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THE *IN VIVO* INDUCTION OF RAT HEPATIC CYTOCHROME P450-DEPENDENT ENZYME ACTIVITIES AND THEIR MAINTENANCE IN CULTURE

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Abstract—Cytochrome P450-dependent enzyme activities were measured in hepatocytes from adult male rats, induced *in vivo* with phenobarbitone, β-naphthoflavone, dexamethasone or isoniazid; the stability of the induced activities in culture was also determined. Each inducer produced a characteristically different pattern of enzyme activities with dexamethasone, isoniazid and β-naphthoflavone selectively inducing erythromycin *N*-demethylase, *p*-nitrophenol hydroxylase and ethoxyresorufin *O*-dealkylase respectively. In general, the induced activities were maintained for 24 hr in culture. This indicates the feasibility of an *in vivo* induction-hepatocyte culture system for the study of metabolism-mediated toxicity.

Cytochromes P450 constitute a superfamily of isozymic haemoproteins involved in the metabolism of a wide range of endogenous and xenobiotic compounds, many of which also induce P450 [1]. Induction of specific isozymic forms can have profound effects on the metabolism and toxicity of xenobiotics. In general, isozymes from different gene families and subfamilies, as classified by Nebert *et al.* [2], are induced by different classes of compound, and also have distinct substrate specificity patterns. By measuring enzyme activities selective for different isozymes, an activity/isozyme profile for each inducer can be obtained, as has been attempted, for example, using stereospecific hydroxylation products of testosterone [3], and the dealkylation of a series of alkoxyresorufins [4].

Primary cultures of rat hepatocytes are now widely used as a model for the study of metabolism-mediated toxicity, and although a range of P450 activities can be maintained in such cultures [5, 6] marked alterations in the inducibility and regulation of P450 occur in culture, especially with respect to the phenobarbitone-inducible forms [5, 7, 8]. This phenotypic abnormality limits the use of hepatocyte cultures for studies of xenobiotic metabolism and toxicity. One possible alternative approach is induction *in vivo* prior to culture of the hepatocytes. Such an approach has recently been demonstrated to increase the sensitivity of cultured hepatocytes as an activation system for the *in vitro* mouse lymphoma mutagenesis assay [9]. However, there appears to be little information available on the stability of the induced isozymes under culture conditions.

Accordingly, in this study we have determined (i) the induction of different enzyme activities in hepatocytes isolated from adult male rats treated with prototypic inducers of the main P450 gene families, using substrates considered to be selective for the appropriate isozyme, and (ii) the stability of these activities over 24 hr in culture. From these data an attempt has been made to derive activity profiles for each type of inducer. The inducers studied comprised phenobarbitone (PB†) and isoniazid (ISO) which induce the P450IIB/IIE subfamilies respectively [1, 10], dexamethasone (DEX) which induces the P450III family [11], and β-naphthoflavone (BNF) which induces P450 IA1 and IA2 [1].

MATERIALS AND METHODS

Animals. The source and maintenance of the Wistar rats used in this study have been described previously [12]. Adult male rats (6–8-weeks-old) were treated i.p. with dexamethasone sodium phosphate (100 mg/kg in saline) for 4 days or BNF (80 mg/kg in arachis oil) for 3 days. PB and ISO (0.1%) were administered in drinking water for 5 and 10 days, respectively.

Materials. William's E medium, foetal calf serum and glutamine were obtained from Flow Laboratories (Irvine, U.K.). Insulin (Actrapid MC) was obtained from Novo Industri A/S (Denmark). Dexamethasone sodium phosphate (Decadron) was purchased from Merck, Sharp and Dohme Ltd (Hoddesdon, U.K.). Fungizone was obtained from Squibb Surgicare Ltd (Hounslow, U.K.) and gentamicin from Roussel Laboratories Ltd (Uxbridge, U.K.). Benzphetamine, erythromycin, *p*-nitrophenol and all enzymes and cofactors were obtained from Sigma Chemical Co. (Poole, U.K.). The 7-ethoxyresorufin was prepared by the method of Prough *et al.* [13]. Plasticware was obtained from Falcon (Becton Dickinson. U.K. Ltd, Oxford, U.K.).

