Induction of Cytochrome P-450 by Pregnenolone-16α-carbonitrile in Primary Monolayer Cultures of Adult Rat Hepatocytes and in a Cell-free Translation System*

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To investigate whether primary cultures of adult rat hepatocytes retain the capacity to respond to inducers of cytochrome P-450 other than polycyclic aromatic compounds, we tested the effects of pregnenolone-16α-carbonitrile (PCN) which induces a novel form of the hemoprotein (PCN cytochrome P-450) in rat liver (Elshourbagy, N. A., and Guzelian, P. S. (1980) J. Biol. Chem. 255, 1279-1285). Cultured hepatocytes incubated for 72 h in serum-free medium containing 10^{-6} M PCN exhibited increases in the concentration of cytochrome P-450 (1.5-fold) and in the activities of ethylmorphine (4-fold) and aminopyrine (2-fold) N-demethylation as compared to values in incubated control cultures. The cytochrome P-450 induced in culture by PCN matched the PCN cytochrome P-450 purified from PCN-treated rats in the sensitivity of its catalytic activity to formamidine (1, 5), adult (5), or malignant (6) liver have been available for many years, but unfortunately, the levels of cytochrome P-450 in these cell cultures are either undetectable or extremely low.

For the past several years, we have tested primary monolayer cultures of nonproliferating hepatocytes prepared from mature rats as an alternative to standard systems of liver cell culture. We have found that, although these cultures retain an impressive array of functions characteristic of the liver in vivo, the level of cytochrome P-450 falls precipitously during the first 24 h of incubation of the hepatocytes under standard culture conditions (9). Not only is there a rapid decline in the basal levels of cytochrome P-450 but also the response of the cultured hepatocytes to exposure to environmental agents is altered when compared with the effects of such inducers on the liver in vivo. For example, although exposure of cultured adult rat hepatocytes to 3-methylcholanthrene results in stimulation of aryl hydrocarbon hydroxylase activity (9) and, under some conditions, in accumulation of cytochrome P-448 (10), there is no evidence that addition of phenobarbital to the culture medium stimulates de novo synthesis of the major form of cytochrome P-450 induced by phenobarbital in vivo (9, 11).

Cytochrome P-450 is a collective term for a group of microsomal hemoproteins, located prominently in the endoplasmic reticulum of the liver, that catalyzes the oxidation of many lipophilic drugs, carcinogens, and environmental chemicals. An important feature of cytochrome P-450 is that it is inducible by many of its substrates. For example, treatment of rats with phenobarbital or 3-methylcholanthrene, traditional representatives of two classes of agents that induce separate, distinct forms of cytochrome P-450 in vivo, results in increased de novo synthesis of hepatic heme and microsomal protein (1, 2). Although cytochrome P-450 may be an important locus of interaction between man and his chemical environment, little is known of the molecular events involved in substrate-mediated induction of cytochrome P-450 or of the modulation of this process by hormonal, nutritional, or metabolic factors. Investigating such complex questions is inherently difficult in living animals and, therefore, there is a need for suitable in vitro systems, for example, liver cell cultures. Continuously dividing cell strains or cell lines prepared from embryonic (3, 4), adult (5), or malignant (6) liver have been available for many years, but unfortunately, the levels of cytochrome P-450 in these cell cultures are either undetectable or extremely low.

