EXPLORATORY RESEAPCH ON MUTAGENIC ACTIVITY OF COAL-RELATED MATERIALS

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D. Warshawsky and R. Schoeny

University of Cincinnati Medical College
Kettering Laboratory
Department of Environmental Health
3223 Eden Avenue
Cincinnati, Ohio 45267

MASTER

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Abstract

Samples ETM-05 and ETM-12 (powdered coal) have been re-evaluated and found to be marginally mutagenic. Organic solvent fractions prepared from these have also been shown to be slightly mutagenic. Upon fractionation of ETM-18, a highly active methylene chloride component was noted when assayed with Aroclor-induced S9.

This report covers data on the following samples:

ETTM-05 Coal
ETTM-12 Coal
ETTM-18 Liquefaction Solid Residual

1. Preparation for Assay

ETTM-05 and ETTM-12 are black powders; ETTM-18 is a black solid easily reduced to powder. Test materials were stored at 5 °C in the dark. They were prepared for assay by weighing 20-70 mg and adding dimethylsulfoxide (DMSO) so as to obtain a presumptive concentration of 10 mg/ml. For no sample did all material dissolve. For ETTM-18 the amount of insoluble sample was subtracted from the total to determine the adjusted concentration used in calculating mutagenic activities. Samples ETTM-05 and ETTM-12 retained the solvent after evaporation to apparent dryness, making it impossible to ascertain the weight of insoluble material. Data from assays of these samples are expressed as function of sample dilution rather than µg sample/plate.

Sample solutions were routinely filter sterilized and applied as 0.1 ml aliquots. Dilutions were made in DMSO so that these percentages of the original solution were tested: 100%, 50%, 10%, 5%, 1%.

Organic solvent fractions of all three samples were prepared and assayed as previously reported (#DOE-ET-0222-4 final report). Three sets of fractions were prepared from ETTM-05 and two from both ETTM-12 and ETTM-18.

2. Chemical Analysis of Mutagenic Fractions

A new set of organic solvent fractions has been prepared from the following coal-related materials:

ETTM-01
ETTM-02
ETTM-08
ETTM-09
ETTM-10
ETTM-11
ETTM-15
ETTM-16

All of these samples have previously been shown to be mutagenic for Salmonella and have three or more mutagenic organic solvent fractions. Limited mutagenicity testing (TA98 only, 4 point dose response with S9) has been done to determine if this new set of fractions is similar in bio-activity to those two sets for which there are extensive data. For ETTM-01 and ETTM-10 it was necessary to prepare a third set of extracts. Fractions are being prepared for analyses of ETTM-18 as well. Samples will be selected from this fraction set for determination of PAH and aromatic amine content by high performance liquid chromatography and GC-MS.

3. Results of Mutagenicity Testing

Quantitative dose response assays of ETTM-18 and fractions have been completed. Neither ETTM-18 nor any of its fractions was mutagenic for TA1535 or for any strain with S9 (Table 1). Unlike ETTM-11 (solid residual from the earlier liquefaction run), ETTM-18 exhibited reproducible distribution into the extracting solvents on a weight basis (Table 2). For both sets of ETTM-18 extracts the bulk of material (63-64%) was non-extractable residue which was somewhat mutagenic for TA98, but which did not revert TA100 in a dose dependent fashion. Both extractions produced a highly mutagenic methylene chloride fraction which accounted for about 10% of the weight of the whole sample. The toluene fraction (22-23% by weight) varied greatly in mutagenicity for both TA98 and TA100 from the first to the second set of extracts. A 3rd set of ETTM-18 extracts is being prepared and will be tested with TA98 in such a way as to preserve the majority of sample for analysis by HPLC. Regression analyses of linear portions of dose response curves provided data in Table 3. ETTM-18 is more mutagenic for both R factor bearing strains than for TA1538, indicating the presence of components dependent upon a type of DNA misrepair for their activity.