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† Abbreviations: BNF, β-naphthoflavone; BZDM, benzphetamine *N*-demethylase; DEX, dexamethasone; EMDM, erythromycin *N*-demethylase; EROD, ethoxyresorufin *O*-deethylase; ISO, isoniazid, PB, phenobarbitone, PCN, pregnenolone 16α-carbonitrile, PNPH, *p*-nitrophenol hydroxylase; RA, relative activity, RSA, relative specific activity.

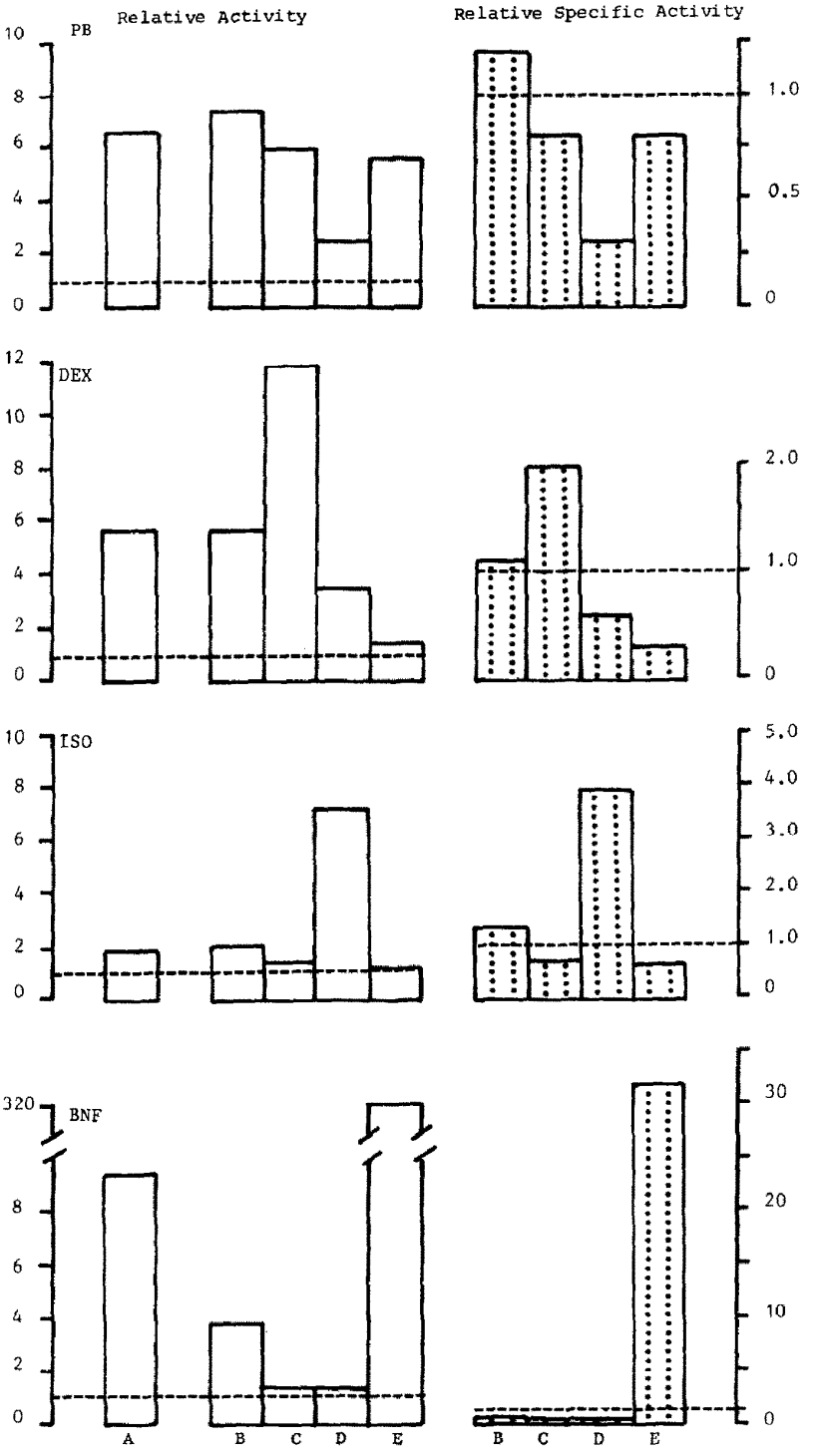

Fig. 1. Induction of P450 and enzyme activities relative to control. Each value is the mean activity (open histogram) or mean specific activity (speckled histogram) measured in hepatocytes from the treated animals relative to that of the untreated animals (dotted line). (A) P450; (B) BZDM; (C) EMDM; (D) PNPH; (E) EROD.

Hepatocyte isolation and culture. Hepatocytes were isolated from untreated and inducer-treated adult male rats by lobe perfusion, essentially as described by Reese and Byard [14]. Half of the cell suspension was sonicated to give an homogenate of fresh cells [5, 15]; the other half was plated out in WEC medium (Williams E containing 5 mM glutamine, 2.5 μg/mL fungizone, 50 μg/mL gentamicin,

Table 1. P450 content and enzyme activities measured in homogenates of freshly isolated hepatocytes from untreated and inducer treated male rats

Parameter	Treatment of animals				
	CON	PB	DEX	ISO	BNF
P450 content (nmol/mg protein)	0.16 ± 0.02	1.05 ± 0.11*	0.90 ± 0.13*	0.29 ± 0.04	1.50 ± 0.39*
BZDM (nmol prod/min/mg)	0.34 ± 0.04	2.50 ± 0.30*	1.90 ± 0.20*	0.69 ± 0.05	1.30 ± 0.30* (5)
EMDM (nmol prod/min/mg)	0.17 ± 0.01	1.00 ± 0.06*	2.00 ± 0.02*	0.23 ± 0.02	0.23 ± 0.09 (3)
PNPH (nmol prod/min/mg)	0.24 ± 0.04	0.58 ± 0.05*	0.81 ± 0.07*(5)	1.70 ± 0.10*	0.30 ± 0.09
EROD (pmol prod/min/mg)	5.0 ± 0.3	28 ± 5	7.4 ± 0.5 (5)	5.6 ± 0.6	1500 ± 400*

