DNA adduct formation and 7-ethoxyresorufin o-deethylase induction in primary culture of rainbow trout hepatocytes exposed to benzo(a)pyrene

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DNA ADDUCT FORMATION AND 7-ETHOXYRESORUFIN *O*-DEETHYLASE INDUCTION IN PRIMARY CULTURE OF RAINBOW TROUT HEPATOCYTES EXPOSED TO BENZO[*a*]PYRENE

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Abstract—The formation of DNA adducts, using the ^{32}P-postlabelling assay, and induction of 7-ethoxyresorufin *O*-deethylase (EROD) were investigated in a primary culture of rainbow trout hepatocytes exposed to benzo[*a*]pyrene (B[*a*]P). Concentrations of 0.1 and 1 μM-B[*a*]P were shown to induce EROD whereas 10 μM was an inhibitory concentration. DNA adducts were detected for 12 hr to 72 hr after exposure to 1 μM-B[*a*]P whereas EROD activity was increased 36 hr after treatment. The pattern of adducts was shown to be identical to that obtained after B[*a*]P treatment of rainbow trout *in vivo*, as demonstrated by co-chromatography of the adducts. Pre-exposure of hepatocytes for 48 hr to β-naphthoflavone (βNF) and subsequent 24-hr exposure to 1 μM-B[*a*]P did not lead to increased DNA adduct formation although βNF treatment led to a 3.4-fold induction of EROD activity at the time of B[*a*]P addition. This study suggests that primary culture of rainbow trout hepatocytes provides a suitable method for studying DNA adduct formation and its modulating factors *in vitro*.

INTRODUCTION

Exposure of living organisms to xenobiotics leads to interactions between those chemicals and biological systems that may give rise to biochemical disturbances or/and adaptive responses (e.g. enzyme induction). Biochemical events resulting from exposure to xenobiotics have been proposed as biomarkers, generally seen as 'changes in a biological system that can be related to an exposure to, or effect from, a specific xenobiotic or type of toxic material' (Henderson *et al.*, 1989).

Among molecular targets of xenobiotics, interest has been focused on DNA, since DNA–xenobiotic binding is considered to be a critical step that may initiate mutagenesis and carcinogenesis (Miller and Miller, 1981). Xenobiotics may interact with DNA either directly or after transformation by metabolizing enzymes into reactive species that react with DNA forming DNA addition products (DNA adducts). Thus, DNA adducts were proposed as a biomarker of exposure to genotoxic compounds (Henderson *et al.*, 1989; Perera, 1987).

Abbreviations: AHH = aryl hydrocarbon hydroxylase; B[*a*]P = benzo[*a*]pyrene; βNF = β-naphthoflavone; BPDE = B[*a*]P 7,8-diol 9,10-epoxide; DMSO = dimethyl sulphoxide; EROD = 7-ethoxyresorufin *O*-deethylase; *P*-450 = cytochrome *P*-450; PAH = polycyclic aromatic hydrocarbon; PEI = polyethyleneimine.

Polycyclic aromatic hydrocarbons (PAHs) are widespread carcinogenic micropollutants that form DNA adducts after activation by cytochrome *P*-450 (*P*-450) enzymes (Conney, 1982; IARC, 1987), which are present in most living organisms, including fish. A main *P*-450 responsible for PAH activation in rodents has been characterized as a member of the *P*450 1A subfamily, namely *P*4501A1 (Ioannides and Parke, 1990). A fish *P*-450 implicated in PAH metabolism and activation is very similar to the rodent *P*4501A1 form, particularly in its induction by planar compounds enhancing the metabolic activation of PAHs (Goksøyr *et al.*, 1991; Stegeman and Lech, 1991). The *P*-450 inducible by planar xenobiotics in fish has been proposed as a biomarker of exposure to planar micropollutants (Stegeman and Lech, 1991).

Cells isolated from mammalian and fish tissues can be used in various cytotoxicity and genotoxicity assays as alternatives to experiments *in vivo* to reduce the use of animals and to lessen the cost/time of testing (Babich and Borenfreund, 1991). Cultured fish cells have been shown to metabolize and activate PAHs into DNA-damaging species (Smolarek *et al.*, 1987).