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It has been suggested that there is a "third class" of inducers exemplified by pregnenolone-16a-carbonitrile and other synthetic steroids which preferentially induce microsomal ethylmorphine N-demethlyase activity in rat liver (22). We have recently confirmed this supposition by isolating a novel form of hepatic cytochrome P-450 protein purified from PCN. The form of PCN cytochrome P-450, differs from the forms induced by phenobarbital or 3-methylcholanthrene as judged by spectral, catalytic, chromatographic, electrophoretic, and immunologic characteristics (23). Encouraged by preliminary results that treatment of hepatocyte cultures with PCN increased cytochrome P-450 (24), we prepared and isolated an antibody specific for purified PCN cytochrome P-450 and developed a specific and quantitative immunassay for measuring the rate of synthesis of PCN cytochrome P-450. Using this technique, we now demonstrate that PCN cytochrome P-450 can be induced by PCN in hepatocyte cultures as it is in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—All studies used female Sprague-Dawley rats weighing 200-250 g purchased from the NIDB Laboratories. Animals were housed individually in wire-bottomed cages with free access to chow and water. Collagenase type I was purchased from Sigma; Eagle's minimal essential vitamins (100X) were from Grand Island Biological Co.; [3H]leucine and [14C]ovalbumin were from New England Nuclear; rabbit reticulocyte lysate was from Amersham; quanidine hydrochloride was from Schwarz-Mann; and complete and incomplete adjuvant was from Difco Laboratories. All other chemicals were of the highest purity available commercially.

**Primary Cultures of Adult Rat Liver Parenchymal Cells in Monolayer**—Hepatocyte cultures were prepared by a method described in detail elsewhere (8). In brief, animals were anesthetized with ether and the liver was perfused in situ first with a calcium-free salt solution containing 0.5 mg/mt EGTA, 25 mM Tricine buffer, followed by complete culture medium containing 0.036% collagenase. The cells (3.5 x 10^7) were placed in 60-mm plastic dishes precoated with rat tail collagen in a final volume of 3 ml of culture medium and were incubated at 35°C in humidified air with 5% CO2. As judged by recovery of DNA, approximately 90% of the inoculated cells remained attached as a confluent monolayer on the bottom of the culture dish after 24 h of incubation. The standard serum-free culture medium consisted of a modified Waymouth MB-752/1 (9) supplemented with estradiol (10^-8 M) and corticosterone (10^-9 M) and was renewed every 24 h in these experiments. PCN was dissolved in dimethyl sulfoxide and was added to the culture medium by a single 1000-fold dilution to give a final concentration of M, unless indicated otherwise.

**Experimental Approach**—Most of the experiments were carried out after 24 h of incubation of the freshly isolated hepatocytes in culture medium because the rapid loss of cytochrome P-450 and of some drug-metabolizing activities occurs largely during this period (9). Thereafter, the level of cytochrome P-450 or drug-metabolizing activity declined more slowly, providing a relatively stable baseline to serve as reference for studies of enzyme induction. In each experiment, hepatocytes prepared from a single donor liver were incubated in standard culture medium for 24 h and then were divided into two groups; the first group was incubated with medium containing PCN, and the second was incubated with medium containing an equal volume of the vehicle, dimethyl sulfoxide. When cytochrome P-450 synthesis or degradation was to be measured, cultures were radioactively labeled for 4 h to culture medium containing 3.3 pCi/ml of [3H]leucine with carrier leucine omitted. The 4-h period was selected for pulse labeling because this interval gave adequate incorporation of radioactivity into cytochrome P-450 protein (10) (see in conclusion). The former was measured by immunocorrelation means (see below), the latter was measured by precipitation with trichloroacetic acid (10%, w/v, final concentration). The acid precipitate was sedimented by centrifugation at 1000 X g for 5 min, washed three times in 0.5% trichloroacetic acid, resuspended in 0.5 ml of Soluene 350 (Packard Instrument Co.), and incubated overnight at 25°C. Dimilume (5 ml) (Packard) was added and radioactivity was determined by liquid scintillation spectrometry (Beckman LS-9000) using external standardization to correct for quenching and random coincidence monitoring to detect the presence of chemoluminescence.

**Purification of Cytochrome (s) P-450**—We used our published procedures to purify hepatic cytochrome P-450 from rats treated with PCN, phenobarbital, or 3-methylcholanthrene (23). The livers of PCN-treated rats killed 4 h after intraperitoneal injection of 40 mg of [3H]PCN (300 millicuries) served as starting material for preparing purified PCN (14C)cytochrome P-450.

