Immunochemical detection of different isoenzymes of cytochrome *P*-450 induced in chick hepatocyte cultures

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This study investigated whether the same cytochrome *P*-450 (*P*-450) isoenzymes were inducible in cultures of chick-embryo hepatocytes as in the liver of chicken embryos. We purified two isoenzymes of cytochrome *P*-450 from the livers of 17-day-old-chick embryos: one of molecular mass approx. 50 kDa induced *in vivo* by the phenobarbital-like inducer glutethimide, and the second of approx. 57 kDa induced by 3-methylcholanthrene. Rabbit antiserum against the 50 kDa protein inhibited benzphetamine demethylase activity in hepatic microsomes (microsomal fractions) from glutethimide-treated chick embryo. Antiserum to the 57 kDa protein inhibited ethoxyresorufin de-ethylase activity in hepatic microsomes from methylcholanthrene-treated chick embryo. Cultured chick hepatocytes were treated with chemicals known to induce isoenzymes of *P*-450 in rodent liver. The induced *P*-450s were quantified spectrophotometrically and characterized by immunoblotting and enzyme assays. From these studies, chemical inducers were classified into three groups: (i) chemicals that induced a *P*-450 isoenzyme of 50 kDa and increased benzphetamine demethylase activity: glutethimide, phenobarbital, metyrapone, mephenytoin, ethanol, isopentanol, isobutanol, lindane, lysodren; (ii) chemicals that induced a *P*-450 isoenzyme of 57 kDa and increased ethoxyresorufin de-ethylase activity: 3-methylcholanthrene and 3,3',4,4'-tetrachlorobiphenyl; and (iii) the mono- α -substituted 2,3',4,4',5-pentabromobiphenyl, which induced both proteins and both activities. The immunochemical data showed that chick-embryo hepatocytes in culture retain the inducibility of glutethimide- and methylcholanthrene-induced isoenzymes of *P*-450 that are inducible in the liver of the chicken embryo.

INTRODUCTION

The cytochrome *P*-450s (*P*-450s) are a family of haemoproteins of the endoplasmic reticulum that catalyse the oxidative metabolism of a number of endogenous and exogenous substances. This oxidative metabolism most often leads to the conversion of a lipophilic compound into a more polar compound that is readily excreted, but the *P*-450s can also convert compounds into more reactive molecules, including activated carcinogens and toxins [for a review, see Guengerich (1988)]. Multiple isoenzymes of *P*-450 have been identified in experimental animals (Black & Coon, 1986) and humans (Beaune *et al.*, 1986; Wrighton *et al.*, 1986). All have protohaem IX as prosthetic group, but differ in their protein moieties. Some isoenzymes of *P*-450 are found in untreated animals, whereas others can be highly induced by exposure to chemicals (Black & Coon, 1986).

As an alternative to animals, primary, non-proliferating, monolayer cultures of hepatocytes have been used by many workers to study the induction of *P*-450. These cultures can be maintained in a chemically defined, serum-free medium, while retaining a diverse array of liver-specific functions (Reid & Jefferson, 1984). Rat hepatocyte cultures have been used to study carcinogen

and hepatotoxin activation that is mediated by *P*-450. However, rat hepatocytes in culture rapidly lose a large portion of their *P*-450, as determined spectrally and enzymically (Forster *et al.*, 1986). By using antibodies to specific isoenzymes of rat cytochrome *P*-450, it has been found that there are differential losses of the various isoenzymes of cytochrome *P*-450 in rat hepatocyte cultures (Steward *et al.*, 1985). By contrast, we found that, in chick-embryo hepatocytes in culture, isoenzymes of *P*-450 remain as inducible as in the intact embryo liver (Althaus *et al.*, 1979; Sinclair *et al.*, 1979, 1981). This conclusion was based on studies in which the inductions of the isoenzymes were detected by increases in specific enzyme activities and their mobility on polyacrylamide-gel electrophoresis (PAGE).

Purification of some isoenzymes of *P*-450 from chick liver has been recently reported. Ades (1983) purified a *P*-450 of 54 kDa from phenobarbital-treated chick embryo, Oron & Bar-Nun (1984*a*) purified a protein of 56 kDa from phenobarbital-treated rooster, and Brooker *et al.* (1983) purified a *P*-450 of 50 kDa from chick embryos treated with allylisopropylacetamide. Using a polyclonal antibody prepared against rat phenobarbital-induced *P*-450, Oron & Bar-Nun (1984*a,b*) detected induction by phenobarbital of a 50 kDa protein in primary cultures of chick-embryo hepatocytes.