Recently, the inducibility of *P*4501A based on 7-ethoxyresorufin *O*-deethylase (EROD) activity was evaluated in primary culture of rainbow trout hepatocytes (Pesonen and Andersson, 1991; Vaillant *et al.*, 1989). Furthermore, the pattern of *P*4501A

induction (rate, time trend) in this cell system appeared to be very similar to that in fish liver after exposure to PAHs (A. Devaux, unpublished data).

The objective of the present study was to examine whether DNA adduction is associated with *P*4501A enzymatic activity in trout hepatocytes exposed to the model PAH benzo[*a*]pyrene (B[*a*]P) with or without pre-exposure to the well known inducer of *P*4501A, β-naphthoflavone (βNF). To examine the biological relevance of our system *in vitro*, we also compared the pattern of hepatic B[*a*]P–DNA adduction *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals. Rainbow trout (*Oncorhynchus mykiss*) with an average body weight of 250 g, were obtained from a local hatchery (Font-Rome, France). Trout were kept in tanks with aerated, charcoal-filtered tap water at a temperature of 10°C and fed with commercial pellets. Fish were acclimatized to laboratory conditions for a minimum of one month before experiments started.

Chemicals. Medium 199, antibiotic–antimycotic solution (10,000 U penicillin G/ml, 10 mg streptomycin/ml, 25 μg amphotericin B/ml, in 0.9% NaCl), L-glutamine and collagenase type IV were from Sigma Chemicals (St Quentin, Fallavier, France). βNF was purchased from Aldrich (St Quentin, Fallavier, France) and 7-ethoxyresorufin from Boehringer-Mannheim (Meylan, France). Proteinase K, and RNases A and T_1 used for DNA isolation were from Sigma Chemicals and α-amylase was from Boehringer-Mannheim. Enzymes used for DNA adduct analysis were from Worthington Biochemical Corp. (Freehold, NJ, USA) (micrococcal nuclease, spleen phosphodiesterase), Boehringer-Mannhein (*Penicillium citrinum* nuclease P_1), US Biochemical Corp. (Cleveland, OH, USA) (polynucleotide kinase T_4) and Sigma Chemicals (potato apyrase). [γ-^{32}P]ATP (sp. act. 3000 Ci/mmol) was supplied by Amersham (Bucks., UK). All other reagents were of analytical grade.

Isolation of hepatocytes and culture conditions. Rainbow trout hepatocytes were isolated by a modification of the two-step perfusion method (Seglen, 1972). Fish were killed by a blow on the head and a ventral incision was made from the pelvic fins to the gills. The portal vein was cannulated and the liver perfused at room temperature (approx. 20°C) using a peristaltic pump at a flow rate of 11 ml/min. The first Ca^{2+}-free perfusion buffer [9.4 g NaCl/litre, 0.235 g KCl/litre, 0.117 g $Na_2HPO_4 \cdot 12\,H_2O$/litre, 2.8 g HEPES/litre, pH 7.4, 300 mosmol] was pumped from a reservoir to a bubble trap. After 20 min perfusion, the liver was perfused for 20 min with the second buffer containing 60 mg collagenase (8 g NaCl/litre, 0.2 g KCl/litre, 0.1 g $Na_2HPO_4 \cdot 12\,H_2O$/litre, 2.38 g HEPES/litre, 0.74 g $CaCl_2$/litre, pH 7.4, 300 mosmol). Cell dispersion was achieved by gently raking with a spatula. After filtration through a cotton gauze and a nylon mesh, cells were centrifuged for 2 min at 80 *g* and washed twice with culture medium (medium 199, 10 ml antibiotic–antimycotic solution, 2 mM-L-glutamine, 1 g HEPES/litre, 0.35 g $NaHCO_3$/litre, 1.6 g $Na_2HPO_4 \cdot 12\,H_2O$/litre, 0.14 g $CaCl_2$/litre, pH 7.4, 300 mosmol). Cell viability was assessed by a dye exclusion test using 0.4% trypan blue. Each perfusion yielded about $0.5–1 \times 10^9$ hepatocytes, with viability over 90%. All liquids and glassware were sterilized before use by filtration or autoclaving. After cells had been counted using a Neubauer chamber, hepatocytes in culture medium were seeded at a density of 2×10^6 cells/ml on to surface-treated plastic petri dishes (Falcon Primaria) and incubated at 12°C.

