Regulation of Cytochrome P-450p by Phenobarbital and Phenobarbital-like Inducers in Adult Rat Hepatocytes in Primary Monolayer Culture and in Vivo†

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ABSTRACT: Treatment of rats with phenobarbital increases the hepatic concentration of P-450p, a form of cytochrome P-450 believed to be controlled primarily by a mechanism that stereospecifically recognizes glucocorticoids like dexamethasone and anti-glucocorticoids like pregnenolone-16α-carbonitrile [Schuetz, E. G., & Guzelian, P. S. (1984) *J. Biol. Chem. 259*, 2007]. To test the possibility that phenobarbital induces P-450p indirectly by increasing the availability of endogenous glucocorticoids in the liver, we added phenobarbital and phenobarbital-like inducers to primary monolayer cultures of adult rat hepatocytes incubated in serum-free medium without glucocorticoids and found stimulated de novo synthesis of P-450p measured as increased incorporation of [^{3}H]leucine into immunoprecipitable P-450p protein. With some of the inducers, notably the organochlorine pesticides chlordane and *trans*-nonachlor, there was a greater accumulation of P-450p measured on quantitative immunoblots than could be accounted for by the increase in P-450p synthesis. "Pulse–chase" experiments confirmed that these compounds significantly lengthen the half-life of P-450p up to 60 h as compared to the values in control (11 h) or dexamethasone-treated (10 h) cultures. Treatment of rats with chlordane, *trans*-nonachlor, or other cyclodiene organochlorine pesticides confirmed that these agents increase the concentration of P-450p in liver microsomes analyzed on immunoblots of two-dimensional electrophoretic gels. The time courses of induction in *trans*-nonachlor-treated rats of P-450p protein and of P-450$_{PB}$ proteins induced by phenobarbital were similar as were the amounts of P-450$_{PB}$ mRNA and P-450p mRNA measured by hybridization to cloned cDNA probes. However, analysis of structure–activity relationships among polychlorinated biphenyls revealed that isomers with two ortho chlorinated positions maximally induced P-450$_{PB}$ whereas isomers with three and four ortho chlorines maximally induced P-450p in rats and in hepatocyte culture, respectively. We conclude that P-450p is induced by the phenobarbital class of inducers through direct contact with the hepatocytes involving decreased degradation of the protein and stimulation of its synthesis in a manner similar but not identical with that of P-450$_{PB}$.

The cytochromes P-450 are a superfamily of microsomal hemoproteins found in abundance in the liver that catalyze the oxidative metabolism of many lipophilic chemicals including both xenobiotics and such endogenous substrates as steroid hormones. It has been possible to purify at least 12 distinct isozymes of cytochrome P-450 from rat liver (Waxman, 1986) each differing in their biochemical, immunochemical, and regulatory characteristics. For example, P-450c and P-450d are immunochemically related cytochromes that are induced in rats treated with 3-methylcholanthrene (MC)[1] or other structurally related polycyclic aromatic hydrocarbons (Kimura et al., 1984), whereas P-450b and P-450e are products of a different cytochrome P-450 gene family (Fujii-Kuriyama et al., 1982; Atchison & Adesnick, 1983) whose expression is activated by phenobarbital and "phenobarbital-like" chemicals. A rational basis for identifying "MC-like" inducers emerged with the discovery that such chemicals are stereospecifically bound by a soluble receptor protein (Poland et al., 1974) that is indispensible for stimulating expression of the MC gene family (Gonzalez et al., 1985a; Jones et al., 1985). In contrast, phenobarbital-like inducers of P-450b and P-450e are a heterogeneous collection of structurally dissimilar drugs, organochlorine pesticides, and other lipophilic chemicals (Conney, 1967) for which no critical unifying characteristic has yet been identified.

Recently, we isolated and purified the major form of liver cytochrome P-450 from rats treated with pregnenolone-16α-carbonitrile (PCN) and showed that this hemoprotein, now called P-450p, is structurally and functionally distinct from cytochromes in the MC or phenobarbital families (Elshourbagy & Guzelian, 1980). We showed that de novo synthesis of P-450p, now known to be a member of a third cytochrome P-450 gene family (Hardwick et al., 1983; Gonzalez et al., 1985b; Simmons et al., 1985), is stimulated by glucocorticoids and the anti-glucocorticoid PCN but not by steroid hormones categorized as estrogens, androgens, mineralocorticoids, or progestational agents (Schuetz et al., 1984). PCN treatment increases the amount of liver P-450p mRNA translatable in

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[1] Abbreviations: PCN, pregnenolone-16α-carbonitrile; MC, 3-methylcholanthrene; PCB, polychlorinated biphenyl; SDS, sodium dodecyl sulfate; SSC, saline and sodium citrate; ssDNA, salmon sperm DNA; SSPE, sodium chloride, sodium phosphate, and EDTA; Denhardt's reagent, Ficoll, poly(vinylpyrrolidone), and BSA; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; PBS, phosphate-buffered saline; DTT, dithiothreitol; P-450p = P-450$_{PCN}$, major form of liver cytochrome P-450 in rats treated with glucocorticoids or PCN; P-450$_{PB}$ (P-450b and P-450e), major forms of cytochrome P-450 in rats treated with phenobarbital.

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a cell-free system (Elshourbagy et al., 1981) or hybridizable to cloned cDNA probes (Hardwick et al., 1983; Wrighton et al., 1985b). On the basis of a detailed analysis of the dose–response and agonist–antagonist relationships among many steroid hormones (Schuetz & Guzelian, 1984), we have proposed that induction of P-450p involves a stereospecific recognition system, possibly a "PCN receptor" similar to (or identical with) the glucocorticoid binding protein that mediates transcriptional regulation of many genes by glucocorticoids (Karin et al., 1984; Schmid et al., 1982; Majors & Varmus, 1983; Scheidereit et al., 1983; Scheidereit & Beato, 1984; Buetti & Diggelmann, 1983).

In the course of testing non-steroidal compounds, we found that phenobarbital, but not MC, is an inducer of P-450p in rats (Heuman et al., 1982). Indeed, others have used liver microsomes prepared from phenobarbital-treated rats as a source for preparing a purified cytochrome P-450 similar to, if not identical with, P-450p (Guengerich et al., 1982a; Waxman, 1985). Moreover, analysis of rat liver microsomes by two-dimensional gel electrophoresis was reported to show that treatment with the organochlorine pesticide chlordane induced P-450$_{PB}$ (i.e., P-450b and P-450e and other isozymes of this family) but not the "PCN-inducible protein", whereas phenobarbital treatment induced all three proteins (Vlasuk et al., 1982a). These findings suggest that there may be different mechanisms involved in the induction of P-450$_{PB}$ and P-450p by the phenobarbital-like compounds. Indeed, P-450p is the first (and only) example of a xenobiotic-inducible cytochrome P-450 for which an endogenous inducer has been found. Thus, phenobarbital might induce P-450p indirectly, by increasing, in some manner, the availability of glucocorticoids. While it would be difficult to address this question in the living animal, primary monolayer cultures of nonproliferating adult rat hepatocytes provide a convenient system to study the direct interaction of foreign chemicals and endogenous inducers with the hepatocyte. The hepatocyte cultures respond to the presence of glucocorticoid hormones with dramatic increases in the rate of de novo synthesis of P-450p (Elshourbagy et al., 1981; Schuetz et al., 1984) and in the amount of P-450p mRNA translatable in a cell-free system (Elshourbagy et al., 1981). We have shown also that addition of phenobarbital to the cultures increases the rate of synthesis of P-450$_{PB}$ (Newman & Guzelian, 1982). We now report that phenobarbital, chlordane, and many other phenobarbital-like inducers stimulate the synthesis of P-450p in the hepatocyte directly and that the remarkable induction of P-450p by organochlorine pesticides in culture prominantly involves decreased degradation of P-450p protein.

Materials and Methods

Materials

Monoclonal H-8 antibody against P-450$_{PB}$ and pR17, a cloned cDNA, were gifts from Milton Adesnick; chlordane, *trans*-nonachlor, dieldrin, aldrin, heptachlor, and compound C were given by Velsicol Corp. (Chicago, IL). Other pesticides were EPA analytical reference standards with purities reported to be greater than 99% for each standard (Research Triangle Park, NC). Nitrocellulose was purchased from Bio-Rad (Richmond, CA); 3,3′-diaminobenzidine tetrahydrochloride was from Pfaltz and Bauer, Inc. (Stamford, CT); silastic medical tubing and adhesive silastic were from Dow Corning Corp. (Midland, MI). Liver microsomes prepared from chlordane-treated male Long-Evans rats were given by Wayne Levin (Hoffmann-La Roche, Nutley, NJ); PCN was a gift from John Babcock, Upjohn Co. (Kalamazoo, MI); PCBs were given by Stephen Safe (Texas A&M, College Station, TX). All other chemicals were of the highest purity available commercially.