Values are mean ± SE (N = 6 animals unless indicated otherwise).
* Significantly different to control, $P < 0.05$ (ANOVA + Dunnett's test).

10% (v/v) foetal calf serum, 10 mUnits/mL insulin, 5 mM nicotinamide and 1 μM dexamethasone) at 2–2.5 × 10^6 cells/plate on 60 mm diameter "Primaria" dishes. The medium was replaced after allowing 2 hr for attachment. After 24 hr, the cells were washed with saline and homogenates prepared by sonication.

Analyses. For the four enzyme assays, samples of homogenate (0.5–1.0 mg protein/mL incubation mix) were incubated in a system similar to that described previously [16]. Ethoxyresorufin deethylase (EROD) activity was measured by the method of Pohl and Fouts [17] as described by Warren and Fry [5]. Nitrophenol hydroxylase (PNPH) and benzphetamine and erythromycin *N*-demethylases (BZDM, EMDM) were assayed by a 30 min incubation at 37° with substrate (100 μM final concentration, nitrophenol; 50 μM final concentration benzphetamine and erythromycin, with 10 mM semicarbazide). Reactions were stopped with 0.1 mL 15% TCA. Nitrocatechol production from nitrophenol was measured spectrophotometrically at 546 nm after addition of 0.1 mL 10 N NaOH to 1 mL protein-free supernatant [18]. Formaldehyde production from benzphetamine and erythromycin was determined using the Nash reagent. After a 50-min incubation of 1 mL protein-free supernatant and 1 mL Nash reagent at 37°, the formaldehyde concentration was measured spectrophotometrically or fluorometrically [19, 20]. Preliminary experiments showed that these reactions proceeded linearly with respect to time and protein concentration in homogenates from untreated and pre-treated animals. Cytochrome P450 content was determined using the method of Omura and Sato [21], and protein by the method of Bradford [22].