Enzyme and protein assays. Cells were lysed in 0.15 M-KCl, 50 mM-phosphate buffer, pH 8.0, using a Potter–Elvehjem homogenizer. EROD activity was measured at 20°C as previously described (Monod *et al.*, 1987) using a 7-ethoxyresorufin final concentration of 5 μM in a 50 mM-phosphate buffer, pH 8.0. Protein concentration was determined according to Hartree (1972) with bovine serum albumin as a standard.

DNA adduct analysis. Hepatocytes were harvested by flushing the petri dishes with culture medium. Cell suspensions were then centrifuged (3 min, 100 *g*) and pellets were resuspended in 1 ml of a 0.1 M-Tris–HCl buffer, pH 7.4, and frozen at −80°C until use. DNA was isolated from hepatocytes according to a procedure involving proteinase K digestion, RNAse and α-amylase digestion, phenol/chloroform extraction and ethoxyethanol precipitation (Dunn *et al.*, 1987). The precipitated DNA was recovered in distilled water and the amount of extracted DNA was determined spectrophotometrically assuming $1\ A_{260} = 50$ μg DNA/ml. Purity of samples was estimated by the A_{260}/A_{280} ratio, which was found to be between 1.6 and 1.8.

DNA was analysed for B[*a*]P adducts using the postlabelling assay (Randerath *et al.*, 1981) (nuclease P_1-mediated enrichment version; Reddy and Randerath, 1986). 20 μg hepatocyte DNA were first hydrolysed to 3′-nucleoside monophosphates by treatment with micrococcal endonuclease and spleen phosphodiesterase. The digest was then treated with nuclease P_1 that dephosphorylated only non-adducted 3′-nucleoside monophosphates to nucleosides. The adducted 3′-nucleoside monophosphates were subsequently labelled by polynucleotide T_4 kinase-catalysed transfer of ^{32}P from [γ-^{32}P]ATP, whereas nucleosides were not substrates for the enzyme. Finally, the ^{32}P-labelled nucleoside biphosphates were purified and separated by anion exchange thin-layer chromatography on polyethyleneimine (PEI)-cellulose plates eluted in several directions, as follows (Lu *et al.*, 1986). Inorganic phosphate and labelled impurities were removed by application of

the labelled digest to the origin of a PEI-cellulose plate, which was eluted overnight with an aqueous electrolyte (D1 = 2.3 M-NaH_2PO_4, pH 5.7). The purified ^{32}P-labelled adducts were then contact-transferred to a new PEI-cellulose plate and separated by bidimensional elution (D2 = 7.7 M-urea/4.8 M-lithium formate, pH 3.5; D3 = 9.3 M-urea/1.6 M-LiCl/0.5 M-Tris, pH 8). A final elution (D4 = 1.7 M-NaH_2PO_4, pH 6) was carried out in order to reduce the background radioactivity of plates.

DNA adducts appeared as black spots on the autoradiographies of the PEI-cellulose plates. The radioactive PEI-cellulose material corresponding to the spots was scraped from the plate and measured by Cerenkov counting. Blank areas were also removed in order to subtract background radioactivity from each count. Quantification of DNA adducts was based on the spectrophotometric measurement of DNA, the radioactivity of the spots and the specific activity of [γ-^{32}P]ATP.

Co-chromatography experiments were carried out using liver DNA from immature rainbow trout treated ip with 80 mg B[a]P/kg body weight (Masfaraud *et al.*, 1992).

Statistics. Statistical analyses were performed by the Mann–Whitney *U*-test (Siegel, 1956).

Experimental procedure. βNF and B[a]P were dissolved in dimethyl sulphoxide (DMSO) and mixed with the culture medium to the appropriate concentrations (the final concentration of DMSO was 0.1% which was shown to be non-cytotoxic). In each experiment the control received DMSO alone.

In experiment I, B[a]P in DMSO (0.01, 0.1, 1 and 10 μM-B[a]P final concentration in assays) was added to the culture medium 24 hr after cell seeding and EROD activity was measured 48 and 72 hr after exposure.

In experiment II, B[a]P (1 μM final concentration in assays) was added 24 hr after cell seeding and measurements of the DNA adducts as well as EROD activity were made 12, 24, 36 and 72 hr after B[a]P exposure.

In experiment III, the DNA adduct level and EROD activity were measured in hepatocytes exposed to B[a]P that had, or had not, received βNF pretreatment (Fig. 1).

