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INDUCTION OF THE RAT HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASES BY TWO AZA-ARENES

A COMPARISON WITH THEIR NON-HETEROCYCLIC ANALOGUES

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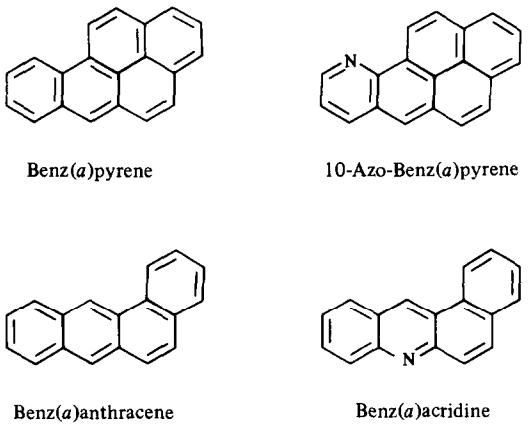
Abstract—The ability of the aza-aromatic polycyclic aromatic hydrocarbons 10-azobenz(*a*)pyrene and benz(*a*)acridine to induce the rat hepatic microsomal mixed-function oxidases was compared to that of their non-heterocyclic analogues benz(*a*)pyrene and benz(*a*)anthracene respectively. All four hydrocarbons markedly increased the *O*-deethylations of ethoxyresorufin and ethoxycoumarin, the non-heterocyclic analogues being the more potent. A more modest increase was seen in the *O*-dealkylation of pentoxyresorufin. All four hydrocarbons induced proteins recognised by antibodies to cytochrome P-450IAI but no increase was seen when antibodies to cytochrome P-450IIB1 were employed. The metabolic activation of benz(*a*)pyrene and Glu-P-1 to mutagenic intermediates in the Ames test was enhanced by all pretreatments. It is concluded that the aza-aromatic polycyclic hydrocarbons, like their non-heterocyclic analogues, selectively induce the cytochrome P-450I family of proteins.

The polycyclic aza-aromatic hydrocarbons (aza-arenes) are products of the pyrolysis of nitrogenous organic materials and, like their non-heterocyclic analogues, are important and ubiquitous environmental pollutants being detected in synthetic fuels [1], airborne particulates [2, 3], marine sediments [4] and in tobacco condensates [5].

In many animal models aza-arenes, such as benz(*c*)acridine and its 7-methyl derivative and a number of dibenzacridines displayed carcinogenic potential [6–8]. Moreover, they exhibited positive mutagenic response in *in vitro* systems employing *Salmonella typhimurium* and *Escherichia coli* bacterial strains [9–13], and clastogenicity in Chinese hamster cells in culture [14].

Similarly to the polycyclic aromatic hydrocarbons the aza-arenes require metabolic activation to form reactive intermediates that manifest their mutagenicity/carcinogenicity, and appear to share similar metabolic pathways for their activation. The ultimate carcinogens are bay-region diol-epoxides which are formed by an initial oxidation of the parent compound to form epoxides that are hydrolysed by epoxide hydrolase to the diols, which finally undergo a second oxidation to yield the diol-epoxides [15]. The latter are potent mutagens and carcinogens [7, 11, 16]. The two oxidation steps in the activation of polycyclic aromatic hydrocarbons are preferentially catalysed by the cytochrome P-450I (cytochromes P-448) family of microsomal haemoproteins and especially the low spin isoenzyme [17]. Similarly the cytochrome P-450I family was the more efficient in converting dibenz(*a*,*h*)acridine to the diols which act as precursors of the bay-region epoxides [15] and was also responsible for the oxidation of 7-methylbenz(*c*)acridine [2, 18].

The polycyclic aromatic hydrocarbons are potent inducers of the cytochrome P450I family, so that on repeated administration they enhance their own bioactivation and this property contributes to their carcinogenic potency. The purpose of the present study was, therefore, to establish if the aza-arenes are (a) inducers of the rat hepatic microsomal mixed-function oxidases, and (b) if they selectively induce the synthesis of cytochrome P450I proteins. The inductive properties of 10-azo-benz(*a*)pyrene (10AB(*a*)P) and benz(*a*)acridine (B(*a*)C) were compared with that of their non-heterocyclic analogues benz(*a*)pyrene (B(*a*)P) and benz(*a*)anthracene (B(*a*)N) respectively (Fig. 1).



Benz(*a*)pyrene — 10-Azo-Benz(*a*)pyrene

Benz(*a*)anthracene — Benz(*a*)acridine

Fig. 1. Chemical structures of the hydrocarbons employed in this study.