Presentation of results and statistical analysis. Enzyme activities were calculated as nmol or pmol product/min/mg protein (the "activity") and as nmol or pmol product/min/nmol P450 (the "specific activity"). Induction of activity relative to control indicated the actual change in activity whereas changes in specific activity relative to control indicated the involvement of induced P450 isozymes in the activity. Statistical analysis was by ANOVA and Dunnett's tests for the induction of fresh activity relative to control, and by use of paired *t*-tests for the maintenance data.

RESULTS

Induction of enzyme activities

Table 1 shows the P450 content and enzyme activities measured in homogenates from hepatocytes isolated from untreated and treated adult male rats.

P450 content and BZDM were both significantly increased by PB, DEX and BNF but not by ISO. EMDM was increased by PB and DEX; PNPH was induced by PB, DEX and ISO. EROD was significantly induced only by BNF. The 5.6-fold induction of EROD by PB was not significant; this was probably because the 320-fold induction of EROD by BNF, being so much greater than that by PB, weighted the Dunnett's test (by yielding a very high estimate of the pooled standard deviation).

Induction relative to control

Figure 1 shows the changes in P450 content, activity and specific activity relative to that measured in untreated rats (control = 1.0).

PB induced BZDM, EMDM and EROD activities to the same extent as the P450 content (7.4-, 5.9-, 5.6- and 6.6-fold, respectively), and accordingly the relative specific activities (RSA) of these enzymes approximated to 1.0 (1.3, 0.8 and 0.8). In contrast, PNPH activity was induced 2.4-fold by PB but the RSA was only 0.3. The demethylase results agreed with microsomal data for the PB-inducible aminopyrine demethylase, in that the activity was induced to the same extent as the P450 content [12]. Also, the induction of PNPH and EROD determined here from whole cell homogenates were similar to the inductions of the same activities determined in microsomes (1.7-fold induction of PNPH [18] and 6-fold inductions of EROD [4]).

DEX induced P450 and BZDM activity 5.6-fold, the RSA of BZDM being 1.1, as with PB induction. EMDM activity was induced 11.8-fold, whereas the RSA was 2.0. Thus, only half of the observed induction of activity could be attributed to the increase in total P450. However, this was the only specific activity measured that was selectively induced by DEX; the RSAs of PNPH and EROD were less than 0.6.

Only PNPH activity was increased by ISO (7.4-fold, similar to the 6.1-fold increase observed in

