

NON-ADDITIVE MUTAGENIC RESPONSES BY COMPONENTS OF COAL-DERIVED MATERIALS

R. Schoeny,¹ D. Warshawsky,² and G. Moore³

¹ U.S. EPA, Cincinnati, Ohio 45268

² Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, Ohio 45267

³ U.S. DOE, Pittsburgh Energy Technical Center, Pittsburgh, PA 15236

In the last decade major efforts have been made to ensure the energy self-sufficiency of this country. Included in that effort has been the development and refinement of coal conversion technologies as a means of permitting increased usage of coal resources without attendant increases in pollution. Processes of coal liquefaction and gasification are not, however, free from hazard risk. Solvent refined coal materials SRCI and SRCII, fractions from Bergius and Fisher-Tropsch operations, and H-coal process products have been shown to produce adverse biological effects, such as tumors in experimental animals or mutations in various test system (e.g. 1-3, 7-10). Analytical data indicate that polycyclic aromatic hydrocarbons (PAH), heterocyclic compounds, aromatic amines and other materials with carcinogenic potential are likely to be produced by liquefaction processes (1, 3-6, 16). Coal conversion materials are highly complex and ill-defined mixtures. Coal liquids, for example, have a more complex composition than do crude petroleum of a similar boiling range. Mutagenic or carcinogenic components may, in fact, constitute a very minor percentage of the mass of these materials.

For these, as for other mixtures of biologically active materials, the question has been raised whether the individual activities of constituents are additive or if there are interactive effects contributing to the overall biological activity observed for the mixture. There are a few examples of non-additive biological responses in the literature. It is known, for instance, that mixtures of PAH and aromatic amines showed greater than additive effects when assayed for mutagenicity of *Salmonella typhimurium* (11). Likewise, a mixture of benzo[a]pyrene (BaP) dihydrodiols was more directly mutagenic than would be expected based on the mutagenic activities of the individual diols. When rat liver homogenates (S9) were added to assay system, the reverse was true (12). Our laboratory has also observed that fractions of coal-derived materials exhibited non-additive mutagenic responses in *Salmonella typhimurium* (14). Current research has been directed toward an examination of synergistic or antagonistic biological activities of coal conversion mixture components.

MATERIALS AND METHODS

Samples and Preparation

Samples were supplied through U.S. DOE PFTC by R.A. Winschel of the Coal Research Division of Conoco. All materials were collected on a daily basis, combined and distilled by Conoco. Sample PDU-9 is a hydroclone overflow material from an H-coal process design unit. Lummus Feed and Lummus Product are the second stage feed and product, respectively from run 3LCF9 of a Lummus Integrated Two Stage Liquefaction process. Samples were stored in the dark at 5°C. They were prepared for assay by weighing 100-500 mg and adding dimethylsulfoxide (DMSO) so as to obtain a presumptive concentration of 10 or 20 mg/ml. Sample solutions were filter sterilized and applied as 0.1 ml aliquots for reverse mutation assays and as 1 ul aliquots for forward mutation assays. Tared vessels containing undissolved materials were dried and weighed, and this figure used in calculation of the true concentration of the test solutions.

Preparation of Solvent Extracts of Samples

Approximately 5g of each sample was weighed and a volume of solvent equal to 5x of the weight was added in capped tubes. This mixture was agitated vigorously in the dark at room temperature for 2 hrs. After centrifugation to settle particulates, the solvent was removed and an equal amount of fresh solvent was added. The mixture was agitated for another 2 hours followed again by centrifugation. The two solvents with extracted material were pooled and evaporated under N₂. 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 and tables as the residue fraction. The DMSO soluble portion of this material and all the dried extracts were prepared for assay in the same manner as the whole sample.

Preparation of "Reconstructed Whole" Samples

"Reconstructed whole" mixtures were prepared by adding individual fractions in the same proportions in which they were extracted. For example, PDU-9 fraction III reconstruction consisted of 1.71% hexane fraction, 42.76% toluene fraction, etc. Materials were dissolved in the original extracting solvents or weighed out, combined, and the solvents evaporated under N₂. The reconstructions were assayed as DMSO solutions on the same occasion as were aliquots of the unfractionated sample and the individual fractions.

