BIOMARKERS, 1998, VOL. 3, NO. 1, 21–33

Conversion of 1-nitropyrene by Brown trout (*Salmo trutta*) and turbot (*Scophthalamus maximus*) to DNA adducts detected by ^{32}P-postlabelling

C. L. MITCHELMORE[1], D. R. LIVINGSTONE[2] and J. K. CHIPMAN[1*]

[1] School of Biochemistry, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, U.K.

[2] NERC Plymouth Marine Laboratory, Citadel Hill, Plymouth, Devon, PL1 2PB, U.K.

The ability of the common aquatic contaminant 1-nitropyrene to form DNA adducts in fish was investigated *in vitro* and *in vivo* using Brown trout (*Salmo trutta*) and turbot (*Scophthalmus maximus*) in comparison to the Wistar rat. *In vitro* studies used Brown trout (control and induced (50 mg kg^{-1} β-naphthoflavone (βNF), i.p. 3 day pre-treatment single injection)) and induced rat (PB; 0·1 % w/v for 7 days in drinking water, βNF; 80 mg kg^{-1}, single injection 2 days prior to sacrifice). Hepatic 9000 *g* supernatant (S9 fractions) were incubated for 2 hours (at 25 °C for fish and 37 °C for rat) with calf thymus DNA (1mg) and 1-NP (100 μM). With all S9 fractions the presence of three distinct 1-NP-related DNA adducts was detected using the butanol enrichment procedure of the ^{32}P-postlabelling assay. A greater level of DNA adducts was observed with the uninduced compared to the induced trout S9 (37, 12 and 8 fold greater for adducts in chromatograph areas 1–3 respectively) suggesting the enhancement of detoxification pathways with respect to bulky adducts following βNF pre-treatment. DNA adduct levels in the induced rat consistently demonstrated approximately two-fold higher levels as compared to the induced fish, reflecting the lower protein levels in the S9 fraction of Brown trout (42 and 22 mg ml^{-1} for rat and fish respectively). Turbot, rat and Brown trout (uninduced and induced (βNF; 50 mg kg^{-1}; i.p. single injection 3 days prior)) were dosed with 100 mg kg^{-1} 1-NP (i.p. single injection, 24 hours). Liver DNA from both turbot and rat exhibited a 1-NP related adduct spot which was similar in position to that of area 1 in the incubations with S9 from rat and Brown trout. However, in contrast to the *in vitro* studies no 1-NP-related adducts were found in liver DNA from induced and uninduced Brown trout. This study highlights the potential, in a marine and a freshwater fish, for 1-NP metabolism to reactive intermediates capable of binding to DNA. However, activation of 1-NP was more optimal in the S9-mediated system, possibly reflecting the influence of detoxification systems.

Keywords: 1-nitropyrene, DNA adducts, Brown trout, turbot, ^{32}P-postlabelling.

Introduction

Nitroaromatic compounds are major products of industrial and combustion processes, with other sources including the nitration of polycyclic aromatic hydrocarbons by atmospheric *N*-oxides (Tokiwa and Ohnishi 1986, IARC 1989, Fu 1990). Such compounds contribute to contamination of sediments in the marine environment and are readily taken up into the tissues of aquatic organisms (Walker and Livingstone 1992). 1-Nitropyrene (1-NP) is found in organic solvent extracts of diesel soot and is commonly used as a model mono-nitro aromatic compound in genetic toxicity studies (IARC 1989, Rosenkranz and Mermelstein 1985, Tokiwa and Ohnishi 1986). It is a potent bacterial mutagen in the absence of an exogenous activation system, suggested as occurring via bacterial nitroreduction (see below and Figure 1) (Mermelstein *et al.* 1981, Lee *et al.* 1994).

* To whom correspondence should be addressed.

1354-750X/98 $12.00

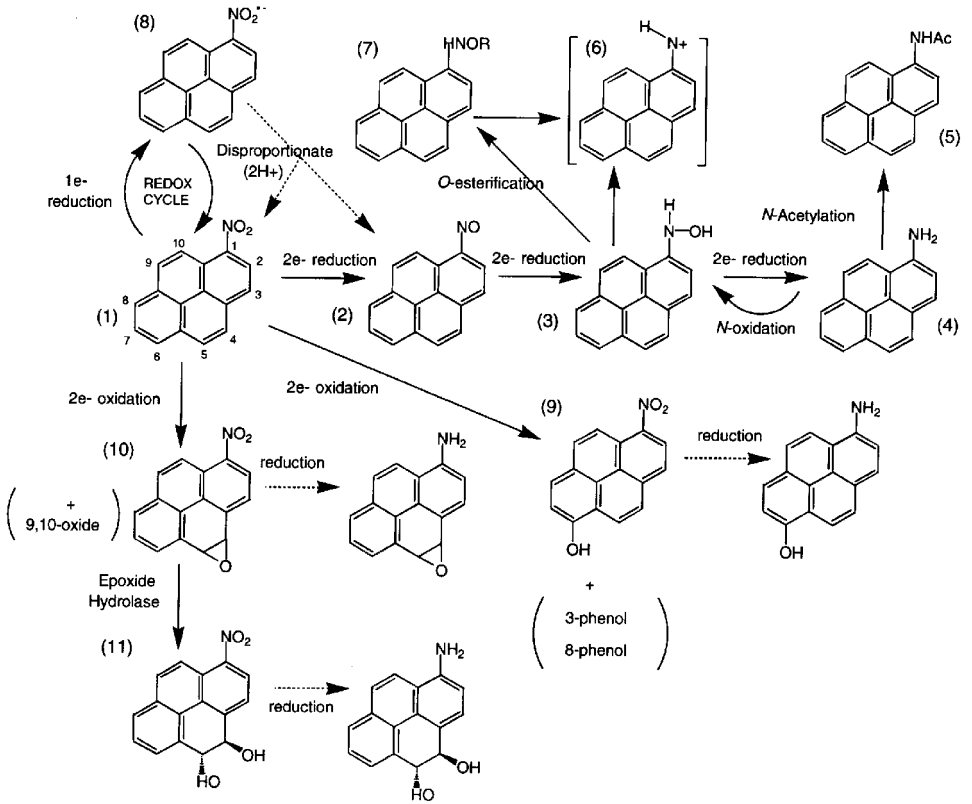

Figure 1. The main pathways involved in the metabolism of 1-nitropyrene. Potential reactive species are the nitrenium ion, various epoxides, nitro-anion species (with or without *C*-oxidation) and resultant reactive oxygen species. (1) 1-Nitropyrene, (2) 1-nitrosopyrene, (3) *N*-hydroxy-1-aminopyrene, (4) 1-aminopyrene, (5) *N*-acetyl-1-aminopyrene (6) nitrenium ion, (7) *O*-esterification products (e.g. *N*-acetoxy-1-aminopyrene), (8) nitro-anion radical, (9) various phenols (e.g. 6-hydroxy-1-nitropyrene), (10) various oxides (e.g. 1-nitropyrene-4,5-oxide), (11) various dihydrodiols (e.g. 1-nitropyrene- 4,5-dihydrodiol).

