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EXPRESSION OF FIVE FORMS OF MICROSOMAL CYTOCHROME P-450 IN PRIMARY CULTURES OF RABBIT HEPATOCYTES TREATED WITH VARIOUS CLASSES OF INDUCERS

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Abstract—In order to investigate the expression of five different forms of microsomal cytochrome P-450 including P-450 2 (P450IIBI), 3b (P450IIC3), 3c (P450IIIA4), 4 and 6 (P450IA2 and A1), hepatocytes were isolated from untreated rabbit and maintained in primary monolayer cultures in serum free modified Waymouth medium in the absence and in the presence of various classes of inducers including phenobarbital (PB), rifampicin (RIF), dexamethasone (DEX) and B-naphthoflavone (BNF). In untreated cultures the level of the various forms of P-450, determined by immunoblot with the use of specific antibodies, generally declined with time but at markedly different rates. In cultures treated with the inducers decline of the various forms was either unaffected, reduced, or even reversed, so that 96 hr after plating some of these forms appeared to be induced several-fold with respect to the untreated cultures. The forms 2 and 3c were co-induced by PB, RIF or DEX; as *in vivo*, BNF induced forms 4 and 6. Induction of forms 2, 3c, 4 and 6 was accompanied by stimulation of related monooxygenase activities, benzphetamine demethylase, progesterone 6B hydroxylase and benzpyrene hydroxylase and ethoxyresorufin deethylase, respectively.

In all cases, induction was accompanied by an increased rate of *de novo* synthesis of the protein, determined by radio-immunoprecipitation assay with the use of specific antibodies on [^{3}H]-Leu labeled cell lysate. Both induction and increased *de novo* synthesis were time- and inducer concentration-dependent. In cultures treated with RIF or BNF *de novo* synthesis of P-450 3c or of P-450 4 and 6 was correlated with the level of their specific mRNA quantitated from northern blots probed with either pLM3c-4.1 or pLM6.1, two plasmids containing inserted cDNA coding for P-450 3c or P-450 6, respectively. We conclude from these experiments that rabbit hepatocytes in primary monolayer cultures represent suitable models for studying regulation induction and pharmacological implications of the microsomal cytochromes P-450.

Primary cultures of hepatocytes are being currently used to investigate various aspects of drug metabolism and microsomal cytochromes P-450 expression [1–19]. These cytochromes constitute a multigenic family of hemoproteins involved in the oxidation of numerous endogeneous and exogeneous compounds like drugs and carcinogens [20]. One of the essential characteristics of these cytochromes is that some of them are inducible. For example, in the rabbit P-450§ 2 is the major form induced by phenobarbital, whereas forms P-450 4 and 6 are induced by polycyclic hydrocarbons like B-naphthoflavone or methylcholanthrene [21–24]. In a recent series of papers we showed that macrolide antibiotics like TAO and rifampicin and glucocorticoïds like dexamethasone induced form P-450 3c [25–29]. In order to investigate further the mechanism of induction and the regulation of this cytochrome as well as its pharmacological implications, we decided to use rabbit hepatocytes in primary monolayer cultures. Only a few reports have been devoted to the study of drug metabolism enzymes in hepatocytes cultures from this animal species [30–32]. Thus, while several forms of P-450 have been shown to be present and inducible in rat hepatocytes cultures [13, 14], nothing is known, in contrast, on the expression of individual forms of P-450 in rabbit hepatocytes cultures.

Here we describe the effect of the age of the culture and of various classes of inducers like phenobarbital, rifampicin, dexamethasone and B-naphthoflavone on the accumulation and *de novo* synthesis of five different forms of P-450, including P-450 2, 3b, 3c, 4 and 6, as well as on the level of P-450 3c, 4 and 6 mRNAs.

§ Abbreviations used: P-450, cytochrome P-450 from hepatocytes or liver microsomes; PB, phenobarbital; DEX, dexamethasone; RIF, rifampicin; TAO, triacetyloleandomycine; BNF, B-naphthoflavone. According to a recently published paper on P-450 nomenclature (Nebert *et al.* 1987 DNA **6** 1–11) the trivial name—gene family correspondence for the forms investigated in this work is as follows: P-450 2, P450IIBI; P-450 3b, P450IIC3; P-450 3c, P450IIIA4; P-450 4, P450IA2; P-450 6, P450IA1.

MATERIALS AND METHODS

Materials. Inducers including, phenobarbital, B-naphthoflavone, rifampicin and dexamethasone were obtained from Specia (Paris, France), Sigma (Saint Louis, MO), Merrel Dow (Neuilly, France) and Merck Sharp & Dohme-Chibret (Paris, France), respectively. All chemicals used to prepare the Waymouth medium and solutions used during perfusion and hepatocytes preparation were tissue culture reagents from Sigma (Saint Louis, MO). Rat tail collagen (type III) and collagenase (type IV) were from Sigma (Saint Louis, MO). All solutions used for hepatocytes isolation and culture were sterilized by filtration through 0.22 μ nitrocellulose membranes from Sartorius GmbH (Göttingen, F.R.G.). Electrophoresis reagents and nitrocellulose filters for immunoblotting were from BioRad (Richmond, CA). Rabbit antigoat IgG, rabbit antisheep IgG and peroxidase labeled goat antirabbit IgG were from Miles Scientific (Elkhart, IN). 4–5 [^{3}H] leucine (130 Ci/mmol), 5-γ-[^{32}P] dCTP (3000 Ci/mmol) and nick translation kit were from Amersham International (Amersham, U.K.).

Animals and treatments. Male and female New Zealand white rabbits weighting between 1 and 1.5 kg were used in this work. In some experiments animals were treated with PB, BNF, RIF, as indicated in a previous report [29], or DEX given *per os* in corn oil at a dose of 300 mg/kg/day for four days. Animals were fasted overnight before sacrifice.