**Hemochromatolytic Determination of the Synthesis and Degradation of PCN Cytochrome P-450**

**Preparation on IgG**—PCN cytochrome P-450 purified from female PCN-treated rats was used to demonstrate that its guanole group is not contained in the cytochrome P-450 protein (11). The protein (1 mg) was dissolved in 5 ml of sterile 0.9% sodium chloride, pH 7.4, and the IgG fraction was added in a 1:200 dilution. The IgG fraction was then incubated overnight at 4°C. The IgG was removed by centrifugation and the solution containing the immunosubunit was washed three times with 0.1 M phosphatase buffer (pH 7.4) and 0.5 M NaCl. The washed IgG was then exposed to 5000 unit of rabbit reticulocyte lysate (25) for 2 h at 30°C. After incubation, the IgG was removed, and the solution containing the immunosubunit was then added to a column of Sepharose SEP-PAK C18 (10 ml) which was washed with methanol (25). Then, the IgG immunosubunit was eluted with 0.1 M ammonium carbonate in 1 M methanol (pH 9.5) at a flow rate of 20 ml/min. The IgG immunosubunit was then dialyzed in a dialysis bag against 0.01 M sodium carbonate (pH 9.5) and then centrifuged in a 1000 X g centrifuge for 30 min. The supernatant was then centrifuged at 1000 X g for 5 min, resuspended in 0.1 M sodium carbonate (pH 9.5), and centrifuged again for 5 min. The supernatant was then subjected to charcoal treatment and dialyzed against 0.01 M sodium carbonate (pH 9.5) for 2 h. After centrifugation, the supernatant was then dialyzed against 0.01 M sodium carbonate (pH 9.5) and then centrifuged for 2 h at 1000 X g. The supernatant was then added to a column of Sepharose SEP-PAK C18 (10 ml) which was washed with methanol (25). Then, the IgG immunosubunit was eluted with 0.1 M ammonium carbonate in 1 M methanol (pH 9.5) at a flow rate of 20 ml/min. The IgG immunosubunit was then dialyzed in a dialysis bag against 0.01 M sodium carbonate (pH 9.5) and then centrifuged at 1000 X g.

We use "protein" to refer to the protein moiety of cytochrome P-450 without specifying whether the heme prosthetic group is present or absent; "apoprotein" refers specifically to the latter situation; "holocytochrome" indicates that the heme is present and fully assembled with the apoprotein.
**Fig. 1. Effect of PCN on microsomal cytochrome P-450 and drug metabolism in hepatocyte cultures.** Freshly isolated hepatocytes were incubated for 24 h in standard medium, and then PCN was added to the culture medium of half the cells according to the protocol described in "Experimental Procedures." The results (bars) and control cultures are expressed as the percent of the values in freshly isolated hepatocytes (mean ± S.E.) which were: cytochrome P-450, 0.234 ± 0.024 nmol/mg of protein, n = 3; ethylmorphine N-demethylation, 2.7 ± 0.54 nmol/min/mg of protein, n = 3; and amiphenazine N-demethylation, 4.1 nmol/min/mg of protein, n = 1. Starred values are significantly different from incubated controls by one-tailed Student's t test.
**Cytochrome P-450 Induction in Hepatocyte Culture**

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of anti-cytochrome P-450 antibodies on ethylmorphine N-demethylation in microsomes from PCN-treated hepatocyte cultures or living rats</th>
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<tr>
<td></td>
<td>Ethylmorphine</td>
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<tr>
<td></td>
<td>N-demethylase</td>
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<tr>
<td><strong>Microsomes from hepatocyte culture</strong></td>
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<tr>
<td>Preimmune antibody</td>
<td>2.59</td>
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<tr>
<td>Anti-PCN cytochrome P-450</td>
<td>1.36</td>
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<tr>
<td>Anti-phenobarbital cytochrome P-450</td>
<td>2.11</td>
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<tr>
<td>Anti-3-methylcholanthrene cytochrome P-448</td>
<td>2.48</td>
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<tr>
<td><strong>Microsomes from rat liver</strong></td>
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<tr>
<td>Preimmune antibody</td>
<td>6.4</td>
</tr>
<tr>
<td>Anti-PCN cytochrome P-450</td>
<td>2.4</td>
</tr>
<tr>
<td>Anti-phenobarbital cytochrome P-450</td>
<td>6.8</td>
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</tbody>
</table>