The metabolism of 1-NP in mammalian species *in vitro* and *in vivo* is complex and species-specific (Howard *et al.* 1990, Kataoka *et al.* 1991, King and Lewtas 1993) with nitroreduction, *C*-oxidation (and a combination of the two), and esterification pathways involved in the formation of reactive metabolites (see figure 1) (Fu 1990, Mason 1990). The nitroreduction pathway results in the sequential formation of the 1-nitrosopyrene, *N*-hydroxy-1-aminopyrene and 1-aminopyrene metabolites. The *N*-hydroxy-1-aminopyrene metabolite (which may also be produced from 1-aminopyrene *via* *N*-hydroxylation (CYP1A2 catalysis)) has been shown to react at the C8 position of guanine forming the *N*-(deoxyguanosine-8-yl)-1-aminopyrene (*N*-dG-1AP) adduct (Roy *et al.* 1989, Smith *et al.* 1990, Chou *et al.* 1996), probably *via* the formation of an electrophilic nitrenium ion (Rosser *et al.* 1996). However, the 2-electron reduction pathway may be limited in the presence of oxygen with a 1-electron reduction pathway predominating and leading to an anion radical (see figure 1) with the potential for redox cycling (Mason 1990, Bauer and Howard 1991, Jung *et al.* 1991, Busby *et al.* 1994). The major pathway in mammalian systems *in vivo* has been suggested to be

via NAD(P)H cytochrome P-450 catalyzed, 2-electron *C*-oxidations (compared to bacterial reduction) producing various carbon oxidation products (phenols, oxides, dihydrodiols), together with nitroreduced derivatives and conjugates of these oxidative products (Roy *et al.* 1989, Roy *et al.* 1991, Thornton-Manning *et al.* 1991, King and Lewtas 1993), some of which are capable of reacting with DNA (Roy *et al.* 1989, Roy *et al.* 1991, Thornton-Manning *et al.* 1991, Beland *et al.* 1992, El-Bayoumy *et al.* 1994). Many studies have suggested this *C*-oxidation to primarily result in detoxification (Howard *et al.* 1988, Howard *et al.* 1990, Bauer and Howard 1991, Kataoka *et al.* 1991, Silvers *et al.* 1994) if not followed by nitroreduction and reactivity may be due to the increased ease of reduction of the nitro functional group with these *C*-oxidation products (Rosser *et al.* 1996).

Regarding aquatic species, it has been demonstrated that 1-NP reduction pathways occur in goldfish (Kitamura and Tatsumi 1996) and invertebrates (Hetherington *et al.* 1996). Mitchelmore and Chipman (1997) and Mitchelmore *et al.* (1997a–c) demonstrated the dose-dependent increase in DNA strand break (SB) formation *in vitro* using Brown trout (*Salmo trutta*) hepatocytes and mussel (*Mytilus edulis* L.) digestive gland cells. However, it is not known if reactions equivalent to those reported in mammals for the production of DNA adducts can occur in aquatic organisms. This study investigates the potential of control and β-naphthoflavone-induced Brown trout derived liver preparations to convert 1-NP to DNA-reactive products. In addition the ability of 1-NP to produce DNA adducts (as potential biomarkers; see Maccubbin 1994) *in vivo* was investigated using two species of fish, Brown trout (*Salmo trutta*) and turbot (*Scopthalamus maximus*) in comparison to the rat.

Materials and methods

Chemicals

Phosphodiesterase (from calf spleen; 2 mg ml^{-1}) and herring sperm DNA were supplied by Boehringer Mannheim (Lewes, East Sussex, U.K.). Micrococcal endonuclease (50 units per 0·52 mg), T4 polynucleotide kinase (isolated from phage T4-infected *Escherichia coli* B.; ~10,000 units ml^{-1}) and potato apyrase (200 units ml^{-1}) were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.). Adenosine 5′-[γ-^{32}P] triphosphate (ATP) triethylammonium salt (approx 3000 Ci $mmol^{-1}$; 1000 μCi per 100 μl in stabilized aqueous solution) was obtained from Amersham International plc (Little Chalfont, Bucks., U.K.). PEI-cellulose TLC plates (20 ×20 cm), phenol, phenol:chloroform and chloroform were supplied from Camlab Ltd (Cambridge, Cambridgeshire, U.K.). All other chemicals were reagent grade obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) or Fisher Scientific U.K. (Loughborough, Leicestershire, U.K.).

Animals

Male Wistar rats (~250 g) were supplied by Charles River (Manston, Kent, U.K.), fed with RMI maintenance diet supplied by Special Diet Services (SDS; Witham, Essex, U.K.). Rats were kept at 20·5 °C ± 1·5 °C, 50 % humidity with a 12 : 12 light : dark regime. Male Brown trout (*Salmo trutta*; 500–800 g) were obtained from Leadmill trout farm (Hathersage, Derby, U.K.). All trout were housed in 2000 l tanks with a 6 l min^{-1} rate of exchange of fresh dechlorinated water with a spraybar flow rate of 240 l min^{-1}. Trout were fed with commercial floating trout pellets (Mazuri zoo foods) supplied by SDS (Witham, Essex, U.K.), kept at 15 °C ± 2 °C with a 12 : 12 light:dark regime and acclimatized to laboratory conditions for at least 2 weeks before use (Pandrangi *et al.* 1995). Turbot (*Scophthalmus maximus*) were purchased from Golden Sea produce (Hunterston, West Kilbride, Scotland; ~500 g wet weight) and maintained at 16 °C ± 2 °C in 1500 l tanks in a re-circulated filtered seawater system and fed on a diet of white fish. Fish were maintained without feeding during short-term experiments (Vigano *et al.* 1993).

Preparation of S9

A male Wistar rat (~250 g) was exposed to phenobarbitone for 7 days (0·1 % w/v in drinking water). After 5 days this rat was given an interperitoneal injection (i.p.) of β-naphthoflavone (80 mg in 1 ml kg^{-1})

dissolved in dimethylsulfoxide (DMSO) and sacrificed after 48 hours. Male Brown trout were injected (i.p.) with either 50 mg kg^{-1} βNF (25 mg ml^{-1} in corn oil) or corn oil control (2 ml kg^{-1}) and sacrificed after 72 hours. Livers were excised and the 9000 *g* supernatants (S9) were prepared as detailed in Maron and Ames (1983) and stored in liquid nitrogen until use. Protein concentration was determined by the Bradford method (Bradford 1976).

In vitro modification of calf thymus DNA

The *in vitro* modification of calf thymus DNA was based on methods described in Chou *et al.* (1996) with minor modifications. Ten percent S9 mixes (500 μl; prepared as detailed in Maron and Ames, 1983) were incubated with 1 mg of calf thymus DNA (500 μl of a 2 mg ml^{-1} solution in ultra-high-quality (UHQ) water) and either 10 μl of DMSO or 100 μM 1-NP (10 μl of 10 mM stock in DMSO). Incubations were at 25 °C (fish) or 37 °C (rat) for 2 hours in triplicate. DNA was re-isolated following phenol extractions and ethanol precipitation based on the method of Gupta (1984) and stored at –70 °C until use.