Animals and Treatment

Female Sprague-Dawley rats weighing either 100–150 g for studies in vivo or 180–210 g for use in preparing hepatocyte cultures were purchased from Flow Laboratories (Dublin, VA) and were housed in pairs in wire-bottomed cages with free access to food and water. *trans*-Nonachlor capsules were prepared by sealing one end of a 70-mm section of silastic tubing (0.062 × 0.125 in.) with medical silastic adhesive. The tube was dried for 24 h and then filled to a length of 63 mm with *trans*-nonachlor. The open end of the tube was plugged with adhesive, and after being allowed to dry for 24 h, the capsule was soaked for 24 h in PBS. Rats were anesthesized with ether, and through a small incision made at the base of the neck, the capsule was inserted intramuscularly parallel to the spine with the use of a trocar. The trocar was removed, Procaine (0.1 mL) was administered locally, and the incision was closed with one to two staples. Although blood and adipose tissue levels of *trans*-nonachlor were not monitored in this study, it has been demonstrated previously that subcutaneous implantation of silastic capsules containing estradiol maintained constant serum levels of the steroid throughout the period of implant (Legan et al., 1975).

Primary monolayer cultures of rat hepatocytes were prepared as described previously (Bissell & Guzelian, 1980; Schuetz et al., 1984). In brief, a laporotomy was performed on a female rat anesthetized with ether, and under septic conditions, the portal vein was cannulated and the liver was perfused with a calcium-free buffer followed by a solution of collagenase. The softened liver was excised and shaken briefly in the collagenase solution, and the hepatocytes were isolated by low-speed centrifugation. The cells were innoculated into 60-mm plastic dishes precoated with collagen in a total volume of 3.0 mL of our standard, serum-free culture medium and were incubated at 35 °C in an atmosphere of 5% CO_2. The medium was renewed every 24 h. Additions to the medium were made by dissolving the chemical in a small amount (<6 μL) of dimethyl sulfoxide. In such experiments, control cultures received an equal volume of the solvent. We have established that this amount of solvent is without effect on cell viability, longevity in culture, and rates of protein, RNA, or P-450p synthesis.

Synthesis of P-450p in hepatocyte cultures was determined as described in detail elsewhere (Schuetz et al., 1984) by measuring the rate of incorporation of a radiolabeled amino acid into P-450p protein. In brief, for a single assay, two to three monolayer dishes were transferred to a medium identical with the composition of the ambient culture medium (including additives, if any) except that [^{3}H]leucine was substituted for unlabeled leucine. After a 2-h "pulse" incubation, the medium was removed, and the monolayers were washed, removed by scraping, pooled, and lysed by sonication. The proteins were solubilized with detergents, and P-450p was quantitatively precipitated with the use of goat anti-P-450p IgG. The immunoprecipitated P-450p was isolated by electrophoresis on polyacrylamide gels containing SDS, and the amount of radioactivity in slices of the unstained, unfixed gel corresponding to the mobility of P-450p was determined by liquid scintillation spectrometry. This value was divided by the amount of radioactivity incorporated into total cell protein and was expressed as percent of total synthesis.

Degradation of P-450p in hepatocyte cultures was determined according to a pulse–chase protocol described previously

(Elshourbagy et al., 1981). In the present studies, all cultures were incubated for 96–120 h in standard culture medium or in medium containing an inducer of P-450p. The cells were then incubated for 3 h in the same medium except [^{3}H]leucine (120 Ci/mmol at 10 μCi/plate) was substituted for unlabeled leucine. The radioactive culture medium was removed and was replaced by the appropriate medium supplemented with 1.9 mM leucine. At appropriate intervals thereafter, cultures were harvested, and the amount of [^{3}H]leucine remaining in immunoprecipitable P-450p was measured as described in the preceding section.

Purification of Cytochromes P-450b and P-450e and Preparation of Specific Antibodies. Cytochromes P-450b (West et al., 1979) and P-450e (Waxman & Walsh, 1982) were purified from phenobarbital-treated male rats as previously described. Preparation of monoclonal H-8 IgG directed against an epitope common to P-450b and P-450e and immunochemical analysis (Kumar et al., 1983) have shown that this monoclonal antibody precipitates three immunochemically related polypeptides from phenobarbital-treated Sprague-Dawley rats corresponding to P-450b, P-450e, and a slower moving polypeptide. Rigorous, repetitive immunoblot analyses of purified P-450b and P-450e either alone or as a mixture and of phenobarbital-treated microsomes demonstrated that the H-8 antibody recognized specifically the purified P-450b and P-450e and their corresponding proteins in liver microsomes. Multiple one-dimensional analyses of phenobarbital-induced liver microsomes with H-8 monoclonal antibody routinely resolved two additional polypeptides (y and x) with faster and slower mobilities, respectively, than P-450b and P-450e.

Accumulation of immunoreactive P-450p and P-450$_{PB}$ in hepatocyte cultures or in liver microsomes was determined by quantitative immunoblot analysis. Immunochemical quantitation of P-450p on blots of electrophoretically separated proteins was performed by a modification of the procedures described (Guengerich et al., 1982b). Microsomes prepared from rat liver or from lysates of cultured hepatocytes (Elshourbagy et al., 1981; Heuman et al., 1982) containing approximately 1 pmol of immunoreactive P-450 were applied to a 1.5-mm slab of 10% Laemmli polyacrylamide gel containing SDS with 10 wells, and electrophoresis was carried out for 2 h at 30 mA/slab. The current was stopped temporarily, and different amounts of purified P-450p (Wrighton et al., 1985a) in the range of 0.25–1.5 pmol of P-450/well or of purified P-450b (Elshourbagy & Guzelian, 1980) in the range of 0.25–2.5 pmol of P-450/well were added, and electrophoresis was continued for 2 more h. The resolved proteins were transferred electrophoretically onto nitrocellulose sheets at a current setting of 200 mA for 2 h at room temperature followed by 700 mA at 4 °C for 1 h in a 25 mM Tris, 192 mM glycine, and 20% methanol buffer. The gels were stained with Coomassie Blue to be certain that transfer of the proteins was complete. Unreacted sites on the nitrocellulose were blocked by incubating the sheets overnight at 25 °C in PBS containing 10% dialyzed calf serum and 3% BSA. The nitrocellulose sheets were then treated sequentially (with intermediate PBS washings) with (a) goat antibody to P-450p, rabbit anti-goat IgG, goat peroxidase anti-peroxidase, and, finally, the peroxidase substrate 3,3′-diaminobenzidine tetrahydrochloride or (b) monoclonal H-8 antibody to P-450PB, rabbit anti-mouse IgG peroxidase conjugated, and the peroxidase substrate. The area and intensity of the colored bands for both the standards and the samples were measured on dried nitrocellulose sheets with the use of a Colorscan C-4100 reflectance densitometer (Optronics, International) controlled by an Optronics computer. A VAX computer then calculated the integrated density from approximately 3000 point readings for a typical band, and subtracted the relevant background values. All results for integrated density fell within the linear response range as determined from densities of the P-450 standards. Repetitive analysis of a given sample agreed within 10%.

Dot Blot Hybridization. Total liver RNA was isolated as reported previously (Deeley et al., 1977; Elshourbagy et al., 1981). Dot blots of total RNA were carried out as described in Schleicher & Schuell Specification Sheet 352-354. Briefly, serial dilutions of total RNA (5–25 μg) in 100 μL of water were added to 300 μL of a solution of 6.15 M formaldehyde and 10× SSC. The RNA was denatured by incubation at 65 °C for 15 min and loaded into wells of a slot blot apparatus, and the wells were rinsed with 400 μL of 10× SSC. The nitrocellulose filter was air-dried and baked 2 h at 80 °C in vacuo.

Nitrocellulose sheets were prehybridized in a solution of 5× Denhardt's regent (final concentration), 0.1% SDS, 5× SSPE, and 50% formamide containing 200 μg/mL ssDNA at 42 °C for 5 h. Plasmids pR17 and pDex12 harboring cloned DNA sequences complimentary to P-450$_{PB}$ mRNA (Adesnick et al., 1981) and to P-450p mRNA (Wrighton et al., 1985b), respectively, were labeled to a high specific activity (about 10^8 cpm/μg of DNA) with [α-^{32}P]dCTP (deoxycytidine triphosphate) by nick translation (Maniatis et al., 1982). The radioactive probe was mixed with ssDNA, denatured by boiling for 5 min followed by a rapid freeze–thawing, and added to a hybridization solution containing 2× Denhardt's reagent, 50% formamide, and 5× SSPE. Hybridization was carried out at 42 °C overnight. The nitrocellulose sheets were rinsed at low stringency (2× SSC and 0.1% SDS at 42 °C for 30 min) followed by high stringency (0.1× SSC and 0.1% SDS at 42 °C for 30 min) and subjected to autoradiography. The amount of hybridized RNA by pDex12 and pR17 was determined densitometrically with a Corning 750 photodensitometer system (transmittance mode), which also integrated the total area under each curve.