*Hepatocyte cultures were exposed to PCN for 72 h, and cells were scraped and washed. Microsomes were prepared from cell lysates, and ethylmorphine N-demethylase activity was determined as described under "Experimental Procedures" in the presence or absence of the indicated antibodies in amounts (4-6 mg of protein/ml) giving maximal inhibition. The antibodies to the three purified forms of cytochrome P-450 were prepared as described previously (23).*  

*Liver microsomes from rats treated with PCN (75 mg/100 g/day for 5 days by intraperitoneal injection) were prepared by differential centrifugation.*

**Fig. 2.** Electrophoretic analysis of microsomes from control and PCN-treated cultured hepatocytes. Cultured hepatocytes were exposed for 72 h to control medium or to medium containing PCN. The media were removed, the cells were washed and scraped, and microsomes were prepared. Microsomal protein (10 μg) from the control cultures or from PCN-treated cells was combined with 10 mM Tris-HCl (pH 8) containing 1 mM EDTA, 1% β-mercaptoethanol, 1% sodium dodecyl sulfate, 10% glycerol and were heated for 15 min at 100 °C. Electrophoresis was carried out using commercially prepared slab gels (4-30% gradient acrylamide purchased from Pharmacia) for 16 h at 100 V in a continuous buffering system containing 40 mM tris-acetate (pH 7.4), 30 mM sodium acetate, 2 mM EDTA. Protein bands were stained with Coomassie blue, destained with butanol-acetic acid: water (10:10:80), and visualized with an Isco Model UA-5 gel scanner (Instrumentation Specialties Co., Lincoln, NE). Minimum molecular weights were estimated by comparisons with the mobility of standard proteins. The indicated mobility of PCN cytochrome P-450 was determined using a purified standard (see "Experimental Procedures").

might react with microsomal constituents other than PCN cytochrome P-450, the IgG fraction of immune serum was made form-specific by absorption against immobilized constituents of hepatic microsomes prepared from rats treated with 3-methylcholanthrene (see "Experimental Procedures"). These absorbed antibodies gave a single line when reacted against purified PCN cytochrome P-450 or solubilized microsomes from PCN-treated rats (Fig. 3). No cross-reaction was detected when purified phenobarbital cytochrome P-450 or 3-methylcholanthrene cytochrome P-448 was used, even when sensitivity of the Ouchterlony technique was heightened by staining for protein (Fig. 3). However, for present purposes, it is more relevant to test antibody specificity under conditions of the immunoprecipitation assay. Accordingly, we found that increments of form-specific anti-PCN cytochrome P-450 IgG produced a concentration-dependent increase in precipitation of purified PCN [14C]cytochrome P-450 reaching a value of more than 70% with saturating amounts of the IgG (Fig. 4). The immunoprecipitation of purified PCN [14C]cytochrome P-450 was unaffected by additions of unlabeled purified phenobarbital cytochrome P-450 or 3-methylcholanthrene cytochrome P-448 but was competively blocked by additions of unlabeled purified PCN cytochrome P-450 (Fig. 5). We

**Fig. 3.** Ouchterlony double diffusion analysis of form-specific anti-PCN cytochrome P-450 IgG versus purified cytochrome(s) P-450 or microsomes. Standard immunodiffusion analysis was made in 1% agarose with 3-mm wells on microscopic slides and incubated at 25 °C in a humidified atmosphere for 2 days (23). The gel was washed with phosphate-buffered saline, dried, stained with 0.5% Coomassie blue in destaining solution (ethanol-acetic acid: water, 35:10:55), and then destained. Upper, the central well contained 10 μl of form-specific anti-PCN cytochrome P-450 IgG and peripheral wells contained 0.10 nmol of PCN cytochrome P-450, phenobarbital (PB) cytochrome P-450, and 3-methylcholanthrene (MC) cytochrome P-448. Lower, the central well contained 10 μl of form-specific anti-PCN cytochrome P-450 IgG and peripheral wells contained 0.20 nmol of cytochrome P-450 solubilized from microsomes prepared from PCN-pretreated rats.
Cytochrome P-450 Induction in Hepatocyte Culture

