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Genotoxic Activity of Particulate Material in Petroleum Refinery Effluents

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The discharge of industrial effluents into waters which are sources of drinking water has prompted concern over the potential health effects of these practices (Zoeteman, 1977). Epidemiological studies have presented evidence for an increased cancer risk to populations drinking water from contaminated sources (Crump and Guess, 1980.)

The petroleum industry is a major user of water for the upgrading and refining of crude oil. An average of 200 l of water is required to refine each 42 gal barrel of crude oil. Canada alone refines about 2 million barrels of crude oil per day.

The purpose of this study was to evaluate the genotoxic hazard associated with the discharge of suspended particulates in oil refinery effluents. Particulate extracts were tested by in vitro assays for mutagenic (Ames test) and clastogenic (sister chromatid exchange assay) activity, both with and without mammalian microsomal activation.

MATERIALS AND METHODS

Effluents were collected from three refineries in Ontario, Canada. Refinery 1 was sampled several times during 1981 to 1983. Refinery 2 was sampled once during February of 1983, and Refinery 3 was sampled in February and June of 1983. "Grab" samples of 4 l were collected in glass solvent bottles, stored at 4°C under nitrogen, and processed within 96 h of collection.

Particulates were separated from samples by continuous centrifugation at 10,000 rpm (250 ml/min flow rate), and placed in a glass extraction thimble (0.4 μm frit) for soxhlet extraction into methanol (6 h). Extracts were evaporated to approximately 15 ml on a rotary evaporator, and dried by passing through sodium sulfate. The dried sample was rotary-evaporated to 2 ml, and then evaporated just to dryness under a stream of nitrogen. After weighing the residue, extracts were made to volume in acetone.

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Extracts were subfractionated into acid, base and neutral fractions by the method of Bryant and McCalla (1982), with the exception that diethyl ether was used instead of cyclohexane as the partitioning solvent. Extracts were also subfractionated by silica gel column chromatography according to the method of Coleman et al. (1980). Concentrated extracts (15-25 mg dry weight were aplied to a micro-column packed with 0.2 g of 5% deactivated silica gel, and fractionated by eluting with: (1) 0.5 ml of hexane, (2) 1 ml of hexane, (3) 4 ml of hexane, (4) 4 ml of hexane/benzene (1:1), (5) 4 ml of benzene, (6) 4 ml of methylene chloride, (7) 4 ml of methanol.

Particulate effluent extracts were analyzed with a Varian 3700 gas chromatograph equipped with a cold on-column injector and flame ionization detector. A fused silica capillary column was used (30 m x 0.32 mm I.D., 0.1 um DB-5), and all analyses were performed with a temperature program of 60^{o} to 320^{o} at 5^{o}/min.

The Salmonella/mammalian microsome mutagenicity assay was conducted essentially as described by Ames et al. (1975) using strains TA98 and TA100. Because of poor solubility of refinery extracts in DMSO, samples were dissolved in acetone. Rat-liver microsome solutions (S-9) were prepared from Aroclor 1254-induced rats as described by Ames et al. (1975). The number of revertant colonies for each treatment was calculated from the mean of 3 replicate plates.

In the sister chromatid exchange (SCE) assay, Chinese hamster ovary (CHO) cells were cultured in αMEM medium with 10% fetal calf serum. To start the assay, cells were inoculated into 25 ml of medium in 250 ml tissue culture flasks, and incubated for 12 h. When metabolic activation was required, fresh medium (without serum), extracts, and 10% S-9 mix (prepared as in the Ames asay) were added. Effluent extracts (0.1 ml) were added to culture flasks and incubated for 1 h, or in some tests for 2.5 h. The medium was then changed and 10^{-5} M 5-bromodeoxyuridine (BrdU) added. The flasks were wrapped in aluminum foil and incubated for 22 h. After 22 h, 2 ug/ml of colchicine was added to each flask, and 2 h later, the cells were harvested by shake-off. The culture was approximately 50% confluent at this point.

