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# INDUCTION OF CYTOCHROME P-448 ACTIVITY AS EXEMPLIFIED BY THE *O*-DEETHYLATION OF ETHOXYRESORUFIN

## EFFECTS OF DOSE, SEX, TISSUE AND ANIMAL SPECIES

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**Abstract**—The effects of tissue, sex, animal species and dose on the induction of cytochrome P-448 activity by various inducing agents were investigated using *O*-ethoxyresorufin as a model substrate. The liver was by far more effective in catalysing the *O*-deethylation of ethoxyresorufin (EROD) than the lung and kidney. The extent of induction was also highest in the liver, with the exception of benzo(a)pyrene and 3-methylcholanthrene where inducibility was more pronounced in the kidney. The benzo(a)pyrene-induced hepatic EROD activity in the rat decayed to reach control levels four days after a single administration. Rat hepatic EROD activity was induced in both sexes but tended to be higher in the male. Marked species differences in the inducibility of hepatic EROD activity by various chemicals was observed, the rat being always more responsive when compared to the hamster or mouse. The induction of rat hepatic EROD activity by benzo(a)pyrene, 2-acetylaminofluorene and safrole was dose-dependent, maximum induction being achieved with single doses of 5, 2 and 5 mg/kg, respectively.

It is now recognised that cytochrome P-450, the terminal oxygenase of the mixed-function oxidase system, exists as a number of families in the mammalian hepatic endoplasmic reticulum [1–3]. These families may be induced by administration of exogenous chemicals; cytochromes P-448 (also designated as P-450c and P-450d in the rat) are induced by polycyclic aromatic hydrocarbons and other chemical carcinogens [4] while the induction of cytochrome P-450 (designated as P-450b and P-450e in the rat) is exemplified by drugs such as phenobarbitone [5]. These cytochromes differ not only in their mode of induction, but also in their immunological and spectral properties [6], developmental patterns [7] and substrate specificity [4]. Cytochrome P-450 directs metabolism towards deactivation while, in contrast, cytochrome P-448 directs metabolism towards the generation of toxic intermediates [4, 8]. Spectral binding studies have revealed that these two cytochromes possess markedly different substrate binding sites [9], cytochrome P-450 accepting substrates of varied molecular size while cytochrome P-448 accommodates a restricted number of substrates. Recent studies from our own laboratory [10] have demonstrated that cytochrome P-448 substrates are essentially planar characterised by large area/depth ratios. Non-planar bulky molecules do not interact with cytochrome P-448 but do so with the phenobarbital-induced cytochrome P-450.

A number of studies have been devoted to the factors that affect induction of cytochrome P-450 activity, such as age, tissue, sex and species. In contrast, the influence of these factors on the induction of cytochrome P-448 activity has been largely neglected, primarily due to the lack of a specific substrate that monitors the activity of this form of the cytochrome. The 7-deethylation of ethoxyresorufin (EROD) constitutes a highly specific and sensitive method for the determination of cytochrome P-448 activity [11, 12]. Using this substrate, we have already established that the development of cytochrome P-450 and P-448 activities and their inducibility with age follow markedly contrasting patterns [7]. The induction of cytochrome P-448 activity has been associated with mutagenic and carcinogenic activity of the inducing agent [4] and with genetic potential for malignancy [13], and may constitute a useful index of the potential carcinogenicity/toxicity of chemicals whose activation is mediated by the mixed-function oxidase system. To this end it was desirable to optimize the animal model and dose of the test chemical, and in this paper species differences, sex and tissue differences, and effect of dose, on the induction of cytochrome P-448 by some established inducing agents has been investigated.

### MATERIALS AND METHODS

2-Aminofluorene, myristicine, acridine orange and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Aldrich Chemical Co., Gillingham, Dorset, U.K.), 3-methylcholanthrene, 2-aminoanthracene, benzo(a)pyrene, β-naphthoflavone, cyclophosphamide, 2-acetylaminofluorene, safrole, biphenyl, dimethylnitrosamine (DMN) and all cofactors (Sigma Co., Poole, Dorset, U.K.), 4-aminobiphenyl (Phase Separations, Queensferry, Flintshire, U.K.), safrole (Hopkins and Williams Ltd., Essex, U.K.) and ethoxyresorufin and resorufin (Molecular Probes Inc., Junction City, OR, U.S.A.)

