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Evidence for enzymatic reduction of 1-nitropyrene by rat liver fractions

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Summary. 1-Nitropyrene, a mutagenic compound found in diesel exhaust and photocopy toners, is reduced anaerobically by rat liver fractions to 1-aminopyrene. This reaction is stimulated in the microsomal fraction by NADPH and in the cytosol by FMN. Addition of both cofactors produced more reduction in S-9, microsomes, or cytosol than either cofactor alone.

The nitroaromatic compounds 4,4′-dinitrobiphenyl[2], 2-nitrofluorene[3] and 4-nitrobiphenyl[4] produce cancer when fed to experimental animals. The mechanisms of carcinogenicity are thought to be mediated by reduction of the nitro group, followed by conversion of the chemical to reactive intermediates similar to those formed from oxidation of the corresponding carcinogenic amines: (4,4′-diaminobiphenyl), 2-aminofluorene[4] and 4,4′-diaminobiphenyl[2]. Nitroreductases have been studied in relationship to the mutagenic and carcinogenic potentials of nitrofurantoins[5], nitroimidazoles[6], and 4-nitroquinoline-N-oxide, but less is known about the reduction of nitroarenes. Earlier work showed minimal conversion of 2-nitrofluorene to 2-aminofluorene by liver preparations[4]. Poirier and Weisburger[7], however, demonstrated the enzymatic reduction of 2-nitronaphthalene to 2-aminonaphthalene by rat and mouse liver fractions.

Recently, potent mutagenic nitroaromatics have been detected in materials such as photocopy toners[8], engine exhausts[9,10] and urban aerosols. The mutagen bioassay used was the Ames *Salmonella typhimurium* test, a system which contains bacterial nitroreductases. 1 compound, 1-nitropyrene, is of particular interest because its presence in diesel engine exhaust particulates may account for up to 30% of the total engine mutagenic activity in the sample. In order to study the toxic potential of 1-nitropyrene, we tested the ability of mammalian enzymes to reduce 1-nitropyrene (1-NP) to 1-aminopyrene (1-AP).

Liver homogenates were prepared from male Sprague-Dawley rats weighing 150–250 g. Animals were decapitated and the livers rapidly excised and weighed. Tissue samples were homogenized with a Polytron ultrasonic homogenizer using 20 mM Tris-HCl containing 1.15% KCl (pH 7.4) buffer, 4 ml/g of liver wet wt. These homogenates were centrifuged at 9000 × g for 10 min to obtain the S-9 fraction. NADPH, $MgCl_2$ and a glucose-6-phosphate dehydrogenase preparation were added according to the method of Poirier and Weisburger[6]. Protein concentrations were measured by the method of Bradford[11]. Cytosol and microsomal fractions were prepared by centrifugation at 105,000 × g for 1 h at 4 °C. To exclude O_2 from the incubation mixture, tissue and co-factor preparations were kept on ice and gassed with N_2 for at least 20 min. Incubation tubes were also gassed with N_2 and sealed with screw-cap tops. The reaction, with 300 nmoles 1-NP as substrate, was conducted by mechanically shaking the mixture at 37 °C for 40 min (final volume = 3 ml). The reaction was terminated by the addition of 0.1 ml of 10 N HCl.

The sample was extracted with 5 ml cyclohexane for 15 min to remove 1-NP, washed with another 5 ml cyclohexane, and then made basic with 0.6 ml of 2 N NaOH. This was re-extracted with 5 ml cyclohexane to obtain the reduced product 1-AP. The cyclohexane extracts were placed in an American Instruments Co. reflecting fluorimeter (Model SPF-125) with excitation wavelength set at 283 nm, and the percent relative fluorescence was measured. Cyclohexane extracts of incubation mixtures that did not contain 1-NP served as blanks. 1-AP spiked buffer samples were extracted to provide a linear standard curve. The reduction rate was linear with time to approximately 40 min.

1-AP, dissolved in cyclohexane, has an emission maximum at 403 nm. The fluorescence spectra of alkaline extracts of

Subcellular distribution of nitroreductase in rat liver

nmole 1-aminopyrene formed mean (SD)/mg protein			
Cofactors Added	S-9	Cytosol	Microsomes
None	0.03 (0.009)	0.06 (0.012)	0.02
NADPH	0.07 (0.01)	0.07 (0.02)	0.30 (0.05)
FMN	0.36 (0.11)	0.16 (0.05)	0.02
NADPH + FMN	0.54 (0.05)	0.73 (0.09)	7.34 (0.8)
mg protein/fraction	1280	1020	32

The reaction mixture consisted of 300 nmoles of 1-nitropyrene in 50 µl ethanol incubated anaerobically (40 min) in 20 mM Tris-HCl, 1.15% KCl (pH = 7.4) buffer. The final volume was 3.0 ml which contained 1.0 µmoles NADPH and/or FMN, co-factors. Microsomes from 120 mg rat liver; cytosol or S-9 from 40 mg rat liver. 2 determinations per point were performed.