Other Methods

Two-dimensional isoelectric focusing/SDS gel electrophoresis of liver microsomes was carried out according to published procedures (Vlasuk et al., 1982a). The concentration of cytochrome P-450 (dithionite-reduced CO difference spectrum) was determined as reported previously (Omura & Sato, 1964). Protein was measured colorimetrically (Schacterle & Pollack, 1973) with bovine serum albumin as the standard. Radioactive proteins separated on polyacrylamide gels were detected by fluorography (Laskey & Mills, 1975).

RESULTS

Induction of P-450p Synthesis and Accumulation in Cultured Hepatocytes. We incubated hepatocyte cultures in medium containing a high concentration of the glucocorticoid dexamethasone and found that at 120 h of culture age, when induction of P-450p is maximal (Schuetz et al., 1984), the rate of de novo synthesis of P-450p protein in the present two experiments (Figures 1 and 2) averaged 40-fold higher than the rate in control cultures incubated for the first 120 h in our standard culture medium, which contains no steroidal hormones. When cultures were incubated in standard culture medium containing phenobarbital at a concentration (2 mM) previously shown to maximally stimulate the synthesis of P-450$_{PB}$ (Newman & Guzelian, 1982), the rate of de novo synthesis of P-450p increased by 7-fold over the value in control

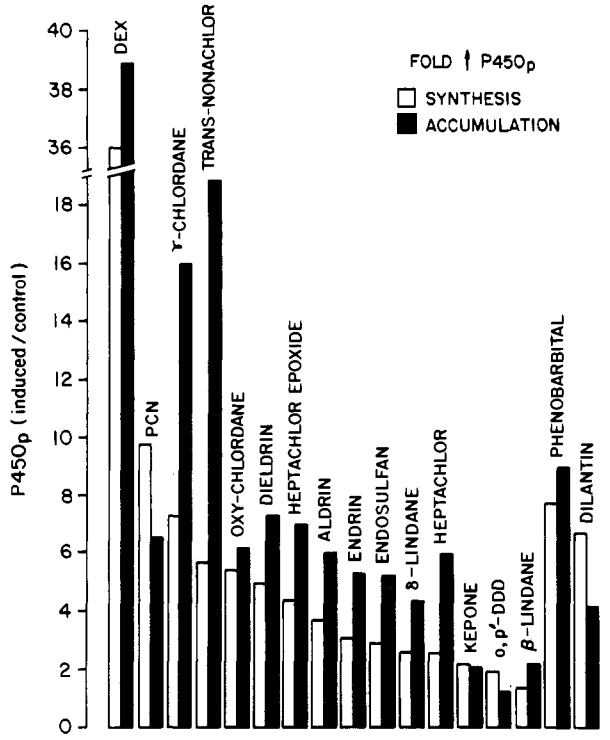

FIGURE 1: Comparison of synthesis and accumulation of P-450p in cultured hepatocytes treated with steroidal or non-steroidal inducers. Cultures of isolated hepatocytes prepared from a single rat were incubated in standard medium for 24 h and then transferred to medium containing one of the indicated steroids or organochlorine pesticides (1×10^{-5} M), Dilantin (1×10^{-5} M), or phenobarbital (2×10^{-3} M). At 120 h of culture age, some of the cultures in each group were incubated with the appropriate medium containing [^{3}H]leucine for assay of the rate of de novo synthesis of immunoprecipitable P-450p (open bar) or were harvested for assay by quantitative immunoblots of the concentration of P-450p (solid bar) as described under Materials and Methods. The results are given as P-450p (*x*-fold increase) induced/control. The values in control cultures were as follows: P-450p synthesis, 0.15% of total protein synthesis; P-450p concentration, undetectable (≤3 pmol/mg of cell lysate protein, the lower limit of detection for P-450p).

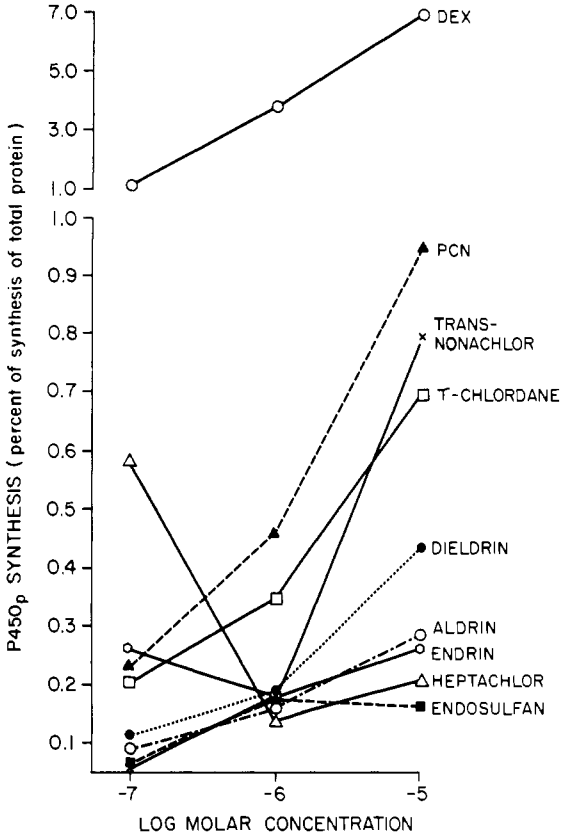

FIGURE 2: Concentration dependence for stimulation of P-450p synthesis by steroids and by cyclodiene organochlorine pesticides in cultured hepatocytes. Cultures of hepatocytes prepared from a single rat were incubated in standard medium for 24 h and then transfered to medium containing one of the indicated pesticides or steroids at one of the three concentrations. At 120 h of culture age, the cells were exposed to medium containing the appropriate pesticide and [^{3}H]leucine, and 4 h later, the cultures were terminated, and P-450p synthesis was determined as described under Materials and Methods. Relative synthesis of P-450p in control cultures was 0.15% of total protein synthesis.

cultures (Figure 1). Addition of the phenobarbital-like inducing drug phenytoin (dilantin) to the culture medium similarly increased P-450p synthesis (Figure 1). We tested the effects of a series of organochlorine pesticides known to be phenobarbital-like inducers of P-450$_{PB}$ in rats (Thomas et al., 1983; Campbell et al., 1983) and found that each stimulated the synthesis of P-450p (2–6-fold) (Figure 1). Chlordane, *trans*-nonachlor, and oxychlordane were equally potent inducers of P-450p when compared to phenobarbital. Stimulation of P-450p synthesis by organochlorine pesticides displayed a typical concentration-dependent pattern (Figure 2). Neither phenobarbital nor the pesticides were superior in potency or efficacy when compared to PCN or to dexamethasone, the best known steroidal inducer of P-450p (Figure 1). For example, in dexamethasone-treated cultures synthesis of P-450p represented approximately 7% of total protein synthesis, a value nearly 9 times higher than that in *trans*-nonachlor-treated cultures (Figure 2).

We tested some polychlorinated biphenyl (PCB) isomers known to induce a pattern of cytochromes P-450 in rat liver similar to that produced by phenobarbital treatment (Parkinson et al., 1983b; Denomme et al., 1983) and found that each one stimulated P-450p (Table I). There emerged a clear rank order of their potency as inducers of P-450p in culture on the basis of the number (4 > 3 > 2) of ortho substitutions with chlorines in these isomers. Surprising was the weak induction produced by 2,2′,4,4′-tetrachlorobiphenyl, a congener previously shown to be one of the best PCB inducers of P-450$_{PB}$ in rat liver (Table II) (Parkinson et al., 1983b).