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were all purchased. *o*-Toluidine hydrochloride and 4-dimethylaminoazobenzene were samples from those used in the International Program for the Evaluation of Short-term Tests for Carcinogenicity.

Wistar albino rats (150–200 g), male adult CD1 mice (Animal Breeding Unit, University of Surrey), and male adult golden Syrian hamsters (100–120 g, Wrights of Essex, Essex, U.K.) were used in all studies. Animals were given daily intraperitoneal doses of the inducing agents (50 mg/kg) for three days, all animals being killed 24 hr after the last administration. The dose of acridine orange was decreased to 10 mg/kg because of acute toxicity. In the studies of different doses of inducing agent, animals received a single intraperitoneal administration (0.5–20 mg/kg) and were killed 24 hr later. In the time-dependent study of induction of cytochrome P-448, animals received a single intraperitoneal dose of benzo(a)pyrene (5 mg/kg) and were killed at regular time intervals. Microsomal fractions (105,000 *g* pellet resuspended) were prepared as previously described [14] and the *O*-deethylation of ethoxyresorufin (EROD) [15], and the 2- and 4-hydroxylations of biphenyl [16], total microsomal cytochromes P-450 [17] and protein [18] were determined. Statistical evaluation was carried out by the Student's unpaired *t*-test.

## RESULTS

### *Selection of species and tissue*

Marked species differences in ethoxyresorufin *O*-deethylase (EROD) activity were observed following treatment with 3-methylcholanthrene, 2-aminoanthracene, safrole and acridine orange (Table 1). In all cases the rat was by far the most sensitive animal to the inducers studied.

Liver EROD activity was induced in both male and female rats by 2-aminoanthracene and 3-methylcholanthrene, but in both cases inducibility was higher in the male (Table 2).

Of the three tissues studied, the liver was by far the most effective in catalysing the *O*-deethylation of ethoxyresorufin (Table 3). With the exception of cyclophosphamide, all compounds studied stimulated liver EROD activity, the polycyclic aromatic hydrocarbons being by far the most potent agents, but marked induction was also observed with aromatic amines and methylenedioxybenzene compounds (Table 3). The polycyclic aromatic hydrocarbons were the only compounds that enhanced lung EROD activity, while kidney EROD activity was increased in addition by 2-aminoanthracene and possibly *o*-toluidine. The extent of induction was highest in the liver, with the exceptions of benzo(a)pyrene and 3-methylcholanthrene which caused a relatively greater extent of induction in the kidney. Lung was the least sensitive to EROD induction (Table 3). β-Naphthoflavone was the most potent inducer of hepatic EROD activity and together with 3-methylcholanthrene were the only compounds that induced this activity in all three tissues studied.

At the low dose of 0.5 mg/kg the *O*-deethylation of ethoxyresorufin, and to a lesser extent the 2-hydroxylation of biphenyl, were the only activities increased by pretreatment with 2-acetylaminofluorene. Maximal induction of both activities were reached at a dose of 2 mg/kg and then decreased with increasing dosage probably due to acute cytotoxicity at high doses (Fig. 1).

Benzo(a)pyrene administration enhanced the *O*-deethylation of ethoxyresorufin and the 2-hydroxylation of biphenyl at all doses and a maximum was reached at 5 mg/kg. The 4-hydroxylation of biphenyl was also increased at high doses of benzo(a)pyrene, but to a lesser extent than was seen for biphenyl 2-hydroxylation or for EROD activity (Fig. 1). The total microsomal cytochromes P-450 remained essentially the same at all doses.