The harvested cells were treated for 7 min with 10 ml of hypotonic solution at 37^{o}C (0.075 M KCl), and hardened by the addition of a further 2 ml of 3:1 methanol-acetic acid fixative. After 15 min hardening, cells were centrifuged and fixed overnight in methanol-acetic acid. Metaphases were spread on slides, and after drying for 1 week, stained with 5 mg/100 ml of Hoechst 33258 fluorochrome. Slides were flooded with Sorensen's buffer (pH = 8.0) and exposed to black light for 3 min at 50^{o}C. Slides were then stained with Geimsa (4% solution for 6 min), dried, and mounted. The number of SCE's were scored for 25 metaphases per treatment. All samples were tested for a dose-response.

RESULTS and DISCUSSION

Effluent streams containing treated "process water" were sampled at all three refineries. Final effluent samples taken at Refineries 1 and 2 consisted of treated process water, and samples from Refinery 3 consisted of mixed cooling and treated process water (3:1 ratio). The oil refined in these facilities was primarily western Canadian light crude, although tar sands synthetic crude was occasionally mixed into conventional crudes at Refinery 1.

Particulate extracts were initially tested over a wide range of concentrations in the Ames assay to define the dose required to yield a maximum mutagenic response with minimum toxicity to the test organism. Extracts from the particulates centrifuged from a volume of 20 ml to 320 ml of effluent (20-320 "ml equivalents") generally gave a linear dose-response in the Ames assay, but sample concentrations above 320 ml equivalents were toxic (Fig. 1). The problem of toxicity in the Ames assay can only be circumvented by adjusting the mutagen dose to a range that produces a linear response below the toxic range (Maron and Ames, 1983).

A comparison of Ames tests of particulate extracts conducted with and without rat S-9 (Fig. 1) indicates that the numbers of revertants were below spontaneous levels when no S-9 was added. This suggests that metabolic activation was necessary for a mutagenic response, and that toxic activity was reduced by the addition of S-9; probably because of adsorption of toxicants to microsomes, or detoxification through metabolic pathways.

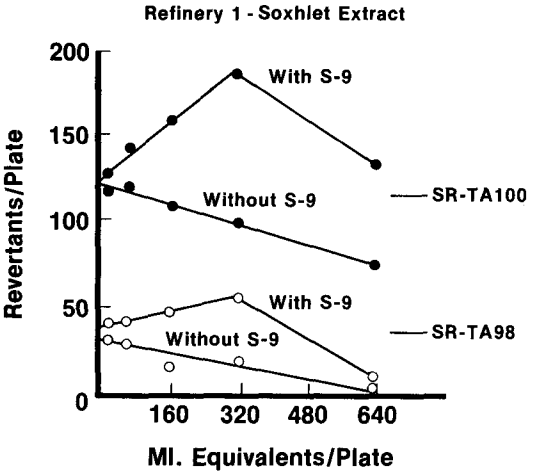

Figure 1 Mutagenic response of Ames tester strains TA98 and TA100 (with and without S-9) to a particulate extract of Refinery 1 effluent collected in October 1981.

Strain TA100 was the most sensitive to reversion by particulate extracts from all three of the refineries. Figure 2 illustrates the relative mutagenic activity of refinery samples tested using strain TA100 with S-9 activation. Data presented here are limited to the highest concentration within the linear portion of the dose-response curves generated for each effluent sample (320 ml

equivalents). The mutagenic activity of particulate extracts from Refinery 1 declined over the sampling period. Extracts from Refineries 2 and 3 were sufficiently mutagenic to yield a doubling over background revertants at the concentrations tested. In Ames tests of single compounds, a doubling of the numbers of revertants over spontaneous levels, and a dose-response are considered evidence of significant mutagenicity (Johnson and Hopke, 1980). Extracts from cooling water (controls) collected at all 3 refineries did not elevate numbers of revertants above spontaneous levels at concentrations used to test effluent samples.