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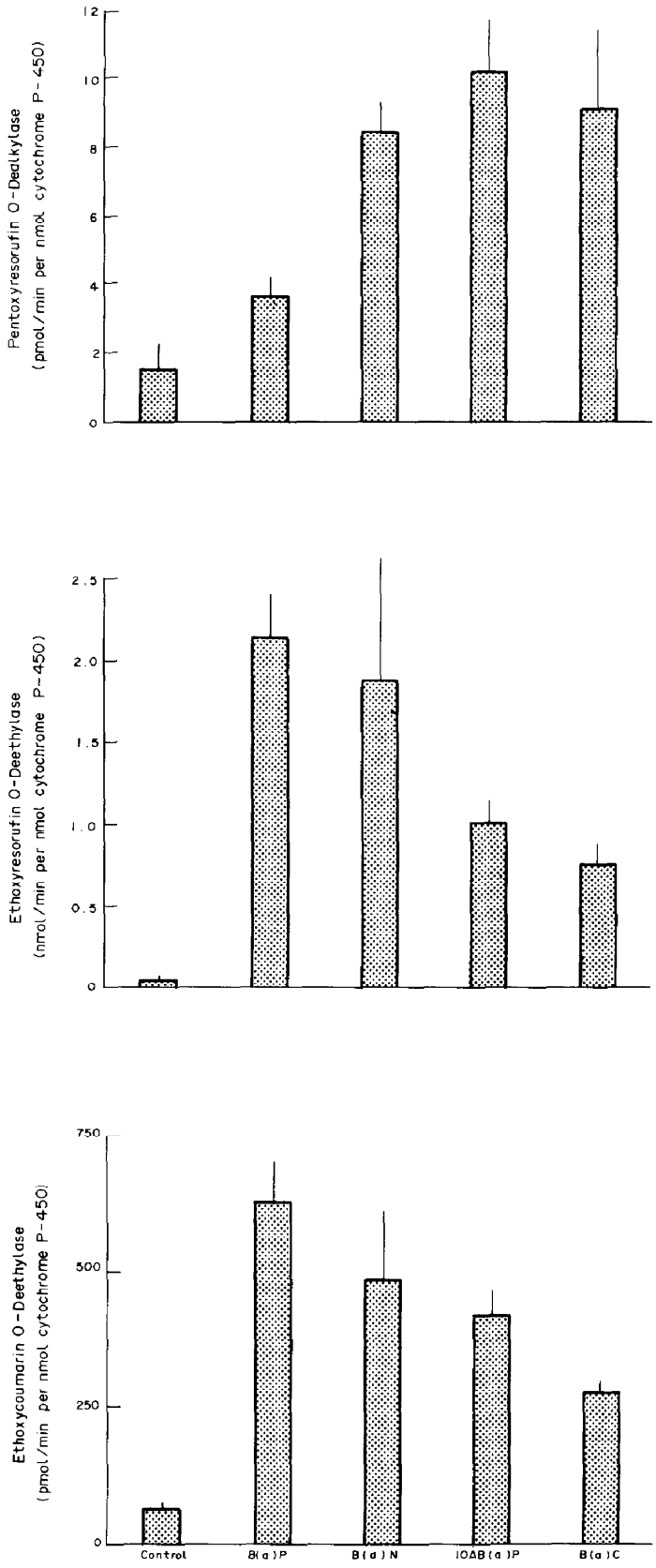

Fig. 2. Hepatic microsomal mixed function oxidase activities in rats pretreated with the various hydrocarbons. Results are presented as mean ± SEM for five animals. The differences between control and treated animals were statistically significant in all cases ($P < 0.05$).

MATERIALS AND METHODS

Ethoxycoumarin and coumarin (Aldrich Chemical Co., Gillingham, Dorset, U.K.), Glu-P-1 (2-amino-6-methyldipyrido[1,2α:3',2'-d]imidazole (Wako Fine Chemicals, Neuss 1, F.R.G.), resorufin and its analogues (Molecular Probes Inc., Eugene, OR), benz(*a*)acridine and 10-azobenz(*a*)pyrene (Electros Developments, Beaconsfield, Bucks., U.K.) and benz(*a*)pyrene, benz(*a*)anthracene and all cofactors (Sigma Co., Poole, Dorset, U.K.) were all purchased. Peroxidase-antiperoxidase, horseradish peroxidase-labelled donkey anti-sheep IgG and donkey anti-rabbit IgG antibodies were obtained from Guildhay Antisera (Guildford, Surrey, U.K.). The *Salmonella typhimurium* strains TA98 and TA100 were donated by Professor B. N. Ames, University of California, Berkeley, U.S.A.