RESULTS

Effect of B[a]P treatment of hepatocytes on EROD activity (experiment I)

The effect of B[a]P treatment after 48 or 72 hr was studied at four different concentrations (0.01, 0.1, 1 and 10 μM-B[a]P final concentration in assays). Table 1 shows a dose-dependent induction of EROD following treatment with B[a]P in the range 0.01 to 1 μM; inhibition was observed for 10 μM-B[a]P.

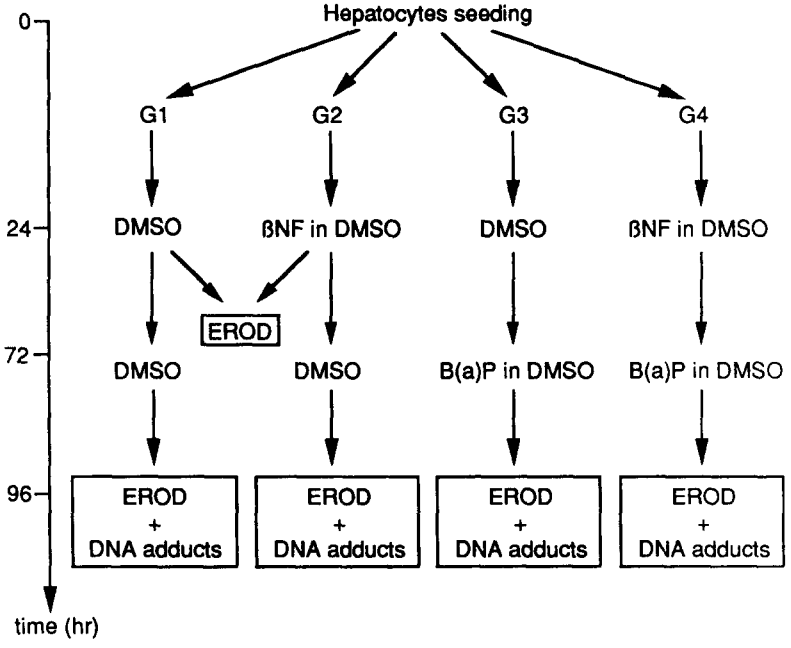

Fig. 1. Protocol of hepatocytes treatment before measurement of activity of 7-ethoxyresorufin *O*-deethylase (EROD) activity and level of DNA adducts. Dishes containing hepatocytes were divided, after seeding, into four groups: G1, G2, G3 and G4. After 24 hr culture, G1 and G3 received DMSO alone while G2 and G4 received 0.36 μM-βNF (final concentration), which previously had been shown to be the optimum concentration for induction (data not shown). After 72 hr culture, EROD activity was measured in some G1 and G2 dishes; DMSO was added to the remaining G1 and G2 dishes; G3 and G4 received 1 μM-B[a]P (final concentration). After 96 hr culture (24 hr after the addition of B[a]P) EROD activity and DNA adduct level were assessed in G1, G2, G3 and G4.

Table 1. EROD activity (pmol · min^{-1} · mg protein^{-1}) as a function of benzo[*a*]pyrene (B[*a*]P) concentration in assays measured after 48 and 72 hr exposure to B[*a*]P

Time after B[*a*]P addition (hr)	EROD activity after exposure to various concentrations (μM) of B[*a*]P†				
	Control	0.01	0.1	1	10
48	80 ± 2	108 ± 1*	128 ± 4*	159 ± 13*	63 ± 6*
72	71 ± 3	101 ± 3*	122 ± 6*	142 ± 6*	57 ± 2*

EROD = 7-ethoxyresorufin *O*-deethylase

†B[*a*]P was added to the culture medium 24 hr after cell seeding. Each value corresponds to mean value ± SD from three dishes. Asterisks indicate significant differences from the control (*$P < 0.05$).

Consequently, 1 μM-B[*a*]P was chosen for the subsequent experiments.

DNA adducts in hepatocytes treated up to 72 hr with 1 μM-B[a]P (experiment II); comparison with adduction in liver in vivo

DNA adducts were assessed 12, 24, 36 and 72 hr after B[*a*]P treatment. Adducts were detected without change in the chromatographic pattern at each sampling time in B[*a*]P-treated hepatocytes and were absent from DMSO-treated hepatocytes. Plate 1 shows the pattern of DNA adducts from 24-hr B[*a*]P-treated hepatocytes, which was shown to be identical to that observed after treatment *in vivo*, as demonstrated by the co-chromatography experiment involving adducted nucleotides from B[*a*]P-treated hepatocytes and from the liver of B[*a*]P-treated rainbow trout.