In vivo animal exposures

Conditions of animal exposures were based on the study by Roy *et al.* (1989). Two male Wistar rats (~250 g) were injected (i.p.) with 100 mg kg^{-1} 1-NP in DMSO (1 ml kg^{-1}) and sacrificed after 24 hours. For positive and negative controls male Wistar rats were injected (i.p.) with either 2-acetylaminofluorene (2AAF; 50 mg kg^{-1} in DMSO, n = 2) or with the vehicle solvent alone (1 ml kg^{-1}, n = 2) and sacrificed after 16 hours. The livers were excised, quickly frozen in liquid nitrogen and stored at –70°C until use. Brown trout (~500–800 g, n = 2 per treatment) were injected (i.p.) with β-naphthoflavone (50 mg kg^{-1}) or corn oil (2 ml kg^{-1}). After 3 days, fish were either injected (i.p.) with 1-NP (100 mg kg^{-1}) or vehicle solvent (2 ml DMSO kg^{-1}). Turbot (n = 2 per treatment) were injected (i.p) with 2AAF (50 mg kg^{-1}), 1-NP (100 mg kg^{-1}) or vehicle solvent alone (1 ml kg^{-1} DMSO). Fish were sacrificed after 24 hours, livers excised, frozen in liquid nitrogen and stored at –70 °C until further use.

Isolation of rat and fish DNA

DNA was isolated and purified by modification of methods described in Gupta (1984) and Roy *et al.* (1989). One gram of rat or fish liver (in triplicate) was homogenized in 8 volumes of buffer (0·15 M NaCl, 15 mM trisodium citrate; pH 7·0) and centrifuged at 1500 *g* for 15 min. The pellet containing nuclei was incubated with equal volumes (2·5 ml) of proteinase K digestion buffer (100 μg ml^{-1}; pH 8·0) and Tris buffer (0·01 M Tris-HCl, 1 mM EDTA, 1 M NaCl; pH 7·0) for 2 hours at 50 °C. The mixture was extracted successively with equal volumes (5 ml) of phenol (containing 0·1 % 8-hydroxyquinoline), phenol:chloroform and Sevag (chloroform:isoamyl alcohol, 24 : 1). Crude DNA was precipitated by the addition of 2·5 volumes ice cold ethanol (–20 °C) and 0·1 volume of 0·3 M sodium acetate. The DNA was pelleted by centrifugation and dried using a vaccum dessicator. Residual RNA was digested by incubation at 37 °C for 2 hours with a Tris buffer (500 μl) containing heat-treated RNAse A (100 μg ml^{-1}) and RNAse T1 (50 units ml^{-1}). DNA was extracted with equal volumes of phenol:chloroform and twice with Sevag and precipitated with ethanol and sodium acetate. DNA was dried and redissolved in ultra high quality (UHQ) water for quantitation and purity estimations.

^{32}P-postlabelling method

DNA purity and concentration was determined using spectrophotometric methods: A_{260}; DNA concentration (as compared to herring sperm DNA where 50 μg = 1 absorbance unit) with purity assessed by A_{230}/A_{260} (~0·4) and A_{260}/A_{280} (~1·8) ratios (Gupta 1984). DNA concentration was adjusted to 1 mg ml^{-1} using UHQ water. DNA (6 μg) was digested to 2′-deoxynucleoside 3′-monophosphates by incubating with 9 μl of spleen phosphodiesterase/micrococcal endonuclease (SP/MN) digestion mix, containing 3 μg SP and 3 μg (0·29 U) MN (in a buffer of 10 mM $CaCl_2$, 20 mM sodium succinate; pH 6·0) for 4·5 hours at 37 °C essentially as described by Gupta (1984) and Roy *et al.* (1989). The ^{32}P-postlabelling assay following the butanol enrichment procedure used in this study is based on that of Roy *et al.* (1989) previously used for the detection of 1-NP-derived DNA adducts in *in vivo* dosed rats and in an *in vitro* system employing xanthine oxidase and calf thymus DNA. An alternative enrichment procedure, nuclease P1 treatment, results in the 3′-dephosphorylation of C8-substituted deoxyguanosine adducts and therefore renders them resistant to T4 polynucleotide kinase catalysed 5′ ^{32}P- postlabelling (Smith *et al.* 1990). The DNA digest was diluted with 45 μl UHQ water with 50 μl of the digest (10 μl was kept for total nucleotide quantification) further diluted with 70 μl UHQ, 15 μl of 100 mM ammonium formate (pH 3·5) and 15 μl of 10 mM tetrabutylammonium chloride. The mixture was extracted twice with 1 volume of butan-1-ol (twice distilled and UHQ water saturated). The combined organic phase was then back-extracted three times with 270 μl UHQ water. The extract was neutralized by adding 2 μl of 200 mM Tris-HCl (pH 9·5), dried and stored at –70 °C until use.

The residue was dissolved in 5 μl UHQ water and then 12 μl of radioactive mixture added (150 μl stock containing: 20 μl of buffer (200 mM bicine-NaOH, pH 9·5), 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM spermidine); 30 μl T4 polynucleotide kinase (20 U); 100 μl [γ-^{32}P]ATP (~3000 mCi ml^{-1})) and incubated for 30 minutes at 37 °C. Following incubation, 1 μl was used to test that the samples had been labelled in the presence of excess ATP (for details see Gupta 1985) and the remainder was incubated for a further 30 minutes (37 °C) with 1 μl of potato apyrase (40 mU ml^{-1}). The adduct mixture was applied to the origin of a 10 ×20 cm thin-layer-chromatography (TLC) plate (with attached filter paper wick) and developed in 1·6 M sodium phosphate (pH 6·0) overnight (D1). The TLC plate was cut in half and further chromatography carried out, in D2 (3 M lithium formate, 7 M urea; pH 3·5), D3 (90 to D2; 0·8 M lithium chloride, 0·5 M Tris-HCl, 7 M urea; pH 8·0) and finally in 1·6 M sodium phosphate (pH 6·0) using a wick. Dried TLC plates were analysed and quantified using phosphorimaging techniques (Imagequant; Molecular Dynamics, U.K.) and levels of adducts calculated following the method of Gupta (1985).

Statistical methods

To determine the effect of pre-treatment on the ability of liver S9 to mediate the formation of DNA adducts in calf thymus DNA from 1-NP, a 2-tailed Student's *t*-test was performed. Statistical significance was determined using alpha values of 0·05, 0·01 and 0·001.

Results

The potential of 1-NP to form DNA adducts in fish was demonstrated, using an *in vitro* model employing β-naphthoflavone-induced and uninduced Brown trout liver S9 for the activation of 1-NP to products that reacted with calf thymus DNA (figure 2 and table 1). All incubations containing 1-NP and S9 (fish and rat; figure 2a–c) demonstrated the formation of three distinct areas of adducts produced in calf thymus DNA which were not present in the respective controls (figure 2d–f). These three 1-NP- induced DNA adducts are similar to the pattern observed by Roy *et al.* (1989) and Chou *et al.* (1996) in rat liver *in vivo* and in an *in vitro* system employing xanthine oxidase and calf thymus DNA. Levels of DNA adducts were much greater in the presence of uninduced compared to induced Brown trout liver S9 preparations (table 1). The largest area of adducts (area 1) was approximately 37 times greater (adduct levels per 10^7 normal nucleotides) with the uninduced compared to induced trout S9 preparations, whilst areas 2 and 3 were approximately 12 and 8 times greater respectively (table 1). The levels of DNA adducts in all 3 areas representing treatment with the induced rat S9 were approximately 2 times greater than those produced by the induced fish S9 in all cases (table 1). This may reflect the differences in protein concentrations of the S9 mixtures (rat S9 was 42 mg ml^{-1} while both fish S9 concentrations were approximately 22 mg ml^{-1}). The presence of a further area of radioactivity was observed with all 1-NP treated and control calf thymus DNA samples (area 4) averaging 7·62 ± 4·11 adducts per 10^7 normal nucleotides (table 1).

