MOLECULAR PHARMACOLOGY, 27:125–132

Changes in the Concentration of Seven Forms of Cytochrome P-450 in Primary Cultures of Adult Rat Hepatocytes

A. RUTH STEWARD,[1] GHAZI A. DANNAN,[2] PHILIP S. GUZELIAN, AND F. PETER GUENGERICH

Division of Clinical Toxicology and Environmental Chemistry, Department of Medicine, Medical College of Virginia, Richmond, Virginia 23298 (A.R.S., P.S.G.) and Department of Biochemistry and Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 (G.A.D., F.P.G.)

Received March 29, 1984; Accepted September 11, 1984

SUMMARY

We prepared primary monolayer cultures of adult rat hepatocytes and measured the losses of cytochromes P-450 with the use of specific antibodies directed against purified forms of hepatic cytochrome P-450 which predominate in untreated rats (P-450$_{UT-A}$, P-450$_{UT-F}$) or in rats treated with phenobarbital (P-450$_{PB-B/D}$, P-450$_{PB-C}$, P-450$_{PB/PCN-E}$) or with 3-methylcholanthrene (P-450$_{\beta NF-B}$, P-450$_{\beta NF/ISF-G}$). In hepatocytes prepared from an untreated rat and incubated in control medium, total cytochrome P-450, measured spectrally as CO-binding hemoprotein, declined 68% during the first 72 hr in culture. However, the sum of the immunoreactive cytochromes P-450 declined only 24%, indicating that loss of heme rather than of protein accounts for much of the well-known loss of cytochromes P-450 in hepatocyte cultures. In cultures prepared from untreated rats or from rats treated with phenobarbital or with 3-methylcholanthrene, individual forms of cytochrome P-450 declined at markedly differing rates. Incubation of cultures in three different media previously reported to maintain levels of total cytochrome P-450 failed to prevent the decline in total cytochrome P-450 during the first 24 to 72 hr in culture. However, in cultures incubated in medium containing metyrapone, the level of holocytochrome P-450 was maintained at the initial value during the first 72 hr, apparently by preventing the net loss of cytochrome P-450 heme and by increasing the concentrations of immunoreactive P-450$_{PB/PCN-E}$ and P-450$_{\beta NF-B}$. Medium containing nicotinamide increased the proportion of P-450$_{\beta NF-B}$ relative to the other forms of cytochrome P-450, whereas cysteine-free medium increased P-450$_{UT-F}$. We conclude that loss of cytochrome P-450 in cultured hepatocytes involves loss of its heme moiety coupled with changes in the concentrations of the individual forms. Recognition of these changes as influenced by specific components of the culture medium is important when using primary hepatocyte cultures for study of xenobiotic metabolism and toxicity in the liver.

INTRODUCTION

In theory, the system of primary monolayer culture of adult rat hepatocytes provides unique advantages for studying the regulation of cytochrome P-450[3] and the role of this enzyme in toxic injury to the liver. The hepatocytes can be maintained as nondividing cells for several weeks in a chemically defined medium free of serum. Under these conditions, the cells retain the ability to carry out a diverse array of liver-specific functions, in many cases at rates comparable to those observed *in vivo* (1–3). However, we and others have found that, when freshly isolated hepatocytes are prepared by collagenase perfusion of liver and are placed into monolayer culture, the concentration of P-450 rapidly declines (for review, see Ref. 4). This loss is selective for P-450 in that the

This research was supported in part by Grants ES 01590, ES 00267, and AM18976 from the National Institutes of Health. P.S.G. is a recipient of Research Career Development Award K04-AM-00570 from the National Institutes of Health. A.R.S. is supported by National Institute of Environmental Health Sciences Grant 1T32ES07087.

[1] Present address: Great Lakes Laboratory, State University of New York College of Buffalo, 1300 Elmwood Avenue, Buffalo, NY 14222.

[2] Present address: Department of Pediatrics-Gastroenterology, Vanderbilt University Medical School, Nashville, TN 37232.

[3] The abbreviations used are: P-450, liver microsomal cytochrome P-450; ADH, equine liver alcohol dehydrogenase; NaDodSO$_4$, sodium dodecyl sulfate; PB, phenobarbital; MC, 3-methylcholanthrene; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetracetic acid; EGTA buffer, 25 mM Tricine buffer (pH 7.4) containing 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 0.14 M NaCl, 5.4 mM KCl, and 0.5 mM EGTA; Way R medium, standard culture medium (defined in text); Way D medium, Way R medium supplemented with bovine serum albumin and a complex mixture of hormones (defined in text) (8); Cys-free Williams medium E, Williams medium E devoid of cysteine and cystine and supplemented with 0.5 mM methionine and 0.1 mM 5-aminolevulinic acid.

0026-895X/85/010125-08$02.00/0

activities of other microsomal enzymes, including glucose-6-phosphatase, are maintained at the initial levels for many days (5). A number of investigators have claimed that this loss of total CO-binding hemoprotein can be prevented by modifying the culture medium (for review, see Ref. 4). However, measurements of P-450 by spectral methods do not establish whether these media prevent the loss of the heme moiety of the cytochrome, prevent the degradation of the protein moiety, or facilitate the synthesis of new holoenzyme. Moreover, even though it might be possible to prevent the decline in the concentration of total P-450 in culture, one cannot assume that the relative amounts of the individual forms of this enzyme remain the same in culture as *in vivo*. In fact, several studies have shown qualitative changes in the profile of P-450-dependent catalytic activities in primary hepatocyte cultures (5, 6).

In this study, we have reexamined changes in P-450 levels in hepatocyte culture with the use of immunochemical techniques to measure seven different polypeptide forms of P-450 which are prominent in the livers of untreated male rats ($P\text{-}450_{UT\text{-}A}$, $P\text{-}450_{UT\text{-}F}$) or in rats treated with PB ($P\text{-}450_{PB\text{-}B/D}$,[4] $P\text{-}450_{PB\text{-}C}$, $P\text{-}450_{PB/PCN\text{-}E}$) or with 3-MC ($P\text{-}450_{\beta NF\text{-}B}$, $P\text{-}450_{\beta NF/ISF\text{-}G}$) (7). We examined the effects of four different culture media which have been previously reported to maintain the level of P-450 in these cultures: Waymouth 752/1 medium supplemented with a mixture of hormones, fatty acids, and bovine serum albumin (8); Williams medium E supplemented with metyrapone (9), nicotinamide (10), or methionine and 5-aminolevulinic acid in the absence of cysteine and cystine (9). The results show that there is marked heterogeneity in the rates at which individual P-450 forms are lost in culture and that the conditions of cell culture exert dramatic and selective effects on the concentrations of some of the P-450 apoproteins and on the retention of their heme prosthetic group.