Table 2 gives confirmation of the identity of adducts resulting from B[*a*]P treatment of hepatocytes and trout, as demonstrated by additivity of the radioactivity of each spot when counting the corresponding spot revealed by co-chromatography. Nevertheless, the relative contribution of the spots was not the same in hepatocytes and in trout liver.

During this experiment, EROD activity measured 12, 36 and 72 hr after B[*a*]P treatment was induced 0.9-, 1.2- and 1.9-fold, respectively, induction being statistically significant at 36 and 72 hr (data not shown).

Effect of βNF pretreatment of hepatocytes on the formation of B[a]P–DNA adducts (experiment III)

Plate 2 shows that no adduct was detectable in control and βNF-treated hepatocytes (0.36 μM final concentration in assays) and that pre-exposure to βNF for 48 hr did not modify the DNA adduct pattern observed following a subsequent 24-hr exposure to 1 μM-B[*a*]P when compared with hepatocytes treated with B[*a*]P alone. Moreover, pre-exposure to βNF inhibited DNA adduct formation without modifying the relative contribution of the spots (Table 3). Nevertheless, Table 4 shows that, at the time of addition of B[*a*]P to the culture medium, EROD activity was significantly induced by βNF (3.4-fold). Moreover, 24 hr after the addition of B[*a*]P, the EROD activity induced was always significantly (2-fold) higher in hepatocytes pre-exposed to βNF than in those pre-exposed to DMSO, while B[*a*]P alone significantly induced EROD activity.

DISCUSSION

This study confirms the inducibility of *P*4501A-dependent enzyme activity in a primary culture of rainbow trout hepatocytes and demonstrates the ability of cultured cells to metabolize B[*a*]P into DNA-damaging species in the same way as occurs *in vivo*; the results also suggest that DNA adduct formation cannot be related simply to the EROD activity level.

The induction of EROD by B[*a*]P in a primary culture of rainbow trout hepatocytes is similar to that found previously with βNF and dioxin as inducers (Pesonen and Andersson, 1991; Vaillant *et al.*, 1989), and parallels the induction of aryl hydrocarbon hydroxylase (AHH; a *P*4501A-dependent activity using B[*a*]P as a substrate) observed with B[*a*]P and other PAH-like compounds in cultured mammalian hepatocytes (Nemoto *et al.*, 1990). In a recent study conducted in rainbow trout from the same fish farm, ip treatment (80 mg B[*a*]P/kg body weight) led to a 2- to 3-fold induction of microsomal hepatic EROD activity (Masfaraud *et al.*, 1992). This, and the present study, indicate that similar responses are obtained in cultured hepatocytes and *in vivo*. The fact that EROD induction depends on the penetration of the inducer into the cell (Okey, 1990) supports the assumption that exposure of hepatocytes to B[*a*]P caused subsequent penetration of this PAH into the cells.

The study demonstrates the inhibitory effect on EROD activity of 10 μM-B[*a*]P in the culture medium. This result suggests that this concentration of B[*a*]P causes cell disturbances rather than competitive occupation of the active site of *P*4501A by B[*a*]P, because similar inhibition was observed when AHH was measured (data not shown). This observation underlines the importance of selecting a xenobiotic concentration in cellular systems when several parameters have to be investigated, and justifies the choice of 1 μM-B[*a*]P for further experiments to study the relationship between EROD induction and the concentration of DNA adducts.

The ability of fish species to metabolize PAHs, especially B[*a*]P, is well known (Tan and Melius, 1986), and activation of B[*a*]P into DNA-binding species has been demonstrated *in vivo* (Kurelec *et al.*, 1991; Shugart *et al.*, 1987; Sikka *et al.*, 1990; Varanasi *et al.*, 1989). Metabolism of B[*a*]P has been demonstrated in several mammalian and fish cultured cells; however, DNA adduct formation in fish has been observed only in cell lines and freshly isolated hepatocytes (Babich and Borenfreund, 1991; Jones *et al.*, 1978; Steward *et al.*, 1989). This study has now demonstrated the occurrence of DNA adducts in a primary culture of fish hepatocytes exposed to B[*a*]P.

