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Induction of cytochrome *P*-450 in cultured rat hepatocytes

The heterogeneous localization of specific isoenzymes using immunocytochemistry

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Primary cultures of rat hepatocytes were exposed to phenobarbitone, clofibric acid, β-naphthoflavone, isosafrole or dexamethasone for 3 days, and the induction of several cytochrome *P*-450 isoenzymes was demonstrated by increased catalytic activity, by Western blotting and by immunocytochemistry. The profiles of isoenzymes induced *in vitro* were compared with those induced in liver microsomes of rats dosed with the same agents. Clofibric acid, an agent which has not been thoroughly investigated previously, was shown to induce both *in vivo* and *in vitro* several *P*-450 isoenzymes normally inducible by phenobarbitone (PB_{1a}, PB_{3a} and PB_{3b}) or steroids (PB_{2c}). Immunocytochemical studies demonstrated that the inducible isoenzymes of cytochrome *P*-450 are not distributed evenly throughout the hepatocyte population, and increasing concentrations of phenobarbitone or β-naphthoflavone in the medium results in an increasing proportion of 'induced' cells. However, whereas maximal concentrations of β-naphthoflavone resulted in virtually all cells containing induced levels of MC_{1b}, a maximal concentration of phenobarbitone resulted in only 30% of the cells containing induced levels of PB_{3a}/PB_{3b}. These results are discussed in relation to the heterogeneous distribution and induction of cytochrome *P*-450 in the intact liver.

INTRODUCTION

The mammalian liver contains high levels of the haemoprotein cytochrome *P*-450 and plays a major role in the detoxification of foreign compounds. Cytochrome *P*-450 is the key component of the microsomal monooxygenase system and is responsible for the oxidative metabolism of many xenobiotics, as well as certain endogenous substrates [1,2]. Cytochrome *P*-450 is not a single haemoprotein, but the name is used as a generic term which encompasses a large number of different isoenzymes, some of which are highly inducible by xenobiotics [3].

In the uninduced rodent liver, the concentration of most cytochrome *P*-450s is higher in the centrilobular hepatocytes than in the periportal hepatocytes [4–6]. Furthermore, isoenzymes of *P*-450 induced by barbiturates, for example, are selectively increased in the centrilobular region. The reason for this heterogeneous distribution of cytochrome *P*-450 in the liver is not known.

Recent studies have shown that rat hepatocytes maintained as primary monolayer cultures can be used to study the metabolism and mechanism of toxicity of xenobiotics [7]. Although cytochrome *P*-450 concentrations decline rapidly in hepatocytes maintained in this way, the levels can be induced by exposure to xenobiotics in the medium [8–10].

The aim of this study is to investigate the expression and distribution of different isoenzymes of cytochrome *P*-450 in cultured rat hepatocytes exposed to various xenobiotics. This information is discussed in relation to the heterogeneous distribution and regulation of cytochrome *P*-450 levels in the liver of the intact animal.

MATERIALS AND METHODS

Materials

Phenobarbitone (PB) sodium was obtained from BDH, Liverpool, U.K. Clofibric acid (CFA) and dexamethasone (DEX) were obtained from Sigma Chemical Company, Poole, Dorset, U.K. β-Naphthoflavone (BNF) was obtained from Aldrich Chemical Company, Gillingham, Dorset, U.K. Isosafrole (ISF) was obtained from Fluka Chemicals, Glossop, U.K. Leibowitz L15 medium, heat-inactivated foetal calf serum and tryptose phosphate broth were obtained from Flow Laboratories, Irvine, U.K. Collagenase, type 1, was obtained from BCL, Lewes, Sussex, U.K. Peroxidase-labelled goat anti-rabbit IgG was obtained from ICN Biomedicals Ltd, Buckingham, U.K. 3,3′-diaminobenzidine was obtained from Polysciences, Northampton, U.K. All other chemicals were of the highest available purity and were obtained from Sigma Chemical Company, BDH or Aldrich Chemical Company.

Purified rat liver *P*-450 isoenzymes and rabbit polyclonal antibodies were prepared as described previously [11–13]. The antibodies were raised to isoenzymes MC_{1a} and MC_{1b}, inducible by polycyclic aromatic hydrocarbons [12], isoenzymes PB_{1a} and PB_{3a}, inducible by phenobarbitone [13], and isoenzyme PB_{2c}, inducible by synthetic steroids and phenobarbitone [13]. For clari-

Abbreviations used: *P*-450, cytochrome *P*-450 enzyme; PB, phenobarbitone; CFA, clofibric acid; BNF, β-naphthoflavone, 3-MC, 3-methylcholanthrene; ISF, isosafrole; DEX, dexamethasone; ECOD, 7-ethoxycoumarin-*O*-de-ethylation; LAH, lauric acid hydroxylation; AE, aldrin epoxidation; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TBS, Tris-buffered saline.

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Table 1. Nomenclature of the rat liver *P*-450 isoenzymes

Wolf	Levin	Guengerich	Gene designation*
PB_{1a}	k	PB-C	*IIC6*
PB_{2c}	–	PCN-E	*IIIA1*
PB_{3a}	b	PB-B	*IIB1*
PB_{3b}	e	PB-D	*IIB2*
MC_{1a}	d	ISF-G	*IA2*
MC_{1b}	c	BNF-B	*IA1*

* See [31].

fication, the nomenclature adopted by other workers is included in Table 1.

