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Measurement of the metabolism of cytochrome *P*-450 in cultured hepatocytes by a quantitative and specific immunochemical method

Sammye L. NEWMAN, Joyce L. BARWICK, Nabil A. ELSHOURBAGY and Philip S. GUZELIAN
Medical College of Virginia, Liver Study Unit, Box 267, MCV Station, Richmond, VA 23298, U.S.A.

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We have defined conditions that permit quantitative and specific measurement of the metabolism of the major phenobarbital-inducible form of cytochrome *P*-450 protein in primary non-proliferating monolayer cultures of adult rat hepatocytes. Isolated antibodies specifically directed against phenobarbital cytochrome *P*-450 are used to immunoprecipitate the cytochrome from lysates of cultured hepatocytes pulse-labelled with [^{3}H]leucine. Phenobarbital cytochrome *P*-450 protein is then isolated from the immunoprecipitate by electrophoresis on polyacrylamide gradient slab gels. Specificity of the assay for phenobarbital cytochrome *P*-450 was established by competition experiments involving other forms of purified cytochrome *P*-450 as well as by testing antibodies directed against these other forms of the cytochrome. Using purified phenobarbital cytochrome *P*-450, radiolabelled in both its haem and apoprotein portions, as an internal standard, we demonstrated that, with this immunoassay, recovery of cytochrome *P*-450 from microsomal samples is nearly complete. Basal rates of synthesis of phenobarbital cytochrome *P*-450 representing as little as 0.02–0.05% of total cellular protein synthesis were reliably and reproducibly detected in hepatocyte culture maintained in serum-free medium for 72h. Moreover, inclusion of phenobarbital in the culture medium for 96h stimulated not only synthesis *de novo* of phenobarbital cytochrome *P*-450 protein, but also accumulation of spectrally and catalytically active cytochrome *P*-450. Advantages of this immunoassay are that metabolism (synthesis or degradation) of the haem or protein of this important form of the cytochrome can be measured conveniently in the small samples available from cultured cells without the necessity of preparing subcellular fractions.

Cytochrome *P*-450 is a collective term for a group of haemoproteins located prominently in the endoplasmic reticulum of the liver which catalyse the oxidation of a variety of endogenous substrates and foreign chemicals including drugs, mutagens, and carcinogens. The concentration of cytochrome *P*-450 in the hepatocyte is inducible, increasing or declining in association with administration or withdrawal of certain environmental agents. The molecular species of cytochrome *P*-450 induced is dictated by the inducing agent. For example, phenobarbital cytochrome *P*-450 (phenobarbital cytochrome *P*-450 refers to the major form of cytochrome *P*-450 purified from rat liver microsomes prepared from animals treated with phenobarbital, while 3-methylcholanthrene cytochrome *P*-448 and pregnenolone-16α-carbonitrile cytochrome *P*-450 represent the major forms of cytochrome *P*-450 purified from 3-methylcholanthrene- and pregnenolone-16α-carbonitrile-treated animals, respectively), differs in its immunological, biochemical, and catalytic properties from 3-methylcholanthrene cytochrome *P*-448 or pregnenolone-16α-carbonitrile cytochrome *P*-450 (Elshourbagy & Guzelian, 1980; Kumar & Padmanaban, 1980; Lu & West, 1980; Toftgard *et al.*, 1980). Efforts to understand the regulation of induction of different forms of hepatic cytochrome *P*-450 by environmental agents would be greatly facilitated if suitable liver cell culture systems were available. However, despite the intensive efforts of many laboratories, there is as yet no conclusive evidence that phenobarbital stimulates synthesis *de novo* of cytochrome *P*-450 protein in continuously dividing cell lines derived from liver or in primary non-proliferating monolayer cultures of adult rat hepato-

Abbreviations used: IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

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cytes as this drug does *in vivo* (Fahl *et al.*, 1979; Guzelian *et al.*, 1977; Owens & Nebert, 1975). Testing for induction of cytochrome *P*-450 by phenobarbital in cultured hepatocytes has generally involved measurements of the concentration of microsomal CO-binding haemoprotein or of drug oxidizing activities (Fry *et al.*, 1980; Michalopoulos *et al.*, 1976; Stenberg & Gustafsson, 1978). These methods reflect net changes in the concentration only of the haem moiety of cytochrome *P*-450 without regard to metabolism of the apoprotein portion of the cytochrome. Since the specificity of each form of cytochrome *P*-450 resides in its apoprotein portion, antibodies have been used as a sensitive and practical means of distinguishing the phenobarbital-inducible form of cytochrome *P*-450 from other forms of the cytochrome (Guengerich *et al.*, 1981; Thomas *et al.*, 1979*a*,*b*, 1981). Immunochemical techniques have been applied to studies of the metabolism (synthesis or degradation) of cytochrome *P*-450 (Bar-Nun *et al.*, 1980; Bhat & Padmanaban, 1978; Craft *et al.*, 1979*a*,*b*; Kumar & Padmanaban, 1980; Ohlsson *et al.*, 1981) without providing direct evidence that these methods are quantitative and specific. We present an immunochemical method for measuring metabolism of phenobarbital cytochrome *P*-450 in hepatocyte culture.

