MOLECULAR PHARMACOLOGY, 21:753–760

Immunochemical Evidence for Induction of a Common Form of Hepatic Cytochrome P-450 in Rats Treated with Pregnenolone-16α-carbonitrile or other Steroidal or Non-Steroidal Agents

DOUGLAS M. HEUMAN,[1] ERIN J. GALLAGHER, JOYCE L. BARWICK, NABIL A. ELSHOURBAGY,[1] AND PHILIP S. GUZELIAN[2]

Liver Study Unit, Department of Medicine, Medical College of Virginia, Richmond, Virginia 23298

Received October 19, 1981; Accepted December 15, 1981

SUMMARY

Recently, we succeeded in isolating and purifying to apparent homogeneity the predominent form of cytochrome P-450 in hepatic microsomes from rats treated with the prototype "catatoxic" steroid, pregnenolone-16α-carbonitrile (PCN), and showed that this form (termed $P450_{PCN}$) is structurally distinct from the major hepatic forms of cytochrome P-450 in rats treated with the traditional inducers, 3-methylcholanthrene or phenobarbital [*J. Biol. Chem.* **255**:1279–1289 (1980)]. In the present study, we prepared antibodies specific for $P450_{PCN}$ and used radial immunodiffusion to measure the concentration of this cytochrome in hepatic microsomes prepared from rats treated with PCN or various other steroidal or nonsteroidal inducers. In untreated control rats, the level of $P450_{PCN}$ was undetectable (<15 nmoles/g of microsomal protein), representing less than 2% of total cytochrome P-450 measured as CO-binding hemoprotein. This finding excludes $P450_{PCN}$ as a major form of cytochrome P-450 in the basal steady state. Following treatment with PCN, the total cytochrome P-450 concentration increased 2-fold, whereas the level of $P450_{PCN}$ rose more than 20-fold (317 nmoles/g of microsomal protein). Accumulation of immunoreactive $P450_{PCN}$ was also observed in microsomes prepared from rats treated with dexamethasone (>30-fold), 6α-methylprednisolone (>10-fold), and spironolactone (>12-fold). In contrast, a variety of other steroids, including glucocorticoids, androgens, estrogens, and progestins, and steroids structurally related to PCN, failed to induce immunoreactive $P450_{PCN}$. The nonsteroidal inducer, phenobarbital, increased the microsomal concentration of immunoreactive $P450_{PCN}$ more than 13-fold, whereas this protein remained undetectable following 3-methylcholanthrene treatment. The dose of dexamethasone (a potent glucocorticoid), required for achieving a maximal increase in immunoreactive $P450_{PCN}$ was at least 10 times higher than that required for maximal induction of tyrosine aminotransferase activity, a measure of glucocorticoid potency. Moreover, doses of PCN that increased the concentration of $P450_{PCN}$ produced a significant decrease in tyrosine aminotransferase activity. We conclude that PCN and selected other steroidal and nonsteroidal inducers promote the accumulation of $P450_{PCN}$ or forms closely related to $P450_{PCN}$ by a process dissociable from events mediated by the classical glucocorticoid receptor.

INTRODUCTION

Cytochrome P-450, a key element of the hepatic microsomal monooxygenase system, plays an important role in the biotransformation of a diverse array of foreign and endogenous compounds. The concentration of cytochrome P-450 in liver is inducible, rising or falling upon the administration or withdrawal of many lipophilic chemicals. Because the amount of cytochrome P-450 may be rate-limiting for the metabolism of many toxic, mutagenic, or carcinogenic substances, there is intense interest in discovering how environmental chemicals promote the accumulation of this hemoprotein. Although it was believed at first that cytochrome P-450 was a single, catalytically unspecific enzyme, recent progress in isolation and purification of individual cytochrome(s) P-450 has revealed a family of hemoprotein isoenzymes with different catalytic specificities (1). The two best-studied

This research was supported by a Program Project in Liver Metabolism from the National Institutes of Health (P02-AM-18976).

[1] Supported by a Training Grant in Gastroenterology from the National Institues of Health (T32-AM-07150).

[2] Recipient of a Research Career Development Award from the National Institutes of Health (K04-AM-00570).

0026-895X/82/030753-08$02.00/0

forms of the cytochrome in rat liver are cytochrome P-450 and cytochrome P-448, which predominate in animals treated with the prototype inducers, phenobarbital or 3-methylcholanthrene, respectively (2–7). It has been suggested that steroids may constitute a "third class" of cytochrome P-450 inducers. Selye (8) identified a group of protective steroids (termed "catatoxic" steroids) capable of accelerating the metabolic clearance of toxic substances administered to pretreated rats. The most potent catatoxic steroid, PCN,[3] also caused proliferation of hepatic smooth endoplasmic reticulum (9) and increased levels of cytochrome P-450 in the liver (10, 11). The possibility that PCN induced a form of cytochrome P-450 differing from those induced by phenobarbital or 3-methylcholanthrene was suggested by the unique profile of microsomal drug-oxidizing activities in PCN-treated rats (11) and also by the fact that antibodies directed against the phenobarbital- or the 3-methylcholanthrene-induced forms of cytochrome P-450 failed to block the enhanced 6β-hydroxylation of testosterone catalyzed by microsomes from PCN-treated rats (12). Recently, we isolated and purified the principal form of hepatic cytochrome P-450 from rats treated with PCN (referred to here as $P450_{PCN}$) (13). We showed that this form is biochemically and immunochemically distinct from $P450_{PB}$ or $P448_{MC}$ (13). By using antibodies as a means for measuring $P450_{PCN}$ specifically, we have reported that PCN markedly accelerates *de novo* synthesis of $P450_{PCN}$ in primary cultures of adult rat hepatocytes (14). In the present study, we have examined the induction of $P450_{PCN}$ by PCN and other steroids in rat liver *in vivo*.

MATERIALS AND METHODS

Materials. Sodium phenobarbital was obtained from Amend Drug & Chemical Company (Irvington, N. J.), and 3-methylcholanthrene from Eastman Kodak Company (Rochester, N.Y.). PCN, fluoxymestrone, and 6α-methylprednisolone were generously provided by The Upjohn Company (Kalamazoo, Mich.). Ethylestrenol and nandrolone were purchased from Organon, Inc. (West Orange, N.J.). All other steroids were purchased from Sigma Chemical Company (St. Louis, Mo.).