Animals

All experiments were performed on adult male Alderley Park rats (Wistar derived) weighing 180–220 g.

Animal treatment and microsome preparation

Rats were given PB (80 mg/kg in saline), BNF (100 mg/kg in corn oil) and ISF (150 mg/kg in corn oil) by intraperitoneal injection, or CFA (250 mg/kg in corn oil) by gavage, each day for four consecutive days. Control animals received vehicle alone (5 ml/kg). At 24 h after the last dose, the animals were killed by cervical dislocation. For each treatment group the livers were removed and pooled, and a 20% homogenate was prepared in 0.25 M-sucrose / 5 mM-EDTA / 20 mM-Tris/HCl (pH 7.4) (SET buffer). Microsomal fractions were prepared by differential centrifugation as described previously [14].

Hepatocyte isolation and culture

Rats were killed by inhalation of excess diethyl ether. Hepatocytes were then isolated by perfusion of the liver *in situ* with collagenase and maintained as primary monolayer cultures in CL15 medium for up to 4 days as described by Mitchell and coworkers [15]. PB dissolved in 0.9% NaCl or BNF, CFA, ISF or DEX dissolved in dimethylformamide were added to the medium when it was changed each day. Unless stated otherwise, hepatocytes were exposed for 3 days to PB, CFA, BNF, ISF or DEX at concentrations of 2 mM, 1 mM, 20 μM, 50 μM and 1 μM respectively. For the dose–response experiments, the maximal concentration of BNF compatible with its solubility in the culture medium was 40 μM. The maximal 'tolerated' dose of PB in the culture medium was 2 mM. At this concentration the hepatocytes showed no signs of cytotoxicity such as blebbing and detachment.

Immunocytochemistry

The cell monolayer was washed thoroughly in saline and then incubated in 0.56% (w/v) KCl for 2 min. The monolayer was then fixed in methanol (4 min), washed in phosphate-buffered saline (PBS; 0.85% (w/v) NaCl/0.085% (w/v) Na_2HPO_4/0.054% (w/v) KH_2PO_4) (5 min) and washed twice in PBS containing 1% (w/v) bovine serum albumin (PBS/BSA). After preincubation in PBS/BSA containing a 1:30 dilution of non-immune swine serum (5 min), the monolayer was then incubated in PBS/BSA containing polyclonal antibody to *P*-450

Table 2. The effects of phenobarbitone, clofibric acid, β-naphthoflavone, isosafrole and dexamethasone on cytochrome *P*-450-mediated mono-oxygenase activities in cultured rat hepatocytes

Hepatocytes were isolated and cultured as described in the Materials and methods section. PB (2 mM), CFA (1 mM), BNF (20 μM), ISF (50 μM) or DEX (1 μM) were added to the medium 24, 48 and 72 h after seeding. At 96 h after seeding, the cells were harvested and ECOD, LAH and AE were determined in cell sonicates. Values are expressed as pmol of product formed·min^{-1}·mg of $protein^{-1}$ and are means ± S.D. ($n = 3$). *Significantly different from control, $P < 0.05$.

	Enzyme activity (pmol·min^{-1}·mg^{-1})		
Inducer	ECOD	LAH	AE
None	49±5	32±3	108±33
PB	280±10*	116±2*	97±12
CFA	340±8*	1358±97*	283±49*
BNF	329±25*	77±10*	64±19
ISF	848±22*	181±13*	28±4*
DEX	202±18*	107±16*	149±5

(2 h). The dilution of the polyclonal antibody varied between 1:200 and 1:1000 depending on the antibody titre. Unbound antibody was then removed by three washes in PBS/BSA (5 min each). The polyclonal antibody was detected by incubation in PBS/BSA containing horseradish-peroxidase-labelled goat anti-rabbit IgG (20 min). Unbound antibody was again removed by three washes in PBS/BSA (5 min each). Peroxidase activity was detected by incubation for 10 min with H_2O_2 (0.04%) and 3,3′-diaminobenzidine (0.5 mg/ml) or 3-amino-9-ethylcarbazole (0.2 mg/ml) in 0.05 M-Tris/HCl, pH 7.6, or 0.1 M-acetate buffer, pH 5.2, respectively. The monolayer was counterstained in Harris's haematoxylin (1 min) and examined by light microscopy.

Control incubations were performed by replacing the polyclonal antibody with non-immune rabbit serum.

Western immunoblotting

SDS/polyacrylamide-gel electrophoresis was performed according to the method of Laemmli [16]; 3% and 7.5% acrylamide was used in the stacking and resolving gels respectively, with 0.1% SDS. After separation, the proteins were transferred from the gels to nitrocellulose sheets according to the method of Towbin *et al.* [17]. For electrophoretic transfers, a current setting of 0.25 A was applied for 15 h at 7 °C. The Western blots were washed in Tris-buffered saline (TBS; 0.15 M-NaCl/20 mM-Tris base/HCl, pH 8.2) for 10 min. To block non-specific binding, the blots were treated for 1 h at 37 °C in TBS containing 5% (w/v) bovine serum albumin (TBS/5% BSA). The blots were then incubated at room temperature for 2 h in TBS containing 0.1% BSA, 1% (v/v) normal swine serum and the polyclonal antibody to a specific *P*-450 form at the same dilution used for immunocytochemistry. The blots were then washed 4 times with this TBS/0.1% BSA (15 min each wash) before incubating with a 1:100 dilution of horseradish-peroxidase-conjugated goat anti-rabbit IgG for 20 min at room temperature. After several 15 min washes in TBS/0.1% BSA, immunoreactive bands were revealed

