome assay. Of the liquefaction materials one can note that samples of a particular material may differ in mutagenicity by as much as an order of magnitude. For example, the ETTM-01 product has a TA98 mutagenic activity of approximately $2X10^4$ revertants/mg; the activity of the ETTM-16 product is about $2X10^3$ revertants/mg. Further testing as well as chemical characterization of products and waste generated by various processes, should aid in the identification of those processes likely to present a health hazard for workers. Such testing should make possible the selection of coal technologies which would not significantly increase the environmental pollutant burden.

TABLE 1

Coal Related Materials for Mutagenicity Testing

ETTM-10	Coal Liquefaction Distillate Oils
ETTM-11	Coal Liquefation Residue
ETTM-13	Coal Liquefaction Untreated Water
ETTM-14	Coal Liquefaction Light Oils
ETTM-15	Coal Liquefaction Heavy Liquid (with solids)
ETTM-16	Coal Liquefaction Product (filtered)
ETTM-17	Coal Liquefaction Distillate Oils

TABLE 2

Relative Mutagenicities of ETTM-10, ETTM-11, ETTM-13, ETTM-14, ETTM-15, ETTM-16, and ETTM-17

Sample	Revertant Colonies/µg[a]				
	TA1535	TA1537	TA1538	TA98	TA100
ETTM-10	-	ND	7.30	10.88	2.10
ETTM-11	-	ND	27.92	27.03	11.36
ETTM-13	-	0.02	-	-	-
ETTM-14	0.02	0.03	0.08	0.10	-
ETTM-15	-	ND	2.39	3.68	1.78
ETTM-16	-	ND	1.37	2.56	1.80
ETTM-17	-	ND	0.79	1.54	0.86

[a]Calculated from linear portion of dose response curves.

n=number of data points; r=coefficient of linear conditions

ETTM-10	TA1538	n = 18	r = 0.9869
	TA98	n = 64	r = 0.9052
	TA100	n = 78	r = 0.9105
ETTM-11	TA1538	n = 28	r = 0.9117
	TA98	n = 98	r = 0.9120
	TA100	n =105	r = 0.9261
ETTM-13	TA1537	n = 14	r = 0.8253
ETTM-14	TA1535	n = 34	r = 0.8229
	TA1537	n = 12	r = 0.7252
	TA1538	n = 32	r = 0.8487
	TA98	n = 69	r = 0.8635
ETTM-15	TA1538	n = 26	r = 0.9828
	TA98	n = 59	r = 0.9546
	TA100	n = 57	r = 0.7739
ETTM-16	TA1538	n = 36	r = 0.9806
	TA98	n = 72	r = 0.9632
	TA100	n = 59	r = 0.7698
ETTM-17	TA1538	n = 32	r = 0.9853
	TA98	n = 67	r = 0.9818
	TA100	n = 61	r = 0.7871

- = no dose response

ND= not determined

TABLE 3

Mutagenicity of ETTM-10 in the Presence of 50 µl/plate Aroclor 1254 Induced S9

Compound	Amount/Plate	Revertant Colonies/Plate[a] TA1535	TA1538	TA98	TA100
DMSO[b]	0.1 ml	23±5	12±12	25±7	121±21
MNNG	spot	±4[c]			
ACNA	150 µg		1129±977	3358±788	
MMS	13.3 mg				1432±436
DMSO[d]	0.1 ml	12±7	23±11	25±8	115±24
2AA	5 µg				1225±683
ETTM-10	9.23 µg			76	181
	9.57 µg	12	132	176	191
	9.63 µg	14	57	110	236
	9.78 µg	17	62	88	143
	46.20 µg			546	279
	47.85 µg	13	353	518	260
	47.15 µg				395
	48.9 µg	91	383	730	341
	92.3 µg			913	493
	95.7 µg	Tox	420	716	375
	96.3 µg	43	601	1062	486
	97.0 µg	Tox	663	1259	339
	462 µg			1725	1078
	478.5 µg	15	1049	1185	390
	481.5 µg	32	2063	2820	820
	489 µg	120	1700	2097	388
	923 µg			1297	910
	957 µg	22	Tox	1905	115
	963 µg		2363	2494	Tox
	970 µg	36	2512	3023	391

(continued) Table 3 Mutagenicity of ETTM-10 in the Presence of 50 l/plate Aroclor 1254 Induced S9

[a] Numbers are means of colony counts, n $\geq$ 2. Standard deviations are included for control plates. Underlined numbers $\geq$ 2 x spontaneous count.

[b] DMSO = dimethylsulfoxide

MNNG = N-methyl-N'-nitro-N- nitroguanidine

ACNA = 1-amino-2-carboxy-4-nitroanthraquinone

MMS = methyl methanesulfonate

2AA = 2-aminoanthracene

Tox = Evident toxicity on plates

[c] +4 = Ring of revertant colonies too numerous to count.

[d] These and subsequent plates received 50 µl/plate Aroclor-induced S9.