The results presented here show that DNA adducts appear after a 12-hr exposure to B[*a*]P, but in

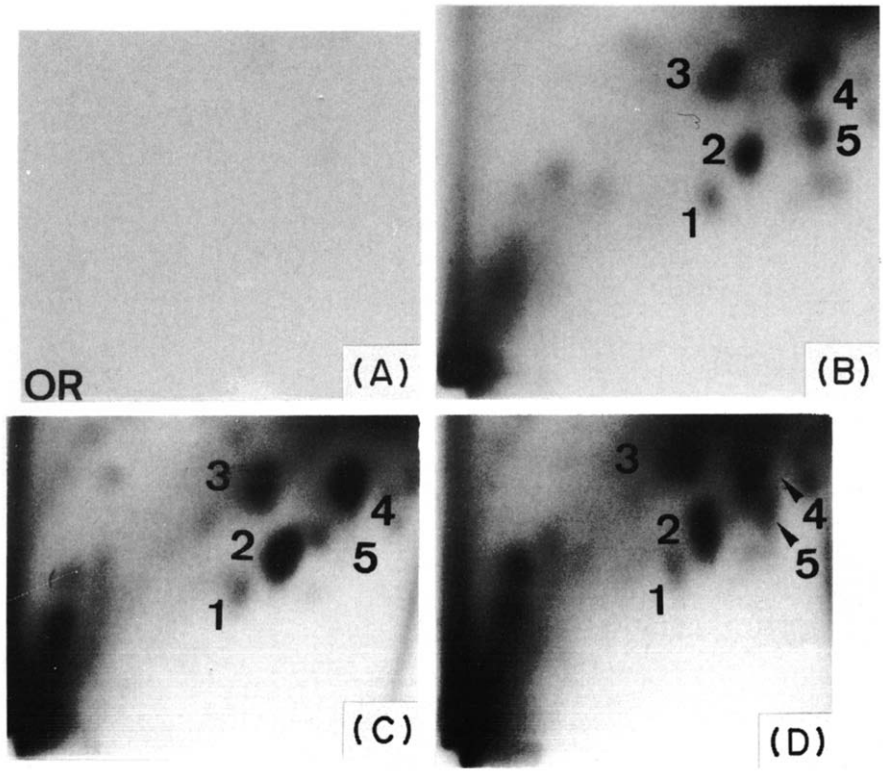

Plate 1. Autoradiograms of chromatograms of DNA adducts from control trout hepatocytes (A), trout hepatocytes (B) and trout liver treated with benzo[*a*]pyrene (B[*a*]P) (C). Hepatocytes were treated with 1 μM-B[*a*]P (final concentration); trout received ip 80 mg B[*a*]P/kg body weight and the liver was removed 48 hr later. For hepatocytes and liver, 20 μg DNA were analysed. For co-chromatography (D), 20-μg aliquots of each DNA sample were spotted on the same thin-layer chromatography plate. Autoradiography was at -80°C for 24 hr using an intensifying screen. OR = origin of chromatography at lower left-hand corner of the chromatogram.