Animals. All experiments were carried out using female Sprague-Dawley rats (Flow Laboratories, Dublin, Va.), weighing 80–160 g, individually maintained in wire-bottomed cages with free access to commercial chow and water. Administered compounds were dissolved in normal saline (10 ml/kg) containing a few drops of Tween 80, with the exception of 3-methylcholanthrene, which was dissolved in corn oil (1.6 ml/kg). All treatments, including the appropriate vehicles for control animals, were given once daily for 4 days by gastric gavage (except where otherwise noted).

Preparation of solubilized liver microsomes. After an overnight fast, rats were killed by decapitation. Livers were perfused retrograde with iced phosphate-buffered saline (pH 7.4), excised immediately, and homogenized at 0° in 0.1 M potassium phosphate (pH 7.4) (4 ml/gm) with the use of a motor-driven Teflon pestle. A portion of each liver homogenate was centrifuged at 31,000 × *g* for 30 min, and the supernatant was frozen for subsequent assay of tyrosine aminotransferase activity. The remaining portion of the homogenate was centrifuged at 18,500 × *g* for 20 min, and the supernatant was transferred to a fresh tube and was centrifuged at 105,000 × *g* for 60 min. The resulting microsomal pellet was resuspended in a small volume of 0.05 M potassium phosphate buffer (pH 7.4) containing 25% glycerol and 0.1 mM sodium EDTA to give a concentration of 15–20 mg of protein per milliliter, and stored at −20°. There was no loss of cytochrome P-450 content as measured spectrally or of $P450_{PCN}$ antigenicity when microsomes were stored for up to 3 months. Microsomes were solubilized on ice by combining four parts of the microsomal suspension with one part of 0.05 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 4.5% NaCl, 1.67% sodium cholate, and 1.0% Nonidet P40 (Bethesda Research Laboratories, Rockville, Md.), using a hand-operated Teflon pestle. The solution was centrifuged for 30 min at 105,500 × *g*, and the supernatant containing solubilized microsomal proteins was used for spectral measurement of total cytochrome P-450 and for the immunodiffusion studies described below. This protocol for solubilization released 85–90% of the total CO-binding hemoprotein in microsomes prepared from control rats and 60–70% of that from steroid-treated animals without detectable denaturation of the hemoprotein to cytochrome P-420. No attempt was made to correct for incomplete release or loss of cytochrome P-450 in the solubilization process, and all data are expressed on the basis of solubilized material.

Gel diffusion techniques. Radial immunodiffusion was performed according to the technique of Mancini *et al.* (15) with certain modifications. The immunodiffusion medium contained 0.9% agarose, 0.9% sodium chloride, 0.1% sodium azide, 10% glycerol, and 0.1 mM sodium EDTA, all dissolved in 0.05 M potassium phosphate buffer (pH 7.4). Freshly prepared gel diffusion medium was heated at 100° in a double boiler to melt the agarose and was then allowed to equilibrate to 55° in a water bath. Form-specific anti-$P450_{PCN}$ IgG (0.6 ml containing 17.5 mg of protein) was warmed to 55° and then combined with 15 ml of the agarose medium. The mixture was poured smoothly and quickly onto the hydrophilic surface of a Gel Bond Film (8.5 × 10 cm) (FMC Corporation, Rockland, Me.), maintained in a level position and allowed to harden. This method gave gels of uniform thickness, and results were identical with those obtained using gels formed with a U-frame spacer. Circular wells were created with use of a 2.5-mm punch, and aliquots (7 μl) of test antigen solution were added to the wells. Preliminary tests were made to estimate the concentration of immunoreactive $P450_{PCN}$ in each solubilized microsomal preparation, and the solution was adjusted to a final concentration of approximately 1 nmole of $P450_{PCN}$ per milliliter. Accordingly, the protein concentration varied widely among different samples (2–15 mg/ml). Serial dilutions of purified $P450_{PCN}$ (1.81 nmoles/ml, based on reduced-CO binding hemoprotein content) served routinely as standards for each gel. Duplicate

[3] The abbreviations used are: PCN, pregnenolone-16α-carbonitrile; $P450_{PCN}$, $P450_{PB}$, and $P448_{MC}$ refer to the major forms of cytochrome P-450 purified from rat liver microsomes prepared from animals treated with PCN, phenobarbital, and 3-methylcholanthrene, respectively (13).

measurements of test samples were made on opposite ends of each gel. Gels were incubated at 4° in a humid chamber for 7–10 days to allow complete equilibration. Because precipitin rings were poorly visible at this stage, the gels were washed by further incubation in phosphate-buffered saline (pH 7.4) for 2–4 days at 4°. After the gels were blotted and dried, they were stained with 0.5% Coomassie Brilliant Blue in 45% ethanol and 10% acetic acid. Precipitin rings were measured against an illuminated white background with the use of a calibrated eyepiece magnifier, and the area within each precipitin ring was calculated. By preparing a linear regression plot of the ring sizes produced by different amounts of the $P450_{PCN}$ standard, the concentration of immunoreactive $P450_{PCN}$ in a test solution could be calculated. A portion of a typical gel is shown in Fig. 1.

Ouchterlony double-immunodiffusion analysis was performed using the same diffusion medium and support. Wells (3 mm) were punched at 10-mm intervals using a hexagonal template, and test solutions were added to each well. Diffusion was allowed to proceed in a humid chamber at 4° for 48 hr. Subsequently, the gels were washed and stained by the same procedure described for radial immunodiffusion gels.