Primary monolayer cultures of rabbit hepatocytes. Untreated animals were used for hepatocytes isolation. After an overnight fast, the animal was anesthesized by i.p. injection of a mixture of Rompun®-xylazine from Bayer (Leverkusen, F.R.G.) and Ketamine chlorhydrate from Division Parke-Davis (Courbevoie, France) at a dose of 10 mg/kg and 50 mg/kg, respectively. The liver was perfused *in situ* under sterile conditions and without recirculation, first with 200 ml of a calcium ion free buffered solution containing 0.5 mM EGTA at a rate of 30 ml/min, followed by 400 ml of a 0.05% solution of collagenase in complete serum-free Waymouth medium 752/1, modified as indicated elsewhere [1] at a rate of 20 ml/min. The softened liver was then excised and placed in a sterile Petri dish where the gall bladder was dissected out and the capsule opened with scissors. The "parenchymal paste" was transferred into a conical flask in which it was shaken for 10 min in a rotary-action (125 rpm) shaking bath at 37° with 80 ml of a fresh 0.025% solution of collagenase in Waymouth medium. The suspension was filtered through a double layer of sterile gauze and the hepatocytes were separated from non-parenchymal cells by centrifugation at 60 g for 2 min at room temperature. Washing of the hepatocytes with 60 ml of complete Waymouth medium was repeated twice and the cells were finally suspended in the culture medium at 10^7 cells/ml. Viability of the cells, assessed by the Trypan blue exclusion test, was always greater than 90% and the yield averaged 10^9. The hepatocytes were inoculated (5×10^6 cells/3 ml medium) in 60 mm plastic plates (Becton-Dickinson Grenoble, France) coated with collagen (50 μg/plate). The plates were then incubated in a humidified atmosphere of 5% CO_2 at 37°. The culture medium was renewed every 24 hr. When present, inducers were added to the serum free culture medium as dissolved in dimethylsulfoxide (DMSO), so that the organic solvent concentration was 0.1%. Control cultures only received DMSO at the same concentration.

Preparation of microsomes and cell lysates. Microsomes from intact liver were prepared as previously described [21]. Microsomes and lysates were prepared from cell cultures (10 and 5 plates, respectively) as follows. Monolayers were washed twice with 3 ml of cold 10 mM potassium phosphate pH 7.4, 0.15 NaCl, (phosphate saline buffer, PBS) and scraped into 10 ml of the same buffer (microsomes) or 100 μl/plate of 0.1 M potassium phosphate pH 7.4, 0.1 mM EDTA, 20% glycerol (lysates). The cell suspension was sonicated at 0° for 45 sec with a 100 W ultra-sonifier Sonimasse S20 (Annemasse, France) and microsomes were obtained as described for intact liver [21] and resuspended in 500 μl of 0.1 M potassium phosphate pH 7.4, 0.1 mM EDTA 20% glycerol. For cell lysates preparation, 2 vol. of solubilisation buffer, 50 mM potassium phosphate pH 7.4, 1 mM EDTA, 0.15 M NaCl, 20% glycerol, 0.3% NP40, 1% sodium cholate were added to the sonicated suspension. After 30 min incubation at room temperature the suspension was centrifuged at 60,000 *g* for 30 min and the supernatant was referred to as the cell lysate.

Preparation of antibodies. Antibodies to highly purified P-450 2, 3b and 4 were raised in sheep, while antibodies to P-450 3c and 6 were raised in goat. The IgG fraction was isolated from the sera by repeated ammonium sulfate precipitations. Precipitated IgG fractions were then dissolved in pure water and stored at −80°. Anti P-450 3c and anti P-450 6 IgG were further purified by immuno-adsorption against liver microsomes from untreated rabbit or highly purified P-450 4 coupled to a Sepharose 4 B gel according to previously described procedures [33] and [34], respectively.

Preparation of RNA from cell cultures. Total RNA was prepared from 30 plates of cultures by the procedure of Auffray and Rougeon [35] adapted as follows. Cells were scraped in 300 μl/plate of 100 mM Tris–HCl pH 7.8, 5 M guanidine thiocyanate, 10 mM EDTA. After a 30 min incubation at room temperature in the presence of 20 μl/plate of B-mercaptoethanol, 600 μl/plate of a solution of 9 M urea, 4.5 M lithium chloride were added to the mixture which was then incubated overnight at 4°. After centrifugation at 10,000 *g* the pellet was treated twice with 5 ml of 10 mM Tris HCl, 1-mM EDTA, 0.1% SDS. This solution was then extracted twice with chloroform: phenol/l: 1 and RNA was precipitated at −80° in 0.3 M sodium acetate pH 5.2, 70% ethanol, centrifuged, re-dissolved in 200 μl of sterile water and stored at −80°. The yield of RNA depended on the age of the culture and averaged from 60 to 10 μg/plate for one and five days of culture, respectively.

Analytical methods. Protein concentration was estimated by the method of Lowry *et al.* [36] with appropriate modifications for buffer components [37]. Concentration of cytochromes P-450 and b_5 was determined spectrally according to Omura and Sato

[38]. NADPH cytochrome P-450 oxydoreductase was assayed according to Vermillion and Coon [39].

Monooxygenase activities. Benzphetamine demethylase [40], ethoxyresorufin deethylase [41], progesterone hydroxylase [26] and benzpyrene hydroxylase [40] activities were determined according to previously published procedures.

Western blots. Cytochromes P-450 2, 3b, 3c, 4 and 6 were quantitated by immunoblot analysis of electrophoretically separated microsomal proteins as described elsewhere [29, 42]. Usually, 5 μg of microsomal proteins were submitted to electrophoresis and the immunoblots were developed in the presence of 0.1 mg/ml of specific IgG. Specific content of each form of P-450 was estimated from densitometric analysis of the blot with a Shimadzu Dual-Wavelength Scanner by reference to a standard concentration range of the authentic antigen on the same blot.

