MUTAGENIC AND GENOTOXIC POTENTIAL OF NITRATED POLYAROMATIC HYDROCARBONS IN COMBUSTION BYPRODUCT MIXTURES

BLAIR PRITCHETT

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by

Blair Pritchett, B.Sc., L.L.B.

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ABSTRACT

The combustion of hydrocarbon fuels generates a considerable amount of reaction byproducts, some of which are mutagenic and DNA-reactive nitro-polyaromatic hydrocarbons. This mutagenic action is generally indirect, such that the compounds require metabolic activation for the exertion of their toxic effect.

Used motor oil extracts prove more mutagenic than crude oil extracts in the Ames *Salmonella* assay for mutagenicity. This mutagenic effect is enhanced by the presence of a rat liver microsomal and cytosolic protein preparation, as well as by the use of a bacterial strain enriched in *O* -acetylase activity. The mutagenic response is diminished in a bacterial strain that is deficient in bacterial nitroreductase. These data implicate nitrocompounds as principal agents in the overall mutagenicity of crankcase oil extracts.

Crankcase oil extracts prove to be good substrates for nitroreductase enzyme activity *in vitro*. The effectiveness as a substrate of nitroreductase enzyme(s) also seems to correlate with the mutagenicity as measured in the Ames *Salmonella* assay. The measured nitroreductase activity exhibits no appreciable difference when NADH or NADPH is used as the reaction cofactor.

The activity of nitroreductase enzymes can produce reactive oxygen species such as hydroxyl radicals ($OH^{\cdot}$), which can generate single strand nicks in DNA. This effect can be initiated by the metabolism of nitrated polynuclear aromatic hydrocarbons by mammalian liver enzymes. 1-nitropyrene and crankcase oil extracts are shown here to be capable of producing

such nicks in the DNA of the plasmid pBR322.

In Memory of

H. Roy Taylor (1916 - 1996)
and
Cecil B. Pritchett (1908 - 1999)

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LIST OF ABBREVIATIONS

Amp	ampicillin
ATP	adenosine triphosphate
B[*a*]P	benzo[*a*]pyrene
cc oil	crankcase
ddH_2O	distilled and deionized water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra acetic acid - disodium salt
HPLC	high performance liquid chromatography
KP	potassium phosphate buffer
3-MC	3-methylcholanthrene
min	minutes
NADH	nicotinamide adenine dinucleotide, reduced form
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NaP	sodium phosphate buffer
1-NP	1-nitropyrene
PAH	polynuclear aromatic hydrocarbons
PEI	polyethylene impregnated

RNA	ribonucleic acid
ROS	reactive oxygen species
TLC	thin layer chromatography

CHAPTER 1

1. INTRODUCTION

1.1 Hydrocarbon combustion

The use of hydrocarbon mixtures as fuel has become a staple in this industrial age of humankind. Hydrocarbons are used to power motorized vehicles, heating systems, and even electricity generating systems, as is done here in Newfoundland. A conventional, and oversimplified, view of the process of hydrocarbon combustion has been that their burning leads to the production of carbon dioxide and water. While this theoretical picture is quite sanitary, the actual business of burning hydrocarbons, especially fossil fuels, is vastly different.

In reality, the combustion of hydrocarbons produces a plethora of compounds, some of which are quite toxic. The reason for this lies in the fact that incomplete combustion is often the rule when considering the use of these compounds in modern society. For example, the intense heat and pressure generated inside the modern automobile engine leads to the presence of many partial combustion products in the petroleum oil lubricant, as compounds which are formed from fuel combustion dissolve in the crankcase oil. Improper disposal of this used oil can then contaminate both the soil and aquatic environments.

The deleterious effects of exposure to combustion byproducts had been seen as far back as 200 years ago, but were poorly understood until recently. The eighteenth century British physician Percival Pott noticed an abnormal incidence of scrotal cancer in young chimney sweeps, which he correctly attributed to the horrendous work environments to which

these boys were exposed. Since that time there have been countless similar discoveries, some of which involve the formation of cancers, and many others which relate genetic mutation and genotoxicity to hydrocarbon combustion byproduct exposure.

1.1.1 Formation of nitrated polyaromatic hydrocarbons

Considerable research has been focussed on such compounds as benzo[*a*]pyrene (B[*a*]P), which has been shown to interact with DNA both *in vitro* (Nishimoto and Varanasi, 1985) and *in vivo* (Culp and Beland, 1994), and other polynuclear aromatic hydrocarbons (PAH), which are commonly formed in combustion reactions. While many of these compounds are quite toxic and often mutagenic, there is a class of compounds formed which are even more reactive and potentially more genotoxic than PAH. These compounds are the nitrated derivatives of PAH which include among them some of the most DNA-reactive and mutagenic compounds yet discovered (Rosenkranz and Mermelstein, 1983).

Nitrated polycyclic aromatic hydrocarbons are formed, in the extreme temperature and pressure conditions of combustion reactions, by the reaction of combustion byproducts with atmospheric nitrogen dioxide (NO_2) and nitric acid (HNO_3)(Pitts et al., 1978). One example of this phenomenon would be the formation of mutagenic nitrated B[*a*]P molecules, which can occur in combustion reactions where B[*a*]P itself is formed (Fu et al., 1994). Similar examples of NO_2-PAH can be found in many combustion residual mixtures including petroleum-based fuel exhaust (Newton et al., 1982; Moller, 1994), cigarette smoke (Jones

et al., 1993), industrial emissions (Khesina et al., 1994) and grilled food (Felton et al., 1994).

The environmental burden of nitro-PAH is considerable. In the early 1980's, it was estimated that passenger cars in the United States would annually generate 15,000 kg of 1-nitropyrene by the turn of the decade (Rosenkranz, 1982). This ominous prediction portrays only part of the story, as there are much more mutagenic forms of nitro-PAH than 1-nitropyrene (McCoy et al., 1983a). It also does not reflect the lion's share of nitro-PAH, which comes from industry. In the United States, a 1980 estimate placed the total mass of NO_2-PAH produced for commercial use at greater than 800 million kg annually (Hartler, 1985). This large scale manufacturing is principally directed toward pesticide production, with minimal contributions from explosives and pharmaceuticals. Industrial activities also produce copious amounts of nitro-PAH in the form of emissions (White, 1985), the total of which is virtually impossible to quantify.

1.2 The metabolism/activation of nitro-PAH

Nitroarenes must undergo metabolic activation to produce the ultimate mutagenic/DNA reactive species. This is illustrated by nitroreductase-knockout bacterial strains which are resistant to nitro compound toxicity (Mason and Josephy, 1985). Williams and Weisburger (1991) illustrate the process of nitro-PAH activation as shown in Figure 1.1. This scheme shows the conversion of the nitro group to the N-hydroxy form, via a nitroso intermediate. Amine groups can also undergo conversion to the N-hydroxy moiety,

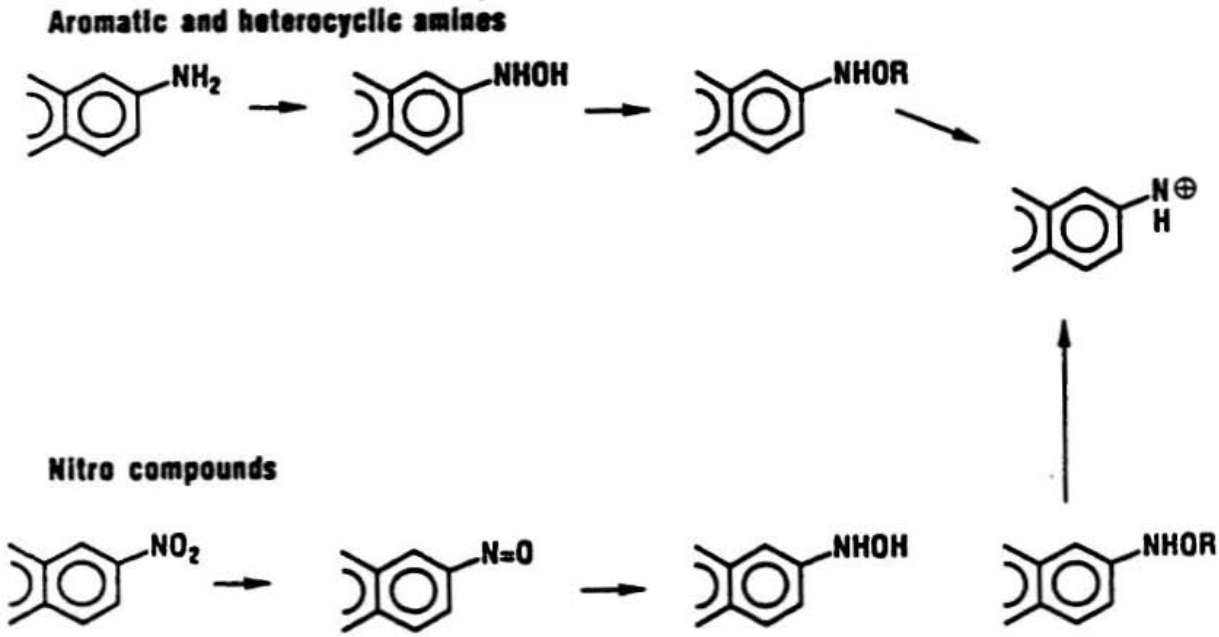


Fig. 1.1 **Metabolism (activation) of nitrated-PAH.**

The pathway of nitroaromatic compound metabolism to the ultimate reactive species, the nitrenium ion.(Taken from: Williams and Weisburger, 1991)

which in either case can be enzymatically conjugated with a suitable functional group (i.e. a sulphate or acetyl group). The sulphate or acetyl group can then nonenzymatically remove itself from the nitrogen atom, under slightly acidic conditions, resulting in the formation of a nitrenium ion. It is the nitrenium ion which subsequently reacts with cellular macromolecules and can cause mutagenic responses in affected cells. This conclusion was also reached by Wild (1990), who suggested that the rate limiting step in the nitroarene-induced mutagenesis in *Salmonella typhimurium* is the reaction of the nitrenium ion with DNA.

1.2.1 The Role of Free Radicals

In addition to the formation of DNA-reactive electrophilic compounds, nitroaromatic compounds can also generate toxic responses via free radical production (as shown in Figure 1.2, below). The initial step in nitroreduction is the two electron reduction of the nitro functionality to yield a nitroso group. Electrons are removed from NADH or NADPH and transferred to the nitro group, via the flavin component of the nitroreductase (Orna and Mason, 1989). When this reduction occurs with only one electron, a nitro radical is formed, which can form superoxide (O_2^-) anion radicals in the presence of molecular oxygen (Mason and Josephy, 1985). The superoxide radical is not in itself a major toxic threat, but it can react with other molecules to form potentially hazardous compounds (Halliwell and Gutteridge, 1989). Alternatively, superoxide can be harmlessly removed via amino thiols such as glutathione (Eyer, 1994).

$$R\text{-}NO_2 + 1e^- \longrightarrow R\text{-}{}^{\bullet}NO_2^-$$

$$R\text{-}{}^{\bullet}NO_2^- + O_2 \longrightarrow R\text{-}NO_2 + {}^{\bullet}O_2^-$$

$${}^{\bullet}O_2^- + {}^{\bullet}O_2^- \longrightarrow H_2O_2 + O_2$$

$$H_2O_2 + Fe^{2+} \longrightarrow OH^- + {}^{\bullet}OH + Fe^{3+}$$

Figure 1.2 **Generation of a hydroxyl radical by nitroreduction reaction in the presence of iron.**

Two superoxide radicals can dismute (combine to produce a more inert molecule) to form hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), either enzymatically, via superoxide dismutase, or nonenzymatically. In the presence of metal ions such as iron(II), copper(I), manganese(II), chromium(V) or nickel (III), hydrogen peroxide can then be cleaved to form one hydroxide anion and one hydroxyl radical (·OH) (Gregus and Klaassen, 1996). The hydroxyl radical itself is a highly reactive molecule which can readily attack cellular macromolecules and, in the case of DNA, can produce strand scission (Rahman et al., 1989).

The effect of DNA strand scission can be easily illustrated in a system of analysis involving plasmid DNA. This technique involves the incubation of plasmid DNA with various chemical or biological preparations. Circular plasmids occur in a supercoiled form which have consistent mobilities when run on an agarose gel. The breakage of a DNA strand results in the relaxing of this supercoil formation, with the result being a circular piece of DNA in a conformation known as a relaxed coil. The relaxed coil DNA is less compact than the supercoiled form and therefore does not run as fast through an agarose gel. This mobility shift can serve as an index of the degree of strand scission levels. In extreme cases, excessive strand scission can produce linear fragments of DNA of varying sizes, and the appearance of these fragments would be indicative of very high rates of hydroxyl radical production (Kukielka and Cederbaum, 1994).

1.2.2 Free radicals and nongenotoxic oxidative damage

In addition to the obvious DNA reactive and genotoxic mechanisms for nitroarene toxicity, a nongenotoxic mechanism has also been identified. The root of this theory is that DNA binding and other genotoxic events cannot explain the specificity seen in terms of species variation and target organs for given carcinogens. Assuming that DNA adduction is the critical event in initiation, the nongenotoxic effects may occur as promotion steps, whereby the growth and conversion to malignancy of an initiated cell is encouraged. Neumann et al. (1994) investigated the known complete carcinogen 2-acetylaminofluorene in comparison with two related compounds which were known to be incomplete carcinogens. They observed only one significant difference, that being the propensity of the complete carcinogen for the stimulation of redox cycling in the mitochondria, possibly as a consequence of disruption of the electron transport chain. This redox cycling created a situation of oxidative stress in affected cells and was presumed to play a vital role in the promotion of liver cancers.

The oxidative stress/respiration interference could promote cancers in any number of ways, as the reduction of ATP formation could reduce the activity of many different enzymes. These enzymes would not necessarily be limited to metabolic functions; they might be responsible for detoxification processes or could be involved in the excision and repair of adducted/damaged nucleotides.

1.3 Nitro-PAH DNA adduction

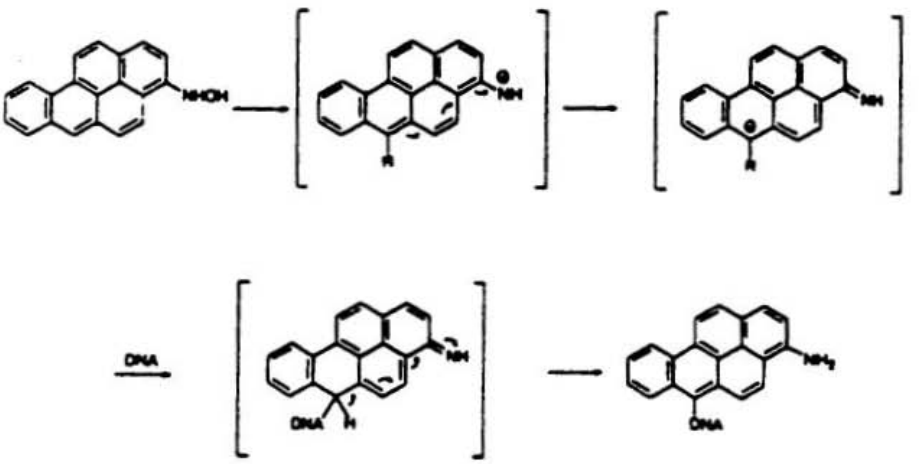
The binding of xenobiotic or endogenous electrophiles to DNA has long been associated with mutagenicity. This adduction of DNA can therefore lead to countless deleterious health conditions for the afflicted organism, including various forms of cancer (Pitot and Dragan, 1996). For this reason, the study of the propensity of compounds to form DNA adducts is of considerable value.

Various species of nitro-PAH have been shown to be potent DNA adducting compounds. 1-Nitropyrene, a commonly occurring nitro-PAH, has been shown to form DNA adducts in Sprague-Dawley rats, CD-1 mice and A/J mice, when injected into the intra peritoneal (i.p.) cavity in tumorigenic doses (Smith et al., 1990). The same effect has also been observed in F344 rats (El-Bayoumy et al., 1994) and *Salmonella typhimurium* bacteria (Messier et al., 1981). 1,6-Dinitropyrene, a disubstituted relative of 1-nitropyrene, has also been identified as a DNA adduct forming compound when administered via the i.p. route to Wistar and Sprague-Dawley rats (Djuric et al., 1993; Wolff et al., 1993). F344 rats also exhibit DNA adducts in lung tissue upon pulmonary exposure of 1,6-dinitropyrene (Beland et al., 1994).

The capability of nitro-PAH to form DNA adducts is not limited to the nitrated pyrenes, though these are some of the more potent species. 2-Nitrofluorene and 2,7-nitrofluorene have been shown to form hepatic DNA adducts in male Wistar rats. This effect can be seen with either oral or intra peritoneal administration (Moller et al., 1993).

As discussed in section 1.2, the nitrenium ion is the usual ultimate reactive species in nitroarene DNA adduction. It is, however, not the only means by which DNA binding can occur. The presence of a nitro substituent can result in the formation of an electrophilic intermediate as portrayed in Figure 1.3, as described by Fu et al. (1994). In this mechanism, a nitro group at the third carbon of B[*a*]P can be hydroxylated, and a nitrenium ion generated. This nitrenium ion, through electron rearrangement, leads to a carbocation at the six position, which can readily react with nucleophilic molecules such as DNA. This mechanism, which leads to a DNA adduct with a free amino group, could be the means by which N-acetylated adducts are formed by nitroarenes.

As alluded to above, it has been determined that two distinct types of DNA adducts, N-acetylated and nonacetylated, can result from *in vivo* exposure to nitro-PAH (Meerman and van de Poll, 1994). The same work also illustrated the differential effects of the two types of adducts. The N-acetylated adducts formed at the C8 position of deoxyguanosine residues are capable of blocking DNA replication. The N-acetyl metabolites of nitroarenes are also associated with the promotional capacity of various carcinogens. This could occur because the suppression of DNA synthesis prevents the DNA editing machinery from correcting mutated adducts while at the same time circumvents the normal process of cellular mitosis. The nonacetylated adducts formed at the C8 position of deoxyguanosine and the N6 position of deoxyadenosine have been correlated with the initiation of preneoplastic cells. This may be the result of the activation of an oncogene or some other deleterious effect of mutagenesis. The relative inability of nonacetylated adducts to block DNA replication may be the result



The proposed SN1 mechanism for the 6-(deoxyguanosin-N^2-yl)-3-aminobenzo[*a*]pyrene formation.

Fig. 1.3: Alternative DNA-reactive pathway of nitroarenes

The generation of a DNA-reactive electrophile from 3-NO_2-B[*a*]P, without direct reaction of the nitro substituent group. (Taken from: Fu et al., 1994)

of the extra bulkiness of the aceylated adduct, which may restrict the action of DNA replication machinery.

1.4 The effects of metabolic routes on nitro-PAH toxicity

Moller (1994) found differential effects in the metabolism of nitroarenes administered by different routes. When 2-nitrofluorene is administered via the pulmonary route, the N-hydroxyl derivative is produced, and it in turn is conjugated to a glucuronide moiety. The relatively harmless glucuronide conjugate can then be excreted in the bile. Cytochrome P450 metabolism is responsible for this pathway, as it metabolizes the nitro group to the N-hydroxyl form. Hence this metabolic pathway is dominant when the lung is the organ of administration, as would often be the case in terms of environmental exposure to industrial emissions. With this route of delivery the liver can modify the xenobiotic during first pass metabolism.

In cases where large amounts of the glucuronide conjugate are present in the bile, however, significant amounts of toxicant can be liberated in the intestine by the enzyme ß-glucuronidase, thus leading to the scenario seen with oral administration. Oral ingestion of 2-nitrofluorene results in its reduction to 2-aminofluorene by the intestinal microflora. 2-Aminofluorene can be subsequently acetylated to form 2-acetylaminofluorene, a very potent mutagen. These results clarify earlier work by the same group where it was determined that 2-nitrofluorene and 2,7-dinitrofluorene exhibited more potency at forming DNA adducts

when administered orally, as opposed to i.p. (Moller et al., 1993). The example of 2-nitrofluorene is useful as it illustrates how both the dose and route of administration of nitroarenes must be considered in the analysis of nitroarene toxicity.

1.5 Nitro-PAH and tumorigenesis

The reactivity and DNA-binding capacity of NO_2-PAH can, in the worst case scenario, lead to the formation of tumours. This has been illustrated with 1,6-dinitropyrene which can produce lung tumours in F344 rats with a single 0.1 mg pulmonary dosage (Tokiwa et al., 1990). The same study found similar effects with the one time pulmonary administrations of 3,7- and 3,9-dinitrofluoranthene, which produced significant levels of lung tumours upon exposure to 0.2 mg of the respective nitro-PAH. The tumorigenicity of 1,6-dinitropyrene (with pulmonary administration) has also been shown to correlate well with DNA adduct formation (Beland et al., 1994), where the percentage of tumour incidence within an experimental group of animals increased from 0% to 80% with a concomitant increase in adduct levels from zero to two fmol adducts/μg DNA. These data suggest that nitro-PAH DNA reactivity (and hence mutagenicity) can translate into the formation of lung tumours.