MATERIALS AND METHODS

Animals. Sprague-Dawley male rats (240–350 g) were obtained from Flow Laboratories (Dublin, VA) and were housed in wire-bottom cages and allowed free access to food and water. They were treated (intraperitoneally) with PB (in saline; 100 mg/kg) once each day for 4 days, with 3-MC (in corn oil; 20 mg/kg) once each day for 3 days, or with Aroclor 1254 (Monsanto Chemical Co.) (in corn oil; 150 mg/kg) once each day for 2 days. The animals were sacrificed 1 day (PB and MC) or 7 days (Aroclor 1254) following the last injection.

Chemicals and media. Unless otherwise indicated, all chemicals were obtained from Sigma (St Louis, MO). Bovine serum albumin (from Sigma) was essentially fatty acid-free.

The standard control medium (Way R) is a modification of Waymouth MB-752/1 prepared as previously described (11); it contains penicillin (100 units/ml) and is supplemented each day with insulin (1 μM) and L-ascorbate (0.3 mM). This medium was modified further (8, 12) by the omission of penicillin and the addition of 0.2% bovine serum albumin, gentamicin (20 mg/liter); insulin (0.1 μM), glucagon (0.01 μM), hydrocortisone (10 μM), D-thyroxine (10 μM), estradiol (1 μM), testosterone (1 μM), D-α-tocopherol (2 μM), linoleic acid (20 μM), oleic acid (20 μM), 5-aminolevulinic acid (1 μM), alanine (0.1 mM), serine (0.1 mM), asparagine (0.2 mM), and L-ascorbate (0.3 mM). This medium, designated Way D, also contained supplementary vitamins to make it equivalent to Waymouth MB 752/1. Way R and Way D contained no serum. Williams medium E was prepared as described (13) with the modifications of Paine *et al.* (9) to contain 5% fetal bovine serum (from GIBCO), hydrocortisone (0.1 mM), insulin (1 μM), and gentamicin (20 mg/liter). Williams medium E was further modified by the addition of 0.5 mM metyrapone or 25 mM nicotinamide as indicated.

Hepatocyte monolayer culture. Hepatocytes were isolated by a two-step *in situ* perfusion based on a modification (11) of the method of Seglen (14). Briefly, the liver was perfused with EGTA buffer, followed by single-pass perfusion with 400 ml of Way R medium containing collagenase (0.37 mg/ml; type I from Sigma). The softened liver was excised and was further digested for 10 min at 37° in medium containing dilute collagenase (0.2 mg/ml). The hepatocytes were separated from nonparenchymal cells by repeated centrifugation and washing with Way R medium as described (11). Hepatocytes were innoculated (3.5×10^6 cells/3 ml of medium) in plastic dishes (Lux Contur, 60-mm diameter) coated with 50 μg of collagen (Vitrogen 100 from Flow Laboratories). The viability of the cells, as assessed by the exclusion of trypan blue, was >90%. The medium was renewed every 24 hr. At the time points indicated, the monolayers were rinsed twice with Ca^{2+}-free 10 mM potassium phosphate buffer (pH 7.4) containing 0.14 M NaCl, removed from the dishes by scraping into the same buffer, pelleted, and resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 20% (v/v) glycerol (0.2 ml/culture). The cells were lysed by sonication and stored at −70°.

Preparation of homogenates and microsomes. When isolated hepatocytes and whole liver homogenates and microsomes were prepared from the same liver, a laparotomy was performed and the portal vein was cannulated; the liver was perfused with EGTA buffer. One lobe of the liver was then ligated, excised, placed in 4 volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.5), and homogenized with a Potter-Elvehjem homogenizer. The remainder of the liver was perfused with medium containing collagenase, and hepatocytes were isolated as described above. Microsomes were prepared from a portion of the liver homogenate by centrifugation of the 18,000 × *g* (15 min) supernatant for 60 min at 105,000 × *g*. The microsomal pellets were resuspended in 10 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA, 20% glycerol, and 20 μM butylated hydroxytoluene. Glycerol, EDTA, and butylated hydroxytoluene were added to the remaining homogenates to give final concentrations of 20%, 0.5 mM, and 20 μM, respectively. The liver perfusion was done at 37°. Tissue samples and cell lysates were kept on ice and then frozen and stored at −70°.

Assays. The concentrations of P-450 in purified enzyme preparations and in whole cell lysates were determined by $Na_2S_2O_4$-reduced CO difference spectroscopy using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ (15). Cell protein was determined by the method of Schacterle and Pollack (16) using bovine serum albumin as the standard.

Concentrations of individual P-450 isoenzymes were estimated immunochemically using modifications of methods described elsewhere (7, 17, 18). Briefly, cells were lysed in buffer containing $NaDodSO_4$ and proteins were separated by $NaDodSO_4$-polyacrylamide gel electrophoresis. The resolved proteins were electrophoretically transferred to sheets of nitrocellulose and the nitrocellulose sheets were treated sequentially (with intermediate washing) with rabbit antisera prepared to the P-450 being quantitated, *Staphylococcus aureus* protein A-horseradish peroxidase conjugate (Sigma), and a mixture of 3,3′-diaminobenzidine and H_2O_2 to visualize the P-450s as brown bands on a light brown background. The intensity of the visualized bands was determined by densitometry and integration using a Kontes Fiber-Optic scanner. Replicate determinations of a single sample agreed to within ±15%. In this work, internal standard curves were prepared by electrophoresing known amounts of ADH and each P-450 and carrying out the visualization procedure with rabbit anti-ADH antisera (1:200 dilution) added to the anti-P-450. Subsequently, 0.2 μg of purified ADH

[4] $P\text{-}450_{PB\text{-}B}$ and $P\text{-}450_{PB\text{-}D}$ were not distinguished in this work. These two forms have nearly identical mobilities in $NaDodSO_4$-polyacrylamide gel electrophoresis and are not distinguished by these immunochemical methods (7).