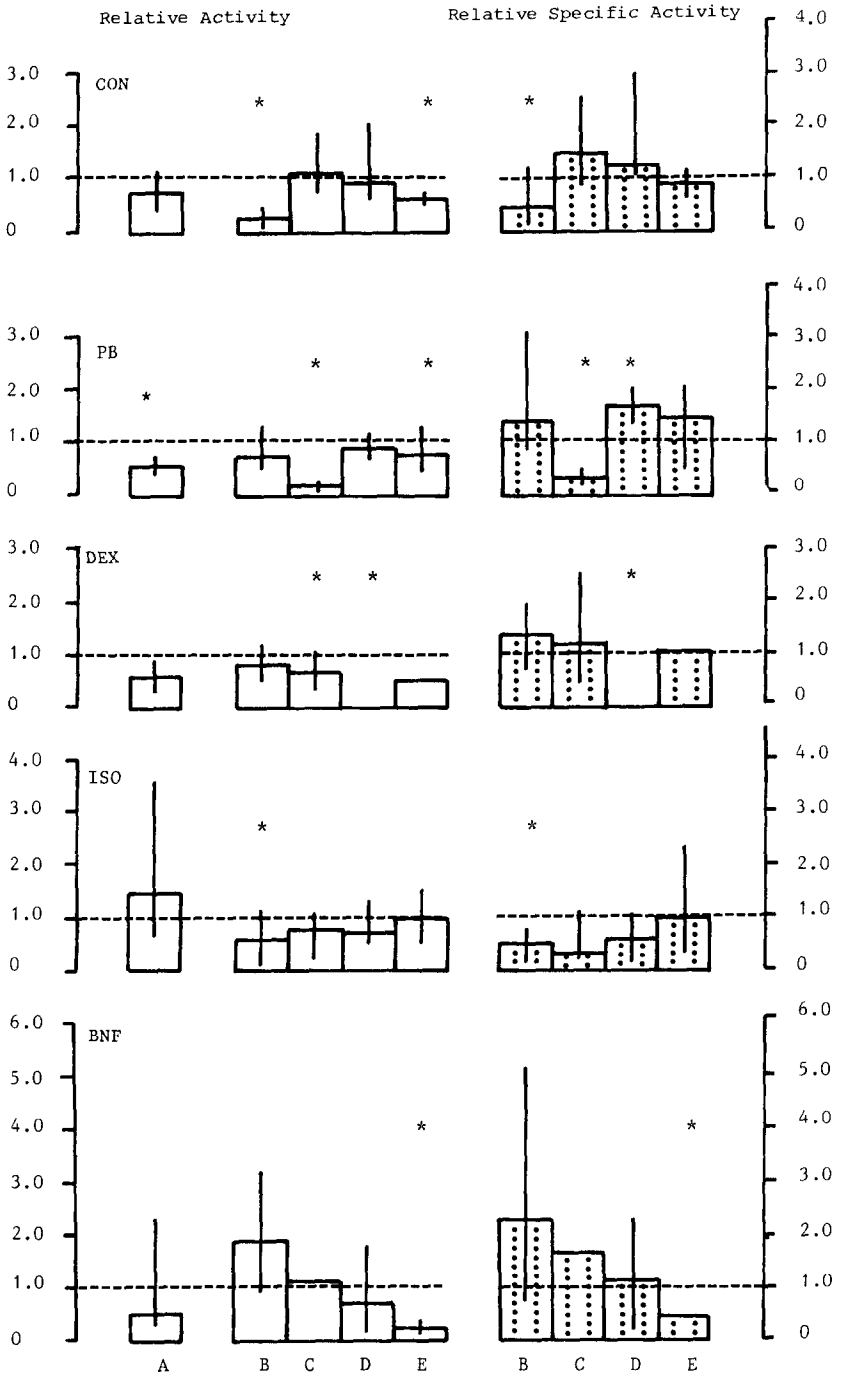

Fig. 2. Maintenance of P450 and enzyme activities after 24 hr in culture. Mean ± range of activities (open bars) and specific activities (speckled bars) after 24 hr in culture relative to the fresh cell values (dotted line = 1.0). * $P < 0.05$ (paired t-test). (A) P450; (B) BZDM; (C) EMDM; (D) PNPH; (E) EROD.

microsomes from ethanol-fed rats [18]). The RSA was 3.9, indicating selective induction of this activity, and again, not all of the increase in activity could be accounted for by the P450 content, which did not increase significantly after ISO pretreatment.

EROD was selectively induced by BNF (relative activity 320; RSA 32), and again, this was much greater than the observed induction of P450 (9.4-fold). The RSAs of the other enzymes were less than 0.6; this suggested that the isozymes induced by BNF are not involved in BZDM, EMDM or PNPH activity.

Thus, BNF selectively induced EROD, ISO selectively induced PNPH and DEX selectively induced

EMDM, whereas PB induced the three demethylase activities to more or less the same level.

Maintenance of activities in culture

The maintenance of the activities after 24 hr in culture, in hepatocytes from untreated and treated animals, is shown in Fig. 2, the data being presented both as activity at 24 hr relative to that of fresh cells and as relative specific activity.

EMDM and PNPH activities were maintained in culture from untreated rats, but BZDM and EROD activities were significantly reduced. P450 content fell by 31%, as a consequence of which there was a selective loss of specific activity due to BZDM. The loss of EROD in culture has been reported previously [15].

BZDM and PNPH activities were maintained in cultures obtained from PB-treated rats in the face of significant reductions in P450 content and EMDM and EROD activities. The specific activity of EMDM was decreased whilst that of PNPH was increased.

PNPH activity could not be detected following 24 hr culture of hepatocytes from DEX-treated rats. P450 content fell by 43%, and the specific activities of the three demethylases were maintained.