Male Wistar albino rats (Animal Breeding Unit, University of Surrey) were used in all studies. Animals (150–200 g) were housed on woodchips (J. C. Lee, Chertsey, Surrey, U.K.) and fed Labsure Rodent Breeding Diet No. 1 and water *ad libitum*. They were administered a single intraperitoneal dose (5 mg/kg) of the polycyclic hydrocarbon dissolved in corn oil (5 mg/ml) while controls received only the vehicle (1 ml/kg). All animals were killed 24 hr after administration by cervical dislocation. Hepatic microsomal fractions were prepared as previously described [19] and the following determinations were carried out: ethoxyresorufin *O*-deethylase [20], pentoxyresorufin *O*-dealkylase [21], ethoxycoumarin *O*-deethylase [22] at substrate concentrations of 0.5, 2.5 and 25 mM respectively, NADPH-cytochrome *c* reductase [23] cytochromes P-450 and b_5 [24] and protein using bovine serum albumin as standard [25].

Cytochromes P-450b (P450IIB1) and P-450c (P450IA1) purified from hepatic microsomes of Wistar albino rats pretreated with phenobarbital and 3-methylcholanthrene respectively have already been characterised [26]. Antibodies to purified cytochrome P-450IA1 were raised in a single sheep after priming with 1.0 mg and boosting with 0.25 mg of antigen. Antisera to cytochrome P-450IIB1 were raised in rabbits and were generously donated by Dr. G. G. Gibson (University of Surrey). Immunoblotting analysis of the proteins following electrophoretic resolution [27] was carried out as described by Towbin *et al.* [28]. Antibodies to cytochrome P-450IIB1 could detect both cytochromes P450IIB1 (P-450b) and P450IIB2 (P-450e) while those to cytochrome P-450IA1 detected cytochromes P450IA1 (P-450c) and P450IA2 (P-450d).

The microsome-mediated metabolic activation of benz(*a*)pyrene and Glu-P-1 to mutagens was determined using the Ames mutagenicity test [29] and activation systems comprising hepatic microsomes (105,000 *g* pellet resuspended in 1.15% KCl), NADP (4 mM), glucose 6-phosphate (5 mM) and glucose 6-phosphate dehydrogenase (1 unit/plate). The concentration of microsomes in the activation system was 10% and 20% for benz(*a*)pyrene and Glu-P-1 respectively. Both carcinogens were dissolved in DMSO so that the solvent concentration never exceeded 100 μl/plate.

RESULTS

All four hydrocarbons gave rise to a marked increase in the *O*-deethylations of ethoxycoumarin and ethoxyresorufin. In both cases the aromatic hydrocarbons were more potent inducers than their aza-aromatic analogues (Fig. 2). A modest increase in the *O*-dealkylation of pentoxyresorufin was also evident but in this case the aza-arenes were at least as potent inducing agents as their non-heterocyclic analogues (Fig. 2). The NADPH-dependent reduction of cytochrome *c* was not markedly affected by treatment of the rats with the hydrocarbons except for a small, but statistically significant increase following treatment with benz(*c*)acridine (Table 1). Cytochrome b_5 levels were not significantly affected by any of the treatments. However, the microsomal levels of total cytochrome P-450 decreased following treatment of the rats with benz(*a*)anthracene or the two aza-arenes (Table 1). There were no significant differences in the microsomal protein levels.

Immunoblots employing polyclonal antibodies against the 3-methylcholanthrene-inducible cytochrome P-450IA1 revealed that all four polycyclic hydrocarbons induced both cytochrome P-450I proteins, benz(*a*)pyrene being the more potent (Fig. 3). No increase in any of the proteins was visible when antibodies against cytochrome P-450IIB1 were employed in the immunoblots (Fig. 4). The positive control, phenobarbital gave the expected response inducing both IIB1 and IIB2. Similarly to previous studies [30], no P-450IIB1 was seen in control animals and a third band of greater mobility was detected by the antibody in the control animal.

Activation systems derived from the animals pretreated with the polycyclic hydrocarbons were markedly more efficient than control in converting benz(*a*)pyrene to mutagens (Fig. 5). Similarly the metabolic activation of Glu-P-1 was induced by all treatments, benz(*a*)pyrene being clearly the most effective (Fig. 5).