Abbreviations used: BPDm, benzphetamine demethylase; DTT, dithiothreitol; EROD, ethoxyresorufin de-ethylase; GLUT, glutethimide; MC, 3-methylcholanthrene; DMSO, dimethyl sulphoxide; *P*-450_{GLUT}, liver microsomal cytochrome *P*-450 induced by treatment with glutethimide; *P*-450_{MC}, liver microsomal cytochrome *P*-450 induced by treatment with MC; PCN, pregnenolone-16 α -carbonitrile; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; *P*-450, cytochrome *P*-450; EROD, ethoxyresorufin de-ethylase; BPDm, benzphetamine demethylase.

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We now report purification of two isoenzymes of *P*-450 from chick-embryo livers treated with glutethimide (GLUT) or 3-methylcholanthrene (MC): an isoenzyme of approx. 50 kDa (designated '*P*-450_{GLUT}') induced by GLUT, a powerful phenobarbital-like *P*-450 inducer in chick embryo (Rifkind *et al.*, 1973), and an isoenzyme of approx. 57 kDa (designated '*P*-450_{MC}') induced by MC. Specific polyclonal antibodies were prepared against these isoenzymes of *P*-450 and were used to establish that the isoenzymes induced by GLUT and MC in chick embryos were also induced in culture by a number of chemicals. The results were compared with the reported abilities of these chemicals to induce specific isoenzymes of *P*-450 in rat liver and in rat hepatocyte cultures.

A preliminary report of this work has been presented in abstract form (P. Sinclair *et al.*, 1986).

EXPERIMENTAL

Chemicals

3-Methylcholanthrene and metyrapone were purchased from Aldrich (Milwaukee, WI, U.S.A.). 3,3',4,4'-Tetrachlorobiphenyl and Aroclor 1254 were purchased from Ultra-Science (Hope, RI, U.S.A.). Lindane was from Chevron Chemical Co. (San Francisco, CA, U.S.A.). Dimethyl sulfoxide (DMSO) and isosafrole were purchased from Fluka (Hauppauge, NY, U.S.A.). Lysodren (2,4'-dichlorodiphenyldichloroethane, also known as mitotane) was a gift from Dr. B. Jensen, University of Maine, Orono, ME, U.S.A. (or was purchased from Aldrich, Milwaukee, WI, U.S.A.). 2,3',4,4',5-Pentabromobiphenyl was a gift from Dr. S. Aust, Utah State University, Logan, UT, U.S.A. Mephentoin was a gift from Sandoz Pharmaceuticals (East Hanover, NJ, U.S.A.). Pregnenolone-16 α -carboxynitrile (PCN) and benzphetamine were gifts from Upjohn Company (Kalamazoo, MI, U.S.A.). 2-Propyl-2-isopropylacetamide was a gift from Hoffmann-La Roche (Nutley, NJ, U.S.A.). Glutethimide (GLUT), Freund's complete adjuvant, Lubrol PX, DEAE-Sephacel, CNBr-Sepharose, cholic acid, 4-chloro-1-naphthol, butylated hydroxytoluene and phenylmethanesulphonyl fluoride were purchased from Sigma (St. Louis, MO, U.S.A.). Whatman DEAE-cellulose 52, CM-cellulose 52, acrylamide and Baker poly(ethylene glycol) 8000 were purchased from VWR Scientific (Rochester, NY, U.S.A.). Nitrocellulose and hydroxyapatite were purchased from Bio-Rad (Richmond, CA, U.S.A.). Horseradish-peroxidase-tagged goat anti-rabbit antibody was purchased from Cooper Biomedical (Malvern, PA, U.S.A.). Ethanol was U.S. Pharmacopeia grade from U.S. Industrial Chemical Co. (Tuscola, IL, U.S.A.); 2-methylpropan-1-ol and 3-methylbutan-1-ol were from Fisher Scientific Co. (Springfield, NJ, U.S.A.) and were glass-distilled before use.

Animals

Fertilized eggs from White Leghorn hens were obtained from Treslow Farms (Chestertown, MD, U.S.A.). New Zealand White adult female rabbits were from Snelling Rabbitry (Claremont, NH, U.S.A.).