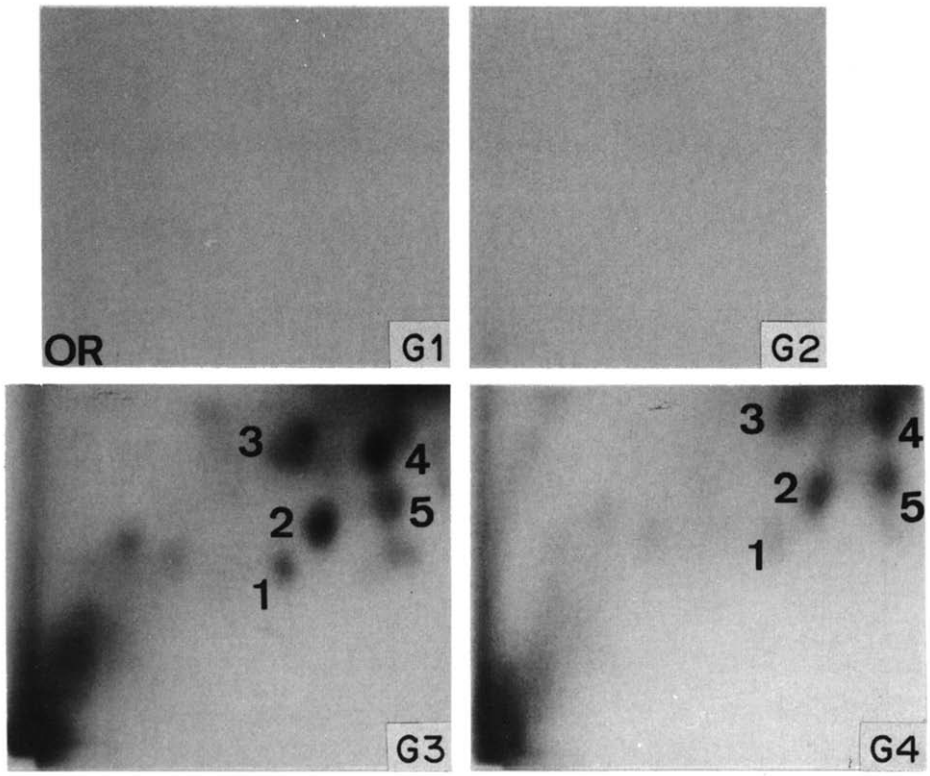

Plate 2. Autoradiograms of chromatograms of DNA adducts from control trout hepatocytes (G1), and those treated with βNF (G2), B[*a*]P (G3) or βNF plus B[*a*]P (G4) (see Fig. 1 for protocol of treatment). 20-μg aliquots of each DNA sample were analysed. Autoradiography was at $-80°C$ for 24 hr using an intensifying screen. OR = origin of chromatography at lower left-hand corner of the chromatogram.

Table 2. Level of the main DNA adducts detected in trout hepatocytes or trout liver following treatment with benzo[a]pyrene

Source of DNA	Level of DNA adducts (cpm)*				
	1†	2	3	4	5
Hepatocytes (20 μg DNA)	117	323	465	436	243
Liver (20 μg DNA)	232	910	772	1333	139
Co-chromatography	361 (349)‡	1153 (1233)	1278 (1236)	1675 (1769)	388 (382)

*Level of DNA adducts is expressed as cpm/20 μg DNA from either hepatocytes or liver. Co-chromatography was carried out with 20 μg DNA hydrolysate from both hepatocytes and liver.
†Adducts 1–5 are those depicted in Plates 1 and 2.
‡Numbers in parentheses indicate the total cpm expected for co-chromatography, assuming additivity of the DNA adduct level in liver and hepatocytes.

Table 3. Level of DNA adducts detected from trout hepatocytes after a 24-hr treatment with benzo[a]pyrene with or without a 48-hr pretreatment with β-naphthoflavone (βNF)*

Pretreatment	Level of different DNA adducts†					
	1‡	2	3	4	5	Total adducts§
None						
Sample 1	2.95	8.16	11.76	11.01	6.14	40.02
Sample 2	3.80	8.23	11.33	10.61	7.55	41.52
βNF						
Sample 1	2.26	5.21	4.97	8.61	6.67	27.72
Sample 2	nd	7.58	9.89	9.39	5.19	32.05¶
Sample 3	2.88	6.00	10.89	10.32	6.80	36.89

*See Fig. 1 for protocol of treatment. Each sample originates from three dishes.
†Adduct level is expressed as fmoles of adduct/mg DNA.
‡Adducts 1–5 are those depicted in Plates 1 and 2.
§A significant difference (Mann–Whitney *U*-test) in total adduct level was shown between pretreated and non-pretreated hepatocytes.
¶Total adducts for sample 2 in the βNF-treated-group were calculated without taking into account adduct 1, which was not determined (nd).

preliminary experiments adducts were detected after only 4 hr (data not shown). DNA adducts could probably be detected even earlier, as suggested by Zaleski *et al.* (1991) who detected DNA adducts only 1 hr after exposure to 40 μM-B[a]P of freshly isolated hepatocytes from mirror carp. Unfortunately, these authors did not describe the effect of this concentration of B[a]P on the activity of *P*4501A. As, in our study, EROD was not induced after 12 hr of exposure, it seems likely that activation of B[a]P by rainbow trout hepatocytes takes place before any significant increase in *P*4501A protein content, which occurs after some delay caused by the synthesis of CYP1A mRNA, as recently shown by Pesonen *et al.* (1992).