The ability of 1-NP to produce DNA adducts *in vivo* was investigated using Brown trout, turbot and rat following similar dosing regimes as described by Roy *et al.* (1989) in the rat. With both turbot and rat an area of radioactivity (similar to area 1 in the incubations with S9) was found, which was not detected in their respective controls (figure 3 a, b, d, e and table 2). Levels in turbot were slightly higher compared to rat, although both levels were low compared to those obtained in the incubations with S9 (7·6 and 4·6 adducts/10^8 normal nucleotides respectively). A background area of radioactivity was also observed and consistently was approximately 10 adducts per 10^8 normal nucleotides for all treatments and controls for both turbot and rat. Turbot and rat dosed with 2AAF

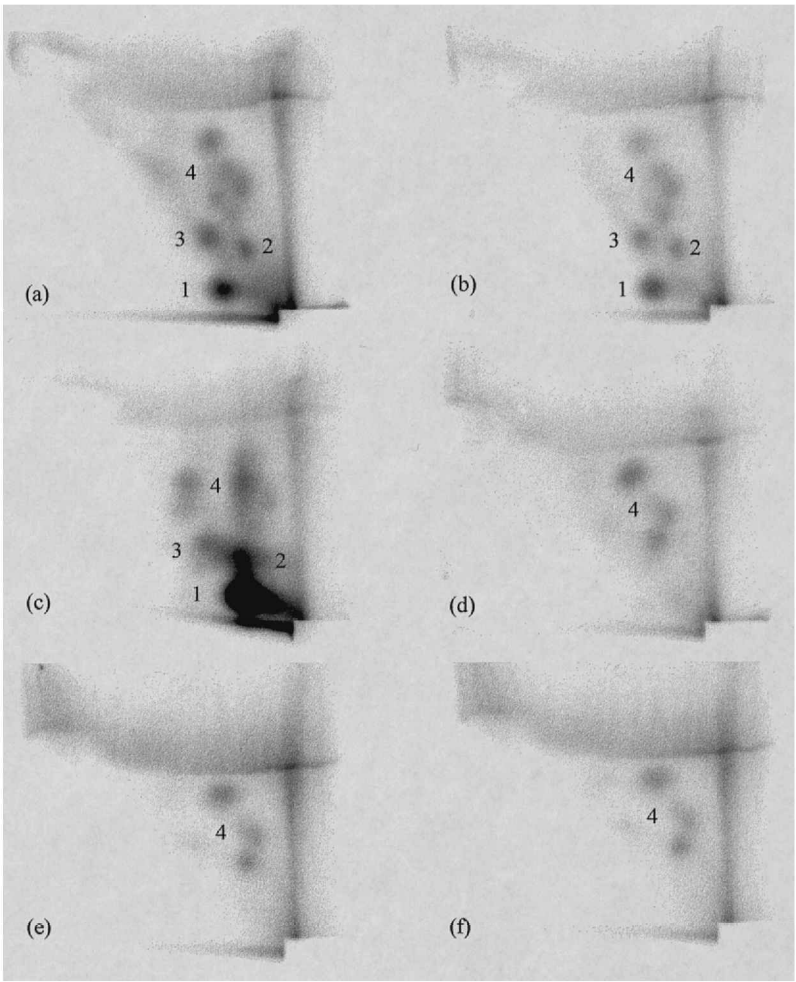

Figure 2. PEI-cellulose TLC maps of ^{32}P-labelled digests of calf thymus DNA incubated *in vitro*. Plate (a): PB and βNF induced rat S9 + 1-NP, (b): βNF induced Brown trout S9 + 1-NP, (c): uninduced Brown trout S9 + 1-NP, (d): 1-NP with no S9, (e): PB and βNF induced rat S9 without 1-NP, (f): calf thymus DNA alone. Areas 1–3 represent specific radioactive areas induced by 1-NP with area 4 representing an area of 'background' radioactivity associated with all plates. Representative chromatograms are shown for the results of triplicate experiments given in table 1.

both demonstrated high levels of multiple DNA adducts (with some, but not total, co-chromatography) (figures 3c, 2f, table 2). This was informative about the fate of 2AAF and also acted as a positive control for the study of 1-NP.

Despite the ability of Brown trout S9 to metabolize 1-NP to DNA-binding species, no 1-NP-derived DNA adducts were detected with 1-NP treated Brown trout following similar dosing regimes to those of rat and turbot (figure 4). Areas of radioactivity observed (including a diagonal radioactive zone) were indicative of a background level of adducts in all treated and control Brown trout. This is consistent with the apparent 'endogenous' adducts reported in fish species by Kurelec *et al.* (1989). This background limited the detection level for potential 1-NP-derived adducts in this region to less than approximately 15 adducts in 10^7 normal nucleotides.

Table 1. DNA adduct levels produced by 1-nitropyrene in calf thymus DNA modified *in vitro* and detected by ^{32}P-postlabelling.

	Adduct levels / 10^7 normal nucleotides		
Plate (in fig. 2)	1	2	3
a	6·80 ± 0·54	2·10 ± 0·10	2·76 ± 0·00
b	3·09 ± 0·14	1·14 ± 0·08	1·30 ± 0·12
c	113·85 ± 2·27***	13·46 ± 2·50***	9·91 ± 0·86***
d	-	-	-
e	-	-	-
f	-	-	-

Results are expressed as means ± SD (n = 3). plate a: PB and βNF induced rat S9 + 1-NP, plate b: βNF induced Brown trout S9 + 1-NP, plate c: uninduced Brown trout S9 + 1-NP, plate d: 1-NP with no S9, plate e: PB and βNF induced rat S9 without S9, plate f: calf thymus DNA alone. Numbers 1–3 represent specific radioactive areas, see figure 1 for details. (- indicates no adducts detected). S9 protein levels (mg ml^{-1}) were 42·1 (rat); 22·2 (induced trout) and 22·6 (uninduced trout). Statistical differences between induced and uninduced trout S9 incubations are determined by 2-tailed Student's *t*-test ($P < 0·05$, *; $P < 0·01$, **; $P < 0·001$, ***). Adduct levels in area 4, seen also in control incubations with DNA alone are not detailed since they were not 1-NP- derived (average levels 7·62 ± 4·11 adducts/10^7 normal nucleotides).