Immunoprecipitation assay. The rate of *de novo* synthesis of the various forms P-450 2, 3b, 3c, 4 and 6 was determined by an immunoprecipitation assay previously described [13, 14] and modified as follows. The cultures were radiolabeled for 3 hours with 4–5 [^{3}H] Leu (10 μCi/plate) in Leu free Waymouth medium in the absence or in the presence of inducers. The cells were then harvested and lysates prepared as indicated in a previous section. The immunoprecipitation assay medium consisted of 100 μl of lysate, 20 μl of 100 mg/ml BSA, and 220 μl of a "dilution buffer" (50 mM potassium phosphate pH 7.4, 0.1 M KCl, 1 mM EDTA, 0.2% NP40). This solution was first incubated 30 min at room temperature in the presence of 20 μl of a 10% (w/w) solution of *Staphylococcus aureus* ghosts; this step allowed removal from the lysate of proteins which unspecifically bind to the *S. aureus* membranes. After centrifugation of the suspension, the supernatant was incubated for 3 hr at 37° and overnight at 4° in the presence of increasing amounts of anti P-450 IgG (from 0 to 10 mg/ml) in a final volume of 400 μl. The immune complex was precipitated by a 30 min incubation at room temperature in the presence of *S. aureus* ghosts, collected by centrifugation, washed three times in the "dilution buffer" and submitted to electrophoresis on SDS polyacrylamide gel (10%). After localisation of the P-450 migration region by the use of appropriate prestained protein molecular weight standards, the gel lanes were cut into 2 mm slices and the radioactivity associated with each slice was determined as previously indicated [14]. The radioactivity associated with the P-450 form tested was estimated by summing the radioactivity of the corresponding slice and substrating the blank value obtained from immunoprecipitation in the presence of non-immune IgG. The results were expressed as percent of total protein synthesis by normalizing the P-450 immunoprecipitate radioactivity to the total (TCA precipitable) cellular proteins radioactivity [13, 14].

Northern blots. Electrophoresis on agarose gel (1.2%) of RNA (7 μg) prepared from cell cultures, transfer to nitrocellulose filter, prehybridization and hydrization with nick translated (1–2 10^8 dpm/μg) appropriate probes, pLM3c-4.1, pLM6-1* or pGAPDH-13 (10 ng), were carried out as indicated in a previous paper [29]. Filters were autoradiographied for 16 hr at −80°. In control experiments, filters were dehybridized and rehybridized with nick translated pGAPDH-13 [29] to ascertain that identical amount of mRNA had been analysed for each sample.

* A cDNA library was prepared from BNF induced rabbit liver mRNA and screened by selective hybridisation with cDNA (pP_1-450) kindly provided by D. W. Nebert. Two positive clones were isolated p4-1 and p6-1. Comparative analysis (involving restriction maps and nucleotide sequence) with p6-34-1 and p6-34-2 inserts recently characterized by Okino *et al.* (44), indicated that p4-1 and p6-1 inserts were cDNAs encoding for P-450 4 and P-450 6, respectively. (Denis Pompon, unpublished results).

RESULTS AND DISCUSSION

Cells cultures

In a developmental study we observed that the specific content of P-450 2, 3b, 3c, 4 and 6 in the 2-month-old rabbit was not significantly different from that determined in the adult (Table 1). Moreover, these forms were inducible in the young animals as they are in the adults under same conditions of treatment (not shown). We therefore selected 2-month-old animals for this work in order to reduce the amount of collagenase used during the perfusion.

The method of isolation of hepatocytes as well as the conditions of culture were the same as those routinely used for rat hepatocytes [1]. Only the volume of solutions used during the perfusion was modified in proportion to the liver weight and the number of cells plated per 60 mm dishes was 5.10^6. Over 25

Table 1. Specific content of five forms of P-450 from liver microsomes of 2 months old and adult untreated rabbits

		P-450 Forms (nmol/mg)				
Age	Sex	2	3b	3c	4	6
2 months	F	0.04 ± 0.02	0.28 ± 0.05	0.10 ± 0.03	0.80 ± 0.05	0.23 ± 0.05
	M	0.06	0.32	0.05	0.66	0.26
Adults	F	0.04 ± 0.02	0.26 ± 0.04	0.05 ± 0.02	0.53 ± 0.05	0.27 ± 0.08
	M	0.05	0.27	0.04	0.29	0.24

Liver from 3 males or 3 females were pooled and microsomes were prepared. The various forms of P-450 were quantitated by immunoelectrophoresis as described under Materials and Methods.

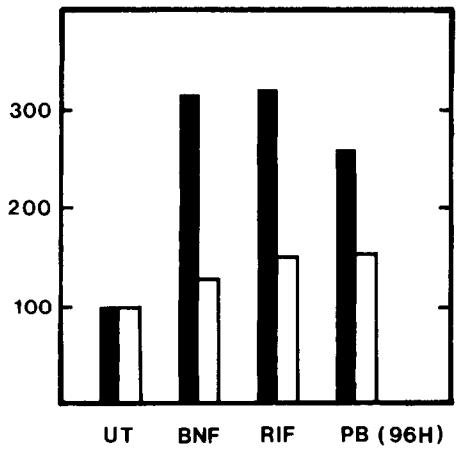

Fig. 1. Effect of inducers on the relative level of cytochromes P-450 and b5 determined spectrally in microsomes from hepatocytes maintained 96 hr in primary culture. Hepatocytes prepared from an untreated rabbit were maintained in primary culture in the absence (UT) or in the presence of 2 mM phenobarbital (PB), 50 μM B-naphthoflavone (BNF) or 50 μM of rifampicin (RIF). Medium (± inducers) was renewed every 24 hr. After 96 hr, cells (10 plates for each sample) were scraped, washed and microsomes were prepared. Specific content of cytochromes P-450 and b5 was determined spectrally according to the method of Omura and Sato, using $\Delta\varepsilon$ (450–490) = 91 $mM^{-1}\,cm^{-1}$ for P-450 (Fe^{2+}–Co–Fe^{2+}) and $\Delta\varepsilon$ (426–410) = 185 $mM^{-1}\,cm^{-1}$ for b5 (Fe^{2+}–Fe^{3+}), respectively. Absolute values for microsomes from untreated cells (UT) were: P-450, 0.3 nmol/mg; b5, 0.6 nmol/mg, (experiments culture no. 9); ■, P-450; □, b5.

preparations the yield averaged 3.32 10^7 cell/g of liver (extreme values 1.7–5.2 10^7) with a viability greater than 90%. This yield was slightly higher than the value reported by Maslansky and Williams [30]. We did not observe any influence of the sex of the animal on either the yield or the viability of the preparations. Cell were attached to the dish within 3 hr after plating and assumed polygonal shape within 24 hr. Approximately 50% of the cells were binucleated. No sign of degradation was apparent during the first 96 hr of culture by light microscopy, irrespective of the absence or the presence of the inducers.