Materials and methods

Materials

Male Sprague–Dawley rats weighing 175–200g were purchased from Flow Laboratories (Dublin, VA, U.S.A.), and were housed in wire-bottomed cages with free access to chow and water. Goats used in the study were $1\frac{1}{2}$–2 year old castrated males obtained from, and housed at, the Virginia Commonwealth University Hanover Research Farm. Complete and incomplete adjuvants were purchased from Difco Laboratories, collagenase type I was from Sigma, Eagle's Minimal Essential Vitamins were from Grand Island Biological Company, Nonidet P-40 was from Bethesda Research Laboratories and U-^{14}C-labelled amino acid mixture, [^{14}C]leucine, [^{3}H]leucine, δ-amino[^{3}H]laevulinic acid and [^{14}C]ovalbumin were from New England Nuclear. All other chemicals were of the highest purity available commercially.

Purification of cytochrome P-450

Previously published procedures were used to purify hepatic cytochrome *P*-450 from rats treated with phenobarbital, pregnenolone-16α-carbonitrile or 3-methylcholanthrene (Elshourbagy & Guzelian, 1980). Purified phenobarbital ^{14}C-labelled cytochrome *P*-450 was prepared from the livers of phenobarbital-treated rats killed 4h after intraperitoneal injection of 40μCi of [^{14}C]leucine (300mCi/mmol). Purified phenobarbital cytochrome *P*-450 labelled in both the haem and apoprotein portions of the molecule was prepared from the livers of phenobarbital-treated rats killed 4h after injection of both 40μCi of U-^{14}C-labelled amino acid mixture and 40μCi of δ-amino[^{3}H]laevulinic acid.

Preparation of form-specific anti-(phenobarbital cytochrome P-450) IgG

We have used the term 'form-specific' to refer to selected antibodies that are directed against one purified form of cytochrome *P*-450 and that do not appear to cross-react with other purified forms of cytochrome *P*-450 or with other hepatic microsomal proteins. We prefer this term to 'monospecific', used in much the same sense by other authors (Thomas *et al.*, 1979*a*), because the latter term is too non-definitive.

Antiserum was prepared from the blood of goats immunized with purified phenobarbital cytochrome *P*-450 as described previously (Elshourbagy & Guzelian, 1980). An IgG-enriched fraction of serum was obtained by precipitation with $(NH_4)_2SO_4$ followed by chromatography on DE-52 cellulose (Elshourbagy & Guzelian, 1980). Control IgG was prepared in the same manner using blood obtained from non-immunized goats. IgG directed against the 3-methylcholanthrene- or pregnenolone-16α-carbonitrile-inducible forms of cytochrome *P*-450 was prepared as above with the appropriate purified cytochromes serving as immunogens. IgG from phenobarbital cytochrome *P*-450-immunized or non-immunized goats was adsorbed against Sepharose-4B linked microsomal proteins prepared from rats treated with 3-methylcholanthrene (20mg/kg by intraperitoneal injection daily for 4 days) (Cuatrecasas, 1970; March *et al.*, 1974; Elshourbagy *et al.*, 1981).

Primary monolayer cultures of adult rat liver parenchymal cells

Hepatocyte cultures were prepared as described previously (Bissell & Guzelian, 1980). To summarize, either untreated or phenobarbital-pretreated (100mg/kg intraperitoneally daily for 4 days) male rats were anaesthetized with ether and the livers were perfused *in situ* with a calcium-free salt solution containing 0.5mM-EGTA and 25mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine} buffer, followed by complete culture medium consisting of a modification of Waymouth MB-752/1 (Bissell & Guzelian, 1980) supplemented with testosterone (1μM) and corticosterone (1μM) and containing 0.036% collagenase. Hepatocytes were isolated by repeated differential centrifugation at 60–80*g* and resuspended in complete culture medium. Viability of the initial cell isolate consistently was

greater than 82% as judged by Trypan Blue exclusion. Hepatocytes (3.5×10^6) were placed in 60mm plastic dishes pre-coated with rat tail collagen and incubated in 3ml of culture medium at 35°C under humidified air/CO_2 (19:1). The complete serum-free medium was renewed every 24h.

Metabolism of cytochrome P-450 protein in hepatocyte culture

We use 'protein' to refer to the protein moiety of cytochrome *P*-450 without specifying whether the haem prosthetic group is present or absent; 'apoprotein' refers specifically to the latter situation; 'holocytochrome' indicates that the haem is present and fully assembled with the apoprotein.

To measure synthesis of the cytochrome, freshly isolated cells or monolayer cultures of various age were 'pulse-radiolabelled' by being exposed for 4h to culture medium containing 3.3μCi of [^{3}H]leucine/ml with carrier leucine omitted. Then, the medium was removed and cells were harvested as described below. A 'pulse–chase' experiment was used to measure degradation of the cytochrome. Freshly isolated cells were incubated with medium containing [^{3}H]leucine for 4h, and then this medium was replaced by complete culture medium containing unlabelled leucine. Care was taken not to disrupt monolayer formation. Cultures were incubated for various times thereafter according to protocol, and then cells were harvested for measurement of radioactivity in phenobarbital cytochrome *P*-450 and in total cellular protein.