ETTM-05 and ETTM-12 both appear to be marginally mutagenic producing numbers of revertants = 2 x the spontaneous revertants (Tables 4 and 5). Due to the absorption of solvent by these materials accurate sample weights have not been obtained. This has made it impossible to ascertain whether or not ETTM-05 and ETTM-12 are mutagenic in a sample-dose dependent manner. Upon extraction both 05 and 12 yielded fractions which were somewhat mutagenic when assayed with S9 (Tables 4 and 5). For both coals, the majority of material (98% and 97%) proved to be nonextractable residue which was not mutagenic. Mutagenic activity was more pronounced for ETTM-05 fractions than for ETTM-12. For neither sample were the fraction mutagenicities well reproduced. Unlike other sample fractions studied to date, the coal fractions were more active for TA100 than for TA98 (Tables 6 and 7). Based on the sums of fractional activities an estimate of coal mutagenicity can be given as follows: ETTM-05, TA98 17 colonies/mg, TA100 40 colonies/mg; ETTM-12, TA100 28 colonies/mg.

4. Results of Bioassay of Fractions for Chemical Analysis

Fractions have been prepared from previously reported mutagenic samples. That these are comparable in distribution and in mutagenic activity to the earlier fractions is indicated in Tables 8 and 9.

TABLE 1 - Dose Dependent Mutagenicity of ETM-18 in the Presence of Aroclor 1254-Induced S-9

Compound	Amount/Plate	TA1535	TA1538	TA98	TA100
DMSO ^b	0.1 ml	16 ⁺ 5	9 ⁺ 3	19 ⁺ 5	123 ⁺ 35
MNNG	Spot	<u>+4^c</u>			
ACNA	150 µg		<u>1167⁺260</u>	<u>3047⁺18</u>	
MMS	1.3 mg				<u>1657⁺506</u>
DMSO ^d	0.1 ml	26 ⁺ 13	20 ⁺ 6	29 ⁺ 9	133 ⁺ 20
2AA	5 µg				<u>1488⁺523</u>
ETM-18	0.8 µg			25	155
	1.5 µg			36	134
	2.0 µg			31	263
	4.2 µg			70	166
	4.5 µg	29	36	47	163
	8.0 µg			<u>155</u>	173
	8.3 µg			47	186
	9.5 µg			<u>65</u>	150
	11.0 µg			<u>152</u>	202
	15.0 µg			<u>436</u>	<u>307</u>
	19.0 µg			<u>128</u>	196
	21.5 µg			<u>235</u>	212
	22.3 µg	27	<u>94</u>	<u>126</u>	190
	41.6 µg			<u>814</u>	<u>556</u>
	44.6 µg	31	<u>179</u>	<u>602</u>	264
	74.7 µg			<u>1599</u>	<u>918</u>
	83.2 µg			<u>1596</u>	<u>613</u>
	95 µg			<u>662</u>	<u>384</u>
	108 µg			<u>1332</u>	<u>730</u>
	149 µg			<u>2274</u>	<u>996</u>
	190 µg			<u>986</u>	<u>694</u>
	215 µg			<u>1793</u>	<u>736</u>
	223 µg	41	<u>733</u>	<u>1373</u>	<u>648</u>
	446 µg	45	<u>1111</u>	<u>2170</u>	<u>1114</u>

^a Numbers are means of colony counts, n ≥ 4. Standard deviations are included for control plates. Underlined numbers are 2 x the spontaneous rate.

^b DMSO = dimethylsulfoxide; MNNG = N-methyl-N'-nitro-N-nitrosoguanidine; ACNA = 1-amino-2-carboxy-4-nitroanthraquinone; MMS = methyl methanesulfonate; 2AA = 2-aminoanthracene.

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

^d These and subsequent plates received 50 µl/plate Aroclor-induced S-9.