Effect of the age of the cultures on the level of five different forms of P-450 in the absence and in the presence of inducers

The decline of cytochrome P-450 concentration in primary culture of hepatocytes is well documented [2, 43] and was also routinely observed in this work. However, this decline exhibited marked differences from one preparation to another (most likely due to interindividual differences), irrespective of the sex of the animal. In some cases it was linear against time, whereas in others it was multiphasic with the largest loss (i.e. 50%) during the first 24 hr. In most cases the P-450 level averaged 20–30% of its initial value after 96 hr in culture. Similar behavior (not reported here) was found with NADPH cytochrome *c* reductase activity. In contrast, the level of cytochrome *b5* exhibited a moderate and linear decline with a 30% reduction or less after 96 hr in culture.

In the presence of the inducers (2 mM PB, 50 μM RIF, DEX or BNF) the concentration of P-450 continued to decline after plating, although in much more moderate proportion so that after 96 hr it was 2–3 times higher than in control culture as shown in Fig. 1. NADPH cytochrome *c* reductase activity exhibited similar behaviour and in some cultures (in the presence of RIF or DEX) it was even higher after 96 hr than before plating (results not shown). The level of cytochrome *b5* was either unaffected or slightly increased (Fig. 1). Whereas in the absence of inducer or in the presence of PB, RIF or DEX the wavelength of maximal absorbance of the microsomal ferrous–carbonyl complex was 452 nm, a blue shift to 449 nm was observed in the microsomes prepared from 96-hr-old BNF-induced cultures. In aggregate, all these observations suggest that the level of one or more form(s) of P-450 could have been increased in rabbit hepatocytes cultures in the presence of the inducers.

The level of five different forms of P-450, including P-450 2, 3b, 3c, 4 and 6, was therefore determined by western blotting in microsomes from hepatocytes maintained in primary culture for different periods of time, in the absence or in the presence of either 2 mM PB, 50 μM RIF, DEX or BNF. Although quantitative differences occured from one preparation to another, the results reported in Fig. 2 represent the most frequently encountered situation. In the absence of inducer, all the forms tested appeared to decline after plating although at different rates. For example, the level of P-450 3b was only decreased by 25% after 96 hr in culture, whereas P-450 3c was barely detectable in most of cultures older than 48 hr. This suggests that the level of the various forms could be dependent on the combination of distinct stimuli some of which are conserved in culture (see for example form 3b) some of which are lost more or less rapidly (P-450 3c). These stimuli could originate from a number of distinct contributions such as strain of the animal, method of cell preparation, culture medium, nutrients, hormones, vitamins, presence or absence of xenobiotics, cell substrata, cell density, presence or absence of non parenchymal cells, etc. Some of these possible contributions have been investigated; the nature of the culture medium, the presence of putative essential compounds were shown to affect significantly the level of some, but not all, of the P-450 forms in the rat hepatocytes cultures [1, 7, 8, 10, 13, 14, 16, 43, 46]. Further exploration of these various contributions is therefore needed and will require extensive studies.

In the presence of the inducers these patterns were modified. The level of form 2 was roughly maintained at its initial value. Form 3c continued to drop with time although at a much lower rate in the presence of RIF, DEX or PB. On the other hand, the level of forms 4 and 6 (both cytochromes P-448) was increased 2- and 4-fold, respectively, by BNF after 96 hr in culture and this could explain the blue shift from 452 to 449 nm observed for the ferrous carbonyl complex in microsomes from these cultures. Although other forms of P-450, not tested in this