Preparation of $P450_{PCN}$ antigen and antibody. Methods used in purification of $P450_{PCN}$, in preparation of form-specific antibody (IgG fractions) directed against $P450_{PCN}$, and in verification of antibody specificity have been detailed in a previous publication (13). The major form of hepatic microsomal cytochrome P-450 from PCN-treated rats was purified to apparent homogeneity as judged by gel electrophoresis and was used to immunize goats. An IgG fraction of the antiserum was prepared by ammonium sulfate precipitation followed by DEAE-cellulose chromatography (13). The IgG fraction was adsorbed against Sepharose-bound microsomes prepared from 3-methylcholanthrene-treated rats to remove antibodies that might recognize either proteins other than cytochrome P-450 or antigenic determinants common to other forms of cytochrome P-450 (13).

Other assays. The concentration of microsomal cytochrome P-450 was determined as its dithionite-reduced CO difference spectrum using an extinction coefficient of 91 mM^{-1} cm^{-1} as described by Omura and Sato (16). Spectra were recorded with an Aminco DW2-A spectrophotometer equipped with a Midan digital electronic baseline correction (American Instrument Company, Silver Spring, Md.). Tyrosine aminotransferase activity was measured by the method of Diamondstone (17) as modified by Granner and Tomkins (18). Activity of the enzyme was expressed as nanomoles of *p*-hydroxyphenyl pyruvate produced per minute per milligram of microsomal protein, with the assumption of 19.9 as the millimolar extinction coefficient. Protein was measured by the procedure of Schacterle and Pollack (19), with the use of crystalline bovine serum albumin as standard.

RESULTS

The radial immunodiffusion assay gave sharp precipitin rings when form-specific anti-$P450_{PCN}$ IgG was allowed to react with either purified $P450_{PCN}$ or solubilized hepatic microsomes prepared from PCN-treated rats (Fig. 1). No precipitin rings appeared when purified

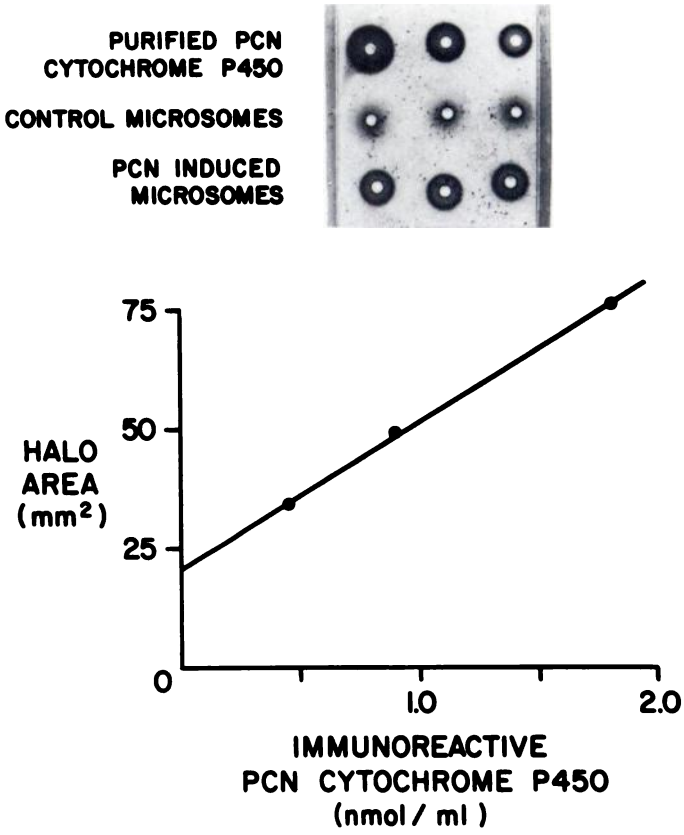

FIG. 1. *Radial immunodiffusion assay for $P450_{PCN}$*

A portion of a radial immunodiffusion gel that had been washed, dried, and stained for protein as described under Materials and Methods is shown at *top*. The *upper row* contained serial dilutions of purified $P450_{PCN}$ (7.8 nmoles/mg of protein). The *lower two rows* contained solubilized hepatic microsomes prepared from control rats (14.0 mg of protein per milliliter) or PCN-treated rats (0.9 mg of protein per milliliter). The area contained within each standard precipitin ring, excluding the central well (halo area), was plotted against the $P450_{PCN}$ concentration to obtain a standard curve (*bottom*).

$P448_{MC}$ or $P450_{PB}$ was tested (data not shown). Repeated determinations of a given sample gave less than 5% variation provided that purified standards were used on each gel. Cholate and nonionic detergents were omitted from the formulation of our gels because, contrary to the experience of Thomas *et al.* (20), who used antibodies directed toward other forms of cytochrome P-450, we found that inclusion of these detergents not only failed to improve the sensitivity of the assay, but actually interfered markedly with the precipitation reaction. Also in contrast to the results of Thomas *et al.* (20), we detected only a diffuse haziness rather than a clear precipitin ring using solubilized hepatic microsomes prepared from untreated rats, even when the concentration of protein added to the wells was as high as 15 mg/ml (Fig. 1).

When we treated rats with PCN and measured the concentration of total CO-binding hemoprotein in hepatic microsomes, the value was approximately 2 times higher than that obtained with microsomes prepared from control animals (Fig. 2). This finding confirms previous reports on the magnitude of induction of total cytochrome P-450 by PCN (10, 14, 21–23). In contrast, the concentration of immunoreactive $P450_{PCN}$ as mea-