Although nitro-PAH are often airborne pollutants, making pulmonary exposure quite common, the lungs are not the only target organ in terms of carcinogenicity. Hepatic tumours have been produced in newborn mice as the result of intra peritoneal exposure to 1-nitropyrene, 1-nitrosopyrene, and 6-nitrochrysene (Wislocki et al., 1986). Interestingly, 1-

nitropyrene was only marginally more potent than pyrene in generating tumours, while 1-nitrosopyrene was substantially more tumorigenic than either pyrene or 1-nitropyrene. This is in keeping with the scheme of nitro metabolism outlined in section 1.2, whereby reduction of the nitro group to a nitroso intermediate is an essential step in nitro-PAH activation.

The establishment of tumorigenic activity of nitro-PAH serves as the culmination of mutagenicity and DNA binding studies. The existence of tumours in treated animals strongly suggests that this is the ultimate toxic manifestation of the deleterious effects of nitro-PAH exposure.

1.6 Mixtures of NO_2-PAH

Nitrated polyaromatic hydrocarbons are rarely seen in isolation in the environment but, instead, are present in complex mixtures of combustion byproducts. For this reason, the investigation of pure samples of these compounds, while still a valid scientific undertaking, does not necessarily examine any interaction between the constituents of the mixture. Combinations of compounds could act synergistically to enhance the toxic effect. Alternatively, the combined effects of mixture constituents could act antagonistically and diminish the toxic effects produced by the individual compounds. For this reason, the study of these mixtures is imperative in the investigation of the true effects of environmental exposure to nitro-PAH.

Some combustion byproduct mixtures have been shown to be genotoxic. Tobacco

smoke has been linked to DNA adduct formation in human lymphocytes (Jahnke et al., 1990) and oral tissue (Jones et al., 1993). The feeding of coal tar to B6C3F$_1$ mice has been shown to form DNA adducts in the liver, lung and forestomach (Culp and Beland, 1994). Diesel emissions, in addition to being bacterial mutagens (Rosenkranz, 1982a; Rosenkranz, 1982b), have also been shown to be DNA reactive, both *in vivo* and *in vitro* (Gallagher et al., 1993). These findings, although they are likely not entirely due to the action of nitro-PAH, certainly mimic the action of various isolated nitro-PAH.

In addition to direct DNA reactivity, combustion byproducts are also capable of generating the reactive oxygen species (ROS) superoxide anion. This has been clearly illustrated with diesel exhaust (Sagai et al., 1993). The same study also found that diesel particulate-induced mortality could be diminished by the elevation of available superoxide dismutase. The potential for oxidative stress, in combination with the DNA-reactivity of combustion byproduct mixtures, lay the foundation for substantial toxicity with *in vivo* exposures.

1.6.1 The use of ^{32}P-postlabelling for identification of DNA adducts generated by NO_2-PAH mixtures

The ^{32}P-postlabelling method for the identification of DNA adducts is well suited for monitoring exposure to complex mixtures. Many studies of DNA adduct formation involved the binding of radioactively labelled compounds to DNA. It would be impossible to undertake

such a study using a mixture of unknown composition. It would also be practically impossible to accurately reconstruct a complex mixture with radioactively labelled constituents. The ^{32}P-postlabelling assay circumvents this problem by selectively labelling adducted nucleotides by a process known as enrichment.

Enrichment can be performed by one of two different methods. Butanol can be used to extract hydrophillic adducted nucleotides from a digested DNA sample, while at the same time selectively leaving behind normal nucleotides (Gupta, 1985). When this step is performed prior to the labelling reaction, only the adducted nucleotides need be radioactively labelled. Nuclease P1 enrichment achieves the same goal via a different mechanism (Reddy and Randerath, 1986). In this system, DNA is digested to 3'-nucleotides. The DNA digest is then subjected to treatment with nuclease P1. This enzyme cleaves the 3'-phosphate from each non-adducted nucleotide. The presence of a large hydrophobic molecule bound to a nucleotide sterically inhibits the nuclease's activity and leaves the 3'-phosphate group intact. Since the polynucleotide kinase required for the labelling reaction only recognizes nucleotides with a 3'-phosphate group, it selectively labels those nucleotides whose hydrophobic attachment prevented the nuclease P1 activity. The end result is again the selective radio-labelling of adducted nucleotides.

By utilizing selective procedures of enrichment, investigators are able to resolve two daunting problems: (1) the incredible number of compounds present in combustion residual mixtures; and (2) the enormous amount of normal DNA compared to the relatively few nucleotides which form adducts with reactive compounds.

1.7 The Ames-*Salmonella* assay and nitro-PAH

There is a considerable body of published work on the mutagenicity of nitro-PAH and like compounds, using the Ames *Salmonella*-mammalian microsome assay (Ames et al. 1975, Maron and Ames, 1983). The Ames assay uses strains of the bacteria *Salmonella typhimurium* that are incapable of producing histidine and biotin. The strains have been genetically engineered to back mutate to the wild type bacteria which can produce both of these essential components for growth. This back mutation could be in the form of a frameshift mutation, as in the TA98 strain, or a base-substitution mutation, as in the case of the TA100 strain (McCoy et al., 1983b). Since the assay is done on agar plates with limiting quantities of biotin and histidine, only the mutants will produce viable colonies. Thus the number of colonies on an agar plate is directly proportional to the mutagenicity of the test compound. The assay also allows for the detection of activation-dependent mutagens; by adding a preparation of mammalian liver enzymes, compounds which are not directly mutagenic but are converted to a mutagenic metabolite in mammals can be assayed.

Experimental evidence has shown that the TA98 strain of *Salmonella typhimurium* is generally more responsive to various nitroarenes and nitrated polyaromatic hydrocarbons than the TA100 strain (McCoy *et al.* 1983a), implicating the process of frame-shift mutagenesis. These data also show a particularly strong mutagenic response of TA98 bacteria to 1,8-dinitropyrene, with 272,000 revertants produced per microgram of 1,8-dinitropyrene. This paper (1983a) also illustrates the use of an *S. typhimurium* strain

designed specifically for work with nitrocompounds. The strain TA98NR, is a derivative of the parent TA98 strain that is deficient in the classical bacterial nitroreductase responsible for the metabolism, and activation, of many nitroarenes (eg. nitrofurans and niridazole). The TA98NR strain is however partially responsive to other nitroarenes such as mono- and di-nitropyrenes (Rosenkranz et al., 1981, Djuric et al., 1986).

Another specially modified version of TA98 *S. typhimurium* is the YG1024 strain, which is enriched in *O*-acetyltransferase activity (Watanabe et al., 1990). This strain has been shown to be much more sensitive to nitroarene exposure than its parent strain, TA98 (Tokiwa et al., 1994) due to the importance of *O*-acetylation in the production of the presumed ultimate reactive species, the nitrenium ion, as outlined in section 1.2.

Bacterial strains such as TA98NR and YG1024 greatly enhance the capability of the Ames assay for diagnostic work, especially in terms of use for complex mixtures which have mutagenic potential. These mixtures contain an abundance of different compounds, which makes the assignment of the species responsible for the mutagenic potential (if any is exhibited) quite difficult. By using bacterial strains which are deficient/enhanced in a certain area of xenobiotic metabolism, more can be determined as to which class of compounds are the primary mutagens in a mixture.

1.8 Rationale and objectives of this study

Considerable research has been directed at the metabolism, DNA-reactivity, and subsequent mutagenicity of nitrated polyaromatic hydrocarbons. Considerably less information is available on these characteristics with respect to their normal environmental occurrence in complex mixtures of hydrocarbon combustion byproducts. The goal of this thesis is to associate nitroreductase activity, bacterial mutagenicity and DNA damage as part of the overall toxicity seen with exposure to nitroarenes as constituents of complex mixtures.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Materials

PEI-Cellulose TLC plates were purchased from Fisher Scientific (Ottawa, ON, Canada).

The *S. typhimurium* strain TA98 was obtained as a gift from Dr. Bruce Ames, University of California at Berkeley, Berkeley, CA, USA. The *S. typhimurium* strain YG1024 was obtained as a gift from Dr. M. Watanabe, National Institute of Hygiene Sciences, Tokyo, Japan. The TA98NR strain of *S. typhimurium* was a gift from Dr. Elena McCoy, Case Western Reserve University, Cleveland, OH, USA.

Crude oils and used crankcase oils were obtained from Dr. Jeremiah Payne, Fisheries and Oceans Canada, St. John's, NF, Canada.

2.1.1 Chemicals

Apyrase, bicine, calf thymus DNA, cytochrome c (type VI, from horseheart), DMSO, DTT, EDTA, 3-methylcholanthrene (MC), micrococcal nuclease, NADH, $NADP^+$, NADPH, nuclease P1, pBr322 plasmid DNA, sodium dithionite, spermidine and succinic anhydride were purchased from Sigma Chemical Co., St. Louis, MO, USA. The enzymes α-amylase,

proteinase k, RNase A, RNase T_1 and spleen phosphodiesterase were obtained from Boehringer Mannhiem, Laval, PQ, Canada. [γ-^{32}P]-ATP (specific activity 6000 Ci/mmol) was purchased from Amersham Canada Ltd., Oakville, ON., Canada. Analytical grade KCl, KH_2PO_4, K_2HPO_4 and KOH were purchased from BDH Ltd., Toronto, ON Canada. Polynucleotide kinase was purchased from US Biochemical Co., Cleveland, OH, USA. Chelex 100 resin was purchased from Bio-Rad, Mississauga, ON, Canada. Aroclor 1254 was purchased from Chem Services, West Chester, PA, USA. All other chemicals were of the highest grade commercially available.

2.2 Methods

2.2.1 Animal Treatment

Male Sprague-Dawley rats (200-250 g) were obtained from Memorial University Animal Care Services.

For Aroclor 1254 pretreatment, rats were dosed once with 500 mg per kg body weight of the polychlorinated biphenyl (PCB) mixture, Aroclor 1254, suspended in corn oil (250 mg/mL). Aroclor pretreated rats were given chow and water *ad libitum* for four days prior to sacrificing. In the case of 3-methyl cholanthrene (3-MC) pretreatment, rats were dosed with 20 mg of 3-MC in corn oil (10 mg/mL) per kg body weight, daily, for three days. The 3-MC pretreated rats were given chow and water *ad libitum* for the first two days. After

the prescribed time, the food, but not the water, was removed. The rats were fasted overnight and sacrificed (by cervical dislocation) the following morning. Control rats, except where otherwise noted, received no pretreatment, but were subjected to the same dietary regimen and sacrificing procedure as the experimental animals. [Earlier experimentation in this lab has illustrated that rats treated with the corn oil vehicle only have exhibited no response.]

For the purposes of ^{32}P-postlabelling analysis, four male rats were administered, by gavage, 0.5 mL of DMSO crankcase oil extract per kilogram of body weight on alternate days over a thirty-one day period. Three control rats were kept for the same time period, but were not given crankcase oil treatment. For all but the last day of this treatment regimen, the rats were provided with chow and water *ad libitum*, then fasted overnight prior and sacrificed the following morning. The brains, hearts, livers, kidneys and stomachs of the rats were excised and subjected to the ^{32}P-postlabelling procedure.

2.2.2 Succinoylation of cytochrome *c*

Partial succinoylation of cytochrome *c* was performed as described by Kuthan *et al.* (1982). 100 mg of cytochrome *c* (horse heart; type VI) was dissolved in 40 mL of ice cold 30 mM KP buffer, pH 7.6. The stirring solution was left on ice and 0.42 mmol of finely ground succinic anhydride was added slowly, over a period of 30 minutes, with constant stirring. The pH of the solution was closely monitored and maintained at 7.6 by the periodic addition of 2 M KOH. Following the completion of the succinic anhydride addition, the

solution was stirred on ice for a further 20 minutes.

The succinoylated cytochrome c solution was transferred to a dialysis bag and dialysed for 16 hours against 2 L 0.1 mM EDTA. The dialysis bag was placed in 2 L of deionized water for 4 hours, after which the solution was concentrated using a Diaflo ultrafiltration apparatus, used under nitrogen gas with a PM 10 filter.

2.2.2.1 Determination of cytochrome c concentration.

The final preparation of succinoylated cytochrome c was assayed to determine its true concentration. Two visible light range cuvettes were identically prepared with 50 mM KP buffer, pH 7.6, and a known volume of succinoylated cytochrome c. The two cuvettes were simultaneously monitored at 550 nm, and the spectrophotometer zeroed. The reference cuvette was left unchanged while the sample cuvette received a 5 mg quantity of sodium dithionite to completely reduce the cytochrome c. The relative absorbance of the sample cuvette was converted to a concentration value for the preparation using an extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$.

2.2.3 Extraction of crude oils and used crank case oils

Extracts were made of four different crude oils (Prudhoe Bay crude, Hibernia crude, Alberta sweet mix crude, and Venezuelan crude) and two separate pools of used crankcase

oils. Oils to be extracted were combined with equal volumes of DMSO in plastic tubes. [DMSO was chosen because is dissolves many hydrophobic compounds, is miscible with water, and does not appear to affected the bacteria used in the mutagenicity studies (Ames et al., 1975).] The tubes were gently mixed by inversion, for 10 minutes, then centrifuged at 2000 rpm (3000 x g) in a benchtop centrifuge. The DMSO phase was recovered by puncturing the bottom of the tube and draining off the bottom layer. The recovered extract was then combined with another equal volume portion of the oil and the process repeated. The bottom layer of the second extraction was retained for future experimentation.

The above procedure was found to be inadequate for the production of a crankcase oil extract for plasmid DNA strand breakage studies. As the hydroxyl radical is essential to this process, steps were required to remove DMSO, a hydroxyl radical scavenger, from the extract. For this preparation the DMSO extract was prepared with a triple extraction procedure, producing a final volume of 90 mL. The final extract was then distilled under vacuum to eliminate the bulk of the DMSO from the preparation. This condensed extract, 20 mL in volume, was combined with 150 mL water in a separatory funnel and extracted with chloroform (2 x 100 mL), dichloromethane (2 x 75 mL) and toluene (2 x 75 mL). The organic extracts were pooled and reduced in volume using a rotary evaporator. The remaining 17.5 mL of organic extract was aliquoted into three 5 mL portions and one 2.5 mL portion, each into a capped test tube. Prior to use, the extracts were evaporated to dryness under nitrogen and then resuspended in the original volume of acetone.

2.2.3.1 Extraction of soots

DMSO extracts of two industrial stack soots (A.T. Cameron and Gulf Star) were prepared. For these extracts, DMSO and the raw soot were combined in a 1:1 weight ratio. These extracts were then prepared as per section 2.2.3.

2.2.4 Nitroreductase activity assay

Nitroreductase activity was determined as described by Djuric et al. (1986). The assay was carried out in 50 mM KP buffer (pH 7.6), with 33 μM succinoylated cytochrome c, and a total volume of 3.0 mL. 1 - 15 μL of DMSO oil/soot extract and 32 - 480 μg of cytosolic protein (from 3-MC pretreated rats) were added to the sample and reference cuvettes. 100 μL of either 10 mM NADH or 10 mM NADPH (330 μM final concentration) was added to start the reaction. The zero oil extract and zero protein levels of enzyme activity were determined by replacing the oil extract with 10 μL DMSO, and the protein with 10 μL of 50 mM KP buffer (pH 7.6), respectively

The reduction of the succinoylated cytochrome c was monitored at 550 nm. All assays were performed in duplicate, and the average of the two rates taken.

All nitroreduction assays were performed using a common rat liver cytosol

preparation

2.2.4.1 Testing of inhibitory action of DMSO extracts

To evaluate any nitroreductase inhibitory action of various extracts, nitroreduction of 1-nitropyrene was assayed with the presence and absence of oil extract, using the method outlined in section 2.2.4. Assays were done in triplicate and the results statistically compared to determine if any change in enzyme activity occurred.

2.2.5 Ames-*Salmonella* mutagenicity assay

This assay was performed as described by Maron and Ames (1983).

2.2.5.1 Preparation of rat liver post-mitochondrial fraction and NADPH-regenerating system.

Aroclor 1254 pretreated rats were killed by cervical dislocation and placed on their backs. The fur on the anterior side of each animal was thoroughly swabbed with 95% ethanol, and pointed scissors were used to cut through the skin without damaging the underlying muscle layer. The skin was pulled back and the exposed muscle tissue swabbed with 95% ethanol before it was cut open with sterile scissors. The rat livers were excised, weighed, and

placed in a volume of ice cold 100 mM KP buffer equalling three times the wet weight of the livers. This mixture was then homogenized using a Polytron homogenizer and the homogenate centrifuged at 9000 x g for 30 minutes at 4°C. The supernatant was stored at -70°C in 3 mL aliquots for future use in the Ames assay.

On the day of the experiment, an aliquot of the frozen supernatant (S9) was used to prepare an NADPH-regenerating system. This solution was a 1 in 10 dilution of the S9, with a final composition of 33 mM KCl; 8 mM $MgCl_2$; 0.1 M KP, pH 7.4; 4 mM $NADP^+$; and 5 mM glucose-6-phosphate. The regenerating system was sterilized by straining through a sterile 0.45 μm filter.

2.2.5.2 Preparation of bacterial tester strain cultures.

Master cultures of TA98, TA98NR and YG1024 were stored at -70°C with 9% of the culture volume of DMSO added for storage. The night before an experiment, approximately 0.1 mL of the frozen master culture was used to inoculate 10 mL of Oxoid culture media. The inoculated culture was left at 37°C overnight (16 hours) in a shaking water bath after which cultures were left on ice for the duration of the sample preparation

2.2.5.3 Sample preparation and incubation.

All tests were done in duplicate, as well as with and without the addition of a rat liver

S9-based NADPH-regenerating system. The test compound (dissolved in acetone or DMSO) was added to a sterile glass culture tube, followed by 2 mL top agar (0.6% Difco agar; 0.5% NaCl), and the appropriate bacterial tester strain.

The combined mixture was briefly vortexed, then aseptically transferred to an agar plate (1.5% Difco agar and 2% glucose in Vogel-Bonner medium E [Vogel and Bonner, 1956]). The plates were left to solidify in the dark at 25°C, then inverted and kept at 37°C for 48 hours. After the 48 hour incubation period the bacterial colonies on each plate were counted.

2.2.6 ^{32}P-Postlabelling of DNA adducts

^{32}P-Postlabelling experiments were performed using the methods of Reddy and Randerath (1986), with modifications.

2.2.6.1 Isolation of DNA

Approximately 250 mg of tissue were excised from test organs and placed in 1.5 mL plastic tubes. In the case of *in vitro* incubations, the entire volume was used for extraction. To each sample, 0.5 mL of SET/SDS solution (100mM NaCl; 20mM EDTA; 50mM Tris base; 0.5% SDS; 1mg/ml proteinase K) was added. The tubes were incubated at 37°C for three hours with gentle mixing by a tube rotator. 0.5 mL tris-saturated phenol was then added

to the mixture and each tube was agitated for 5 minutes in a horizontal position. The tubes were then centrifuged at 14,000 rpm (12,800 x g) for 5 minutes to separate the two phases. The bottom phenol layer was pipetted away, the tubes recentifuged at 14,000 rpm for 1 minute, and the top aqueous layer removed to a clean labelled tube.

The aqueous layer was extracted twice more as described above, with different organic solvents. The second and third extractions were performed with phenol: chloroform: isoamyl alcohol (50:48:2), and chloroform: isoamyl alcohol (24:1), respectively. The final aqueous extracts were combined with 2 volumes of cold ethoxyethanol, and gently mixed. The samples were then placed at -20°C for at least 2 hours to precipitate the DNA, and centrifuged for 5 minutes at 14,000 rpm. The supernatant was discarded, the pellet washed with 1 mL 70% ethanol, and resuspended in 0.5 ml SET buffer (100 mM NaCl; 20 mM EDTA; 50 mM Tris base) with RNase A (0.1 mg/ml), RNase T_1 (1000 U/ml) and ∝-amylase (1 mg/ml). These mixtures were incubated at 37°C for 1 hour.

10 μL of SET buffer with proteinase K (10 mg/mL) was added to each tube, after which all were incubated at 37°C for a further 1 hour. The samples were then extracted as before, with phenol, phenol: chloroform: isoamyl alcohol, and chloroform: isoamyl alcohol. The remaining aqueous fraction was combined with 2 volumes of cold ethoxyethanol, incubated at -20°C for at least 2 hours, and centrifuged at 14,000 rpm for 5 minutes to yield a DNA pellet.

The DNA pellet was washed with 70% ethanol, dissolved in 0.5 mL SET buffer and combined with 2 volumes of cold ethoxyethanol to precipitate any remaining small RNA

fragments. The mixture was again left at -20°C, centrifuged at 14,000 rpm, and the pellet washed with 70% ethanol as before. The final pellet was resuspended in distilled water, and the absorbance of the solution measured at 260 nm and 280 nm. The absorbance at 260 nm was used to determine the concentration of DNA, and the Abs@260/Abs@280 ratio was used as an index of DNA purity.

2.2.6.2 Digestion of DNA

From the purified DNA samples, the volume corresponding to 2.5 μg DNA was added to 1.5 mL Eppendorf centrifuge tubes, and the sample evaporated to dryness under vacuum. 5 μg (0.60 U) micrococcal nuclease and 5 μg spleen phosphodiesterase were added, and each was sample made up to 12.0 μL total volume (20 mM succinate, 10 mM $CaCl_2$). The DNA digestion reaction was carried out at 37°C for 3 hours.