TABLE 4

Mutagenicity of ETTM-11 in the Presence of 50 µl/plate Aroclor 1254-induced S9

Compound	Amount/Plate	Revertant Colonies/Plate[a] TA1535	TA1538	TA98	TA100
DMSO[b]	0.1 ml	23±5	12±12	25±7	121±21
MNNG	spot	+4[c]			
ACNA	150 µg		1129±977	3358±788	
MMS	13.3 mg				1432±436
DMSO[d]	0.1 ml	12±7	23±11	25±8	115±24
2AA	5 µg				1225±683
ETTM-11	1.4 µg			88	160
	1.85 µg	101	64	81	139
	2.27 µg			75	154
	2.71 µg	81	27	47	120
	4.34 µg	8	49	75	85
	4.69 µg			71	149
	6.8 µg			424	298
	9.25 µg	26	377	519	274
	11.3 µg			334	188
	13.5 µg	21	100	730	480
	18.5 µg	24	966	1026	300
	21.7 µg	13	311	351	304
	22.7 µg			947	427
	23.4 µg			651	255
	27.1 µg	27	345	415	239
	43.4 µg	11	398	499	339
	46.9 µg			1041	394
	68 µg			2090	1499
	92.5 µg	162	2936	2863	1444
	113 µg			3124	1820
	135.5 µg	37	1925	2556	1484
	185 µg		3954	3233	2151
	217 µg	26	2384	1443	1661
	227 µg			4100	1555

(continued)

TABLE 4 (continued)

Mutagenicity of ETTM-11 in the Presence of 50 µl/plate Aroclor 1254-induced S9

Compound	Amount/Plate	Revertant Colonies/Plate[a] TA1535	TA1538	TA98	TA100
	234 µg			2194	1052
	271 µg	67	2616	4304	1405
	434 µg	Tox	2750	2903	2390
	469 µg			1920	1681

[a] Numbers are means of colony counts, $n \geq 2$. Standard deviations are included for control plates. Underlined numbers are ≥ spontaneous counts.

[b] DMSO = dimethylsulfoxide
MNNG = N-methyl-N'-nitro-N- nitrosoguanidine
ACNA = 1-amino-2-carboxy-4-nitroanthraquinone
MMS = methyl methanesulfonate
2AA = 2-aminoanthracene
Tox = evident toxicity on plates

[c] +4 = ring of revertant colonies too numerous to count.

[d] These and subsequent plates received 50 µl/plate Aroclor-induced S9.

TABLE 5

Mutagenicity of ETTM-13 in the Presence of 50 µl/Plate Aroclor 1254-induced S9

		Revertant Colonies/Plate[a]				
Compound	Amount/Plate	TA1535	TA1537	TA1538	TA98	TA100
DMSO[b]	0.1 ml	31±22	12±7	16±7	27±8	166±30
MNNG	spot	+4[c]	108±47			
ACNA	150 µg			1428±840	3304±1129	
MMS	13.3 mg					1799±526
DMSO[d]	0.1 ml	16±7	14±6	26±15	32±9	121±30
2AA	5.0 µg					1404±1209
ETTM-13	10 µg	30	10	19	19	117
	50 µg	12	6	12	24	114
	100 µg	22	8	14	32	128
	500 µg	14	25	27	32	133
	944.6 µg	52	30	27	52	193
	1000 µg	17	16	21	31	128
	4723 µg	62	40	14	62	181
	9446 µg	Tox	28	14	31	152
	47230 µg	Tox	37	Tox	Tox	146
	94460 µg	52	Tox	Tox		172

[a]Numbers are means of colony counts, $n \geq 2$. Standard deviations are included for control plates. Underlined counts $\geq$ 2 x spontaneous counts.

[b]DMSO = dimethylsulfoxide

MNNG = N-methyl-N'-nitro-N-nitrosoguanidine

ACNA = 1-amino-2-carboxy-4-nitroanthraquinone

MMS = methyl methanesulfonate

2AA = 2-aminoanthracene

Tox = evident toxicity on plates

[c]+4 = ring of revertant colonies too numerous to count.

[d]These and subsequent plates received 50µl/plate Aroclor-induced S9.

TABLE 6

Mutagenicity of ETTM-14 in the Presence of 50 µl/Plate Aroclor 1254-induced S9

Compound	Amount/Plate	Revertant Colonies/Plate[a]				
		TA1535	TA1537	TA1538	TA98	TA100
DMSO[b]	0.1 ml	31±22	12±7	16±7	27±8	166±30
MNNG	spot	+4[c]	108±47			
ACNA	150 µg			1428±840	3304±1129	
MMS	13.3 mg					1799±526
DMSO[d]	0.1 ml	16±7	14±6	26±15	32±9	121±30
2AA	5.0 µg					1404±1209
ETTM-14	10 µg	12		12	26	140
	50 µg	19	18	16	30	121
	100 µg	25		19	28	140
	250 µg	11	6	21	32	193
	500 µg	22	18	46	64	190
	832.9 µg	29	44	94	71	Tox
	1000 µg	14		110	138	Tox
	2500 µg	20	Tox	Tox	141	Tox
	4164.5 µg	130	Tox	Tox	592	Tox
	5000 µg	Tox	Tox	Tox	224	Tox
	8329 µg	Tox	Tox	Tox	647	Tox
	25000 µg	Tox		Tox	Tox	
	41645 µg	Tox	Tox	Tox	Tox	Tox
	83290 µg	Tox	Tox	Tox	Tox	Tox

[a] Numbers are means of colony counts, $n \geq 2$. Standard deviations are included for control plates. Underlined numbers ≥ 2 x spontaneous counts.

[b] DMSO = dimethylsulfoxide
MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
ACNA = 1-amino-2-carboxy-4-nitroanthraquinone
MMS = methyl methanesulfonate
2AA = 2-aminoanthracene
Tox = evident toxicity on plates

[c] +4 = ring of revertant colonies too numerous to count.

[d] These and subsequent plates received 50 µl/plate Aroclor-induced S9.