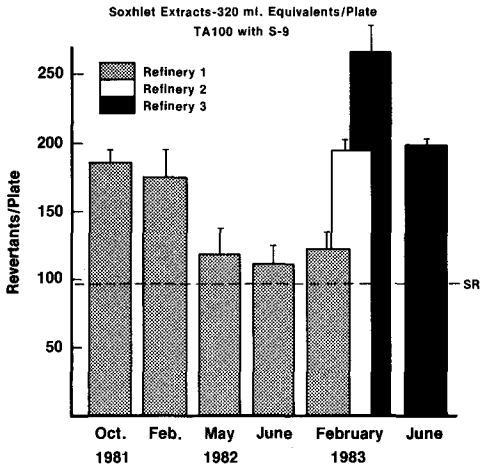

Figure 2 Mutagenicity of particulate extracts from effluents sampled at three refineries (1981-1983) using strain TA100 with S-9, at a dose of 320 ml equivalents per plate.

A mutagenic soxhlet extract from Refinery 3 (February, 1983 sample) significantly increased the numbers of sister chromatid exchanges in CHO cells with S-9 activation (Table 1). Incubation of CHO cells with extract and S-9 for 2.5 h produced the greatest response. All other samples taken from the three refineries in 1983 did not give a statistically significant positive response in the SCE assay, with or without S-9.

Table 1 Results of a sister chromatid exchange assay with S-9 activation for a February, 1983 particulate extract from Refinery 3. Standard deviations for mean SCE's are presented in brackets.

Sample	Ml Equiv/Flask	Mean and S.D. of SCE's per Metaphase	
		1h incubate	2.5h incubate
Control	-	4.3(2.2)	4.6(2.0)
Refinery 3	160	6.4(1.8)*	10.7(2.0)*
	80	6.7(1.6)	9.1(2.9)*
	40	4.8(2.4)	8.8(1.8)*

*Mean SCE's significantly different from controls

The acid/base/neutral fractionation of a refinery 3 sample indicated that mutagenicity was retained in the neutral fraction (Figure 3). However, a portion of the mutagenic activity (as well as 26% of the residue weight) was lost during the fractionation process. In other refinery samples fractionated by this method, mutagenicity was always retained in the neutral fraction.

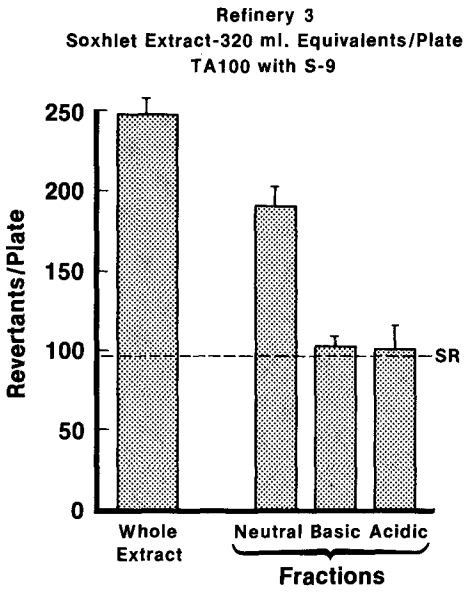

Figure 3 Mutagenicity of whole particulate extract, and acid/base/neutral subfractions from a Refinery 3 sample (February, 1983) tested with TA100 and S-9 at a dose of 320 ml equivalents per plate.

Capillary column gas chromatography (GC) with flame ionization detection was performed on neutral fractions of selected particulate extracts. As illustrated in Figure 4, the samples were complex, with a few major peaks superimposed on an envelope of unresolved components. Differences were observed between particulate extracts from different refineries. For example, the neutral fraction of a Refinery 3 particulate extract, with relatively high mutagenic activity (Fig.3), gave the GC pattern illustrated in Figure 4b. A neutral fraction from Refinery 1, with little mutagenic activity contained less material, with a lower range of molecular weights (Fig. 4c). With such complex mixtures, however, it is not possible to draw any correlations between GC patterns and genotoxic activity. Preliminary analyses by combined GC-MS have established the components of these mixtures to be primarily aliphatics, alkyl-substituted aromatics, and di-alkyl phthalates. Di-alkyl phthalates were a major component of the mutagenic sample from Refinery 3 (Fig. 4b) and are commonly found in ppb quantities in Canadian refinery effluents (PACE, 1981).