Mutagenicity Assays in Salmonella Typhimurium

Liver homogenates (S9) for mammalian metabolism were prepared from male Sprague-Dawley rats which had been injected i.p. with 500 mg/kg Aroclor 1254 and killed five days thereafter. Livers were homogenized in 3 volumes cold buffer (0.15 M KCl, 0.05M Tris HCl). The supernatant fraction from 20 min. centrifugation at 9000g was dispensed into 0.5 ml aliquots and stored at -70°C until use.

Reverse mutation plate incorporation assays using Salmonella typhimurium strains TA97 and TA98 were done according to published procedures (9). All assays included use of 50 ul S9/plate plus appropriate buffers and cofactors.

Strain TM677 was used in forward mutation assays wherein resistance to 8-azaguanine toxicity was measured as the endpoint (15). Fresh cultures (from frozen aliquots) of approximately 3×10^7 organisms were used in microvolume suspension assays, total volume 100 ul. The test material was added as 1 ul aliquots and S9 comprised 1% of the total assay volume. Duplicate assay tubes per experimental point were incubated for 2 hr at 37°C in a gyrotory bath. At the end of the incubation period cells were diluted 1/5 with phosphate buffered saline and 145 ul plated in triplicate for selection of mutant organisms. An aliquot was further diluted and plated in the absence of selection for determination of bacterial survival. Numbers of revertants/ 10^5 surviving cells were calculated from the 6 mutagenicity and 6 toxicity plate counts per experimental point. A positive response in this assay is one in which the number of mutants/ 10^5 survivors is greater than the upper 99% confidence limit on both day of assay and mean historical spontaneous controls.

Results and Discussion

Sample PDU-9 is a vacuum still bottom material from an H-coal process design unit using Kentucky 11 coal and run in the Syncrude mode. The Lummus process which provided the Feed and Product samples consisted of a short contact time liquefaction, followed by antisolvent deashing and subsequent upgrading of

the deashed coal liquids in a Lummus-Cities Service LC fining unit. The Lummus Feed is the LC-Finer feed stock, and Lummus Product the recycle oil produced by this unit.

All materials were assayed for mutagenicity as DMSO solutions. All were mutagenic in both reverse mutation assays to histidine prototrophy (Ames test) and in a forward mutation assay using selection of 8-azaguanine resistant clones. Note that the latter test incorporates a 2-hour period wherein the Salmonella and test items are incubated in liquid suspension. For all assays mutagenicity was detectable only when rat liver homogenates (S9) were included to provide mammalian enzymes for metabolism of the materials to reactive forms.

Of the three samples, PDU-9 was most mutagenic, and is, in fact the most mutagenic fuel material assayed in our laboratory. This sample, and all others, was diluted and assayed at 5 concentrations. The assay was repeated on this set of solutions and then a fresh set of sample solutions was prepared and tested on two occasions. As these coal materials are heterogenous in composition, there are variations both in the amount of material soluble in DMSO and in the mutagenicity observed. Data from assay of PDU-9 graphed in Figure 1, illustrate the variation in mutagenic response. It is also clear from this figure that, at low sample concentrations, there is a linear sample-dose/mutagenic response relationship. It is possible, therefore, to obtain an estimate of the mutagenic response per unit sample by applying regression analysis to those points and obtaining the slope of the regression line. For the PDU-9 data graphed in Figure 1, one obtains an estimate of 50.6 TA98 colonies/ug sample and 48.1 TA97 colonies/ug. Mutagenicity observed for Lummus Feed was 13.4 TA97 and 4.2 TA98 Colonies/ug and for Lummus Product 17.9 TA97 and 11.7 TA98 colonies/ug. Strain TA98 is known to respond to a variety of compounds which cause frameshift mutations, including PAH and aromatic amines. Strain TA97 also is reverted by frameshift mutagens but is particularly sensitive to acridine compounds and probably to other heterocyclics as well (8). The difference in response by the two strains of the Lummus vs the H-coal (PDU-9) samples is suggestive of variations in their composition. It is expected that the Lummus materials will be shown to be composed of a greater percentage acridines and related compounds than the H-coal sample.