2.2.6.3 Nuclease P1 enrichment and determination of nucleotide levels

Each DNA digestion mixture was combined with 3 μL 0.25 M sodium acetate, 1.8 μL 0.3 mM $ZnCl_2$, and 1.2 μL 5μg/μL nuclease P1. The enrichment reaction was allowed to occur for 1 hour at 37°C. To neutralize the enrichment reaction, 2.4 μL 0.5 M Tris buffer (pH 9.0) was added to each tube. 2.0 μL of each solution was transferred to a separate tube containing 18.0 μL ddH_2O. This mixture was retained for future HPLC analysis, while the

original enrichment mixture was used for the postlabelling reaction.

HPLC analysis of 3'-nucleotide content was done with a solvent system of 5% methanol/95% 10 mM NaP buffer, pH 7.5. The deoxycytidine-3'-phosphate peak was compared to a 2 nmol deoxycytidine-3'-phosphate standard to determine 3'-nucleotide levels.

2.2.6.4 Preparation of standards and blanks

For each set of experiments, two blank reactions and four nucleotide standards were prepared. The blanks were 18.0 μL ddH_2O, and the standards were 18.0 μL of a 1 x 10^{-7} M deoxyadenosine-3'-phosphate solution. The standards and blanks were treated in the same fashion in the postlabelling reaction as were the test samples.

2.2.6.5 ^{32}P-Postlabelling reaction

A kinase mix solution was prepared by the combination of 41 μL ddH_2O, 100 μL γ-^{32}P-ATP solution (1 mCi), 3.3 μL (5 units) polynucleotide kinase, and 56 μL kinase buffer (0.2 M bicine, 0.1 M $MgCl_2$, 0.1 M DTT, 10 mM spermidine). Ten μL of this kinase mixture was added to each sample and incubated at 37°C for 45 minutes. To these tubes, 4 μL of a bicine (20 mM, pH 9.5) and apyrase (20 U/mL) mixture was added and the samples further incubated at 37°C for 30 minutes. All additions were made to one blank and two standards, then to the test samples, and finally to the remaining standards and blank. This step is taken

to ensure that any diminishing of kinase activity over the course of the sample preparation can be detected.

2.2.6.6 PEI-Cellulose thin layer chromatography of nucleotides

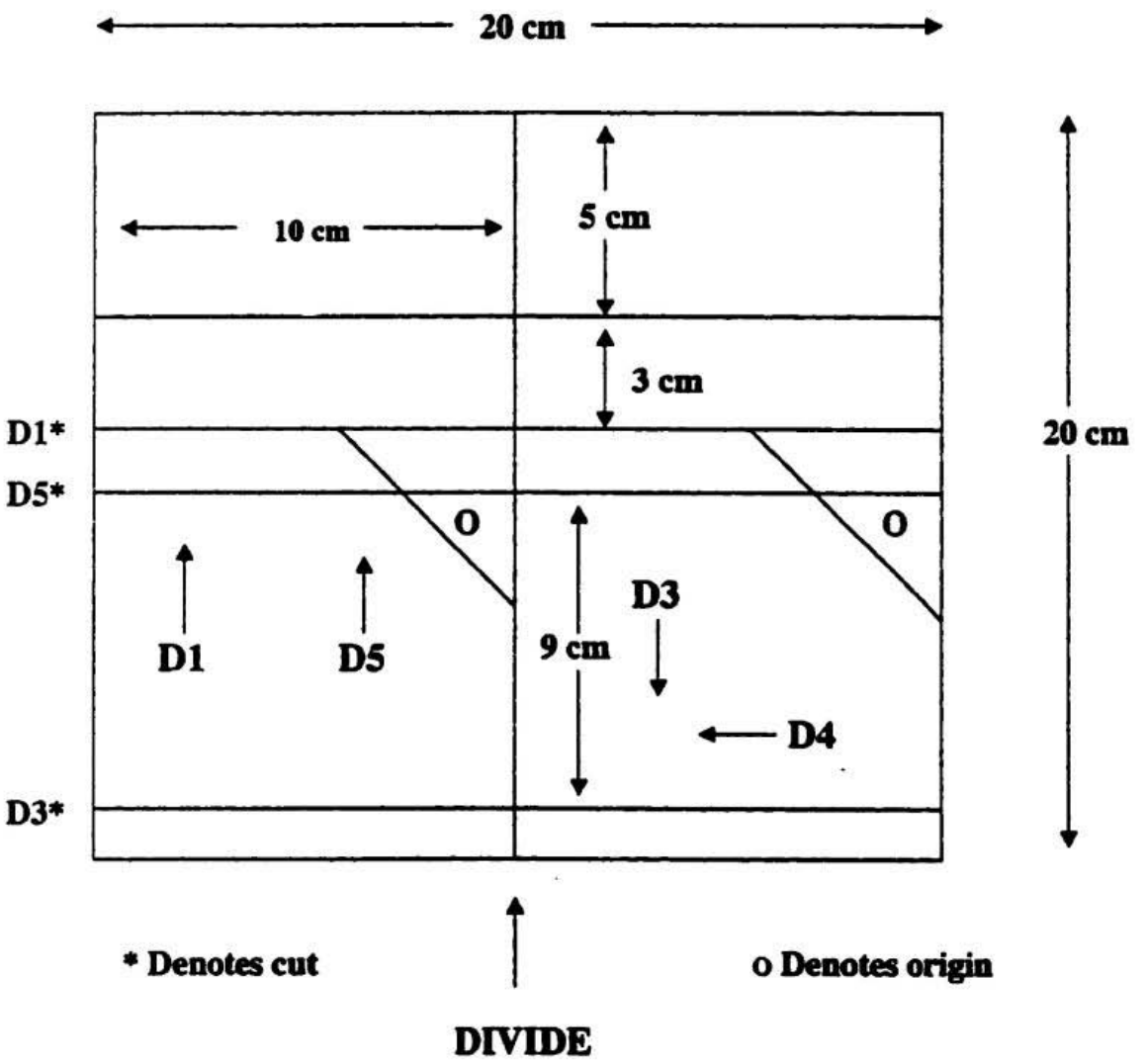
PEI-Cellulose TLC plates were washed with 100% methanol and ddH_2O, then air dried prior to use.

The entire test sample volumes, approximately 33 μL, were spotted onto a PEI-Cellulose TLC plate at the origin as indicated in Figure 2.1. The plates were developed overnight in 1.0 M NaH_2PO_4, pH 6.5, in the D1 direction (towards the top of Figure 2.1). The next morning the filter paper wicks and top third of the TLC plates were removed (cut along the line marked D1* in Figure 2.1) and discarded. The remainder of the plates were rinsed twice with ddH_2O and air dried.

The dry plates were then developed in 5.3 M lithium formate, 8.5 M urea, pH 3.5, in the D3 direction (towards the bottom of Figure 2.1). The plate was developed until the solvent front reached the edge of the plate. The top 1 cm was removed (cut along the line marked D3* in Figure 2.1) and discarded. The cut and developed plates were rinsed with ddH_2O and then soaked for 10 minutes in 10mM Tris-HCl. The plates were then further rinsed with ddH_2O, and air dried.

The plates were then developed, in the D4 direction (towards the left of Figure 2.1), again to the edge of the plate, in 1.2 M LiCl, 0.5 M Tris-base and 8.5 M urea, pH 8.0. The

Fig. 2.1 **TLC plate markings and areas of removal in multidimensional chromatography**



plates were twice rinsed in ddH_2O and air dried.

Filter paper wicks were attached to the top of the plates prior to their final, overnight, development in 1.7 M NaH_2PO_4, pH 6.0, in the D5 direction (towards the top of Figure 2.1). The wick and the area of attachment to the TLC plate were removed (cut along the line marked D5*), and the residual plates rinsed with ddH_2O air dried.

2.2.6.7 Standard, blank and sample residue analysis

Following the apyrase:bicine incubation, 970 μl ddH_2O was added to each of the standards and blanks. Ten μL of these mixtures were then spotted onto PEI-Cellulose plates. The tubes which had contained the test samples received 100 μl ddH_2O, and 10 μL of each of these residual solutions were spotted onto PEI-Cellulose plates (six sample residues per plate) and the plates were developed in 0.3 M ammonium sulphate.

The developed plates were dried and then labelled with a fluorescent marker. The labelled TLC plates were exposed for autoradiography using x-ray plates at -70°C for one hour. The x-ray films were developed and the adenosine nucleotide standard radioactive spots marked on the film. The areas in the sample blank lanes with R_f values corresponding to the adenosine spots were also marked. The areas on the TLC plates which corresponded to the marked spots on the autoradiogram were excised and scintillation counted. The relationship between standard nucleotide added and radioactivity counted was used to establish the specific activity of the labelling reaction.

2.2.6.8 Analysis of test samples

Following the final chromatography step, the plates were labelled with a fluorescent marker and placed in autoradiography cassettes with x-ray film for 16 hours at -70^{0}C.

The x-ray films were developed after exposure and any visible radioactive spots were marked. In addition to the visible spots, a 1 cm^2 portion of each film was marked in an area where no radioactivity was detected. This spot was used to calculate the background levels of radioactivity per square centimetre of TLC plate.

The areas on the TLC plates which corresponded to the marked spots on the film were excised and weighed to determine their area as compared to a standard sample. The radioactivity blanks as well as sample spots were then scintillation counted.

The radioactive counts detected in the test samples were converted to a measure of the adducted nucleotides present using the previously established specific activity of labelling. This number was then expressed as a molar ratio of the total 3'-nucleotides present in the sample. The means (+/- S.D.) of triplicate samples were compared using a 2-way ANOVA test.

2.2.7 Plasmid DNA strand cleavage experiments

The investigation of plasmid DNA nick production was done according to the methods outlined by Kukielka and Cederbaum (1994), with modifications.

2.2.7.1 Preparation of solutions

The solutions used in preparation of rat liver fractions and in plasmid nicking incubations were prepared with deionized water (filtered through Chelex 100 resin) and analytical grade reagents, so as to minimize the presence of free metal ions in the solutions.

2.2.7.2 Preparation of rat liver fractions

Aroclor 1254 pretreated and control rats were killed via cervical dislocation. The outer skin and the muscle layers were cut to expose the internal organs and the portal vein was used to perfuse the livers with 0.9% KCl. Following the perfusion, the livers were excised, weighed, and combined with a volume equalling three times the liver weight of sterile 50 mM KP buffer (pH 7.4). The livers were minced with clean scissors and the mixture homogenized using a Polytron homogenizer. The resulting homogenate was centrifuged at 9000 x gravity, the pellet discarded and the supernatant retained as the post-mitochondrial fraction.

2.2.7.3 Generation of pBR322 plasmid

Fifty μL *Escherichia coli* competent cells (DH5α strain) was diluted with 450 μL 0.1 M sterile $CaCl_2$. One μg pBr322 plasmid DNA was combined with 50 μL of the diluted

E. coli cells. The mixture was kept on ice for 30 minutes and then heat shocked for 90 seconds at 42°C. One mL sterile LB broth was added to the plasmid/bacteria mixture and the reaction tube was incubated at 37°C for 1 hour. The resultant culture was centrifuged at 14,000 rpm for 1 minute to pellet the cells, the supernatant discarded, and the pellet spread on an LB/ ampicillin plate, which was incubated at 37°C for 16 hours.

Colony scrapings from the overnight plate were used to inoculate 15 mL sterile LB broth (with ampicillin), and the cultures were grown at 37°C in a gyratory incubator for 24 hours. The 15 mL cultures were used to inoculate 500 mL sterile LB/Amp broth preparations, which were also incubated at 37°C in a gyratory incubator for 24 hours.

The 500 mL cultures were centrifuged at 4500 rpm for 15 minutes to pellet the transformed cells. Each culture pellet was resuspended in 20 mL sterile GTE solution (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0), containing 100 μg/mL RNase A, to which 40 mL sterile NaOH-SDS (0.2 N NaOH, 1% SDS) was added. This solution was gently mixed and left on ice for 5 minutes, after which 30 mL sterile KAcF (5 M potassium acetate, 6.7% formic acid) was added, the mixture swirled, and left on ice for a further 10 minutes. The resulting slurry was strained through 2 thicknesses of cheesecloth into sterile centrifuge tubes. The contents of each tube were combined with 0.6 x volume of isopropanol and centrifuged at 10,000 x g for 10 minutes. The resulting supernatant was discarded and the pellets resuspended in 4 mL sterile TE solution (10 mM Tris, 1 mM EDTA, pH 8.0).

The TE solution was combined with 2 x volume of Tris-saturated phenol and thoroughly mixed. The mixture was centrifuged for 5 minutes at 2500 rpm (3000 x g) in a

benchtop centrifuge to separate the organic and aqueous phases, and the top aqueous layer was pipetted into a clean glass tube. The extraction procedure was repeated twice: once with Tris-saturated phenol and once with chloroform:isoamyl alcohol (24:1). The final aqueous extract was combined with 7.5 M sterile ammonium acetate and incubated on ice for 15 minutes. The solution was centrifuged at 10,000 x g for 5 minutes and the pellet discarded. The supernatant was combined with 2 x volume of 100% ethanol and kept at -20°C for 90 minutes. This suspension was centrifuged at 10,000 x gravity and the supernatant discarded. The pellet was dried under N_2 gas and resuspended in 0.5 mL TE buffer.

The DNA content and purity was checked by recording the absorbance of the solution at 260 and 280 nm and calculating the Abs@260/Abs@280 ratio. The DNA was also run on a 0.5% agarose gel to verify both the absence of RNA and the purity of the plasmid.

2.2.7.4 Incubation and analysis of plasmid DNA strand cleavage

The test compounds (dissolved in an organic solvent) were added to 1.5 mL plastic tubes which were left at 37°C to evaporate the sample to dryness. Sterile 50 mM KP buffer, pH 7.4, was added to each tube in a quantity sufficient to make the total reaction volume 50 μL. 2.5 μL of 2.5 μM $FeSO_4$ (final concentration = 0.125 μM) was added to each reaction tube, followed by 1 μg of plasmid, 0 - 5 μg postmitochondrial fraction protein, and 5 μL 10 mM NADPH (in sterile 50 mM KP buffer, pH 7.4) to start the reaction. Capped sample tubes were incubated at 37°C for 1 - 24 hours.

Following the incubation, samples were removed from the incubator and briefly centrifuged to remove condensation from the cap and sides of the tube. 5μL of 6 x tracking dye was added to each tube, followed by 5 μL of a solution 1 mg/mL in protease and RNase T_1. This sample was carefully mixed and incubated again at 37°C for a further 45 minutes.

The samples were then loaded onto a 0.5% agarose gel (in 0.5 x TBE buffer, with ethidium bromide) with the incubation samples flanked on the gel, to the extreme right and left, by a molecular weight marker of Hind III/EcoRI digested λ phage DNA. The gels were run at 20 - 100 V for 4 to 16 hours, or sufficient time such that clear plasmid separation became visible on the gel. Gels were viewed under u.v. light in a darkroom, and photographed. The negative from the gel photograph was scanned using an LKB scanning laser densitometer, to reveal the relative intensities of the plasmid forms evident on the gel. Samples were compared by relating the percentage of total plasmid DNA present in the relaxed coil form.

2.2.7.5 Statistical Analysis of Scanning Densitometer Data

Statistical analysis was performed using the Sigma Plot computer program. The data collected with the scanning laser densitometer were subjected to a paired t-test statistical analysis. Sample means that were not different at the level $p < 0.05$ were rejected as not significantly different.

CHAPTER 3

3. RESULTS

3.1 Nitroreductase activity

3.1.1 Crude oil extracts as nitroreductase substrates

Nitroreductase assays showed extremely limited enzyme activity with crude oil extracts as substrates. The DMSO extract of Prudhoe Bay crude oil exhibited no effect on nitroreductase enzyme activity (Figure 3.1.1), as neither tested quantity of extract, with either NADH or NADPH as a cofactor, produced above control levels of nitroreduction.

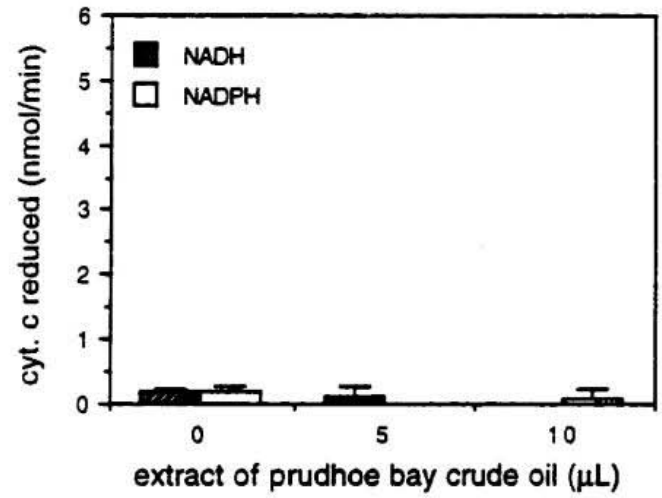
Alberta sweet mix crude oil and Hibernia crude oil extracts both generated less than 1 nmol/min nitroreductase activity, which were both minimally, if at all, greater than DMSO control levels (Figures 3.1.2 and 3.1.3).

Of the crude oils, the DMSO extract of Venezuelan crude oil exhibited the greatest potency as a nitroreductase substrate (Figure 3.1.4). With 15 μl of the oil extract and NADH as the cofactor in the reaction cuvette, a reaction rate of 0.937 nmol/min. was exhibited. This was a 5 fold increase over the DMSO control reaction rate of 0.188 nmol/min.

Fig. 3.1.1 **Nitroreductase activity generated with DMSO extract of Prudhoe Bay crude oil as enzyme substrate.**

Fig. 3.1.2 **Nitroreductase activity generated with DMSO extract of Alberta sweet mix crude oil as enzyme substrate.**

Cuvettes contained a total volume of 3 mL in 50 mM KP buffer, pH 7.6, with 330 μM succinoylated cytochrome c. Cuvettes also contained 10 μL cytosol (320 μg protein), either 0 (10 μL DMSO), 5, or 10 μL oil extract, and 100 μL of 10 mM NADH/ NADPH to start the reaction. Reactions were carried out at 37°C. Chart recordings of reactions were monitored for a minimum of 5 minutes per assay.


NADH
NADPH
cyt. c reduced (nmol/min)
0
1
2
3
4
5
6
0
5
10
extract of prudhoe bay crude oil (µL)

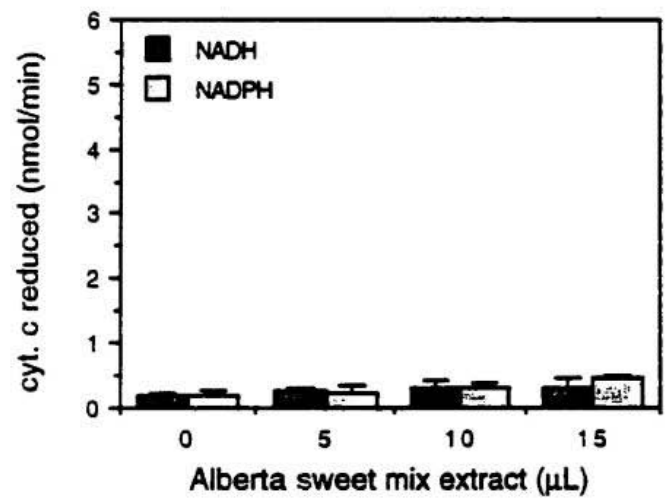
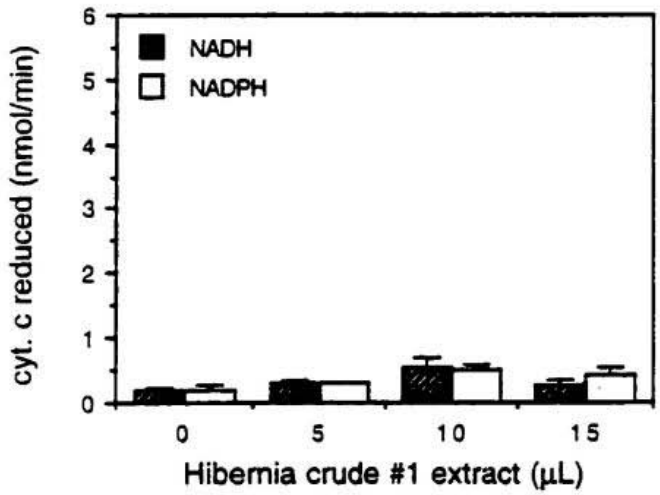
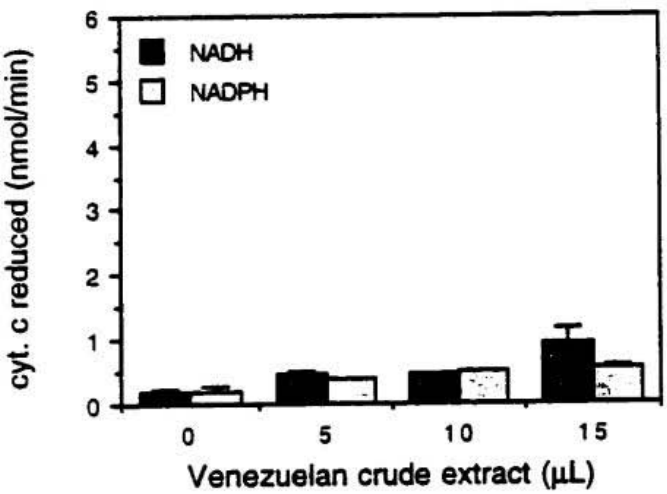

NADH
NADPH
cyt. c reduced (nmol/min)
0
1
2
3
4
5
6
0
5
10
15
Alberta sweet mix extract (µL)

Fig. 3.1.3 **Nitroreductase activity generated with DMSO extract of Hibernia crude oil as enzyme substrate.**

Fig. 3.1.4 **Nitroreductase activity generated with DMSO extract of Venezuelan crude oil as enzyme substrate.**

Cuvettes contained a total volume of 3 mL in 50 mM KP buffer, pH 7.6, with 330 μM succinoylated cytochrome c. Cuvettes also contained 10 μL cytosol (320 μg protein), either 0 (10 μL DMSO), 5, or 10 μL oil extract, and 100 μL of 10 mM NADH/ NADPH to start the reaction. Reactions were carried out at 37°C. Chart recordings of reactions were monitored for a minimum of 5 minutes per assay.