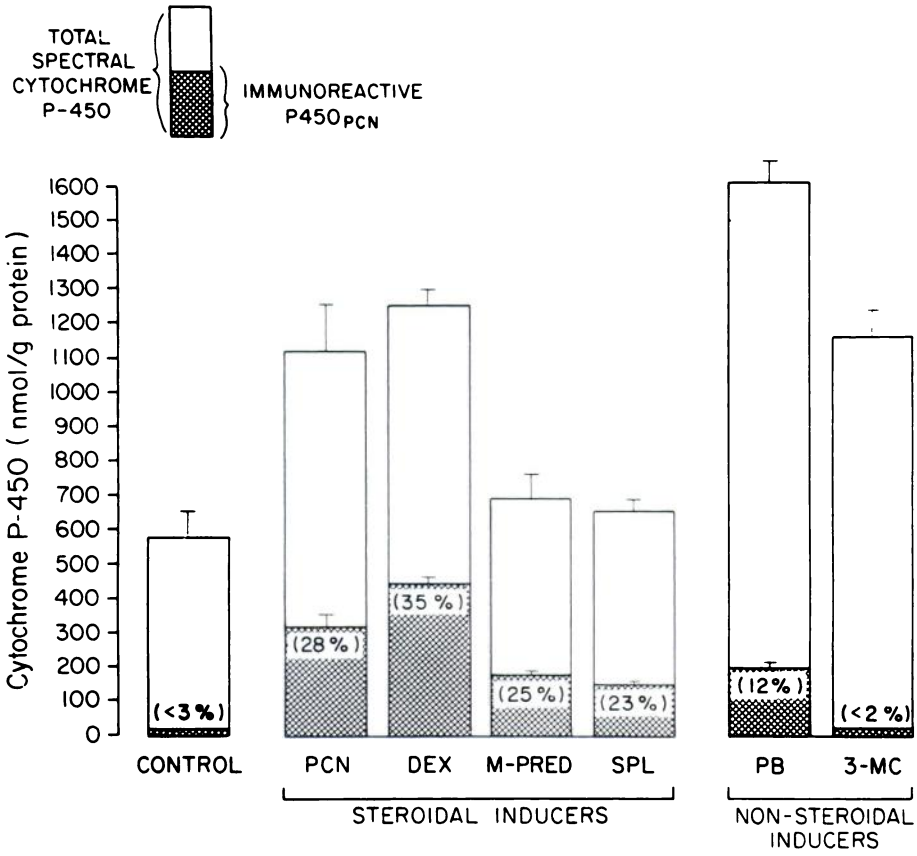

FIG. 2. *Comparison of the effects of PCN and other agents on induction of total cytochrome P-450 and immunoreactive P450PCN*

Groups of rats were treated by gavage with the indicated agents at a dose of 100 mg/kg daily for 4 days, with the exception of 3-methylcholanthrene, which was given as a daily dose of 20 mg/kg (see Materials and Methods). The rats were then killed, liver microsomes were prepared and solubilized, and measurements were made of total cytochrome P-450 (*bar*) and immunoreactive P450PCN (*hatched area*). Results are expressed as nanomoles of cytochrome P-450 per gram of microsomal protein. Numbers in parentheses indicate immunoreactive P450PCN expressed as a percentage of the total cytochrome P-450. Brackets indicate standard error of the mean for three to four animals.

sured by radial immunodiffusion in microsomes from PCN-treated rats was 317 nmoles/g of microsomal protein, while being undetectable in microsomes from control animals (Fig. 2). Since the lower limit of accuracy of the immunoassay is about 15 nmoles/g of microsomal protein, this change represents at least a 20-fold induction of P450PCN. Whereas P450PCN appeared to represent 28% of the total cytochrome P-450 in PCN-treated rats, it constituted less than 3% of total cytochrome P-450 in control animals. This finding excludes P450PCN as a major form of hepatic cytochrome P-450 in the basal steady state.

Three steroidal agents other than PCN—spironolactone, 6α-methylprednisolone, and dexamethasone—gave readily detectable levels of immunoreactive P450PCN in hepatic microsomes. Each agent induced an antigen that was indistinguishable from P450PCN as judged by Ouchterlony double-immunodiffusion (Fig. 3). Thus, when purified P450PCN was compared with solubilized liver microsomes from rats treated with PCN, dexamethasone, spironolactone, 6α-methylprednisolone, or phenobarbital, we observed fusion of the precipitin lines without spur formation, consistent with antigenic identity. In contrast, we were unable to demonstrate precipitin lines using liver microsomes prepared from control or 3-methylcholanthrene-treated rats. Dexamethasone was a more potent inducer than PCN, increasing total cytochrome P-450 more than twice that in controls, and increasing immunoreactive PCN cytochrome P-450 to 441 nmoles/g of microsomal protein as measured by radial immunodiffusion (Fig. 2). In contrast, spironolactone and 6α-methylprednisolone produced minimal (if any) increases in total cytochrome P-450, and yet increased immunoreactive P450PCN significantly (148 and 175 nmoles/g of microsomal protein, respectively).

We also studied 3-methylcholanthrene and phenobarbital as traditional representatives of two classes of cytochrome P-450 inducers. Both agents produced the expected increases in total cytochrome P-450 (Fig. 2). However, immunoreactive P450PCN remained undetectable following 3-methylcholanthrene treatment, whereas this protein was increased by phenobarbital treatment to a value (192 nmoles/g of microsomal protein) in the same range as that seen following spironolactone or 6α-methylprednisolone treatments (Fig. 2). However, because phenobarbital increased total cytochrome P-450 to a far greater extent than did the steroid inducers, immunoreactive P450PCN represented only 12% of total cytochrome P-450 in the phenobarbital-treated animals, as compared with more than 23% for all steroidal treatments (Fig. 2).