TABLE 7

Mutagenicity of ETTM-15 in the Presence of 50 µl/Plate Aroclor 1254-Induced S9

Compound	Amount/Plate	Revertant Colonies/Plate[a] TA1535	TA1538	TA98	TA100
DMSO[b]	0.1 ml	24±13	12±6	22±7	96±24
MNNG	Spot	4+[c]			
ACNA	150 µg		875±740	3619±868	
MMS	13.3 mg				1359±329
DMSO[d]	0.1 ml	17±11	26±18	23±8	123±44
2AA	5.0 µg				1588±672
ETTM-15	8 µg	37	61	92	185
	9 µg			84	201
	10 µg	21	34	61	152
	40 µg	82	142	227	Tox
	46 µg			181	226
	49 µg	21	128	269	270
	50 µg			260	233
	80 µg	Tox	290	519	204
	93 µg			Tox	269
	98 µg	21	250	397	332
	100 µg			424	294
	398 µg	28	971	1458	292
	404 µg			959	402
	492 µg	27	895	1277	318
	499 µg			1383	520
	795 µg	27	1450	2178	475
	927 µg			Tox	349
	983 µg	Tox	1072	1974	383
	998 µg			2255	628

[a]Numbers are means of colony counts, n ≥ 2. Standard deviations are included for control plates. Underlined numbers are ≥ 2 x spontaneous counts.

[b]DMSO = dimethylsulfoxide
MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
ACNA = 1-amino-2-carboxy-4-nitroanthraquinone
MMS = methyl methanesulfonate
2AA = 2-aminoanthracene
Tox = evident toxicity on plates
[c]+4 = ring of revertant colonies too numerous to count.

[d]These and subsequent plates received 50 µl/plate Aroclor-induced S9.

TABLE 8

Mutagenicity of ETTM-16 in the Presence of 50 µl/Plate Aroclor 1254-Induced S9

Compound	Amount/Plate	Revertant Colonies/Plate[a]			
		TA1535	TA1538	TA98	TA100
DMSO[b]	0.1 ml	24±13	12±6	22±7	96±24
MNNG	Spot	4+[c]			
ACNA	150 µg		875±740	3619±868	
MMS	13.3 mg				1359±329
DMSO[d]	0.1 ml	17±11	26±18	23±8	123±44
2AA	5.0 µg				1588±672
ETTM-16	9 µg	24	33	46	186
	10 µg	25	50	80	169
	46 µg	22	133	224	282
	47 µg			78	195
	48 µg	38	159	190	252
	92 µg	25	196	308	294
	93 µg			241	303
	95 µg	23	276	428	292
	97 µg			372	253
	459 µg			1080	254
	462 µg	29	681	1347	316
	466 µg			915	425
	476 µg	38	982	1559	333
	485 µg			1393	433
	918 µg			1723	299
	924 µg	Tox	1225	1957	390
	932 µg			1401	489
	951 µg	34	1440	2134	387
	970 µg			2216	472

[a] Numbers are means of colony counts, $n \geq 2$. Standard deviations are included for control plates.

[b] DMSO = dimethylsulfoxide
MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
ACNA = 1-amino-2-carboxy-4-nitroanthraquinone
MMS = methyl methanesulfonate
2AA = 1-aminoanthracene
Tox = evident toxicity on plates

[c] +4 = ring of revertant colonies too numerous to count

[d] These and subsequent plates received 50 µl/plate Aroclor-induced S9.

TABLE 9

Mutagenicity of ETTM-17 in the Presence of 50 µl/Plate Aroclor 1254-Induced S9

Compound	Amount/Plate	Revertant Colonies/Plate[a]			
		TA1535	TA1538	TA98	TA100
DMSO[b]	0.1 ml	24±13	12±6	24±8	106±25
MNNG	Spot	+4[c]			
ACNA	150 µg		743±737	3627±810	
MMS	13.3 mg				1389±270
DMSO[d]	0.1 ml	17±11	26±18	27±8	124±27
2AA	5 µg		34±4		1565±603
ETTM-17	5 µg	19	32	21	171
	9 µg			30	119
	10 µg	19	30	40	144
	24 µg	26	34	62	173
	43 µg			79	115
	49 µg	20	78	97	155
	86 µg			174	156
	97 µg			188	238
	98 µg	27	97	194	216
	245 µg	36	266	355	337
	430 µg			698	258
	487 µg			569	242
	490 µg	38	430	853	111
	490 µg	19	400	706	148
	492 µg			264	187
	861 µg			1318	Tox
	974 µg			947	Tox
	980 µg	27	568	1101	Tox
	984 µg			770	204

[a]Numbers are means of colony counts, $n \geq 2$. Standard deviations are included for control plates.

[b]DMSO = dimethylsulfoxide
MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
ACNA = 1-amino-2-carboxy-4-nitroanthraquinone
MMS = methyl methanesulfonate
2AA = 1-aminoanthracene
Tox = evident toxicity on plates

[c] +4 = ring of revertant colonies too numerous to count.

[d]These and subsequent plates received 50 µl/plate Aroclor-induced S9.