P450 content was maintained in hepatocyte cultures obtained from ISO-treated rats, this being accompanied by a selective decrease in BZDM activity.

BZDM, EMDM and PNPH activities were maintained in cultures obtained from BNF-treated rats, which was accompanied by losses in P450 content and EROD activity.

DISCUSSION

Each inducer produced an unique profile in terms of P450 content and relative enzyme activity. Thus, PB produced inductions of 5.5–7.5-fold in P450 content and BZDM/EMDM/EROD activities with a lesser induction of PNPH. DEX produced inductions of similar magnitude in P450 content and BZDM/PNPH activities with a greater (12-fold) induction of EMDM activity. ISO produced a selective induction of PNPH activity, whilst BNF produced a marked selective induction of EROD activity with a lesser induction of P450 content. These data are consistent with results of previous studies which have demonstrated BZDM activity to be principally mediated by P450IIB [23], EMDM activity by P450 III [24], PNPH activity by P450IIE [18] and EROD activity by P450IA1 [4], although, to our knowledge, a comprehensive induction/activity profile such as that determined in this study has not been presented previously. Unique induction profiles were also obtained if the data were expressed in terms of relative specific activity. The apparent discrepancies between induction of activity and of specific activity in some cases (eg. PB and ISO inductions of PNPH), reflects the multiplicity of the P450 system with respect to substrate specificity and isozymic content. Induction of high affinity forms and/or induction of relatively minor isozymes, constituting a small proportion of the total P450, would account for the increases in relative activity that are accompanied by much lower relative specific activities.

There was some overlap in the induction profiles obtained with PB and DEX. It has recently been demonstrated that DEX, but not pregnenolone-16α-carbonitrile (PCN), induced the main PB-inducible forms in addition to the P450III proteins [24], and also that PB can induce the proteins of the P450III family [1]. These findings probably explain the similar magnitudes of induction of BZDM and EMDM by PB and DEX.

The profile of maintenance of P450 content and relative (specific) enzyme activity also varied between inducers (and the control state), with no consistent pattern emerging. BZDM activity induced by PB and DEX was maintained in culture for 24 hr whereas the constitutive activity and that in cells cultured from ISO-treated rats was much less stable. In contrast, the EMDM activity induced by PB and DEX was significantly reduced following culture whereas the constitutive form was stable, this being consistent with the rapid degradation of P450 forms induced by DEX [25]. EROD activity induced by BNF was markedly reduced following culture. Nevertheless, the activity of the principal inducible forms following induction *in vivo* with the appropriate inducer and culture of hepatocytes isolated therefrom was in each case greater than that measured in the hepatocytes isolated and cultured from untreated rats. By this criterion, the inducer profiles were maintained on culturing the hepatocytes, which, in turn, suggests the feasibility of an *in vivo* induction — *in vitro* toxin exposure protocol for study of metabolism-mediated cytotoxicity. Preliminary experiments (Hammond, unpublished) have confirmed this assertion.

In conclusion, unique induction profiles were obtained when liver cell homogenates were assayed for P450 content and four commonly-used selective enzyme activities, for each of the prototype inducers of the principal inducible P450 forms. These induction profiles were generally well maintained during culture for 24 hr of hepatocytes isolated from appropriately-treated animals.

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REFERENCES

1. Nebert DW and Gonzalez FJ, P450 genes: structure, evolution and regulation. *Annu Rev Biochem* **56**: 945–993, 1987.
2. Nebert DW, Nelson DR, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato R and Waterman MR, The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* **8**: 1–13, 1989.
3. Darby NJ, Lodola A and Burnet F, Testosterone metabolite profile differences in the spectrum of cytochrome P450 isozymes induced by phenobarbitone, 2-acetylaminofluorene and 3-methylcholanthrene in the chick embryo liver. Biochem Pharmacol **35**: 4073–4076, 1986.
4. Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of