Safrole administration (0.5–20 mg/kg) again markedly increased EROD activity even at the low dose of 0.5 mg/kg; the 2-hydroxylation of biphenyl was also increased, but to a lesser extent (Fig. 1).

Table 1. Species differences in the induction of hepatic EROD activity by three chemical carcinogens

| Treatment | Liver ethoxyresorufin *O*-deethylase activity of (nmol/min per nmol P-450) | | |
|---|---|---|---|
| | Rat | Hamster | Mouse |
| Control | 0.06 ± 0.03 | 0.08 ± 0.02 | 0.08 ± 0.02 |
| 3-Methylcholanthrene | 1.8 ± 0.4* (30) | 0.23 ± 0.08* (2) | 0.42 ± 0.11* (5) |
| Control | 0.05 ± 0.04 | 0.09 ± 0.01 | 0.20 ± 0.09 |
| 2-Aminoanthracene | 0.26 ± 0.09* (4) | 0.07 ± 0.01 | 0.22 ± 0.12 |
| Safrole | 0.49 ± 0.12* (9) | 0.19 ± 0.02* (1) | 0.07 ± 0.05 |
| Control | 0.11 ± 0.02 | 0.06 ± 0.02 | 0.06 ± 0.01 |
| Acridine orange | 0.26 ± 0.12* (1) | 0.12 ± 0.05 (1) | 0.07 ± 0.02 |

* $P < 0.05$.

Animals were given single daily intraperitoneal administrations of the chemical (50 mg/kg except for acridine orange 10 mg/kg) for three days and were killed 3 days after last injection.

Results are presented as mean ± SD for 5 animals. Numbers in parentheses represent fold increases in activity.

Table 2. Sex differences in the induction of hepatic ethoxyresorufin *O*-deethylase activity by 3-methylcholanthrene and 2-aminofluorene in the rat

| Treatment | Liver ethoxyresorufin *O*-deethylase activity in (nmol/min per nmol P-450) | |
|---|---|---|
| | Male rat | Female rat |
| Control | 0.11 ± 0.03 | 0.14 ± 0.04 |
| 2-Aminofluorene | 0.51 ± 0.03* (4) | 0.56 ± 0.06* (3) |
| Control | 0.05 ± 0.01 | 0.07 ± 0.03 |
| 3-Methylcholanthrene | 1.60 ± 0.26* (35) | 1.80 ± 0.53* (25) |

* $P < 0.05$.

Animals received daily i.p. administrations of the chemical (50 mg/kg) for three days and were killed 24 hr after the last dose.

Results presented as mean ± SD for 5 animals. Numbers in parentheses represent fold increases in activity.