TABLE 1

Comparative concentrations of four P-450s in liver homogenates, microsomes, and freshly isolated hepatocytes obtained from untreated rats

In each case, liver homogenate, whole liver microsomes, and isolated hepatocytes were obtained from the same rat (see Materials and Methods).

P-450 form	Concentration of immunoreactive cytochrome P-450		
	Cell lysates	Liver homogenates	Liver microsomes
	pmol/mg protein		
$P\text{-}450_{UT\text{-}A}$	45 ± 3	39 ± 2	280 ± 40[a]
	(54 ± 4)	(54 ± 4)	(53 ± 11)[b]
$P\text{-}450_{PB\text{-}C}$	24 ± 3	23 ± 4	190 ± 60
	(29 ± 4)	(32 ± 6)	(36 ± 12)
$P\text{-}450_{PB/PCN\text{-}E}$	4.0 ± 0.5	3.5 ± 0.5	25 ± 3
	(5 ± 0.6)	(4.8 ± 0.8)	(5 ± 0.9)
$P\text{-}450_{UT\text{-}F}$	11 ± 2	7.0 ± 1.0	34 ± 3
	(13 ± 2)	(9.6 ± 1.5)	(6 ± 1)
Totals	84 ± 42	73 ± 5	529 ± 72

[a] Means ± SD ($n = 3$ rats).

[b] Percentage of total immunochemically determined P-450.

was added to each hepatocyte lysate aliquot which was electrophoresed, and anti-ADH was added to the anti-P-450 used for visualization.

RESULTS

Forms of P-450 identified in liver and in freshly isolated hepatocytes from untreated rats. The relative concentrations of the four forms of P-450 measured immunochemically in lysates of freshly isolated hepatocytes were similar to the concentrations in liver homogenates prepared from the same liver and in microsomes prepared from those homogenates (Table 1). $P\text{-}450_{UT\text{-}A}$ and $P\text{-}450_{PB\text{-}C}$ together comprised more than half of the immunochemically measurable P-450 in freshly isolated hepatocytes from untreated rats, while the other two detectable forms ($P\text{-}450_{UT\text{-}F}$ and $P\text{-}450_{PB/PCN\text{-}E}$) were present at lower concentrations (although $P\text{-}450_{UT\text{-}F}$ was unusually prominent in one cell preparation, i.e., Fig. 4). $P\text{-}450_{\beta NF/ISF\text{-}G}$, $P\text{-}450_{PB\text{-}B/D}$ and $P\text{-}450_{\beta NF\text{-}B}$ were undetectable (<8 pmol/mg total cell protein) in most of the cell preparations. Thus, we conclude that the procedures for preparation of isolated hepatocytes do not alter the P-450 isoenzyme profile. Furthermore, results obtained with cell lysates or homogenates are comparable to those obtained with microsomes.

Changes in P-450s with time in primary hepatocyte culture. Consistent with previously reported data (for review, see Ref. 4) in cultures prepared from untreated rats, total P-450 (measured spectrally as CO-binding hemoprotein) declined and after 72 hr in control Way R medium had fallen to 32% of the initial value (Table 2). However, the sum of the P-450 proteins measured immunochemically declined during this 72 hr in culture proportionally less, only to 76% of the initial value. This change was not expressed uniformly among the individual P-450s. Whereas the levels of $P\text{-}450_{PB/PCN\text{-}E}$ and $P\text{-}450_{PB\text{-}C}$ changed little during the first 72 hr in culture, $P\text{-}450_{UT\text{-}A}$ increased while $P\text{-}450_{UT\text{-}F}$ decreased rapidly during the first 24 hr to 12% of its initial value and remained at this diminished level (Fig. 2). Although considerable variability was observed from one cell preparation to another, both in the initial levels of the P-450 formed and in the rates at which they declined in cultures (legend to Fig. 1), in all cases, $P\text{-}450_{UT\text{-}A}$ increased with time in culture relative to the other forms. Therefore, $P\text{-}450_{UT\text{-}A}$ became the predominant form in 72-hr cultures.

TABLE 2

Changes with time in culture in the levels of spectrally determined holocytochrome P-450 versus total immunoreactive P-450 in cultured rat hepatocytes

Hepatocytes were isolated from an untreated rat (Figs. 1 and 4) or from rats treated with PB (Fig. 2), MC (Fig. 3), or Aroclor 1254 (Fig. 5) and were incubated in control medium. At the indicated times, cultures were taken for measurement of spectral or immunochemical P-450. Additional data are given in Table 3 for the untreated rats (Experiments 1 and 2) and the Aroclor-treated rat (Experiment 1).

Treatment of rat	Age of cultures	Experiment No.	Spectral P-450 (A)	Total immunochemical P-450 (B)	Ratio (A/B)
	hr		*pmol/mg protein*	*pmol/mg protein*	
Untreated	0	1	220	170	1.29
		2	200	ND[a]	
		3	256	390	0.66
	24	1	100 (45)[b]	120 (71)	0.83
		2	94 (48)	ND	
	72	1	70 (32)	130 (76)	0.54
		2	39 (20)	ND	
PB	0	1	420	980	0.43
	24	1	120 (29)	890 (91)	0.14
MC	0	1	420	630	0.67
	24	1	330 (79)	350 (56)	0.95
Aroclor 1254	0	1	510	770	0.66
		2	450	ND	
	24	1	360 (71)	1020 (132)	0.35
		2	330 (73)	ND	

[a] ND, not determined.

[b] Numbers in parentheses represent percentages of zero-hr values.