DISCUSSION

The polycyclic aromatic hydrocarbons are established inducers of the mixed-function oxidases, selectively enhancing the synthesis of P450I (cytochromes P-448) family of proteins, which are closely associated with the bioactivation of chemicals and carcinogenicity [31]. The induction of this family of cytochromes P-450 is mediated through interaction with a cytosolic receptor [32]. The inducing agent binds to the receptor and the complex translocates into the nucleus where it activates the structural genes giving rise to increased mRNA synthesis coding for cytochrome P-450I apoproteins. The receptor appears to interact with rigid, planar molecules and a good correlation exists between the extent of induction of cytochrome P-450I activity and the avidity with which the inducer binds to the receptor [33, 34]. Since the aza-arenes retain the planarity of their non-heterocyclic analogues they would be expected also to interact with this receptor and induce the P450I family of isoenzymes. In order to test this hypothesis animals were pretreated with a single intraperitoneal dose of 5 mg/kg, as such a dose was shown to achieve

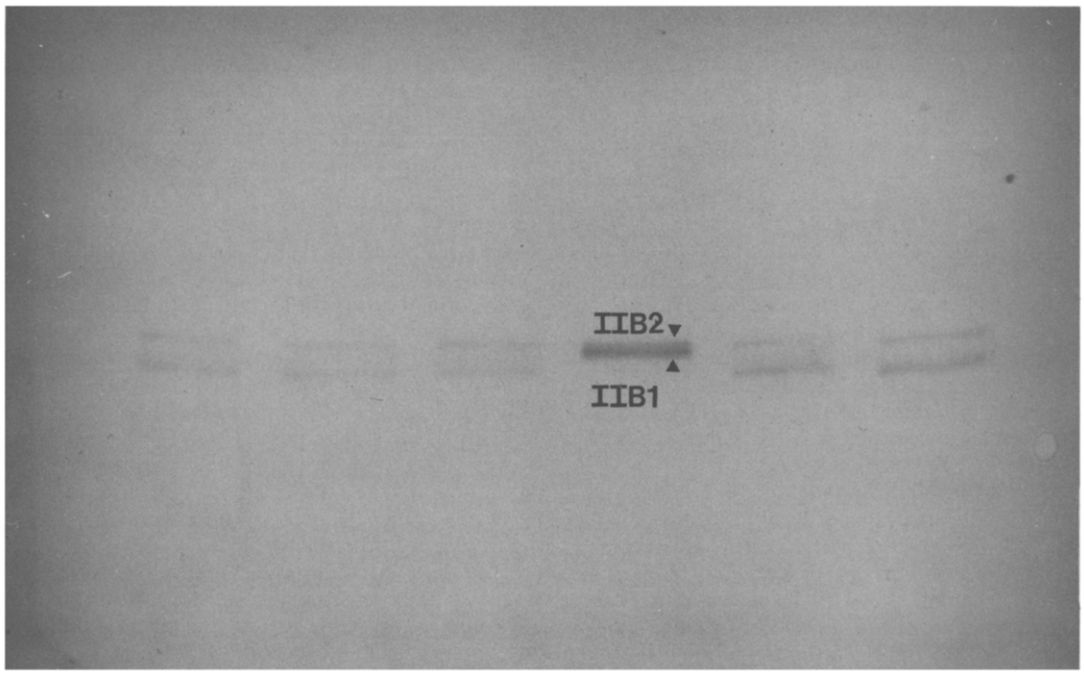

Fig. 3. Immunoblot employing antibodies against P-450IA1. Following resolution in a 10% (w/v) SDS-polyacrylamide gel the protein samples were transferred electrophoretically to nitrocellulose. The immunoblot was performed with sheep anti-cytochrome P-450IA1 (diluted 1:12,000), followed by donkey anti-sheep 1gG (diluted 1:100) and sheep peroxidase-antiperoxidase (diluted 1:1000). Solubilised proteins (10 μg protein) were derived from (A) benz(*a*)acridine, (B) control, (C) benz(*a*)pyrene, (D) 3-methylcholanthrene, (E) benz(*a*)anthracene, and (F) 10-azobenz(*a*)pyrene.