Next, we compared the effects of phenobarbital and the organochlorine pesticides on the *synthesis* of P-450p to their effects on the *accumulation* of P-450p protein in the same cultures. For example, in cultures treated with dexamethasone, the increases over control values in synthesis and in accumulation of P-450p protein were similar (36–40-fold) (Figure 1). In contrast, in cultures treated with chlordane or *trans*-nonachlor the increase in P-450 protein synthesis was less than $^1/_3$ of the increase in accumulation of P-450p protein (Figure 1). Slightly greater accumulation vs. synthesis of P-450p was observed in cultures treated with other cyclodiene organochlorine pesticides, with their stable metabolites (heptachlor epoxide and oxychlordane), or with lindane isomers (Figure 1). Inasmuch as the increases in synthesis and accumulation of P-450p were similar in cultures treated with phenobarbital, *o,p′*-DDD, kepone (Figure 1), and some PCB isomers (Table I), it may be concluded that the exaggerated accumulation of P-450p is a selective effect exerted by only some of the organochlorine pesticides. It is unlikely that these results reflect nonspecific effects of high concentrations of lipophilic substances since there was no exaggerated accumulation of P-450p in cultures treated with the organochlorine pesticides toxaphene or compound C (1×10^{-5} M), with the MC-type inducer benzanthracene (5×10^{-6} M), with the super murine

Table I: Stereospecificity of Induction of P-450p by Polychlorinated Biphenyl Isomers in Cultured Hepatocytes[a]

position of chlorines	total no. of chlorines	no. of chlorines			P-450p synthesis (% of total)	P-450p accumulation (nmol/mg of protein)
		ortho	meta	para		
2,2′,3′,4,4′,6,6′[b]	7	4	1	2	0.85	0.022
2,2′,3′,4,5′,6,6′	7	4	2	1	0.70	0.031
2,2′,3,3′,4′,5,6,6′	8	4	3	1	0.63	0.020
2,2′,4,4′,6,6′	6	4	0	2	0.64	0.014
2,2′,3,3′,4,4′,5′,6	8	3	3	2	0.47	0.014
2,2′,3′,4′,5,5′,6′	7	3	3	1	0.44	0.017
2,2′,3′,4,4′,5′,6	7	3	2	2	0.42	0.015
2,2′,4,4′[b]	4	2	0	2	0.27	<0.003
2,2′,3,3′,4,4′,5	7	2	3	2	0.11	<0.003
2,3′,4,4′	4	1	1	2	0.25	<0.003
control					0.14	<0.003

[a] Cultured hepatocytes were incubated in standard medium for 24 h and then transferred to medium containing one of the PCBs (10^{-5} M). At 120 h of culture age, some of the cultures in each group were exposed to [^{3}H]leucine for assay of the rate of de novo synthesis of immunoprecipitable P-450p or were exposed for 2 h to fresh medium with the appropriate pesticide and then harvested for assay by quantitative immunoblots of the concentration of P-450p as described under Materials and Methods. [b] See Table III for in vivo values.

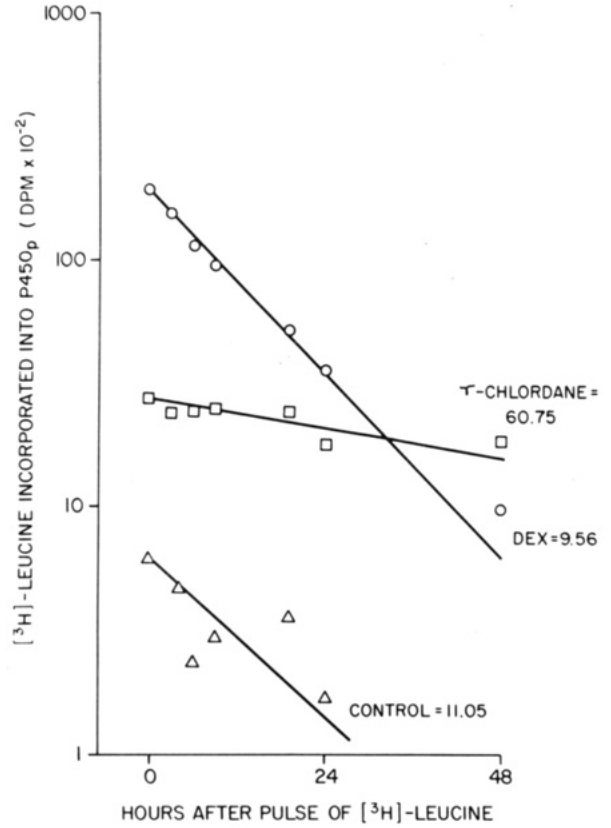

FIGURE 3: Degradation of P-450p in hepatocyte cultures treated with dexamethasone or chlordane. Freshly isolated hepatocytes prepared from a single rat were incubated in control culture medium or in medium containing 1×10^{-5} M chlordane or dexamethasone. At 96 h of culture age, the monolayers were exposed to medium containing [^{3}H]leucine plus an inducer where appropriate. Four hours later, this pulse was terminated by replacing the radioactive culture media with media containing 1.9 mM unlabeled leucine plus chlordane or dexamethasone where appropriate. Incubation of the cultures was continued, and at the times indicated, monolayers were harvested, and the amount of radioactivity remaining in immunoprecipitable P-450p was determined as described under Materials and Methods. The half-lives were calculated from linear regression analysis of the rate of disppearance of isotope incorporated in P-450p.

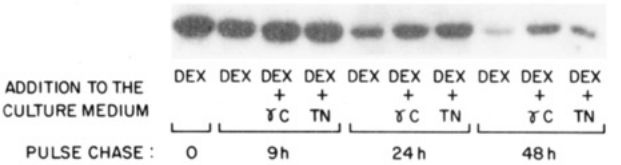

FIGURE 4: Effect of dexamethasone plus organochlorine pesticides on degradation of P-450p in hepatocyte cultures. The protocol differed from that in Figure 3 in that freshly isolated hepatocytes were incubated in medium containing dexamethasone (10^{-5} M) for the first 120 h and then pulse-labeled for 4 h in dexamethasone medium containing [^{3}H]leucine followed by continued incubation in medium containing 1.9 mM leucine and dexamethasone alone or in combination with 1×10^{-5} M chlordane (γC) or *trans*-nonachlor (TN). At time zero (124 h of culture age) and at the indicated data points, cultures were harvested, and P-450p was isolated by immunoprecipitation as described under Materials and Methods. A fluorograph of the immunoprecipitates following their electrophoresis on a 10% Laemmli 1.5-mm slab gel is shown. From counts in the gel slices of this experiment, the calculated half-lives of P-450p wee as follows: dexamethasone, 9.8 h; dexamethasone + γC, 20.0 h; dexamethasone + TN, 20.7 h; control cultures incubated in standard medium without dexamethasone for the entire 188 h of this experiment, 9.0 h.

PB-type inducers tetrabromo, tetrachloro, or pentachloro derivatives of TCPOP (10^{-5} M) (Poland et al., 1981, 1980), with substituted pyridines such as chlorpromazine, isonicotinamide, or 3-aminopyridine (each at 1×10^{-4} M), or with one of the following bile acids (1×10^{-4} M): cholic acid, chenodeoxycholic acid, taurochenodeoxycholic acid, or ursodeoxycholic acid (not shown). Furthermore, none of the P-450p inducers significantly altered the viability of the cultures as judged by Trypan Blue exclusion, by the rate of total protein synthesis, or by the recovery of protein per dish at 120 h of incubation (not shown).

In the preceding experiments, the synthesis and the concentration of P-450p were assayed at only 120 h of culture age. Therefore, the results suggest, but by no means prove, that organochlorine pesticides decrease the rate of degradation of P-450p. We used a pulse–chase protocol to radioactively label proteins in hepatocyte culture and examined the effects of cyclodiene organochlorine pesticides on the rate of disappearance of radioactivity in immunoprecipitable P-450p protein. In control cultures, [^{3}H]-P-450p disappeared rapidly with an apparent half-life of 11 h (Figure 3). In dexamethasone-treated cultures, the apparent half-life of P-450p (9.5 h) was slightly, although not significantly, faster (Figure 3). In contrast, in cultures treated with chlordane the rate of disappearance of radioactive P-450p was markedly prolonged to a half-life of 60 h (Figure 3). In a similar experiment in cultures treated with *trans*-nonachlor, the apparent half-life of P-450p was 49.0 h while in control or dexamethasone-treated cultures derived from the same liver the values were 8.1 and 9.4 h, respectively (not shown). Even when chlordane or *trans*-nonachlor was added to the cultures *after* they had been radiolabeled with [^{3}H]leucine, these pesticides prolonged the half-life of P-450p by a factor of 2 (Figure 4). Although it has been reported that glucocorticoids stimulate the rate of

Table II: Accumulation of Cytochrome P-450 and Immunoreactive P-450p in Liver Microsomes Prepared from Rats Treated with Cyclodiene Organochlorine Pesticides[a]

pesticide	total no. of chlorines	total spectral P-450 (nmol/mg of protein)	immunoreactive P-450p (nmol/mg of protein)	immunoreactive P-450p (% of total spectral P-450)
trans-nonachlor	9	1.99 ± 0.33	0.409 ± 0.259	20.6
γ-chlordane	8	2.06 ± 0.12	0.347 ± 0.93	16.8
heptachlor	7	1.12 ± 0.02	0.131 ± 0.044	11.7
dieldrin	6	1.22 ± 0.20	0.068 ± 0.007	5.6
aldrin	6	1.16 ± 0.26	0.124 ± 0.027	10.7
control		0.50 ± 0.16	≤0.003	<1

[a] Female rats received organochlorine pesticides at single daily doses for 4 days equivalent to 11% of their reported LD_{50}: *trans*-nonachlor (46 mg/kg), chlordane (42 mg/kg), dieldrin (5.1 mg/kg), heptachlor (2.5 mg/kg), and aldrin (7.7 mg/kg). The compounds were dissolved in corn oil and were injected intraperitoneally. Animals were then starved overnight and killed on day 5. Liver microsomes were prepared as described previously (Heuman et al., 1982), and the amounts of total cytochrome P-450 and immunoreactive P-450p were determined as described under Materials and Methods. P-450p was undetectable in corn oil treated control female rats (<3 pmol/mg of microsomal protein). Values are the means ± SD of three separate rats.