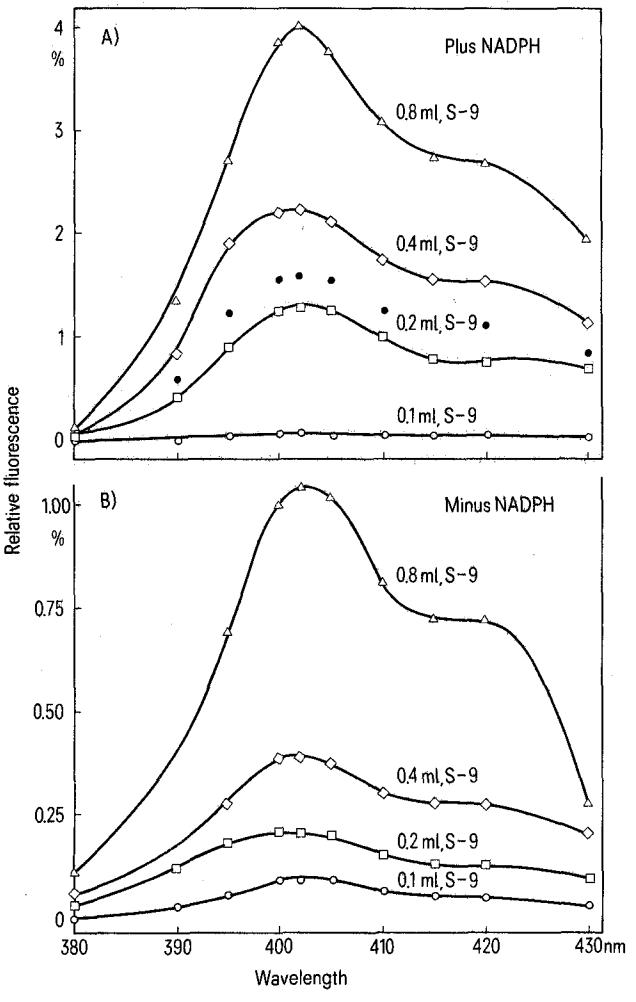

Figure 1. Fluorescence spectra of 1-aminopyrene (1-AP) extracted from anaerobic incubations of 1-nitropyrene and various amounts of S-9 fraction. A shows the yield with all co-factors including NADPH; B shows the yield with all co-factors except NADPH. ● refers to the fluorescence spectrum of 60 nmoles 1-AP standard.

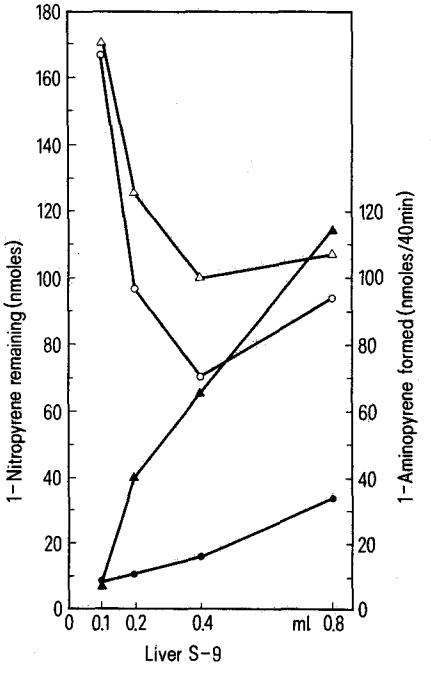

Figure 2. The open symbols represent the amounts of 1-nitropyrene remaining after a 40 min anaerobic incubation with S-9 at 37 °C. The closed symbols represent the amount of 1-aminopyrene formed under the same reaction conditions. ○ + ▲ refer to reactions run with all co-factors present; ● + △ refer to reactions run with all co-factors except NADPH.

the incubation mixture of S-9 and 1-NP are shown in figure 1. It is clear that addition of liver homogenates to 1-NP results in the formation of 1-AP. The reaction is enhanced by the addition of NADPH which increased the yield of 1-AP by a factor of 4. NADPH alone or enzymes which had been heated to 100 °C for 5 min did not produce 1-AP after incubation with 1-NP and co-factors. These results indicate that the formation of 1-AP is enzymatic. Alkaline extracts of 1-NP incubated with S-9 and co-factors were analyzed by TLC (Silica gel G, solvent system toluene:dichloromethane:methanol = 25:10:1). These extracts showed a fluorescent spot with color and R_f identical to standard 1-AP (R_f = 0.31 for 1-AP).

The relationship of remaining 1-NP concentrations to newly formed 1-AP concentrations is shown in figure 2. The amount of 1-AP formed increases with the amount of liver homogenate in the incubation medium and is enhanced by NADPH. The disappearance of 1-NP also increases with the amount of liver up to 0.4 ml of S-9. It should be noted, however, that the total amount of 1-NP + 1-AP is less than the 300 nmoles of 1-NP added. It is possible that other products of this reaction, e.g. 1-nitrosopyrene and 1-hydroxylaminopyrene, are formed and are not measured by these analytical procedures. To further characterize the enzymes for 1-NP reduction, microsomal and cytosol fractions were incubated with 1-NP and flavin mononucleotide (FMN). FMN stimulated reduction by the cytosol fraction but not the microsomal fraction; whereas NADPH stimulated reduction by both fractions (table). In all 3 fractions, simultaneous addition of NADPH and FMN stimulated more reduction than either co-factor alone. Note that the microsomal fraction contains the highest specific activity although the cytosol contains more total activity (see table). These results indicate that more than one class of enzymes is responsible for nitroreduction.

In conclusion, we have shown that 1-NP can be reduced anaerobically to 1-AP by liver homogenates. The presence of 1-NP in diesel engine exhaust particulates therefore may be of toxicological significance because mammalian enzymes may be able to convert this chemical to more toxic forms.

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