substrates to distinguish between different induced cytochromes P450. *Biochem Pharmacol* **34**: 3337–3345, 1985.
5. Warren M and Fry JR, Influence of medium composition on 7-alkoxycoumarin *O*-dealkylase activities of rat hepatocytes in primary maintenance culture. *Xenobiotica* **18**: 973–981, 1988.
6. Hammond AH and Fry JR, The influence of donor age and sex on the activity, and maintenance in culture, of 7-alkoxycoumarin *O*-dealkylases of rat isolated hepatocytes. *In Vitro Toxicology* in press.
7. Miyazaki M, Handa Y, Oda M, Yabe T, Miyano K and Sato J, Long-term survival of functional hepatocytes from adult rat in the presence of phenobarbital in primary culture. *Exp Cell Res* **159**: 176–190, 1985.
8. Turner NA, Wilson NM, Jefcoate CR and Pitot HC, The expression and metabolic activity of cytochrome P450 isozymes in control and phenobarbital-induced primary cultures of rat hepatocytes. *Arch Biochem Biophys* **263**: 204–215, 1988.
9. Oglesby LA, Brock KH and Moore MM, Induced hepatocytes as a metabolic activation system for the mouse lymphoma assay. *Mutation Res* **223**: 295–302, 1989.
10. Ryan DE, Koop DR, Thomas PE, Coon MJ and Levin W, Evidence that isoniazid and ethanol induce the same microsomal cytochrome P450 in rat liver, an isozyme homologous to rat liver cytochrome P450 isozyme 3a. *Arch Biochem Biophys* **246**: 633–644, 1986.
11. Hardwick JP, Gonzalez FJ and Kasper CB, Cloning of DNA complementary to cytochrome P450 induced by pregnenolone-16α-carbonitrile. *J Biol Chem* **258**: 10182–10186, 1983.
12. Fry JR, A comparison of biphenyl 4-hydroxylation and 4-methoxybiphenyl O-demethylation in rat liver microsomes. *Biochem Pharmacol* **30**: 1915–1919, 1981.
13. Prough RA, Burke MD and Mayer RT, Direct fluorometric methods for measuring mixed function oxidase activity. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol. 52, pp. 372–377. Academic Press, New York, 1978.
14. Reese JA and Byard JL, Isolation and culture of adult hepatocytes from liver biopsies. *In Vitro* **17**: 935–940, 1981.
15. Grant MH, Melvin MAL, Shaw P, Melvin WT and Burke MD, Studies on the maintenance of cytochromes P450 and b_5, monooxygenases and cytochrome reductases in primary cultures of rat hepatocytes. *FEBS Letts* **190**: 99–103, 1985.
16. Paterson P, Fry JR and Horner SA, Influence of cytochrome P450-type on the pattern of conjugation of 7-hydroxycoumarin generated from alkoxycoumarins. *Xenobiotica* **14**: 849–859, 1984.
17. Pohl RJ and Fouts JR, A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal Biochem* **107**: 150–155, 1980.
18. Reinke LA and Moyer MJ, *p*-Nitrophenol hydroxylation. A microsomal oxidation which is highly inducible by ethanol. *Drug Metab Disp* **13**: 548–552, 1985.
19. Nash T, The colourimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* **55**: 416–421, 1953.
20. Belman S, The fluorometric determination of formaldehyde. *Anal Chim Acta* **29**: 120–126, 1963.
21. Omura T and Sato R, The carbon monoxide binding pigment of liver microsomes. 1. Evidence for its haemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
22. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Anal Biochem* **72**: 248–254, 1976.
23. Ryan DE, Thomas PE, Korzeinowski D and Levin W, Separation and characterisation of highly purified forms of liver microsomal cytochrome P450 from rats treated with polychlorinated biphenyls, phenobarbital and 3-methylcholanthrene. *J Biol Chem* **254**: 1365–1374, 1979.
24. Namkung MJ, Yang HL, Hulla JE and Juchau MR, On the substrate specificity of cytochrome P450IIIA1. *Mol Pharmacol* **34**: 628–637, 1988.
25. Watkins PB, Wrighton SA, Schuetz EG, Maurel P and Guzelian PS, Macrolide antibiotics inhibit the degradation of the glucocorticoid-responsive cytochrome P450p in rat hepatocytes *in vivo* and in primary monolayer culture. *J Biol Chem* **261**: 6264–6271, 1986.