Preparation of cell fractions

Cells were harvested by washing each plate twice with 3ml of cold phosphate-buffered saline and then scraping the cells into 3ml of the same buffer. Several (3–5) plates were pooled for each group. Each complete experiment represents the cells from a single donor liver. Cells were centrifuged at 1000*g* for 5min, resuspended in 0.1M-potassium phosphate buffer (pH7.4) and disrupted by brief sonication (35s at a setting of 35 using an Artek Sonic 300 dismembrator with microtip attachment). The cell lysates were homogenized in 2vol. of 20mM-potassium phosphate buffer (pH7.4) containing 0.1mM-EDTA, 20% (v/v) glycerol, 0.2% Nonidet P-40, 1% sodium cholate, and 0.15M-NaCl (20 strokes by hand using a Teflon/glass homogenizer). Preliminary experiments confirmed that these concentrations of detergents released >90% of cytochrome *P*-450 (measured spectrally) from both freshly isolated and cultured cells and yet did not impair immunoprecipitation of purified phenobarbital cytochrome *P*-450 by form-specific IgG. The insoluble material in the cell lysates was sedimented by centrifugation at 106500*g* for 30min. The supernatant was used as the source of solubilized cytochrome *P*-450 protein. In some instances, microsomes were prepared by centrifuging the 0.1M-potassium phosphate/cell lysate mixture at 18000*g* for 15min (Beckman J-21C centrifuge, JA-20 rotor, 4°C). The supernatant was removed and centrifuged at 106500*g* for 60min. The microsomal pellet was resuspended in 50mM-phosphate buffer containing 0.1mM-EDTA and 25% (v/v) glycerol, and solubilized as described above for cell lysates.

Analytical methods

Protein was determined according to the method of Schacterle & Pollack (1973), using bovine serum albumin (fraction V powder, 9.2% water; Sigma) as standard. DNA was measured colorimetrically (Richards, 1974). The concentration of cytochrome *P*-450 was measured in microsomes as its dithionite reduced–CO minus reduced difference spectrum assuming an absorption coefficient of 91 $mM^{-1} \cdot cm^{-1}$ and an isosbestic point of 490nm (Omura & Sato, 1964).

To measure the amount of radioisotope incorporated into total cellular protein, cell lysates were combined with trichloroacetic acid (10% w/v, final concn.), and incubated first at 0°C for 20min and then at 90°C for 15min. The acid precipitate was sedimented by centrifugation at 1000*g* for 5min, washed three times in 5% (w/v) trichloroacetic acid, resuspended in 1.0ml of Soluene 350 (Packard), and incubated overnight at 25°C. Dimilume (5ml; Packard) was added and radioactivity was quantified by liquid-scintillation spectrometry (Beckman LS-9000) using external standardization to correct for quenching and random coincidence monitoring to detect the presence of chemical luminescence.

Immunoprecipitation was used to determine the amount of radioisotope incorporated into phenobarbital cytochrome *P*-450 protein. Immunoreactions were carried out in 1.5ml conical polypropylene tubes containing between 25 and 300μg of protein (representing purified cytochrome *P*-450 or material solubilized from microsomes or cell lysates), form-specific anti-(phenobarbital cytochrome *P*-450) IgG or non-immune adsorbed IgG (between 3.33 and 40mg of IgG/mg of cytochrome *P*-450-containing protein), and 20μg of bovine serum albumin, all dissolved in a final volume of 0.4ml in 50mM-potassium phosphate (pH7.4) containing 0.2% Nonidet P-40, 1.0mM-EDTA, and 0.1M-KCl ('incubation buffer'). The mixture was incubated at 25°C for 1h. Then, 10μl of a 10% suspension of formalin-fixed, heat-inactivated *Staphylococcus aureus* (Kessler, 1975, 1976) was added to achieve quantitative precipitation of the antigen–antibody complex (Brunda *et al.*, 1977; Ivarie & Jones, 1969); 30min after addition of *S. aureus*, the contents of the immunoreaction mixture were sedimented for 1min in a Beckman Microfuge. The pellet was

washed three times in 0.5ml of incubation buffer. The final supernatant was aspirated and the pellet was stored dry at −20°C for no more than 1 week. The frozen immunoprecipitate was warmed rapidly to 25°C, combined with 100μl of 63mM-Tris/HCl (pH 6.8) containing 2.3% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 10% (v/v) glycerol, and heated at 100°C for 90s. Tracking dye (3μl of 0.05% Bromophenol Blue in water) was added and the *S. aureus* was removed by centrifugation for 1min in the Microfuge. To measure total radioactivity in the immunoprecipitate, 20μl of the supernatant were digested at 25°C overnight in 0.5ml of Soluene 350, and 5ml of Dimilume was added for liquid-scintillation spectrometry. The remaining 80μl were placed on a polyacrylamide gradient (10–13%) slab gel containing 0.093% SDS and subjected to electrophoresis (125V for approx. 3h) using a discontinuous buffer system of 25mM-Tris/0.192M-glycine/0.1% SDS. Electrophoresis was terminated when the tracking dye had migrated to within 5mm of the base of the gel. Each gel lane was immediately divided into sequential 2mm slices, and these were individually digested overnight in 0.5ml of Soluene 350 at 25°C in a glass liquid-scintillation minivial (Research Products International, Elk Grove Village, IL, U.S.A.). Dimilume (5ml) was added and each sample was counted by liquid-scintillation spectrometry when chemical luminescence had fully subsided. Recovery of [^{14}C]ovalbumin standards from gel slices was consistently greater than 90%. For each sample a control immunoreaction was carried out in which non-immune IgG was substituted for immune IgG. At least 100d.p.m. over background in each gel slice was necessary for detection of phenobarbital cytochrome *P*-450 in the immunoprecipitate, and the ratio of radioactivity in the gel slices containing phenobarbital cytochrome *P*-450 in immune versus non-immune gel lanes was at least 2:1. Radioisotope incorporated into phenobarbital cytochrome *P*-450 protein was calculated as follows: total radioactivity migrating in gel bands in adjacent lanes with mobility corresponding to that of purified phenobarbital cytochrome *P*-450 was determined for both the immune and control reactions. The difference between these values was then divided by total acid-precipitable radioactivity in the sample and expressed as the rate of synthesis (or degradation) of the phenobarbital cytochrome *P*-450 protein relative to that of total cellular protein.