Fig. 4. Immunoprecipitation of purified PCN [14C]cytochrome P-450 by form-specific anti-PCN cytochrome P-450 IgG. A series of immunoprecipitation reactions was carried out using 0.05 nmol of purified PCN [14C]cytochrome P-450 and the indicated amount of form-specific anti-PCN IgG under conditions described under "Experimental Procedures." Results were expressed as the percentage of the added radioactivity remaining in the washed immunoprecipitate.

Fig. 5. Effect of purified cytochrome(s) P-450 on immunoprecipitation of purified PCN [14C]cytochrome P-450 by form-specific anti-PCN cytochrome P-450 IgG. Incubations were carried out using 0.05 nmol of purified PCN [14C]cytochrome P-450 and 7.1 mg of form-specific anti-PCN cytochrome P-450 IgG (see Fig. 4). Other incubations also contained the indicated amounts of unlabeled, purified phenobarbital (PB) cytochrome P-450, 3-methylcholanthrene (MC) cytochrome P-448, or PCN cytochrome P-450. Results were expressed as the percentage of the added radioactivity remaining in the washed immunoprecipitate.

pre pared endogenously radiolabeled PCN cytochrome P-450 for use in these tests to avoid the possibility that exogenous labeling techniques might affect the immunoreactivity of the native cytochrome protein. As a final test of specificity, we reacted anti-PCN cytochrome P-450 IgG with solubilized cytochrome P-450 from lysates of cells from control or PCN-treated hepatocyte cultures pulse-labeled with [3H]leucine. The radioactivity in immunoprecipitates as analyzed by gel electrophoresis followed by gel slicing or fluorography appeared as a single band at Mₐ = 51,000, corresponding in mobility to that of purified PCN cytochrome P-450 (Fig. 6). Similar results were obtained using radiolabeled microsomes prepared from these cell lysates (data not shown).

Whereas the preceding results demonstrate that cytochrome P-450 can be solubilized and precipitated quantitatively with antibody, recovery on polyacrylamide gels of applied radiolabel in the immunoprecipitate proved to be low (sometimes less than 25%) and variable among several experiments. Two explanations were found. First, the step of staining and destaining of gels reduced recovery of standard [14C]ovalbumin or PCN [14C]cytochrome P-450 from greater than 90 to approximately 50%. An additional cause of low recovery was that the washed immunoprecipitates contained variable amounts of radiolabeled low molecular weight material (possibly free amino acids) which migrated in advance of the tracking dye (Fig. 6), in some instances leaving the gel entirely. The amount of this nonspecific radioactivity increased progressively with cells of increasing age in culture. Hence, measurement of radioactivity directly in washed immunoprecipitate gave systematically erroneous results and, therefore, as a routine quantitative procedure, immunoprecipitated proteins were isolated on unstained polyacrylamide gels.

Measurement of Cytochrome P-450 Synthesis in Culture—With freshly isolated hepatocytes, incorporation of [3H]leucine into immunoprecipitable PCN cytochrome P-450 was 0.07% of that incorporated into total cellular protein (Fig. 7). This rate declined to less than 0.05% at 24 h after the cells were plated and was maintained at that level during the next 72 h. Addition of PCN to the medium of 24-h-old cultures produced no detectable increase in the rate of synthesis of PCN cytochrome P-450 during the next 24-h interval. However, this rate increased 9-fold after 48 h of exposure of the
PCN. The data were expressed routinely as relative rates of isotopic precursor incorporation. Despite these theoretical considerations, a similar time course of isotopic precursor incorporation was observed when the data from the same experiment were expressed as radioactivity incorporated into immunoprecipitable PCN cytochrome P-450 protein relative to that in total and precipitable cell lysate protein.