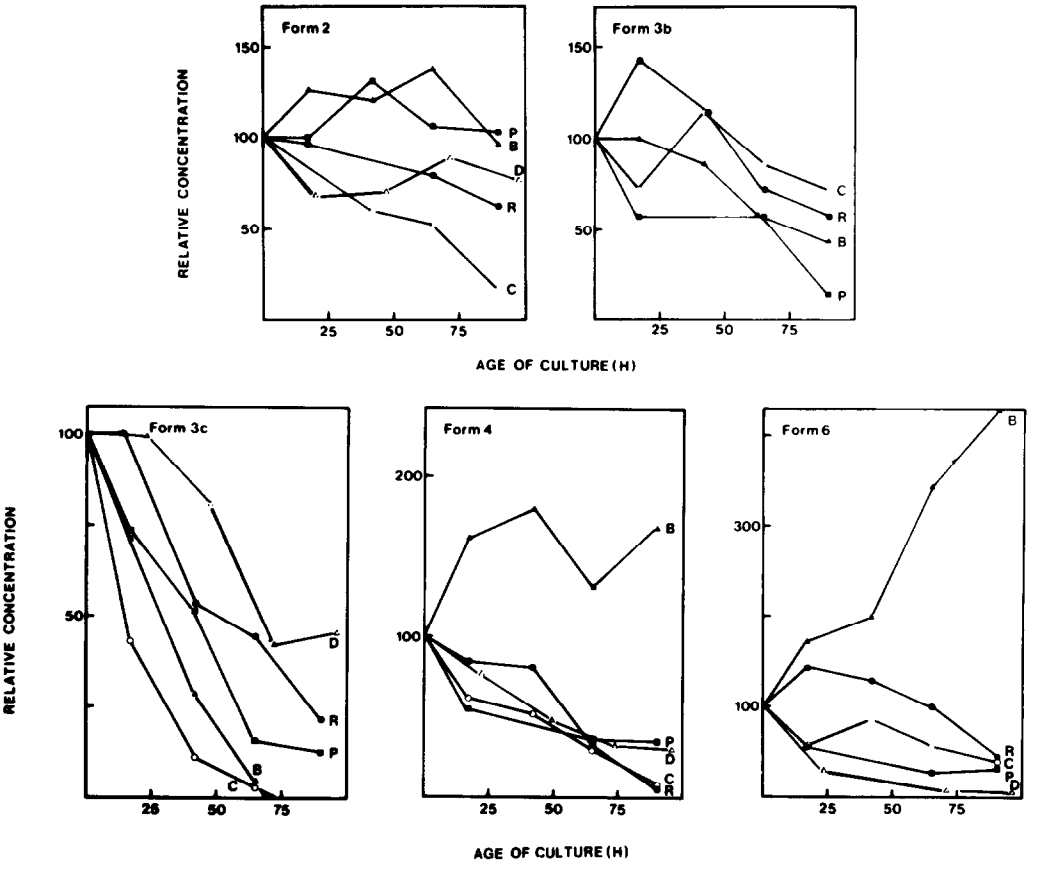

Fig. 2. Effect of time on the relative level of five forms of cytochrome P-450 from microsomes of rabbit hepatocytes maintained in primary culture in the absence and in the presence of inducers. Hepatocytes prepared from an untreated rabbit were maintained in primary culture in the absence (C) or in the presence of various inducers: 2 mM phenobarbital (P), 50 μM B-naphthoflavone (B), 50 μM rifampicin (R) 50 μM dexamethasone (D). Medium (±inducers) was renewed every 24 hr. At various intervals of time cells were scraped (5 plates each sample), washed and microsomes were prepared. The various forms of P-450 were quantitated by western blot as indicated in Materials and Methods. Absolute values at time 0 (before platting) in this culture were = P-450 2: 0.07 nmol/mg; 3b: 0.20 nmol/mg; 3c: 0.14 nmol/μg; 4: 0.30 nmol/mg; 6: 0.10 nmol/mg (experiments culture no: 21).

Table 2. Monooxygenase activities of microsomes from rabbit hepatocytes in primary culture: effect of inducers

Inducers	Monooxygenase activities (nmol/min/mg)			
	Benzphetamine demethylase	6B Progesterone hydroxylase	Benzpyrene hydroxylase*	Ethoxyresorufine deethylase*
Before plating	4.6–1.6†	3.9	0.32	0.15
None	1.2	1.2	0.13	0.025
PB	4.3	2.9	0.19	0.03
RIF	5.4	3.4	0.08	0.075
DEX	3.2†	3.0	0.21	0.045
BNF	1.3	0.8	0.23	0.14

Hepatocytes were prepared from untreated rabbit, plated and maintained in primary culture for 96 hr or 48 hr* in the absence or in the presence of inducers: 2 mM phenobarbital (PB), 50 μM rifampicin (RIF), 50 μM dexamethazone (DEX) or 50 μM B-naphthoflavone (BNF). The medium (±inducer) was renewed every 24 hr. At 48 hr* or at 96 hr after plating, cells were scraped, washed and microsomes were prepared. Monooxygenase activities were determined as indicated under Materials and Methods. Average values from duplicate or triplicate experiments. (Experiments from cultures no. 9, 10, 21, 23, 25†.)

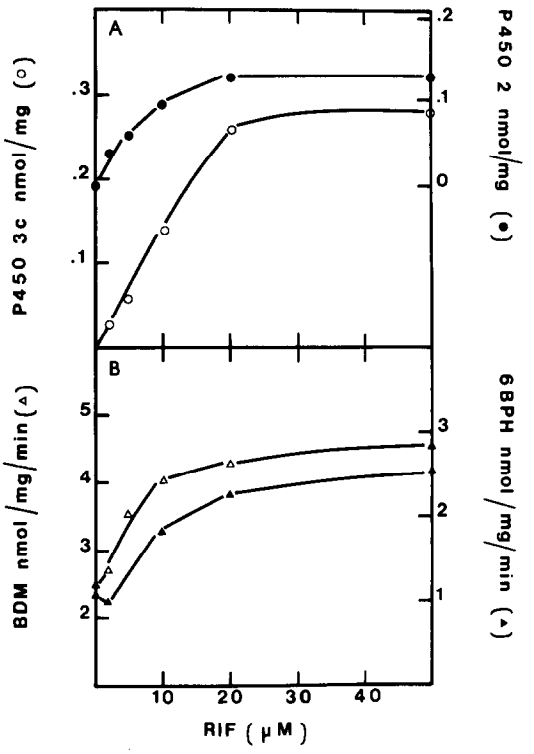

Fig. 3. Effect of increasing concentration of rifampicin on the level of P-450 2 and 3c and on related monooxygenase activities in microsomes from rabbit hepatocytes maintained 96 hr in primary culture. Hepatocytes from an untreated rabbit were maintained in primary culture in the absence or in the presence of increasing concentration of RIF. Medium was renewed every 24 hr. After 96 hr, cells (8 plates each sample) were scraped, washed and microsomes were prepared. Immunoquantitation of P-450 2 and 3c and monooxygenase activities, benzphetamine demethylase (BDM) and 6 B progresterone hydroxylase (6-BPH) were determined as indicated in Materials and Methods. (Experiments culture no: 19).

work, could have also been induced, the results presented in Fig. 2 reasonably account for the increased level of P-450 observed in induced cultures (Fig. 1). Moreover these observations suggest that under the culture conditions used in this work, the heme pool is apparently sufficient to respond to increased accumulation of P-450 apoprotein. This point is further substantiated by the observations (Table 2) that various microsomal monooxygenase activities are indeed retained in 96-hr-old cultures.

In Fig. 3, A and B we report the effect of increasing concentration of RIF on the level of P-450 2 and 3c in microsomes isolated from primary cultures of hepatocytes which have been exposed 96 hr to the inducer. Both forms appear to be induced with a similar dose-response pattern. Similar results, not shown here, were obtained with DEX and PB, the only difference being the concentration for which a maximal effect was obtained: 10–20 μM with RIF, 50 μM with DEX; with PB much higher concentration was needed and at 2 mM the plateau was not reached. The results presented in Fig. 3B show that, concomitant with the induction of both forms, benzphetamine demethylase and 6 B progesterone hydroxylase, two monooxygenase activities specific of forms 2 and 3c, respectively [21, 27], were increased according to the same dose–response pattern. Similar observations revealing the parallelism between induction of forms 2 and 3c and benzphetamine demethylase and 6B progesterone hydroxylase were made with PB and DEX.