Results

Isolation of specific anti-(phenobarbital cytochrome P-450) antibodies

When IgG isolated from the serum of goats immunized against purified phenobarbital cytochrome *P*-450 was cross-reacted against phenobarbital cytochrome *P*-450 antigen in Ouchterlony double-diffusion analysis, a single immunoprecipitation line appeared (Fig. 1). No immunoprecipitation lines were apparent when the phenobarbital cytochrome *P*-450 IgG was cross reacted against either 3-methylcholanthrene or pregnenolone-16α-carbonitrile cytochrome *P*-450 antigen in this system. The IgG fraction isolated from the serum of non-immunized goats did not visibly precipitate any of the above three antigens when Ouchterlony double-diffusion analysis was performed. Because this IgG preparation might contain antibodies that react with microsomal constituents other than phenobarbital cytochrome *P*-450, we adsorbed the IgG fractions from both

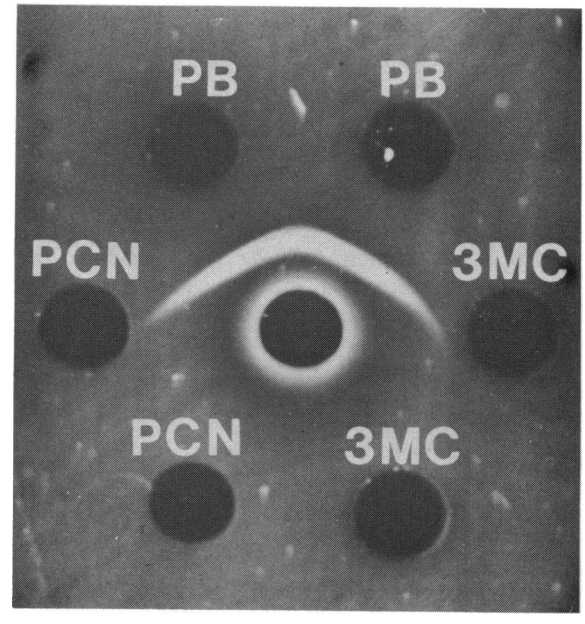

Fig. 1. *Ouchterlony double-diffusion analysis of form-specific anti-(phenobarbital cytochrome P-450) IgG or non-immune IgG versus purified cytochrome(s) P-450*
Standard immunodiffusion analysis was made in 1% agarose with 3mm wells on microscopic slides and incubated at 25°C in a humidified atmosphere for 2 days (Elshourbagy & Guzelian, 1980). The gel was washed with phosphate-buffered saline, dried, stained with 0.5% Coomassie Blue in destaining solution (ethanol/acetic acid/water, 7:2:11, by vol.), and then destained. The centre well contains 1.06mg of anti-(phenobarbital cytochrome *P*-450) IgG; the upper wells contain 0.20nmol of purified phenobarbital cytochrome *P*-450 (PB); the left and lower left wells contain 0.15nmol of purified pregnenolone-16α-carbonitrile cytochrome *P*-450 (PCN); the right and lower right wells contain 0.22nmol of purified 3-methylcholanthrene cytochrome *P*-448 (3MC).

immunized and non-immunized goats against immobilized constituents of hepatic microsomes prepared from 3-methylcholanthrene-treated rats (Thomas *et al.*, 1979*a*) in which the concentration of phenobarbital cytochrome *P*-450 is low (Thomas *et al.*, 1981) (see the Materials and methods section).

Under conditions for immunoprecipitation of solubilized cytochrome *P*-450, increments of form-specific anti-(phenobarbital cytochrome *P*-450) IgG caused a concentration-dependent increase in the precipitation of purified phenobarbital [^{14}C]cytochrome *P*-450. As much as 89% (corresponding to 45 pmol) of added radioactive cytochrome was precipitated by using saturating amounts of IgG (Fig. 2).

To determine whether the holocytochrome remains intact during immunoprecipitation, we prepared a dual-labelled standard. The protein and haem portions of phenobarbital cytochrome *P*-450 were labelled respectively by injecting phenobarbital-treated rats with both a ^{14}C-labelled amino acid mixture and δ-amino[^{3}H]laevulinic acid, a specific precursor of cytochrome *P*-450 haem (Bissell & Hammaker, 1976). ^{14}C/^{3}H-dual-labelled phenobarbital cytochrome *P*-450 was purified from the livers of these rats and was incubated with anti-(phenobarbital cytochrome *P*-450) IgG. We observed a parallel increase in the amounts of both radioisotopes in the immunoprecipitate as the concentration of added IgG was increased (Fig. 2). Furthermore, the ^{14}C/^{3}H ratio of the precipitate was identical to that of the starting material (results not shown). Hence, it is unlikely that significant denaturation of cytochrome *P*-450 with loss of its haem moiety occurred in the presence of IgG. Although the assay described gave quantitative immunoprecipitation of purified cytochrome *P*-450, it cannot be assumed that the cytochrome solubilized directly from cellular membranes will behave the same. Since we lacked an independent means for measuring the total solubilized phenobarbital cytochrome *P*-450 protein added to the immunoreaction, we chose an alternative approach, using the available purified ^{14}C/^{3}H-phenobarbital cytochrome *P*-450 as an internal standard. Radiolabelled hepatic microsomes were prepared from phenobarbital-treated rats injected with 40 μCi of a ^{14}C-labelled amino acid mixture 3 h prior to killing. The microsomal proteins were solubilized and combined with the dual-radiolabelled cytochrome *P*-450, and increments of anti-(phenobarbital cytochrome *P*-450) IgG were added to define a curve for maximal radioactivity in the immunoprecipitate. By subtracting the contribution of the internal standard to total radioactivity at each IgG concentration, a saturation curve was established for the endogenously labelled protein (Fig. 3). Since maximal precipitation of radioactivity occurred at the same IgG concentration for both the purified and the labelled solubilized microsomal proteins, it may be concluded that this assay measured the bulk of endogenous phenobarbital cytochrome *P*-450.