Cultures to PCN and 30-fold after 96 h of incubation with PCN. The data were expressed routinely as relative rates of cytochrome P-450 synthesis to circumvent the possibility that culture age or exposure to PCN might alter either the uptake of isotopic precursor or the size of the pool of endogenous leucine. Despite these theoretical considerations, a similar time course and magnitude of induction of PCN cytochrome P-450 was observed when the data from the same experiment were expressed as radioactivity incorporated into immunoprecipitated PCN cytochrome P-450/μg of cellular DNA (data not shown). In two additional experiments, we found that the relative rates of PCN cytochrome P-450 synthesis measured in lysates of cultured cells exposed to PCN for 72 h (1.5 and 3.0%) were similar to the values obtained using microsomes prepared from portions of the cell lysates.

Cell-free Synthesis of PCN Cytochrome P-450 mRNA—Additional evidence for stimulation of de novo synthesis of PCN cytochrome P-450 by PCN in hepatocyte cultures is the accumulation of mRNA encoding for immunoreactive PCN cytochrome P-450. RNA was extracted from PCN-treated hepatocyte cultures and translated under standard conditions in a reticulocyte lysate system containing [3H]leucine as isotopic precursor. The radioactivity incorporated into translation products first, precipitated with anti-PCN cytochrome P-450 IgG and then isolated on SDS-polyacrylamide gels, was confined largely to a peptide with mobility (M₀ = 51,000) corresponding to that of purified PCN cytochrome P-450 (Fig. 8). This similarity in apparent molecular weights makes it unlikely that PCN cytochrome P-450 is synthesized in vivo as a substantially larger precursor which is then converted to the resident microsomal enzyme. The lack of truncated immunoreactive products suggests that premature termination of translation of the mRNA for PCN cytochrome P-450 in this system was not an important problem.

Using this cell-free system, we found that the amount of translatable mRNA encoding for PCN cytochrome P-450 in cultured hepatocytes exposed to PCN for 72 h was at least 6 to 8 times higher than that in control cultures. Because the latter contained less than detectable activity, the magnitude of this increase is uncertain (Table II). Nevertheless, values for synthesis of PCN cytochrome P-450 relative to total pro-

**TABLE II**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell-free translation (mRNA)</th>
<th>Hepatocyte cultures</th>
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<tbody>
<tr>
<td></td>
<td>Total protein</td>
<td>PCN-CP450</td>
</tr>
<tr>
<td></td>
<td>dpm/μg of RNA</td>
<td>dpm/μg of RNA</td>
</tr>
<tr>
<td>Control 1a</td>
<td>18,106</td>
<td>&lt;20</td>
</tr>
<tr>
<td>PCN</td>
<td>51,200</td>
<td>426</td>
</tr>
<tr>
<td>Control 2b</td>
<td>15,400</td>
<td>&lt;20</td>
</tr>
<tr>
<td>PCN</td>
<td>86,613</td>
<td>559</td>
</tr>
</tbody>
</table>

a Measurements were also made of the rate of synthesis of PCN cytochrome P-450 relative to total cellular protein in some of the intact cultured hepatocytes (see "Experimental Procedures").

b The data for hepatocyte cultures were obtained from cells derived from a separate liver but were treated similarly in culture.
For more than a decade, investigators have been attracted to the possibility of using liver cell culture to elucidate the mechanism underlying substrate-mediated induction of cytochrome P-450. Because it became apparent that cultured cells derived from embryonic mammalian liver (3) or hepatomas (6) contained low or undetectable amounts of cytochrome P-450, early studies of induction in cell culture relied heavily on measurements of aryl hydrocarbon hydroxylase activity, a ubiquitous cytochrome P-450-dependent enzyme for which there is a sensitive assay (39). Genetic studies and use of selective inhibitors revealed that this hydroxylase activity in rodent liver was supported by at least two separate forms of cytochrome P-450. These forms were induced respectively by barbiturates (cytochrome P-450) or by polycyclic aromatic compounds (cytochrome P-448 or possibly cytochrome P-450 (40). However, cultures of hepatoma cells or of cells from fetal rat liver contained only the latter form of the hydroxylase activity (cytochrome P-450) regardless of which many hydrophobic inducers was tested including phenobarbital (41).