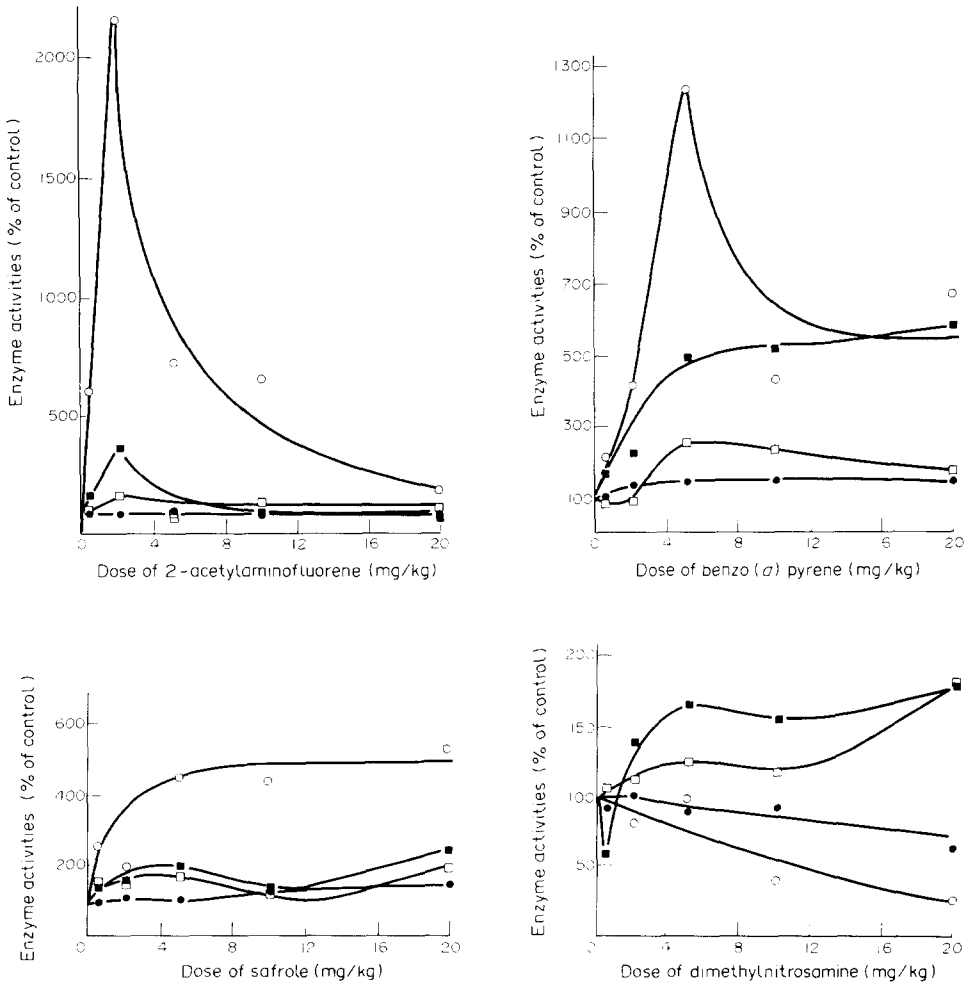


Fig. 1. Effect of dose on the induction of rat hepatic mixed-function oxidases by various chemical carcinogens. Animals received a single intraperitoneal dose of the carcinogen and were killed 24 hr later. Each point represents the mean of four animals. Control activities ranged for biphenyl 4-hydroxylase (□) 1.5–7.5, biphenyl 2-hydroxylase (■) 0.11–0.35 and ethoxyresorufin *O*-deethylase (○) 0.04–0.17 nmol/min per nmol of cytochrome P-450 while cytochrome P-450 levels (●) were 0.69–1.1 nmol/mg protein.

Table 3. Tissue differences in the induction of ethoxyresorufin *O*-deethylase activity by various chemicals in the rat

| Treatment | Ethoxyresorufin *O*-deethylase activity in Liver (nmol/min per mg of protein) | Lung (pmol/min per mg of protein) | Kidney (pmol/min per mg of protein) |
|---|---|---|---|
| Control | 0.03 ± 0.01 | 1 ± 0 | 1 ± 1 |
| 3-Methylcholanthrene | 1.9 ± 0.3* (60) | 8 ± 4* (7) | 100 ± 21* (100) |
| Control | 0.06 ± 0.02 | 1 ± 1 | 1 ± 0 |
| 2-Aminofluorene | 0.35 ± 0.02* (5) | 1 ± 2 | 2 ± 1 |
| Control | 0.02 ± 0.02 | 2 ± 1 | 1 ± 0 |
| Benzo(a)pyrene | 1.1 ± 0.3* (50) | 5 ± 3 | 66 ± 30* (65) |
| 4-Aminobiphenyl | 0.07 ± 0.01* (4) | 1 ± 1 | 1 ± 1 |
| Safrole | 0.19 ± 0.04* (8) | 2 ± 1 | 2 ± 2 |
| Control | 0.04 ± 0.02 | 2 ± 2 | 2 ± 2 |
| β-Naphthoflavone | 3.6 ± 1.2* (100) | 12 ± 8* (5) | 120 ± 72* (60) |
| 2-Aminoanthracene | 0.36 ± 0.02* (9) | 3 ± 1 | 26 ± 2* (12) |
| Myristicine | 0.16 ± 0.01* (4) | 2 ± 2 | 3 ± 1 |
| Control | 0.01 ± 0.01 | <1 | 1 ± 1 |
| *o*-Toluidine | 0.05 ± 0.01* (4) | <1 | 4 ± 2 |
| 4-Dimethylaminoazo benzene | 0.03 ± 0.01* (2) | <1 | 1 ± 0 |
| Cyclophosphamide | 0.02 ± 0.01 | <1 | 1 ± 0 |

* $P < 0.05$.