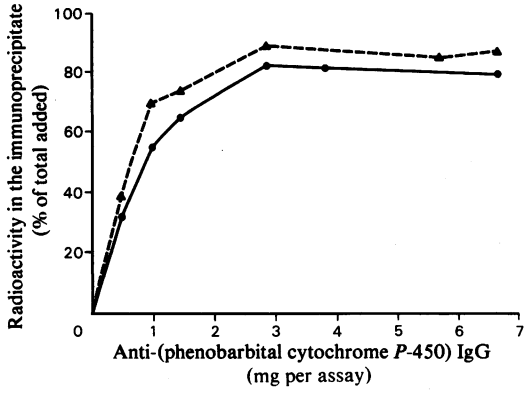

Fig. 2. *Immunoprecipitation by anti-(phenobarbital cytochrome P-450) IgG of radiolabelled, purified phenobarbital cytochrome P-450*

A series of immunoprecipitation reactions were carried out using purified phenobarbital cytochrome *P*-450 (0.05 nmol) prepared from rats injected with both ^{14}C-labelled amino acids and δ-amino[^{3}H]-laevulinic acid as described in the Materials and methods section. The specific radioactivity of the preparation was 177 d.p.m. of ^{14}C and 481 d.p.m. of ^{3}H per 0.05 nmol. Each reaction contained the indicated amount of form-specific anti-(phenobarbital cytochrome *P*-450) IgG. Results are expressed as the % of added radioactivity remaining in the washed immunoprecipitate. ●, Immunoprecipitated [^{14}C]protein; ▲, immunoprecipitated [^{3}H]haem.

Several observations indicated that our form-specific anti-(phenobarbital cytochrome *P*-450) IgG retains its specificity for phenobarbital-inducible cytochrome *P*-450 under immunoprecipitation assay conditions. The use of microsomes from control, 3-methylcholanthrene- or pregnenolone-16α-carbonitrile-treated rats labelled *in vivo* with ^{14}C-labelled amino acids was associated with precipitation by anti-(phenobarbital cytochrome *P*-450) IgG of less than 5% of added label. Moreover, unlabelled cytochrome *P*-450 purified from 3-methylcholanthrene- or pregnenolone-16α-carbonitrile-treated rats, when combined with phenobarbital[^{14}C]cytochrome *P*-450, had no effect at any concentration tested on the amount of radioactivity precipitated by anti-(phenobarbital cytochrome *P*-450) IgG (Fig. 4). In contrast, addition of the purified phenobarbital cytochrome *P*-450 to the immunoassay mixture caused a decrease of more than 50% in the amount of radiolabel appearing in the immunoprecipitate compared with controls where no unlabelled cytochrome *P*-450 was added (Fig. 4).

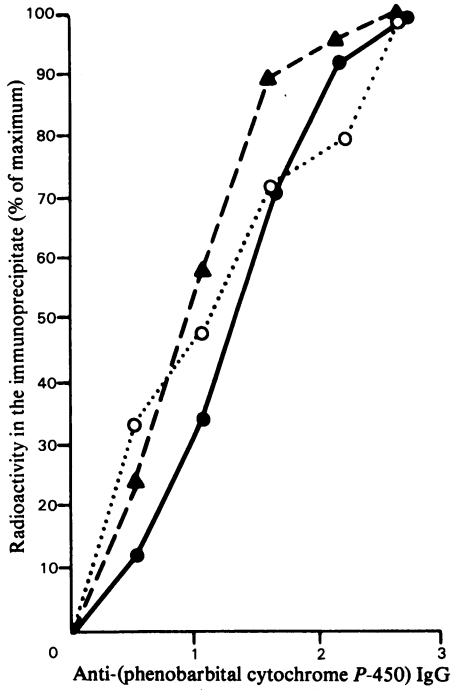

Fig. 3. *Immunoprecipitation of radiolabelled, purified cytochrome P-450 plus microsomes by anti-(phenobarbital cytochrome P-450) IgG*

Dual-labelled cytochrome *P*-450 (0.05 nmol) purified from the livers of phenobarbital-treated rats (see the Materials and methods section and the legend to Fig. 2) was combined with [^{14}C]cytochrome *P*-450 (0.075 nmol) solubilized from microsomes prepared from phenobarbital-treated rats and subjected to immunoprecipitation by increments of form-specific anti-(phenobarbital cytochrome *P*-450) IgG. Results are expressed as % of the maximum radioactivity appearing in the immunoprecipitate with saturating amounts of IgG (3 mg per assay). ●, [^{14}C]protein; ▲, [^{3}H]haem; ○, [^{14}C]protein from microsomes.