In more recent studies of cultures of adult rat hepatocytes, it was reported that 3-methylcholanthrene or phenobarbital induced separate forms of cytochrome P-450 in culture as they do in rats. However, a more thorough reappraisal disclosed that the cytochrome induced by phenobarbital in these cultures resembled a fetal (3-methylcholanthrene-inducible) form rather than the cytochrome P-450 present in barbiturate-treated rat liver (11). From these findings, the concept has arisen that hepatocytes in culture undergo "fetalization" (5, 11, 42), losing the capacity to express such differentiated adult functions as induction of the phenobarbital cytochrome P-450, while retaining the form(s) of cytochrome P-450 which predominate in fetal liver (11, 41, 43, 44) and in extrahepatic tissues (45) and can be induced by 3-methylcholanthrene.

Based on our results, it is apparent that modification of this concept is necessary. Cultures of adult rat hepatocytes retain the capacity to respond to PCN appropriately by increasing synthesis of a distinct form of cytochrome P-450 similar in catalytic activity and in spectral, electrophoretic, and immunologic characteristics to the major form of cytochrome P-450 induced by PCN in adult rats. Because these cultured hepatocytes are capable of synthesizing heme (46) and apoprotein (present studies) and assembling these moieties into a fully functional holocytochrome (Fig. 1 and Table I), we believe that the apparent failure of such cultures to synthesize phenobarbital cytochrome P-450 is not likely due to a generalized defect in expression of differentiated genes in the liver. Rather, it is likely that the presence or absence of specific factors in the culture system alters selectively the formation of some, but not all, of the forms of cytochrome P-450 found in adult rat liver. Indeed, recent studies of chick embryo hepatocytes treated with 3-methylcholanthrene or phenobarbitol demonstrated induction of separate microsomal proteins (47), although the significance of this finding must await isolation and characterization of the forms of cytochrome P-450 in avian liver.

Our results demonstrate unequivocally that the mechanism of "induction" of cytochrome P-450 by PCN in hepatocyte cultures is stimulated de novo synthesis of PCN cytochrome P-450 protein. This was established by measuring quantitatively the rate of incorporation of a radiolabeled amino acid precursor into immunoprecipitable PCN cytochrome P-450 protein. Previously, "induction" of cytochrome P-450 in cultured hepatocytes (5) derived from adult (9, 20, 48, 49) or fetal (3, 41, 50-52) liver or hepatomas (41, 53, 54) was inferred from less specific tests. Such tests include measuring the accumulation of spectrally assayed cytochrome P-450 or the rise in oxidizing activity for model substrates when cells are exposed to media containing chemical, hormonal, nutritional, or metabolic "inducers." However, this approach, even when combined with the use of protein synthesis inhibitors or "diagnos-
tic inhibitors" of drug-oxidizing activity, fails to distinguish among many possible causes for "induction" of cytochrome P-450. These include changes in degradation versus synthesis of cytochrome P-450 protein, changes in relative amounts of heme, apoprotein, and holocytochrome, or formation of non-cytochrome peptide factors which may alter the catalytic activity or substrate specificity (or both) of the cytochrome P-450 in control cultures (55). It is possible that some of these ambiguities may be avoided by using a "double isotope" technique to measure relative net incorporation of amino acid precursors into microsomal proteins extracted from control and "induced" cell cultures and separated on polyacrylamide gels (47). However, it is open to question whether such gels sufficiently resolve the many radiolabeled proteins in the M, = 45,000 to 55,000 region to permit their unequivocal identification as forms of cytochrome P-450. The approach for measuring synthesis of cytochrome P-450 protein that we have employed takes advantage of the specificity provided by antibodies. This approach has been used by others also (57, 58), but it should be noted that for technical reasons (see "Experimental Procedures" and "Results"), uncritical application of immunoprecipitation techniques may yield erroneous results.