NADH
NADPH
cyt. c reduced (nmol/min)
0
1
2
3
4
5
6
0
5
10
15
Hibernia crude #1 extract (µL)


NADH
NADPH
cyt. c reduced (nmol/min)
0
1
2
3
4
5
6
0
5
10
15
Venezuelan crude extract (µL)

3.1.2 Crankcase oil extracts as nitroreductase substrates

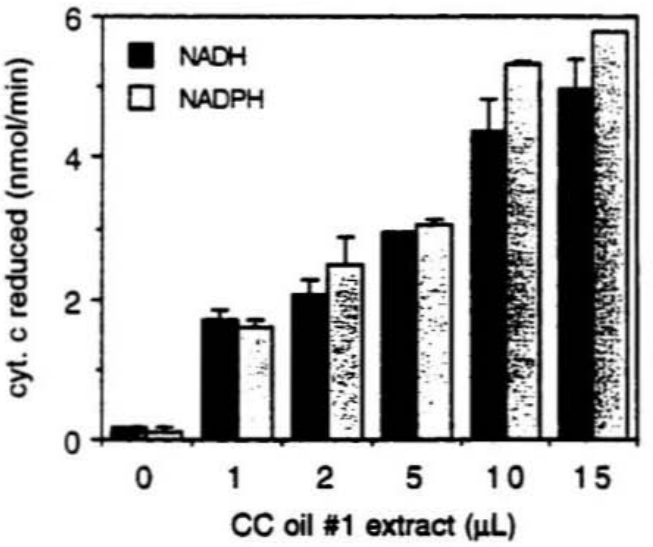
The extract of crankcase oil #1 exhibited considerable potency as a nitroreductase substrate (Figure 3.2.1) and there seemed to be no marked difference or visible trend in terms of the preference for either NADH or NADPH as the reaction cofactor. The DMSO blank for crankcase oil #1 gave 0.179 nmol/min and 0.121 nmol/min of cytochrome c reduction with NADH and NADPH as the respective cofactors. The lowest level of extract addition to the reaction cuvettes (1μl in 3 mL total volume) produced marked increases in the rate of nitroreduction to 1.7 nmol/min for the NADH reaction and 1.58 for the NADPH reaction. The rates increased in a dose dependent manner to relative maximums of 4.96 nmol/min (NADH) and 5.77 nmol/min (NADPH) in the presence of 15 μl crankcase oil extract #1.

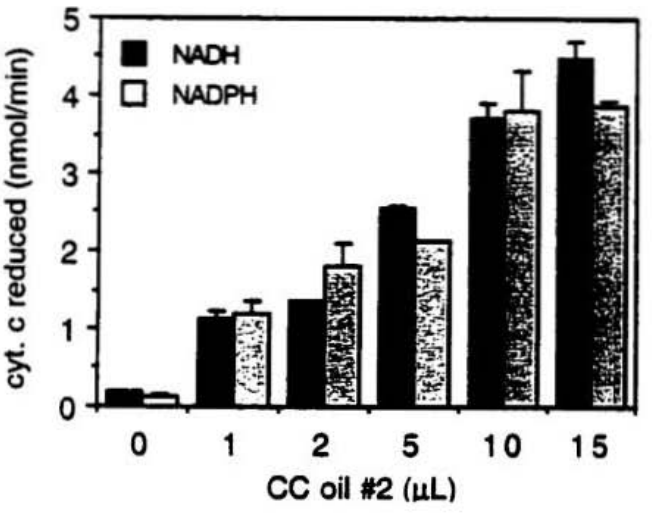
The DMSO extract of crankcase oil #2 illustrated similar properties to those seen with the first crankcase oil extract, although the level of enzyme activity produced by the second extract was slightly diminished (Figure 3.2.2). As observed with extract #1, neither NADH nor NADPH was a visibly superior cofactor in generating nitroreduction with extract #2 as the substrate. The DMSO reaction blank levels of enzyme activity were identical to those seen in the first set of assays. One μl of the second extract produced 1.14 nmol/min (NADH) and 1.19 nmol/min (NADPH), again a marked increase above control levels. As before, the rate of nitroreduction increased in a dose dependent fashion, reaching maximal activity levels with the addition of 15 μl of extract #2: 4.47 nmol/min (NADH) and 3.87 nmol/min (NADPH).

Fig. 3.2.1 **Nitroreductase activity generated with DMSO extract of Crankcase oil #1 as enzyme substrate.**

Fig. 3.2.2 **Nitroreductase activity generated with DMSO extract of Crankcase oil #2 as enzyme substrate.**

Cuvettes contained a total volume of 3 mL in 50 mM KP buffer, pH 7.6, with 330 μM succinoylated cytochrome c. Cuvettes also contained 10 μL cytosol (320 μg protein), either 0 (10 μL DMSO), 1, 2, 5, 10, or 15 μL oil extract, and 100 μL of 10 mM NADH/ NADPH to start the reaction. Reactions were carried out at 37°C. Chart recordings of reactions were monitored for a minimum of 5 minutes per assay.


6
4
2
0
NADH
NADPH
cyt. c reduced (nmol/min)
0
1
2
5
10
15
CC oil #1 extract (µL)


5
4
3
2
1
0
NADH
NADPH
cyt. c reduced (nmol/min)
0
1
2
5
10
15
CC oil #2 (µL)

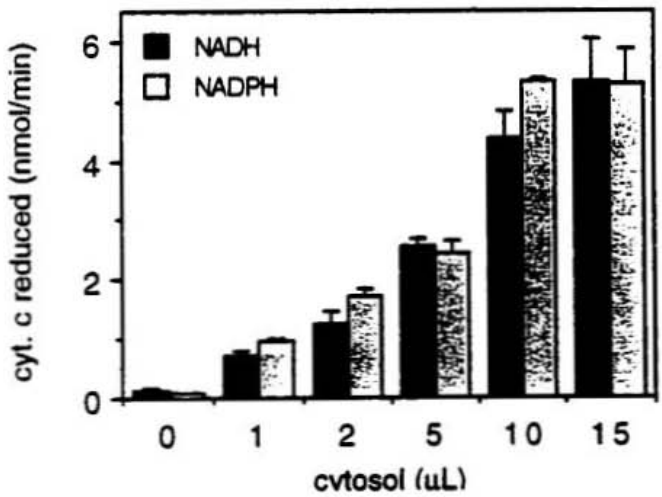
Nitroreductase activity was also measured for both crankcase oil extracts while varying the levels of cytosolic protein added and maintaining a constant level of crankcase oil extract (Figure 3.3.1 and 3.3.2). Both oil extracts behaved similarly as increases in protein levels corresponded to increased rates of nitroreduction. As previously seen, nitroreductase activity was generated equally well with NADH and NADPH as the enzyme cofactor.

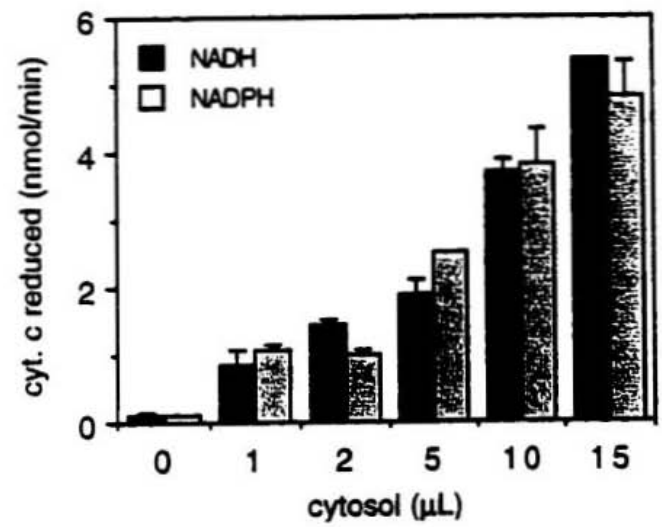
The cytosol deficient control reactions exhibited very low levels of activity, with values less than 0.140 nmol/min, while maximal levels of nitroreductase activity were seen in the presence of 480 μg of rat liver cytosol. For extracts #1 and #2 the relative maximum values were 5.33 nmol/min (NADH), 5.90 nmol/min (NADPH) and 5.36 nmol/min (NADH), 4.82 nmol/min (NADPH), respectively.

Fig. 3.3.1 **Response of nitroreductase activity (generated with DMSO extract of Crankcase oil #1 as enzyme substrate) to varying levels of cytosolic protein.**

Fig. 3.3.2 **Response of nitroreductase activity (generated with DMSO extract of Crankcase oil #2 as enzyme substrate) to varying levels of cytosolic protein.**

Cuvettes contained a total volume of 3 mL in 50 mM KP buffer, pH 7.6, with 330 μM succinoylated cytochrome c. Cuvettes also contained 10 μL oil extract and either 0, 1, 2, 5, 10, or 15 μL cytosol (0, 32, 64, 160, 320, or 480 μg protein, respectively), and 100 μL of 10 mM NADH/ NADPH to start the reaction. Reactions were carried out at 37°C. Chart recordings of reactions were monitored for a minimum of 5 minutes per assay.


NADH
NADPH
cyt. c reduced (nmol/min)
0
2
4
6
0
1
2
5
10
15
cytosol (µL)


NADH
NADPH
cyt. c reduced (nmol/min)
0
2
4
6
0
1
2
5
10
15
cytosol (µL)

3.1.3 Soot extracts as nitroreductase substrates

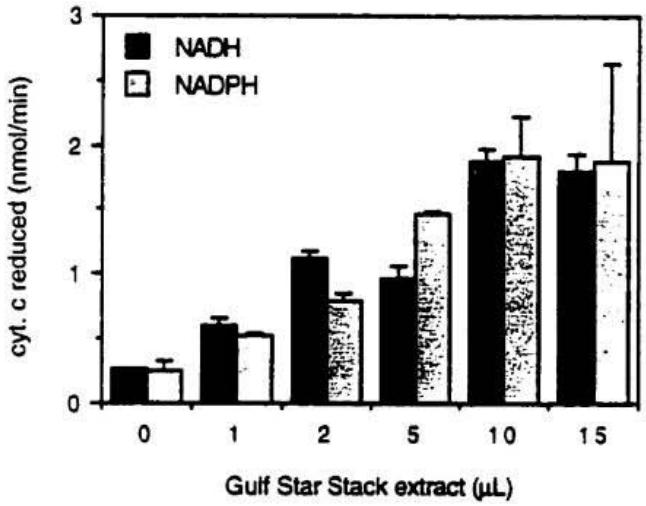
The nitroreductase activities generated by the DMSO extract of Gulf Star stack soot are illustrated in Figure 3.4.1. Increasing levels of the extract produced nitroreductase activity in a dose-dependent manner with 1 μl of extract generating a level of activity which is over 2 times greater than control levels, both with NADH and NADPH as the cofactor. 10 μl of the extract produced the highest recorded activities at 1.87 nmol/min (NADH) and 1.92 nmol/min (NADPH). There was no preference for NADH or NADPH as the electron donor for the reaction.

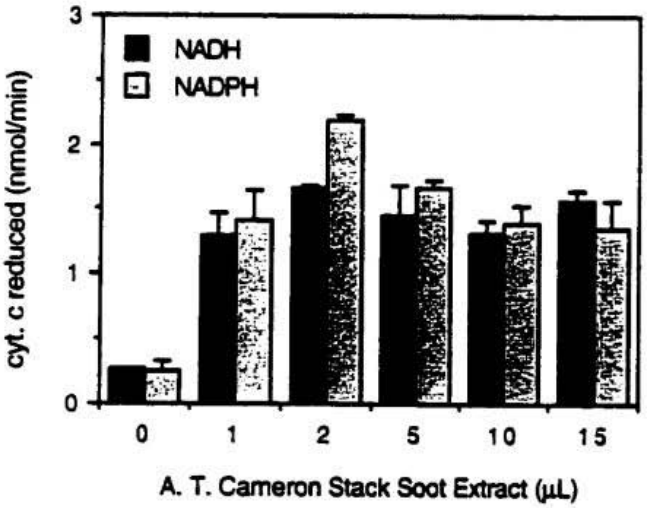
The A.T. Cameron stack soot extract exhibited similar enzymatic responses to the first extract (Figure 3.4.2). One μl of the A.T. Cameron extract, with either NADH or NADPH as the cofactor, produced nitroreduction at rates triple that of controls. The highest levels of activity were seen with 2 μl of the extract: 1.66 nmol/min (NADH); 2.18 nmol/min (NADPH). The industrial stack soot extract showed little, if any, preference for NADH or NADPH as the electron source.

Fig. 3.4.1 **Nitroreductase activity generated with DMSO extract of Gulf Star stack soot as enzyme substrate.**

Fig. 3.4.2 **Nitroreductase activity generated with DMSO extract of A.T. Cameron stack soot as enzyme substrate.**

Cuvettes contained a total volume of 3 mL in 50 mM KP buffer, pH 7.6, with 330 μM succinoylated cytochrome c. Cuvettes also contained 10 μL cytosol (320 μg protein), either 0 (10 μL DMSO), 1, 2, 5, 10 or 15 μL soot extract, and 100 μL of 10 mM NADH/ NADPH to start the reaction. Reaction were carried out at 37°C. Chart recordings of reactions were monitored for a minimum of 5 minutes per assay.


NADH
NADPH
cyt. c reduced (nmol/min)
0
1
2
3
0
1
2
5
10
15
Gulf Star Stack extract (µL)


NADH
NADPH
cyt. c reduced (nmol/min)
0
1
2
3
0
1
2
5
10
15
A. T. Cameron Stack Soot Extract (µL)

3.2 Ames *Salmonella* determination of extract mutagenicity

3.2.1 Mutagenicity of crude oil extracts

Neither of the four crude oil extracts tested produced substantial amounts of revertants. For the Prudhoe Bay (Figures 3.5.1; 3.5.2; 3.5.3), Hibernia (Figures 3.6.1; 3.6.2; 3.6.3), Alberta sweet mix (Figures 3.7.1; 3.7.2; 3.7.3) and Venezuelan (Figures 3.8.1; 3.8.2; 3.8.3) crude oil extracts, there was a prevailing pattern. The TA98 and TA98NR strains showed minimal increases beyond control rates of reversion, with no growth plate displaying greater than 100 colonies. Incubations with the YG1024 strain actually produced less revertants than those seen in experimental controls.

3.2.2 Mutagenicity of soot extracts

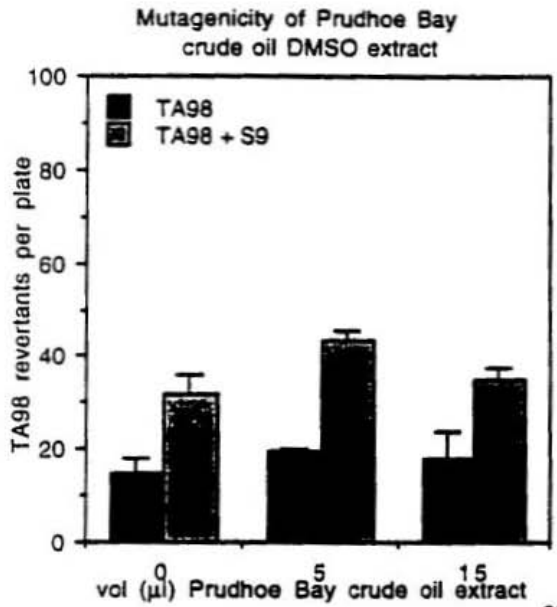
The DMSO extract (5μl) of Gulf Star stack soot proved capable of generating revertants in *Salmonella typhimurium* bacteria (Figure 3.9). The TA98NR strain of the bacteria, a nitroreductase deficient strain of the parent TA98 produced the least number of revertants of the three strains tested. The TA98NR strain alone produced 30 revertants/plate, while the addition of S9 increased the mutation rate to 192 revertants/plate. The parent strain, TA98, produced substantially more mutations: 496 revertants/plate. Unlike the

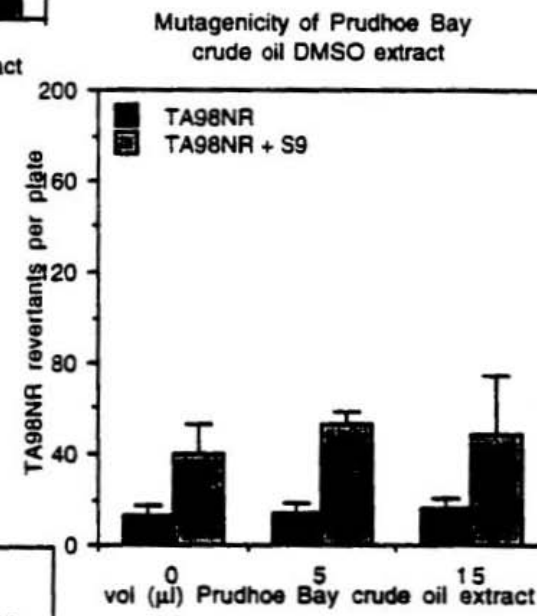
Fig. 3.5.1 ***Salmonella typhimurium*** **TA98NR revertants produced by incubation with Prudhoe Bay crude oil DMSO extract**

Fig. 3.5.2 ***Salmonella typhimurium*** **TA98 revertants produced by incubation with Prudhoe Bay crude oil DMSO extract**

Fig. 3.5.3 ***Salmonella typhimurium*** **YG1024 revertants produced by incubation with Prudhoe Bay crude oil DMSO extract**

Samples were prepared in sterile culture tubes. 0-15 μL of the test compound was placed in each tube, followed by 2 mL liquid (approximately 45°C) top agar. 100 μL of an active culture of the tester strain was added to each tube. Every reaction condition was prepared in duplicate as well as in the presence and absence of a rat liver S9-NADPH regenerating system. Plates were incubated at 37°C for 48 hours prior to counting the colonies.


