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SHORT COMMUNICATIONS

Interaction of isosafrole *in vivo* with rat hepatic microsomal cytochrome P-450 following treatment with phenobarbitone or 20-methylcholanthrene

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Isosafrole [1,2-(methylenedioxy)-4-propenylbenzene] is both an inhibitor and inducer of hepatic cytochrome P-450 mediated monooxygenation. Inhibition by methylenedioxyphenyl compounds is of a dual nature with both competitive inhibition, and non-competitive inhibition associated with the formation of a methylenedioxyphenyl metabolite–cytochrome P-450 complex (which is manifested optically in the reduced form by absorption maxima at 427 and 455 nm) being observed [1, 2]. A similar interaction of isosafrole and safrole (4-allyl-1,2-methylenedioxybenzene) with hepatic microsomal cytochrome P-450 has been demonstrated after administration *in vivo* to rats [3, 4]. Isosafrole as an inducing agent has some properties in common with barbiturate and polycyclic aromatic hydrocarbon inducers. However, the full extent of its induction is not apparent until the isosafrole metabolite is displaced from the cytochrome P-450 [5]. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis, which has been used to examine multiplicity of cytochrome P-450 forms [6, 7], was used to demonstrate that pretreatment of rats with isosafrole results in the formation of a novel species of cytochrome P-450 with molecular weight intermediate between those induced by phenobarbitone and 20-methylcholanthrene [8]. In this paper the effects of combining isosafrole with phenobarbitone or 20-methylcholanthrene on the induction of hepatic cytochrome P-450 are examined.

Male Wistar albino rats (180–200 g) were pretreated with three daily i.p. doses of either corn oil (4 ml/kg), 20-methylcholanthrene (20 mg/kg) in corn oil (4 ml/kg), or phenobarbitone (80 mg/kg) in 0.9%(w/v) saline. This was followed by a single i.p. dose of isosafrole (150 mg/kg) in corn oil (2.5 ml/kg) on the fourth day. The animals were killed by cervical dislocation 24 hr after the last dose, the livers excised, and microsomes prepared by the method of Netter [9]. The microsomal fraction was suspended in 0.25 M sucrose, 5.4 mM EDTA, 20 mM Tris–HCl buffer (pH 7.4) and stored at −40° until required. Microsomal protein was measured by the method of Lowry *et al.* [10], using bovine serum albumin as standard.

Cytochrome P-450 was determined by the method of Omura and Sato [11]. The concentration of the 455 nm absorbing complex was determined by difference spectroscopy following reduction in the sample cuvette by addition of a few grains of dithionite [5].

Displacement of the metabolite complex was carried out by the addition of 2-n-heptylbenzimidazole (200 μM), a compound with optimal displacing properties [12], to the microsomal suspension (1 mg microsomal protein/ml) in 66 mM Tris–HCl buffer (pH 7.4), followed by incubation at 37° for 20 min. Measurement of cytochrome P-450 and the metabolite complex were then made as described above. All spectral determinations were made using either a Perkin–Elmer 356 dual beam dual wavelength spectrophotometer, or a Varian–Carey 219 dual beam spectrophotometer.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was carried out by the discontinuous buffer system of Laemmli [13] using a slab gel apparatus with gel dimensions of 1.5 mm × 120 mm × 80 mm. Whole microsomes (1 mg/ml) were solubilized in 62.5 mM Tris–HCl buffer (pH 6.8), containing 15% (v/v) glycerol, 5% (v/v) mercaptoethanol, 2.3% (w/v) sodium dodecyl sulphate and 0.001% (w/v) bromophenol blue, and placed in a boiling water bath for three min prior to application to the gel. Up to 20 samples of 15 μg microsomal protein were applied to the gel, and electrophoresis carried out at 15 mA in the stacking gel, and 30 mA in the running gel. The gels were then stained for protein with Coomassie Blue. Molecular weight standardization was carried out by plotting $\log_{10}$ of molecular weight against mobility of six purified proteins: cytochrome *c*, chymotrypsinogen A, hen egg albumin, aldolase, catalase and bovine serum albumin.

