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Aryl Hydrocarbon Hydroxylase Induction in Mammalian Liver-Derived Cell Cultures

Stimulation of "Cytochrome $P_1$450-Associated" Enzyme Activity by Many Inducing Compounds

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SUMMARY

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Increases in aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase activity are found in fetal rat liver primary cultures and in cell lines derived from rat Reuber Hepatoma H-35 or mouse Hepatoma BW 7756, when the cells are treated with any one of a large number of hydrophobic compounds in the growth medium. Compounds which are effective inducers include 3-methylcholanthrene, sodium phenobarbital, isoproterenol, α-naphthoflavone, β-naphthoflavone, 2,5-diphenyloxazole, 2-(4'-chlorophenyl)benzothiazole, 2-(4'-formylphenyl)benzothiazole, metyrapone, 1-(2-isopropylphenyl)imidazole, 2-diethylaminoethyl-2,2-diphenyl valerate HCl (SKF 525-A), piperonyl butoxide, diethylstilbestrol, sodium laurate, allylisopropylacetamide, aniline, and aminopyrine. Regardless of which inducer is used, the hydroxylase activity found in liver- or hepatoma-derived cell cultures seems to be (a) particularly sensitive to such inhibitors *in vitro* as α-naphthoflavone or 2,5-diphenyloxazole, (b) less inhibited *in vitro* by such drugs as metyrapone or SKF 525-A, and (c) associated with a blue spectral shift in the Soret peak of the reduced hemoprotein-CO complex. The hydroxylase activity in control cultures and the hydroxylase activity induced by phenobarbital in liver- or hepatoma-derived cell cultures thus differ from the hepatic enzyme activities in the control and phenobarbital-treated intact animal; our findings presumably indicate that cytochrome $P_1$450 is induced by some factor in the control medium or by phenobarbital in culture, whereas other P450 species predominate in the control or phenobarbital-treated adult intact animal. Certain compounds are known to be metabolized differently by $P_1$450 than by P450. For studies with liver- or hepatoma-derived cultures and concerning evaluation of cytotoxicity, drug metabolism, or chemical carcinogenesis by certain compounds known to be metabolized *via* the monooxygenase metabolic pathways, we therefore suggest caution in the extrapolation of certain results found in cultured cells to the actual situation existing in the intact animal.

Some of this work was presented at the Meeting of the American Society for Pharmacology and Experimental Therapeutics, Atlantic City, N. J., April 1973 (1).

INTRODUCTION

In the previous papers of this series, aryl hydrocarbon hydroxylase activity[1] in fetal rat hepatocytes in culture was shown (2–4) to be stimulated by PB,[2] the insecticide 2,2 - bis(*p*-chlorophenyl) - 1,1,1 - trichloroethane (*p,p'*-DDT), benz[*a*]anthracene, MC, or biogenic amines such as norepinephrine and isoproterenol. PB, MC, and biogenic amines all produce rises in the hydroxylase activity that are additive or synergistic when two or three of these types of inducers are combined in the culture medium (2, 4). The action of PB, polycyclic hydrocarbons, and biogenic amines on the hydroxylase induction appears to be transcriptional,[3] because each of the induction processes is inhibited when actinomycin D is added simultaneously with the inducer initially (3, 4). With PB, MC, or norepinephrine, there is also a post-translational effect in which the regular rate of decay of the induced hydroxylase activity is impeded (3, 4). Either PB or MC or norepinephrine as a second inducer can also direct at some post-transcriptional level a further rise in hydroxylase activity after treatment of the hepatocytes with the first inducer (4). In our last paper (5) of this series, the kinetics of hydroxylase induction in five established cell lines—three derived from hepatomas and two from normal rat liver—was reported, and the advantages of such established cell lines over primary cultures of fetal rat liver were discussed.

There appears to be an association between aromatic hydrocarbon-inducible hydroxylase activity and cytochrome P_1450 formation[4] in hepatic and in several nonhepatic tissues of the mouse and rat. This change in the CO-binding hemoprotein, and presumably in the enzyme active site(s), can be detected spectrophotometrically (8, 13–17) and is associated with changes in the EPR spectra at temperatures below 10°K (16, 17), with preferential inhibition of benzo[*a*]pyrene hydroxylation by certain chemicals *in vitro* (18–20), and with a cytochrome-containing moiety when several microsomal solubilized subfractions are combined to reconstitute aryl hydrocarbon hydroxylase activity (20–22). In PB-treated animals the hydroxylase activity more closely approximates that of the control animal (13–22), although differences between PB-treated and control animals are detectable. The purpose of this report is to show that, in cell cultures derived from fetal rat liver or from rat or mouse hepatoma, the hydroxylase activity is induced by any of a wide variety of compounds. Moreover, the induced enzyme activity—even in control cells or in cultures treated with PB—appears always to be associated with cytochrome P_1450.