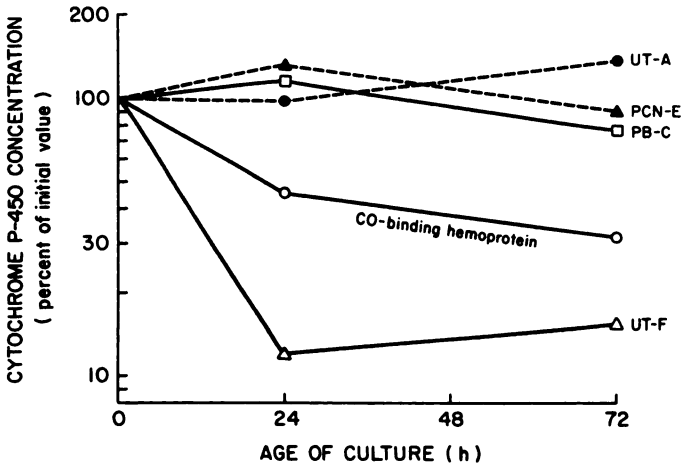

FIG. 1. *Changes in concentration of total and specific cytochromes P-450 in cultures of hepatocytes from an untreated rat*

Freshly isolated hepatocytes were prepared from the rat of Table 2 (Experiment 1) by collagenase perfusion and were inoculated into monolayer culture (time 0) in control medium Way R. At time 0 and at the indicated times, freshly isolated hepatocytes and some of the monolayer cultures were harvested and whole cell lysates were prepared for spectral determination of CO-binding hemoprotein and for immunochemical determination of specific forms of P-450 (see Materials and Methods). The data are given as percentages of the initial values at time 0 which were (in picomoles/mg of cell protein): CO-binding hemoprotein, 220; P-450$_{UT\text{-}A}$ (*UT-A*), 70; P-450$_{PB/PCN\text{-}E}$ (*PCN-E*), 17; P-450$_{PB\text{-}C}$ (*PB-C*), 20; P-450$_{UT\text{-}F}$ (*UT-F*), 63. Initial concentrations (picomoles/mg of cell protein) and final values after 72 hr (percentage of initial concentration) for hepatocytes from three rats were (mean ± SE): P-450$_{UT\text{-}A}$, 73 ± 20, 134% ± 51%, P-450$_{PB\text{-}C}$, 76 ± 46, 53% ± 22%; P-450$_{PB/PCN\text{-}E}$, 23 ± 8, 35% ± 24%; P-450$_{UT\text{-}F}$, 37 ± 27, 18% ± 2% (n = 2); CO-binding hemoprotein, 208 ± 11, 26% ± 6% (n = 2).

Several forms of P-450 are not readily detectable in hepatocytes (or in microsomes) prepared from untreated rats. Therefore, isolated hepatocytes were prepared from rats pretreated with PB or MC and were incubated in Way R medium in the absence of the inducing chemical. As expected from previously reported data obtained with liver microsomes (7), a fraction of the P-450 protein measured immunochemically in freshly isolated hepatocytes from these induced rats was devoid of heme, as indicated by ratios (less than 1.0) of spectral to total immunoreactive P-450 (Table 2). Comparisons between total immunoreactive P-450 in hepatocytes from untreated versus induced rats must be made with caution since the apocytochrome content of the standards may not be the same from one form to another. However, comparisons of changes with time in a given culture are less subject to this limitation. Thus, when hepatocytes from the PB-treated rat were incubated in culture for 24 hr there was a dramatic decline in spectrally measured holocytochrome P-450 (to 29% of the initial value), but little change in total immunoreactive P-450 and, hence, a decrease in the ratio of spectral to total immunoreactive P-450 (from 0.43 to 0.14), indicating a loss of heme from the holocytochrome. In contrast, in cultures of hepatocytes prepared from an MC-treated rat, the proportion of holocytochrome actually increased during the first 24 hr of culture (from 0.67 to 0.96), due to a small decrease in holocytochrome P-450 (to 79% of the initial value) accompanied by a larger decrease in total immunoreactive P-450 (to 56% of the initial value). Inspection of Figs. 2 and 3 reveals that under standard conditions of cell culture the concentrations of the individual forms of cytochrome P-450 decline at markedly different rates.

Effects of culture media on the cytochrome P-450 con-

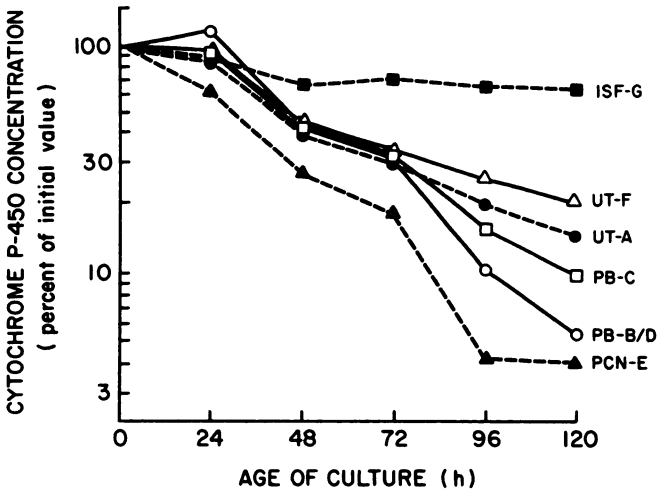

FIG. 2. *Changes in concentration of specific P-450s in cultures of hepatocytes from a PB-treated rat*

Freshly isolated hepatocytes were prepared from a single rat (Table 2) and were inoculated into monolayer culture (time 0) in control medium Way R (containing no PB). At the indicated time, cells were harvested and whole cell lysates were prepared for immunochemical determination of the specific forms of P-450. Data are given as percentages of initial (time 0) values which were (in picomoles/mg of cell protein): P-450$_{\beta NF/ISF\text{-}G}$ (*ISF-G*), 10; P-450$_{UT\text{-}F}$ (*UT-F*), 10; P-450$_{UT\text{-}A}$ (*UT-A*), 40; P-450$_{PB\text{-}C}$ (*PB-C*), 400; P-450$_{PB\text{-}B/D}$ (*PB-B/D*), 250; P-450$_{PB/PCN\text{-}E}$ (*PCN-E*), 250.