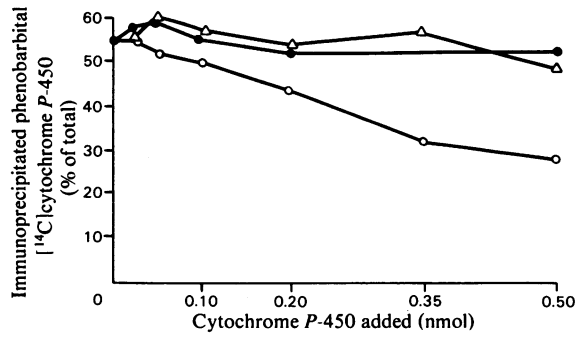

Fig. 4. *Effect of adding purified cytochrome(s) P-450 on immunoprecipitation of purified phenobarbital cytochrome P-450*

Incubations were carried out using 0.15 nmol of purified phenobarbital [^{14}C]cytochrome *P*-450 and 2.05 mg of form-specific anti-(phenobarbital cytochrome *P*-450) IgG (see the legend to Fig. 2). Certain incubations also contained the indicated amounts of unlabelled, purified phenobarbital cytochrome *P*-450 (○), 3-methylcholanthrene cytochrome *P*-448 (●) or pregnenolone-16α-carbonitrile cytochrome *P*-450 (△). Results were expressed as % of the added radioactivity remaining the washed immunoprecipitate.

Measurement of synthesis and degradation of phenobarbital cytochrome P-450 in cultured hepatocytes

To stimulate synthesis of phenobarbital cytochrome *P*-450 protein, rats were treated with phenobarbital. Then, isolated hepatocytes were prepared and incubated for 4 h in culture medium containing [^{3}H]leucine. The medium was removed, and the cells were lysed and solubilized with detergent. Immunoprecipitates of phenobarbital cytochrome *P*-450 were prepared by adding 4 mg of form-specific anti-(phenobarbital cytochrome *P*-450) IgG to 200 μg of cell lysate protein and were subjected to electrophoresis on polyacrylamide gels. The radioactivity in sequential slices of these gels was confined to a single band migrating with the mobility of phenobarbital cytochrome *P*-450 (Fig. 5). Furthermore, fluorography of an identical unsliced gel revealed a single band of radioactivity corresponding in mobility to purified phenobarbital cytochrome *P*-450 run in an adjacent gel lane (results not shown). Nevertheless, recovery of radiolabel in the gel slices corresponding to phenobarbital cytochrome *P*-450 was only 40% of that in the washed immunoprecipitate applied to the gel. This could not be attributed to lixiviation of the labelled antigen from the gel, since no staining/destaining process was employed and because recovery of [^{14}C]ovalbumin standard from the gels was greater than 90%. Since the missing radioactivity was not seen in any other gel slice (Fig. 5), it was presumed due to non-specific, low-molecular-weight cellular constituents or free amino acid migrating ahead of the tracking dye off the gel and into the electrophoresis running buffer. For this reason, labelled immunoreactive phenobarbital cytochrome *P*-450 could not be quantified merely by liquid-scintillation counting of radioactivity in the immunoprecipitate. Therefore, gels were routinely run for each assay, and the appropriate phenobarbital cytochrome *P*-450-containing gel slice excised and analysed as described in the Materials and methods section. In some instances, we noted the occurrence of immunoprecipitable radioactivity which corresponds in mobility on polyacrylamide gels to a much larger

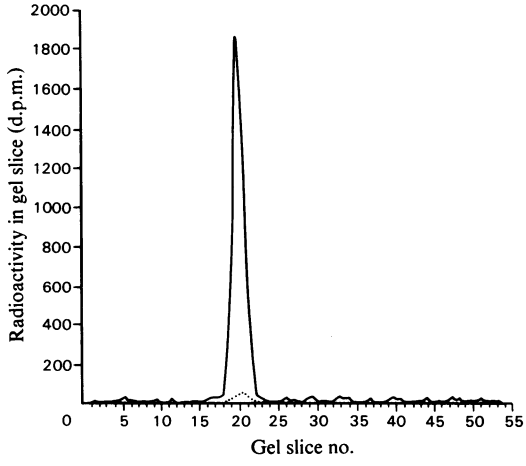

Fig. 5. *Electrophoretic analysis of immunoprecipitated radioactivity from lysates of hepatocyte cultures incubated with [^{3}H]leucine*

Freshly isolated hepatocytes prepared from a phenobarbital-pretreated animal were incubated for 4h in culture medium containing [^{3}H]leucine as described in the Materials and methods section. Cells were then harvested, washed, and lysed, and cytochrome *P*-450 was solubilized and precipitated with form-specific anti-(phenobarbital cytochrome *P*-450) IgG or non-immune IgG. The immunoprecipitates were applied to SDS/polyacrylamide gradient gels and radioactivity was measured by counting sequential 2mm slices of the gel. ——, Form-specific anti-(phenobarbital cytochrome *P*-450) IgG; ····, form-specific non-immune IgG.