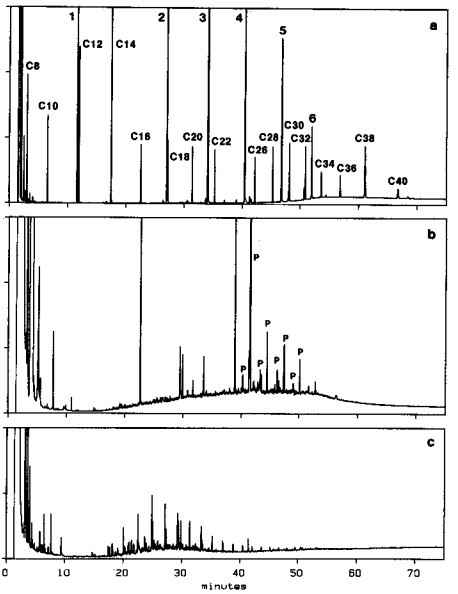

Figure 4 a) Gas chromatogram of standards: n-alkanes (C8 to C40), napthalene (1), phenanthrene (2), pyrene (3), chrysene (4), benzo[a]-pyrene (5), and picene (6). b) Gas chromatogram of particulate extract from Refinery 3 (February, 1983). Peaks marked P are di-alkyl phthalates. c) Gas chromatogram of particulate extract from Refinery 1 (February, 1983).

Selected extracts were subfractionated by column chromatography on silica gel into seven subfractions of increasing polarity. In a particulate sample from Refinery 3 (February, 1983), activity was detected, in order of increasing mutagenicity, in fractions 1, 3, 5 and 7 (Fig. 5a). A sample from Refinery 2 (February, 1983) showed mutagenic activity in fractions 5 and 7, only (Fig. 5b). Dose-response data were generated for all fractions, but only mutagenic activity at 320 ml equivalents per plate is presented. Over 90% of the material applied to silica-gel columns was recovered in the various fractions. GC analysis proved unsuitable for the analysis of the most mutagenic fraction (Fraction 7), but high performance liquid chromatography (HPLC) analyses of the Refinery 3 sample showed that this fraction contained a complex mixture of hydrocarbons.

In 2 of 3 conventional refineries tested we found significant mutagenic activity in the particulate component of effluents, and one particulate extract gave a positive response in an SCE assay. The mutagenic activity of extract residues, calculated on a revertants per mg basis (Table 2), was lower than the activities reported for many other complex mixtures. For example, XAD-2 resin extracts from drinking water gave a response with strain TA100 (without S-9 activation) of 412 to 708 revertants per mg of residue (Nestmann et al., 1979). Neutral tar extracts from shale oil induced 50-4000 revertants per mg with TA98 (Pelroy et al., 1981). When a Refinery 3 particulate sample was tested in the SCE assay with S-9 activation (2.5 hr), 160 ml equivalents of sample increased SCE's to 10.7 from a control value of 4.6 (Table 1). According to the criteria of Latt et al (1981), this sample is a moderately positive inducer of SCE's, since it induced a three point dose-response with one dose showing a two-fold increment in

SCE's above controls.