TABLE 10

Distribution of ETTM-10 into Organic Solvents

Sample	Extraction Solvent	Amount[a] Extracted (gm)	Amount[a] Extracted (%)	Colonies/mg[b] TA98	Colonies/mg[b] TA100	Normalization of Colonies/mg Based on Whole Sample[c] TA98	Normalization of Colonies/mg Based on Whole Sample[c] TA100
ETTM-10_A	hexane	4.1561	97.82	10,220	4040	9997	3952
	toluene	0.0337	0.79	525,190	265,000	4419	2094
	methylene chloride	0.0341	0.80	22,990	19,770	584	158
	acetonitrile	0.0021	0.05	64,470	11,800	32	6
	non-extractable	0.0228	0.54	10,340	6,688	56	36
	Sum of Fractions		100			15,088 (154%)	6,246 (227%)
	Entire Sample		100			9,850	2,750
ETTM-10_B	hexane	4.4358	97.12	7254	1930	7045	1874
	toluene	0.0419	0.92	135,880	62,860	1250	578
	methylene chloride	0.0741	1.62	37,700	10,840	611	176
	acetonitrile	0.0025	0.05	36,860	12,510	184	62
	non-extractable	0.0129	0.28	13,810	5,640	39	16
	Sum of Fractions		100			9,129 (139%)	2,706 (109%)
	Entire Sample		100			6,580	2,474
ETTM-$10_{A\&B}$	hexane	97.47±0.49[d]		9,500[e]	4.080	9260	3977
	toluene	0.86±0.09		271,370	152,010	2334	1307
	methylene chloride	1.21±0.58		63,890	12,640	773	153
	acetonitrile	0.05±0.00		63,510	12,290	32	6
	non-extractable	0.41±0.18		13,260	4,460	54	18
	Sum of Fractions		100			12,453 (134%)	5,461 (233%)
	Entire Sample		100			9,280	2,340

[a] Initial weights prior to extraction: ETTM-10_A, 5.0212 gm; ETTM-10_B, 4.7879. The sum of the organic extract weights for ETTM-10_A was 4.2488 gm, a loss of 15.4%; for ETTM-10_B, 4.5672, a loss of 4.61%.

[b] Calculated from the linear portion of dose response curves, 4 plates/dose point; -= no linear response.

[c] $\frac{\text{Sum of fractions}}{\text{Entire Sample}} \times 100$

[d] Numbers are means ± range of percentages in above 2 sections.

[e] Calculated from linear regression analysis of all dose data points from both extractions.

TABLE 11

Distribution of ETTM-11 into Organic Solvents--Repeated Extractions

Extraction solvent	% extracted				
	A	B	C	D	E
hexane	0.58	0.79	0.91	0.17	0.79
toluene	19.24	21.05	12.81	34.90	46.45
methylene chloride	47.31	33.80	38.90	12.23	17.14
acetonitrile	10.86	43.73	0.78	0.73	0.51
residue	22.02	0.64	47.24	51.95	35.11
	% weight gain after extraction				
	27.0	25.60	2.20	71.13	24.70

TABLE 12

DISTRIBUTION OF ETTM-11_A and $_B$ INTO ORGANIC SOLVENTS

Sample	Extraction Solvent	Amount[a] Extracted (gm)	Amount[a] Extracted (%)	Colonies/mg[b] TA98	Colonies/mg[b] TA100	Normalization of Colonies/mg Based on Whole Sample[c] TA98	Normalization of Colonies/mg Based on Whole Sample[c] TA100
ETTM-11_A	hexane	0.0271	0.58	14,500	3700	84	22
	toluene	0.9037	19.24	16,600	8000	3194	1539
	methylene chloride	2.2230	47.31	208,600	54,600	98689	25831
	acetonitrile	0.5101	10.86	15,900	4300	1727	467
	non-extractable	1.0344	22.02	1,400	-	308	0
	Sum of Fractions		100			103,918 (433%)	27,859 (223%)
	Entire Sample		100			24,000	12,500
ETTM-11_B	hexane	0.0341	0.79	19.900	7300	157	58
	toluene	0.9111	21.05	16,100	4700	3389	989
	methylene chloride	1.4633	33.80	11,900	4600	4022	1555
	acetonitrile	1.8931	43.73	30,600	12,600	13.381	5510
	non-extractable	0.0275	0.64	1,600	-	1024	0
	Sum of Fractions		100			21,973 (78%)	8112 (68%)
	Entire Sample		100			28,030	11,914

[a] Initial weights prior to extraction: ETTM-11_A, 3.6974 gm; ETTM-11_B, 3.4475. The sum of the organic extract weights for ETTM-11_A was 4.6981, a gain of 27.1%; for ETTM-11_B, 4.3291, a gain of 25.6%.

[b] Calculated from the linear portion of dose response curves, 4 plate/dose point; - = no linear response.

[c] $\frac{\text{Sum of fractions}}{\text{Entire Sample}} \times 100$

TABLE 13

DISTRIBUTION OF ETTM-11_C AND $_E$ INTO ORGANIC SOLVENTS

Sample	Extraction Solvent	Amount[a] Extracted (gm)	Amount[a] Extracted (%)	Colonies/mg[b] TA98	Colonies/mg[b] TA100	Normalization of Colonies/mg Based on Whole Sample[c] TA98	Normalization of Colonies/mg Based on Whole Sample[c] TA100
ETTM-11_C	hexane	0.0403	0.91	19,020	11,820	173	108
	toluene	0.5414	12.81	2501	1420	305	173
	methylene chloride	1.7282	38.90	7660	3290	2980	1280
	acetonitrile	0.0345	0.78	25,060	9960	195	78
	non-extractable	2.0987	47.24	536	-	253	252
	Sum of Fractions		100			3906 (14%)	1891 (7%)
	Entire Sample		100			28,840	25,700
ETTM-11_E	hexane	0.0522	0.79	15,167	8997	120	71
	toluene	3.0742	46.45	24,719	10,204	11,482	4739
	methylene chloride	1.1344	17.14	12,043	5008	2064	858
	acetonitrile	0.0336	0.51	33,295	14,240	170	73
	non-extractable	2.3238	35.11	2495	1892	876	664
	Sum of Fractions		100			14,712 (67%)	6405 (128%)
	Entire Sample		100			22,037	5022

[a] Initial weights prior to extraction: ETTM-11_C, 4.3464 gm; ETTM-11_E, 5.309. The sum of the organic extract weights for ETTM-11_C was 4.4431 gm, a gain of 2.2%; for ETTM-11_E, 6.6182, a gain of 24.7%.