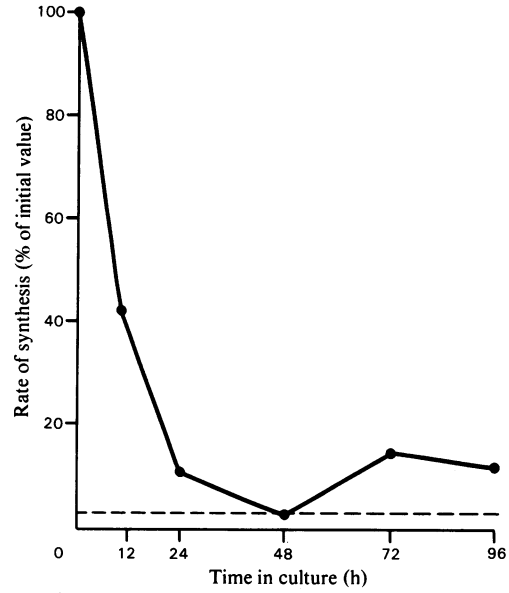

Fig. 6. *Rate of synthesis by cultured hepatocytes of phenobarbital cytochrome P-450 protein relative to total protein*

Rate of synthesis of phenobarbital cytochrome *P*-450 protein was measured in hepatocytes prepared from a phenobarbital-treated animal and maintained in culture for various times as described in the Materials and methods section. Rate of synthesis is expressed as % of the initial value (2.34%) in freshly isolated cells pulsed from 0–4h. The broken line represents the limit of detection.

molecule than phenobarbital cytochrome *P*-450 (results not shown). The appearance of this radioactivity in the gels was quite variable and infrequent, and we attribute it to a non-specific contaminant similar to that observed by Negishi *et al.* (1981).

Radioactivity in excised gel slices containing phenobarbital cytochrome *P*-450 was $2.34 \pm 1.59\%$ ($n = 11$) of that in total acid-precipitable cellular protein when lysates of hepatocytes freshly isolated from phenobarbital-treated animals were used in the immunoassay. The rate of synthesis in these cells then fell rapidly, and was undetectable (<0.05%) by 48h after isolation (Fig. 6). However, the rate of synthesis of phenobarbital cytochrome *P*-450 protein recovered spontaneously by 72h and was maintained at a low level [$0.21 \pm 0.08\%$ ($n = 4$)] up to 96h of age in culture (Fig. 6). The dramatic cessation and recovery of synthesis of phenobarbital cytochrome *P*-450 could not be attributed to removal of the inducer from the cell. Even when as much as 2mM-phenobarbital was incorporated into the culture medium, synthesis of the cytochrome became undetectable in 48h-old cultures (results not shown).

The rate of degradation of phenobarbital cytochrome *P*-450 protein relative to that of total cellular protein was measured in hepatocyte cultures derived from phenobarbital-treated rats. Freshly isolated cells were radiolabelled during the first 4h in culture, and the amount of [^{3}H]leucine remaining in phenobarbital cytochrome *P*-450 protein was measured 20h later. This value represented $3.45 \pm 2.55\%$ ($n = 4$) of the radioactivity remaining in total cellular protein (Fig. 7). This indicates that the cytochrome protein was degraded more slowly than were the general cellular proteins (comparably radiolabelled).

When untreated animals served as hepatocyte donors, the rate of synthesis of phenobarbital cytochrome *P*-450 in freshly isolated cells was $0.36 \pm 0.23\%$ ($n = 3$) of the synthesis rate for total cellular protein. Like cultures prepared from phenobarbital-treated animals, this value fell to less than 0.05% after 24h in culture. However, synthesis of phenobarbital cytochrome *P*-450 failed to recover into the detectable range in cultures prepared from control animals. The rapid decline in rate of synthesis seen during the first 24h in culture in cells prepared from

both control and phenobarbital-treated animals agrees with spectrally measured levels of cytochrome *P*-450 (Fig. 7), and with certain cytochrome *P*-450-dependent drug metabolizing activities which exhibit a similar diminution when hepatocytes are placed in culture (Bissell & Guzelian, 1975). However, these cells retain the ability to respond to phenobarbital induction, since inclusion of 2mM-phenobarbital in the medium for 96h induced both synthesis and accumulation of immunochemically and catalytically active phenobarbital cytochrome *P*-450 (Table 1).

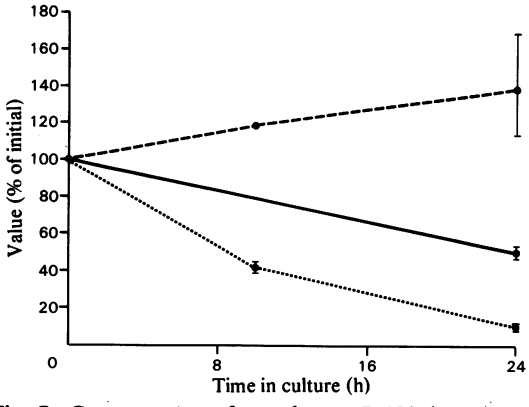

Fig. 7. *Concentration of cytochrome P-450* (——) *and rates of synthesis* (····) *and degradation* (– – –) *of phenobarbital cytochrome P-450 protein relative to total protein in cultured hepatocytes*

Hepatocytes were prepared from a phenobarbital-treated animal, and the concentration of cytochrome *P*-450 and rates of synthesis and degradation were measured at various times thereafter as described in the Materials and methods section. Results are expressed as % of the initial values in freshly isolated cells which were (± S.D.): synthesis of phenobarbital cytochrome *P*-450 protein relative to total protein at termination of 0–4h pulse, 2.34% ± 1.59% ($n = 11$); cytochrome *P*-450 concentration (nmol of cytochrome *P*-450/mg of microsomal protein), 0.52 ± 0.14 ($n = 9$).