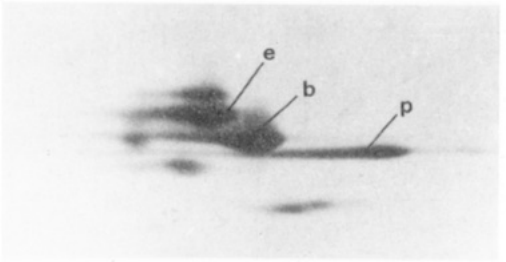

FIGURE 5: Double antibody analysis of chlordane liver microsomes subjected to two-dimensional gel electrophoresis and immunoblotting. A total of 15 μg of microsomes from a chlordane-treated rat were focused isoelectrically from the left (acidic end) to the right (basic end) (Vlasuk et al., 1982a) followed by electrophoresis on a 10% Laemmli slab gel. The resolved proteins were transferred electrophoretically to nitrocellulose sheets. Following development with anti-P-450p IgG, only P-450p (p) was visualized. Then, the sheets were washed and developed with H-8 monoclonal antibody to $P\text{-}450_{PB}$, which recognized a cluster of proteins including P-450b (b) and P-450e (e).

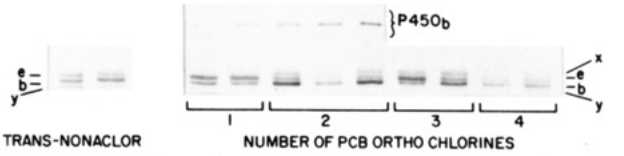

FIGURE 6: Induction of cytochrome P-450 immunoreactive with anti-$P\text{-}450_{PB}$ IgG by polychlorinated biphenyls in rat liver. Liver microsomes prepared from individual rats treated with *trans*-nonachlor (Figure 7) or PCBs (Table III) were resolved by SDS gel electrophoresis. The electrophoresis was temporarily interrupted to allow a second loading of 0.25–2.5 pmol of purified P-450b (see Materials and Methods). The proteins were then transferred electrophoretically to nitrocellulose paper and localized with H-8 monoclonal antibody to $P\text{-}450_{PB}$. From left to right, lanes 1 and 2 contain 10 μg each of microsomes from *trans*-nonachlor-treated rats, lanes 3 and 4 contain 10 μg each of microsomes from the 2,3,3′,4,4′,5′-PCB-treated rats, lanes 5–7 and 8–9 contain respectively 7 μg of microsomes from the 2,2′,4,4′-PCB- and the 2,2′,4,4′,5,6′-PCB-treated rats, and lanes 10 and 11 contain respectively 15 and 20 μg of microsomes from the 2,2′,3′,4,4′,6,6′-PCB-treated rats. Proteins y and x correspond to the fastest and slowest moving immunoreactive polypeptides, respectively.

total protein degradation in hepatocyte cultures (Hopgood & Ballard, 1980), under the conditions of these experiments neither dexamethasone nor the organochlorine pesticides significantly affected the recovery or the rate of disappearance of total radioactive cellular protein (data not shown). Thus, organochlorine pesticides retard the degradation of P-450p selectively.

Induction of P-450p and P-450$_{PB}$ in Vivo. To confirm the results of experiments in hepatocyte culture, we administered five cyclodiene organochlorine pesticides to rats and found, as expected, that each compound increased by 2–4-fold over control values the concentration of total CO-binding hemoprotein in liver microsomes (Figure 5). The concentration of immunoreactive P-450p protein, measured by quantitative immunoblot analysis, was undetectable (<3 pmol/mg of protein) in microsomes prepared from female control rats. There was a dramatic accumulation of P-450p in the rats treated with *trans*-nonachlor (at least 133-fold) or the other cyclodiene organochlorine pesticides (Table II). The efficacy of induction of P-450p could be ranked by the degree of chlorination of the compounds: *trans*-nonachlor (nine Cl's) > chlordane (eight Cl's) > heptachlor (seven Cl's) > aldrin (six Cl's) > dieldrin (six Cl's, epoxide of aldrin formed metabolically). Since this rank order is similar to that induced in cultured hepatocytes (Figure 1), the number of chlorines may determine the intrinsic effectiveness of the pesticide as an inducer of P-450p although differences in absorption, transport, or metabolism of these compounds may be important in vivo. Despite the significant accumulation of P-450p in rats treated with these pesticides (Figure 5) or with phenobarbital (Heuman et al., 1982), P-450p still represented a minor fraction (<20%) of the total CO-binding hemoprotein.

We noted that induction of P-450p by chlordane as demonstrated presently in cultured hepatocytes and in rats contradicts a recent report that phenobarbital but not chlordane induces P-450p (Vlasuk et al., 1982a). To investigate this discrepancy, we used two-dimensional gel electrophoresis followed by immunoblotting to analyze microsomes from one of the chlordane-treated Sprague-Dawley rats (Figure 5). The blot was first developed with anti-P-450p IgG, which revealed a single spot (p, Figure 5) with a mobility (p*I* = 8.0) the same as that of purified P-450p (not shown). When the blot was washed and then redeveloped with H-8 monoclonal antibody directed specifically against an epitope common to P-450b and P-450e (Kumar et al., 1983), a cluster of four proteins with similar p*I*'s could be visualized. The first corresponds (top to bottom) to an uncharacterized high molecular weight protein, possibly protein x (see Figure 6). The middle two proteins correspond to P-450e and P-450b on the basis of the mobilities of purified P-450b and P-450e in other blots of two-dimensional gels (not shown). The fourth immunoreactive polypeptide (possibly protein y, see Figure 6) is present in untreated male and female Sprague-Dawley rats and does not appear to be significantly induced by phenobarbital-like agents (not shown). Since Vlasuk and co-workers used immature Long-Evans rats in their study, it was possible that age or interstrain and intrastrain differences in rat P-450 phenotype, (Vlasuk et al., 1982a,b; Rampersaud & Walz, 1983; Walz et al., 1982; Guengerich et al., 1981; Dent et al., 1980) could explain the different results. However, when the same prep-

Table III: Stereospecificity of Induction of P-450p and P-450$_{PB}$ by Polychlorinated Biphenyl Isomers in Rat Liver in Vivo[a]

position of chlorines	total no. of chlorines	no. of chlorines			P-450p accumulation (nmol/mg of protein)	P-450$_{PB}$ accumulation (nmol mg/of protein)		
		ortho	meta	para		P-450e	P-450b	y + e + b + x[c]
2,3′,3,4,4′,5′	6	1	3	2	0.0255 ± 0.0112	0.201	0.147	0.43
2,2′,4,4′[b]	4	2	0	2	0.0424 ± 0.0270	0.231	0.416	0.96
2,2′,4,4′,5,6′	6	3	1	2	0.0737 ± 0.0081	0.222	0.249	0.78
2,2′,3′,4,4′,6,6′[b]	7	4	1	2	0.0020 ± 0.0012	0.017	0.041	0.08

[a] Female rats received the PCB congeners at a dose of 500 mol/kg of body weight in corn oil (0.5 mL) by a single intraperitoneal injection on day zero and were killed 4 days later by decapitation. Liver microsomes were prepared as described previously (Heuman et al., 1982), and the amount of immunoreactive P-450p and P-450$_{PB}$ was determined as described under Materials and Methods. Values are the averages of two to three separate rats. [b] See Table I for in vitro values. [c] Sum of all bands (see Figure 7).