Mutagenicity of Prudhoe Bay
crude oil DMSO extract
TA98
TA98 + S9
TA98 revertants per plate
100
80
60
40
20
0
0
5
15
vol (µl) Prudhoe Bay crude oil extract


Mutagenicity of Prudhoe Bay
crude oil DMSO extract
TA98NR
TA98NR + S9
TA98NR revertants per plate
200
160
120
80
40
0
0
5
15
vol (µl) Prudhoe Bay crude oil extract

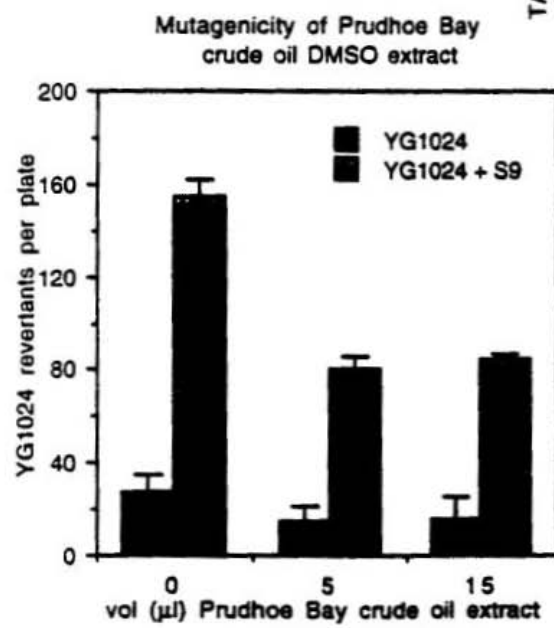
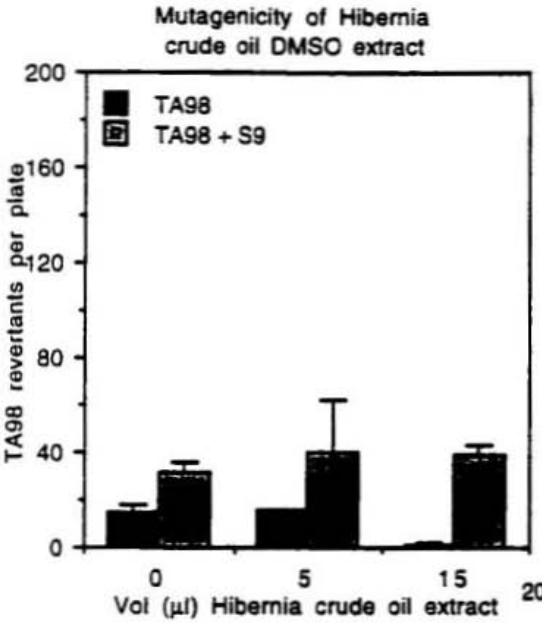

Mutagenicity of Prudhoe Bay
crude oil DMSO extract
YG1024
YG1024 + S9
YG1024 revertants per plate
200
160
120
80
40
0
0
5
15
vol (µl) Prudhoe Bay crude oil extract

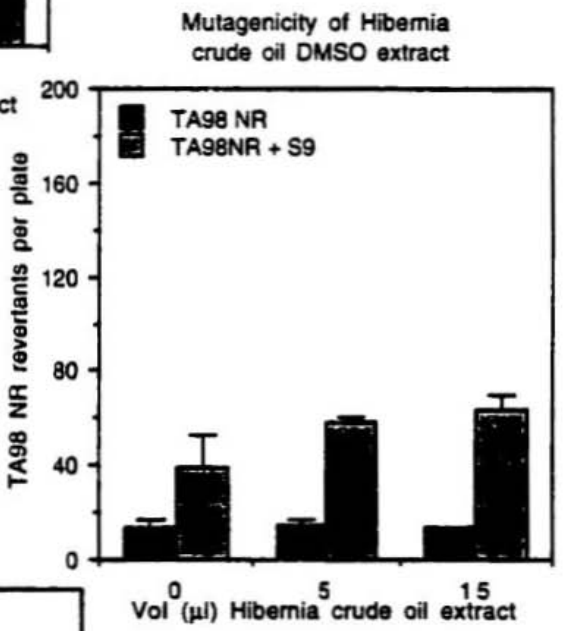
Fig. 3.6.1 ***Salmonella typhimurium*** **TA98NR revertants produced by incubation with Hibernia crude oil DMSO extract**

Fig. 3.6.2 **Salmonella typhimurium TA98 revertants produced by incubation with Hibernia crude oil DMSO extract**

Fig. 3.6.3 ***Salmonella typhimurium*** **YG1024 revertants produced by incubation with Hibernia crude oil DMSO extract**

Samples were prepared in sterile culture tubes. 0-15 μL of the test compound was placed in each tube, followed by 2 mL liquid (approximately 45°C) top agar. 100 μL of an active culture of the tester strain was added to each tube. Every reaction condition was prepared in duplicate as well as in the presence and absence of a rat liver S9-NADPH regenerating system. Plates were incubated at 37°C for 48 hours prior to counting the colonies.


Mutagenicity of Hibernia
crude oil DMSO extract
TA98
TA98 + S9
TA98 revertants per plate
200
160
120
80
40
0
0
5
15
Vol (µl) Hibernia crude oil extract


Mutagenicity of Hibernia
crude oil DMSO extract
TA98 NR
TA98NR + S9
TA98 NR revertants per plate
200
160
120
80
40
0
0
5
15
Vol (µl) Hibernia crude oil extract

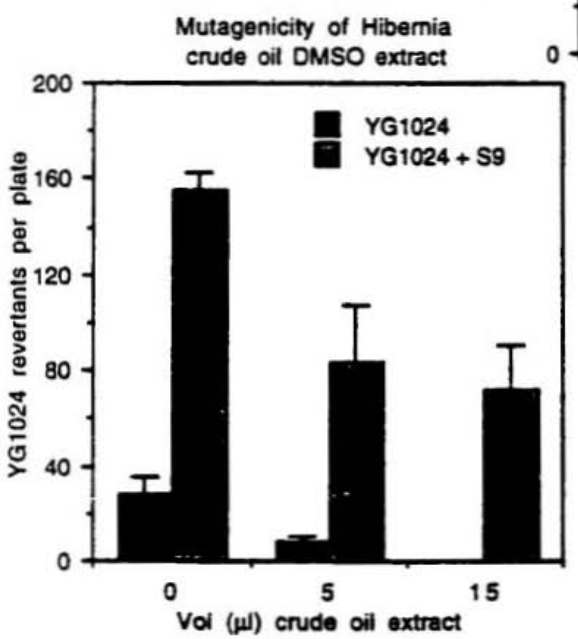
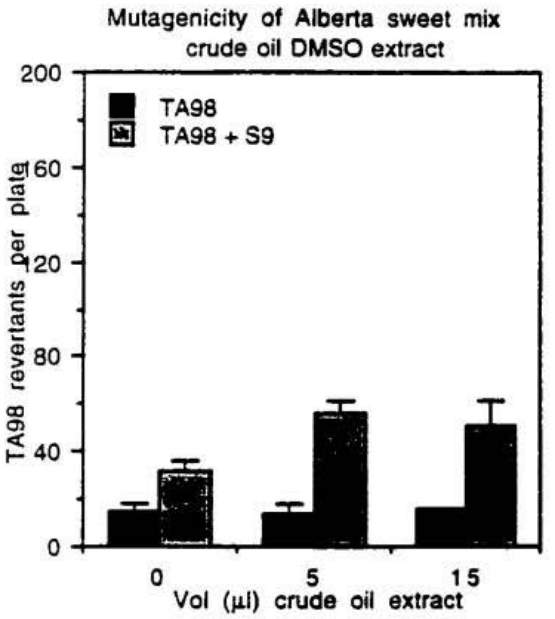

Mutagenicity of Hibernia
crude oil DMSO extract
YG1024
YG1024 + S9
YG1024 revertants per plate
200
160
120
80
40
0
0
5
15
Vol (µl) crude oil extract

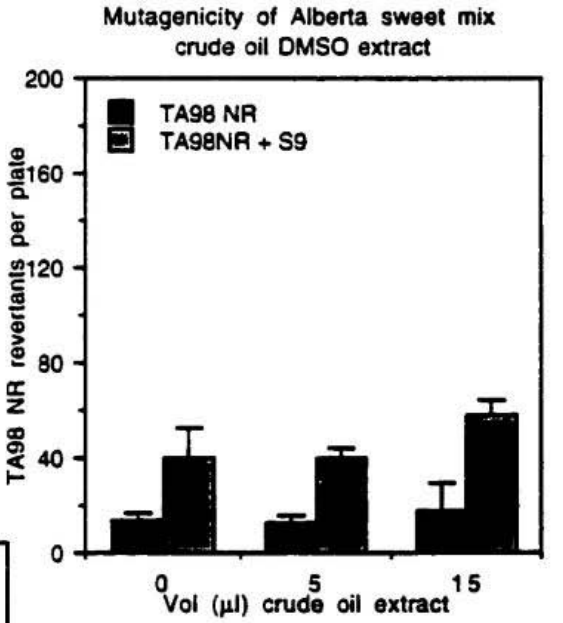
Fig. 3.7.1 ***Salmonella typhimurium*** **TA98NR revertants produced by incubation with Alberta sweet mix crude oil DMSO extract**

Fig. 3.7.2 ***Salmonella typhimurium*** **TA98 revertants produced by incubation with Alberta sweet mix crude oil DMSO extract**

Fig. 3.7.3 ***Salmonella typhimurium*** **YG1024 revertants produced by incubation with Alberta sweet mix crude oil DMSO extract**

Samples were prepared in sterile culture tubes. 0-15 μL of the test compound was placed in each tube, followed by 2 mL liquid (approximately 45°C) top agar. 100 μL of an active culture of the tester strain was added to each tube. Every reaction condition was prepared in duplicate as well as in the presence and absence of a rat liver S9-NADPH regenerating system. Plates were incubated at 37°C for 48 hours prior to counting the colonies.


Mutagenicity of Alberta sweet mix
crude oil DMSO extract
TA98
TA98 + S9
TA98 revertants per plate
200
160
120
80
40
0
0
5
15
Vol (µl) crude oil extract


Mutagenicity of Alberta sweet mix
crude oil DMSO extract
TA98 NR
TA98NR + S9
TA98 NR revertants per plate
200
160
120
80
40
0
0
5
15
Vol (µl) crude oil extract

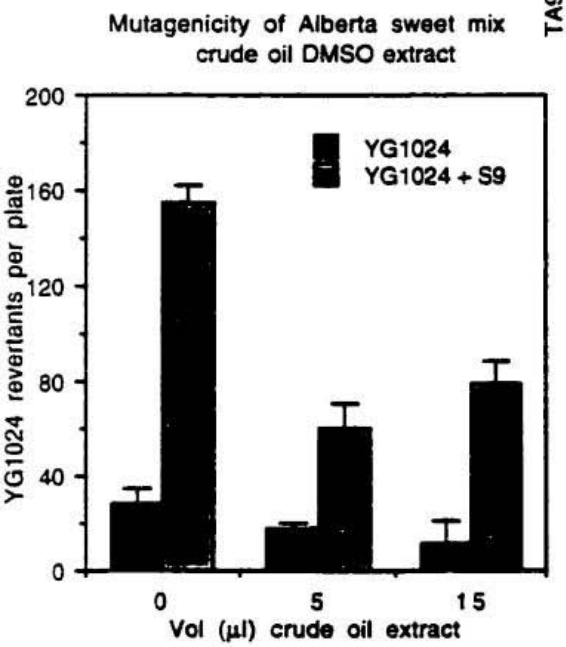
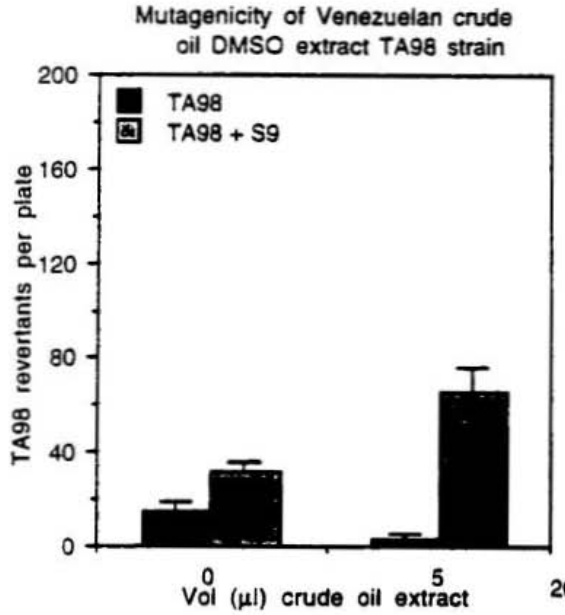

Mutagenicity of Alberta sweet mix
crude oil DMSO extract
YG1024
YG1024 + S9
YG1024 revertants per plate
200
160
120
80
40
0
0
5
15
Vol (µl) crude oil extract

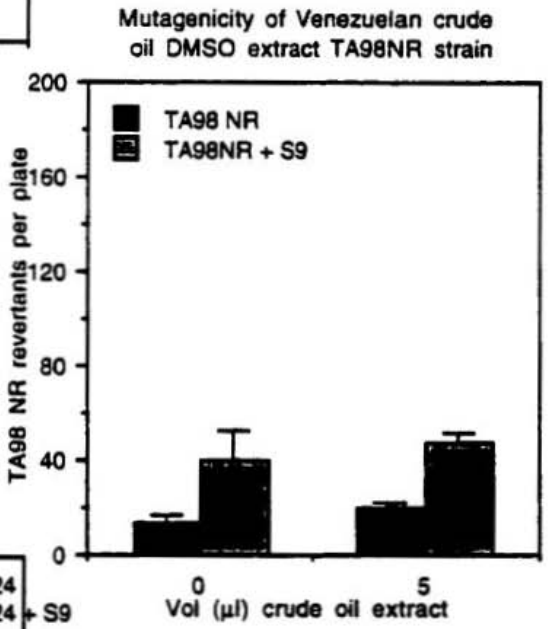
Fig. 3.8.1 ***Salmonella typhimurium*** **TA98NR revertants produced by incubation with Venezuelan crude oil DMSO extract**

Fig. 3.8.2 ***Salmonella typhimurium*** **TA98 revertants produced by incubation with Venezuelan crude oil DMSO extract**

Fig. 3.8.3 ***Salmonella typhimurium*** **YG1024 revertants produced by incubation with Venezuelan crude oil DMSO extract**

Samples were prepared in sterile culture tubes. 0-15 μL of the test compound was placed in each tube, followed by 2 mL liquid (approximately 45°C) top agar. 100 μL of an active culture of the tester strain was added to each tube. Every reaction condition was prepared in duplicate as well as in the presence and absence of a rat liver S9-NADPH regenerating system. Plates were incubated at 37°C for 48 hours prior to counting the colonies.


Mutagenicity of Venezuelan crude oil DMSO extract TA98 strain
TA98
TA98 + S9
TA98 revertants per plate
200
160
120
80
40
0
0
5
Vol (µl) crude oil extract


Mutagenicity of Venezuelan crude oil DMSO extract TA98NR strain
TA98 NR
TA98NR + S9
TA98 NR revertants per plate
200
160
120
80
40
0
0
5
Vol (µl) crude oil extract

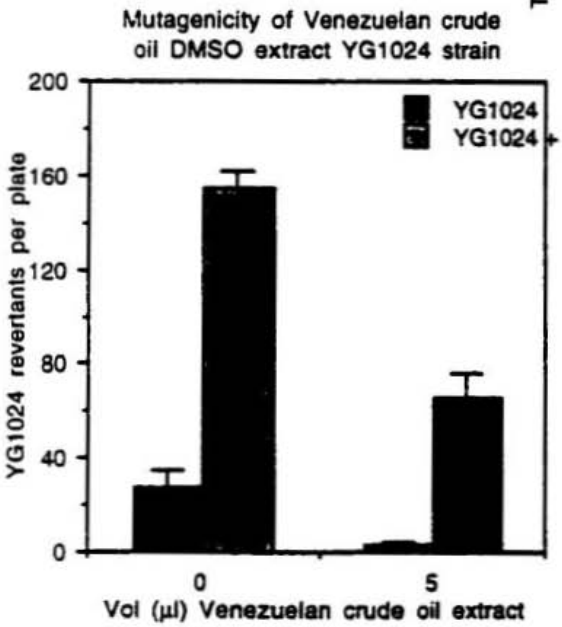
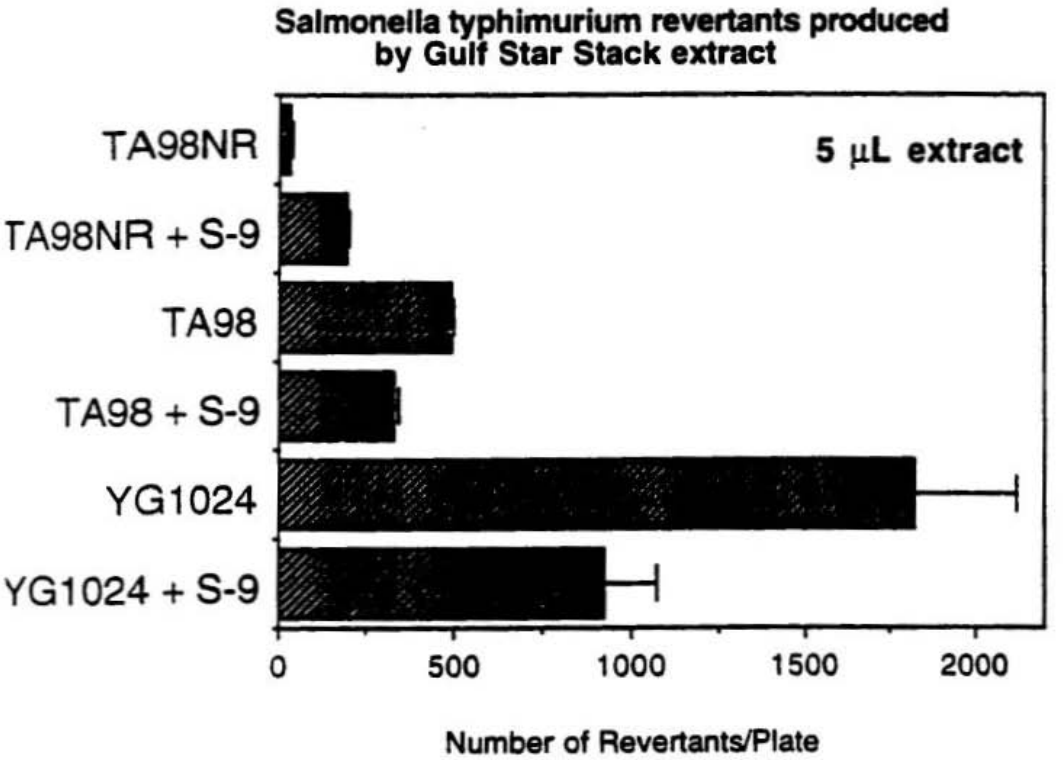

Mutagenicity of Venezuelan crude oil DMSO extract YG1024 strain
YG1024
YG1024 + S9
YG1024 revertants per plate
200
160
120
80
40
0
0
5
Vol (µl) Venezuelan crude oil extract

Fig. 3.9 ***Salmonella typhimurium* TA98NR, TA98, and YG1024 revertants produced by incubation with Gulf Star stack soot DMSO extract**

Samples were prepared in sterile culture tubes. 0-15 μL of the test compound was placed in each tube, followed by 2 mL liquid (approximately 45°C) top agar. 100 μL of an active culture of the tester strain was added to each tube. Every reaction condition was prepared in duplicate as well as in the presence and absence of a rat liver S9-NADPH regenerating system. Plates were incubated at 37°C for 48 hours prior to counting the colonies.


Salmonella typhimurium revertants produced
by Gulf Star Stack extract
TA98NR
TA98NR + S-9
TA98
TA98 + S-9
YG1024
YG1024 + S-9
5 µL extract
0
500
1000
1500
2000
Number of Revertants/Plate

TA98NR strain, the TA98 strain exhibited less mutations in the presence of rat liver S9, at a rate of 330 revertants/plate.

By far the highest level of mutations were seen with the YG1024 strain. Again the mutation rate was higher in the absence of S9. Without the mammalian enzyme supplement, 1820 revertants/plate were generated, while the presence of S9 dropped the mutation level to 920 revertants/plate.

The A.T. Cameron stack soot extract generated very high numbers of revertants (Figures 3.10.1; 3.10.2; 3.10.3). With the bacterial strain TA98NR, minimal mutations occurred in the absence of rat liver S9. In the presence of S9, these levels rose to 192 revertants/plate and 295 revertants/plate, with 5 and 15 μl of extract, respectively. The TA98 strain proved much more susceptible to mutation as 15 μl extract produced 1443 revertants/plate. The presence of S9 had little effect on this total, as 1306 revertants/plate were observed.

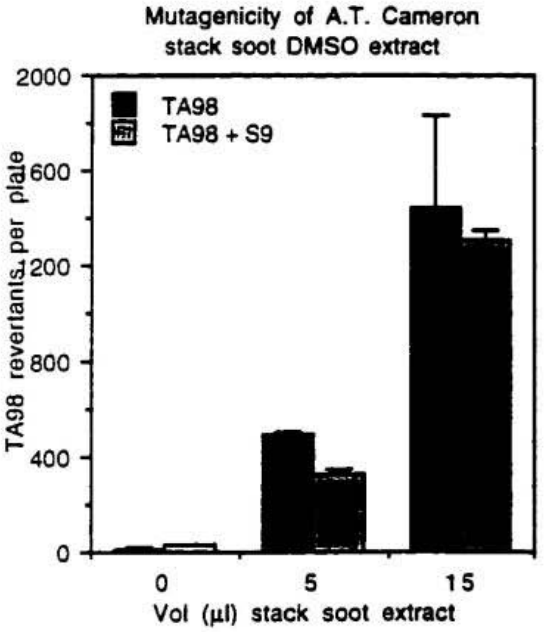
By far the highest reversion levels were seen with the YG1024 strain. Five μl of the extract increased the number of colonies from a control level of 28 revertants/plate to 2390 revertants/plate. The addition of 15 μl extract further increased the revertant total to 6052 per plate. As with the Gulf Star extract, the addition of S9 diminished the mutagenic effects of extract #2. Five μl and 15 μl of the extract produced 893 revertants/plate and 3767 revertants/plate, respectively; a considerable reduction in mutagenesis.