From extensive studies with inhibitors of protein synthesis, it has been inferred that the primary events in induction of aryl hydrocarbon hydroxylase activity in cell culture are associated with the step of gene transcription (55, 56). We elected to investigate the mechanism of PCN-stimulated \emph{de novo} synthesis of PCN cytochrome P-450 by measuring the mRNA in cultured hepatocytes that specifies this cytochrome protein. Cell-free translation of RNA extracted from hepatocyte cultures revealed that PCN treatment resulted in at least a 6- to 8-fold accumulation of mRNA activity encoding PCN cytochrome P-450. It would be desirable to confirm this finding by measuring the number of such mRNA sequences using molecular hybridization probes. Nevertheless, our results strongly suggest that PCN intervenes at a pretranslational step in cytochrome synthesis because there is no evidence for large pools of untranslated ("masked") mRNA in mammalian tissue.

The cell-free translation products contained no detected immunoreactive precursor polypeptides of substantially larger size than the PCN cytochrome P-450 found in intact hepatic microsomes. This finding is in keeping with the lack of formation of such precursors in cell-free translation of mRNA directing synthesis of rat liver PCN cytochrome P-450,\footnote{N. A. Elshourbagy, and P. S. Guzelian, manuscript in preparation.} phenobarbital cytochrome P-450 (59-61), adrenal cortical mitochondrion and microsomal cytochrome(s) P-450 (62), or mouse liver cytochrome P, 450 (63). Conflicting results for the existence of a precursor of rodent liver 3-methylcholanthrene cytochrome P-448 have been reported (63, 64).

Although RNA extracted from control hepatocyte culture contained insufficient mRNA encoding for PCN cytochrome P-450 to be detected in cell-free translation (Table II), it should be emphasized that \emph{de novo} synthesis of PCN cytochrome P-450 was detected in freshly isolated hepatocytes prepared from untreated adult rats and in monolayer cultures of these cells maintained in standard medium (Fig. 7). This finding stands in contrast to the reported absence of cytochrome P, 450 in normal mouse liver (63). Assuming the latter result is unrelated to sensitivity of detection and assuming also that our antibodies are strictly specific for PCN cytochrome P-450, then the intriguing possibility is raised that some forms of cytochrome P-450 may be continuously expressed in the basal steady state (constitutive), whereas others are totally inactive. This in turn implies that some inducers may intervene by amplifying an ongoing process of cytochrome P-450 synthesis, whereas other inducers may evoke or perhaps direct cytochrome P-450 gene expression (65).

More than 24 h of exposure to PCN were required before stimulation of PCN cytochrome P-450 synthesis could be detected in cultured hepatocytes. In contrast, a rise in cytochrome P-450 concentration and drug-metabolizing activity is prominent within 18 h in rats given a single injection of PCN (66). We are currently testing some of the many possible explanations for this discrepancy. One suggestion we have offered previously (9) is that primary cultures of adult rat hepatocytes may be refractory to the effects of some inducers during the first 24 to 48 h of incubation. Associated with the procedures for isolation and primary culture of hepatocytes is a rapid fall in the concentration of cytochrome P-450 (9). This fall prominently involves accelerated degradation of the heme moiety (38) of selected forms of cytochrome P-450 (67). Hence, the attendant accumulation of intracellular heme (9) may impart induction of cytochrome P-450 as has been observed in hemin-treated rats (68). Alteration of the inducibility of cytochrome P-450 may be one of many phenotypic changes in hepatocyte functions reflecting physiologic responses of cells adapting to culture conditions (5, 7, 8). In conclusion, we believe that the availability of primary hepatocyte culture systems provides an opportunity to begin examining the fine details of the interaction between environmental agents and the metabolism of the cytochrome P-450 system.

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