Table 3. The profile of cytochrome *P*-450 isoenzymes in rat liver microsomes and cultured hepatocytes following exposure to various chemical inducers

Primary monolayer cultures were exposed to vehicle only, PB (2 mM), CFA (1 mM), BNF (20 μM), ISF (50 μM) or DEX (1 μM) for 3 days. Adult male rats were given four daily doses of vehicle only (5 ml/kg), PB (80 mg/kg), BNF (100 mg/kg), ISF (150 mg/kg) or CFA (250 mg/kg). Samples of hepatocyte sonicates (50 μg of protein), liver microsomes (3 μg of protein) and purified *P*-450 isoenzymes (0.8 pmol) were separated by SDS/polyacrylamide-gel electrophoresis and immunoblotting was used to detect specific *P*-450 isoenzymes as described in the Materials and methods section. Key: −, isoenzyme not detected; ±, isoenzyme weakly detected; +, isoenzyme easily detected; ++, isoenzyme strongly detected; +++, isoenzyme very strongly detected; ND, not determined.

		P-450 isoenzyme					
		PB_{1a}	PB_{2c}	PB_{3a}	PB_{3b}	MC_{1b}	MC_{1a}
Control	Hepatocytes	+	−	−	−	±	±
	Microsomes	++	+	−	±	−	−
PB	Hepatocytes	++	++	±	±	+	±
	Microsomes	+++	+++	+++	++	−	−
CFA	Hepatocytes	++	++	+++	+	+	+
	Microsomes	+++	+++	++	++	−	−
BNF	Hepatocytes	±	−	−	−	+++	±
	Microsomes	+	+	−	−	+++	+++
ISF	Hepatocytes	±	±	±	±	+++	+
	Microsomes	±	++	++	++	−	+++
DEX	Hepatocytes	±	+++	−	−	±	±
	Microsomes	ND	ND	ND	ND	ND	ND

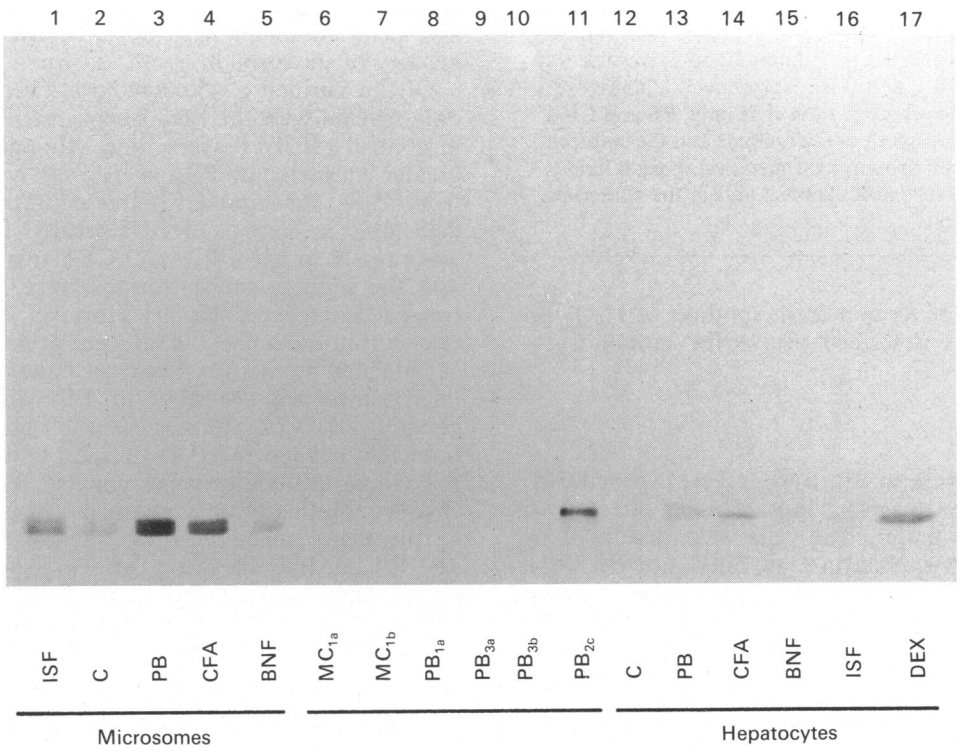


Fig. 1. Immunoblot of rat liver microsomes and cultured hepatocytes incubated with anti-PB_{2c}

Lanes 1–5, liver microsomes from rats treated with ISF, vehicle only, PB, CFA and BNF respectively; lanes 6–11, purified *P*-450 isoenzymes MC_{1a}, MC_{1b}, PB_{1a}, PB_{3a}, PB_{3b} and PB_{2c} respectively; lanes 12–17, cultured hepatocytes exposed to vehicle only, PB, CFA, BNF, ISF and DEX respectively. The treatment of the animals and the cultured hepatocytes, and the protein concentration in each lane is as described in the legend to Table 3 and in the Materials and methods section.