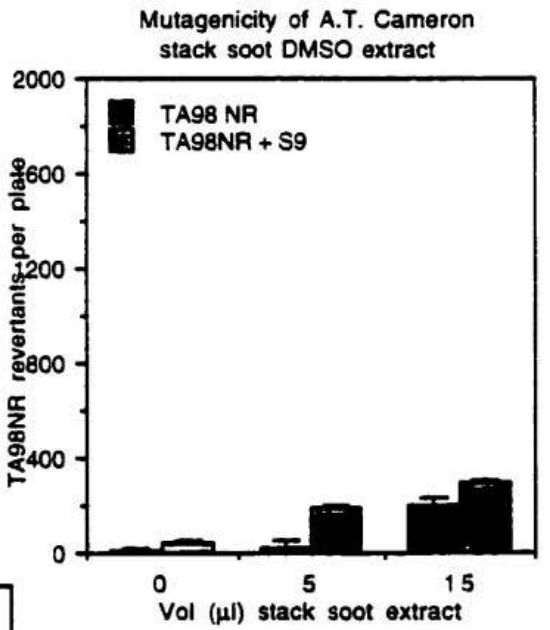
Fig. 3.10.1 ***Salmonella typhimurium*** **TA98NR revertants produced by incubation with A.T. Cameron stack soot DMSO extract**

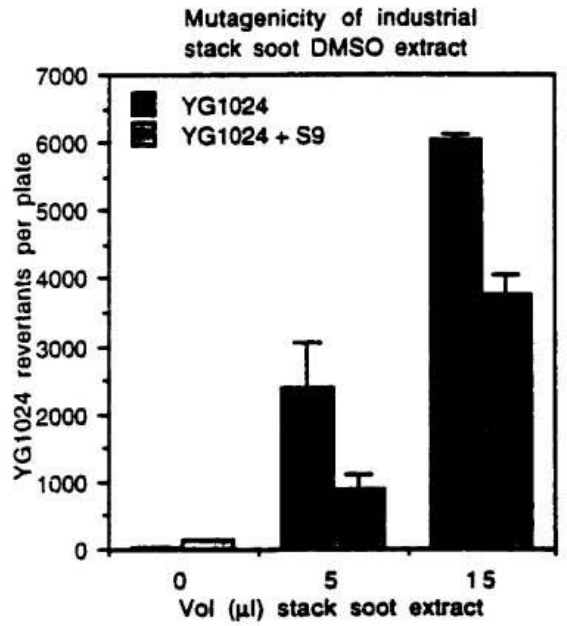
Fig. 3.10.2 ***Salmonella typhimurium*** **TA98 revertants produced by incubation with A.T. Cameron stack soot DMSO extract**

Fig. 3.10.3 ***Salmonella typhimurium*** **YG1024 revertants produced by incubation with A.T. Cameron stack soot DMSO extract**

Samples were prepared in sterile culture tubes. 0-15 μL of the test compound was placed in each tube, followed by 2 mL liquid (approximately 45°C) top agar. 100 μL of an active culture of the tester strain was added to each tube. Every reaction condition was prepared in duplicate as well as in the presence and absence of a rat liver S9-NADPH regenerating system. Plates were incubated at 37°C for 48 hours prior to counting the colonies.


Mutagenicity of A.T. Cameron
stack soot DMSO extract
TA98
TA98 + S9
TA98 revertants per plate
2000
600
200
800
400
0
0
5
15
Vol (µl) stack soot extract


Mutagenicity of A.T. Cameron
stack soot DMSO extract
TA98 NR
TA98NR + S9
TA98NR revertants per plate
2000
600
200
800
400
0
0
5
15
Vol (µl) stack soot extract


Mutagenicity of industrial
stack soot DMSO extract
YG1024
YG1024 + S9
YG1024 revertants per plate
7000
6000
5000
4000
3000
2000
1000
0
0
5
15
Vol (µl) stack soot extract

3.2.3 Mutagenicity of crankcase oil extracts

Tests of the DMSO extract of crankcase oil #1 with the bacterial strain TA98NR indicated a limited mutagenic capability under the reaction conditions (Figure 3.11). In the absence of rat liver S9, the maximal level of mutation was 44 revertants/plate (with 15 μl extract) as compared to a control (no addition) level of 21 revertants/plate. The presence of the rat liver S9 preparation increased the mutation rate to a maximum of 295 revertants/plate with the addition of 15 μl of the crankcase oil extract. In the presence of the S9 preparation, there was also a dose-dependent increase in the rate of mutation as more extract was added to the reaction.

The TA98 bacteria (Figure 3.12) produced elevated levels of mutations relative to the TA98NR strain. As was the case with the nitroreductase deficient strain, there was a negligible mutation rate in the absence of the S9 preparation. 15 μl of extract only increased the level from a control value of 30 revertants/plate to 98 revertants/plate. In the presence of the S9 preparation, the mutation frequency increased substantially, in a dose dependent fashion, to the maximum level of 888 revertants/plate with the addition of 15 μl oil extract.

The highest levels of mutation were seen in the YG1024 strain (Figure 3.13). All tested doses of the extract, in the absence of the S9, produced at least ten times the 21 revertants/plate seen in the control reaction. The maximal value observed was 587 revertants/plate, when 5 μl extract were administered.

Fig. 3.11 ***Salmonella typhimurium* TA98NR revertants produced by incubation with crankcase oil #1 DMSO extract**

Samples were prepared in sterile culture tubes. 0-15 μL of the test compound was placed in each tube, followed by 2 mL liquid (approximately 45°C) top agar. 100 μL of an active culture of the tester strain was added to each tube. Every reaction condition was prepared in duplicate as well as in the presence and absence of a rat liver S9-NADPH regenerating system. Plates were incubated at 37°C for 48 hours prior to counting the colonies.

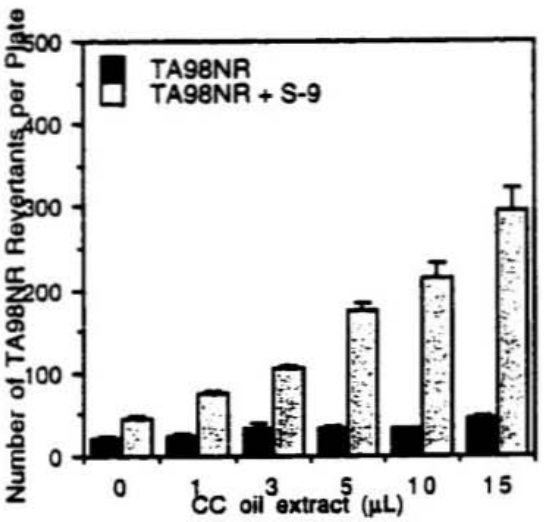

TA98NR
TA98NR + S-9
Number of TA98NR Revertants per Plate
500
400
300
200
100
0
0
1
3
5
10
15
CC oil extract (µL)

Fig. 3.12 ***Salmonella typhimurium* TA98 revertants produced by incubation with crankcase oil #1 DMSO extract**

Samples were prepared in sterile culture tubes. 0-15 μL of the test compound was placed in each tube, followed by 2 mL liquid (approximately 45°C) top agar. 100 μL of an active culture of the tester strain was added to each tube. Every reaction condition was prepared in duplicate as well as in the presence and absence of a rat liver S9-NADPH regenerating system. Plates were incubated at 37°C for 48 hours prior to counting the colonies.

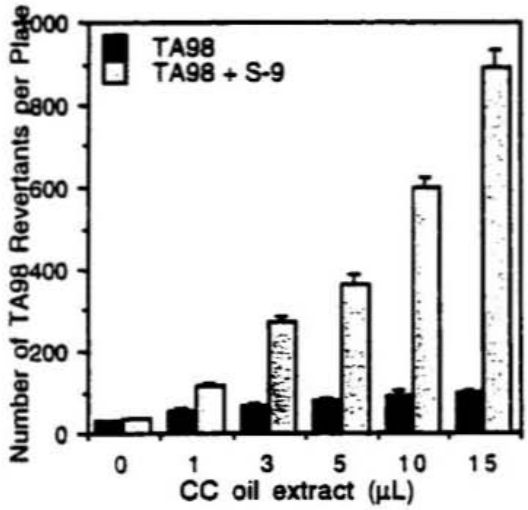
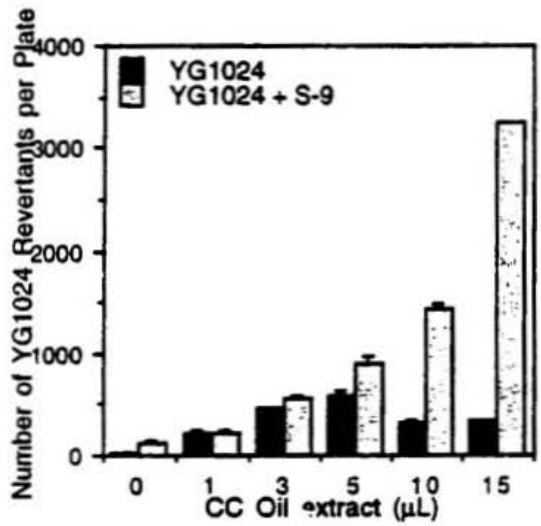

TA98
TA98 + S-9
Number of TA98 Revertants per Plate
1000
800
600
400
200
0
0
1
3
5
10
15
CC oil extract (µL)

Fig. 3.13 ***Salmonella typhimurium* YG1024 revertants produced by incubation with crankcase oil #1 DMSO extract**

Samples were prepared in sterile culture tubes. 0-15 μL of the test compound was placed in each tube, followed by 2 mL liquid (approximately 45°C) top agar. 100 μL of an active culture of the tester strain was added to each tube. Every reaction condition was prepared in duplicate as well as in the presence and absence of a rat liver S9-NADPH regenerating system. Plates were incubated at 37°C for 48 hours prior to counting the colonies.


Number of YG1024 Revertants per Plate
4000
3000
2000
1000
0
YG1024
YG1024 + S-9
0
1
3
5
10
15
CC Oil extract (µL)

When the S9 mixture was present, the results were drastically different. Mutation frequency increased from a control value of 130 revertants/plate to a maximum of 3250 revertants/plate, with 15 μl of the oil extract. This increase occurred in a dose dependent fashion over the range of treatments.

3.3 ^{32}P-postlabelling studies

3.3.1 *In vivo* crankcase oil exposure

The male Sprague-Dawley rats that had been orally administered a DMSO extract of crankcase oil exhibited detectable levels of DNA adducts in the liver, kidney, heart, stomach and brain. However, control animals exhibited similar levels of adducts in the five tissues analyzed. The DNA adduct values for all of these assays are shown in Table 3.1. Samples of autoradiography films for treated liver and control liver are shown in Figures 3.14 and 3.15 respectively.

There was no significant difference between DNA adduct levels of control and treated rats for any of the organs assayed. There also were no adduct spots which appeared exclusively or in distinctly elevated levels, in either set of animals.

The general trend in adduct levels was liver > stomach > kidney > heart > brain,

Table 3.1

DNA adduct levels generated by crankcase oil administration

		Total DNA Adduct Levels (nmol/mol normal nucleotide)				
RAT #	Treatment	LIVER	KIDNEY	HEART	STOMACH	BRAIN
1	T	132.5	94.6	23.1	266.9	22.2
2	T	196.5	168.3	29.4	75.3	27.5
3	T	211.8	37.0	24.6	136.6	25.2
4	T	160.2	50.9	199.2	132.5	32.5
5	U	144.4	59.3	21.6	154.8	29.0
6	U	163.0	18.0	20.1	63.8	30.2
7	U	212.5	124.7	42.8	131.4	28.0

T denotes animals that received crankcase oil dosage.

U denotes control animals.

Fig. 3.14 Adduct profile of crankcase oil extract treated rat livers

Contact print of 32P-postlabelling autoradiogram for two crankcase oil treated rat livers. Rats were orally administered a DMSO extract of crankcase oil every other day for 31 days. Isolated, enriched and radiolabelled DNA was subjected to multidimensional thin layer chromatography and the resulting TLC plate developed in autoradiography. Distinct regions of radioactivity are labelled here and were analyzed separately.

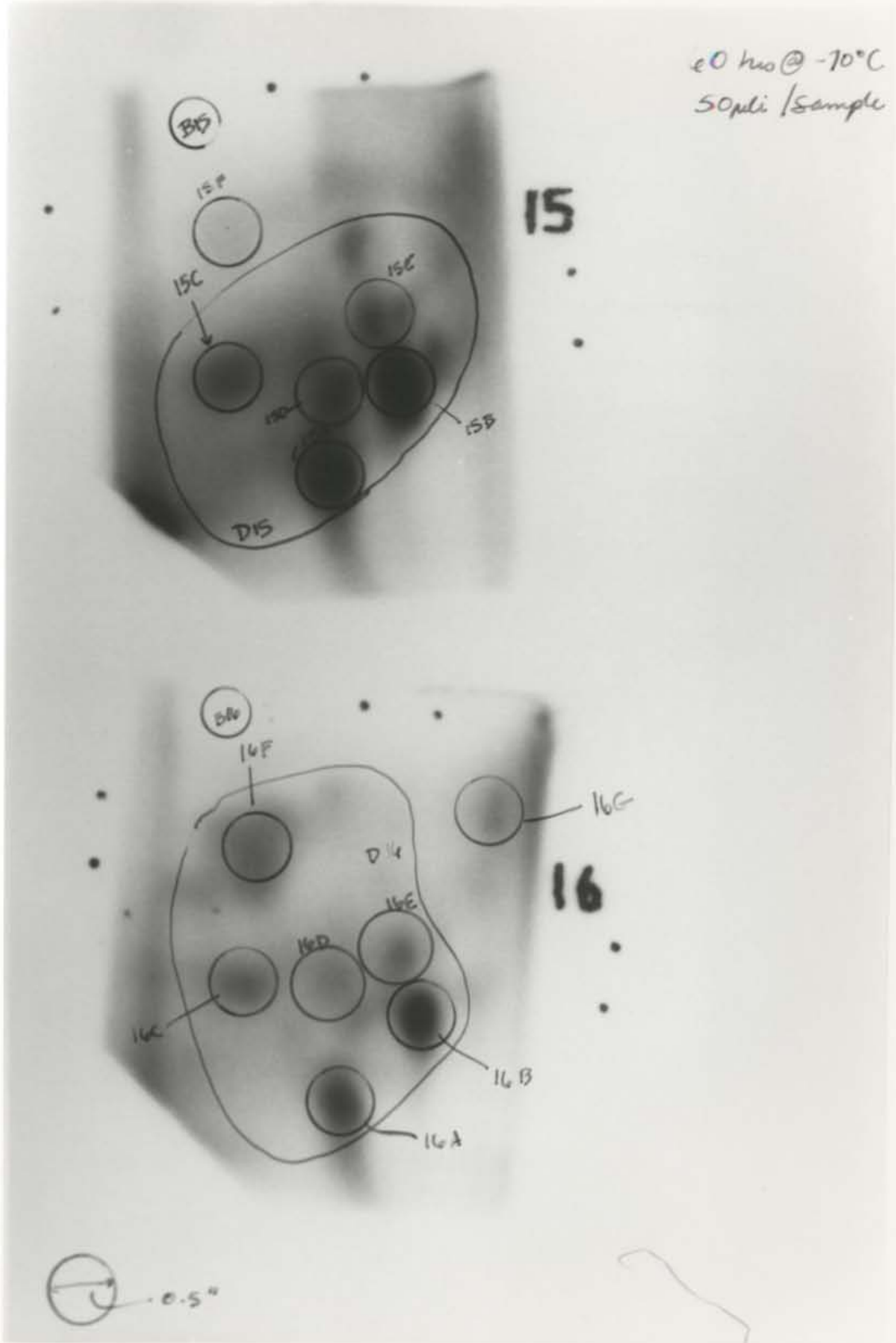
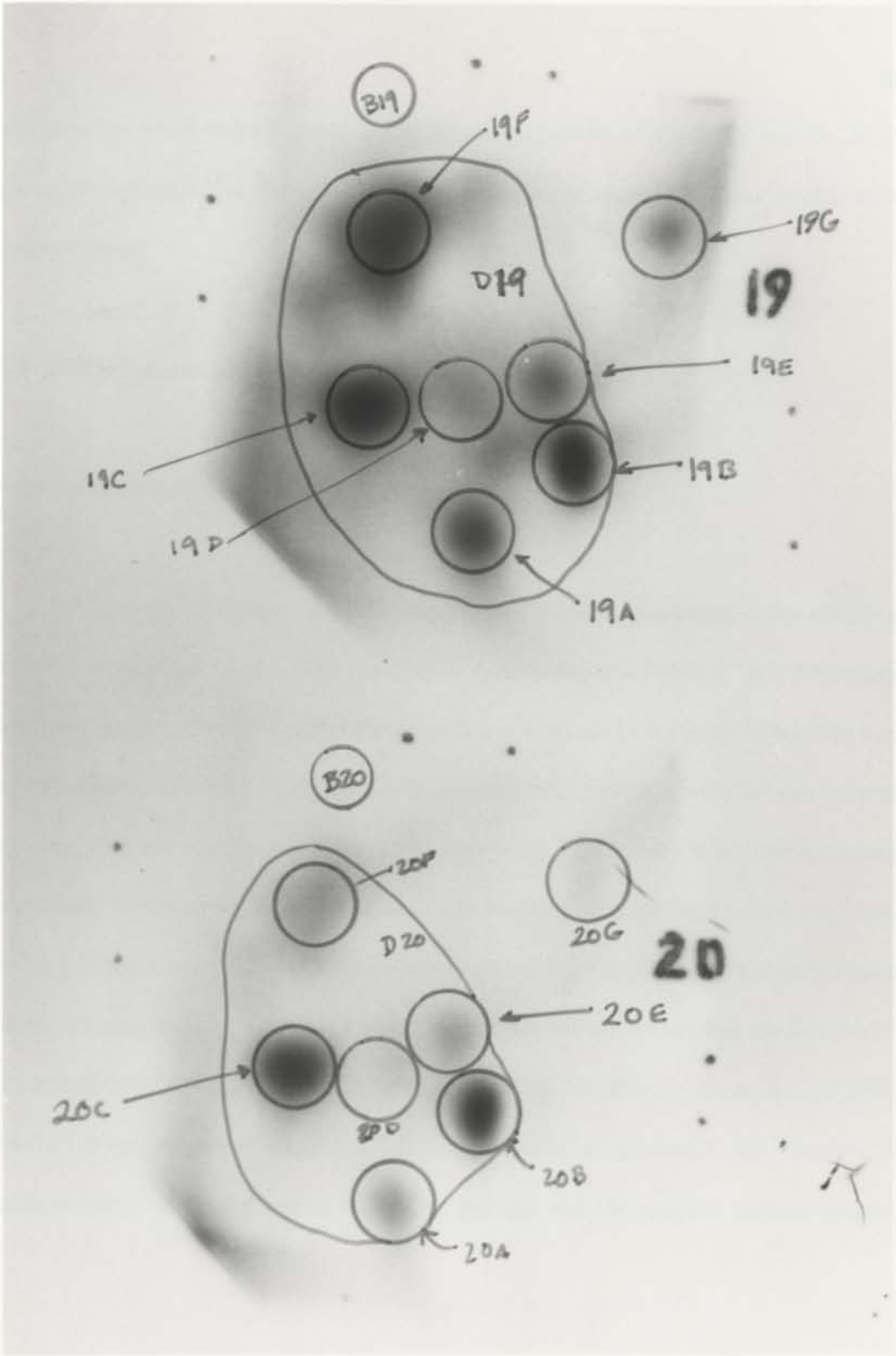

e0 hrs @ -70°C
50µli /sample
B15
15F
15C
15E
15D
15B
D15
15
B16
16F
16G
D16
16
16E
16D
16C
16B
16A
0.5"

Fig. 3.15 **Adduct profile of control rat livers**

Contact print of 32P-postlabelling autoradiogram for two control rat livers. Isolated, enriched and radiolabelled DNA was subjected to multi-dimensional thin layer chromatography and the resulting TLC plate developed in autoradiography. Distinct regions of radioactivity are labelled here and were analyzed separately.


B19
19F
19G
D19
19
19E
19C
19B
19D
19A
B20
20F
20G
D20
20
20E
20C
20D
20B
20A

although individual variability was quite high, and only one of the tested animals fit the general trend exactly. The extreme values seen in the various organ sets did not appear in the same animal(s).