To exclude the possibility that induction of immuno-

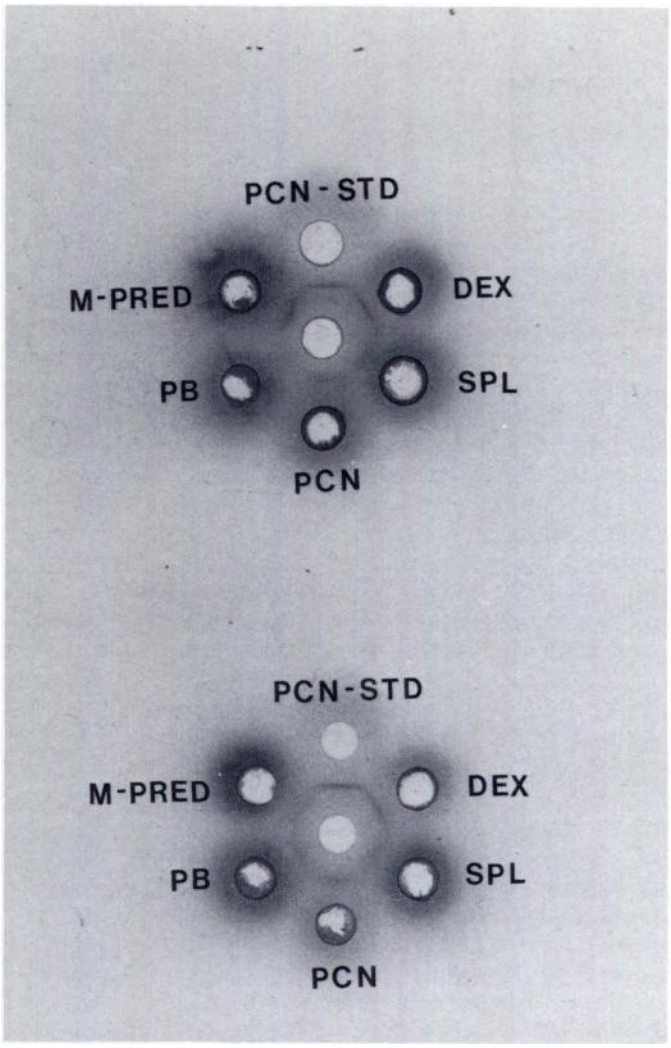

FIG. 3. *Ouchterloney double-immunodiffusion analysis*
In each of the two experiments shown, the *center well* contained form-specific anti-P450$_{PCN}$ IgG. *Outer wells* contained approximately 50 pmoles of purified P450$_{PCN}$ (PCN-STD) (*top*) or immunoreactive P450$_{PCN}$ in solubilized hepatic microsomes prepared from rats pretreated (*clockwise* from *top*) with dexamethasone (*DEX*), spironolactone (*SPL*), PCN, phenobarbital (*PB*), or 6α-methylprednisolone (*M-PRED*). The gel had been washed, dried, and stained with Coomassie Blue to enhance contrast.

reactive P450$_{PCN}$ was a nonspecific effect of steroid treatment, we tested a variety of androgens, progestins, anabolic steroids, estrogens, glucocorticoids, and mineralocorticoids. None of the steroidal agents listed in Table 1 induced P450$_{PCN}$ when given i.p. at a daily dose of 50 mg/kg. It should be noted that this list includes strongly "catatoxic steroids" (ethylestrenol, fluoxymestrone) (8), steroids reported to induce cytochrome P-450-dependent drug metabolism [testosterone (24), methyltestosterone (25)], and steroids that are structurally similar to PCN. Some representative structures of inducers and noninducers of immunoreactive P450$_{PCN}$ *in vivo* are shown in Fig. 4.

A final series of experiments tested the hypothesis that induction of immunoreactive P450$_{PCN}$ by dexamethasone or 6α-methylprednisolone represented a previously unrecognized manifestation of the well-documented glucocorticoid properties of these steroids. However, we found that other well-established glucocorticoids, including hydrocortisone, triamcinolone, prednisone, and cortisol, failed to induce immunoreactive microsomal P450$_{PCN}$ at daily doses of 50 mg/kg (Table 1). Furthermore, dose-response comparisons of the effects of dexamethasone treatment on P450$_{PCN}$ and on tyrosine aminotransferase activity, a widely accepted index of glucocorticoid potency (26), showed that the onset and maximal induction of tyrosine aminotransferase activity were elicited by at least 10-fold lower doses of dexamethasone than were required for induction of P450$_{PCN}$ (Fig. 5). Moreover, PCN and dexamethasone had opposite effects on tyrosine aminotransferase activity. Doses of PCN that were insufficient to induce P450$_{PCN}$ produced a significant decrease in tyrosine aminotransferase activity (Fig. 5). It had been reported previously that PCN lacks glucocorticoid properties as monitored by tyrosine aminotransferase activity in mouse liver (27) or by hepatic glycogen deposition or thymus involution in rats (28). Thus, induction of PCN cytochrome P-450 by dexamethasone or PCN can clearly be dissociated from glucocorticoid effects.

DISCUSSION

Beginning with the earliest years of cytochrome P-450 research, it was suspected that the cytochrome existed as multiple isoenzyme forms. One reason for this belief

TABLE 1

Steroids that failed to induce immunoreactive P450$_{PCN}$

Each agent was given to a minimum of two rats at a dose of 50 mg/kg by i.p. injection daily for 4 days. The animals were killed, hepatic microsomes were prepared and solubilized, and immunoreactive P450$_{PCN}$ was measured by radial immunodiffusion as described under Materials and Methods. In each case, the microsomes contained undetected quantities of P450$_{PCN}$ (less than 15 nmoles/g of microsomal protein) when tested at protein concentrations as high as 15 mg/ml.

Adrenal corticosteroids
Corticosterone
Cortisol
Fludrocortisone
Hydrocortisone
Prednisone
Progestins
Norethindrone
19-Nortestosterone
Progesterone
Estrogens
17α-Estradiol
17β-Estradiol
Mestranol
Androgens
Androstenedione
Fluoxymestrone
17α-Methyltestosterone
Nandrolone
Testosterone
Pregnenolone derivatives
Pregnenolone
16-Dihydropregnenolone
16α-Methylpregnenolone
16α-Methyl-16,17-epoxypregnenolone
16β-Methyl-16,17-epoxypregnenolone