Microsomal monooxygenase activities from rabbit hepatocyte cultures maintained 96 hr in the absence or in the presence of PB, RIF, DEX or BNF are reported in Table 2. In addition to benzphetamine demethylase and 6 B progesterone hydroxylase data on benzpyrene hydroxylase and ethoxyresorufin deethylase, two activities believed to be specific of forms 4 and 6 [24], are also reported. It is clear that these activities are indeed maintained in cultures in the presence of adequate inducer.

Effect of inducers on the de novo *synthesis of the P-450 forms in primary culture of rabbit hepatocytes*

Immunoprecipitation experiments were carried out on lysates prepared from [^{3}H] leucine radiolabeled hepatocytes, maintained in primary culture for 96 hr in the absence or in the presence of PB, RIF, DEX or BNF, in order to estimate the contribution of *de novo* protein synthesis to the increased level of forms 2, 3c, 4 and 6 under these conditions. As *in vivo*, P-450 3b was not inducible by any of the chemicals tested. In a preliminary series of experiments, optimal conditions of immunoprecipitation were determined for each of the antibodies used in this work. Aliquots of radiolabeled cells lysate were combined with increasing amounts of the various antibodies and the immunoprecipitated proteins were separated by SDS polyacrylamide gel electrophoresis. The radioactivity associated with the respective form of P-450 was then plotted against IgG concentration as reported in Fig. 4. These experiments revealed the presence of only one band of radioactivity in each immunoprecipitate. Although this finding was as expected with anti 2, and 3c IgG, the presence of two bands was suspected with anti P-450 4 and unadsorbed anti P-450 6 IgGs, both P-450 4 and 6 sharing common antigenic determinants [44]. Our experiments showed that under the assay conditions used, no cross-reaction occurred presumably because of the presence of a relatively high level of detergent (sodium cholate) in the precipitation medium which, as documented elsewhere, might weaken antibody-antigen interactions [34]. This was further confirmed by immunoprecipitation experiments in which the total radioactivity associated with immunoprecipitate obtained in the presence of a mixture of anti-P-450 4 plus anti-P-450 6 IgG was shown to be exactly the summation of the radioactivity precipitated with anti P-450 4 IgG alone plus the radioactivity precipitated with anti P-450 6 IgG alone.

Results obtained on the *de novo* relative synthesis of forms 2, 3c, 4 and 6 in hepatocytes maintained 7, 24 or 96 hr in culture in the absence or in the presence of 2 mM PB, or 50 μM RIF, DEX or BNF showed that whereas 96 hr after plating none of these forms was synthesized in the untreated hepatocytes, 96-hr-old induced cultures retained their ability to synthesize several forms of P-450. The *de novo* syn-

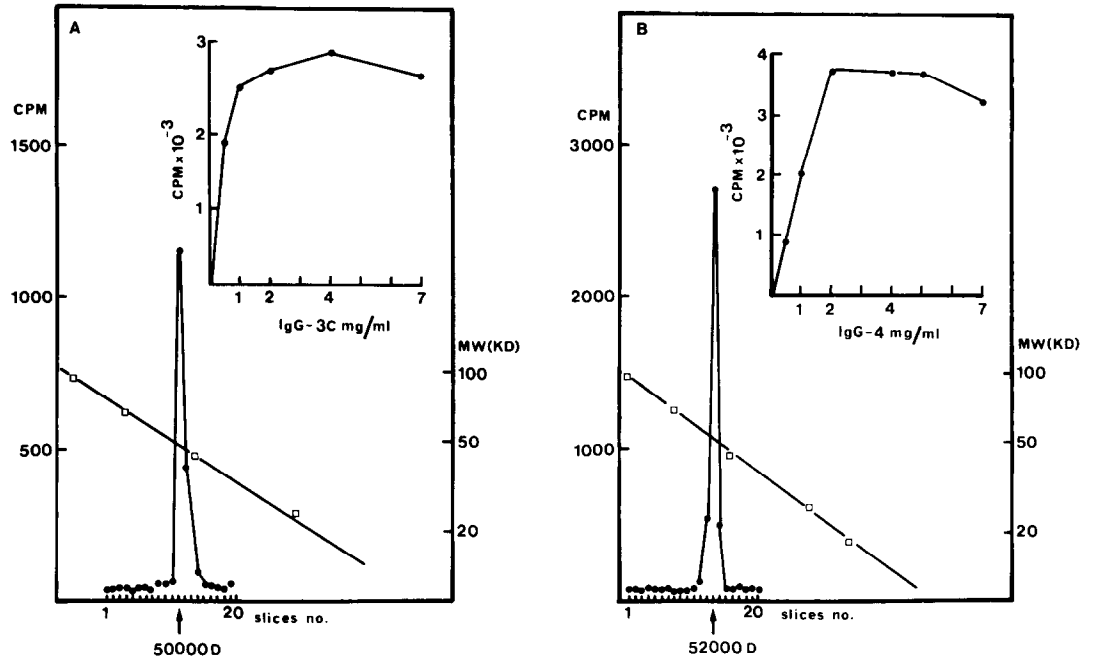

Fig. 4. Immunoprecipitation of [^{3}HLeu]radiolabeled P-450 3c and 4 from rabbit hepatocytes. Hepatocytes prepared from an untreated rabbit were maintained in primary culture in the absence or in the presence of 50 μM rifampicin (A) or B-naphthoflavone (B) for 96 hr, the medium (± inducer) being renewed every 24 hr. At 96 hr of culture, the cells (3 plates each sample) were radiolabeled with 4-5[^{3}H]Leu (10 μCi/plate) in Leu free medium for 3 hr. Cells were then scraped, washed and lysates prepared (Materials and Methods). *De novo* synthesis of P-450 2, 3c, 4 and 6 was estimated by immunoprecipitation assay described under Materials and Methods. (A) Electrophoretic profile of radioactivity associated with a P-450 3c immunoprecipitate obtained with 0.5 mg/ml of specific anti P-450 3c IgG. Insert: Effect of increasing concentration of IgG on the radioactivity associated with P-450 3c after immunoprecipitation assay carried-out on 100 μl aliquots of cell lysate (total protein radioactivity 205,000 cpm). (B) Electrophoretic profile of radioactivity associated with a P-450 4 immunoprecipitate obtained with 2 mg/ml of specific anti P-450 4 IgG. Insert: effect of increasing concentration of IgG on the radioactivity associated with P-450 4 after immunoprecipitation assay carried-out on 100 μl aliquots of cell lysate (total protein radioactivity: 185,000 cpm). In both A and B the straight line refers to stained molecular weight standards which have been run on the same gel: phosphorylase b (97,400 D), bovine serum albumine (68,000 D), ovalbumine (43,000 D), α-chymotrypsinogen (25,700 D) and B-lactoglobulin (18,400 D) (experiments cultures no: 12 and 13).