Male rats received daily intraperitoneal administrations of the chemical (50 mg/kg) for three days and were killed 24 hr after the last administration.

Results are presented as mean ± SD for 5 animals. Numbers in parentheses represent fold increases in activity.

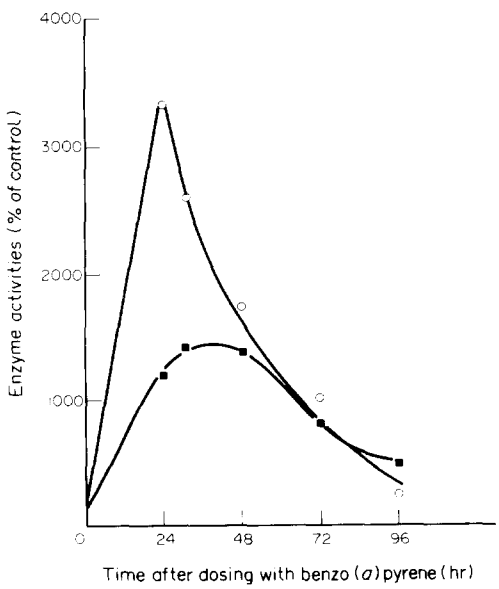


Fig. 2. Time-dependent decay of benzo(a)pyrene-induced hepatic mixed-function oxidases in the rat. Animals received a single intraperitoneal dose of benzo(a)pyrene (5 mg/kg) and were killed at regular time intervals after administration. Control values were for biphenyl 2-hydroxylase (■) 0.08 ± 0.01 and ethoxyresorufin *O*-deethylase (○) 0.02 ± 0.01 nmol/min per nmol cytochrome P-450.

No marked changes were seen in either the 4-hydroxylation of biphenyl or the concentrations of total cytochromes P-450.

In contrast, dimethylnitrosamine (0.5–20 mg/kg) did not enhance EROD activity and at the highest doses (10 and 20 mg/kg) actually caused a decrease in activity. The 2- and 4-hydroxylations of biphenyl were increased but only slightly (Fig. 1).

Finally, with the direct-acting carcinogen MNNG, none of the activities studied was enhanced and the 2-hydroxylation of biphenyl was even decreased at doses of 5 mg/kg and over (results not shown).

The increased EROD activity (33-fold) seen 24 hr after a single dose of benzo(a)pyrene, decayed to approach control values four days after administration (Fig. 2). A similar pattern was seen with biphenyl 2-hydroxylase.

## DISCUSSION

Mixed-function oxidation by cytochrome P-448 results in the metabolism of chemicals to reactive intermediates which give rise to toxicity [4]. It may be expected, therefore, that animals exhibiting high levels of cytochrome P-448 activity would be more susceptible to the toxicity of certain chemicals. Indeed, the neonatal rat, which exhibits higher cytochrome P-448 activity than the adult animal, as measured by EROD activity [7], is more efficient in

converting paracetamol to its reactive intermediates [19], a drug whose activation is catalysed only by cytochrome P-448 [20] and oxygen free radicals [21], but not by the phenobarbital-induced cytochrome P-450. Furthermore, it has been recently demonstrated that rats resistant to the carcinogenicity of 3-methyl-4-dimethylaminobenzene were refractive to cytochrome P-448 induction when compared to rats which were susceptible [22]. Cytochrome P-448 activity is induced by a variety of chemicals, at very small doses compared to those generally required to induce the synthesis of other isozymes [8], especially by chemical carcinogens, of various chemical structures as well as non-carcinogens such as β-naphthoflavone. It is essential, therefore, that the factors which determine the induction of cytochrome P-448, the enzyme responsible for the activation of many toxic chemicals and carcinogens, be elucidated.