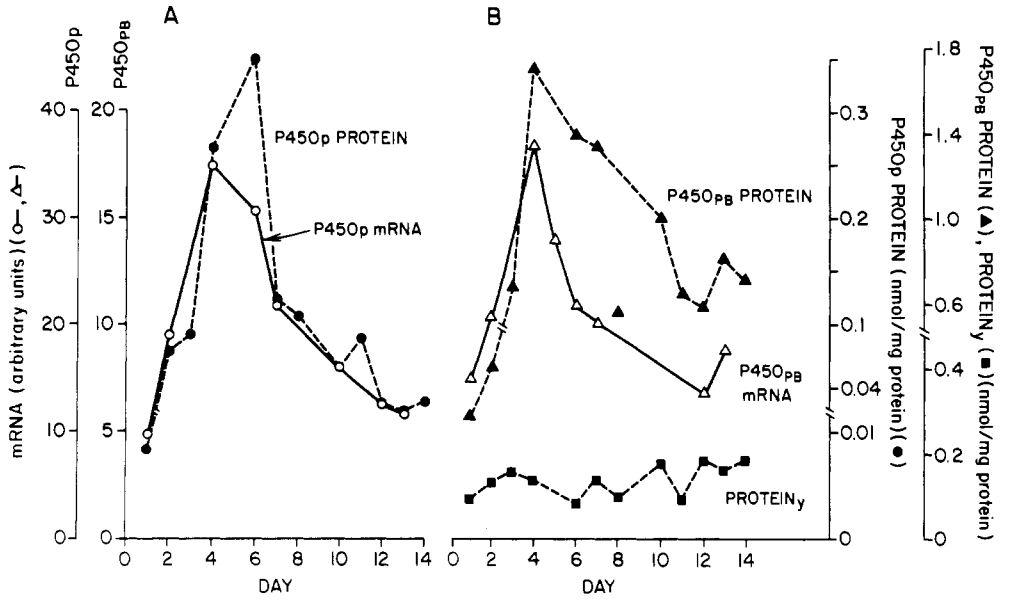

FIGURE 7: Time course of induction of P-450$_{PB}$ and P-450p proteins and their respective mRNA by *trans*-nonachlor in rat liver. Silastic capsules containing 0.03 in.^{3} of *trans*-nonachlor were implanted intramuscularly, and on days 1–14 thereafter, rats were killed, and liver microsomes and total RNA were prepared. The amounts of P-450$_{PB}$ (A) and P-450p mRNA (B) were determined by dot blot hybridizations to pR17 and pDex12, respectively, as described under Materials and Methods. The amounts of P-450$_{PB}$, P-450p, and protein y in microsomes were determined by quantitative immunoblot analysis. The values for P-450b and P-450e were summed and are given as P-450$_{PB}$. Protein y is the fastest moving of the resolved proteins recognized by the H-8 monoclonal antibody (see Figure 6).

aration of chlordane microsomes used by Vlasuk et al. (Vlasuk et al., 1982a; Thomas et al., 1983) was analyzed by one-dimensional immunoblotting with anti-P-450p IgG, the results showed a significant amount of an immunoreactive protein with the same mobility as that of purified P-450p from Sprague-Dawley rats (not shown). Other structurally diverse agents shown to be inducers of the putative "P-450p" (Vlasuk et al., 1982a) such as isosafrole, *trans*-stilbene oxide, and SKF-525A have been found by others (Guengerich et al., 1983) and ourselves (data not shown) to induce P-450p.

PCBs, like the organochlorine pesticides and phenobarbital, simultaneously induced P-450$_{PB}$ and P-450p in rats (Table III). Whereas the degree of chlorination of the organochlorine pesticides was directly related to the extent of induction of P-450p in vivo (Table II), there was no relationship between the number of chlorine substituents on the PCB isomers and their ability to induce P-450p (Tables I and III). Inducibility of P-450p by PCBs in vivo was related to the number of ortho chlorine substituents (3 > 2 > 1) except for the four ortho chlorinated isomer. This PCB (2,2′,3′,4,4′,6,6′) was the most potent inducer in cultured hepatocytes (Table I) but was the weakest inducer in rats (Table III). Indeed, the trichloro-substituted PCB is quantitatively a better inducer of P-450p than P-450$_{PB}$.

When microsomes prepared from PCB-treated rats were analyzed by immunoblotting with H-8 monoclonal antibody against P-450$_{PB}$, four proteins were detected (Figure 6). The central two (major) bands, P-450e and P-450b, were sufficiently well separated to permit their quantitation by high-resolution densitometry. In contrast to reports (Vlasuk et al., 1982a) that P-450b and P-450e are present in the same relative proportion, regardless of the PB inducer tested, our data suggest that the ratio of b:e was significantly elevated by the two ortho chlorine isomer, whereas the one ortho chlorine PCB congener preferentially increased P-450e (Table III). The discrepancy in the ratio of b:e between the two animals treated with the three ortho chlorine PCB (Figure 6) may be due to interanimal variability. All samples contained an immunoreactive protein (y) with more rapid mobility than P-450b, and in some there was a fourth immunoreactive protein (x) with mobility less rapid than P-450e. These results are consistent with the findings in hepatocyte culture that the stereospecificities of these compounds for induction of P-450$_{PB}$ and P-450p are not identical. This difference could not be attributed to the kinetics of induction of P-450p and P-450$_{PB}$. When we implanted silastic capsules containing *trans*-nonachlor into a group of rats to achieve a uniform, sustained release of the inducer, there was a prompt increase in the concentration of P-450$_{PB}$ detectable within 24 h of the start of treatment, which reached a peak after 4 days and declined thereafter for 10 days (Figure 7). This pattern was closely paralleled by changes in the amount of P-450$_{PB}$ mRNA that hybridized to the pR17

cDNA probe. In contrast, there was no change in concentration of protein y, the immunoreactive polypeptide that exhibited the fastest mobility on gel electrophoresis (Figure 6). The concentration of P-450p in the same microsomes and in the amount of P-450p mRNA hybridizable to pDex12 increased with almost the same time course except that the peak accumulation of P-450p was on day 6 (Figure 7).

Discussion

These studies were prompted by the recent intriguing observation that phenobarbital is an inducer of P-450p in rat liver (Heuman et al., 1982). Prior evidence suggested that phenobarbital and related inducers stimulated coordinate expression of the $P\text{-}450_{PB}$ gene family (Atchison & Adesnick, 1983) to the exclusion of the "constituative" forms of cytochrome P-450 (Guengerich et al., 1982a) and the members of the MC gene family (Kimura et al., 1984). P-450p is structurally dissimilar to $P\text{-}450_{PB}$ (Elshourbagy & Guzelian, 1980; Wrighton et al., 1985a; Gonzalez et al., 1985b), is regulated primarily by synthetic and endogenous glucocorticoids, and is encoded by a member of a gene family located on a chromosome different from the $P\text{-}450_{PB}$ locus (Simmons et al., 1985). Therefore, it seemed possible that phenobarbital might induce P-450p indirectly. This explanation has been ruled out by the present demonstration that phenobarbital, organochlorine pesticides, and some PCBs stimulate in a concentration-dependent manner the de novo synthesis of P-450p in cultured hepatocytes incubated for many days in the absence of steroid hormones. These chemicals evoke enhanced synthesis and accumulation of P-450p in hepatocytes in culture and therefore exert their effects by direct contact with the hepatocyte and not as the result of a compensatory response of the whole animal. Since P-450p and $P\text{-}450_{PB}$ and their respective mRNAs increase in parallel in *trans*-nonachlor-treated rats (Figure 7), it may be concluded that although P-450p and $P\text{-}450_{PB}$ genes are structurally distinct, they share some regulatory characteristics.