thesis occurred early after the addition of the inducers to the culture, maximal effect being obtained after 24 hr of treatment with RIF and DEX on form 3c (3.5 and 4.6% of total protein, respectively) and 7 hr with BNF on forms 4 and 6 (0.95 and 0.5% respectively). At 96 hr, relative synthesis of form 2 was 0.35, 1.0 and 0.1% after treatment of cultures with RIF, DEX and PB respectively, while that of form 3c was 0.15% with PB. Among the forms tested, P-450 3c was the only one to be apparently actively synthesized in freshly isolated hepatocytes (from 2.4 to 4.5%). In addition to the results presented in Figs 2 and 3, the data on *de novo* synthesis suggest that forms 2 and 3c are co-induced in cultures exposed to PB, RIF or DEX. This appears to be different from the situation prevailing *in vivo* where PB and RIF or DEX are representative of two different classes of inducers stimulating specifically forms 2 and 3c, respectively, in both adult and young (2 months old) male and female rabbits. While this finding could reasonably be accounted for by phenotypic changes accompanying the adaption of the hepatocytes to culture conditions, it is reminiscent of the fact that in the rat and human P-450 p and P-450 HLp—the putative orthologous forms of P-450 3c in these species—are inducible *in vivo* not only by DEX and RIF but also by PB [47–49].

It should be noted here that although TAO and erythromycin are two potent inducers of P-450 3c *in vivo* [28, 29], neither increased accumulation nor *de novo* synthesis of this form—nor of the other forms tested in this work—in cultures, even at the highest concentration compatible with their solubility in the Waymouth medium, (10^{-4} M). Similar observation was recently reported on rat hepatocyte cultures with P-450 p, the putative orthologous form of P-450 3c in this species [45].

Effect of RIF and BNF on the accumulation of mRNAs specific for P-450 3c and 4 and 6

Full length cDNAs coding for P-450 3c (pLM3c 4.1) [29] P-450 4 (pLM4.1) and P-450 6 (pLM6.1) have been recently isolated and characterized. It appeared therefore of interest to determine whether

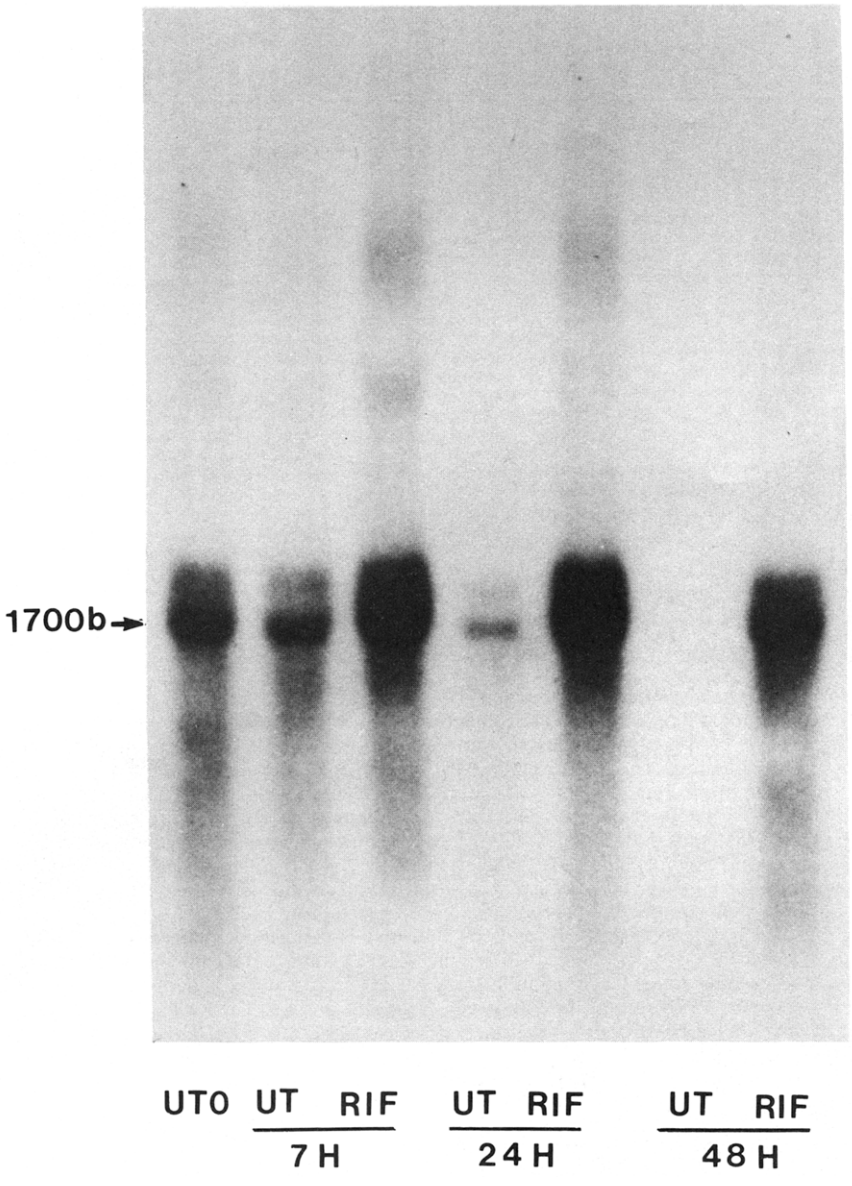

Fig. 5. Effect of rifampicin on the level of P-450 3c mRNA from primary culture of rabbit hepatocytes. Hepatocytes from an untreated rabbit were plated and maintained in primary culture in the absence (UT) or in the presence of 50 μM rifampicin (RIF). Just before plating (UTO) and at various intervals of time (7, 24 and 48 hr) after culture were started total RNA was extracted (30 plates). Seven μg (each lane) of RNA was submitted to electrophoresis on a 1% agarose gel, transferred to nitrocellulose filter and probed with radiolabeled [^{32}P]pLM3c-4.1 (10^7 cpm). The filter was autoradiographied for 16 hr at $-80°$.