Discussion

Currently, there is a shortage of information regarding 1-NP metabolism (which is known to be species-specific (see Introduction)) in aquatic organisms. Recently it has been demonstrated that goldfish (*in vivo*; Kitamura and Tatsumi 1996) and invertebrates (*Mytilus edulis*, *Carcinus maenas* and *Asterias rubens* (*in vitro*); Hetherington *et al.* 1996) are capable of metabolising 1-NP to nitro-reduced products, however, there is a lack of data on metabolism to other products and in particular on the formation of DNA adducts by reactive intermediates. Recently, the formation of DNA strand breaks (SB) by 1-NP has been demonstrated in Brown trout hepatocytes and *Mytilus edulis* digestive gland cells, suggesting the potential for the formation of reactive products, although the exact nature of the observed DNA SB was not determined (Mitchelmore and Chipman 1997, Mitchelmore *et al.* 1997a–c). The metabolism of 1-NP to reactive intermediates capable of binding to DNA was indicated in this study using *in vitro* (aerobic) conditions utilizing induced and uninduced Brown trout liver S9 (and rat induced liver S9 as control). The resultant DNA adduct profiles in this study were similar for liver preparations from both species with the observed adduct pattern similar to that obtained by Roy *et al.* (1989) and Chou *et al.* (1996) with *in vivo*-dosed rats and an *in vitro* calf thymus/xanthine oxidase system. A major adduct (area 1) may be the result of nitroreduction forming the *N*-(deoxyguanosine-8-yl)-1-aminopyrene (*N*-dG-1AP) DNA adduct (Roy *et al.* 1989, Chou *et al.* 1996). In the latter studies there were also two (Roy *et al.* 1989) or more (Chou *et al.* 1996) further DNA adduct areas which may represent other DNA modifications, including imidazol ring-opened derivatives of *N*-dG-1AMP produced by the basic conditions of the ^{32}P-postlabelling procedure (Thornton-Manning *et al.* 1991). Howard *et al.* (1983) also demonstrated the presence of three DNA adducts in calf thymus DNA, using an *in vitro* xanthine oxidase system, derived from 1-NP nitroreduction including *N*-dG-1AP and two decomposition products of this adduct.

It has, however, been suggested that in aerobic (and *in vivo*) conditions, 2-electron nitroreduction is only a minor pathway (due to the preferred 1-electron

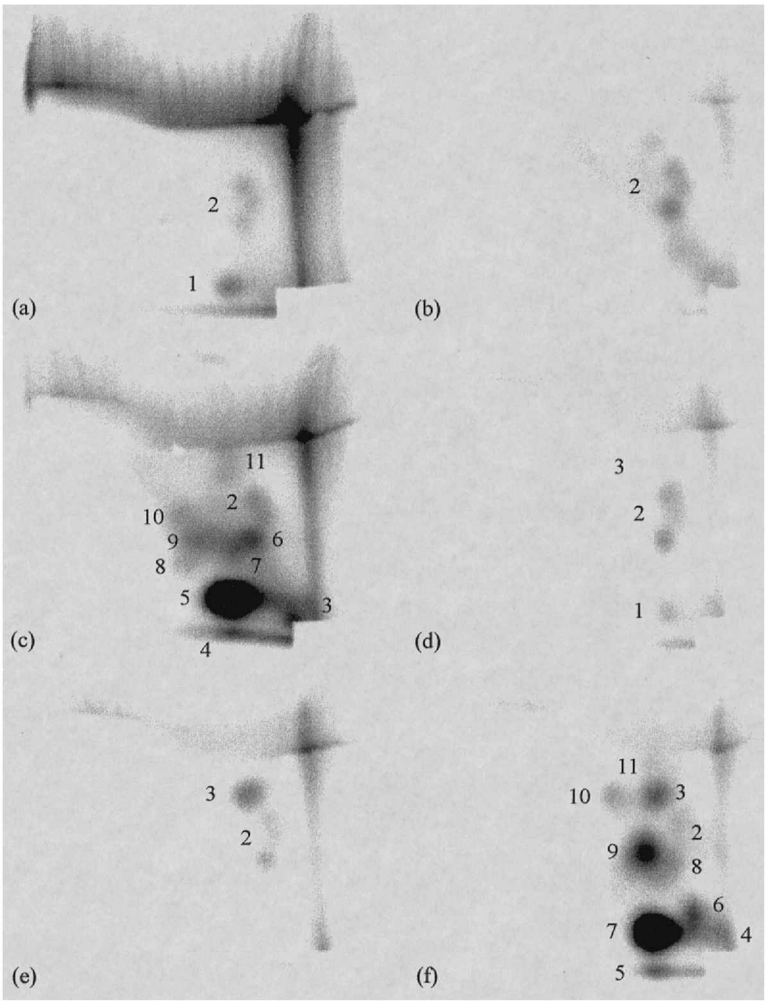

Figure 3. PEI-cellulose TLC maps of ^{32}P-labelled digests from *in vivo* studies of turbot (*Scophthalmus maximus*) and male Wistar rats. Plate (a): turbot, 1-NP (100 mg kg^{-1}; 24 hours), (b): turbot, vehicle control (1 ml kg^{-1}; 24 hours), (c): turbot, 2AAF (50 mg kg^{-1}; 24 hours), (d): rat, 1-NP (100 mg kg^{-1}; 24 hours), (e): rat vehicle control (1 ml kg^{-1}; 16 hours), (f): rat, 2AAF (50 mg kg^{-1}; 16 hours). Note: for 1-NP and control plates, adduct numbers are in equivalent areas for both rat and fish, with area 1 representing a 1-NP derived DNA adduct, and area 2 a 'background' area of radioactivity (rat DNA additional background adduct 3). Both 2AAF dosed rat and fish (c and f) demonstrated multiple DNA adducts however, numbering of adducts do not totally match eqivalent areas in each species. Representative chromatograms are shown for results in table 2.

reduction of the nitro function to the nitro anion radical) with *C*-oxidation predominating (see Introduction). Various studies have demonstrated the formation of DNA adducts resulting from reactions of 1-NP 4, 5- and 9,10- oxides with CHO cells and calf thymus DNA, some of which co-chromatographed in the same area as the *N*-dG-1AP DNA adduct (Roy *et al.* 1991, Beland *et al.* 1992, El-Bayoumy *et al.* 1994, Thornton-Manning *et al.* 1991). However, there are conflicting results as to the predominance of DNA adducts resulting from reduced or oxidative metabolites (Roy *et al.* 1989, Smith *et al.* 1990). There is some limited,

Table 2. Levels of liver DNA adducts produced *in vivo* in turbot (*Scophthalamus maximus*) and rat as analysed by ^{32}P-postlabelling.

Adduct (indicated in fig. 3)	Adduct levels / 10^7 normal nucleotides					
	a	b	c	d	e	f
1	0·76	-	-	0·46	-	-
2	0·85	1·10	1·19	1·28	0·92	0·89
3	-	-	3·23	0·15	0·54	2·90
4	-	-	35·04	-	-	31·91
5	-	-	2·01	-	-	0·83
6	-	-	2·06	-	-	4·99
7	-	-	1·63	-	-	1·56
8	-	-	0·65	-	-	0·89
9	-	-	1·22	-	-	1·08
10	-	-	0·71	-	-	0·83
11	-	-	0·91	-	-	0·55
12	-	-	-	-	-	0·42

Results are expressed as means of adducts derived from two animals in each case. Plate a: turbot, 1-NP (100 mg kg^{-1}; 24 hours), b: turbot, vehicle control (1 ml kg^{-1}; 24 hours), c: turbot, 2AAF (50 mg kg^{-1}; 24 hours), d: rat, 1-NP (100 mg kg^{-1}; 24 hours), e: rat, vehicle control (1 ml kg^{-1}; 16 hours), f; rat 2AAF (50 mg kg^{-1}; 16 hours). The numbers 1–10 represent specific radioactive areas, see figure 2 for details (-, no adducts detected). Note: for 1-NP and control plates, adduct numbers are in equivalent areas for both rat and fish. However, numbering of adducts do not totally match equivalent areas in each species.

circumstantial evidence for adducts to be derived from products which have undergone both *C*-oxidation and nitroreduction (Roy *et al.* 1989).