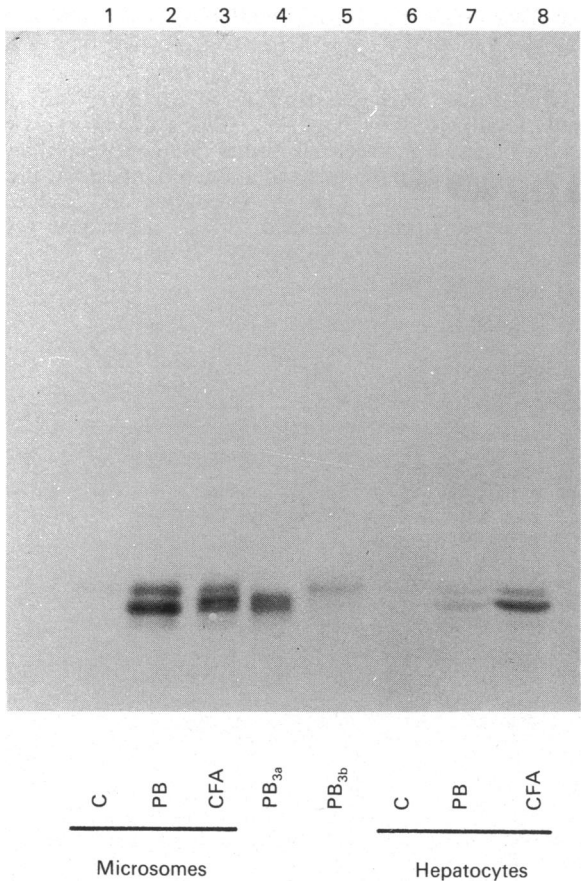


Fig. 2. Immunoblot of rat liver microsomes and cultured hepatocytes incubated with anti-PB_{3a}/PB_{3b}

Lanes 1–3, liver microsomes from rats treated with vehicle only, PB and CFA respectively; lanes 4 and 5, purified *P*-450 isoenzymes PB_{3a} and PB_{3b} respectively; lanes 6–8, cultured hepatocytes exposed to vehicle only, PB and CFA respectively. The treatment of the animals and the cultured hepatocytes, and the protein concentration in each lane is as described in the legend to Table 3 and in the Materials and methods section.

by incubating the blots in a fresh solution of H_2O_2/diaminobenzidine as described above for immunocytochemistry.

Enzyme activities

After 72 h exposure to PB, BNF, CFA, ISF or DEX, the cell monolayer was washed in SET buffer and scraped into 1 ml of SET buffer. The cells were disrupted by sonication. 7-Ethoxycoumarin-*O*-de-ethylation (ECOD), aldrin epoxidation (AE) and lauric acid hydroxylation (LAH) were determined as described previously [18–20].

Statistics

Statistical comparisons were carried out by Student's *t* test. A level of significance of $P < 0.05$ (two-tailed) was chosen.

Protein determination

The protein content of the samples was determined by the method of Lowry *et al.* [21] employing bovine serum albumin as a standard.

RESULTS

Enzyme activities

Exposure of cultured rat hepatocytes to PB (2 mM), CFA (1 mM), BNF (20 μM), ISF (50 μM) or DEX (1 μM) in the medium for 3 days resulted in marked increases in cytochrome *P*-450-dependent mono-oxygenase activities (Table 2). ECOD was induced 5.7-, 6.9-, 6.7-, 17.3- and 4.1-fold by PB, CFA, BNF, ISF and DEX respectively. LAH was induced 3.6-, 42.4-, 2.4-, 5.6- and 3.3-fold by PB, CFA, BNF, ISF and DEX respectively. AE was only induced by CFA (2.6-fold).

Immunoblotting

Polyclonal antibodies raised to specific isoenzymes of cytochrome *P*-450 were used to detect the presence of those isoenzymes in rat liver microsomes and sonicated whole hepatocytes (Table 3).