Discussion

We have previously discussed the many theoretical reasons for believing that primary monolayer cultures of non-proliferating adult rat hepatocytes may provide a valuable experimental system for studying the events involved in the induction of hepatic cytochrome *P*-450 (Bissell & Guzelian, 1975, 1980). Addition of phenobarbital to primary hepatocyte cultures has been reported to produce an accumulation of total cytochrome *P*-450 measured by spectral techniques (Fahl *et al.*, 1979; Michalopoulos *et al.*, 1976) or drug metabolizing activity (Stenberg & Gustafsson, 1978; Fry *et al.*, 1980; Toftgard *et al.*, 1980). However, these methods are non-specific in that they reflect only the haem (not apoprotein) of cytochrome *P*-450, do not distinguish changes in cytochrome *P*-450 concentration from changes in synthesis or degradation, and do not identify the form(s) of cytochrome *P*-450 being measured. Furthermore, the use of immunochemical techniques to measure quantitatively changes in specific content of individual forms of cytochrome *P*-450 (Thomas *et al.*, 1981), although certainly applicable to cell culture, would again fail to identify changes in synthesis or degradation of these proteins. The ability to measure specifically changes in synthesis or degradation of cytochrome *P*-450 protein in hepatocyte culture should greatly facilitate a more complete understanding of these events *in vivo*.

We have relied on the specificity of isolated, form-specific antibodies to measure rates of synthesis or degradation of the protein portion of phenobarbital cytochrome *P*-450 in culture. The immunoprecipitation assay is specific for the phenobarbital-inducible form of cytochrome *P*-450 as judged by

Table 1. *Stimulation of synthesis de novo of cytochrome P-450 and accumulation of benzphetamine demethylase activity in cultured hepatocytes exposed to phenobarbital for 96 h*

Cultures were incubated in the standard serum-free medium for 24h, and then one-half of the cells were transferred to medium containing phenobarbital (2.0mM) and the other half were maintained in control medium for an additional 96h. The cultures were then apportioned for measurement of synthesis *de novo* of phenobarbital cytochrome *P*-450 protein relative to total cellular protein (see the Materials and methods section), concentration of cytochrome *P*-450 (nmol/mg of microsomal protein; see the Materials and methods section), or activity of benzphetamine demethylase (nmol of formaldehyde formed/min per mg of microsomal protein; Elshourbagy & Guzelian, 1980). Results are expressed as the mean ± S.D. with the number of observations in parentheses. In each instance, the difference between control and phenobarbital-induced values is significant by Student's *t*-test ($P < 0.05$).

Additions to culture medium	Relative synthesis of cytochrome *P*-450	Concentration of cytochrome *P*-450	Benzphetamine demethylase activity
None (control)	<0.05% (11)	0.21 ± 0.06 (3)	1.43 ± 0.43 (3)
Phenobarbital (2mM)	0.33 ± 0.11% (11)	0.46 ± 0.07 (3)	2.67 ± 0.27 (3)

gradient polyacrylamide gel electrophoresis. Unquestionably, this method greatly enhances specificity as compared with fractionation of radiolabelled microsomes or partially purified cytochrome *P*-450 by gel electrophoresis (Althaus *et al.*, 1979; Haugen *et al.*, 1976; Rajamanickam *et al.*, 1973). Nevertheless, the specificity of our method and, indeed, the definition of a 'form' of cytochrome *P*-450 is ultimately determined by the resolving power of gel electrophoresis. It should be noted that, since as much as 60% of the radioactivity in immunoprecipitates does not appear to be incorporated into cytochrome *P*-450 protein (probably existing in free amino acids or small peptides instead), it is important to perform subsequent electrophoresis to isolate and quantify the cytochrome. In fact, this may explain the discrepancy between our detected rate of phenobarbital cytochrome *P*-450 protein synthesis in cells isolated from phenobarbital-pretreated animals (2.34%) and the value (15%) reported by Bhat & Padmanaban (1978), since these investigators merely counted radioactivity in the immunoprecipitate without performing subsequent electrophoresis. Our method is particularly well suited for applications in primary hepatocyte cultures because the cells can be extensively radiolabelled with amino acid precursors of cytochrome *P*-450 protein and because very little starting material is required to yield quantitatively reproducible results. In addition to its utility in studies of cytochrome *P*-450 protein metabolism, this method is potentially useful in studies of cytochrome *P*-450 haem metabolism through labelling of the haem portion of the cytochrome *P*-450 molecule followed by immunoprecipitation.

We observed that synthesis of phenobarbital cytochrome *P*-450 protein declined immediately upon incubation of cells in culture, in parallel with the observed loss of spectrally measured cytochrome *P*-450, whereas degradation of this protein actually was decreased. These observations may reveal the mechanism behind the well-documented loss of cytochrome *P*-450 from cultured hepatocytes. The fact that primary hepatocytes in culture retain the ability to synthesize phenobarbital cytochrome *P*-450 protein is significant. The previously reported lack of induction of cytochrome *P*-450 by phenobarbital in culture cannot be attributed to a loss of the cellular machinery for synthesis of the protein. Indeed, our data indicate that cultured hepatocytes are capable of responding to phenobarbital with an increased rate of synthesis *de novo* of phenobarbital cytochrome *P*-450 protein. The use of the primary hepatocyte culture system and the immunoassay described here should provide the means to acquire better understanding of the molecular events involved in induction of cytochrome *P*-450 and the factors controlling these events at a cellular level.

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