One mechanism of induction of P-450p revealed in these studies is that some phenobarbital-like inducers, especially the cyclodiene organochlorine pesticides like chlordane and *trans*-nonachlor, decrease the rate of degradation of P-450p. Unlike dexamethasone, organochlorine pesticides increased P-450p accumulation disproportionately more than its synthesis, and in standard pulse–chase experiments these chemicals selectively decrease the rate at which P-450p is degraded (Figures 3 and 4). Chlordane and *trans*-nonachlor exerted a lesser (albeit still significant) inhibitory effect on degradation of P-450p when they were added after dexamethasone-induced cultures were pulse-labeled (Figure 4). This difference may reflect a competitive interaction between dexamethasone and the pesticides, a requirement for metabolism of the pesticides or some other adaptation of the cells to these compounds. It would have been desirable to confirm the results in cultured hepatocytes by measuring the turnover of P-450p in vivo, but we were unable to achieve an induced, steady-state level of P-450p in *trans*-nonachlor-treated rats (Figure 7). Even though it has been reported that phenobarbital treatment of rats causes a 2-fold decrease in the specific rate of P-450p degradation, failure to achieve an induced steady state prevented the authors from concluding that the effect of the drug was significant (Shiraki & Guengerich, 1984). Nevertheless, there is indirect evidence that organochlorine pesticides decrease the degradation of P-450p in vivo as they do in hepatocyte culture. First, chlordane and *trans*-nonachlor were the most efficacious inducers of P-450p concentration in vivo (Table II) and in cultured hepatocytes (Figure 1) and, yet, were only slightly superior to the other pesticides at stimulating the synthesis of P-450p (Figure 1). Second, whereas the amounts of $P\text{-}450_{PB}$ protein and mRNA increased in parallel, the peak accumulation of P-450p protein occurred 24–48 h after the peak of P-450p mRNA (Figure 7). Finally, P-450p reaches an induced steady state within 2 days of dexamethasone administration to rats (Watkins et al., 1984) while P-450p levels increase linearly for 6 days after *trans*-nonachlor treatment (Figure 7). These results are consistent with observations in cultured hepatocytes that dexamethasone induces P-450p primarily by increasing its rate of synthesis (Figures 1 and 3) whereas *trans*-nonachlor also decreases significantly the rate of P-450p degradation.

Our finding that organochlorine pesticides selectively inhibit the rate of P-450p degradation should be interpreted in light of additional new data indicating that P-450p degradation is specifically and dramatically inhibited by macrolide antibiotics such as triacetyloleandomycin or erythromycin that are specifically converted by P-450p into metabolites that bind strongly to P-450p heme (Watkins et al., 1984). Therefore, by analogy to the classic model in which tryptophan stabilizes tryptophan pyrrolase by facilitating hematin binding (Feigelson & Greengard, 1962), stabilization of P-450p heme might prevent catabolism of P-450p protein. However, since neither organochlorine pesticides nor their metabolites appear to form P-450p heme complexes and yet comparably inhibit the breakdown of P-450p protein, stabilization of the heme prosthetic group may not be the key event controlling degradation of P-450p protein. However, both organochlorine pesticides and macrolide antibiotics bind to microsomal P-450p producing a "type I" spectral change (unpublished results) indicative of an interaction with the substrate binding site. Therefore, analogous to stabilization of tryptophan oxygenase by its substrate tryptophan (Schimke, 1964), the turnover of P-450p, the most rapid of any cytochrome P-450 yet reported, may be controlled by the amounts and types of bound native and exogenous substrates.

Phenobarbital-like inducers increase the concentration of P-450p not only by decreasing its catabolism but also by stimulating its synthesis as assayed directly in cultured hepatocytes and indirectly by measurement of P-450p mRNA in vivo. We have proposed that P-450p gene expression is mediated by glucocorticoid hormones binding to a stereospecific "PCN receptor" or "recognition site" similar to, but not identical with, the classic glucocorticoid receptor (Schuetz & Guzelian, 1984). Even though there is little apparent structural relationship between the phenobarbital-like inducers and the glucocorticoids, it is still possible that these compounds induce P-450p synthesis by interacting with the postulated "receptor" just as chlordane and *o,p'*-DDD, compounds structurally distinct from estrogens, bind to the estradiol receptor and evoke hormonal responses (Hammond et al., 1979). Indeed, dilantin, a phenobarbital-like inducer of P-450p, binds to the same receptor as does dexamethasone (Katsumata et al., 1982) while other phenobarbital-like inducers displace glucocorticoids in whole cell binding assays (Goldman et al., 1978).

In testing PCBs having one or more ortho chlorines and known to resemble phenobarbital as inducers of liver cytochromes P-450 (Parkinson et al., 1983b; Denomme et al., 1983), we found that P-450p and $P\text{-}450_{PB}$ were not induced in parallel. PCBs with more than two ortho chlorines were the most efficacious inducers of P-450p in hepatocyte cultures and in rats, whereas others have reported maximum $P\text{-}450_{PB}$ induction was produced by congeners with two ortho chlorine

substituents (Parkinson et al., 1983b; Denomme et al., 1983).

It should be noted that 2,2′,4,4′-tetrachlorobiphenyl, one of the best inducers of P-450$_{PB}$ in vivo (Parkinson et al., 1983b), caused no accumulation of P-450p in hepatocyte culture and also that the 2,2′,3′,4,4′,6,6′ form was the most potent stimulator of P-450p synthesis in culture and, yet, was the weakest inducer of P-450p in vivo. These discrepancies may be due to differences in solubility, uptake, distribution, and/or metabolism of the PCB isomers in the two experimental systems. Nevertheless, on the basis of the available data, it may be reasonably concluded that induction of P-450$_{PB}$ and P-450p is mediated by pathways that, although similar, are not identical.

The most dramatic *qualitative* difference in structure–activity relationships among phenobarbital-like inducers is a recent report of a PCN-inducible protein tentatively identified as P-450p that is also inducible by phenobarbital but *not* by chlordane (Vlasuk et al, 1982a). By combining two-dimensional gel electrophoresis with immunoblotting, we have clearly demonstrated that chlordane induces not only P-450b and P-450e but also P-450p, both in our rats and in the same microsomes from the Long-Evans strain used in that report (Vlasuk et al., 1982a; Thomas et al., 1982). This eliminates the possibility that the reported insensitivity of Long-Evans rats to chlordane is due to a strain-related variation in the P-450p phenotype or in gene control of the P-450p gene. Given the induction characteristics and molecular weight of the protein identified by Vlasuk and co-workers, it is possible that this is another member of the P-450p family. Even though it appears that only one PCN-inducible protein has to date been purified from rat liver (Wrighton et al., 1985a), there are two proteins immunoreactive with anti-P-450p IgG and inducible by dexamethasone in rabbits (Wrighton et al., 1985b) and in some inbred strains of mice (unpublished observation). Moreover, the NH_2-terminal sequence determined for purified P-450p protein (Wrighton et al., 1985a) is different from that derived from a cloned cDNA sequence encoding a P-450$_{PCN}$ mRNA (Gonzalez et al., 1985a). Finally, there are 50–60 kilobases of the rat genome that hybridize to P-450$_{PCN}$ cDNA, indicative of multiple genes (Hardwick et al., 1983). Indeed, preliminary data show a second isozyme in the P-450/PCN gene family is refractory to induction by chlordane (unpublished results). Thus, chlordane may prove valuable not for discriminating between the regulation of P-450$_{PB}$ and P-450p but for distinguishing mechanisms of induction of members of the P-450p gene family.

ACKNOWLEDGMENTS

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Registry No. *o,p′*-DDD, 53-19-0; 2,2′,3′,4,4′,6,6′-heptachloro-1,1′-biphenyl, 74472-48-3; 2,2′,3′,4,5′,6,6′-heptachloro-1,1′-biphenyl, 74487-85-7; 2,2′3,3′,4′,5,6,6′-octachloro-1,1′-biphenyl, 40186-71-8; 2,2′,4,4′,6,6′-hexachloro-1,1′-biphenyl, 33979-03-2; 2,2′,3,3′,4,4′,5′,6-octachloro-1,1′-biphenyl, 42740-50-1; 2,2′,3′,4′,5,5′,6′-heptachloro-1,1′-biphenyl, 52712-05-7; 2,2′,3′,4,4′,5′,6-heptachloro-1,1′-biphenyl, 60145-23-5; 2,2′,4,4′-tetrachloro-1,1′-biphenyl, 2437-79-8; 2,2′,3,3′,4,4′,5-heptachloro-1,1′-biphenyl, 35065-30-6; 2,3′4,4′-tetrachloro-1,1′-biphenyl, 32598-10-0; 2,3,3′,4,4′,5′-hexachloro-1,1′-biphenyl, 69782-90-7; 2,2′,4,4′,5,6′-hexachloro-1,1′-biphenyl, 60145-22-4; cytochrome P-450, 9035-51-2; phenobarbital, 50-06-6; dexamethasone, 50-02-2; dilantin, 630-93-3; γ-chlordane, 5566-34-7; *trans*-nonachlor, 39765-80-5; oxychlordane, 27304-13-8; pregnenolone-16α-carbonitrile, 1434-54-4; dieldrin, 60-57-1; heptachlor epoxide, 1024-57-3; aldrin, 309-00-2; endrin, 72-20-8; endosulfan, 115-29-7; δ-lindane, 319-86-8; heptachlor, 76-44-8; kepone, 143-50-0; β-lindane, 319-85-7.

REFERENCES

Adesnick, M., Bar-Nun, S., Maschio, F., Zunich, M., Lippman, A., & Bard, E. (1981) *J. Biol. Chem. 256*, 10340–10345.

Atchison, M., & Adesnick, M. (1983) *J. Biol. Chem. 258*, 11285–11295.