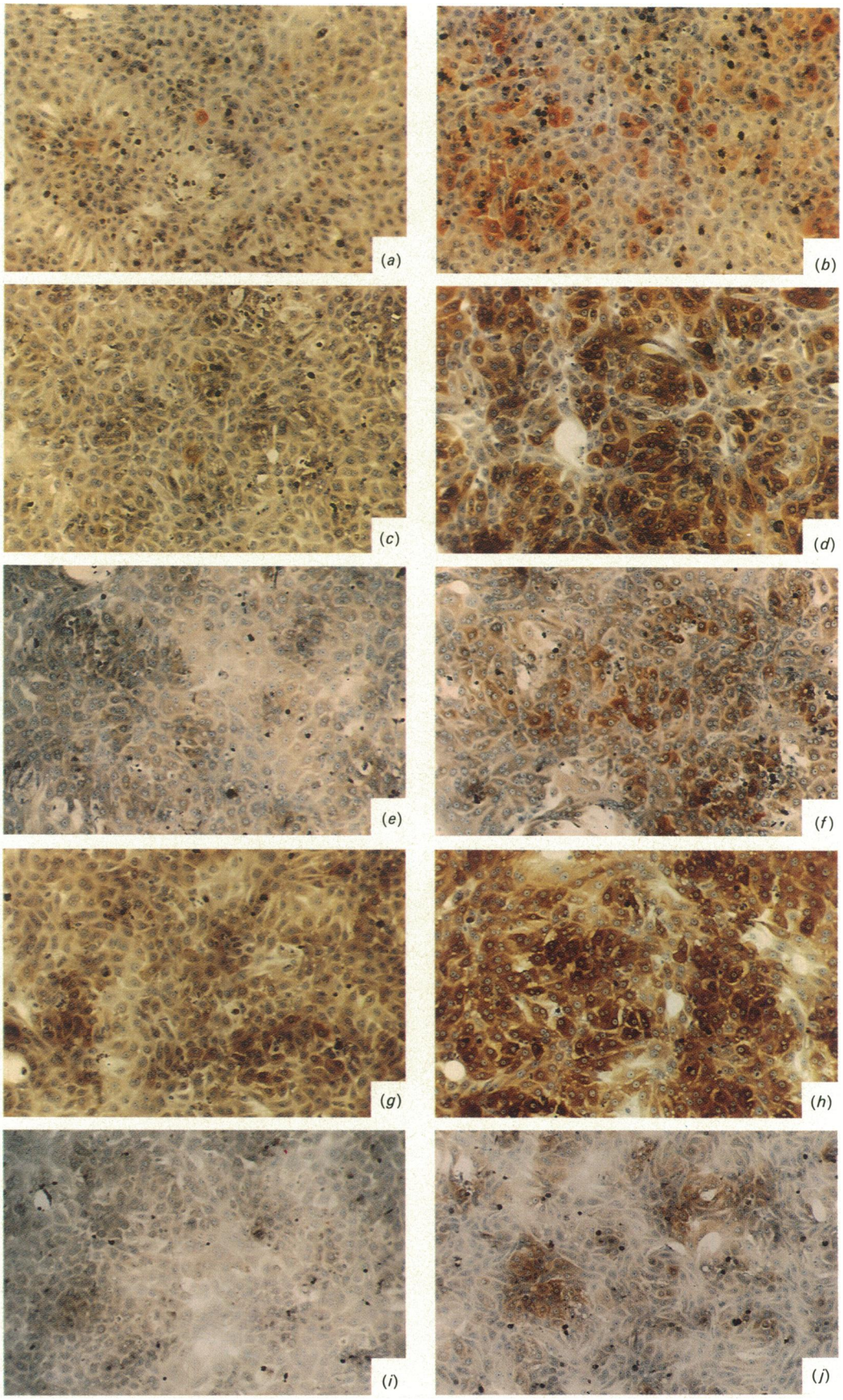
Anti-PB_{1a} reacted with isoenzyme PB_{1a}. PB_{1a} was detected in all the samples of microsomes and hepatocytes, although those resulting from exposure to PB or CFA demonstrated the highest concentrations of PB_{1a}. A second protein with a molecular mass lower than PB_{1a} was detected in all hepatocyte samples, although the identity of this protein is not known.

Of the purified *P*-450 standards examined, anti-PB_{2c} only reacted with the PB_{2c} isoenzyme. PB_{2c} was present in control rat liver microsomes, though comparatively greater amounts of PB_{2c} were present in microsomes from ISF-, PB- and CFA-treated rats. PB_{2c} was not detectable in control or BNF-treated hepatocytes, but was present in ISF-, PB- and CFA-treated hepatocytes, and the highest concentrations were found in DEX-treated hepatocytes (Fig. 1). However it is possible that two steroid-inducible *P*-450s were detected by anti-PB_{2c} as indicated by the two very close bands revealed in liver microsomes and possibly in PB- and DEX-treated hepatocytes. Considering the multiplicity of steroid-inducible hepatic rat *P*-450 [22,23] and the inability of polyclonal antibodies to distinguish those very similar *P*-450s [23], this result may not be surprising.

Anti-PB_{3a} reacted with two purified isoenzymes, PB_{3a} and PB_{3b}. Although these two *P*-450 forms have 97% amino-acid sequence similarity [24] they were separated on a 7.5% polyacrylamide gel (Fig. 2). PB_{3b} was detected

Fig. 3. Immunocytochemical localization of inducible *P*-450 forms in cultured hepatocytes following exposure to various chemical inducers