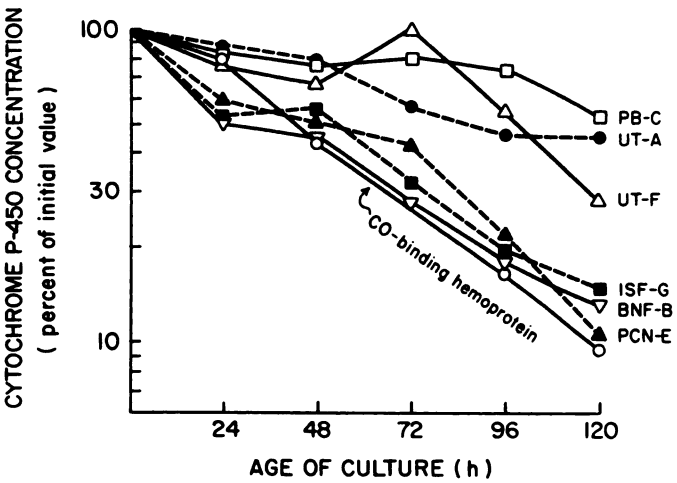

FIG. 3. *Changes in concentration of specific P-450s in cultures of hepatocytes from a rat treated with MC*

Freshly isolated hepatocytes were prepared from a single rat (Table 2) and were inoculated into monolayer culture (time 0) in control medium Way R (containing no MC). At the indicated times, cells were harvested and whole cell lysates were prepared for the spectral determination of the specific forms of P-450. Data are given as percentages of initial (time 0) values which were (in picomoles/mg of cell protein): P-450$_{PB\text{-}C}$ (*PB-C*), 15; P-450$_{UT\text{-}A}$ (*UT-A*), 16; P-450$_{UT\text{-}F}$ (*UT-F*), 20; P-450$_{\beta NF/ISF\text{-}G}$ (*ISF-G*), 91; P-450$_{\beta NF\text{-}B}$ (*BNF-B*), 453; P-450$_{PB/PCN\text{-}E}$ (PCN-E), 28.

tent of cultured hepatocytes. We tested media previously reported to prevent the loss of P-450 in primary hepatocyte cultures for their effects on loss not only of holocytochrome but also on loss of specific forms of apocytochrome P-450 in cultures prepared from untreated rats, or from rats treated with Aroclor 1254, a mixture of polychlorinated biphenyls. Of these media, only Williams medium E supplemented with metyrapone maintained the level of spectrally determined P-450 at 80–110% of the initial level for up to 72 hr (Table 3), whereas the values in cultures incubated in Way D, nicotinamide-supplemented Williams medium E, or Cys-free Williams medium E were in the same range as the values in control cultures incubated in Way R or Williams E medium and fell to 36–67% of the initial levels after 24 hr in culture (Table 3). In cultures prepared from an untreated rat and incubated in control medium, the ratio of spectrally measurable P-450 to total immunoreactive P-450 decreased, whereas in cultures incubated in metyrapone-supplemented Williams medium E this ratio was maintained at the initial value of 1.3. A ratio larger than 1.0 indicates either that there are additional forms of P-450 in the untreated rat which were not quantified in our immunochemical assays, or that the concentration of apo-P-450 in our form-specific standards was underestimated (7). Thus, in the absence of metyrapone, the well-known decline in P-450 which occurs with time in culture appears to represent primarily a loss of heme and only secondarily a loss of apocytochrome.

TABLE 3

The effects of various culture media on changes in spectrally determined holocytochrome P-450 and total immunoreactive P-450 occurring with time in hepatocyte cultures prepared from an untreated rat and a rat treated with Aroclor 1254

Experimental details are given in Table 2 for untreated rats and for a rat treated with Aroclor 1254. Induction protocols and culture media are described in Materials and Methods.

Culture medium	Age of cultures	Spectral P-450 (A)	Total immunochemical P-450 (B)	Ratio A/B
	hr	*pmol/mg protein*	*pmol/mg protein*	
I. Untreated rat	0	220 (100)[a]	170 (100)	1.30
		200 (100)	ND[b]	
Way R	24	100 (45)	120 (71)	0.83
		95 (48)	ND	
	72	70 (32)	130 (76)	0.54
Way D	24	110 (50)	110 (65)	1.00
		100 (50)	ND	
	72	60 (27)	110 (65)	0.55
Williams E	24	130 (59)	150 (88)	0.87
	72	70 (32)	110 (65)	0.64
Williams E plus metyrapone	24	180 (82)	130 (76)	1.34
	72	230 (105)	180 (106)	1.28
Williams E plus nicotinamide	24	140 (64)	140 (82)	1.00
	72	90 (41)	120 (71)	0.75
Cys-free Williams E	24	110 (50)	140 (82)	0.79
	72	80 (36)	150 (88)	0.53
II. Rat treated with Aroclor 1254	0	510 (100)	770 (100)	0.66
Way R	72	240 (47)	570 (74)	0.42
Way D	72	230 (45)	710 (92)	0.32
Williams E	72	240 (47)	610 (79)	0.39
Williams E plus metyrapone	72	570 (112)	710 (92)	0.80
Williams E plus nicotinamide	72	340 (67)	520 (68)	0.65
Cys-free Williams E	72	320 (63)	620 (81)	0.52

[a] Numbers in parentheses represent percentages of zero-hr values.
[b] ND, not determined.

Even though most of the culture media examined failed to prevent fully the loss of total spectrally measured P-450, these media did exert selective effects on three specific forms of P-450. The same effects were observed in cultures prepared from an untreated (Fig. 4) and an Aroclor 1254-treated rat (Fig. 5). First, incubation of the cultures in Cys-free Williams medium E prevented the dramatic decline in the concentration of $P\text{-}450_{UT\text{-}F}$ observed in hepatocyte cultures incubated in other media. Second, incubation of cultures in the presence of metyrapone for 72 hr resulted in 2.5–3.0-fold increases in the levels of $P\text{-}450_{PB/PCN\text{-}E}$ as compared to the level of this form in cultures incubated in control Williams medium E. This effect was also observed in an experiment in which hepatocyte cultures from an untreated rat were incubated in our standard Way R medium in the presence or absence of metyrapone (0.1 mM) (data not shown). Third, addition of metyrapone or nicotinamide to the medium increased the level of $P\text{-}450_{\beta NF\text{-}B}$. In cultures prepared from an untreated rat, this increase after 72 hr was small (final level, 8–15 pm/mg of cell protein, compared to <2 pm/mg in cultures incubated in control medium). In cultures from a rat pretreated with Aroclor 1254, on the other hand, treatment with metyrapone or nicotinamide greatly increased the level of $P\text{-}450_{\beta NF\text{-}B}$ to a value 90-110 pm/mg over and above the level in cultures incubated in control medium. Despite the similar effects of these two additives on $P\text{-}450_{\beta NF\text{-}B}$, nicotinamide, unlike metyrapone, had no effect on the level of $P\text{-}450_{PB/PCN\text{-}E}$ (Fig. 5). Thus, the isoenzyme profiles of P-450 in the hepatocytes change markedly with time and depend on the age of the culture and the composition of the culture medium.