Bissell, D. M., & Guzelian, P. S. (1980) *Ann. N.Y. Acad. Sci. 349*, 85–98.

Buetti, E., & Diggelmann (1983) *EMBO J. 8*, 1423–1429.

Campbell, M. A., Gyorkos, T., Leece, B., Homonko, K., & Safe, S. (1983) *Gen. Pharmacol. 14*, 445–454.

Conney, A. H. (1967) *Pharmacol. Rev. 19*, 317–366.

Dannan, G. A., Guengerich, F. P., Kaminsky, L. S. & Aust, S. D. (1983) *J. Biol. Chem. 258*, 1282.

Dannan, G. A., Waxman, D. J., & Guengerich, F. P. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 1813.

Deeley, R. G., Gordon, J. I., Burns, A. T. H., Millinix, K. P., Benastein, M., & Goldberger, R. F. (1977) *J. Biol. Chem. 252*, 8310–8319.

Denome, M. A., Bandiera, S., Lambert, I., Copp, L., & Safe, S. (1983) *Biochem. Pharmacol. 32*, 2955–2963.

Dent, J. G., Graichen, M. E., Schnell, S., & Lasker, J. (1980) *Toxicol. Appl. Pharmacol. 52*, 45–53.

Elshourbagy, N. A., & Guzelian, P. S. (1980) *J. Biol. Chem. 255*, 1279–1285.

Elshourbagy, N. A., Barwick, J. L., & Guzelian, P. S. (1981) *J. Biol. Chem. 256*, 6060–6068.

Feigelson, P., & Greengard, O. (1962) *J. Biol. Chem. 237*, 1908.

Fujii-Kuriyama, Y., Mitzukami, Y., Kawajiri, K., Sogawa, K., & Muramatsu, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2793–2797.

Goldman, A. S., Shapiro, B. H., & Katsumata, M. (1978) *Nature (London) 272*, 464–466.

Gonzalez, F. J., Kimura, S., & Nebert, D. N. (1985a) *J. Biol. Chem. 260*, 5040–5049.

Gonzalez, F. J., Nebert, D. W., Harwick, J. P., & Kasper, C. B. (1985b) *J. Biol. Chem. 260*, 7435–7441.

Guenerich, F. P., Wang, P., Mason, P. S., & Mitchell, M. B. (1981) *Biochemistry 20*, 2370–2378.

Guenerich, F. P., Dannan, G. A., Wright, S. T., Martin, W. V., & Kaminsky, L. S. (1982a) *Biochemistry 21*, 6019–6030.

Guenerich, F. P., Wang, P., & Davidson, N. K. (1982b) *Biochemistry 21*, 1698–1706.

Hammond, B., Katzenellenbogen, B. S., Krauthammer, N., & McConnell, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6641.

Hardwick, J. P., Gonzalez, F. J., & Kasper, C. B. (1983) *J. Biol. Chem. 258*, 10182–10186.

Heuman, D. M., Gallagher, E. J., Barwick, J. L., Elshourbagy, N. A., & Guzelian, P. S. (1982) *Mol. Pharmacol. 21*, 753–760.

Hopgood, M. F., & Ballard, F. J. (1980) *Ciba Found. Symp. 75*, 205–218.

Jones, P. B. C., Galeazzi, D. R., Fisher, J. M., & Whitlock, J. P. (1985) *Science (Washington, D.C.) 227*, 1499–1502.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R. I., Karuter, P., Westphal, H. M., & Beato, M. (1984) *Nature (London) 308*, 513–519.

Katsumata, M., Gupta, C., Baker, M. K., Sussdorf, C. E., & Goldman, A. S. (1982) *Science (Washington, D.C.) 218*, 1313–1315.

Kimura, S., Gonzalez, F. J., & Nebert, D. W. (1984) *J. Biol. Chem. 259*, 10705–10713.
Kumar, A., Raphael, C., & Adesnik, M. (1983) *J. Biol. Chem. 258*, 11280–11284.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.
Legan, S. A., Coon, G. A., & Karsch, F. J. (1975) *Endocrinology (Philadelphia) 96*, 50–56.
Majors, J., & Varmus, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5866–5870.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Newman, S., & Guzelian, P. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2922–2926.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2379–2385.
Parkinson, A., Robertson, L., Safe, L., & Safe, S. (1981) *Chem.-Biol. Interact. 31*, 1.
Parkinson, A., Thomas, P. E., Ryan, D. E., Reik, L. M., Safe, S. H., Robertson, L. W., & Levin, W. (1983a) *Arch. Biochem. Biophys. 225*, 203–215.
Parkinson, A., Safe, S. H., Robertson, L. W., Thomas, P. E., Ryan, D. E., Reik, L. M., & Levin, W. (1983b) *J. Biol. Chem. 9*, 5967–5976.
Poland, A., Glover, E., Robinson, J. R., & Nebert, D. N. (1974) *J. Biol. Chem. 249*, 5599–5606.
Poland, A., Mak, I., Glover, E., Boatman, R. J., Ebetino, F. H., & Kende, A. S. (1980) *Mol. Pharmacol. 18*, 571–580.
Poland, A., Mak, I., & Glover, E. (1981) *Mol. Pharmacol. 20*, 442–450.
Rampersaud, A., & Walz, F. G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6542–6546.
Rudy, A. M., Rashad, M. N., & Mi, M. P. (1984) *Environ. Pharmacol. 1*, 93.
Ryan, D. E., Thomas, P. E., Koreniowski, D., & Levin, W. (1979) *J. Biol. Chem. 254*, 1365–1374.
Sadano, H., & Omura, T. (1983) *Biochem. Biophys. Res. Commun. 116*, 1013–1019.
Schaterle, G. R., & Pollack, R. L. (1973) *Anal. Biochem. 51*, 654–655.
Scheidereit, C., & Beato, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3029–3033.
Scheidereit, C., Geisse, S., Westphal, H. M., & Beato, M. (1983) *Nature (London) 304*, 749–752.
Schimke, R. T. (1964) *J. Biol. Chem. 239*, 3808–3817.
Schmid, W., Scherer, G., Danesch, U., Zentgraf, H., Matthias, P., Strange, C. M., Rowekamp, W., & Schutz, G. (1982) *EMBO J. 10*, 1287–1293.
Schuetz, E. G., & Guzelian, P. S. (1984) *J. Biol. Chem. 259*, 2007–2012.
Schuetz, E. G., Wrighton, S. A., Barwick, J. L., & Guzelian, P. S. (1984) *J. Biol. Chem. 259*, 1999–2006.
Shiraki, H., & Guengerich, F. P. (1984) *Arch. Biochem. Biophys. 235*, 86–96.
Simmons, D. L., Lalley, P. A., & Kasper, C. B. (1985) *J. Biol. Chem. 260*, 515–521.
Thomas, P. E., Reik, L. M., Ryan, D. E., & Levin, W. (1983) *J. Biol. Chem. 258*, 4590–4598.
Vlasuk, G. P., Ryan, D. E., Thomas, P. E., Levin, W., & Walz, F. G. (1982a) *Biochemistry 24*, 6288–6292.
Vlasuk, G. P., Gharayeb, J., Ryan, D. E., Reik, L., Thomas, P. E., Levin, W., & Walz, F. G. (1982b) *Biochemistry 21*, 789–798.
Walz, F. G., Vlasuk, G. P., Omiecinski, C. J., Bresnick, E., Thomas, P. E., Ryan, D. E., & Levin, W. (1982) *J. Biol. Chem. 257*, 4023–4026.
Watkins, P. B., Schuetz, E. G., Wrighton, S. A., Maurel, P., & Guzelian, P. S. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 335.
Watkins, P. B., Wrighton, S. A., Maurel, P., Schuetz, E. G., Mendez-Picon, G., Parker, G. A., & Guzelian, P. S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6310–6314.
Waxman, D. J. (1986) *Cytochrome P-450: Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., Ed.) Plenum, New York (in press).
Waxman, D. J., & Walsh, C. (1982) *J. Biol. Chem. 257*, 10446–10457.
West, S. B., Huang, M.-T., Miwa, G. T., & Lu, A. Y. H. (1979) *Arch. Biochem. Biophys. 193*, 42–50.
Wrighton, S. A., Maurel, P., Schuetz, E. G., Watkins, P. B., Whitley, B., & Guzelian, P. S. (1985a) *Biochemistry 24*, 2171–2178.
Wrighton, S. A., Maurel, P., Schuetz, E. G., Watkins, P. B., Barwick, J., Bailey, B. S., Hartle, H. T., Young, B., & Guzelian, P. S. (1985b) *Mol. Pharmacol. 28*, 312–321.