The forward mutation assay presumably detects all types of mutagenic events, including deletions and translocations, that are compatible with bacterial viability. The use of a suspension incubation gives the added advantage of providing a relevant quantitation of test item toxicity. None of the test materials when assayed with S9 were toxic to the Salmonella; that is, caused a reduction of more than 40% viability. All three proved to give positive results, as measured by increases in numbers of mutants/ 10^5 survivor greater than the upper 99% confidence limit on both day-of-assay and mean historical spontaneous controls. Furthermore, the magnitude of the mutagenicity response was seen to increase in a linear fashion with sample concentration, allowing the same sort of estimation as for the reverse assay data. Sample PDU-9 was also the most mutagenic sample as measured in the forward mutation assay, with 2.94 mutants/ 10^5 survivors/ug compared to Lummus Feed (0.11 mutants/ 10^5 survivors/ug) and Lummus Product (0.45 mutants/ 10^5 survivors/ug).

As a first step in the analysis of these fuel materials, a simple fractionation process was undertaken. Sequential organic solvent extracts were prepared using solvents of increasing polarity; namely, hexane, toluene, methylene chloride, and acetonitrile. The DMSO-soluble portion of the non-extractable material (residue) left at the end of the process, and all fractions were assayed for mutagenicity in forward and reverse assays as described for the uncut samples. Two sets of fractions were prepared and assayed.

Generally these materials were similar in their extraction properties to other H-coal vacuum bottoms we have assayed (13, 14, 17). Little material is extracted by hexane and less than 1% of the total mass was acetonitrile extractable. From 20-36% of the material was left at the end of the procedure as the residue fraction. In the course of the fractionation, the sample gains weight, presumably due to retained solvent or water. Use of solvents which had been dried by the addition of a water sieve agent did not reduce this weight gain.

The fractions differ markedly from one another in their mutagenic activity (Tables 1-5). As one would expect, the residue fraction of the extraction process is the least mutagenic material. The components extracted by acetonitrile are generally highly mutagenic (e.g. PDU-9 100,940 TA98 colonies/mg; 73,272 TA97 colonies/mg). This latter fraction, presumably the most polar components of the coal-conversion materials, is present in small amounts. The proportionate activity of the whole mixture is, therefore, very small, assuming additivity. If one multiplies the calculated mutagenicity of a fraction in colonies/mg by the per cent of the whole mixture that fraction represents, a determination of fraction specific activity can be made. For example, in Table 1, the TA98 mutagenicity of the residue fraction was low, 2544 colonies/mg, but these components constitute 23.84% of the sample. The specific activity of the residue, or its contribution to the mutagenicity of the whole sample, was 606 colonies/mg whole sample. The highly mutagenic acetonitrile fraction contributed to the same extent to whole sample mutagenicity (596 colonies/mg whole sample) as it constituted only 0.59% of the PDU-9 sample mass.

If there are no interactive effects among fractions of these coal-conversion materials, the sum of the specific activities should equal the mutagenicity observed for the unfractionated material. When these summations have been calculated for a variety of coal-conversion samples the percentages have been found to be comparable to the whole sample mutagenicity. There have also been observed sums of fractional activities less than the whole as well as greater than the whole (14). PDU-9, Lummus Product and Lummus Feed fall into the former category. This non additivity of fraction mutagenic activities is unlikely to be due to dilution as a result of weight increase of the material during the extraction process. In no instance was the weight gain more than 20% of the original sample weight (PDU-9). Table 1 indicates that the "loss" of PDU-9 mutagenicity with fractionation was about 50% for the reverse mutation assay (Table 2). The data for Lummus Feed are similar (Tables 4,5), whereas dilution cannot be discounted as accounting for the majority of the non-additivity observed with the Lummus Product sample (Table 3).

Another explanation for non-additivity is the loss or alteration of mutagenic compounds as a consequence of the extraction process. It was believed that this mild extraction, which entails no changes in pH or temperature of the material, would be unlikely to generate oxygenated or otherwise modified compounds. The protocol was undertaken under yellow lights to minimize photoreactions of PAHs and other aromatic compounds. A third possibility is that the fractional components act as co-mutagens.