[1] With benzo[*a*]pyrene as the substrate *in vitro*, the aryl hydrocarbon hydroxylase activity is equated with the rate of formation of 3-hydroxybenzo[*a*]pyrene; this phenolic product may be formed either by a direct hydroxylation or in a two-step process via an arene oxide. "Induction" simply denotes a relative accumulation of hydroxylase activity. This may represent an increase in the rate of synthesis of a protein *de novo*, in the rate of activation of enzyme activity from pre-existing components, or both, compared with the rate of degradation. Until technical difficulties regarding the solubilization of the enzyme system are resolved further, one cannot at present distinguish among these three possibilities.

[2] The abbreviations used are: PB, sodium phenobarbital; MC, 3-methylcholanthrene; ANF, α-naphthoflavone; metyrapone, 2-methyl-1,2,3,3-pyridyl-1-propanone; SKF 525-A, 2-diethylaminoethyl-2,2-diphenyl valerate HCl. Throughout this report a careful distinction is made: (a) *in vivo* denotes the intact animal; (b) in cell culture, inside the intact cells; and (c) *in vitro*, cell-free reactions which take place in a flask or other vessel.

[3] Whereas the inducing compounds may be stimulating the rate of synthesis of specific mRNA species, these studies (3, 4) do not in fact distinguish between a mechanism whereby the compounds act to amplify specific genes and one that allows transport or stabilization of (otherwise) rapidly degraded induction-specific RNA which is continuously synthesized yet rapidly degraded within the nucleus.

[4] We refer to cytochrome P_1450 (6)—also called P-448 (7) or P-446 (8)—as that species of CO-binding hemoprotein which increases in concentration in response to aromatic hydrocarbon treatment either *in vivo* or in cell culture. It is increasingly evident (9–12) that the Soret maximum in the 450 nm region comprises a group of CO-binding hemoproteins with different enzymatic and spectral properties and with different responses to inducers of monooxygenase activity.

EXPERIMENTAL PROCEDURE

Materials

The tissue culture materials (2–5) and all chemicals (4, 18) used in this study were obtained from the sources cited. Nominally labeled L-[3-^{3}H]phenylalanine (12.69 mCi/μmole) was purchased from New England Nuclear Corporation, and instrumental grade CO gas was bought from Matheson Company, Inc. Taconic Farms, Inc. (Germantown, N. Y.), provided us with pregnant Sprague-Dawley rats. H-4-II-E, a rat cell line derived (23) from Reuber Hepatoma H-35, was generously given to us by Dr. E. Brad Thompson, National Cancer Institute. Hepa-1, a mouse cell line derived from the transplantable BW 7756 originally produced in the C57L/J mouse (24), was kindly provided in 1971 by Dr. Gretchen Darlington, Department of Biology, Yale University.

Methods

Preparation of cell cultures. Livers from fetal rats estimated to be 18–20 days of gestational age were used in the preparation of the primary cultures of dispersed cells, as described in detail previously (2, 4). The inducing compounds were always added about 48 hr after plating of the primary hepatocytes or the H-4-II-E or Hepa-1 established hepatoma cell lines. At this time the cultures were in logarithmic growth, and there were typically between 1 $\times$ 10^6 and 2 $\times$ 10^6 cells/60-mm tissue culture dish during the period when the effects of the inducing compounds were examined (2–5). For any exposure longer than 24 hr the medium was replaced every 24 hr with fresh medium containing the compound.

Preparation of compounds in medium. Those chemicals which did not dissolve readily in the culture medium were initially dissolved in a minimal amount of acetone, ethanol, or dimethyl sulfoxide and then added to the growth medium. These organic solvents at concentrations not greater than 0.5% in the medium were shown not to have any effect on the parameters under investigation. Because of the propensity for MC to bind to glass or plastic, the actual concentration of MC dissolved in the medium was always determined by spectrophotofluorometry. The possible toxic effects of these various compounds at every concentration tested were evaluated daily by means of light microscopy, gross RNA synthesis (4), gross protein synthesis (4), and protein determinations when the cultures were harvested.

Treatment of animals. The treatment of the pregnant mother or of young rats with the inducer MC consisted of a single intraperitoneal dose (80 mg/kg of body weight) in corn oil 48 hr before death; controls received corn oil alone. PB treatment consisted of daily intraperitoneal doses (40 mg/kg of body weight) in sodium phosphate buffer, pH 7.4, for 5 days prior to death. Preparation of liver homogenates or of liver microsomal fractions was performed exactly as described previously (13, 14).

Enzyme assay. Both the hydroxylase activity and protein concentration were determined in duplicate for the homogenate from cells scraped from one cell culture dish 60 mm in diameter (2, 4) or from liver homogenates (13) as described in the references cited. Usually two values of hydroxylase specific activity were obtained from each of two dishes, the cells of which were harvested at each time point. One unit of aryl hydrocarbon hydroxylase activity is defined (8, 13) as that amount of enzyme catalyzing, per minute at 37°, the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[*a*]pyrene recrystallized standard. Unless otherwise indicated, the specific activity is expressed in units per milligram of protein of the total cellular homogenate.