TABLE 2

DISTRIBUTION OF ETTM-18 INTO ORGANIC SOLVENTS

Sample	Extraction Solvent	Amount ^a Extracted		Colonies/mg ^b		Normalization of Colonies/mg Based on Whole Sample	
		(gm)	(%)	TA98	TA100	TA98	TA100
ETTM-18A	hexane	0.1096	1.95	8466	5940	165	116
	toluene	1.2279	21.88	6930	3130	1516	685
	methylene chloride	0.6934	12.35	107420	47400	13266	5854
	acetonitrile	0.0447	0.8	8310	2780	66	22
	non-extractable	3.5372	63.02	4360	-	2747	-
Sum of Fractions		100				17760 (94.8%) ^c	6677 (116.5%)
Entire Sample		100				18740	5730
ETTM-18B	hexane	0.0966	1.93	4130	10340	80	200
	toluene	1.1719	23.37	99980	70300	23365	16429
	methylene chloride	0.4985	9.94	232600	118000	23120	11729
	acetonitrile	0.0339	0.68	8270	3370	56	23
	non-extractable	3.2133	64.08	7450	-	477	-
Sum of Fractions		100				47098 (698.8%)	28381 (960.4%)
Entire Sample		100				6740	2955
ETTM-18A+B	hexane	1.94±0.01 ^d		4240 ^e	9440 ^e	82	183
	toluene	22.63±1.05		6230	46530	1410	10530
	methylene chloride	11.14±1.70		271800	139600	30278	15551
	acetonitrile	0.74±0.08		8260	3050	61	23
	non-extractable	63.55±0.75		4710	-	2993	-
Sum of Fractions		100				34824 (235.3%)	26287 (411.4%)
Entire Sample		100				14800	6390

^aInitial weights prior to extraction: ETTM-18A, 5.0903 gm; ETTM-18B, 4.6171. The sum of the organic extract weights for ETTM-18A was 5.6128 gm, a gain of 10.26%; for ETTM-18B, 5.0142, a gain of 8.60%.

^bCalculated from the linear portion of dose response curves, 4 plate/dose point; - = no linear response.

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

^dNumbers are means ± range of percentages in above 2 sections.
^eCalculated from linear regression analysis of all dose data points from both extractions.

TABLE 3
Mutagenicity of ETM-18

His ⁺ ^a Colonies/ μ g			
TA1535	TA1538	TA98	TA100
- ^b	3.18	13.95	8.66

^aCalculated from linear portion of dose response curve:

TA1538	n = 20	r = 0.9905
TA98	n = 91	r = 0.9106
TA100	n = 78	r = 0.8790

^b- = No dose response.

TABLE 4

Mutagenicity of ETTM-05 and Fractions in the Presence
of 50 µl/Plate Aroclor 1254-Induced S9

Compound	Starting Concentration (mg/ml)	Amount/plate	Revertant Colonies/Plate ^a			
			TA1535	TA1538	TA98	TA100
DMSO ^b		0.1 ml	16 ⁺ 5	9 ⁺ 3	21 ⁺ 5	118 ⁺ 22
		spot	<u>+4^c</u>			
		150 µg		<u>1167⁺ 260</u>	<u>3113⁺ 652</u>	
		1.3 mg				<u>1539⁺ 471</u>
		0.1 ml	26 ⁺ 13	20 ⁺ 6	31 ⁺ 13	128 ⁺ 20
		5 µg				<u>1380⁺ 449</u>
Whole Sample	10	0.1 ml of 1%			32	120
		5%			37	136
		10%			31	
		50%			43	158 ^e
		100%			52	139 ^e
Whole Sample	50	0.1 ml of 1%	20	32	48	181
		5%	28	<u>42</u>	<u>66</u>	168
		10%	36	<u>43</u>	54	183
		50%	38	<u>41</u>	52	147
		100%	30	<u>41</u>	<u>64</u>	249
Hexane Extract		5.9 µg			34	122
		9.6			27	163 ^e
		10			50	ND
		30			47	134 ^e
		48			29 ^e	122 ^e
		59			38	139 ^e
		96			26	118
		480			35	144 ^e
Toluene Extract		1.75 µg			52	147
		3.5			50	200
		6.7			35	132
		8.0			28	152
		17.5			58	150
		33.6			32	133
		35.0			<u>104</u>	248

TABLE 4

Mutagenicity of ETM-05 and Fractions in the Presence of 50 μ l/Plate Aroclor 1254-
Induced S9 (continued)