(*a*)–(*f*) Hepatocyte cultures immunostained with anti-PB_{3a}/PB_{3b}: (*a*) control, (*b*) 2 mM-PB; (*c*) control, (*d*) 1 mM-CFA; (*e*) control, (*f*) 50 μM-ISF. (*g*)–(*h*) Hepatocyte cultures immunostained with anti-MC_{1b}: (*g*) control, (*h*) 20 μM-BNF. (*i*)–(*j*) Hepatocyte cultures immunostained with anti-PB_{2c}: (*i*) control, (*j*) 1 μM-DEX. Hepatocytes were exposed to the inducer for 3 days. For each experiment the corresponding control is shown, because of variations in the staining intensity. Magnification $\times 200$.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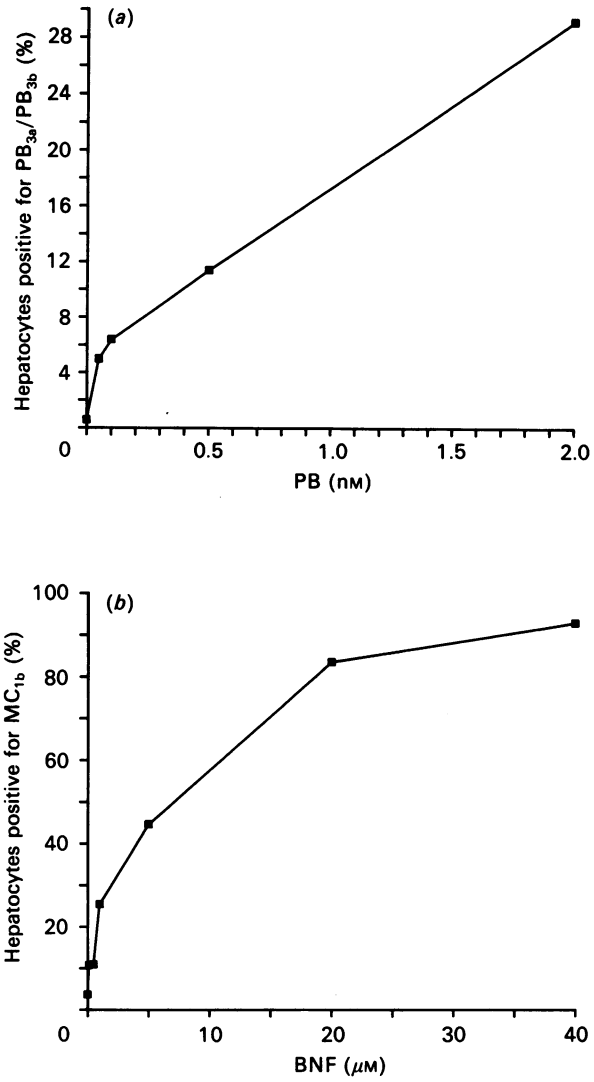

Fig. 4. Effect of (*a*) PB and (*b*) BNF concentrations on the percentage of hepatocytes containing the specific *P*-450 isoenzymes PB_{3a}/PB_{3b} and MC_{1b} respectively

Hepatocytes were isolated and cultured as described in the Materials and methods section with various concentrations of (*a*) PB (0.05 mM, 0.1 mM, 0.5 mM and 2 mM) or (*b*) BNF (0.125 μM, 0.5 μM, 1 μM, 5 μM, 20 μM, and 40 μM). 96 h after seeding, immunocytochemical detection with (*a*) anti-PB_{3a}/PB_{3b} or (*b*) anti-MC_{1b} was performed and evaluated by light microscopy. Values are means of four randomly selected areas, each of approx. 300 cells.

in control rat liver microsomes but PB_{3a} was not. Both proteins were strongly detected in microsomes from ISF-, CFA- and PB-treated rats. No PB_{3a} or PB_{3b} was detected in microsomes from BNF-treated rats. Though not detectable in control hepatocytes, PB_{3a} and PB_{3b} were present in PB- and ISF-treated hepatocytes, but the highest concentrations of PB_{3a} and PB_{3b} were found in CFA-treated hepatocytes.

Anti-MC_{1b} reacted only with purified MC_{1b}. MC_{1b} was detected only in microsomes prepared from BNF-treated rats. In contrast, however, MC_{1b} was detected in control hepatocytes and in hepatocytes treated with the various inducers, the highest concentrations being found in BNF- and ISF-treated cells. Anti-MC_{1b} also reacted slightly with an unknown protein of a lower molecular mass than *P*-450 in ISF-treated hepatocytes.

Anti-MC_{1a} reacted with two related isoenzymes, MC_{1a} and MC_{1b}. Both isoenzymes were clearly detected in microsomes from BNF-treated rats, whereas only MC_{1a} was detected in microsomes from ISF-treated rats. The levels of MC_{1a} in control or induced hepatocytes were very low, although some indication of induction by ISF was apparent.

Immunocytochemistry

Immunocytochemistry was used to investigate the distribution of *P*-450 isoenzymes in control hepatocytes and in hepatocytes exposed to various inducers (Fig. 3). In control hepatocytes PB_{1a}, PB_{3a} and MC_{1b} were localized in a small number of hepatocytes whereas PB_{2c} was not detectable. When hepatocytes were exposed to PB, both the number and the staining intensity of cells containing PB_{1a}, PB_{2c} and PB_{3a} was markedly increased, but some unstained hepatocytes were clearly visible. Thus, marked heterogeneity in the response of the hepatocyte population to PB was evident. Similar heterogeneous induction of PB_{1a}, PB_{2c} and PB_{3a} was evident after exposure to CFA. However, there was no significant increase in the number of cells containing 3-methylcholanthrene (3-MC)-inducible isoenzymes following exposure to PB or CFA.

Following exposure of cultured hepatocytes to BNF, there was no evidence of induction of the PB-inducible isoenzymes, whereas marked increases in the number of cells containing 3-MC-inducible isoenzymes were noted. Heterogeneous distribution of these isoenzymes in the hepatocyte population was again apparent.

When hepatocytes were exposed to DEX, PB_{2c} was shown to be present in many cells, although unstained cells were clearly visible.

When hepatocytes were exposed to ISF, MC_{1b} was shown to be present in most of the cells (results not shown) whereas PB_{3a}/PB_{3b} was present in only a certain fraction of the hepatocyte population.