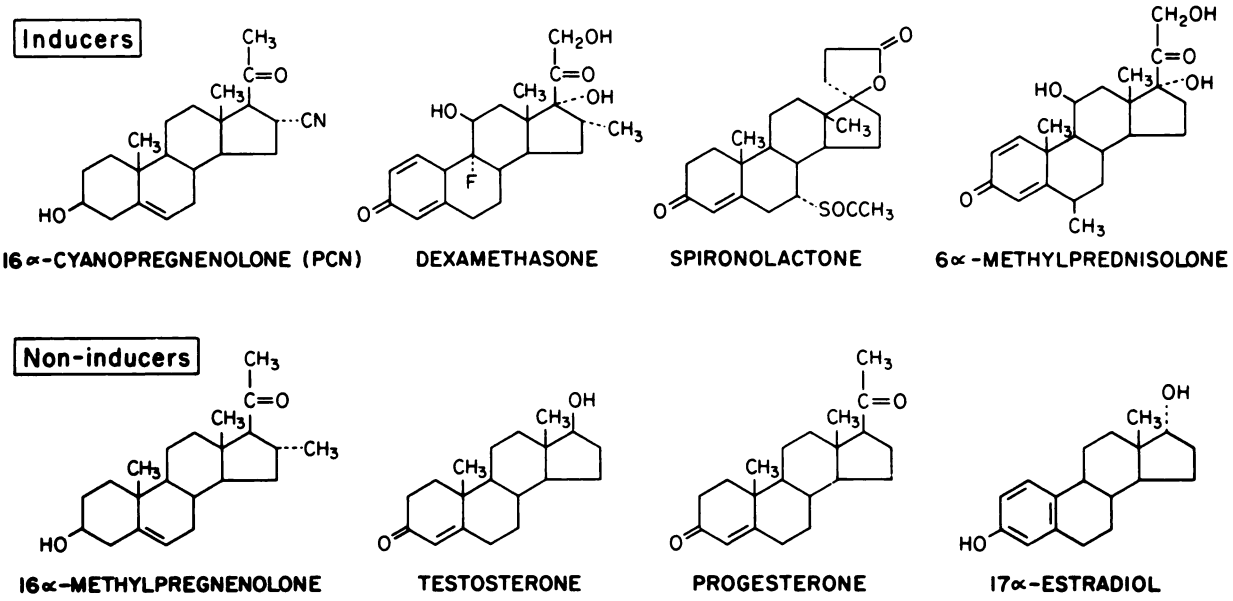

FIG. 4. *Structures of some inducers and noninducers of P450$_{PCN}$ in vivo*

was that many types of experimental manipulations that produced changes in the content of total CO-binding hemoprotein in hepatic microsomes (e.g., treatment of rats with an inducer) often failed to produce proportional changes in the profile of microsomal drug-oxidizing activities. For example, treatment of female rats for 3 days with spironolactone increases hepatic microsomal ethylmorphine demethylase activity by 7-fold, but has no significant effect on the concentration of total cytochrome P-450 measured spectrally (25). The explanation for this type of discrepancy remained ambiguous until the advent of techniques to isolate and purify individual molecular forms of cytochrome P-450, coupled with the demonstration that antibodies could serve as specific reagents (20, 29) for examining the metabolism of cytochrome P-450 isoenzymes individually. Using an immunochemical approach for the present studies, we found that treatment of rats with PCN leads to a dramatic accumulation of P450$_{PCN}$ in hepatic microsomes, whereas this drug produces only a modest increase in total cytochrome P-450 concentration. Moreover, we have identified other steroids (e.g., spironolactone and 6α-methylprednisolone) that increase the microsomal concentration of immunoreactive P450$_{PCN}$ accompanied by little or no increase in total cytochrome P-450. Our results are entirely consistent with analogous immunochemical examinations of hepatic microsomes prepared from rats treated with phenobarbital or 3-methylcholanthrene (3, 6, 7). For example, Thomas and co-workers (6) found that these agents promoted the accumulation of P450$_{PB}$ and P448$_{MC}$, apparently at the expense of other basal forms of the cytochrome present in untreated animals. Furthermore, analogous to our results using steroids other than PCN, they reported that chemicals other than the prototype inducers, phenobarbital or 3-methylcholanthrene, were capable of promoting the accumulation of these immunoreactive cytochrome(s) P-450. In summary, present and published studies provide direct evidence that the induction process involves radical alterations in the isoenzyme profile of cytochrome(s) P-450, all of which are subsumed under much less conspicuous changes in total CO-binding hemoprotein content. It is not yet clear to what extent the accumulation or loss of the individual cytochrome(s) reflects changes in their rates of synthesis or degradation (or both) in the basal steady state.

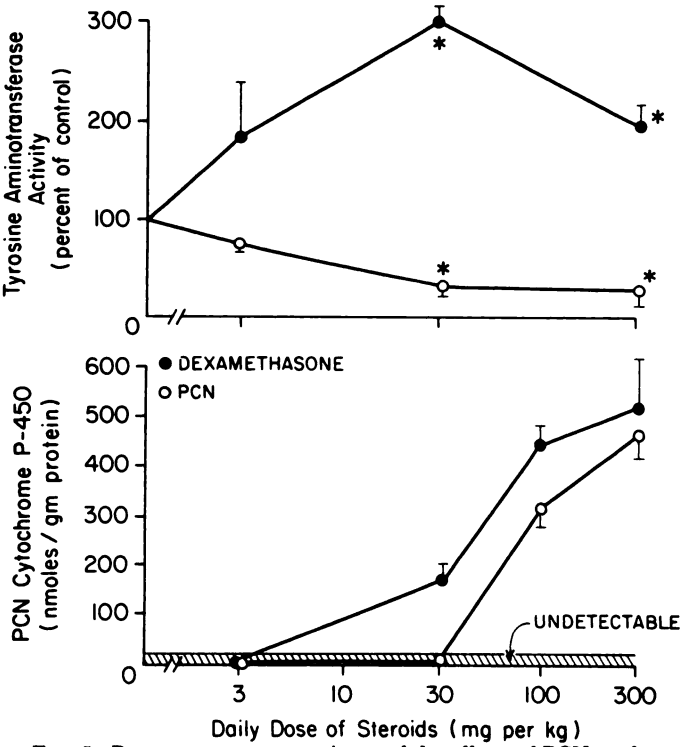

FIG. 5. *Dose-response comparisons of the effects of PCN or dexamethasone on liver tyrosine aminotransferase activity and P450$_{PCN}$ specific content*

Groups of rats received by gastric gavage the indicated doses of PCN or dexamethasone suspended in normal saline (10 ml/kg) daily for 4 days. The animals were then killed, and the cytosolic and microsomal fractions of liver homogenates were prepared for measurements of tyrosine aminotransferase activity and immunoreactive P450$_{PCN}$, respectively, as outlined under Materials and Methods. Brackets indicate standard error of the mean for three animals. *Asterisks* indicate a value significantly different from control ($p < 0.05$) by Student's *t*-test.