Gross protein synthesis. The incorporation of [^{3}H]phenylalanine into perchloric acid-precipitable material was used as an estimation of total cellular protein synthesis. To the cells of one 60-mm tissue culture dish was added 0.5 μCi of [^{3}H]phenylalanine for a 30-min pulse, as described previously in detail (4). The specific radioactivity for three identical tissue culture dishes

harvested at the same time point varied less than 15%.

Effects of test compounds on benzo[a]-pyrene hydroxylation in vitro. The test compound—ANF, metyrapone, SKF 525-A, or 2,5-diphenyloxazole—was added in 10 μl of organic solvent (e.g., acetone or ethanol) to the 1.0-ml reaction mixture and incubated for 1 min at 37° prior to addition of the substrate benzo[a]pyrene, as previously described (18). The hydroxylase activity in the presence of 10 μl of the solvent alone was considered as 100% (18). When these test compounds were added after the enzyme had been incubated for 10 min with the substrate benzo[a]pyrene, the quantitative extraction of the hydroxylated benzo[a]pyrene product(s) formed was unaffected (18).

Spectrophotometry. Between 4.0×10^8 and 1.4×10^9 cells were harvested for a single determination of CO-binding hemoprotein. The cells were homogenized in 0.25 M potassium phosphate buffer, pH 7.4, containing 5 mM dithiothreitol, as described previously (8). The homogenate was then centrifuged at 1075 × *g* for 10 min; the large pellet at this stage was predominantly nuclei and mitochondria. In certain experiments 10-min centrifugations at 650 × *g* or 800 × *g* were carried out instead. The supernatant fraction was then centrifuged at 78,000 × *g* for 90 min. The "microsomal" pellet was resuspended in 0.25 M potassium phosphate buffer–30% glycerol, pH 7.25, and the concentration of CO-binding cytochrome was determined by the usual method (8, 13–17, 25). An extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorbance between the Soret maximum in the 450 nm region and the 490 nm baseline was used for all samples (25).

The 10-min centrifugation at 650 × *g*, 800 × *g*, or 1075 × *g* proved necessary, because of the presence of a large amount of mitochondrial cytochrome a_3 (with Soret peak at about 428 nm) in these rapidly growing cell cultures that otherwise obscured the 450 nm Soret maximum representing the "P450-type" cytochromes. An alternative method (26), whereby the baseline is adjusted with CO in both the experimental and reference solutions, cancels out differences caused by cytochrome a_3 or hemoglobin contamination; with H-4-II-E microsomal fractions, we obtained complementary results with the use of this method (26). A Shimadzu model MPS-50L multipurpose recording spectrophotometer (American Instrument Company, Travenol Laboratories, Inc., Silver Spring, Md.) was used at room temperature.

RESULTS

Induction of hydroxylase activity in fetal rat hepatocyte cultures or in rat or mouse hepatoma-derived cell lines by numerous compounds. We studied many hydrophobic compounds which are known substrates or inhibitors (see ref. 18 for discussion) of cytochrome P450-mediated monooxygenase activities. Each of the 15 compounds illustrated in Figs. 1–3 caused at least some induction of hydroxylase activ-

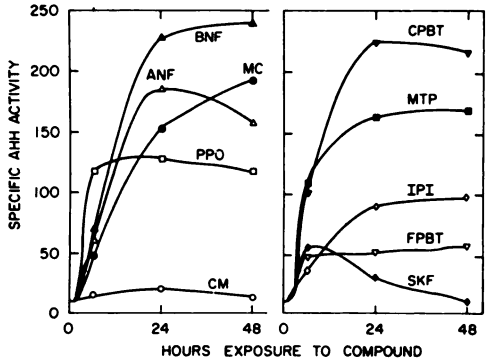

FIG. 1. *Induction of aryl hydrocarbon hydroxylase (AHH) activity in fetal rat liver primary cultures by various compounds dissolved in growth medium*

No cytotoxicity was observed with the use of optimal inducing concentrations [50 μM β-naphthoflavone (BNF), 100 μM ANF, 1.0 μM MC, 100 μM 2,5-diphenyloxazole (PPO), 1.0 mM 2-(4'-chlorophenyl)benzothiazole (CPBT), 100 μM metyrapone (MTP), 50 μM 1-(2-isopropylphenyl)imidazole (IPI), 100 μM 2-(4'-formylphenyl)benzothiazole (FPBT), 10 μM SKF 525-A (SKF)] or the control medium (CM) alone. In this and subsequent figures each symbol represents the average of duplicate determinations on each of two tissue culture dishes and represents the results of a typical experiment which had been repeated between one and five times to ensure reproducibility.