Further investigations revealed that the number of hepatocytes responding to inducers such as PB or BNF was in fact related to the concentration of inducer in the medium. Following exposure to increasing concentrations of PB (0.05–2 mM), the number of hepatocytes stained for PB_{3a}/PB_{3b} increased linearly up to 30% of the population. Following exposure to increasing concentrations of BNF (0.125–40 μM), however, the number of hepatocytes stained for MC_{1b} increased to approx. 95% of the population (Figs. 4*a* and 4*b*).

DISCUSSION

Previous investigations on the inducibility of cytochrome *P*-450 in cultured hepatocytes have utilized cellular disruptive techniques [8–10] and give no information on the localization of *P*-450 in the hepatocyte population. This is of great significance when considering the heterogeneous distribution and induction of cytochrome *P*-450 in the intact liver. Consequently this work has concentrated on the qualitative aspects of *P*-450 induction, by studying the profile of isoenzymes induced, and the localization of these different isoenzymes in the hepatocyte population.

When cultured hepatocytes were exposed to PB, CFA,

BNF, ISF or DEX in the medium, marked increases in *P*-450 levels were demonstrated both by immunochemical techniques and by enzymic activities. However, in some cases the profile of isoenzymes induced by xenobiotics *in vitro* differed from the profile induced in the liver *in vivo*. For example, although ISF is reported to induce both MC_{1b} and MC_{1a} in the liver [25,26], in this study only MC_{1a} induction was observed. Nevertheless, when cultured rat hepatocytes are exposed to ISF, MC_{1b} induction clearly predominates. Thus the ratio of MC_{1a} to MC_{1b} induction by ISF is inverted in the model *in vitro*. Also, epoxidation of aldrin, a substrate which is selective for PB-inducible isoenzymes in the intact liver [27], was not increased in hepatocytes exposed to PB.

The profile of *P*-450 isoenzymes induced in the liver by CFA and other peroxisome proliferators has not been thoroughly investigated. However, it has been shown that these compounds have in common the ability to markedly induce a specific isoenzyme of cytochrome *P*-450, termed *P*-450IV, which is involved in the ω-oxidation of fatty acids [28–31]. That cytochrome *P*-450IV was induced in cultured hepatocytes exposed to CFA is demonstrated by increased LAH and immunodetection with anti-*P*-450IV on Western blot (results not shown). However, increased ECOD and AE activities, and immunoblots, revealed that several isoenzymes, namely PB_{1a}, PB_{2c}, PB_{3a} and PB_{3b}, were induced by CFA both *in vitro* and *in vivo*, and by comparing the intensity of staining of the detected bands, CFA appeared to be the most potent inducer of these PB-inducible isoenzymes in cultured hepatocytes. Recently Hardwick and coworkers [30] have reported that a single intraperitoneal dose (500 mg/kg) of clofibrate resulted in only a slight increase in hepatic *P*-450b (PB_{3a}) and no change at all in the level of *P*-450e (PB_{3b}). These results are in disagreement with our data, where four daily oral doses of 250 mg of CFA/kg resulted in marked increases in these two isoenzymes. The discrepancy may be explained by the different doses used, whereby repeated doses of this peroxisome proliferator may induce a wider variety of *P*-450 forms from distinct gene subfamilies.

Our immunocytochemical studies showed very clearly that these inducible isoenzymes of *P*-450 were not evenly distributed throughout the hepatocyte population. It has been proposed that the previously reported uneven distribution and inducibility of cytochrome *P*-450 in the intact liver [4,6,11,32,33] are related to the different microenvironments which are generated by the hepatic circulation across the liver lobule [34]. However, our work has demonstrated that even when hepatocytes are exposed to the same environment by maintaining them as primary monolayer cutlures, a heterogeneous distribution of inducible isoenzymes of *P*-450 is still apparent. It is noteworthy that one such factor which was found to affect this pattern of distribution was the concentration of inducer to which the hepatocytes were exposed. Following exposure to BNF or PB the number of hepatocytes containing increased levels of MC_{1b} or PB_{3a}/PB_{3b} respectively was related to the concentration of inducer in the medium. For example, the percentage of hepatocytes containing PB_{3a}/PB_{3b} increased linearly from 0.6% in control hepatocytes to 30% in hepatocytes exposed to 2 mM-PB. Hepatocytes exposed to PB at concentrations higher than 2 mM showed signs of cytotoxicity. When rats are dosed with PB at maximal inducing doses, PB_{3a}/PB_{3b} induction is observed essentially within the centrilobular region of the liver lobule [11]. Thus under conditions of maximal induction, PB_{3a}/PB_{3b} is highly induced only in a certain fraction of the hepatocyte population both in the liver lobule and in cultured hepatocytes. When hepatocytes were exposed to increasing concentrations of BNF, the percentage of cells containing MC_{1b} also increased from 3.7% in control cells to virtually 100% at 40 μM-BNF. Thus, unlike PB, maximal concentrations of BNF produced an 'homogeneous' distribution of MC_{1b} with all hepatocytes containing increased levels of this cytochrome. The homogeneous pattern of induction of MC_{1b} by 40 μM-BNF observed in cultured hepatocytes reflects the pan-lobular induction of this isoenzyme reported in the livers of rats given maximal doses of inducers [35]. However, some workers have reported a preferential induction of MC_{1b} by BNF in the periportal region [11]. Our data obtained *in vitro* suggest that these contradictory observations *in vivo* may be explained by different concentrations of BNF reaching the liver. Hence it is possible that the periportal hepatocytes are more sensitive to BNF induction in the liver and that they may be represented by the fraction of hepatocytes responding to low concentrations of BNF *in vitro*.