The higher activity of cytochrome P-448 in rat liver, compared to kidney and lung, is in agreement with radioimmunoassay studies using monoclonal antibodies to the major 3-methylcholanthrene-induced cytochromes [23]. Tissue differences in the induction of mixed-function oxidases by phenobarbitone, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have previously been demonstrated [24], but the non-specific aryl hydrocarbon hydroxylase assay [11] was used as a measure of cytochrome P-448 activity, whereas in the present study the specific EROD assay was employed. Kidney cytochrome P-448 activity was more extensively induced than liver activity by 3-methylcholanthrene (see Table 3), similar to previous studies using benzo(a)pyrene as substrate [25].

Sex differences in the induction of cytochrome P-450 by compounds such as 3-methylcholanthrene and TCDD have been reported [26–28]. These differences were substrate dependent, e.g. TCDD stimulated benzo(a)pyrene hydroxylase (AHH) activity in rats of both sexes but the *N*-demethylations of aminopyrine, benzphetamine and ethylmorphine were decreased in the male and unaffected in the female [27]. These findings indicate that these inducing agents may selectively induce different cytochrome P-450 isozymes in male and female rat. In this study, the induction of cytochrome P-448 activity by 2-aminofluorene and 3-methylcholanthrene showed no significant sex differences in the rat.

Marked species differences in the response of animals to the toxic effects of chemicals have repeatedly been reported [29]. Some of these differences reflect qualitative and quantitative differences in drug metabolism and in the induction of these enzymes by drugs and other chemicals [30]. Marked species variations have been reported in the induction of AHH by phenobarbitone [31]. A chemical may act as an inducer in one species but have no effect or even cause inhibition in another [27, 32]. Similarly, in the present study cytochrome P-448 activity was induced by all agents in the rat but 2-aminoanthracene had no such effect in the hamster; however, in all cases the extent of induction in the hamster was much less than in the rat. In contrast, the CD1 mouse, a strain associated with the Ah locus that encodes the receptor regulating cytochrome P-448 activity and therefore responsive to induction by 3-methylcholanthrene, showed induction of this activity only with 3-methylcholanthrene, while aminoanthracene and acridine orange had no effect and safrole even caused inhibition. The rat therefore appears to be the most sensitive, of the three species studied, to induction of cytochrome P-448.

The induction of cytochrome P-448 activity is not restricted to polycyclic aromatic hydrocarbons and polychlorinated biphenyls, as other chemical groups, such as aromatic amines [33], methylenedioxybenzene derivatives [34] and aromatic amides [35] also stimulate this activity at doses as low as 0.5 mg/kg. However, maximal induction occurs at doses around 2–5 mg/kg and it is possible that the inductive effect of a compound may be missed because of the use of an inappropriate dose regimen. For example, maximal induction of cytochrome P-448 activity by 2-acetylaminofluorene occurs at about 2 mg/kg and at higher doses the extent of induction is drastically decreased, (see Fig. 1) possibly because of the hepatotoxicity of the inducing agent. The induced activity, at least following treatment with benzo(a)pyrene, takes more than 4 days to return to control values.

In summary, in the present study we have demonstrated that (a) the liver is markedly more active than other tissues in the enzymic dealkylation of 7-ethoxyresorufin (b) the liver is the most responsive tissue to induction of cytochrome P-448 by some agents when compared with lung and kidney, (c) the rat appears to be markedly more sensitive to cytochrome P-448 inducers than the CD1 mouse or hamster, and (d) maximal induction of cytochrome P-448 activity by some agents may occur at relatively low doses of 2.5–5 mg/kg. Finally, this study confirms our previous findings [11] that the *O*-deethylation of ethoxyresorufin is a more sensitive index for cytochrome P-448 activity than the 2-hydroxylation of biphenyl.

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