Comparison of spectrally detectable binding of metyrapone to P-450s with changes in the isoenzyme profiles occurring in hepatocyte culture. Others have proposed that metyrapone "maintains" P-450 levels in hepatocyte cultures by forming a complex with the heme moiety of the cytochrome (19, 20). Hence, it seemed possible that selective binding of metyrapone to $P\text{-}450_{PB/PCN\text{-}E}$ and $P\text{-}450_{BNF\text{-}B}$ could account for the increased concentrations of these forms following incubation of hepatocyte cultures with metyrapone-supplemented Williams medium E. However, in testing this idea with purified P-450s incubated with metyrapone (at concentrations up to 10–40 μM) *in vitro*, we found that spectrally detectable complexes (in either the oxidized or reduced difference spectrum between 390 and 450 nm) were found only with $P\text{-}450_{PB\text{-}B}$, $P\text{-}450_{PB\text{-}D}$, and $P\text{-}450_{\beta NF/ISF\text{-}G}$ data not shown)

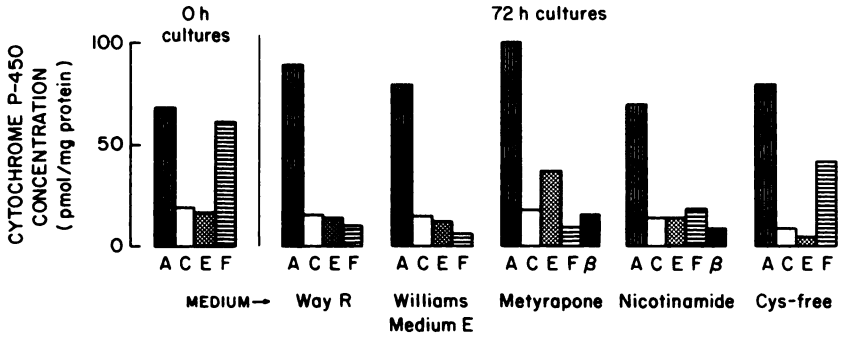

FIG. 4. *Effects of various media on the levels of immunospecific P-450s in hepatocyte cultures from an untreated rat*

Freshly isolated hepatocytes were prepared from a single rat (Fig. 1; Tables 2 and 3, Experiment 1) and were inoculated into monolayer culture (time 0) in the medium indicated: Way R and Williams Medium E, control media (see Materials and Methods); metyrapone, Williams Medium E supplemented with 0.5 mM metyrapone; nicotinamide, Williams Medium E supplemented with 25 mM nicotinamide; Cys-free, cysteine and cystine-free Williams Medium E supplemented with 0.5 mM methionine and 0.1 mM 5-aminoelevulinic acid. At the indicated times, cells were harvested and whole cell lysates were prepared for the immunochemical determination of the specific forms of P-450 designated as follows: A, P-450$_{UT-A}$; C, P-450$_{PB-C}$; E, P-450$_{PB/PCN-E}$; F, P-450$_{UT-F}$.

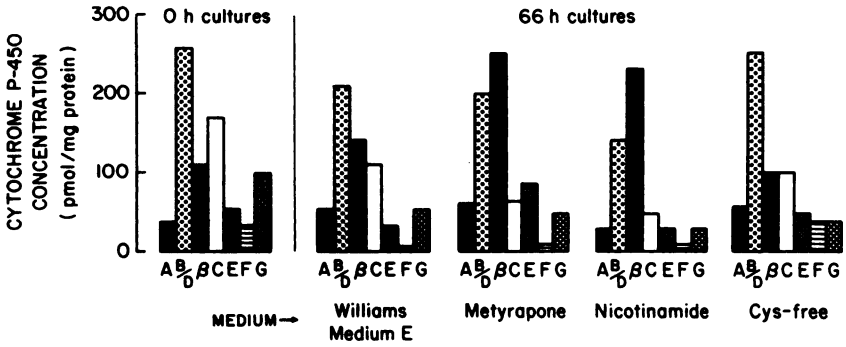

FIG. 5. *Effects of various media on the levels of immunospecific P-450s in hepatocyte cultures prepared from a rat treated with Aroclor 1254*

Freshly isolated hepatocytes were prepared from a single rat (Tables 2 and 3, Experiment 1) and were inoculated into monolayer culture (time 0) in the medium indicated (see Fig. 4). At the indicated times, cells were harvested and whole cell lysates were prepared for the immunochemical determination of the specific forms of P-450 designated as follows: A, P-450$_{UT-A}$; B/D, sum of P-450$_{PB-B}$ and P-450$_{PB-D}$; β, P-450$_{\beta NF-B}$; C, P-450$_{PB-C}$; E, P-450$_{PB/PCN-E}$; F, P-450$_{UT-F}$; G, P-450$_{\beta NF/ISF-G}$.

despite the fact that none of these cytochromes increased in hepatocyte cultures exposed to metyrapone (Fig. 5). On the other hand, incubation of purified cytochromes P-450$_{PB/PCN-E}$ and P-450$_{\beta NF-B}$ with metyrapone failed to form spectrally detectable complexes whereas these two forms did increase in cultures incubated in medium containing metyrapone (Figs. 4 and 5). Thus, the ability of metyrapone to bind to certain purified cytochromes does not appear to account for its ability to stimulate the selective accumulation of these P-450s in the intact hepatocyte.

DISCUSSION

Many laboratories are working to devise a chemically defined culture medium which effectively maintains the concentration of P-450 in primary hepatocyte cultures at levels comparable to those in the liver *in vivo*. Progress has been problematic. Successes reported by one laboratory have not been reproduced by others (11, 21, 22), due perhaps to differences among strains of animals and differences among laboratories in cell isolation procedure, culture techniques, and analytical methods. Of the experimental "maintenance" media tested in this investigation (Way D, Williams media E supplemented with metyrapone or nicotinamide, and Cys-free Williams medium E) only metyrapone-supplemented Williams medium E prevented the well-known decline in CO-binding hemoprotein. In line with previous suggestions that qualitative changes in the P-450 isoenzyme profile may also occur with time in culture (5, 6), we found that individual immunoreactive forms of P-450 differ in their response to the conditions of cell cultures. Therefore, lack of change in the concentration of spectrally determined P-450 is not an adequate criterion for demonstrating that cultured hepatocytes have retained the same phenotypic expression of the P-450s as is present in the parent liver. For example, despite the fact that in the presence of metyrapone both total immunoreactive P-450 and heme content of the P-450 proteins were maintained in culture, significant changes in the profile of the P-450s still occurred. Therefore, uncritical use of maintenance media for such purposes as toxicity or carcinogenicity testing in the hepatocyte culture system may give misleading results.