Table 1. Effect of various pretreatment on levels of cytochrome P-450 and the isosafrole–metabolite complex before and after displacement

Treatment	$\Delta E_{427-490}$/mg protein	$\Delta E_{455-490}$/mg protein	Cytochrome P-450 (nmoles/mg protein)
Before displacement			
Corn oil–isosafrole	0.076 ± 0.002	0.028 ± 0.006	0.79 ± 0.08
Phenobarbitone–isosafrole	0.082 ± 0.012	0.026 ± 0.010	1.53 ± 0.07
20-Methylcholanthrene–isosafrole	0.086 ± 0.004	0.111 ± 0.014	1.43 ± 0.13
After displacement			
Corn oil–isosafrole	0.076 ± 0.002	0.028 ± 0.006	0.79 ± 0.08
Phenobarbitone–isosafrole	0.093 ± 0.002	0.006 ± 0.002	2.05 ± 0.07
20-Methylcholanthrene–isosafrole	0.096 ± 0.010	0.006 ± 0.004	2.74 ± 0.30

Values represent the mean ± standard deviation of three animals per group. Dissociation of the metabolite complex was effected by the addition of 2-n-heptylbenzimidazole (200 μM) to 5 ml microsomal suspension (1 mg microsomal protein/ml) which were preincubated for 5 min at 37°. Incubation was continued for a further 20 min, the suspension was divided equally between two cuvettes and spectra were recorded as described.

The results in Table 1 indicate that incubation of microsomes with 2-n-heptylbenzimidazole causes a decrease in the 455 nm peak and an increase in the amount of cytochrome P-450 measured by the reduced carbon monoxide spectrum. The 427 nm peak, which is thought to be due to a metabolite complex of greater stability than the 455 nm complex, is slightly increased after displacement of the 455 nm complex. The reason for this is under investigation. Methylcholanthrene–isosafrole animal pretreatment produces a considerably greater induction of cytochrome P-450 than phenobarbitone–isosafrole. Pretreatment with 20-methylcholanthrene–isosafrole also produces the largest proportion of the total cytochrome P-450 present as the 455 nm complex (47.8 per cent), compared with phenobarbitone–isosafrole (25.3 per cent) and oil–isosafrole (44.4 per cent). These results suggest that a form of cytochrome P-450 which is able either to produce and bind, or to bind the metabolite formed by other P-450 species, is strongly induced by 20-methylcholanthrene–isosafrole, but to a lesser extent by phenobarbitone–isosafrole.

Use of sodium dodecyl sulphate–polyacrylamide gel electrophoresis has shown that isosafrole apparently induces a protein which differs from those induced by either phenobarbitone (mol. wt 50,000) or 20-methylcholanthrene (mol. wt 55,000). Pretreatment with oil–isosafrole induces this novel protein which has a molecular weight of 53,000 (see Fig. 1). Pretreatment with phenobarbitone–isosafrole produces an additive effect in that it induces both the characteristic phenobarbitone (mol. wt 50,000) and isosafrole (mol. wt 53,000) bands. However, 20-methylcholanthrene–isosafrole produces a decrease in the amount of the typical 20-methylcholanthrene 55,000 mol. wt band, and a large increase in the 53,000 mol. wt band. This effect does not seem to be due to any direct action of isosafrole on the haemoproteins, since incubation of microsomes from 20-methylcholanthrene or phenobarbitone-pretreated animals with isosafrole and NADPH *in vitro* to produce the isosafrole–metabolite complex did not result in any change in the electrophoretic pattern.

The extent of formation of the displaceable isosafrole–metabolite complex *in vivo* seems to be related to the extent of induction of the 53,000 mol. wt band. Phenobarbitone–isosafrole and oil–isosafrole have similar amounts of complexed cytochrome P-450 (0.63 and 0.52 nmoles/mg protein respectively), and produce similar amounts of the 53,000 mol. wt band, whereas 20-methylcholanthrene–isosafrole (with 1.31 nmoles cytochrome P-450 complexed/mg protein) produced the largest increase in the 53,000 mol. wt band. The magnitude of the increase in amount of total cytochrome P-450 following 20-methylcholanthrene–isosafrole treatment compared to 20-methylcholanthrene pretreatment alone (2.74 nmoles/mg compared with 1.3–1.4 nmoles/mg protein) suggests that the isosafrole induced band may be the same gene product as the minor band induced by 20-methylcholanthrene.