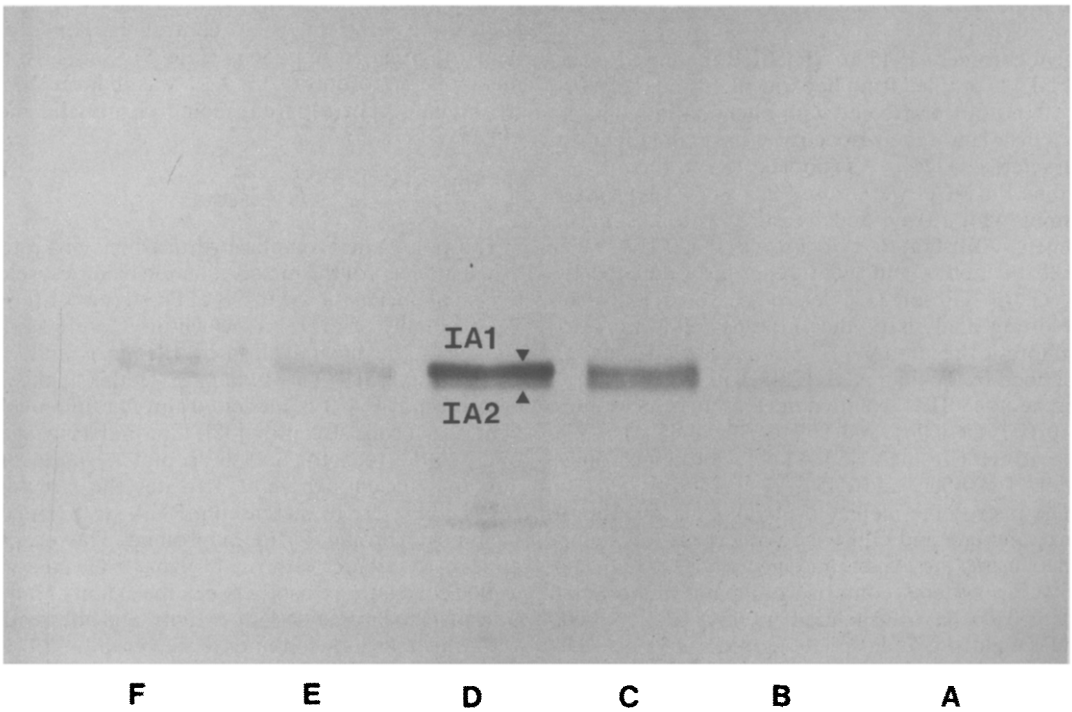

Fig. 4. Immunoblot employing antibodies against P-450IIBI. The immunoblot was performed with rabbit anti-cytochrome P-450IIB1 (diluted 1:2000) followed by donkey anti-rabbit 1gG (diluted 1:100) and rabbit peroxidase-antiperoxidase complex (diluted 1:1000). Conditions as in legend to Fig. 3, except that protein sample in lane D was derived from phenobarbitone-treated animals.

Table 1. Effect of treatment with polycyclic hydrocarbons on some rat liver parameters

Treatment	Cytochromes P-450 (nmol/mg protein)	Cytochromes b_5 (nmol/mg protein)	NADPH-cyt.*c* reductase (nmol/min per mg protein)	Microsomal protein (mg/g liver)
Control	0.51 ± 0.06	0.71 ± 0.11	5.5 ± 0.2	30.1 ± 3.2
Benz(*a*)pyrene	0.65 ± 0.11	0.60 ± 0.14	3.7 ± 1.5	28.1 ± 2.0
Benz(*a*)anthracene	0.35 ± 0.03*	0.56 ± 0.06	4.8 ± 1.0	34.1 ± 1.1
10-Azobenz(*a*)pyrene	0.27 ± 0.05**	0.52 ± 0.03	6.1 ± 0.8	27.6 ± 2.1
Benz(*a*)acridine	0.32 ± 0.02**	0.52 ± 0.03	7.5 ± 0.4**	28.2 ± 1.0

* $P < 0.05$; ** $P < 0.01$.
Animals were given a single intraperitoneal administration of the chemical (5 mg/kg) and were killed 24 hr later. Results are presented as mean ± SEM for 5 animals.