To elucidate the mechanism of the observed non-additivity, "reconstructed whole" mixtures were prepared. This was done by recombining the sample fractions in the same proportions which they were extracted. The reconstructions were assayed in reverse and forward mutation assays on the same occasions as the unfractionated whole and the individual fractions. If there are, in fact, co-mutagenic interactions among fractions, the mutagenicity of the reconstructed mixture should be increased relative to the fraction sums. For PDU-9 this proved not to be the case (Table 1). For both strains TA97 and TA98, the reconstruction

mutagenicity was not increased, but was roughly equivalent to the fraction sum. This was also the case for forward mutation assay of the reconstructed sample (Table 2). Data derived from assay of the set B fractions corroborate these results (not shown). The Lummus Product reconstruction assay showed only a very little increase in TA97 mutagenicity. For both of these samples it would appear that non-additivity of organic fraction mutagenicity is due to artifacts of extract preparation.

This does not appear to be the case for the Lummus Feed material. Data from reverse mutation assay of both sets of fractions (Tables 4,5) indicate an increase in mutagenesis on the order of 28% to 65% relative to the fraction sums. That there were alterations in the fractions as artifacts of preparation cannot be discounted, but it is also clear that there is evidence of co-mutagenicity among fractions of the Lummus Feed sample. It should be noted parenthetically that the data in these tables also illustrate the potential for loss or change of biological activity that occurs in these complex mixtures as a function of time. Fraction sets A & B were prepared in assayed over a period of several months.

The premise that single mixture compounds behave independently or additively when mixed is a subject of continuing discussion. The work described here indicates that complex mixtures of compounds may not have biological activity which can be estimated on the basis of summation of activities of known components. It also points out, however, the difficulty of separating mixture components in a way which leaves them unchanged.

ACKNOWLEDGEMENTS

This work was supported by U.S. DOE Contract #DE-AC22-83PC62999. We thank Ms. Lois Hollingsworth for her expert technical assistance and Ms. Carol Haynes for typing of the manuscript.

REFERENCES

1. Battelle Pacific Northwest Laboratories (1979). Biomedical studies on solvent refined coal (SRC II) liquefaction materials: A status report. PNL-3189.
2. Cowser, K.E. (Ed.) (1984). Synthetic Fossil Fuel Technologies. Results of Health and Environmental Studies. Proceedings of the Fifth Life Sciences Symposium, Gatlinburg, TN, Butterworth Publishers, Boston, MA.
3. Freudenthal, R.I., Lutz, G.A., Mitchell, R.I. (1975). Carcinogenic potential of coal and coal conversion products. Battelle-Columbus Laboratories, Columbus, OH.
4. Guerin, M.R., Ho, C-h, Rao, T.K., Clark, B.R. and Epler, J.L. (1980). Polycyclic aromatic primary amines as determinant chemical mutagens in petroleum substitutes. Environ. Res. 23: 42-53.
5. Guerin, M.R., Rubin, I.B., Rao, T.K., Clark, B.R., and Epler, J.L. (1981). Distribution of mutagenic activity in petroleum and petroleum substitutes, Fuel 60: 282-288.
6. Ho, C-h., Ma, C.Y, Clark, B.R., Guerin, M.R., Rao, T.K. and Epler, J.L. (1980). Separation of neutral nitrogen compounds from synthetic crude oils for biological testing. Environ. Res. 22: 412-422.