[b] Calculated from the linear portion of dose response curves, 4 plate/dose point; - = no linear response.

[c] $\frac{\text{Sum of fractions}}{\text{Entire Sample}} \times 100$

TABLE 14
DISTRIBUTION OF ETTM-15 into Organic Solvents

Sample	Extraction Solvent	Amount[a] Extracted (gm)	(%)	Colonies/mg[b] TA98	TA100	Normalization of Colonies/mg Based on Whole Sample[c] TA98	TA100
ETTM-15$_A$	hexane	3.0790	85.07	2470	1037	2101	882
	toluene	0.4788	12.31	2450	860	302	106
	methylene chloride	0.8370	2.15	2950	430	63	9
	acetonitrile	0.0090	0.23	4820	-	11	0
	non-extractable	0.0089	0.23	-	-	0	0
	Sum of Fractions		100			2477 (59.4%)	997 (60.1%)
	Entire Sample		100			4170	1660
ETTM-15$_B$	hexane	3.2059	53.74	2130	1355	1145	728
	toluene	0.6199	10.39	3500	1810	364	188
	methylene chloride	0.3472	5.82	7690	1940	478	113
	acetonitrile	0.0137	0.23	28010	6040	64	14
	non-extractable	1.7792	29.82	50	-	15	0
	Sum of Fractions		100			2066 (82.6%)	1043 (51.4%)
	Entire Sample		100			2500	2030
ETTM-15$_{A\&B}$	hexane	69.40±22.16[d]		2230[e]	1080	1548	749
	toluene	11.35±1.36		3380	1230	384	140
	methylene chloride	3.98±2.60		7310	2020	291	80
	acetonitrile	0.23±0.00		27150	3320	62	8
	non-extractable	15.02±20.92		-	-	0	0
	Sum of Fractions		100			2285 (63.6%)	977 (53.7%)
	Entire Sample		100			3590	1820

[a] Initial weights prior to extraction: ETTM-15$_A$, 4.4623 gm; ETTM-15$_B$, 5.7345. The sum of the organic extract weights for ETTM-15$_A$ was 3.8883, a loss of 12.9%; for ETTM-15$_B$, 5.9659, a gain of 4.0%.

[b] Calculated from the linear portion of dose response curves, 4 plate/dose point; - = no linear response.

[c] $\frac{\text{Sum of fractions}}{\text{Entire Sample}} \times 100$

[d] Numbers are means ± range of percentages in above 2 sections.

[e] Calculated from linear regression analysis of all dose data points from both extractions.

TABLE 15
DISTRIBUTION OF ETTM-16 INTO ORGANIC SOLVENTS

Sample	Extraction Solvent	Amount[a] Extracted (gm)	Amount[a] Extracted (%)	Colonies/mg[b] TA98	Colonies/mg[b] TA100	Normalization of Colonies/mg Based on Whole Sample[c] TA98	Normalization of Colonies/mg Based on Whole Sample[c] TA100
ETTM-16$_A$	hexane	3.4530	76.36	1050	329	802	251
	toluene	0.4202	9.29	1660	767	154	71
	methylene chloride	0.6211	13.74	70	-	10	0
	acetonitrile	0.0012	0.03	6050[d]	-	2	0
	non-extractable	0.0263	0.58	297	-	2	0
	Sum of Fractions		100			970 (41.8%)	322 (54.9%)
	Entire Sample		100			2320	586
ETTM-16$_B$	hexane	2.9437	77.09	870	135	671	104
	toluene	0.5976	15.65	7440	2370	695	371
	methylene chloride	0.1690	4.43	6810	1790	302	79
	acetonitrile	0.0069	0.18	5380	1280	10	2
	non-extractable	0.1011	2.65	180	-	5	0
	Sum of Fractions		100			1683 (132.5%)	556 (182.9%)
	Entire Sample		100			1270	304
ETTM-16$_{A\&B}$	hexane		76.72±0.52[e]	999[f]	324	766	248
	toluene		12.47±4.50	4120	1110	514	138
	methylene chloride		9.08±6.58	6520	1750	592	159
	acetonitrile		0.10±0.11	5380	1310	5	1
	non-extractable		1.62±1.46	157	-	3	0
	Sum of Fractions		100			1880 (88.7%)	546 (133.2%)
	Entire Sample		100			2120	410

[a] Initial weights prior to extraction: ETTM-16$_A$, 4.6341 gm; ETTM$_B$, 4.1686. The sum of the organic extract weights for ETTM-16$_A$ was 4.5218 gm, a loss of 2.4%; for ETTM-16$_B$, 3.8183, a loss of 8.4%.

[b] Calculated from the linear portion of dose response curves, 4 plate/dose point; - = no linear response.

[c] $\frac{\text{Sum of fractions}}{\text{Entire Sample}} \times 100$

[d] Calculated from one dose point plus spontaneous counts.

[e] Numbers are means ± range of percentages in above 2 sections.

[f] Calculated from linear regression analysis of all dose data points from both extractions.