Immunochemical determination of individual forms of P-450 provides a practical new way to examine in greater detail the changes in P-450 content which accompany transfer of the hepatocyte from the living animal to the

conditions of cell culture. Previously, changes in the levels of different forms of P-450 in cell culture were inferred from spectral determinations plus enzyme assays using multiple substrates. For example, Fahl *et al.* (6) reported increased prominence with time in culture of a form of P-450 which resembled "P-448" in the profile of benzopyrene metabolites it produced but which differed from P-448 in its spectral properties. Antibodies provide a less ambiguous way to identify separate forms of P-450, to examine the changes in their concentrations in cell culture, and, with the use of immunoprecipitation, to measure their rates of *de novo* synthesis and degradation (23, 24). Even from the present, limited number of observations surveying seven forms of P-450, it is clear that the forms display fascinating nonuniformity in their response to culture conditions that will require more extensive future study on a form by form basis. For example, P-450$_{UT\text{-}F}$ may be uniquely sensitive to the toxic effects of cysteine (25). Cys-free Williams medium E prevented the loss of P-450$_{UT\text{-}F}$ (Figs. 4 and 5), but not the loss of total P-450 heme (Table 3), even though this medium contains a high concentration of 5-aminolevulinic acid which stimulates heme synthesis (26).

Metyrapone is a well-known inhibitor of P-450 catalytic activity. We found that it also exerts noteworthy effects on the metabolism of the P-450 molecule. This investigation shows that the effects of metyrapone are complex and involve at least two distinctly different mechanisms. First, metyrapone prevents the loss of heme from P-450s, and, second, metyrapone enhances the accumulation of P-450$_{\beta NF\text{-}B}$ and P-450$_{PB/PCN\text{-}E}$. Because administration of metyrapone to $CoCl_2$-treated rats prevented loss of spectrally determined P-450 even when heme oxygenase activity was high (20), it was proposed that metyrapone forms ligand bonds with P-450 and thus protects the cytochrome from degradation. We found no positive correlation between high affinity binding of metyrapone to purified P-450 proteins (as measured spectrally) and increased levels of these proteins in cultured hepatocytes. Nevertheless, at the concentration of metyrapone present in the culture medium (0.5 mM), lower affinity binding of metyrapone to P-450s may occur (27), and this could prevent the loss of heme from cytochrome P-450. Alternatively, metyrapone may have increased the rate of heme synthesis by increasing the rate of synthesis of aminolevulinic acid synthase (20), the rate-limiting enzyme.

It will be noted that in the present study we have found readily detectable amounts of P-450$_{PB/PCN\text{-}E}$ in hepatocytes isolated from untreated rats. This is in contrast to a previous report from this laboratory in which we were unable to detect this form in cultures prepared from untreated female rats (28). The reason for this apparent discrepancy is that P-450$_{PB/PCN\text{-}E}$ appears to be a male-specific form of cytochrome P-450. In liver microsomes from untreated female rats, the concentration of P-450$_{PB/PCN\text{-}E}$ is consistently undetectable (<15 pmol/mg of microsomal protein) by either Western blot immunoquantitation[5] or by radial immunodiffusion (28). In sexually mature untreated male rats, on the other hand, which were used in the present study, P-450$_{PB/PCN\text{-}E}$ is consistently present at measurable levels, averaging around 80–200 pmol/mg of microsomal protein, although values as low as 25 pmol/mg of microsomal protein have also been observed (Table 1).

The accumulation of P-450$_{\beta NF\text{-}B}$ in hepatocyte cultures treated with either metyrapone or nicotinamide corroborates a previous report (29) that in hepatocyte cultures metyrapone induces *O*-deethylation of 7-ethoxyresorufin, an activity specifically catalyzed by P-450$_{\beta NF\text{-}B}$ (7). In cultures from an Aroclor 1254-treated rat, the effects of these media on the regulation of P-450$_{\beta NF\text{-}B}$ appear complex (Fig. 5). The apparent induction of P-450$_{\beta NF\text{-}B}$ in cultures of hepatocytes from a rat pretreated with Aroclor 1254 and then treated in culture with metyrapone (or nicotinamide), is far greater than the sum of the separate effects of metyrapone (or nicotinamide) (Fig. 4) and Aroclor (control cultures of Fig. 5). This synergy suggests that metyrapone and nicotinamide may be acting by mechanisms different from that of the polychlorinated biphenyl isomers which presumably bind to a cytosolic receptor (the *Ah* receptor) (30) and stimulate the transcription of specific mRNA. One possibility is that metyrapone and nicotinamide specifically inhibit the degradation of P-450$_{\beta NF\text{-}B}$. The system of primary monolayer hepatocyte culture is well suited for examining such effects and for making comparisons of the control of P-450s in cultures prepared from various species including humans. Such information is urgently needed by those seeking cell culture systems for toxicity testing.

[5] F. P. Guengerich, unpublished data.

ACKNOWLEDGMENTS

We thank M. V. Martin for assistance in preparation of the P-450s and antisera and Ellen F. Cardlin for preparing the isolated hepatocytes.