3.4 pBR322 plasmid nicking studies

3.4.1 DNA strand breaks with 1-nitropyrene exposure

Four separate 20 hour reactions, each done in triplicate, were used to test the DNA strand breakage potential of 1-NP. The agarose gel is pictured in Figure 3.16.1. Percentage of relaxed coil formation for each type of reaction is represented in Figure 3.16.2. The first reaction combination was the pBR322 plasmid DNA with the reaction buffer only (control sample). This combination resulted in an average of 22.3 ± 0.51% of total DNA occurring in the relaxed coil form. When the plasmid was incubated with the buffer and 5 μg 1-NP, 24.7 ± 1.77% of the pBR322 DNA appeared in the relaxed coil form. The difference between the control samples and the control + 1-nitropyrene samples was not statistically significant. The complete reaction system (plasmid, buffer, rat liver S9, NADPH), in the absence of 1NP, resulted in the conversion of 51.1 ± 0.60% of the pBR322 plasmid to the relaxed coil configuration. The highest rate of conversion was seen with the complete reaction system

Fig. 3.16.1 Agarose gel of pBR322 plasmid with exposure to 1-nitropyrene

The test compounds were added to 1.5 mL plastic tubes and evaporated to dryness. The reactions were carried out in sterile 50 mM KP buffer, pH 7.4, with a total volume of 50 μL. Each reaction tube contained $FeSO_4$ (0.125 μM), 1 μg of plasmid, and 0-5 μg postmitochondrial fraction protein. Five μL 10 mM NADPH was used to start each reaction. Samples were incubated at 37°C for 1 - 24 hours. Following the incubation, 5μL of 6 x tracking dye was added to each tube, followed by 5 μL of a solution 1 mg/mL in protease and RNase T_1. These samples were incubated at 37°C for a further 45 minutes, and then loaded onto a 0.5% agarose gel. The gels were run at 20 - 100V for 4 to 16 hours. Gels were viewed under u.v. light, in a darkroom, and photographed. Lanes 1, 2 and 3 are control samples. Lanes 4, 5 and 6 are control samples with 1-nitropyrene added. Lanes 7, 8 and 9 are complete reaction systems in the absence of 1-nitropyrene. Lanes 10, 11 and 12 are complete reaction systems in the presence of 1-nitropyrene.

mw 1 2 3 4 5 6 7 8 9 10 11 12 mw

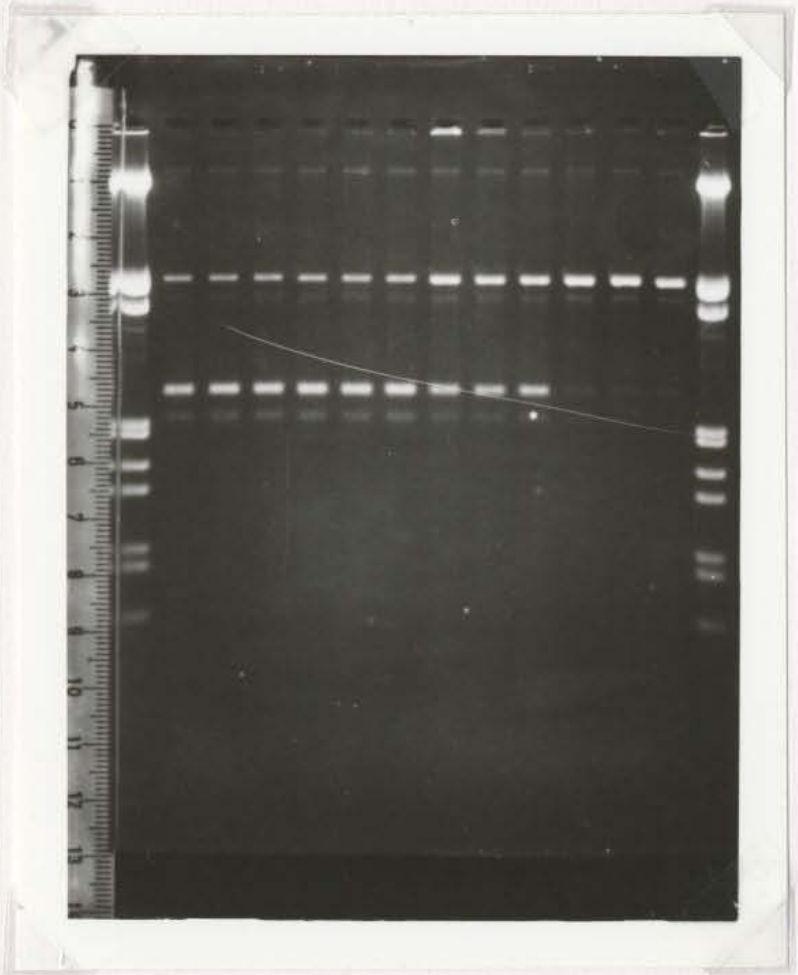
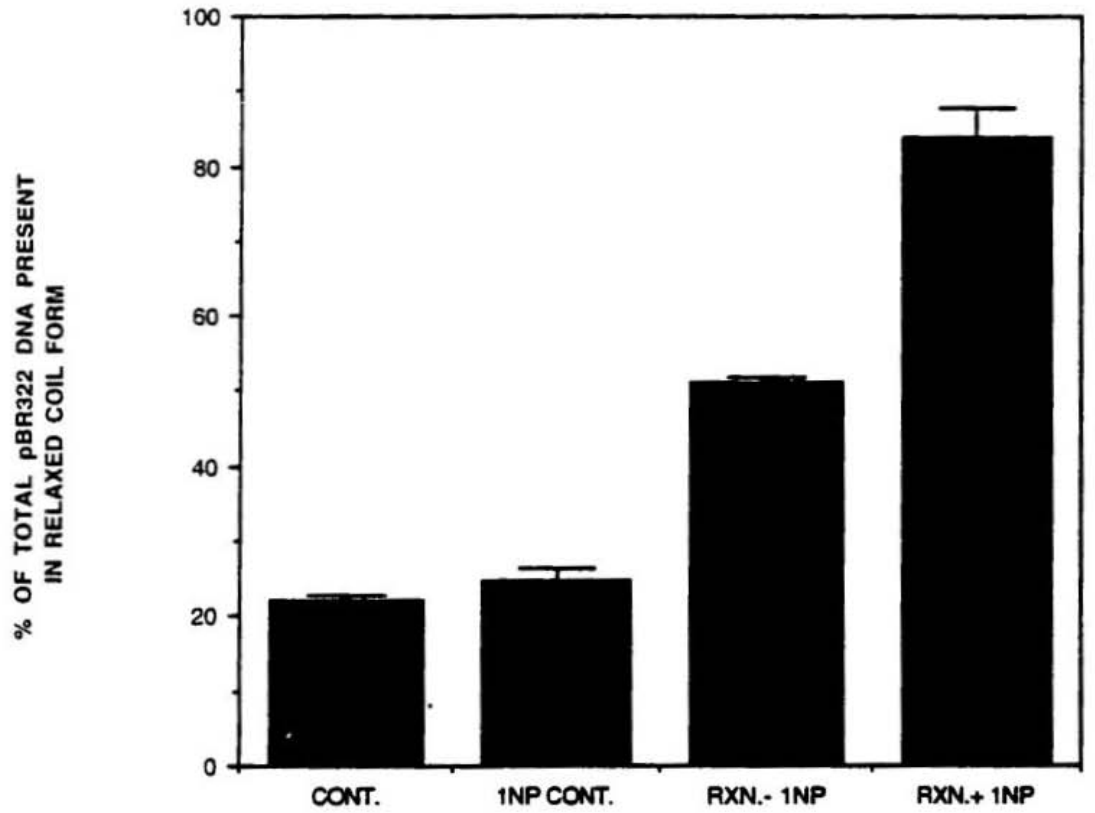


Fig. 3.16.2 **Formation of relaxed coil pBR322 plasmid with exposure to 1-nitropyrene**

The test compounds were added to 1.5 mL plastic tubes and evaporated to dryness. The reactions were carried out in sterile 50 mM KP buffer, pH 7.4, with a total volume of 50 μL. Each reaction tube contained $FeSO_4$ (0.125 μM), 1 μg of plasmid and 0-5 μg postmitochondrial fraction protein. Five μL 10 mM NADPH was used to start each reaction. Samples were incubated at 37°C for 1 - 24 hours. Following the incubation, 5μL of 6 x tracking dye was added to each tube, followed by 5 μL of a solution 1 mg/mL in protease and RNase T_1. These samples were incubated at 37°C for a further 45 minutes, and then loaded onto a 0.5% agarose gel. The gels were run at 20 - 100V for 4 to 16 hours. Gels were viewed under u.v. light, in a darkroom, and photographed. The negatives were scanned using an LKB scanning laser densitometer to reveal the relative intensities of the plasmid forms evident on the gel. Samples were compared by relating the percentage of total plasmid DNA present in the relaxed coil form ($n = 3$; +/- S.D.; $p < 0.05$).


% OF TOTAL pBR322 DNA PRESENT
IN RELAXED COIL FORM
100
80
60
40
20
0
CONT.
1NP CONT.
RXN.- 1NP
RXN.+ 1NP

plus 5 μg 1-NP, with 83.9 ± 3.96% of the plasmid DNA present in the modified state. The complete reaction system plus 1-NP conversion was significantly different from the levels exhibited in each of the three other sets of conditions ($p<0.005$).

3.4.2 DNA strand breaks with crankcase oil extract exposure

Over the course of a 20 hour incubation (at 37°C), the effects of crankcase oil extract on pBR322 DNA were assayed (agarose gel pictured in Figure 3.17.1; results of scanning data illustrated in Figure 3.17.2), with each set of reaction conditions performed in triplicate. The plasmid, when incubated with buffer only, exhibited only 24.7 ± 1.34 % of total DNA in the relaxed coil form. When the same reaction was performed in the presence of crankcase oil extract, relaxed coil plasmid accounted for 37 ± 7.62 % of the total DNA. The combination of plasmid, 0.25 μM Fe^{2+}, 5 μg protein and 1 mM NADPH (complete system) resulted in 35.8 ± 1.65 % of total DNA appearing as relaxed coil. The highest percentage was seen with the complete system plus crankcase oil extract, with an average of 61.7 ± 3.93 % conversion. The complete system + crankcase oil reactions generated an amount of relaxed coil plasmid that was statistically significant from the other three sets of experimental conditions ($p < 0.02$).

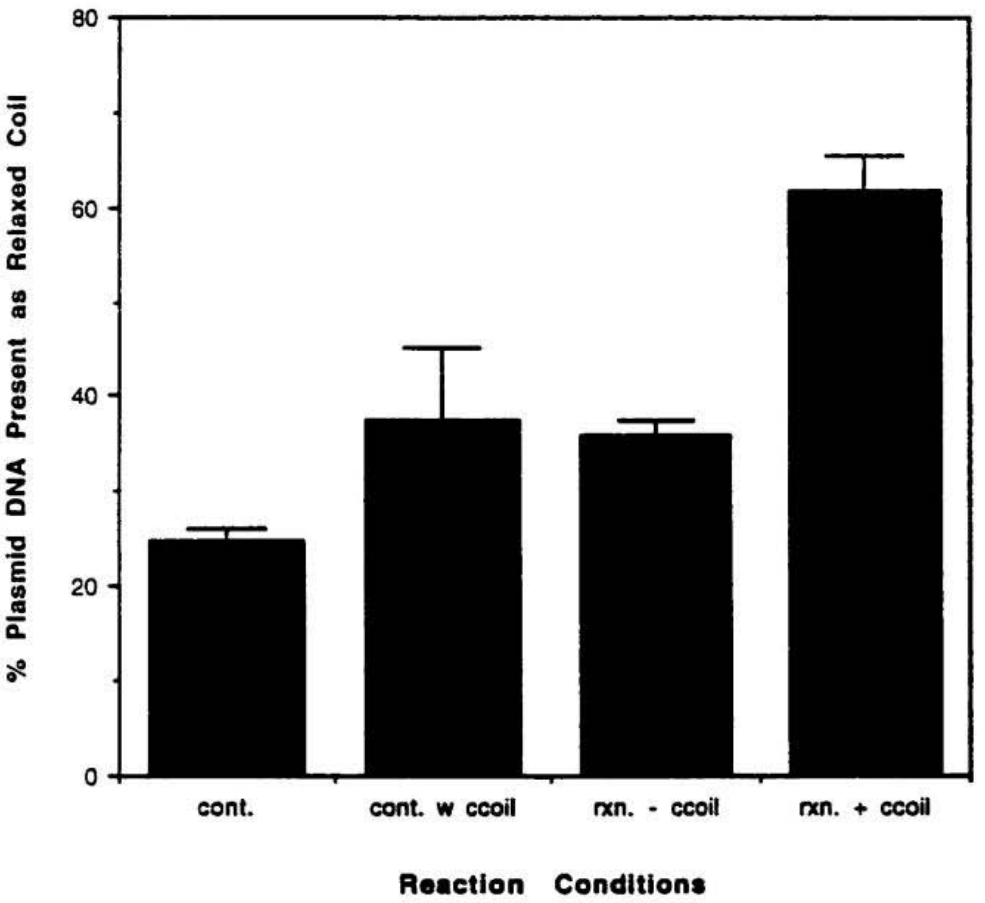
Fig. 3.17.1 Agarose gel of pBR322 plasmid with exposure to crankcase oil

The test compounds were added to 1.5 mL plastic tubes and evaporated to dryness. The reactions were carried out in sterile 50 mM KP buffer, pH 7.4, with a total volume of 50 μL. Each reaction tube contained $FeSO_4$ (0.125 μM), 1 μg of plasmid and 0-5 μg postmitochondrial fraction protein. Five μL 10 mM NADPH was used to start each reaction. Samples were incubated at 37°C for 1 - 24 hours. Following the incubation, 5μL of 6 x tracking dye was added to each tube, followed by 5 μL of a solution 1 mg/mL in protease and RNase T_1. These samples were incubated at 37°C for a further 45 minutes, and then loaded onto a 0.5% agarose gel. The gels were run at 20 - 100V for 4 to 16 hours. Gels were viewed under u.v. light, in a darkroom, and photographed. Lanes 1, 2 and 3 are control samples. Lanes 4, 5 and 6 are control samples with crankcase oil added. Lanes 7, 8 and 9 are complete reaction systems in the absence of crankcase oil. Lanes 10, 11 and 12 are complete reaction systems in the presence of crankcase oil.

mw 1 2 3 4 5 6 7 8 9 10 11 12 mw

Fig. 3.17.2 **Formation of relaxed coil pBR322 plasmid with exposure to crankcase oil extract**

The test compounds were added to 1.5 mL plastic tubes and evaporated to dryness. The reactions were carried out in sterile 50 mM KP buffer, pH 7.4, with a total volume of 50 μL. Each reaction tube contained $FeSO_4$ (0.125 μM), 1 μg of plasmid and 0-5 μg postmitochondrial fraction protein. Five μL 10 mM NADPH was used to start each reaction. Samples were incubated at 37°C for 1 - 24 hours. Following the incubation, 5μL of 6 x tracking dye was added to each tube, followed by 5 μL of a solution 1 mg/mL in protease and RNase T_1. These samples were incubated at 37°C for a further 45 minutes, and then loaded onto a 0.5% agarose gel. The gels were run at 20 - 100V for 4 to 16 hours. Gels were viewed under u.v. light, in a darkroom, and photographed. The negatives were scanned using an LKB scanning laser densitometer to reveal the relative intensities of the plasmid forms evident on the gel. Samples were compared by relating the percentage of total plasmid DNA present in the relaxed coil form ($n = 3$; +/- S.D.; $p < 0.05$).


80
60
40
20
0
% Plasmid DNA Present as Relaxed Coil
cont.
cont. w ccoil
rxn. - ccoil
rxn. + ccoil
Reaction Conditions

3.4.2.1 DNA strand breaks with crankcase oil and 1-nitropyrene exposure

Crankcase oil extract was also incubated with pBR322 plasmid DNA in the presence of 1-NP (agarose gel shown in Figure 3.18.1; results of scanning data illustrated in Figure 3.18.2). The reaction control samples (contained neither of 1-NP and crankcase oil extract) resulted in 41.3 ± 2.29 % of the DNA appearing in the relaxed coil form. When crankcase oil or 1-NP were added, the amount of DNA converted rose to 63.4 ± 2.22 % ($p = 0.02$) and 72.3 ± 0.38 % ($p = 0.003$). The totals for the crankcase oil reaction and the 1-NP reaction were significantly different ($p = 0.02$). When the 1-NP and the crankcase oil were added in combination the conversion level was 70.6 ± 1.71 %. This result was significantly different from the reaction control ($p=0.006$) and the reaction with crankcase oil alone ($p=0.007$). There was no significant difference between the 1-NP reaction and the 1-NP/cc oil reaction.

3.4.2.2 DNA strand breaks with crankcase oil and DMSO exposure

Plasmid DNA (pBR322) was incubated with crankcase oil and the hydroxyl radical scavenger DMSO (agarose gel shown in Figure 3.19.1; results of scanning data illustrated in Figure 3.19.2). The first set of reaction tubes contained plasmid, buffer and crankcase oil condensate. Under these conditions, 45.9 ± 2.50% of the plasmid appeared in the relaxed coil form. When the plasmid was incubated under reaction control conditions, the relaxed coil

Fig. 3.18.1 Agarose gel of pBR322 plasmid with exposure to 1-nitropyrene and crankcase oil extract

The test compounds were added to 1.5 mL plastic tubes and evaporated to dryness. The reactions were carried out in sterile 50 mM KP buffer, pH 7.4, with a total volume of 50 μL. Each reaction tube contained $FeSO_4$ (0.125 μM), 1 μg of plasmid and 0-5 μg postmitochondrial fraction protein. Five μL 10 mM NADPH was used to start each reaction. Samples were incubated at 37°C for 1 - 24 hours. Following the incubation, 5μL of 6 x tracking dye was added to each tube, followed by 5 μL of a solution 1 mg/mL in protease and RNase T_1. These samples were incubated at 37°C for a further 45 minutes, and then loaded onto a 0.5% agarose gel. The gels were run at 20 - 100V for 4 to 16 hours. Gels were viewed under u.v. light, in a darkroom, and photographed. Lanes 1, 2 and 3 are control samples in the presence of crankcase oil. Lanes 4, 5 and 6 are complete reaction systems in the presence of crankcase oil. Lanes 7, 8 and 9 are complete reaction systems in the presence of 1-nitropyrene. Lanes 10, 11 and 12 are complete reaction systems in the presence of both crankcase oil and 1-nitropyrene.

mw 1 2 3 4 5 6 7 8 9 10 11 12 mw

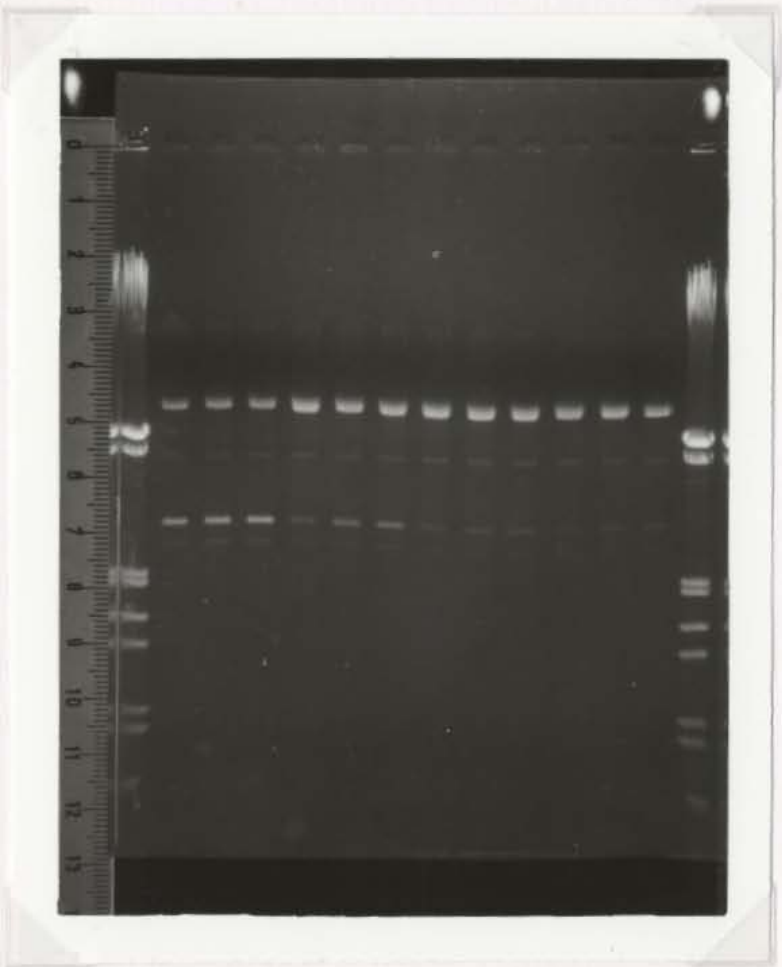


Fig. 3.18.2 Formation of relaxed coil pBR322 plasmid with exposure to crankcase oil extract

The test compounds were added to 1.5 mL plastic tubes and evaporated to dryness. The reactions were carried out in sterile 50 mM KP buffer, pH 7.4, with a total volume of 50 μL. Each reaction tube contained $FeSO_4$ (0.125 μM), 1 μg of plasmid and 0-5 μg postmitochondrial fraction protein. Five μL 10 mM NADPH was used to start each reaction. Samples were incubated at 37°C for 1 - 24 hours. Following the incubation, 5μL of 6 x tracking dye was added to each tube, followed by 5 μL of a solution 1 mg/mL in protease and RNase T_1. These samples were incubated at 37°C for a further 45 minutes, and then loaded onto a 0.5% agarose gel. The gels were run at 20 - 100V for 4 to 16 hours. Gels were viewed under u.v. light, in a darkroom, and photographed. The negatives were scanned using an LKB scanning laser densitometer to reveal the relative intensities of the plasmid forms evident on the gel. Samples were compared by relating the percentage of total plasmid DNA present in the relaxed coil form ($n = 3$; +/- S.D.; $p < 0.05$).