TABLE 16
DISTRIBUTION OF ETTM-17 INTO ORGANIC SOLVENTS

Sample	Extraction Solvent	Amount[a] Extracted (gm)	Amount[a] Extracted (%)	Colonies/mg[b] TA98	Colonies/mg[b] TA100	Normalization of Colonies/mg Based on Whole Sample[c] TA98	Normalization of Colonies/mg Based on Whole Sample[c] TA100
ETTM-17$_A$	hexane	4.4146	98.80	928	1640	917	1620
	toluene	0.2950	0.76	4680	2910	36	22
	methylene chloride	0.0005	0.01	39500[d]	16200[d]	4	2
	acetonitrile	0	0.00	-	-	0	0
	non-extractable	0.0162	0.42	-	-	0	0
	Sum of Fractions		100			957 (57.0%)	1644 (132.6%)
	Entire Sample		100			1680	1240
ETTM-17$_B$	hexane	2.6493	98.27	1098	1430	1079	1405
	toluene	0.0246	0.91	3180	2306	29	21
	methylene chloride	0.0041	0.15	1340	-	2	0
	acetonitrile	0.0016	0.06	-	-	0	0
	non-extractable	0.0164	0.61	-	-	0	0
	Sum of Fractions		100			1110 (71.6%)	1426 (185.7%)
	Entire Sample		100			1550	768
ETTM-17$_{A\&B}$	hexane		98.54 ± 0.37[e]	1068[f]	1440	1052	1419
	toluene		0.84 ± 0.11	4579	2050	38	17
	methylene chloride		0.08 ± 0.10	39290	15560	31	12
	acetonitrile		0.03 ± 0.04	-	-	0	0
	non-extractable		0.52 ± 0.13	-	-	0	0
	Sum of Fractions		100			1121 (72.3%)	1442 (136.6%)
	Entire Sample		100				

[a] Initial weights prior to extraction: ETTM-17$_A$, 5.5102 gm; ETTM-17$_B$, 3.1136. The sum of the organic extract weights for ETTM-17$_A$ was 3.8608, a loss of 29.9%; for ETTM-17$_B$, 2.696, a loss of 13.41%.

[b] Calculated from the linear portion of dose response curves, 4 plate/dose point; - = no linear response.

[c] $\frac{\text{Sum of fractions}}{\text{Entire Sample}} \times 100$

[d] Calculated from one dose point plus spontaneous counts.

[e] Numbers are means ± range of percentages in above 2 sections.

[f] Calculated from linear regression analysis of all dose data points from both extractions.

TABLE 17

RELATIVE MUTAGENIC ACTIVITIES OF COAL-RELATED MATERIALS

Sample		Revertant Colonies/µg[a]		
		TA1538	TA98	TA100
ETTM-11	Liquefaction Residual	27.92	27.03	11.36
ETTM-01	Liquefaction Product	30.12	18.54	6.89
ETTM-08	Liquefaction Heavy Liquid	ND	16.66	8.38
ETTM-09	Liquefaction Product	ND	11.55	6.76
ETTM-10	Liquefaction Distillate Oils	7.30	10.88	2.10
ETTM-02	Gasification Tar	11.16	6.75	6.49
ETTM-15	Liquefaction Heavy Liquid	2.39	3.60	1.78
ETTM-16	Liquefaction Product	1.37	2.56	1.80
ETTM-17	Liquefaction Distillate Oils	0.79	1.54	0.86
ETTM-14	Liquefaction Light Oils	0.08	0.10	-

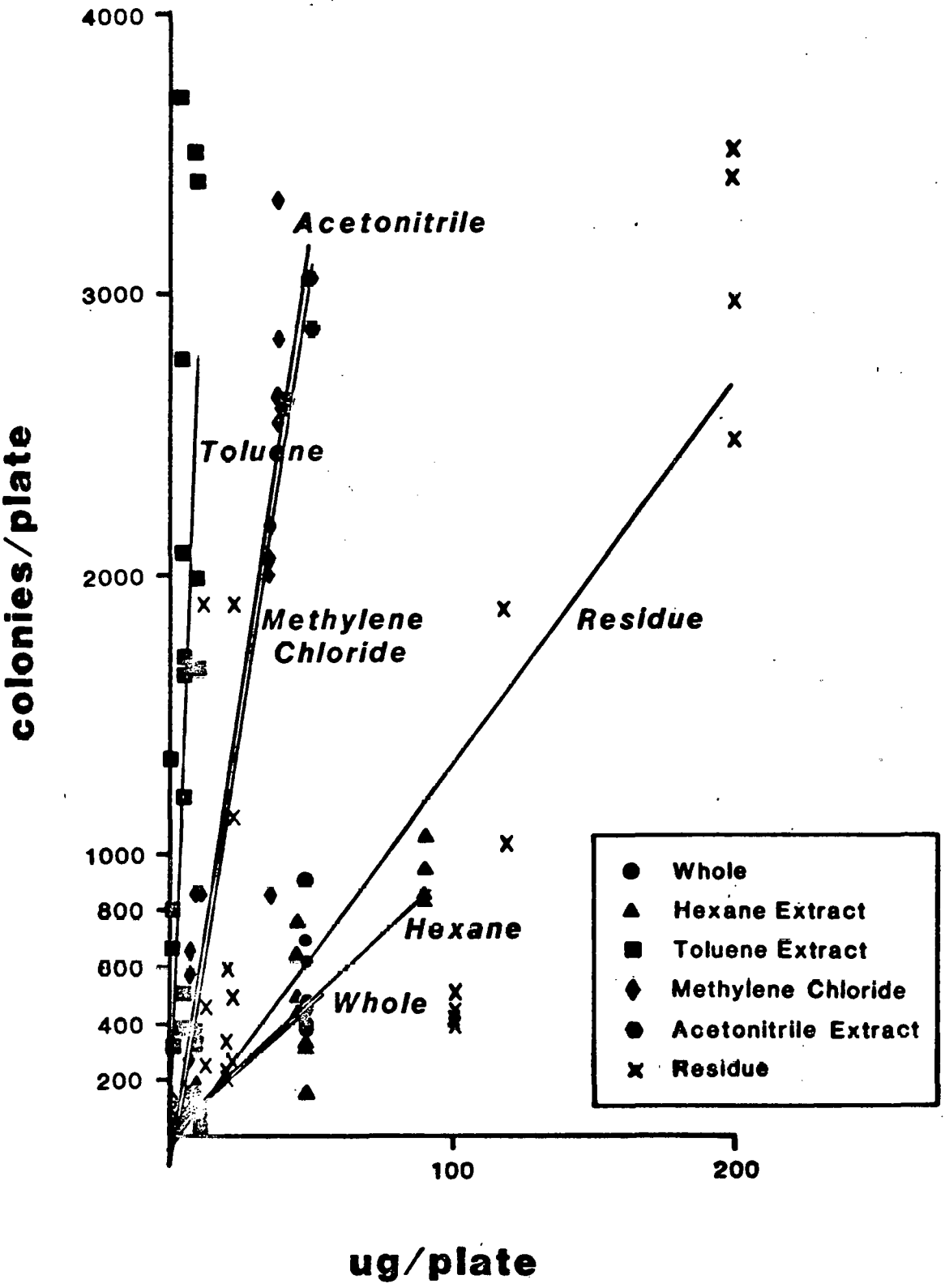
[a]Calculated from linear portion of dose response curves.