<u>Compound</u>	<u>Starting Concentration (mg/ml)</u>	<u>Amount/plate</u>	<u>Revertant Colonies/Plate^a</u>			
			<u>TA1535</u>	<u>TA1538</u>	<u>TA98</u>	<u>TA100</u>
Toluene Extract (cont'd.)		40.0 μ g			41	154
		67.2			30	136
		80.2			47	202
		336			46	216
		401			<u>91</u>	<u>315</u>
		672			50	231
Methylene Chloride		0.6 μ g			33	140
		2.5			47	120
		3.2			39	145
		4.5			51	244
		6.4			32	177
		12			48	101
		23			56	165
		25			56	162
		32			<u>62</u>	<u>286</u>
		45			57	176
		64			<u>71</u>	<u>264</u>
		124			<u>115</u>	<u>272</u>
		227			<u>86</u>	226
		247			<u>76</u>	224 ^e
		454			<u>108</u>	<u>305</u>
Acetoni- trile Extract		1.0 μ g			39	126
		3.2			<u>85</u>	156
		5			42	122
		6			54	248
		10			48	144
		16			58	133
		30			<u>71</u>	254
		32			50	150
		50			<u>76</u>	231
		60			<u>68</u>	138
		99			54	ND
		160			59	<u>278</u>
					62	196*

TABLE 4 (continued)

Mutagenicity of ETTM-05 and Fractions in the Presence of
50 μ l/Plate Aroclor 1254-Induced S9

Compound	Starting Concentration (mg/ml)	Amount/plate	Revertant Colonies/Plate ^a			
			TA1535	TA1538	TA98	TA100
Residue		0.27 μ g			26	133
		1.4			28	129
		2.5			38	119
		2.7			41	108
		3.6			30	129
		12.4			56	132
		13.5			43	152
		18			34	124
		25			52	119
		27			40	121
		36			37	130
		124			54	136
		182			<u>71</u>	136
		247			<u>73</u>	161
		364			45	146

^a Numbers are means of colony counts, $n \geq 2$. Standard deviations are included for control plates. Underlined numbers are at least 2 x the spontaneous rate. (*n = 1)

^b DMSO = dimethylsulfoxide
MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
ACNA = 1-amino-2-carboxy-4-nitroanthraquinone
MMS = methyl methanesulfonate
2AA = 2-aminoanthracene
ND = Not Done

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

^d These and subsequent plates received 50 μ l/plate Aroclor-induced S9.

^e Evident toxicity on some plates counted.

TABLE 5

Mutagenicity of ETM-12 in the Presence of
50 µl/Plate Aroclor-Induced S9

Compound	Starting Concentration (mg/ml)	Amount/plate	Revertant Colonies/Plate ^a			
			TA1535	TA1538	TA98	TA100
DMSO ^b		0.1 ml	16 ⁺ 5	9 ⁺ 3	19 ⁺ 5	177 ⁺ 25
MNNG		spot	<u>+4^c</u>			
ACNA		150 µg		<u>1167⁺260</u>	<u>3071⁺882</u>	
MMS		1.3 mg				<u>1533⁺456</u>
DMSO ^d		0.1 ml	26 ⁺ 13	20 ⁺ 6	37 ⁺ 13	137 ⁺ 15
2AA		5 µg				<u>1300⁺481</u>
Whole Sample	10	0.1 ml of 1%			32	118
		5%			33	140
		10%			37	134
		50%			45	140
		100%			54	149
Whole Sample	50	0.1 ml of 1%	30	34	38	136
		5%	28	<u>41</u>	59	163
		10%	40	<u>42</u>	40	178
		50%	42	<u>54</u>	<u>84</u>	214
		100%	35	37	<u>79</u>	181
Hexane Extract		5 µg			29 ^e	81
		7.14			37	132
		26			32 ^e	101 ^e
		36			48	151
		51			39	115
		71.4			47	130
Toluene Extract		1.4 µg			38	134
		1.5			56	132
		7.2			48	156
		7.5			49	171
		14.4			53	170
		15			51	171
		72			57	240
		75			50	161
		144			56	ND

(continued)

TABLE 5 (continued)
 Mutagenicity of ETM-12 in the Presence of 50 μ l/Plate
 Aroclor-Induced S9

Compound	Starting Concentration (mg/ml)	Amount/plate	Revertant Colonies/Plate ^a			
			TA1535	TA1538	TA98	TA100
Methylene Chloride Extract		3.2 μ g			44	184
		4.6			56	151
		16			44	153
		23			51	169
		32.4			52	146
		45.6			68	189
		162			51	200
		228			54	186
		324			70	213 ^e
		456			76	198
Acetoni- trile Extract		0.14			42	143
		0.7			38	176
		1.4			40	182
		5.8			50	114
		7			48	179
		14			62	ND
		29			54	121
		58.5			59	180
		293			64	190
		585			72	172
Non-extract- able Material		0.02 μ g			28	118
		0.1			27	120
		0.2			28	126
		1			39	155
		2			47	180
		6.5			48	181
		32.4			46	204
		64.8			43	129
		324			53	151
		648			54	162

^a Numbers are means of colony counts, $n \geq 2$. Standards deviations are included for control plates. Underlined numbers are at least 2 x the spontaneous rate.