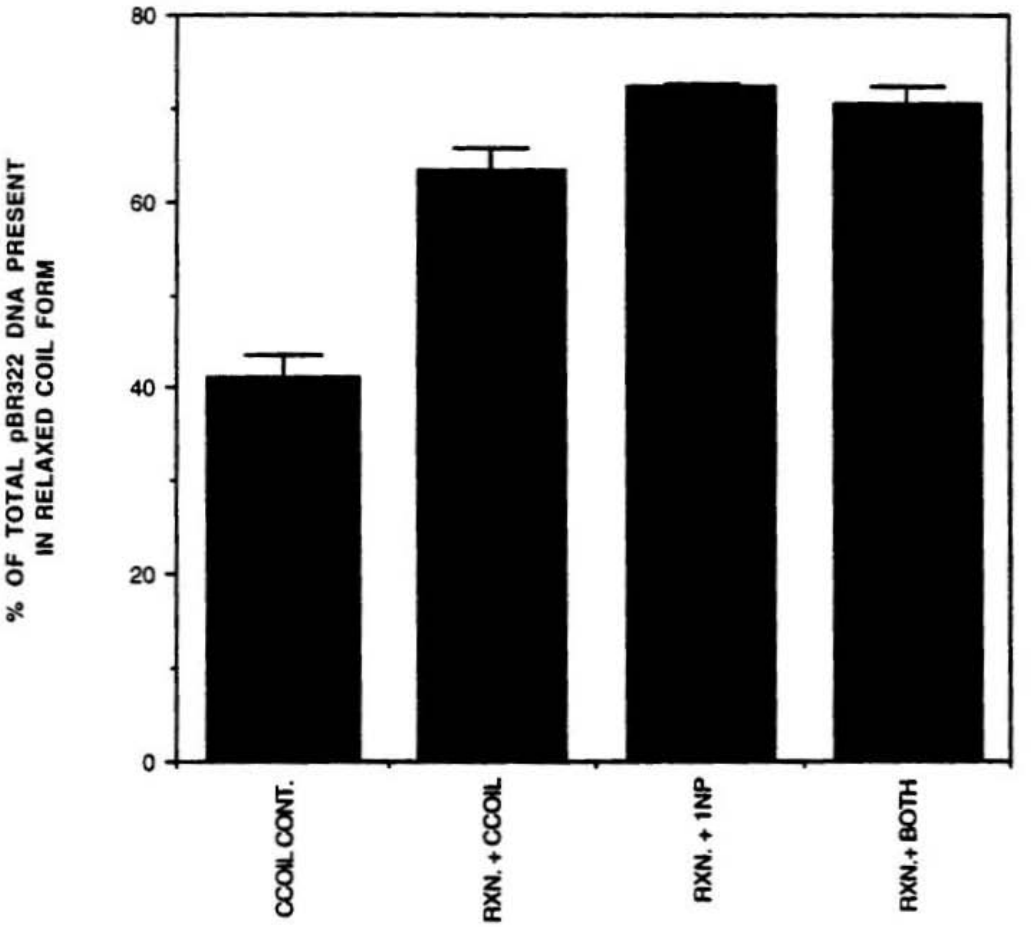

% OF TOTAL pBR322 DNA PRESENT
IN RELAXED COIL FORM
80
60
40
20
0
CCOIL CONT.
RXN. + CCOIL
RXN. + 1NP
RXN.+ BOTH

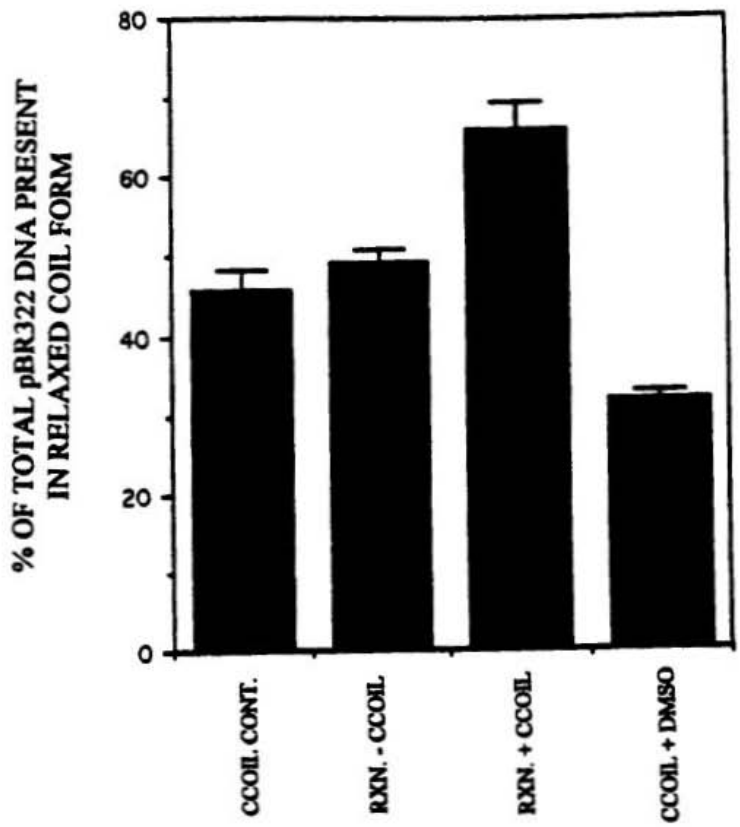
Fig. 3.19.1 Agarose gel of pBR322 plasmid with exposure to crankcase oil extract and DMSO.

The test compounds were added to 1.5 mL plastic tubes and evaporated to dryness. The reactions were carried out in sterile 50 mM KP buffer, pH 7.4, with a total volume of 50 μL. Each reaction tube contained $FeSO_4$ (0.125 μM), 1 μg of plasmid and 0-5 μg postmitochondrial fraction protein. Five μL 10 mM NADPH was used to start the reaction. Samples were incubated at 37°C for 1 - 24 hours. Following the incubation, 5μL of 6 x tracking dye was added to each tube, followed by 5 μL of a solution 1 mg/mL in protease and RNase T_1. These samples were incubated at 37°C for a further 45 minutes, and then loaded onto a 0.5% agarose gel. The gels were run at 20 - 100V for 4 to 16 hours. Gels were viewed under u.v. light, in a darkroom, and photographed. Lanes 1, 2 and 3 are control samples in the presence of crankcase oil. Lanes 4, 5 and 6 are complete reaction systems in the absence of crankcase oil. Lanes 7, 8 and 9 are complete reaction systems in the presence of crankcase oil. Lanes 10, 11 and 12 are complete reaction systems in the presence of crankcase oil and DMSO.

mw 1 2 3 4 5 6 7 8 9 10 11 12 mw

Fig. 3.19.2 Formation of relaxed coil pBR322 plasmid with exposure to crankcase oil extract and DMSO

The test compounds were added to 1.5 mL plastic tubes and evaporated to dryness. The reactions were carried out in sterile 50 mM KP buffer, pH 7.4, with a total volume of 50 μL. Each reaction tube contained $FeSO_4$ (0.125 μM), 1 μg of plasmid and 0-5 μg postmitochondrial fraction protein. Five μL 10 mM NADPH was used to start the reaction. Samples were incubated at 37°C for 1 - 24 hours. Following the incubation, 5μL of 6 x tracking dye was added to each tube, followed by 5 μL of a solution 1 mg/mL in protease and RNase T_1. These samples were incubated at 37°C for a further 45 minutes, and then loaded onto a 0.5% agarose gel. The gels were run at 20 - 100V for 4 to 16 hours. Gels were viewed under u.v. light, in a darkroom, and photographed. The negatives were scanned using an LKB scanning laser densitometer to reveal the relative intensities of the plasmid forms evident on the gel. Samples were compared by relating the percentage of total plasmid DNA present in the relaxed coil form ($n = 3$; +/- S.D.; $p < 0.05$).


% OF TOTAL pBR322 DNA PRESENT
IN RELAXED COIL FORM
80
60
40
20
0
CCOIL CONT.
RXN. - CCOIL
RXN. + CCOIL
CCOIL + DMSO

band accounted for 49.1 ± 1.56% of the detected plasmid. This total was not significantly different from that seen from the plasmid, buffer and crankcase oil incubations. When crankcase oil was added to the reaction control mixture, relaxed coil plasmid totals rose to 66.1 ± 3.30% of the total ($p < 0.02$). When this reaction was repeated in the presence of DMSO, the percentage of total plasmid appearing as relaxed coil shrunk to 31.6 ± 1.04% ($p < 0.02$).

CHAPTER 4

4. DISCUSSION

4.1 Nitroreductase activity studies

4.1.1 Crude Oil studies

The four crude oil extracts, which were from ecologically diverse locations, all proved to be poor nitroreductase substrates *in vitro*. Neither extract could produce as much as one sixth of the activity seen in crankcase oil extract assays.

Since little, if any, nitroreductase activity can be generated with crude oil extracts, it follows logically that the more potent substrates found in partially combusted oils are not naturally occurring, but in fact are the result of their mechanical or industrial use. The alternate explanation is that nitroreductase inhibiting compounds are present in the crude oil extracts, but are not present in the crankcase oil extracts. However, the fact that reported levels of nitro-PAH are highest in the residue of organic material combustion (Newton et al., 1982; Jones et al., 1993; Felton et al.; 1994 Khesina et al., 1994; and, Moller, 1994), would suggest that this is not the case. While inhibitory compounds might play a role in the low levels of nitroreduction activity generated with crude oil extracts as enzyme substrates, the fact remains that crude oils have not been "burned" to the point of gasoline or diesel fuel

exhaust (the residue of which is collected in the crankcase oil of automobiles). Therefore, the crankcase oils are richer in nitro-PAH than the crude oils, and as a result the enzyme activity levels are higher with crankcase oil extracts as the substrate.

4.1.2 Industrial Stack Soot Studies

The tested industrial stack soot extracts exhibited considerably more potency (than crude oil extracts) as mammalian nitroreductase substrates. This fact is certainly in keeping with the hypothesis that combustion is the root cause of the formation, from hydrocarbons, of nitro-PAH. Both extracts seemed to reach a threshold where enzyme activity was not enhanced by the addition of more substrate. This stage was reached much earlier for extract #2, as the maximal activity was achieved with one fifth the amount required of the first extract. Because these extracts were both incredibly complex mixtures, these observation could be due to any of a number of factors. Since extract #2 produced maximal enzyme rates at a lower concentration, it could simply have a higher concentration of suitable substrates. Similarly, any nitroreductase-inhibitory compounds present in these extracts could be more concentrated in extract #1. The existence of an apparent activity threshold, which was not evident at much higher activity levels with the crankcase oil extracts, could imply the latter. While this would seem to best explain the observed results, it does not eliminate the possibility that the mechanics of nitroreductase varies considerably between the substrates

present in the soot extracts and the oil extracts.

4.1.3 Crankcase Oil Studies

The DMSO extracts of crankcase oil produced nitroreductase activity levels of up to 3 times those seen with the soot extracts. While, as before, higher activity levels might be indicative of either higher levels of substrate or lower levels of inhibitors (or a combination of the two), the more likely explanation would seem to be the former. When gasoline or diesel fuel is burned in an automobile engine, the residual compounds can collect in the motor oil. As such, over time, the motor oil becomes very rich in combustion by-products, including nitrated polyaromatic hydrocarbons. Therefore, it would follow that extracts of such materials would prove to be excellent nitroreductase substrates. In any event, the potency of crankcase oil extracts to induce the formation of nitroreduction products would tend to implicate this mixture as being potentially more toxic than the other mixtures tested in this study.

The levels of cytochrome c reduced with varying levels of enzyme and (crankcase oil) substrate present suggest that the enzyme(s) responsible for the activity had a considerable capacity to facilitate the reaction, as no apparent activity threshold was obvious from the data. The limiting factor in these assays would appear to be the availability of substrate, as higher concentrations of substrate and enzyme each increased activity in a dose-dependent fashion.

4.1.4 Identity of the Nitroreductase

In all the nitroreductase assays, there was no apparent preference for NADH or NADPH as a cofactor. One possible explanation for this is that the enzyme responsible for the activity is DT Diaphorase, a known nitroreductase. However, DT Diaphorase is known to be present in the endoplasmic reticulum of mammalian cells, meaning that is should be present primarily in the microsomal product of a sub-cellular fractionation procedure (Nilsson and Dallner, 1977). Given that rat liver cytosol was the enzyme source for these experiments, DT Diaphorase may not be the only nitroreductase present in the protein source.

An alternate explanation for the apparent cofactor-indifference of the nitroreductase enzyme is that, like DT Diaphorase, some other single (in this case cytosolic) enzyme can reduce nitro moieties present on PAH molecules, by utilizing either NADH or NADPH as a cofactor. If this is the case, such an enzyme has not yet been identified. Finally, there could be a number of nitroreduction-capable enzymes that are present in rat liver cytosol, which use either (or both) of NADH and NADPH as cofactors.

4.2 Ames *Salmonella* Mutagenicity Studies

4.2.1 Crude Oil Studies

All crude oil extracts exhibited little or no mutagenic activity when tested with the TA98, TA98NR, or YG1024 bacterial strains. This data mirrored that collected in the nitroreductase activity studies where minimal enzyme activity was detected. Therefore, crude oils, which are poor sources of nitroaromatic hydrocarbons, are also poor substrates for nitroreductase activity. These facts suggest that the lack of mutagenic activity was the result of the relative absence of DNA-reactive species which can arise from the metabolic activation of nitrated polyaromatic hydrocarbons. The fact that the addition of a rat liver preparation did not produce substantial colonies of mutant bacteria suggests that the crude oil extracts would have a minimal mutagenic effect *in vivo*, a thesis which is supported by the data from the nitroreductase studies.

4.2.2 Industrial Stack Soot Studies

As between the two stack soot extracts studied, the A.T. Cameron soot extract was by far the more mutagenic. However, it should not be considered odd to find two separate stack soots with widely ranging mutagenic potentials. As has been discussed, such mixtures

are complex by their very nature, and differing compositions in the levels and varieties of mutagens and inhibitors can produce profoundly different effects in biological systems.

The stack soot extracts exhibited a trend, with respect to the response of the different bacterial strains, which was in keeping with that produced by the crankcase oils. Minimal numbers of revertant colonies were produced in the TA98NR strain tests of both extracts. When the TA98 strain was exposed to the extracts, significantly more revertants were produced. These results suggest a significant role of nitrated compounds in the mutagenic process. When a nitroreductase deficient strain of bacteria was used, scarcely more revertants were produced than those seen in control tests. Since experiments done under identical conditions but for the nitroreductase deficiency (eg. the TA98 tests) produced many more revertants, nitroreduction appears essential to generating mutations.

A further increase, beyond the number of revertants produced by the TA98 strain, was exhibited when the YG1024 strain was tested. Since YG1024 is essentially TA98 bacteria with elevated *O*-acetylase activity, the elevated levels of mutation exhibited with the YG1024 strain support the overall theory of mutagenicity whereby *O*-acetylation is required (after nitroreduction) to generate the ultimate DNA reactive species. Thus nitroreduction and *O*-acetylation seem essential to the DNA-reaction/mutation pathway as per the hypothesis presented in Figure 1.1 (at page 4).

The final observation arising from the stack soot extract mutagenicity studies is the fact that the presence of the mammalian protein actually diminished the mutagenic response.

This is contrary to the trend exhibited in the other mutagenicity studies, where the mammalian protein proved essential to produce the highest levels of revertant formation. This is likely a testament to the fact that there are numerous competing chemicals, enzymes, and reaction pathways in such a complex mixture. As such, the mammalian enzymes may be binding and possibly modifying species which are essential to the production of the ultimate mutagen at work in the non-S9 experiments. Alternately, the mammalian enzymes may be producing compounds which inhibit the mutagenic pathway. This does not detract from the theory that nitrocompounds are the parent mutagenic species at work here. It merely illustrates that complex mixtures can have unpredictable effects *in vivo*, and that while the general trend may be toward nitro-PAH-induced mutagenicity, this does not preclude the diminution of toxicity of some combustion byproduct mixtures by mammalian enzymatic modification.

4.2.3 Crankcase Oil Extract Studies

The crankcase oil extracts produced a clear trend with respect to the response of the different bacterial strains. The TA98NR strain produced minimal amounts of revertant colonies, while the TA98 strain produced almost three times as many revertants. This level of mutation was increased a further three fold when the tester strain was YG1024. Clearly, the absence of nitroreductase enzymes drastically reduces the mutagenicity of the extract administration. Furthermore, the combination of elevated *O*-acetylase activity and normal

nitroreductase levels greatly increases the mutagenic effect. Both observations offer strong support for the hypothesis that, in the bioactivation of nitro-PAH, nitroreduction is the initial step, followed by *O*-acetylation. In the absence of either of these steps, mutagenicity is significantly reduced.

It is also clear from the results that the presence of rat liver S9 is key in producing maximal levels of revertant production. Again, this is in keeping with the theory of nitro-PAH DNA-reactivity in mammalian systems, as bioactivation by mammalian enzymes greatly increases the mutagenic potential of this particular combustion byproduct mixture. Obviously some of the rat liver enzymes play a key role in generating the ultimate DNA-reactive species. The fact that this elevation of mutagenicity occurs with all tester strains make it unclear which aspect of the mutagenic pathway is being enhanced by the mammalian enzymes. Suffice it to say that is could be increased nitroreduction, increased *O*-acetylation, diminished inhibition or any combination thereof, that gives the observed result.

4.3 ^{32}P-Postlabelling Studies

The postlabelling studies yielded no data which was of any comparative value. This could be the result of any number of factors. The first is that the animals tested could just be exhibiting their natural biological variance. While this is certainly a valid concern, attempts were made to minimize this effect, as tested animals were of the same sex, age, and were of

similar body weights. Furthermore, in some experiments, all the animals tested were of the same litter. In any event, the results were always wildly inconsistent.

The more likely, and some respects unavoidable, sources of error in these experiments arise from the method itself, namely the extreme sensitivity of the technique (Randerath et al., 1985). Because the method is theoretically capable of detecting such miniscule amounts of adducted DNA, the slightest human error or reagent problem could give grossly inaccurate results. Furthermore, the time required to do the experiments, the financial costs, and the complexity of the system all severely limit the viability of a comprehensive evaluation of the protocol. Simply put, once severe protocol problems were suspected, the attempted resolution of the difficulties was beyond the scope of this work. Finally, problems in reproducing postlabelling results have been documented in the past. This inherent difficulty with the technique, combined with the biological variation of samples, makes the production of meaningful results very difficult. It may well be that the technique requires experienced technicians with the time and resources to refine the system, elements which were lacking in this particular study.

4.4 pBR322 plasmid nicking studies

When incubated with a complete reaction system and 1-nitropyrene or crankcase oil condensate, pBR322 plasmid was almost completely converted to the relaxed coil form. This

is strong evidence that metabolites of both 1-nitropyrene and crankcase oil condensate are capable of producing hydroxyl radicals which can result in DNA stand scission. These data are consistent with the theory that the nitroaromatic compounds being studied here are capable of producing potent DNA-reactive species *in vivo*. This DNA damaging effect can be said to occur *in vivo* on the strength of the observation that, in the absence of rat liver protein, significantly lower levels of DNA strand scission were observed.

When crankcase oil condensate and 1-nitropyrene were used in concert (in the presence of a complete reaction system), there was no significant elevation in strand scission beyond that exhibited with 1- nitropyrene. This means that was no synergistic, cumulative or inhibitory effect being observed, unless the DNA conversion levels were maximal. This might mean that a threshold exists in terms of production of radical-producing species, and that this threshold had been reached by 1-nitropyrene and/or crankcase oil condensate alone. If this were the case, then increased levels of the parent compound would not produce any appreciable effect. Furthermore, oxygen radicals are quite short-lived. Thus, an increased production of radicals only means a short-term increase in their concentration, as the highly reactive species are quickly consumed. As such, there is no continuing and lingering effect to be observed when more raw materials are present.

The final experiment conducted in the plasmid breakage series was that involving the crankcase oil condensate and DMSO. The presence of DMSO reduced the strand breakage level to approximately half of that seen in the complete system reaction. Since DMSO is a

known hydroxyl radical scavenger, these data offer strong support to the theory that the cause of the strand breakage exhibited in the plasmid experiments was the production of hydroxyl radicals. Thus, the undeniable capacity of crankcase oil and 1-nitropyrene to cause DNA strand scission would seem to arise from the propensity of *in vivo* systems to create oxygen radicals in the metabolic modification of these complex chemicals, as discussed in Chapter 1..

CHAPTER 5

5. CONCLUSIONS

5.1 Conclusions

In this study, three principal types of studies were undertaken to measure the possible *in vivo* effects of exposure to nitro-PAH. In the nitroreductase assays, crankcase oil and stack soot extracts were shown to be excellent substrates for rat liver cytosolic nitroreductase. Likewise, in the Ames tests, crankcase oil and stack soot extracts were found to be potent mutagens. For purposes of comparison, crude oil extracts were used in nitroreductase and Ames *Salmonella* assays, and proved capable of producing neither nitroreduction nor revertant forms of *salmonella* bacteria. These results are strongly suggestive that it is the partial combustion reactions which have generated the chemical species responsible for the nitroreductase activity and mutation/revertant production. Furthermore, the Ames results using differing bacterial strains illustrate that, as hypothesized, nitroreduction and *O*-acetylation are critical steps in the mutagenic pathway.

In Chapter One it was suggested that after the nitroreduction and *O*-acetylation steps, the compound underwent a chemical de-acetylation to produce a chemically active species. Taken one step further, this chemically active species could interact with oxygen molecules to produce hydroxyl radicals. The hydroxyl radicals could then produce strand scission in

DNA which could be responsible for genetic mutations and other toxic responses. To test this theory, studies were done to measure the strand scission of pBR322 plasmid upon exposure to nitro-PAH. These studies illustrate that nitro-PAH compounds can produce DNA strand scission. Furthermore, when these reactions were attempted in the presence of DMSO, a scavenger of hydroxyl radicals, the scission effect was greatly diminished. Thus it would seem that nitro-PAH can produce the ultimate genotoxic effect via the production of hydroxyl radicals and subsequent DNA strand scission.

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