The radial immunodiffusion assay is inexpensive and simple to perform. However, the test was not sufficiently sensitive to measure the concentration of $P450_{PCN}$ in untreated female rats, although other workers have been able to measure small amounts of $P450_{PB}$ and $P448_{MC}$ in untreated rats of both sexes (6, 7). Indeed, because our antibody precipitated $P450_{PCN}$ weakly, we found it necessary to increase the sensitivity of the procedure by washing and staining the gels for protein precipitates. Because we have demonstrated *de novo* synthesis of $P450_{PCN}$ as measured by immunoprecipitation of the radiolabeled cytochrome in freshly isolated hepatocytes prepared from untreated rats (14), we believe that $P450_{PCN}$ exists in liver in the basal steady state. A second limitation which applies to all immunochemical tests is that, despite efforts to prepare specific antibodies, the immunodiffusion assay may measure not just a single form of cytochrome P-450, but rather a group of similar forms sharing one or more major antigenic determinants in common with $P450_{PCN}$. Finally, it should be noted that the absolute concentration of cytochrome P-450 measured by radial immunodiffusion is dependent on the spectrally determined concentration of hemoprotein in the purified standard. To the extent that the standard may contain immunoreactive apocytochrome P-450, the radial immunodiffusion assay will underestimate the concentration of immunoreactive cytochrome P-450 in microsomes. This consideration may pertain particularly to highly purified $P450_{PCN}$ which has a spectrally measured specific hemoprotein content, approximately one-half that of highly purified $P450_{PB}$ or $P448_{MC}$ (13). The problem may be further compounded if the results for immunoreactive $P450_{PCN}$ are expressed as a percentage of total CO-binding hemoprotein because spectral assays detect neither apocytochrome P-450 nor small amounts of cytochrome P-420, the denatured form of cytochrome P-450. Notwithstanding these limitations, the assay provides a basis for accurately comparing changes in the microsomal content of immunoreactive $P450_{PCN}$ produced by different inducers at various doses.

Induction of $P450_{PCN}$ *in vivo* by various steroids administered at a uniform high dose did not appear to be related to steroid structure or to classical steroid functions. $P450_{PCN}$ induction did not seem to be associated with androgenic, estrogenic, progestational, glucocorticoid, or mineralocorticoid activities. For example, not all inducers of $P450_{PCN}$ are steroids (e.g., phenobarbital), and not all steroids that increase cytochrome P-450-dependent drug-oxidizing activity induced $P450_{PCN}$. Furthermore, some "catatoxic" steroids failed to induce $P450_{PCN}$, whereas others (dexamethasone, spironolactone) induced the cytochrome only when given in doses higher than those required for catatoxic activity (8). Thus, although PCN, dexamethasone, spironolactone, and 6α-methylprednisolone may alter metabolism of drugs and toxins at least in part by induction of $P450_{PCN}$, this mechanism is not a universal explanation for the protective effects of all catatoxic steroids. The availability of a hepatocyte culture system capable of expressing induction of $P450_{PCN}$ (14) will greatly facilitate future detailed studies of steroid structure-activity relationships.

Recognition of dexamethasone as a potent inducer of $P450_{PCN}$ opens the way for future investigations of the mechanism of the induction process. Dexamethasone is an extensively studied pure glucocorticoid having minimal androgenic or mineralocorticoid activities (30). There is solid evidence that the glucocorticoid activities of dexamethasone are mediated through a high-affinity cytoplasmic receptor that binds the hormone, translocates the steroid into the nucleus (30), and then in some manner elicits expression of several genes, including tyrosine aminotransferase. There is a precedent for an analogous receptor-mediated induction of cytochrome P-450 in the recent demonstration of a genetically controlled, stereospecific cytoplasmic receptor that interacts with polycyclic aromatic hydrocarbons for induction of hepatic arylhydrocarbon hydroxylase activity (31). It might be argued that induction of $P450_{PCN}$ by dexamethasone reflects simply another of the "pleiotropic" glucocorticoid responses (32). However, higher doses of dexamethasone were required for induction of $P450_{PCN}$ than for induction of tyrosine aminotransferase activity (Fig. 5). Furthermore, PCN actually depressed tyrosine aminotransferase activity (Fig. 5) while at the same time increasing $P450_{PCN}$. Finally, there was no relationship between the relative potency of glucocorticoid analogues such as prednisone and triamcinolone and induction of $P450_{PCN}$. These results suggest that $P450_{PCN}$ induction is mediated by a mechanism other than the glucocorticoid receptor. Conceivably this mechanism could involve a second, lower-affinity dexamethasone "receptor(s)," as has been described in liver microsomes (33). This postulated lower-affinity mechanism may have relevance to such physiological states as stress, or to clinical situations in which large doses of steroids are employed therapeutically.

ACKNOWLEDGMENTS

We thank Dr. Shaun Ruddy for his helpful advice about immunochemical techniques. We also thank Marcia Tetlak for providing expert secretarial assistance.

REFERENCES

1. Lu, A. Y. H., and S. West. Multiplicity of mammalian cytochromes P-450. *Pharmacol. Rev.* **31**:277–295 (1980).
2. Ryan, D., A. Y. H. Lu, J. Kawalek, S. B. West, and W. Levin. Highly purified cytochrome P-448 and P-450 from rat liver microsomes. *Biochem. Biophys. Res. Commun.* **64**:1134–1141 (1975).
3. Ryan, D. E., P. E. Thomas, D. Korzeniowski, and W. Levin. Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. *J. Biol. Chem.* **254**:1365–1374 (1979).
4. Guengerich, F. P. Separation and purification of multiple forms of microsomal cytochrome P-450. *J. Biol. Chem.* **253**:7931–7939 (1978).
5. Masuda-Mikawa, R., Y. Fujii-Kuriyama, M. Negishi, and Y. Tashiro. Purification and partial characterization of hepatic microsomal cytochrome P-450s from phenobarbital- and 3-methylcholanthrene-treated rats. *J. Biochem.* **86**:1383–1394 (1979).
6. Thomas, P. E., L. M. Reik, D. E. Ryan, and W. Levin. Regulation of three forms of cytochrome P-450 and epoxide hydrolase in rat liver microsomes. *J. Biol. Chem.* **256**:1044–1052 (1981).
7. Harada, N., and T. Omura. Selective induction of two different molecular species of cytochrome P-450 by phenobarbital and 3-methylcholanthrene. *J. Biochem.* **89**:237–248 (1981).
8. Selye, H. Hormones and resistance. *J. Pharm. Sci.* **60**:1–28 (1971).
9. Garg, B. D., K. Kovacs, J. A. Blascheck, and H. Selye. Ultrastructural changes induced by pregnenolone nitrile in the rat liver. *J. Pharm. Pharmacol.* **22**:872–873 (1970).
10. Solymoss, B., J. Werringloer, and S. Toth. The influence of pregnenolone-16α-carbonitrile on hepatic mixed-function oxygenases. Steroids **17**:427–433 (1971).
11. Lu, A. Y. H., A. Somogyi, S. West, R. Kuntzman, and A. H. Conney.