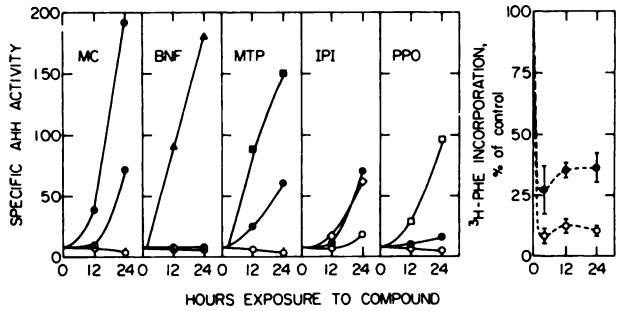

FIG. 2. *Effect of 0.7 μM* (○) *or 3.5 μM* (●) *cycloheximide on aryl hydrocarbon hydroxylase (AHH) induction and gross protein synthesis in fetal rat liver primary cultures*

Cycloheximide was added simultaneously with the inducers 1.0 μM MC, 50 μM β-naphthoflavone (BNF), 500 μM metyrapone (MTP), 100 μM 1-(2-isopropylphenyl)imidazole (IPI), or 200 μM 2,5-diphenyloxazole (PPO). No cytotoxicity was observed during the 24-hr experiment. Gross protein synthesis was determined as described in *Methods* and in refs. 2 and 4; 30-min pulses at 4, 12, and 24 hr were given. None of the compounds had any significant effect on the degree of inhibition of protein synthesis caused by cycloheximide. For the illustration at far right, each symbol and brackets represent the mean ± standard deviation for 10 tissue culture dishes (two each per inducing compound).

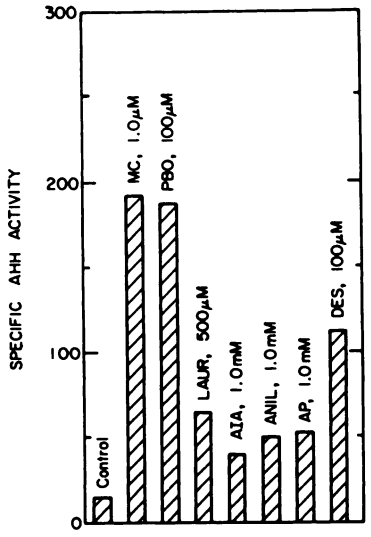

FIG. 3. *Induction of aryl hydrocarbon hydroxylase (AHH) activity in fetal rat liver primary cultures by various hydrophobic compounds*

The optimal inducing concentrations in this experiment are indicated for MC, piperonyl butoxide (PBO), sodium laurate (LAUR), allylisopropylacetamide (AIA), aniline (ANIL), aminopyrine (AP), and diethylstilbestrol (DES). In each case except sodium laurate, the hydroxylase activity after 24 hr of exposure to the compound was much greater than that after 48 hr of exposure. Slight cytotoxicity was detectable with diethylstilbestrol or aniline present; otherwise no cytotoxicity was found at these optimal inducing concentrations.

ity in fetal rat liver primary cultures and in the H-4-II-E or Hepa-1 established cell lines. Representative examples from fetal rat hepatocytes are illustrated in Fig. 1.

Several effects for each of these chemicals as inducers of hydroxylase activity were quite similar and worthy of note. (a) Addition of an inducer to the growth medium for 2–3 hr produced increases in the enzyme activity, whereas exposure of the cultures to an inducer for 15–30 min did not. (b) The kinetics of hydroxylase induction was about the same among all inducers, with a maximal level being reached after 24 or 48 hr of treatment of the cells with the compound; similar kinetics of enzyme induction caused by PB (2–4) or biogenic amines (4) has been illustrated previously. A plateau or decline was reached with certain chemicals [e.g., 2,5-diphenyloxazole, 2-(4′-formylphenyl)benzothiazole, and SKF 525-A], and with a light microscope this could not be attributed morphologically to cytotoxicity. (c) When either cycloheximide (0.7 or 3.5 μM) or actinomycin D (0.4 or 4.0 nM) was added simultaneously with an inducer, the process of enzyme induction was always blocked to some degree by the inhibitors of protein or RNA synthesis, respectively. Among the various inducers, there were minor variations in the sensitivity of the

induction process to the different concentrations of cycloheximide (Fig. 2) or actinomycin D (data not illustrated). No predictable pattern of sensitivity to either cycloheximide or actinomycin D was discernible. (d) Optimally inducing concentrations ranged from 1.0 μM for MC to 1.0 mM for several other inducers. The optimal concentration for each inducer probably represents a balance between specificity in the mechanism of action of the compound and its cytotoxic properties.