(footnotes continued)

TABLE 5 - (continued) Mutagenicity of ETM-12 in the Presence of
50 μ l/Plate Aroclor-Induced S9

^b
DMSO = dimethylsulfoxide
MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
ACNA = 1-amino-2-carboxy-4-nitroanthraquinone
MMS = methyl methanesulfonate
2AA = 2-aminoanthracene
ND = Not Done

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

^dThese and subsequent plates received 50 μ l/plate Aroclor-induced S9.

^eEvident toxicity on some plates counted.

TABLE 6
DISTRIBUTION OF ETM-05 INTO ORGANIC SOLVENTS

Sample	Extraction Solvent	Amount ^a Extracted		Colonies/mg ^b		Normalization of Colonies/mg based on Whole Sample	
		(gm)	(%)	TA98	TA100	TA98	TA100
ETM-05A	hexane	0.0049	0.10	-	-	-	-
	toluene	0.0129	0.26	1680	20670	4.4	53.7
	methylene chloride	0.0434	0.89	194	356	1.7	3.2
	acetonitrile	0.0253	0.52	-	922	-	4.8
	non-extractable	4.7959	98.23	-	-	-	-
Sum of Fractions			100			6.1	61.7
Entire Sample			100			-	-
ETM-05B	hexane	0.0001	0.02	-	-	-	-
	toluene	0.0152	0.32	146	457	0.5	1.5
	methylene chloride	0.0511	1.08	627	1240	6.8	13.4
	acetonitrile	0.0212	0.45	800	2150	3.6	9.7
	non-extractable	4.6648	98.14	-	-	-	-
Sum of Fractions			100			10.9	24.6
Entire Sample			100			-	-
ETM-05C	hexane	0.0175	0.37	-	-	-	-
	toluene	0.0427	0.90	-	280	-	2.5
	methylene chloride	0.1736	3.68	656	5080	24.1	186.9
	acetonitrile	0.0021	0.04	1290	4410	0.5	1.8
	non-extractable	4.4844	95.00	-	-	-	-
Sum of Fractions			100		24.6		191.2
Entire Sample			100		-		-
ETM-05A,B,C	hexane	0.16 ⁺ 0.18 ^c		-	-	-	-
	toluene	0.49 ⁺ 0.35		1110 ^d	344	5.4	1.7
	methylene chloride	1.88 ⁺ 1.56		609	1350	11.4	25.4
	acetonitrile	0.34 ⁺ 0.26		- ⁺	3640	-	12.4
	non-extractable	97.12 ⁺ 1.84		-	-	-	-
Sum of Fractions			100			16.8	39.5
Entire Sample			100			-	-

^aInitial weights prior to extraction:

ETM-05A, 4.8824 g; 05B, 4.9519 g; 05C, 5.0896. The sum of the organic extract weights for ETM-05B was 4.7533 g, a loss of 401%; for 05C, 4.7203 g, a loss of 7.25%. 05A nonextractable fraction could not be sufficiently dried so as to obtain an accurate weight.

^b+ = mutagenic but not linear.

^cNumbers are means ⁺ range of percentages in above 2 sections.

^dCalculated from linear regression analysis of all dose data points from both extractions.