A close similarity was shown between DNA adduct patterns generated in cultured hepatocytes and *in vivo*, suggesting that the metabolic activation of PAHs in primary culture of rainbow trout hepatocytes is representative of that *in vivo*. Studies have demonstrated the predominance of the adduct derived from B[a]P 7,8-diol 9,10-epoxide (BPDE), in the liver, the isolated hepatocytes or the cell lines obtained from several fish species exposed to B[a]P (Kurelec *et al.*, 1991; Smolarek *et al.*, 1987; Steward *et al.*, 1989; Varanasi *et al.*, 1989). However, it seems that the pattern of DNA adducts occurring after exposure to B[a]P is species specific, since we and other authors have observed that the major adduct in trout detected after B[a]P treatment *in vivo* (liver) and in the RTG-2 cell line (a fibroblast line) exposed to B[a]P, differed from the adduct derived from BPDE (Masfaraud *et al.*, 1992; Smolarek *et al.*, 1987).

The induction of EROD activity has been proposed as a biomarker of exposure to planar polycyclic hydrocarbons (PAHs, dioxins, furans and planar PCBs). Because of the involvement of *P*4501A in the activation of PAHs into their ultimate carcinogens, EROD might also be considered as a biomarker of the ability of a system to activate PAHs into DNA-damaging compounds (Stegeman and Lech, 1991). The primary culture of rainbow trout hepatocytes, in which EROD activity is inducible and DNA adducts are generated on exposure to B[a]P, enables the relationship between EROD activity and DNA adduct pattern and level to be examined.

The fact that the pattern of DNA adducts was the same as that observed in the absence of EROD induction (see experiment II) supports the assumption that *P*4501A was present in control hepatocytes, as recently shown by Pesonen *et al.* (1992) and that EROD induction did not result from the synthesis of new forms of *P*-450 involved in B[a]P activation, but from reinforcement of the formation/stabilization of *P*-4501A present in the cells before treatment with βNF.

As implied by these results, the lack of association between EROD activity (*P*4501A level) and DNA adduct levels suggests a significant involvement of

Table 4. 7-Ethoxyresorufin *O*-deethylase (EROD) activity (pmol · min^{-1} · mg protein^{-1}) in control hepatocytes or in hepatocytes treated with β-naphthoflavone (βNF), benzo[a]pyrene (B[a]P), or βNF + B[a]P

Time in culture (hr)	EROD activity after treatment			
	Control	βNF	B[a]P	βNF + B[a]P
72	77.3 ± 8.9	264.0 ± 16.6*	—§	—§
96	28.6 ± 2.9	72.8 ± 8.8*	66.0 ± 4.7*	121.8 ± 15.9*‡

†See Fig. 1 for protocol of treatment. Each value corresponds to mean value ± SD from three dishes.
‡At 96 hr, significantly different from βNF- and B[a]P-treated hepatocytes ($P < 0.05$).
§B[a]P added only after 72 hr culture.
Significantly different from the control at 72 and 96 hr (*$P < 0.05$).

conjugating as well as DNA-repair enzymes in the regulation of DNA adduct formation and elimination, respectively, as discussed elsewhere (James, 1987; Klaunig, 1984; Zaleski *et al.*, 1991). An increase in DNA adduct formation may require an induction threshold of *P*4501A that was not attained in our study (3.4-fold); thus, Monteith *et al.* (1990) observed that an 11-fold preinduction in AHH activity was correlated with no more than a 1.7-fold increase in DNA-binding in a primary culture of human hepatocytes treated with radiolabelled B[*a*]P.

In conclusion, this study has demonstrated the formation of DNA adducts in a primary culture of rainbow trout hepatocytes exposed to B[*a*]P. The DNA-adduct pattern is very similar to that in the liver after treatment *in vivo*. Pre-exposure of hepatocytes to a *P*4501A inducer failed to demonstrate a relationship between the level of DNA adduct and EROD activity, under the conditions of the present study. EROD activity is a good indicator of exposure to PAHs, but the conditions under which *P*4501A is the limiting factor for DNA adduct formation following PAH exposure remain to be elucidated. For this purpose, a primary culture of rainbow trout hepatocytes provides a suitable method *in vitro*.

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