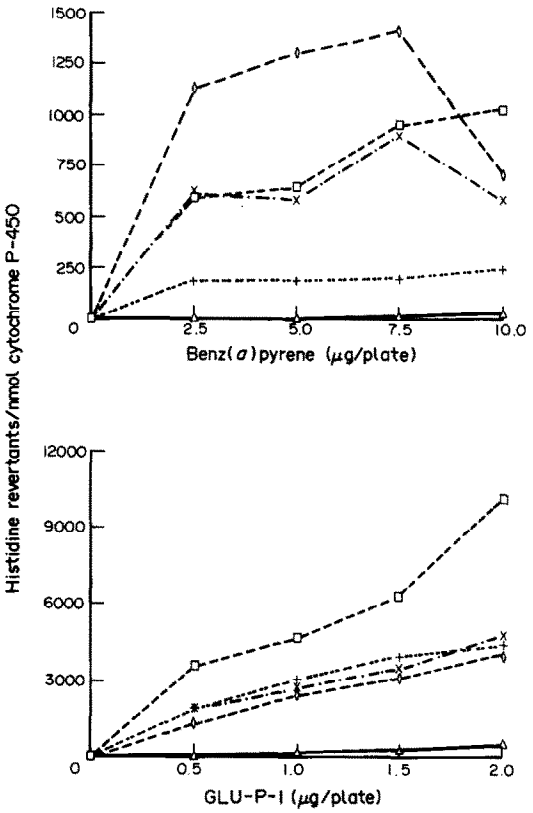

Fig. 5. The bioactivation of benz(*a*)pyrene and Glu-P-1 by hepatic S9 preparations from rats treated with various polycyclic hydrocarbons. The bioactivation of benz(*a*)-pyrene and Glu-P-1 to mutagens was investigated using *Salmonella typhimurium* strains TA 100 and TA 98 respectively. S9-Activation systems, 10% and 20% for benz(*a*)pyrene and Glu-P-1 respectively, were derived from control (△), B(a)N (+), B(a)C (×), B(a)P (□) and 10AB(a)P (◇) pretreated animals. Results are presented as the average of triplicates. The spontaneous reversion rates of 75 ± 6 and 19 ± 3 for TA 100 and TA 98 respectively have already been substrated. The experiment was repeated with activation systems from different, but similarly treated animals with the same results.

maximum induction of cytochrome P-450 activity, as exemplified by the *O*-deethylation of ethoxyresorufin, with polycyclic aromatic hydrocarbons [35]. All four polycyclics induced the *O*-deethylation of ethoxyresorufin, a reaction which serves as an excellent marker for cytochrome P-450I activity [36, 37]. A similar effect was obtained with ethoxycoumarin *O*-deethylase, which also reflects primarily cytochrome P-450I activity [36]. In both cases the aromatic hydrocarbons were more potent inducers than their corresponding aza-arenes. These results are supported by the immunoblots with anti-cytochrome P-450IAI where induction of both cytochrome P-450I isoenzymes was evident with all four compounds, benz(*a*)pyrene being clearly the most potent, even when the amount of total cytochrome P-450 loaded is taken into account.

The metabolic activation of Glu-P-1 to mutagens, a reaction selectively catalysed by the high spin isoenzyme of the cytochrome P450I family [38], was

increased by all treatments with benz(*a*)pyrene being once again clearly the most effective. Similarly, when benz(*a*)pyrene itself was used as the mutagen, all activation systems were more efficient than control in converting it to mutagens, but in this case treatments with benz(*a*)pyrene and its 10-azo-benz(*a*)pyrene were the most effective. The mutagenicity of benz(*a*)pyrene is the result of many metabolites, including the highly mutagenic diol-epoxides [39]. Part of the increase in the mutagenicity of benz(*a*)pyrene may be due to enhancement of epoxide hydrolase, the enzyme that converts the primary epoxides to the diols, which are then oxidised to form the diol-epoxides. Such a mechanism may explain why, when benz(*a*)pyrene is the mutagen, benz(*a*)acridine is as potent an inducer as benz(*a*)pyrene, presumably by enhancing epoxide hydrolase activity and this possibility is currently under investigation.

The *O*-dealkylation of pentoxyresorufin, an enzyme reaction catalysed primarily by the phenobarbital-induced cytochrome P450IIB family of proteins [21], was also stimulated by the four hydrocarbons but to a much lesser extent when compared to the *O*-deethylation of ethoxyresorufin. No increase in the levels of these proteins was evident on immunoblot analysis employing anti-cytochrome P-450IIB1. The *O*-dealkylation of pentoxyresorufin is to a small extent increased also by polycyclic aromatic hydrocarbons [21], and the modest increase observed in the present study may reflect, at least partly, the induction of cytochrome P-450I proteins.

In conclusion the present study demonstrates that (a) aza-aromatic polycyclic aromatic hydrocarbons, like their non-heterocyclic analogues, induce the hepatic microsomal mixed-function oxidases, selectively inducing the cytochrome P450I family, and (b) the aromatic hydrocarbons are generally more potent inducers when compared to their heterocyclic analogues.

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