7. Heuper, W.C. (1953). Experimental studies on carcinogenesis of synthetic liquid fuels and petroleum substitutes. AMA Arch. Indust. Hygiene Occup. Med. 8: 307-327.
8. Levin, D.E., Yamasaki, E. and Ames, B.M. (1982). A new Salmonella tester strain, TA97, for the detection of frameshift mutagens. Mutat. Res. 94: 315-330.
9. Maron, D., Ames, B.M. (1983). Revised method for the Salmonella mutagenicity test. Mutat. Res. 113: 173-215.
10. Munro, N.B., Fry, R.J.M., Gammage, R.B., Haschek, W.M., Calle, E.F., Klein, J.A., Schultz, T.W. (1983). Indirect Coal Liquefaction: A Review of Potential Health Hazards and Worker Exposure During Gasification and Synthesis. ORNL-5938, Oak Ridge National Lab., Oak Ridge, TN.
11. Salamone, M.F., Heddle, J.A., Katz, M. (1979). The use of Salmonella/microsomal assay to determine mutagenicity in paired chemical mixtures. Can. J. Genet. Cytol. 21: 101-107.
12. Schoeny, R., Cody, T., Radtke, M. and Warshawsky, D. (1985). Mutagenicity of algal metabolites of benzo(a)pyrene for Salmonella typhimurium. Environ. Mutagen., in press.
13. Schoeny, R. and Warshawsky, D. (1983). In Vitro mutagenicity testing of Ohio coal-derived materials. In: M. Waters, S. Sandhu, J. Lewtas, L. Claxton, N. Chernoff and S. Nesnow (Eds.). Short-term Bioassays in the Analysis of Complex Environmental Mixtures, III, pp. 285-295.
14. Schoeny, R., Warshawsky, D., Hollingsworth, L., Hund, M. and Moore, G. (1981). Mutagenicity of products from coal gasification and liquefaction in the Salmonella microsome assay. Environ. Mutagen 3: 181-195.
15. Skopek, T.R., Liber, H.L., Krolewski, J.J., Thilly, W.G. (1978). Quantitative forward mutation assay in Salmonella typhimurium using 8-azaquanine resistance as a genetic marker. Proc. Natl. Acad. Sci. USA, 75: 410-414.
16. Tanita, R., Telesca, D., Walker, J., Beradinelli, S. (1980). Organic contaminants in direct coal liquefaction - a preliminary assessment. Amer. Indust. Hygiene Assoc. J. 41: 851-853.
17. Warshawsky, D., Schoeny, R. and Moore, G. Evaluation of coal liquefaction technologies by Salmonella mutagenesis. Toxicol. Lett. 10: 121-127, 1982.

Table 1

COMPARISON OF PDU-9 FRACTION (SET A) AND RECONSTRUCTED MIXTURE
MUTAGENICITIES: REVERSE MUTATION PLATE INCORPORATION ASSAYS

SAMPLE	PERCENT EXTRACTION	COLONIES/mg ^a		SPECIFIC ACTIVITY COLONIES/mg ^b	
		TA97	TA98	TA97	TA98
Hexane	1.71	16,850	18,881	288	323
Toluene	42.76	9,516	15,903	4,069	6,800
Methylene Chloride	31.11	29,319	49,017	9,121	15,249
Acetonitrile	0.59	73,272	100,940	432	596
Residue	23.84	1,404	2,544	335	606
Whole				28,056	49,092
Sum of Fractions				14,245 (50.8%)	23,574 (48.0%)
Reconstruction				15,829 (56.4%)	16,389 (33.4%)

^a Calculated from linear portions of dose response curves

^b Colonies/mg x per cent extraction

Table 2

COMPARISON OF PDU-9 FRACTION (SET A) AND RECONSTRUCTED
MIXTURE MUTAGENICITIES: FORWARD MUTATION ASSAY

SAMPLE	PERCENT EXTRACTION	TM677 COLONIES/ 10 SURVIVORS/mg	SPECIFIC ACTIVITY COLONIES/10 ⁵ SURVIVORS/mg
Hexane	1.71	187	3
Toluene	42.76	405	173
Methylene Chloride	31.11	1,084	337
Acetonitrile	0.59	2,514	15
Residue	23.84	57	14
Whole			2,911
Sum of Fractions			542 (18.6%)
Reconstruction			313 (10.8%)

^a Calculated from linear portion of dose response curves

^b Colonies/10⁵ survivors/mg x per cent extracted

Table 3

COMPARISON OF LUMMUS PRODUCT FRACTION (SET A) AND RECONSTRUCTED MIXTURE
MUTAGENICITIES: REVERSE MUTATION PLATE INCORPORATION ASSAYS