Other lipophilic compounds which induced the hydroxylase activity in culture are illustrated in Fig. 3. Whereas only the optimal inducing concentrations of the compounds are shown in Figs. 1–3, between three and five different concentrations of each chemical were studied during a 48-hr period. Generally, a dose slightly less than that which caused morphological changes indicative of cytotoxicity was the optimal inducing dose. Palmitoleic acid ethyl ester, palmitoyl chloride, palmitoleyl acetate, or palmityl acetate—alone or in combination with such inducers as MC, PB, or isoproterenol and over a concentration range of 0.01–1.0 mg/ml of growth medium—did not significantly alter the induction of hydroxylase activity, when compared with cells exposed to control medium, MC, PB, or isoproterenol alone. Spermine and crude extracts of bovine brain, both over a range of 0.01–1.0 mg/ml of culture medium, depressed rather than enhanced the induction of hydroxylase activity by MC, PB, or isoproterenol. Spironolactone was cytotoxic and depressed rather than induced the hydroxylase activity. These results were found in the fetal rat liver primary cultures and also in the H-4-II-E and Hepa-1 established cell lines.

When two or three inducers such as MC, PB, and certain biogenic amines are added in culture simultaneously or sequentially (4), each of these agents produces apparent additive effects, indicative of different mechanisms of action. Among the 14 other inducers illustrated in Figs. 1–3, similarities in mechanism of action were demonstrable in only a few instances. For example, hydroxylase activity induced by ANF, β-naphthoflavone, MC, or 2,5-diphenyloxazole was additive or synergistic with enzyme activity induced by PB. Also, the hydroxylase activity induced by 1-(2-isopropylphenyl)imidazole or metyrapone was additive with that induced by MC but not with that induced by PB.

Induction in cell culture of only one of two different forms of hydroxylase activity. At least two distinct aryl hydrocarbon hydroxylase activities appear to exist. In the intact mouse or rat compounds such as ANF, β-naphthoflavone, 2,5-diphenyloxazole, and lindane inhibit *in vitro* the MC-inducible or *a** hydroxylase activity[5] (18, 19), and we suggest that such compounds interact with the cytochrome P_1450 enzyme active site(s). The compounds metyrapone and SKF 525-A inhibit the basal hydroxylase activity *in vitro* in mouse or rat (18, 19), and we suggest that these drugs interact with other cytochrome P450 enzyme active site(s). The PB-inducible hydroxylase activity behaves more like the basal enzyme activity than the MC-induced enzyme (18), since metyrapone and SKF 525-A are much better inhibitors of the PB-induced and basal hydroxylase activities than are ANF or 2,5-diphenyloxazole. We thus posed the question: In cell culture, is the *a** or *b** hydroxylase activity induced by these various compounds?

Fetal rat primary hepatocytes (Fig. 4) were treated for 2 days with PB, MC, 1-(2-isopropylphenyl)imidazole, or metyrapone. Whether the hydroxylase activities from these cultures were preferentially inhibited by *a** or *b** compounds *in vitro* was then determined. Figure 4 shows that the enzyme activity induced by any of these four chemicals is more sensitive to inhibition *in vitro* by the *a** compounds ANF and 2,5-diphenyloxazole than by the *b** compounds metyrapone and SKF 525-A. The

[5] For purposes of conserving space, we wish to refer to the hydroxylase activity which is more sensitive to inhibition by ANF or 2,5-diphenyloxazole *in vitro* as *a** hydroxylase activity, and to the enzyme which is more sensitive to inhibition by metyrapone or SKF 525-A *in vitro* as *b** hydroxylase activity (14). We suggest that the former is more closely associated with cytochrome P_1450 and that the latter is more closely associated with some other P450 species. However, only indirect evidence supports this hypothesis.

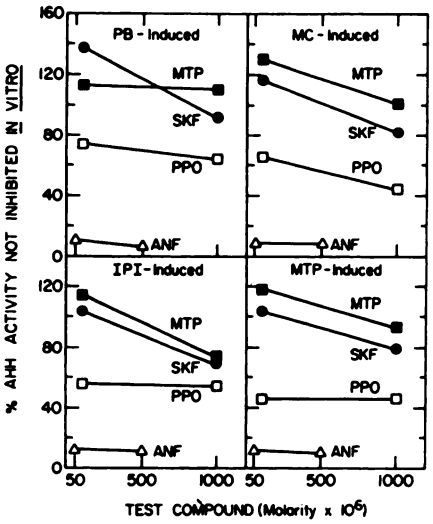

FIG. 4. *Effects of various compounds as inhibitors in vitro of aryl hydrocarbon hydroxylase (AHH) activity induced by PB, MC, 1-(2-isopropylphenyl)imidazole (IPI), or metyrapone (MTP)*

The fetal rat liver primary cultures were first treated for 48 hr with 2.0 mM PB, 1.0 μM MC, 50 μM 1-(2-isopropylphenyl)imidazole, or 500 μM metyrapone. The specific hydroxylase activity, which represents 100%, was 76, 136, 51, and 160 units, respectively, per milligram of total cellular protein. To cell homogenates in the 1.0-ml reaction mixture *in vitro*, an *a** compound [ANF or 2,5-diphenyloxazole (PPO)] or a *b** compound [metyrapone or SKF 525-A (SKF)] was added prior to addition of the substrate benzo[*a*] pyrene, as described previously (18) and under *Methods*.

enzyme in cells grown in control medium alone was also more sensitive to inhibition by *a** compounds than *b** compounds *in vitro* (data not illustrated). We also found that the induced enzyme activities were preferentially more inhibited by two other *a** compounds, β-naphthoflavone and lindane, than by the *b** compounds. These results were reproducible not only in fetal rat liver cultures but also in the H-4-II-E and Hepa-1 established cell lines.