Similar experiments have been carried out on the formation of metabolite complexes following the administration of SKF-525A (β-diethylaminoethyl diphenylpropylacetate) to phenobarbitone and 20-methylcholanthrene-treated rats [14]. The phenobarbitone–SKF-525A treatment produced an increase in cytochrome P-450 levels and 41 per cent of the haemoprotein was in the complexed form. Animals receiving SKF-525A alone had 33 per cent of the haemoprotein as complex whereas 20-methylcholanthrene–SKF-525A treatment produced 3 per cent of the total cytochrome P-450 as complex. SKF-525A induces a protein with the same molecular weight as that induced by phenobarbitone as measured by sodium dodecylsulphate–polyacrylamide gel electrophoresis (T. Fennell, unpublished observation). These results suggest that the subpopulation of cytochrome P-450 which forms the *in vivo* metabolic complex with SKF-525A is not the same species which forms the *in vivo* isosafrole metabolite–cytochrome P-450 complex, and therefore at least two different species of haemoprotein are capable of forming metabolite complexes *in vivo*.

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REFERENCES

1. M. R. Franklin, *Biochem. Pharmac.* **21**, 3287 (1972).
2. R. M. Philpot and E. Hodgson, *Molec. Pharmac.* **8**, 204 (1972).
3. M. R. Franklin, *Xenobiotica* **1**, 581 (1971).
4. D. V. Parke and H. Rahman, *Biochem. J.* **123**, 9P (1971).
5. C. R. Elcombe, J. W. Bridges, T. J. B. Gray, R. H. Nimmo-Smith and K. J. Netter, *Biochem. Pharmac.* **24**, 1427 (1975).
6. A. P. Alvares and P. Siekevitz, *Biochem. biophys. Res. Commun.* **54**, 923 (1973).
7. A. F. Welton and S. D. Aust, *Biochem. biophys. Res. Commun.* **56**, 898 (1974).

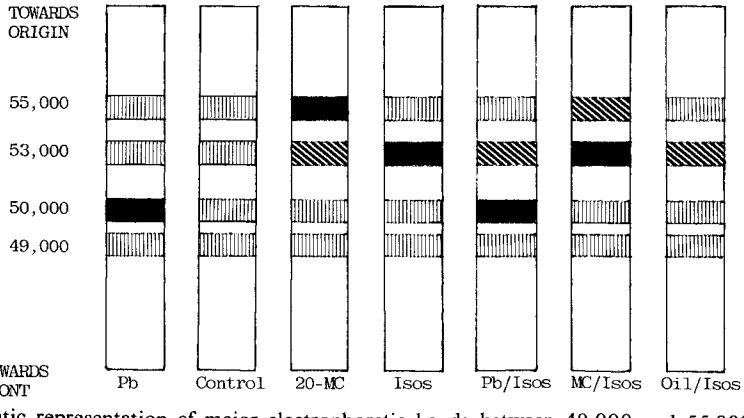

Fig. 1. Diagramatic representation of major electrophoretic bands between 49,000 and 55,000 mol. wt following treatment with inducing agents. The solid, diagonally and vertically lined regions represent decreasing intensities of protein staining. The pretreatments used were: phenobarbitone (Pb), 3 doses of 80 mg/kg; 20-methylcholanthrene (20-MC), 3 doses of 20 mg/kg; isosafrole (Isos), 3 doses of 150 mg/kg; phenobarbitone followed by isosafrole (Pb/Isos); 20-methylcholanthrene followed by isosafrole (MC/Isos); corn oil followed by isosafrole (Oil/Isos).

8. M. Dickins, J. W. Bridges, C. R. Elcombe and K. J. Netter, *Biochem. biophys. Res. Commun.* **80**, 89 (1978).
9. K. J. Netter, *Naunyn-Schmiedeberg's Arch. Pharmak.* **238**, 292 (1960).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randal, *J. biol. Chem.* **193**, 265 (1951).
11. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
12. M. Dickins, C. R. Elcombe, S. J. Moloney, K. J. Netter and J. W. Bridges, *Biochem. Pharmac.* **28**, 231 (1979).
13. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
14. M. K. Buening and M. R. Franklin, *Drug Metab. Dispos.* **4**, 244 (1976).