In addition to the presence of 1-NP derived DNA adducts an area of 'background' adducts was observed in each incubation (area 4). Roy *et al.* (1989) also demonstrated the presence of 'background' radioactivity in this region and suggested them to result from 'I compounds' (see Garg *et al.* 1992, De Flora *et al.* 1996).

The difference observed between βNF-induced and uninduced Brown trout is interesting and suggests that pre-induction with βNF results in increased detoxification of 1-NP. This may involve the induction of cytochrome P-450s, resulting in the increased production of non-reactive (in terms of the formation of bulky DNA adducts) *C*-oxidation products (e.g. see Howard *et al.* 1988). Indeed various mammalian studies have demonstrated the increased production of 6- and 8-phenols with βNF or 3-methylchloanthracene (3MC) induction in addition to decreased metabolism to the nitro-reduced 1-aminopyrene metabolite and decreased formation of the *N*-dG-1AP DNA adduct (e.g Howard *et al.* 1988, Silvers *et al.* 1994). Similarly in the present study, area 1 (which has already tentatively been suggested to be a result of the *N*-dG-1AP DNA adduct), DNA adduct levels were decreased by 97 % with pre-induction of Brown trout with βNF.

There is some debate as to the mutagenicity of the various phenolic metabolites of 1-NP (Ball *et al.* 1984, Heflich *et al.* 1990, Rosser *et al.* 1996); the production of DNA adducts may be low or absent if not followed by further nitroreduction (Heflich *et al.* 1990, Thornton-Manning *et al.* 1991, El-Bayoumy *et al.* 1994). The *C*-oxidation pathway, therefore, represents a balance between activation (when associated with nitro-reduction) and detoxification pathways as suggested by others (Howard *et al.* 1990).

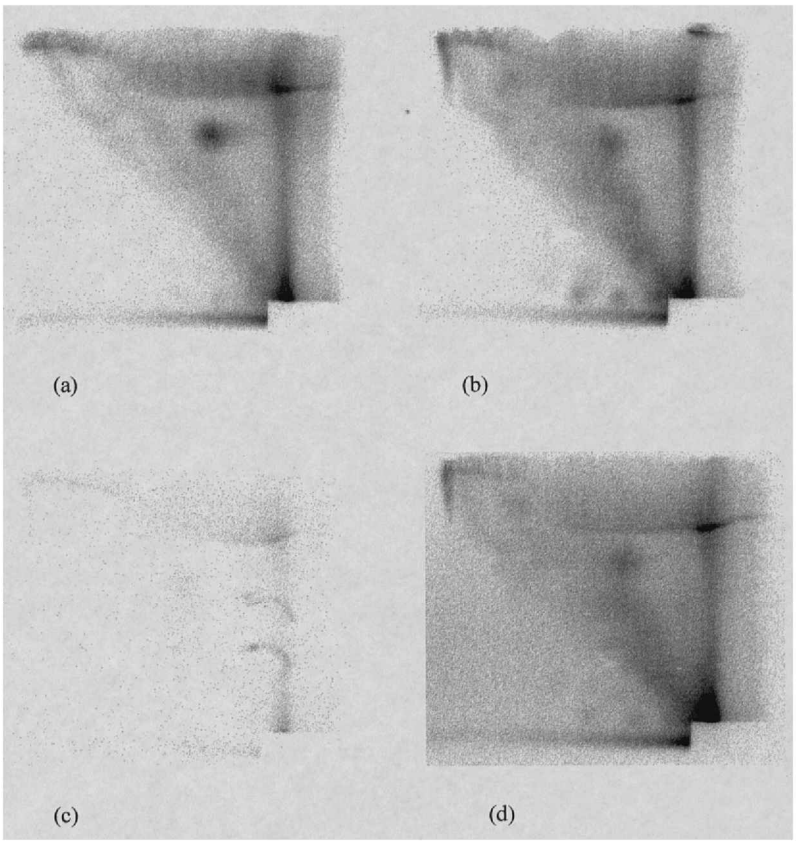

Figure 4. PEI-cellulose TLC maps of ^{32}P-labelled digests from *in vivo* studies of Brown trout (*Salmo trutta*). Plate (a) βNF- induced (50 mg kg^{-1} βNF (2 ml kg^{-1} corn oil), 3 days) + 1-NP (100 mg kg^{-1} (2 ml kg^{-1} DMSO), 24 hours), (b) 2ml/kg corn oil, 3 days plus 1-NP (100 mg kg^{-1} (2 ml kg^{-1} DMSO), 24 hours), (c) control (2 ml kg^{-1} corn oil 3 days plus 2 ml kg^{-1} DMSO 24 hours), (d) βN F control (50 mg kg^{-1} βNF (2 ml kg^{-1} corn oil), 3 days plus 2 ml kg^{-1} DMSO 24 hours). Note: no DNA adducts were detected from 1-NP above the background control level. Representative chromatograms are shown for duplicate experiments.

To demonstrate the effect of 1-NP *in vivo*, various species were injected i.p. with 100 mg kg^{-1} 1-NP for 24 hours. In contrast to the results observed *in vitro*, no 1-NP derived DNA adducts were detected in Brown trout although rat and turbot demonstrated one faint area of 1-NP derived radioactivity similar to area 1 in the *in vitro* study. Recently, the presence of DNA SB were detected in Brown trout hepatocytes following *in vitro* incubations with 1-NP, however, these may have been primarily as a result of 1-nitropyrene redox cycling as opposed to the excision repair or alkali-lability of bulky DNA adducts (Mitchelmore and Chipman 1997). The results in this study parallel those of some other vertebrate studies which were unable to demonstrate 1-NP derived DNA adducts *in vivo* (Djuric *et al.* 1988), or the formation of mutagenic species (Heflich *et al.* 1990). The adduct detection in turbot contrasts with an earlier preliminary study (Mitchelmore *et al.* 1996) in which adducts could not be detected, and may reflect differences in the dosing regime (50 mg kg^{-1} 1-NP, in 20 % acetone and corn oil, for 48 hours versus 100 mg kg^{-1} 1-NP, in DMSO, for 24 hours), the ^{32}P-postlabelling assay (8·5 M urea

compared to 7 M urea solvents in this study), or a seasonal difference (July 1994 versus April 1995) which is known to influence metabolism and DNA adduct levels (Stegeman *et al.* 1992, Garg *et al.* 1992).

This study has shown that liver enzymes from two fish species can activate 1-NP to DNA-reactive products. It is possible that additional DNA modifications (e.g. unstable depurination products) are also present, but not detected using the specific DNA isolation and adduct enrichment procedures employed (see Venier and Canova 1996). Following a single *in vivo* dose, 1-NP-derived adducts were evident at a low level in the turbot but not in Brown trout. These differences may reflect different Phase I and II enzyme contents in the two species. For example, the levels of UDP-glucuronyltransferases in trout are quite high compared to other fish species (Förlin *et al.* 1995). Similarly glutathione-*S*-transferase activity has been demonstrated to be higher in Brown trout compared to turbot (see George 1994). The contrast between *in vitro* and *in vivo* findings in the latter species may simply relate to a quantitative difference, although it is possible that the formation of DNA-reactive products, such as nitrenium ions may be favoured *in vitro* in the absence of phase II enzymes.