In conclusion, cytochrome *P*-450 may be induced *in vitro* by xenobiotics; however, the profile of isoenzymes induced may differ from that observed in the intact liver. Furthermore, this study suggests strongly that the heterogeneous expression of *P*-450 isoenzymes in the intact liver is not due simply to the immediate microenvironments of the cells, but perhaps reflects the programmed individuality of the mature hepatocyte, which is retained during primary culture.

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REFERENCES

1. Lu, A. Y. H. & West, S. B. (1978) Pharmacol. Ther. **2**, 337–358
2. Conney, A. H. (1982) Cancer Res. **42**, 4875–4917
3. Eisen, H. J. (1986) in Cytochrome *P*-450: Structure, Mechanism, and Biochemistry (Ortiz de Montellano, P. R., ed.), pp. 315–344, Plenum Press, New York
4. Gooding, P. E., Chayen, J., Sawyer, B. & Slater, T. F. (1978) Chem.-Biol. Interact. **20**, 299–310
5. Baron, J., Redick, J. A. & Guengerich, F. P. (1981) J. Biol. Chem. **256**, 5931–5937
6. Ji, S., Lemasters, J. J. & Thurman, R. G. (1981) Mol. Pharmacol. **19**, 513–516
7. Sirica, A. E. & Pitot, H. C. (1980) Pharmacol. Rev. **31**, 205–228
8. Elshourbagy, N. A., Barwick, J. L. & Guzelian, P. S. (1981) J. Biol. Chem. **256**, 6060–6068
9. Newman, S. & Guzelian, P. S. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 2922–2926
10. Schuetz, E. G., Wrighton, S. A., Safe, S. H. & Guzelian, P. S. (1986) Biochemistry **25**, 1124–1133
11. Wolf, C. R., Moll, E., Friedberg, T., Oesch, F., Buchmann, A., Kuhlmann, W. D. & Kunz, H. W. (1984) Carcinogenesis **5**, 993–1001

12. Wolf, C. R. & Oesch, F. (1983) Biochem. Biophys. Res. Commun. **111**, 504–511
13. Wolf, C. R., Seilman, S., Oesch, F., Mayer, R. T. & Burke, D. (1986) Biochem. J. **240**, 27–33
14. Vodicnik, M. J., Franklin, R. B., Elcombe, C. R. & Lech, J. J. (1981) Biochem. Pharmacol. **30**, 1091–1097
15. Mitchell, A. M., Bridges, J. W. & Elcombe, C. R. (1984) Arch. Toxicol. **55**, 239–246
16. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
17. Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
18. Ullrich, V. & Weber, P. (1972) Hoppe-Seyler's Z. Physiol. Chem. **353**, 1171–1177
19. Wolff, T., Deml, E. & Wanders, H. (1979) Drug Metab. Dispos. **7**, 301–305
20. Mitchell, A. M., Lhuguenot, J. C., Bridges, J. W. & Elcombe, C. R. (1985) Toxicol. Appl. Pharmacol. **80**, 23–32
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
22. Hostetler, K. A., Wrighton, S. A., Kremers, P. & Guzelian, P. S. (1987) Biochem. J. **245**, 27–33
23. Graves, P. E., Kaminsky, L. S. & Halpert, J. (1987) Biochemistry **26**, 3887–3894
24. Yuan, P. M., Ryan, D. E., Levin, W. & Shively, J. E. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 1169–1173
25. Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V. & Kaminsky, L. S. (1982) Biochemistry **21**, 6019–6030
26. Waxman, D. J., Dannan, G. A. & Guengerich, F. P. (1985) Biochemistry **24**, 4409–4417
27. Wolff, T. & Guengerich, F. P. (1987) Biochem. Pharmacol. **36**, 2581–2588
28. Orton, T. C. & Parker, G. L. (1982) Drug Metab. Dispos. **10**, 110–115
29. Tamburini, P. P., Masson, H. A., Bains, S. K., Makowski, R. J., Morris, B. & Gibson, G. G. (1984) Eur. J. Biochem. **139**, 235–246
30. Hardwick, J. P., Song, B. J., Huberman, E. & Gonzalez, F. J. (1987) J. Biol. Chem. **262**, 801–810
31. Nebert, D. W., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzales, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R. & Waterman, M. R. (1987) DNA **6**, 1–11
32. Moody, D. E., Taylor, L. A., Smuckler, E. A., Levin, W. & Thomas, P. E. (1983) Drug Metab. Dispos. **11**, 339–343
33. Gumucio, J. J., DeMason, L. J., Miller, D. L., Krezoski, S. O. & Keener, M. (1978) Am. J. Physiol. **234**, C102–C109
34. Jungermann, K. & Katz, N. (1982) in Metabolic Compartmentation (Siess, H., ed.), pp. 411–435, Academic Press, New York
35. Baron, J., Redick, J. A. & Guengerich, F. P. (1982) J. Biol. Chem. **257**, 953–957

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