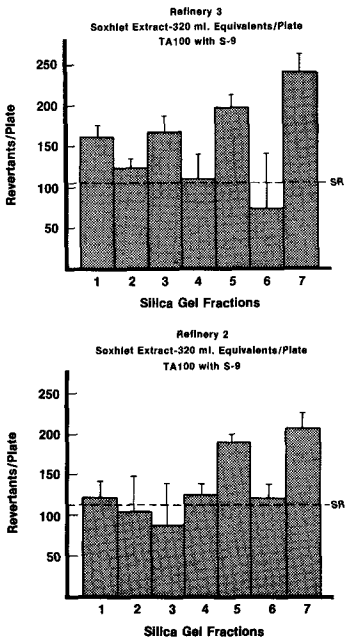

Figure 5 Mutagenicity of silica-gel subfractions from particulate extracts of effluents from Refineries 2 and 3. Results are shown for test strain TA100 plated with S-9 at a dose of 320 ml equivalents per plate.

Table 2 Mean and range of mutagenic activity (revertants per mg) of particulate residues, and rate of discharge from refineries. Residues were tested with TA100 (with S-9).

Refinery	No. Samples	Residue Mutagenicity (Revert./mg)	Residue Per Litre (mg)	Residue Discharge (kg/day)
1	5	64(52-73)	25(17-37)	100[a]
2	1	123	24	290[b]
3	2	101(97-105)	40(36-43)	960[c]

a) Effluent discharge from refinery 1 averages 4×10^6 l/day
b) Effluent discharge from refinery 2 averages 12×10^6 l/day
c) Effluent discharge from refinery 3 averages 24×10^6 l/day

The low genotoxic activity of residues from particulates would seem to indicate that the discharge of mutagens in refinery effluents is low. However, the amount of this residue released in particulates from a refinery may total tonnes per day (Table 2). Based upon a mutagenic response of 52 revertants per µg of benzo[a]

pyrene in the Ames test (TA100 with S-9), each mg of residue extracted from Refinery 3 particulates contains mutagenic activity equivalent to 1.9 μg of benzo[a]pyrene. Therefore, discharges from this refinery are equivalent in mutagenic yield to approximately 1.9 kg of benzo[a]pyrene per day. It is possible that long-term accumulation of genotoxic compounds from refineries may represent a hazard to sources of drinking water. The magnitude of this hazard would depend upon parameters affecting the fate of the suspended particulates and associated genotoxic compounds.

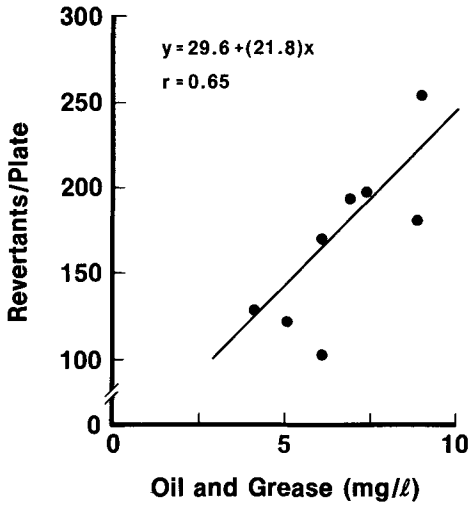

Figure 6 Relationship between mutagenicity of particulate extracts at a dose of 320 ml equivalents per plate (TA100 with S-9) and the concentration of oil and grease in the effluents of 3 refineries (1981-1983).

When particulates were removed from effluent samples by centrifugation, over 95% of the oil and grease were lost from the effluent. It is probable that most of material classified as particulates in this study consisted of immiscible oil and grease. Figure 6 indicates that the mutagenic activity of refinery particulate extracts is positively associated with the levels of oil and grease in the sample ($r = 0.65$).

GC analyses have shown that neutral components of effluent extracts contain complex mixtures of aliphatic and aromatic hydrocarbons. The majority of mutagenic activity in extracts from Refineries 2 and 3 was present in the most polar silica gel fraction (Fraction 7). The polar nature of mutagenic subfractions indicates that previous studies of the levels of genotoxic compounds in refinery effluents (Andelman and Suess, 1970; Ershova, 1967) have been too selective in limiting chemical analysis to non-polar PAH compounds. Studies of the mutagenicity of various synthetic fuels have indicated that Ames-positive mutagens are concentrated in polar, nitrogen-rich fractions (Pelroy, et al., 1981).

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