Pregnenolone-16α-carbonitrile: a new type of inducer of drug metabolizing enzymes. *Arch. Biochem. Biophys.* **152**:457–462 (1972).
12. Thomas, P. E., A. Y. H. Lu, S. B. West, D. Ryan, G. T. Miwa, and W. Levin. Accessibility of cytochrome P-450 in microsomal membranes: inhibition of metabolism by antibodies to cytochrome P-450. *Mol. Pharmacol.* **13**:819–831 (1977).
13. Elshourbagy, N. A., and P. S. Guzelian. Separation, purification, and characterization of a novel form of hepatic cytochrome P-450 from rats treated with pregnenolone-16α-carbonitrile. *J. Biol. Chem.* **255**:1279–1285 (1980).
14. Elshourbagy, N. A., J. L. Barwick, and P. S. Guzelian. Induction of cytochrome P-450 by pregnenolone-16α-carbonitrile in primary monolayer cultures of adult rat hepatocytes and in a cell-free translation system. *J. Biol. Chem.* **256**:6060–6068 (1981).
15. Mancini, G., A. O. Carbonora, and J. F. Hefermans. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* **2**:235–254 (1964).
16. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2385 (1964).
17. Diamondstone, T. I. Assay of tyrosine aminotransferase activity by conversion of *p*-hydroxyphenyl pyruvate to *p*-hydroxybenzaldehyde. *Anal. Biochem.* **16**:395–401 (1966).
18. Granner, D. K., and G. M. Tomkins. Tyrosine aminotransferase (rat liver). *Methods Enzymol.* **17**:633–634 (1970).
19. Schacterle, G. R., and R. L. Pollack. A simplified method for the quantitative assay of small amounts of protein in biologic material. *Anal. Biochem.* **51**:654–655 (1973).
20. Thomas, P. E., D. Korzeniowski, D. Ryan, and W. Levin. Preparation of monospecific Ab against two forms of rat liver cytochrome P-450 and quantitation of these forms in microsomes. *Arch. Biochem. Biophys.* **192**:524–532 (1979).
21. Stripp, B., R. H. Menard, N. G. Zampaglione, M. E. Hamrick, and J. R. Gillette. Effect of steroids on drug metabolism in male and female rats. *Drug Metab. Dispos.* **1**:216–223 (1973).
22. Talcott, R. E., and S. J. Stohs. Enzyme induction with pregnenolone-16α-carbonitrile: onset of action and effect of thioacetamide. *Biochem. Pharmacol.* **23**:1221–1223 (1974).
23. Stohs, S. J., and H. Rosenberg. Onset of enzyme induction with pregnenolone-16α-carbonitrile in male and female rats. *J. Pharm. Sci.* **65**:610–611 (1976).
24. Booth, J., and J. R. Gillette. The effect of anabolic steroids on drug metabolism by microsomal enzymes in rat liver. *J. Pharmacol. Exp. Ther.* **137**:374–379 (1962).
25. Hamrick, M. E., N. G. Zampaglione, B. Stripp, and J. R. Gilette. Investigation of the effects of methyltestosterone, cortisone and spironolactone on the hepatic microsomal mixed function oxidase system in male and female rats. *Biochem Pharmacol.* **22**:293–310 (1973).
26. Samuels, H. H., and G. M. Tomkins. Relation of steroid structure to enzyme induction in hepatoma tissue culture cells. *J. Mol. Biol.* **52**:57–74 (1970).
27. Lazar, G., S. Sekiya, and M. K. Agarwal. Interaction between syntoxic and catatoxic steroids on endotoxin lethality in relation to liver metabolism in mice. *J. Reticuloendothal. Soc.* **22**:13–20 (1977).
28. Szabo, S., P. Kourounakis, H. Selye, and O. D. Silva. Pharmacodynamic interactions among gluco-, mineralo- and glucomineralocorticoids, pregnenolone-16α-carbonitrile and various drugs. *J. Pharmacol. Exp. Ther.* **188**:45–54 (1974).
29. Thomas, P. E., A. Y. H. Lu, D. Ryan, S. West, and W. Levin. Specificity of antisera directed against rat liver cytochrome P-448. *Life Sci.* **15**:1475–1483 (1974).
30. Rousseau, G. G., and J. D. Baxter. Glucocorticoid receptors, in *Glucocorticoid Hormone Action* (J. D. Baxter and G. G. Rousseau, eds.). Springer-Verlag, New York, 49–77 (1979).
31. Poland, A., E. Glover, and A. S. Kende. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol: evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. *J. Biol. Chem.* **251**:4936–4946 (1976).
32. Hershko, A., P. Mamont, R. Shields, and G. M. Tomkins. Pleiotypic response. *Nature [New Biol.]* **232**:206–211 (1971).
33. Ambellan, E., M. Swanson, and A. Davidson. Glucocorticoid binding to rat liver microsomal fractions *in vitro*. *J. Steroid Biochem.* **14**:421–428 (1981).

Send reprint requests to: Dr. Philip S. Guzelian, Liver Study Unit, Department of Medicine, Medical College of Virginia, Richmond, Va. 23298.