The observations in Fig. 4 indicate that, with respect to preferential inhibition *in vitro*, the PB-induced and basal hydroxylase activities in culture differ from those in the intact adult mouse or rat (18). We therefore wondered whether these enzyme activities in PB-treated and control fetal rat liver cultures are similar to the hydroxylase activities in liver of the neonatal rat. Figure 5A demonstrates the development of the basal, PB-inducible, and MC-inducible hydroxylase activities in the rat as a function of age, and Fig. 5B illustrates the sensitivities of these three enzyme activities to ANF or metyrapone. The *b** hydroxylase activity in the control rat and the *a** enzyme activity in the MC-treated rat are very distinct, beginning at birth. However, the hydroxylase activity in PB-treated rats appears to be a combination of the two until about 2 weeks post partum, at which time the adult-type *b** hydroxylase activity becomes apparent.

Induction in cell culture of only cytochrome P_1450. The data in Figs. 4 and 5 would be supported by the finding that PB—and other inducing compounds which do not induce cytochrome P_1450 *in vitro*—cause the formation of cytochrome P_1450 in cell culture. Spectrophotometric evidence in favor of this hypothesis is shown in Figs. 6 and 7. The degree to which the Soret maximum was shifted to the blue was highly correlated with the content of CO-binding cytochrome in the 450 nm region and also with the hydroxylase activity induced in H-4-II-E cultures by PB, metyrapone, and MC. Similar data were observed in Hepa-1 cultures with MC, isoproterenol, PB, and 1-(2-isopropylphenyl)imidazole as the inducing compounds. The Soret maximum of the hemoprotein-CO reduced complex from control Hepa-1 cells is about 2 nm nearer the blue than that from the control H-4-II-E cells. This is of particular interest, because the basal hydroxylase activity is 15–30 times higher in the Hepa-1 cells than in the H-4-II-E cells. Cytochrome P420 content (25, 26) was estimated to be less than 20% of the concentration of cytochrome P450 or P_1450 in these studies.

The necessity for low-speed centrifugation in order to separate a large portion of the mitochondria from the microsomal fraction is aptly illustrated in Fig. 6. There is a large contribution of mitochondrial cytochrome a_3 to the CO difference spectrum. Consequently, without the low-speed centrifugation, the total cellular homogenate could not be accurately examined for

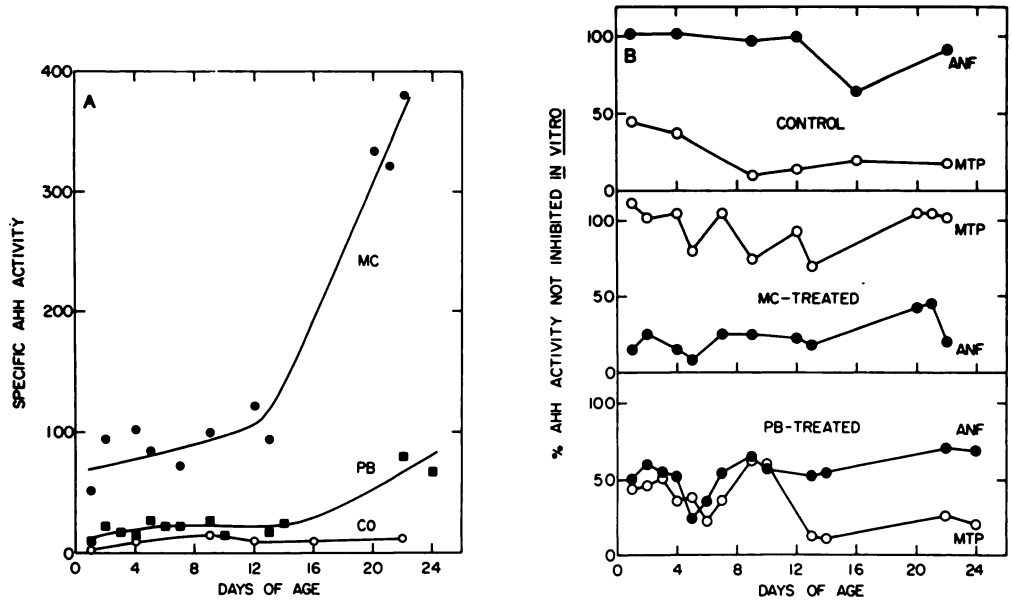

FIG. 5. *Ontogenetic expression of hepatic aryl hydrocarbon hydroxylase (AHH) activity in vivo in immature rats (A) and ANF-sensitive or metyrapone-sensitive hepatic hydroxylase activity as a function of age (B)*