the increase in accumulation and *de novo* synthesis of P-450 3c and 4 and 6 in the presence of RIF and BNF, respectively, was related to increased accumulation of their specific mRNA. For this purpose, rabbit hepatocytes prepared from uninduced animal were maintained in primary culture in the absence or in the presence of 50 μM RIF or BNF. In order to avoid the effect of repetitive addition of the inducer, the medium was not renewed in this series of experiments. At various intervals of time total RNA was extracted and analyzed by northern blots, probed either with [^{32}P]pLM3c 4.1 for P-450 3c mRNA or with [^{32}P]pLM6.1 for both P-450 4 and 6 mRNAs. Results are presented in Figs 5 and 6 respectively.

As already observed with poly(A) RNA from control or RIF induced rabbit liver [29], two distinct species of RNA of 1700 and 1850 bases cohybridizing with pLM3c-4.1 cDNA were present in the cultures. These mRNAs appeared to be constitutively expressed, both being detected in isolated hepatocytes and shortly after plating. The origin of the P-450 3c mRNA length heterogeneity as well as its implication in terms of regulation of P-450 3c are

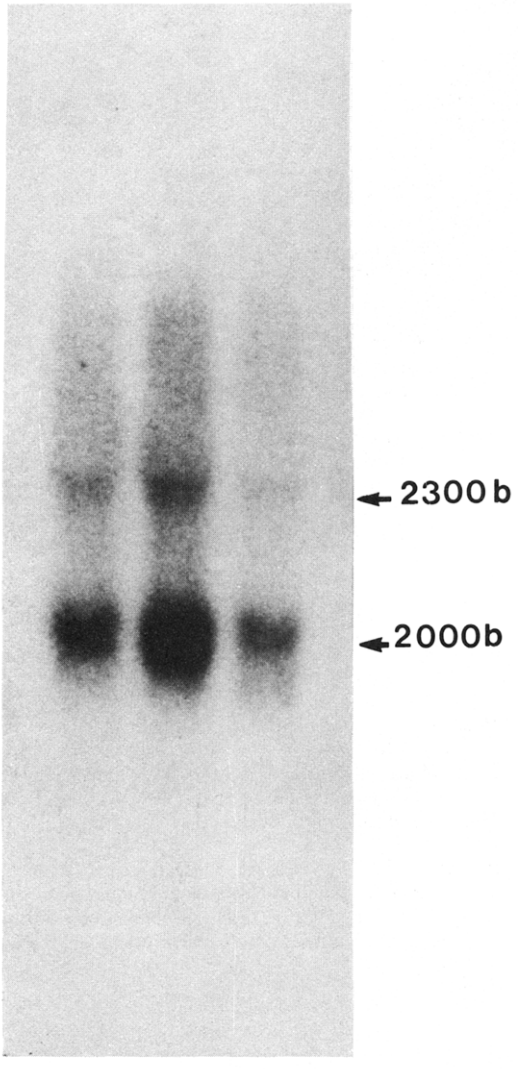

Fig. 6. Effect of B-naphthoflavone on the level of P-450 4 and 6 mRNAs from primary culture of rabbit hepatocytes. Hepatocytes from an untreated rabbit were plated and maintained in primary culture in the absence or in the presence of 50 μM B-naphthoflavone (BNF). Just before plating UTO and at various intervals of time (4, 8, 24 hr) after cultures were started total RNA was extracted (30 plates). Seven μg (each lane) of RNA was submitted to electrophoresis on a 1% agarose gel, transferred to nitrocellullose filter and probed with radiolabeled [^{32}P]pLM6.1 (10^7 cpm). The filter was autoradiographed for 16 hr at −80°.

not yet understood and are the subject of extensive investigation in this laboratory. The results presented in Fig. 5 suggest that both mRNAs decline at comparable rate in untreated cells (both are no longer detectable at 48 hr) and are similarly induced in the RIF treated cultures. These results are in good agreement with the data presented on P-450 3c accumulation in Fig. 2.

Cytochromes P-450 4 and 6 are two closely related forms which appear to exhibit 75% homology in their primary sequence [44]. Both P-450 4 and P-450 6 cDNAs have been shown to hybridize with two mRNAs of 2000 and 2300 bases coding respectively for forms 4 and 6. In the experiments reported in Fig. 6, pLM6-1, a plasmid containing a cDNA coding for P-450 6 was used as a probe. In contrast to form 3c both P-450 4 and 6 mRNAs were barely detectable before plating of the hepatocytes or in control cultures (not shown). However, in BNF induced cultures both P-450 4 and 6 mRNAs sharply accumulated, reaching a maximum at 8 hr of treatment. Their level then decreased at 24 hr. It seems reasonable to suggest that gene transcription has been sharply increased by BNF.

The data reported in Table 1 indicate that specific concentration of form 4 is several times higher than that of P-450 3c in untreated animals. This observation appears to be in disagreement with the data on corresponding mRNA level, comparing Figs 5 and 6 (untreated culture) and suggests that *in vivo*, half life of P-450 4 could be several times larger than that of P-450 3c. This seems to be also true in culture (Fig. 2).

Finally the results presented in this paper provide conclusive evidence in favor of the use of rabbit hepatocyte primary cultures to investigate the regulation and mechanism of induction of cytochromes P-450, as well as their pharmacological implications. In this respect, a study devoted to form P-450 3c is being currently carried out in our laboratory.

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