TABLE 7 - DISTRIBUTION OF ETM-12 INTO ORGANIC SOLVENTS

Sample	Extraction Solvent	Amount ^a Extracted		Colonies/mg ^b		Normalization of Colonies/mg based on Whole Sample	
		(gm)	(%)	TA98	TA100	TA98	TA100
ETM-12A	hexane	0.0070	1.45	-	-	-	-
	toluene	0.0160	0.33	-	5320	-	17.6
	methylene chloride	0.0428	0.89	-	1220	-	10.8
	acetonitrile	0.0590	0.81	-	-	-	-
	non-extractable	4.9644	96.5	-	-	-	-
	Sum of Fractions		100			-	28.4
Entire Sample							
ETM-12B	hexane	0.0041	0.09	-	-	-	-
	toluene	0.0250	0.57	-	1460	-	8.3
	methylene chloride	0.0578	1.32	-	2227	-	
	acetonitrile	0.0211	0.48	-	-	-	-
	non-extractable	4.2590	97.53	-	-	-	-
	Sum of Fractions		100				8.3
Entire Sample							
ETM-12A+B	hexane	0.77 ⁺ 0.96 ^c		-	-	-	-
	toluene	0.45 ⁺ 0.17		-	1460 ^d	-	6.6
	methylene chloride	1.10 ⁺ 0.30		-	1902	-	20.9
	acetonitrile	0.64 ⁺ 0.23		-	-	-	-
	non-extractable	97.00 ⁺ 0.71		-	-	-	-
	Sum of Fractions		100			-	27.5
Entire Sample							

^aInitial weights prior to extraction: ETM-12A, 4.9883 gm; ETM-12B, 51.361. The sum of the organic extract weights for ETM-12A was 4.8110 gm, a loss of 3.7%; for ETM-12B, 4.3670, a loss of 14.97%.

^bCalculated from the linear portion of dose response curves, 4 plate/dose point; - = no linear response.

^cNumbers are means $\pm$ range of percentages in above 2 sections.

^dCalculated from linear regression analysis of all dose data points from both extractions.

TABLE 8

Distribution by Weight into Organic Solvents

Extraction Solvent	Amount Extracted (%)						
	01	02	08	09	11	15	16
Previously Reported Extracts ^a							
Hexane	24.47	0.46	53.18	53.45	0.71	65.67	79.72
Toluene	67.96	22.88	25.13	29.93	42.42	13.70	12.17
Methylene Chloride	3.87	56.36	14.32	13.69	19.31	4.92	6.82
Acetoni- trile	0.19	1.60	2.33	0.30	0.78	0.15	0.13
Non-extract- able	3.51	18.72	5.03	2.63	36.80	15.55	1.17
Extracts for Chemical Analysis ^b							
Hexane	12.53	0.44	67.86	61.60	0.62	58.20	85.71
Toluene	75.06	31.49	20.04	20.43	38.39	18.40	11.57
Methylene Chloride	4.67	39.86	8.69	13.68	21.47	6.80	2.28
Acetoni- trile	0.23	1.73	0.49	0.35	1.04	.002	0.17
Non-extract- able	7.51	26.48	7.91	3.94	38.49	16.60	0.28

^a Values are means of percentages by weight from one set of extracts for ETM-02, five sets for 11 and three sets for all other samples.

^b Values are percentages.

TABLE 9

Comparative Mutagenic Activities of Fraction Sets

Extraction Solvent	Colonies/ μ g						
	01	02	08	09	11	15	16
Previously Reported ^a Extracts							
Hexane	7.64	4.24	5.86	7.70	15.17	2.22	0.99
Toluene	16.49	5.47	20.63	27.24	24.72	3.38	4.12
Methylene Chloride	5.22	7.20	13.05	21.06	12.04	7.31	6.52
Acetonitrile	- ^b	6.37	4.43	108.40	33.30	27.15	5.38
Non-extractable	-	0.35	0.48	0.77	2.50	0.05	0.16
Extracts for Chemical ^c Analysis							
Hexane	6.81	3.11	5.18	12.11	24.49	1.98	1.69
Toluene	12.70	5.92	13.41	24.59	49.26	4.86	7.11
Methylene Chloride	13.27	10.26	19.53	13.67	19.50	17.24	3.35
Acetonitrile	3.60	16.56	13.87	215.60	65.34	ND ^d	ND
Non-extractable	-	1.05	0.28	0.89	7.68	-	1.86

^a Values are slopes of least squares lines derived from colony counts of repeated assays on two sets of extracts for all but ETTM-11 and 02 which were from repeat assays on one set of extracts.

^b - = No dose dependent mutagenicity.

^c Values are slopes of least squares lines from repeat assays on one set of extracts.

^d ND = Not Done in order to conserve sample for chemical analysis.