A. Rats were treated with MC, PB, or corn oil (CO) alone. MC treatment consisted of a single dose 48 hr prior to assay; PB treatment consisted of daily doses for 5 days. Thus values for 1-day-old rats indicate that the mother received MC or corn oil intraperitoneally 1 day prior to giving birth or received PB intraperitoneally for the final 5 days of gestation. B. The inhibition of benzo[*a*]pyrene hydroxylation *in vitro* by 50 μM ANF or 100 μM metyrapone (MTP) was studied with whole liver homogenates in the reaction mixture in the same manner as described in Fig. 4 and in ref. 18. The same results were also found with liver microsomal fractions from postnatal rats. Differences greater than 30% are statistically significant ($p < 0.05$). For the data illustrated, a total of 18 PB-treated, 15 MC-treated, and 6 control litters were studied. The livers from all fetuses in one litter were combined to make a single homogenate. Each symbol therefore represents the results of a single experiment or the mean value of several experiments performed on similarly treated rats of the exact same age. One hundred per cent of the specific hydroxylase activity from control, MC-treated, and PB-treated rats aged 9 days, for example, represents 15, 100, and 27.6 units, respectively, per milligram of liver homogenate protein.

cytochrome P450 and P_1450 content because of the large contamination of this CO-binding component in the 428-nm region.

DISCUSSION

From the data shown in this report we conclude that the PB-induced and basal hydroxylase activities in the liver of the intact adult animal are not the same as the PB-induced and basal enzyme activities in liver- or hepatoma-derived cell cultures. This conclusion supports the finding[6] that no proliferative changes in the endoplasmic reticulum were detectable in fetal rat liver primary cultures which had been treated with PB. This conclusion is important to those scientists interested in using liver or hepatoma cultures as experimental models for evaluating hepatotoxicity, drug metabolism, or chemical carcinogenesis. It is increasingly likely that for certain compounds the metabolite formation by one species of P450 can differ from the metabolite profile produced by another species of P450, e.g., cytochrome P_1450. Several laboratories have demonstrated that hydroxylations may occur in different chemical positions on the molecule for such substrates as biphenyl (27), testosterone (28), bromobenzene (29), and *n*-hexane (30). Such differences in the metabolite profile of a drug or polycyclic hydrocarbon might

[6] H. L. Moses, J. E. Gielen, and D. W. Nebert, unpublished data, 1971.

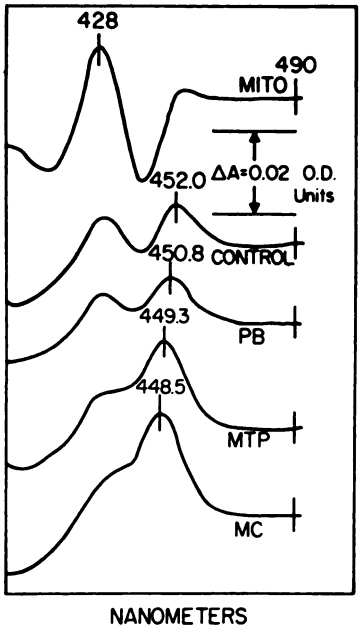

FIG. 6. *CO difference spectra of fractions from rat Reuber Hepatoma H-4-II-E cultures treated with control medium alone, 2.0 mM PB, 500 μM metyrapone (MTP), or 3.3 μM MC for 48 hr*

Specific hydroxylase activities were 1.4, 36, 88, and 122 units, respectively, per milligram of total cellular protein. The fraction containing nuclei and mitochondria (MITO) and sedimenting on centrifugation at 1075 × *g* for 10 min is shown at the top; this fraction is from control cells. Protein concentrations of the suspensions in the cuvettes (from top to bottom) were 6.2, 9.2, 4.4, 4.2, and 4.8 mg/ml. Wavelength measurements were standardized with the use of a holmium oxide crystal.

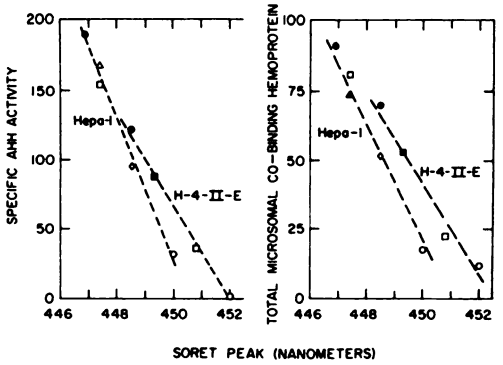

FIG. 7. *Specific aryl hydrocarbon hydroxylase (AHH) activity (left) and total CO-binding hemoprotein (right) as a function of Soret maximum found in CO difference spectrum from fractions of H-4-II-E or Hepa-1 cells*

Specific activity is expressed in units per milligram of microsomal protein; CO-binding cytochrome of the P450 type is expressed in picomoles per milligram of microsomal protein. The cultures during logarithmic growth were exposed for 48 hr to 10 μM MC (●), 0.50 mM isoproterenol (△), 2.0 mM PB (□), 50 μM 1-(2-isopropylphenyl)imidazole (◇), 100 μM metyrapone (■), or control medium alone (○). The H-4-II-E results shown here are from the same samples whose spectra are illustrated in Fig. 6.

result in marked differences in the reactivity of intermediates and therefore might cause marked dissimilarities in the cytotoxicity or carcinogenicity of a given compound. Hence, because the hepatic cytochrome in the adult animal is predominantly P450, experiments with drug- or aromatic hydrocarbon-treated liver or hepatoma cultures may not reflect the actual situation in the intact animal with respect to hepatotoxicity or carcinogenesis.