- = No dose response.

ND = Not Determined.

FIGURE 1

MUTAGENICITY OF ORGANIC SOLVENT EXTRACTS OF ETTM-10 FOR STRAIN TA98 WITH AROCLOR-INDUCED S9 (50 UL/PLATE). CORRELATION COEFFICIENTS (R) FOR THE LINEAR REGRESSION PLOTS ARE AS FOLLOWS: WHOLE SAMPLE, R = 0.8154; HEXANE EXTRACT, R = 0.9366; TOLUENE EXTRACT, R = 0.7774; METHYLENE CHLORIDE EXTRACT, R = 0.9338; ACETONITRILE EXTRACT, R = 0.9583; RESIDUE, R = 0.8683.


4000
3000
2000
1000
800
600
400
200
100
200
colonies/plate
ug/plate
Acetonitrile
Toluene
Methylene Chloride
Residue
Hexane
Whole
Whole
Hexane Extract
Toluene Extract
Methylene Chloride
Acetonitrile Extract
Residue

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APPENDIX

Protocol For Bacterial Mutagenesis and Bacterial Toxicity:

I. Text Organisms:

The mutagen indicator strains of Salmonella typhimurium were supplied by Dr. B. N. Ames, University of California at Berkeley. The set, derived from wild type stock LT-2, is described below:

Table 1

Tester Strain Set

his operon mutation	∇bio uvrB	∇gal bio uvrB	rfa, ∇gal bio uvrB	rfa, ∇gal bio uvrB/pkM101	/pkM101
hisG46	TA1950	TA1530	TA1535	TA100	TA92
hisC207	TA1951	TA1531	TA1536		
hisC3076	TA1952	TA1532	TA1537	TA2637	
hisD3052	TA1534	TA1964	TA1538	TA98	TA94

II. Protocols

A. Media and Methods

1. Media

N.B. = Nutrient Broth (Difco)

E. = Medium of Vogel and Bonner (J. Biol. Chem. 218: 97-106, 1956).

HG = E made 2.0% with respect to glucose

Agar plates contain 1.5% Difco Bacto agar. Soft agar used with HG plates is made with 0.6% Difco agar, 0.5% NaCl, and 0.05 mM histidine HCl, 0.05 mM biotin according to Ames. For quantitative toxicity testing, soft agar is made with 0.6% Difco agar, 0.5% NaCl, and 1.55 mM histidine HCl, and 0.05 mM biotin.

2. Culture Stocks

Culture are maintained at -70°C as NB cultures (1.6 ml) in DMSO (0.14 ml) in sterile vials. Subcultures are made once a week by inoculating NB ith ice scrapings of frozen stocks and incubating overnight at 37°C in a shaking water bath. Alternatively subcultures are made from isolated colonies streaked on typicase soy agar. This overnight culture is subcultured 1/10 in 50 ml NB and incubated by shaking at 37°C in a forced air incubator. The organisms are grown to a density of 10^9 cells/ml as indicated by a reading of 140 on a Klett-Summerson colorimeter with a green filter. The tester strains are assayed with known mutagens as summarized in Table 2. (MMNG: N-methyl-N'-nitro-N-nitrosoguanidine, 2AA: 2-aminoanthracene, ACNA: 1-amino-2-carboxy-4-nitroanthraquinone, AFB_1: Aflatoxin B_1, MMS: methyl methanesulfonate, 9AA: 9-aminoacridine).

TABLE 2

Strain	Mutagens	Plating Conditions	Characteristic Response
TA1535	MNNG (crystal)	Spot Test	Solid Ring of Revertant Colonies
TA1537	MNNG (crystal)	Spot Test	Ring of Discrete Colonies
	9AA (100 µg in 0.1 ml DMSO)	Pour Plate	$\geq$ 400 Revertant Colonies
TA1538	2AA (5 µg in 0.1 ml DMSO)	Pour Plate 12.5 l/pl of PCB induced S9 in activation mixture	$\geq$ 1000 Revertant Colonies
TA98	ACNA (150 µg in 0.1 ml DMSO)	Pour Plate	$\geq$ 2000 Revertant Colonies
	AFB_1 (0.1 µg in 0.1 ml DMSO)	Pour Plate 20 µl/pl of PCB induced S9 in activation mix	$\geq$ 700 Revertant Colonies
TA100	MMS (13.3 mg in 0.1 ml DMSO)	Pour Plate	$\geq$ 1000 Revertant Colonies

For the purpose of quality control, the weekly protocol is as follows:

Day 1: Subcultures are made in the afternoon from stocks as described in A2.