The demonstration herein of the formation of DNA- reactive products from 1-NP by fish enzymes, coupled with the recent demonstration of DNA strand breakage by 1-NP in Brown trout hepatocytes (Mitchelmore and Chipman 1997) emphasizes the ability of this nitroaromatic pollutant to cause genotoxicity in aquatic species. The use of DNA adducts as a biomarker of exposure to this genotoxicity is possible, though restricted sensitivity (at least following a single injection) may be a limitation, in comparison to the marked response to the model aromatic amide 2AAF. Sustained exposure to agents that give rise to persistent adducts may increase detection *in vivo*.

Acknowledgements

We wish to acknowledge L. Peters, J. Davies, A. Gardner and P. Jones for help with animal dosing. This work was carried out under the tenure of a BBSRC Ph.D. Studentship to C. L. Mitchelmore, and was also partially funded by the NERC.

References

BALL, L. M., KOHAN, M. J., INMON, J. P., CLAXTON, L. D. and LEWTAS, J. 1984, Metabolism of 1-nitro [^{14}C] pyrene *in vivo* in the rat and mutagenicity of urinary metabolites. *Carcinogenesis*, **5**, 1557–1564.

BAUER, S. L. and HOWARD, P. C. 1991, Kinetics and cofactor requirements for the nitroreductive metabolism of 1-nitropyrene and 3-nitrofluoranthene by rabbit liver aldehyde oxidase. *Carcinogenesis*, **12**, 1545–1549.

BELAND, F. A., SMITH, B. A., THORNTON-MANNING, J. R. and HEFLICH, R. H. 1992, Metabolic activation of 1-nitropyrene to a mammalian cell mutagen and a carcinogen. *Xenobiotica*, **22**, 1121–1133.

BRADFORD, M. M. 1976, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248–254.

BUSBY, W. F., SMITH, H., BISHOP, W. W. and THILLY, W. G. 1994, Mutagenicity of mono- and dinitropyrenes in the *Salmonella typhimurium* TM677 forward mutation assay. *Mutation Research*, **322**, 221–232.

CHOU, L. S-S., CHAO, S-M., BIAN, S-S., CHERNG, S-H., CHOU, M-Y. and LEE, H. 1996, Suppressive effects of methyl methacrylate on the mutagenicity and DNA adduct formation induced by 1-nitropyrene and benzo(a)pyrene. *Mutagenesis*, **11**, 177–181.

DE FLORA, S., IZZOTTI, A., RANDERATH, K., RANDERATH, E., BARTSCH, H., NAIR, J., BALANSKY, R., VAN SCHOOTEN, F., DEGAN, P., FRONZA, G., WALSH, D. and LEWTAS, J. 1996, DNA adducts and

chronic degenerative diseases. Pathogenetic relevance and implications in preventative medicine. *Mutation Research*, **366**, 197–238.

DJURIC, Z., FIFER, E. K., YAMAZOE, Y. and BELAND, F. A. 1988, DNA binding by 1-nitropyrene and 1,6-dinitropyrene *in vitro* and *in vivo*: effects of nitroreductase induction. *Carcinogenesis*, **9**, 357–364.

EL-BAYOUMY, K., JOHNSON, B., ROY, A. K., UPADHYAYA, P., PARTIAN, S. and HECHT, S. S. 1994, Development of methods to monitor exposure to 1-nitropyrene. *Environmental Health Perspectives*, **102**, 31–37.

FÖRLIN, L., LEMAIRE, P. and LIVINGSTONE, D. R. 1995, Comparative studies of hepatic xenobiotic metabolising and antioxidant enzymes in different fish species. *Marine Environmental Research*, **39**, 201–204.

FU, P. P. 1990, Metabolism of nitro-polycyclic aromatic hydrocarbons. *Drug Metabolism Reviews*, **22**, 209–268.

GARG, A., KRCA, S., KURELEC, B. and GUPTA, R. C. 1992, Endogenous DNA modifications in aquatic organisms and their probable biological significance. *Comparative Biochemistry and Physiology*, **102 B**, 825–832.

GEORGE, S. G. 1994, Enzymology and molecular biology of Phase II xenobiotic-conjugating enzymes in fish. In *Aquatic Toxicology, Molecular, Biochemical and Cellular Perspectives*, D. C. Malins and G. K. Ostrander, eds. (CRC Press, Boca Raton, Florida), pp. 37–86.

GUPTA, R. C. 1984, Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA *in vivo*. *Proceedings of the National Academy of Science USA*, **81**, 6943–6947.

GUPTA, R. C. 1985, Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen DNA adducts. *Cancer Research*, **45**, 5656–5662.

HEFLICH, R. H., THORNTON-MANNING, J. R., KINOUCHI, T. and BELAND, F. A. 1990, Mutagenicity of oxidised microsomal metabolites of 1-nitropyrene in Chinese Hamster Ovary cells. *Mutagenesis*, **5**, 5151–157.

HETHERINGTON, L. H., LIVINGSTONE, D. R. and WALKER, C. H. 1996, Two- and one-electron dependent *in vitro* reductive metabolism of nitroaromatics by *Mytilus edulis*, *Carcinus maenas* and *Asterias rubens*. *Comparative Biochemistry and Physiology*, **113C**, 231–239.

HOWARD, P. C., HEFLICH, R. H., EVANS, F. E. and BELAND, F. A. 1983, Formation of DNA adducts *in vitro* and in *Salmonella typhimurium* upon metabolic reduction of the environmental mutagen 1-nitropyrene. *Cancer Research*, **43**, 2052–2058.

HOWARD, P. C., REED, K. A. and KOOP, D. R. 1988, Oxidative metabolism of 1-nitropyrene by rabbit liver microsomes and purified microsomal cytochrome P-450 isoenzymes. *Cancer Research*, **48**, 4261–4265.

HOWARD, P. C., AOYAMA, T., BAUER, S. L., GELBOIN, H. V. and GONZALEZ, F. J. 1990, The metabolism of 1-nitropyrene by human cytochromes P450. *Carcinogenesis*, **11**, 1539–1542.

IARC 1989, IARC monographs on the evaluation of carcinogenic risks to humans: *Diesel and gasoline engine exhausts and some nitroarenes*. (IARC, Lyon), **46.**

JUNG, H., SHAIKH, A. U., HEFLICH, R. H. and FU, P. P. 1991, Nitro group orientation, reduction potential, and direct-acting mutagenicity of nitropolycyclic aromatic hydrocarbons. *Environmental and Molecular Mutagenesis*, **17**, 169–180.

KATAOKA, K., KINOUCHI, T. and OHNISHI, Y. 1991, Species differences in metabolic activation and inactivation of 1-nitropyrene in the liver. *Cancer Research*, **51**, 3919–3924.

KING, C. L. and LEWTAS, J. 1993, An evaluation of the comparative metabolism and kinetics of 1-nitropyrene by rabbit, rat, and hamster tracheal epithelial cells. *Toxicology and Applied Pharmacology*, **122**, 149–158.