The rise in aryl hydrocarbon hydroxylase activity in control cell cultures has been a common observation (2, 4, 31, 32). Factors in the calf serum or the phenol red pH indicator are possible reasons for this significant increase in the enzyme activity in control cultures. Preliminary reports by Paine and McLean (33, 34) indicate that hydroxylase induction in tissue culture by compounds of such diverse structures may be explainable by a common factor such as the oxidative generation of $\cdot O_2^-$ or ·OH inside the cells. This intriguing hypothesis obviously requires further study. However, numerous lines of evidence from this laboratory[7] indicate that metabolism of highly specific aromatic hydrocarbon inducers such as MC, benz[*a*]anthracene, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is not required for the hydroxylase induction.

As shown in Fig. 4, and also found in previous studies (18), addition of a test compound *in vitro* can lead to an apparent enhancement of benzo[*a*]pyrene hydroxylation. The most likely explanation is that a hydrophobic compound may bind to nonspecific sites on the microsomal membranes or on the glass surface of the flask,

[7] I. S. Owens, J. E. Gielen, and D. W. Nebert, unpublished data; A. Niwa and D. W. Nebert, manuscript submitted for publication.

thereby resulting in fewer such sites being available for the substrate benzo[*a*]pyrene when it is added subsequently. The later addition of the substrate then might result in a more efficient metabolism of the benzo[*a*]pyrene and therefore an apparent increase in hydroxylase activity. A second possible explanation for a seemingly augmented hydroxylase activity *in vitro* is that the enzyme system may be tightly coupled to a membrane-bound epoxide hydrase or glucuronyl transferase (see refs. 35–38 for discussion); a greater inhibition of these enzymes which act subsequently on the products formed by the monooxygenase, compared with inhibition of the P450-mediated enzyme system, could result in a test compound causing an increased production of hydroxylated benzo[*a*]pyrene and therefore a seemingly enhanced hydroxylase activity.

An advantage of a hepatoma-derived cell line over fetal rat liver primary cultures is shown in Fig. 6. The number of fetal rat livers necessary to obtain the number of cells needed for the spectral studies makes the primary hepatocyte cultures impractical from the standpoint of both time and effort. Each spectrophotometric determination, such as those illustrated in Fig. 6, requires 20–30 pregnant rats.

Cytochrome P_1450 formation and its associated hydroxylase activity may function primarily in the organism's portals of entry (skin, lung, and intestine) and in liver, where the system mediates the biotransformation of numerous noxious hydrophobic exogenous compounds (39). Survival of the mammalian fetus may require the presence of this inducible monooxygenase system in fetal tissues (40) and placenta (41). It is therefore of interest that the predominant form of CO-binding cytochrome in fetal hamster secondary cultures (8) in fetal rat liver primary cells, and in hepatoma-derived established cell lines appears to be cytochrome P_1450 and that this form even predominates in control cultures and in liver or hepatoma cultures exposed to large amounts of PB. The capacity for cells to grow well in culture appears to be directly correlated with the dedifferentiated state of the tissue. Thus fetal tissues, anaplastic tumors, and transformed cells grow much more easily than well-differentiated or adult tissues. This phenomenon may explain why PB in culture does not cause proliferative changes in the endoplasmic reticulum or induction of P450-mediated enzyme activities in the same manner in which it acts in the liver of the intact animal. It will be interesting to discover whether these "differentiated" responses of a tissue to PB will be found in any cell culture system.

NOTE ADDED IN PROOF: Very recent experimental evidence [Rasmussen, R. E. and Wang, I. Y. (1974) *Cancer Res.* **34**, 2290–2295] demonstrates that the profile of benzo[a]pyrene metabolites formed by microsomes from MC-treated rats—in which the P_1450 monooxygenase system is considerably increased—differs from the profile of benzo[a]pyrene metabolites formed by microsomes from control or PB-treated rats in that oxygenation in the 7-, 8-, 9-, and 10-positions occurs more frequently. Moreover, it appears that these P_1450-mediated metabolites of benzo[a]pyrene in the 7-, 8-, 9- and 10-positions interact covalently with DNA more so than K-region metabolites of benzo[a]pyrene [Sims, P., Grover, P. L., Swaisland, A., Pal, K., and Hewer, A. (1974) *Nature New Biol.* **252**, 326–328].

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