Day 2: The overnight subcultures are subcultured 1/10 in 50 ml NB as described in A2. When the cells grow to a density of 10^9 cells/ml, they are assayed with known mutagens as described above. Cultures are partitioned into 5 ml aliquots and refrigerated.

Day 4: Assay plates are checked for characteristic response to known mutagens. Refrigerated cultures are used until the following week.

B. Plating Method for Mutagenic Testing

HG plates are used. Strains prepared as described above are added in 0.1 ml molten soft agar which is then poured onto the agar plate. Mutagens are added either to the soft agar for the quantitative test or as crystals or small amounts of liquid to the hardened overlay for the spot test. All plates are done in duplicate with the exception of spontaneous controls which are done in triplicate.

Plates are incubated for 48 hours at 37°C in an air incubator, and then counted using an automatic plate counter. Those counts registering over 2500 colonies/plate are verified by hand count.

DMSO is routinely used as the solvent in volumes ranging from 0.01 to 0.30 ml/plate. At this level the DMSO is non-toxic to the tester strains. Other solvents which have been used successfully in our laboratory include the following: hexane, acetone, ethanol, dimethylformamide, benzene:isooctane, and emulphor:saline:ethanol.

Positive controls with known mutagens are included in each assay as listed in the following table.

TABLE 3

Positive Controls

Strain	Compound	µl/Plate	Activation µl S9 /Plate
TA1535	MNNG	spot	-
TA1537	MNNG	spot	-
TA1538	9-aminoacridine	100	-
	ACNA	150	-
	2AA	5	12.5
TA98	ACNA	150	-
	2AA	5	12.5
TA100	MMS	12.9	-
	2AA	5	12.5

Apparent toxicity of samples is indicated by clearing of the background lawn of organisms, appearance of his⁻ pinpoint colonies, or reduction spontaneous counts. Mutagenicity is indicated by colony counts at least 2 x the spontaneous count for a particular strain on the day of assay.

C. Microsomal Activation

Tests for activated intermediates are performed as described in Section 6a above except for the addition of 0.5 ml S9 mix to the agar overlay. S9 mix is made according to Ames as follows:

S9	.0002 - 0.4 ml/ml
$MgCl_2$	8 micromoles/ml
KCl	33 micromoles/ml
Glucose-6-phosphate	5 micromoles/ml
NADP	4 micromoles/ml
Sodium Phosphate	pH 7.4, 100 micromoles/ml

The stock salt solution (0.4N $MgCl_2$, 1.64M KCl) and phosphate buffer (0.2M, pH 7.4) are stored separately at room temperature. On the day of the assay these are combined with the co-factors and filter sterilized. Frozen S9 is thawed at room temperature, pooled as appropriate, added to the salts on ice to generate S9 mix. Unused S9 mix is discarded at the end of the day.

D. Preparation of S9

1. Induction

Male Sprague-Dawley rats averaging 150-250 g at death are used. Animals are housed two per hanging cage during the 2-4 day acclimation period and singly thereafter. Rats receive water and Purina lab chow ad libitum and are on a 12-hour light-dark schedule.

Induction is by intraperitoneal injection of corn oil solutions of the inducing compounds. Treatment schedules are in Table 4. Rats are weighed daily and dosages calculated so as to administer 2 ml inducing solution per kg weight. Rats are fasted 24 hours prior to harvest. Uninduced S9 is obtained from rats treated with corn oil 2 ml/kg for four successive days.

2. Harvest of Livers and Preparation of S9

The rats are killed by a blow to the head and decapitation. Livers are removed asceptically to sterile cold tared petri dishes and weighed. After washing with cold buffer (0.15 MKCl, 0.05 Tris HCl), the livers are homogenized in 3 volumes of the same buffer using a Potter Elvehjem motor driven apparatus with a Teflon pestle. The homogenate is transferred to 50 ml sterile centrifuge tubes and spun for 20 minutes at 9,000 x g in a Sorvall RC2B. After centrifugation, the supernatant S9fraction is pipetted into sterile bottles,swirled to insure an even mix of microsomal material, and dispended in 1 ml aliquots in sterile vials. These are immediately frozen in liquid nitrogen and stored at -70° C.

Typically, one to three dozen rats are sacrificed at one time. An average ten gram liver generates approximately 30-1 aliquots. To insure sterility, one aliquot of each liver is thawed, plated on nutrient agar, and incubated for 24 hours. Typically in a large plating assay, 1 ml volumes of S9 of comparable activation capacity from two or more rats are pooled. S9 has been shown to maintain activity for at least one year when stored at -70° C.

E. Analysis of Data

For routine screening a minimum of 2 five-point dose-response curves are run on each sample. Linear regression analysis of these data are done using a programable calculator. Positive-negative control data are reported and means ± standard deviation.

TABLE 4

Schedule of Inducing Treatments

Treatment	Amount/Kg/Day	# Days	Total Dose (Amount Kg)
A (Aroclor 1254)	500 mg	1	500 mg
B (Aroclor 1254)	50 mg	5	250 mg
Corn Oil	2 ml	4	8 ml
Water	2 ml	4	8 ml
3-Methylcholanthrene	20 mg	2	40 mg
Phenobarbital	75 mg	4	300 mg

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