SAMPLE	PERCENT EXTRACTED	COLONIES/mg ^a		SPECIFIC ACTIVITY COLONIES/mg ^b	
		TA97	TA98	TA97	TA98
Hexane	4.76	31,373	6,309	1,493	300
Toluene	26.39	13,128	11,880	3,463	3,135
Methylene Chloride	16.50	28,341	38,141	4,676	6,293
Acetonitrile	1.26	18,862	23,775	238	300
Residue	51.08	2,545	4,686	1,300	2,394
Whole				15,730	21,844
Sum of Fractions				11,170 (71.0%)	12,422 (56.9%)
Reconstruction				12,718 (80.9%)	13,482 (61.7%)

^a Calculated from linear portion of dose response curves

^b Colonies/mg x per cent extracted

Table 4

COMPARISON OF LUMMUS FEED FRACTION AND RECONSTRUCTED MIXTURE MUTAGENICITIES:
REVERSE MUTATION PLATE INCORPORATION ASSAYS, STRAIN TA97

SAMPLE	PER CENT EXTRACTED		TA97 COLONIES/mg ^b		SPECIFIC ACTIVITY TA97 COLONIES/mg ^c	
	A	B	A	B	A	B
	Hexane	2.48	2.13	8,862	376	220
Toluene	25.94	17.88	9,603	8,721	2,491	1,559
Methylene Chloride	34.28	37.76	15,106	10,200	5,178	3,852
Acetonitrile	1.06	0.75	22,265	28,732	236	215
Residue	36.24	41.48	2,540	726	920	301
Whole					17,952	13,411
Sum of Fractions					9,045 (50.4%)	5,935 (44.2%)
Reconstruction					15,385 (85.7%)	12,162 (90.7%)

^a Refers to fraction set A or B

^b Calculated from linear portions of dose response curves

^c Colonies/mg x per cent extractor

Table 5

COMPARISON OF LUMMUS FEED FRACTION AND RECONSTRUCTED MIXTURE MUTAGENICITIES:
REVERSE MUTATION PLATE INCORPORATION ASSAYS, STRAIN TA98

SAMPLE	PER CENT EXTRACTED		TA98 COLONIES/mg ^b		SPECIFIC ACTIVITY TA98 COLONIES/mg ^c	
	A ^a	B	A	B	A	B
Hexane	2.48	2.13	4,376	458	108	10
Toluene	25.94	17.88	4,876	3,454	1,265	618
Methylene Chloride	34.28	37.76	4,436	3,973	1,521	1,500
Acetonitrile	1.06	0.75	20,630	10,192	902	151
Residue	36.24	41.48	2,490	364		
Whole					11,127	5,002
Sum of Fractions					4,015 (36.1%)	2,355 (47.1%)
Reconstruction					7,183 (64.6%)	5,643 (112.8%)

^a Refers to fraction set A and B

^b Calculated from linear portion of dose response curves

^c Colonies/mg x per cent extracted

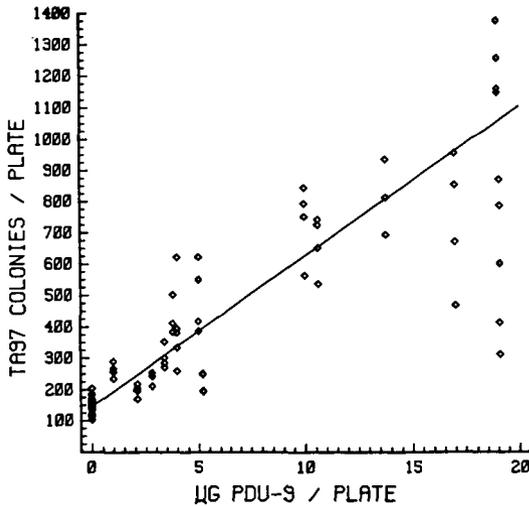


Figure 1. Mutagenicity of coal hydrogenation sample PDU-9 for *Salmonella typhimurium* strain TA98. The solid lines the least squares line for these data, $n = 97$, $r = 0.9259$.