REFERENCES

1. Bissell, D. M., L. E. Hammaker, and U. A. Meyer. Parenchymal cells from adult rat liver in nonproliferating monolayer culture. *J. Cell Biol.* **59**:722–734 (1973).
2. Savage, C. R., and R. J. Bonney. Extended expression of differentiated function in primary cultures of adult liver parenchymal cells maintained on nitrocellulose filters. *Exp. Cell Res.* **114**:307–315 (1978).
3. Lin, R. C., and P. J. Snodgrass. Primary culture of normal adult rat liver cells which maintain stable urea cycle enzymes. *Biochem. Biophys. Res. Commun.* **64**:725–734 (1975).
4. Sirica, A. E., and H. C. Pitot. Drug metabolism and effects of carcinogens in cultured hepatic cells. *Pharmacol. Rev.* **31**:205–228 (1980).
5. Guzelian, P. S., D. M. Bissell, and U. A. Meyer. Drug metabolism in adult rat hepatocytes in primary monolayer culture. *Gastroenterology* **72**:1232–1239 (1977).
6. Fahl, W. E., G. Michalopoulos, G. L. Sattler, C. R. Jefcoate, and H. C. Pitot. Characteristics of microsomal enzyme controls in primary cultures of rat hepatocytes. *Arch. Biochem. Biophys.* **192**:61–72, 1979.
7. Guengerich, F. P., G. A. Dannan, S. T. Wright, M. V. Martin, and L. S. Kaminsky. Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**:6019–6030 (1982).
8. Decad, G. M., D. P. H. Hsieh, and J. L. Byard. Maintenance of cytochrome P-450 and metabolism of aflatoxin B_1 in primary hepatocyte cultures. *Biochem. Biophys. Res. Commun.* **78**:279–287 (1977).
9. Paine, A. J., L. J. Hockin, and C. M. Allen. Long term maintenance and induction of cytochrome P-450 in rat liver cell culture. *Biochem. Pharmacol.* **31**:1175–1178 (1982).
10. Villa, P., L. J. Hockin, and A. J. Paine. The relationship between the ability of pyridine and substituted pyridines to maintain cytochrome P-450 and inhibit protein synthesis in rat hepatocyte cultures. *Biochem. Pharmacol.* **29**:1773–1777 (1980).

11. Bissell, D. M., and P. S. Guzelian. Phenotypic stability of adult rat hepatocytes in primary monolayer culture. *Ann. N. Y. Acad. Sci.* **349**:85–98 (1980).
12. Salocks, C. B., D. P. H. Hsieh, and J. L. Byard. Butylated hydroxytoluene pretreatment protects against cytotoxicity and reduces covalent binding of aflatoxin B_1 in primary hepatocyte cultures. *Toxicol. Appl. Pharmacol.* **59**:331–345 (1981).
13. Williams, G. M., E. K. Weisburger, and J. H. Weisburger. Isolation and long-term cell culture of epithelial-like cells from rat liver. *Exp. Cell Res.* **69**:106–112 (1971).
14. Seglen, P. O. Preparation of isolated rat liver cells. *Methods Cell Biol* **13**:29–83 (1976).
15. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J. Biol. Chem.* **239**:2379–2385 (1964).
16. Schacterle, G. R., and R. L. Pollack. A simplified method for the quantitative assay of small amounts of protein in biologic material. *Anal. Biochem.* **51**:654–655 (1973).
17. Guengerich, F. P., P. Wang, and N. K. Davidson. Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits, and humans using immunochemical staining coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochemistry* **21**:1698–1706 (1982).
18. Dannan, G. A., and F. P. Guengerich. Immunochemical comparison and quantitation of microsomal flavin-containing monooxygenases in various hog, mouse, rat, rabbit, dog and human tissues. *Mol. Pharmacol.* **22**:787–794 (1982).
19. Paine, A. J., P. Villa, and L. J. Hockin. Evidence that ligand formation is a mechanism underlying the maintenance of cytochrome P-450 in rat liver cell culture. *Biochem. J.* **188**:937–939 (1980).
20. Hockin, L. J., and A. J. Paine. The role of 5-aminolaevulinate synthase, haem oxygenase and ligand formation in the mechanism of maintenance of cytochrome P-450 concentration in hepatocyte culture. *Biochem. J.* **210**:855–857 (1983).
21. Holme, J. A., E. Soderlund, and E. Dybing. Drug metabolism activities of isolated rat hepatocytes in monolayer culture. *Acta Pharmacol. Toxicol.* **52**:348–356 (1983).
22. Dickins, M., and R. E. Peterson. Effects of a hormone-supplemented medium on cytochrome P-450 content and mono-oxygenase activities of rat hepatocytes in primary culture. *Biochem. Pharmacol.* **29**: 1231–1238 (1980).
23. Newman, S. L., J. L. Barwick, N. A. Elshourbagy, and P. S. Guzelian. Measurement of the metabolism of cytochrome P-450 in cultured hepatocytes by a quantitative and specific immunochemical method. *Biochem. J.* **204**:281–290 (1982).
24. Schuetz, E. G., S. A. Wrighton, J. L. Barwick, and P. S. Guzelian. Induction of cytochrome P-450 by glucocorticoids in rat liver. I. Evidence that glucocorticoids and pregnenolone-16α-carbonitrile regulate *de novo* synthesis of a common form of cytochrome P-450 in cultures of adult rat hepatocytes and in the liver *in vivo*. *J. Biol. Chem.* **259**:1999–2006 (1984).
25. Vina, J., G. T. Saez, D. Wiggins, A. F. C. Roberts, R. Hems, and H. A. Krebs. The effect of cysteine oxidation on isolated hepatocytes. *Biochem. J.* **212**:39–44 (1983).
26. Guzelian, P. S., and D. M. Bissell. Effects of cobalt on synthesis of heme and cytochrome P-450 in the liver. *J. Biol. Chem.* **251**:4421–4427 (1976).
27. Mitani, F., E. A. Shephard, I. R. Phillips, and B. R. Rabin. Complexes of cytochrome P-450 with metyrapone: a convenient method for the quantitative analysis of phenobarbital-inducible cytochrome P450 in rat liver microsomes. *FEBS Lett.* **148**:302–306 (1982).
28. Heuman, D. M., E. J. Gallagher, J. L. Barwick, N. A. Elshourbagy, and P. S. Guzelian. Immunochemical evidence for induction of a common form of hepatic cytochrome P-450 in rats treated with pregnenolone-16α-carbonitrile or other steroidal or non-steroidal agents. *Mol. Pharmacol.* **21**:753–760 (1982).
29. Lake, B. G., and A. J. Paine. The effect of hepatocyte culture conditions on cytochrome P-450 linked drug metabolising enzymes. *Biochem. Pharmacol.* **31**:2141–2144 (1982).
30. Poland, A., W. F. Greelee, and A. S. Kende. Studies on the mechanism of action of the chlorinated dibenzo-*p*-dioxins and related compounds. *Ann. N. Y. Acad. Sci.* **320**:214–230 (1979).

Send reprint requests to: either F. Peter Guengerich, Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37232 or Philip S. Guzelian, Box 267, MCV Station, Medical College of Virginia, Richmond, VA 23298-0001.