KITAMURA, S. and TATSUMI, K. 1996, Metabolism *in vivo* of 1-nitropyrene, an environmental pollutant in fish. *Biological and Pharmaceutical Bulletin*, **19**, 1524–6158.

KURELEC, B., GARG, A., KRCA, S., CHACKO, M. and GUPTA, R. A. 1989, Natural environment surpasses polluted environment in inducing DNA damage in fish. *Carcinogenesis*, **10**, 1337–1339.

LEE, H., CHERNG, S-H. and LIU, T-Y. 1994, Bacterial mutagenicity, metabolism, and DNA adduct formation by binary mixtures of benzo(a)pyrene and 1-nitropyrene. *Environmental and Molecular Mutagenesis*, **24**, 229–234.

MACCUBBIN, A. E. 1994, DNA adduct analysis in fish: laboratory and field studies. In *Aquatic Toxicology, Molecular, Biochemical and Cellular Perspectives*, D. C. Malins and G. K. Ostrander, eds. (CRC Press, Boca Raton, Florida), pp. 267–294.

MARON, D. M. and AMES, B. N. 1983, Revised methods for the *Salmonella* mutagenicity test. *Mutation Research*, **113**, 173–215.

MASON, R. P. 1990, Redox cycling of radical anion metabolites of toxic chemicals and drugs and the Marcus theory of electron transfer. *Environmental Health Perspectives*, **87**, 237–243.

MERMELSTEIN, R., KIRIAZIDES, D. K., BUTLER, M., MCCOY, E. C. and ROSENKRANZ, H. S. 1981, The extraordinary mutagenicity of nitropyrenes in bacteria. *Mutation Research*, **89**, 187–196.

MITCHELMORE, C. L., CHIPMAN, J. K., GARCIA-MARTINEZ, P., LEMAIRE, P., PETERS, L. D. and

LIVINGSTONE, D. R. 1996, Normal status of hepatic 7-ethoxyresorufin O-deethylase (EROD) activity, antioxidant enzymes and DNA oxidation in turbot (*Scophthalamus maximus*) and other flatfish species following exposure to nitroaromatic compounds. *Marine Environmental Research*, **42**, 329–333.

MITCHELMORE, C. L., BIRMELIN, C., LIVINGSTONE, D. R. and CHIPMAN, J. K. 1997a, Polycyclic- and nitro-aromatic compounds produce DNA strand breakage in Brown Trout (*Salmo trutta*) and mussel (*Mytilus edulis* L.) cells. *Mutagenesis*, **12**, 101.

MITCHELMORE, C. L. and CHIPMAN, J. K. 1997, Detetection of DNA strand breaks in Brown Trout (*Salmo trutta*) hepatocytes and blood cells using the single cell gel electrophoresis (comet). *Aquatic Toxicology,* in press.

MITCHELMORE, C. L., BIRMELIN, C., LIVINGSTONE, D. R. and CHIPMAN, J. K. 1997b, The detection of DNA strand breaks in isolated mussel (*Mytilus edulis* L.) digestive gland cells using the 'comet' assay. *Ecotoxicology and Environmental Safety*, in press.

MITCHELMORE, C. L., BIRMELIN, C., LIVINGSTONE, D. R. and CHIPMAN, J. K. 1997c, Evidence for free radical involvement in the production of DNA strand breaks by benzo(a)pyrene, 1-nitropyrene and nitrofurantoin in mussel (*Mytilus edulis* L.) digestive gland cells. *Aquatic Toxicology*, in press.

PANDRANGI, R., PETRAS, M., RALPH, S. and VRZOC, M. 1995, Alkaline single cell gel (comet) assay and genotoxicity monitoring using bullheads and carp. *Environmental and Molecular Mutagenesis*, **26**, 345–356.

ROSENKRANZ, H. S. and MERMELSTEIN, R. 1985, The genotoxicity, metabolism and carcinogenicity of nitrated polycyclic aromatic hydrocarbons. *Journal of Environmental Science and Health*, **C3**, 221–272.

ROSSER, P. F., RAMACHANDRAN, P., SANGAIAH, R., AUSTIN, R. N., GOLD, A. and BALL, L. M. 1996, Role of *O*-acetyltransferase in activation of oxidised metabolites of the genotoxic environmental pollutant 1-nitropyrene. *Mutation Research*, **369**, 209–220.

ROY, A. K., EL-BAYOUMY, K. and HECHT, S. S. 1989, ^{32}P-postlabeling analysis of 1-nitro-pyrene-DNA adducts in female Sprague-Dawley rats. *Carcinogenesis*, **10**, 195–198.

ROY, A. K., UPADHYAYA, P., EVANS, F. E. and EL-BAYOUMY, K. 1991, Structural characterisation of the major adducts formed by reaction of 4,5-epoxy-4,5-dihydro-1-nitropyrene with DNA. *Carcinogenesis*, **12**, 577–581.

SILVERS, K. J., EDDY, E. P., MCCOY, E. C., ROSENKRANZ, H. S. and HOWARD, P. C. 1994, Pathways for the mutagenesis of 1-nitropyrene and dinitropyrenes in the human hepatoma cell line HepG2. *Environmental Health Perspectives*, **102**, 195–199.

SMITH, B. A., KORFMACHER, W. A. and BELAND, F. A. 1990, DNA adduct formation in target tissues of Sprague-Dawley rats, CD-1 mice and A/J mice following tumorigenic doses of 1-nitropyrene. *Carcinogenesis*, **11**, 1705–1710.

STEGEMAN, J. J., BROUWER, M., DI GIULIO, R. T., FORLIN, L., FOWLER, B. A., SANDERS, B. M. and VAN VELD, P. A. 1992, Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In *Biomarkers. Biochemical, Physiological and Histological Markers of Anthropogenic Stress*, R. J. Huggett, R. A. Kimerle, P. M. Mehrle Jr and H. L. Berman, eds. (Lewis Publishers, Chelsea, MI, USA), pp. 125–153.

THORNTON-MANNING, J., SMITH, B. A., BELAND, F. A. and HEFLICH, R. H. 1991, S9 mediated metabolism of 1-nitropyrene to a mutagen in Chinese hamster ovary cells by ring-oxidation under aerobic conditions and by nitroreduction under anaerobic conditions. *Carcinogenesis*, **12**, 2317–2323.

TOKIWA, H. and OHNISHI, Y. 1986, Mutagenicity and carcinogenicity of nitroarenes and their sources in the environment. *CRC Critical Reviews in Toxicology*, **17**, 23–60.

VENIER, P. and CANOVA, S. 1996, Formation of DNA adducts in the gill tissue *of Mytilus galloprovincialis* treated with benzo[*a*]pyrene. *Aquatic Toxicology*, **34**, 119–133.

VIGANO, L., ARILLO, A., BAGNASCO, M., BENNICELLI, C. and MELODIA, F. 1993, Xenobiotic metabolizing enzymes in uninduced and induced rainbow trout (*Oncorhynchus mykiss*): effects of diets and food deprivation. *Comparative Biochemistry and Physiology*, **104C**, 51–55.

WALKER, C. H. and LIVINGSTONE, D. R. 1992, *Persistent pollutants in marine ecosystems*. (Pergamon Press, Oxford), p. 272.