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Long term induction of microsomal drug oxidizing system in mice following prenatal exposure to barbiturate

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Rodent neonates have a poor ability to metabolize many drugs including barbiturates [1, 2]. Hart *et al.* [3] have demonstrated that following phenobarbital administration to pregnant female rabbits, the fetuses and the neonates showed microsomal drug oxidizing system activities similar to those found in adults. The increased enzyme activity was accompanied by an increased ability to tolerate and metabolize barbiturates [4]. These studies included only the short term effects and the possibility that the metabolic changes were long lasting was not investigated. This is perhaps since biochemical events resulting from administration of sedative hypnotics to adults are mostly transient. This fact is particularly true for the induction of the microsomal drug oxidizing system [5]. Recently, however, it has been shown that the outcome of prenatal administration of sedative hypnotics lasts into adulthood. Thus, early administration of ethanol and barbiturate induced long lasting neural [6, 7] and behavioral changes in mice [8–10]. Most pertinent, mice which received ethanol prenatally showed a higher activity of hepatic alcohol dehydrogenase (ADH) and microsomal ethanol oxidizing system (MEOS) at adulthood [11]. Consequently, the present experiment was conducted to study the long lasting effects of prenatal administration of a barbiturate on the activity of the microsomal drug oxidizing system in adulthood.

HS mice were used. This strain was derived through crosses of eight inbred strains and was deliberately maintained genetically heterogeneous for many generations [12]. Adult animals which were used as parents in the present experiment were housed in mating groups of two males and five females and maintained under standard laboratory conditions. Females were checked daily at 0800 and those that conceived, as evidenced by the existence of a vaginal plug, were separated from the males and housed with other pregnant females. Phenobarbital (PhB, acid form) administration commenced on gestation day 6 (GD6; the day in which the plug was found was considered GD1) and lasted until parturition (P). The females were then housed in individual cages. From GD6 to P, treated females received milled food (Ralston Purina, St. Louis, MO) containing PhB as their only food source and water, both available *ad libitum*; control females received milled food and water. Dose schedule was as follows: GD 6–9, 0.526 g PhB/kg food; GD 9–18, 3 g/kg; GD 18–P, 1 g/kg. Blood PhB levels were monitored on a sample group on GD 8, 10, 12, 15, 18, and 20. Thus, levels from all periods and doses were studied. The tail blood (100 μl) was taken at 0900, suspended in 50 μl 1M H_3PO_4, the PhB extracted into 5 ml toluene and assayed by gas chromatography on OV 17, after being derivatized on column with 20 μl of tetramethylammonium hydroxide. This method is described elsewhere [13].

In order to control for the possible carry-over effect of PhB on maternal milk production or behavior, half of the offspring born to mothers who received PhB during pregnancy (B) were fostered by lactating control females (C) within 12 hr post partum. Half of the C litters were also fostered by C mothers other than their own. Since preliminary analysis revealed no such carry-over effects on any of the variables studied, the results of the fostered and the non-fostered offspring were pooled. The male offspring which were the subject of the present experiment were weaned on day 23. They were tested for enzyme activity on days 45 to 50. The animals were weighed and decapitated and the livers quickly removed. The activity of the microsomal drug oxidizing system was determined in a supernatant fraction which contained the microsomes and soluble proteins according to the method described by Zannoni [14]. Briefly, livers were washed in 0.25 M ice cold sucrose solution, blotted and weighed. A 20% (w/v) homogenate was prepared with the sucrose in a glass Potter–Elvehjem homogenizing tube and centrifuged at 15,000 *g* for 30 min at 5°. The supernatant (0.3 ml) was added to a 2.2 ml mixture of 6.0 μmoles nicotinamide, 10 μmoles glucose-6-PO_4, 3.0 μmoles *p*-nitroanisol and $MgCl_2$ all made up in 0.07 M monophosphate buffer pH 7.8 and incubated at 37°. The reaction was initiated with the addition of 3 μmoles NADP at 37°. The activity of the enzymes, as indicated by the formation of *p*-nitrophenol, which is the demethylated product of *p*-nitroanisol, was followed spectrophotometrically for 8 min at 420 nm. The protein content of the supernatant was assayed according to the method of Lowry *et*

Table 1. The activity of microsomal drug metabolizing system and seminal vesicle weight in control offspring and offspring whose mother consumed barbiturate during pregnancy

Prenatal treatment	Enzyme activity (ΔOD.10^3/min/mg protein)	Seminal vesicle weight (mg)
Control	2.92 ± 0.24 (11)	98.9 ± 4.6 (14)
Barbiturate	3.84 ± 0.19* (10)	81.6 ± 3.6* (15)

Figures in parentheses represent sample sizes.
* $P < 0.01$, *t* test [16].