Purification of the *P*-450_{GLUT} isoenzyme

Chick embryos (300 in all, 15 days old) were each injected with 6 mg of GLUT in 0.2 ml of DMSO. After 48 h, livers were removed, rinsed in saline (0.9% NaCl)

and homogenized (25%, w/v) in Tris/acetate/KCl (0.1 M-Tris base/0.1 M-KCl/1 mM-EDTA, adjusted to pH 7.4 with acetic acid) by eight to ten strokes of the pestle in a Potter-Elvehjem homogenizer. After homogenization, butylated hydroxytoluene (final concn. 45 μ M) and phenylmethanesulphonyl fluoride (115 μ M) were added.

Microsomes were isolated as described by Sinclair *et al.* (1979) and stored as a suspension at -60°C in 20 mM-potassium phosphate/20% (w/v) glycerol/0.1 mM-EDTA, pH 7.4. The purification of *P*-450_{GLUT} was essentially that described by Klotz *et al.* (1983) with column chromatography being performed at room temperature. The microsomal concentration was adjusted to 10 mg of protein/ml in phosphate/glycerol, solubilized with 0.2% Lubrol PX/1.5% sodium cholate, and the protein that precipitated between 6 and 20% (w/v) poly(ethylene glycol) was collected. The poly(ethylene glycol) pellet was resuspended in a small volume of buffer A (10 mM-Hepes/20% glycerol/0.2% Lubrol PX/0.5% sodium cholate/0.1 mM-EDTA/1 mM-DTT, pH 7.5) and applied to a column (2.5 cm $\times$ 20 cm) of DE-52 cellulose equilibrated in buffer A at room temperature. The column was washed with one bed volume of buffer A to elute unbound material and then with one bed volume of buffer B (20 mM-potassium phosphate/20% glycerol/0.2% Lubrol PX/0.5% cholate/0.1 mM-EDTA/1 mM-DTT, pH 7.5), followed by a linear gradient of KCl (0 to 0.5 M) in buffer B. The main band of *P*-450 (fractions 18–20), eluted at the start of the gradient, was dialysed overnight against 20 vol. of buffer A without cholate and applied to a hydroxyapatite column which had been equilibrated in buffer A without cholate. The column was washed with 5 bed volumes of buffer C (20 mM-potassium phosphate/20% glycerol/0.1 mM-EDTA/0.2% Lubrol PX/1 mM-DTT, pH 7.5) with step-wise increases of potassium phosphate in buffer C of 20, 50 and 150 mM. *P*-450 was eluted with 150 mM-potassium phosphate.

After dialysis against 20 vol. of buffer A without cholate, Lubrol PX was removed by repeating the hydroxyapatite chromatography, but replacing the Lubrol PX of buffer C with 0.6% cholate. The column was washed with 20 bed volumes of this buffer C and then the *P*-450 was eluted with 150 mM-potassium phosphate in buffer C. The eluted protein was extensively dialysed against 20 vol. of 100 mM-potassium phosphate/20% glycerol/0.1 mM-EDTA, pH 7.5, to remove cholate.

Purification of *P*-450_{MC}

Chick embryos (300; 15 days old) were each injected with 0.5 mg of MC in 0.2 ml of DMSO. After 48 h, livers were removed and a microsomal fraction was prepared and stored as previously described for purification of *P*-450_{GLUT}. Microsomes were resuspended at 6.5 mg of protein/ml in 20 mM-potassium phosphate/20% glycerol, solubilized with 0.2% Lubrol PX/1.0% cholate, and the protein precipitating between 6 and 20% poly(ethylene glycol) was collected. The poly(ethylene glycol) pellet was resuspended in buffer A and was applied to a DEAE-Sephacel column which had been equilibrated in buffer A. The column was washed with one bed volume of buffer A, which eluted most of the *P*-450 with other unbound proteins. The column was then washed with a one bed volume of buffer B, which eluted a minor band of *P*-450. The unbound fraction

containing most of the cytochrome *P*-450 was adjusted to pH 6.5 with HCl and applied to a CM-52 cellulose column which had been equilibrated in 5 mM-potassium phosphate/20 % glycerol/0.2 % Lubrol/1 mM-DTT/0.1 mM-EDTA, pH 6.5. The column was then washed with stepwise increases of potassium phosphate (20, 75 and 200 mM) in the above buffer. The main band of *P*-450 was eluted at 75 mM-potassium phosphate and was used for production of rabbit antibodies, because attempts at further purification by hydroxyapatite chromatography led to unacceptable loss of *P*-450.

Production of antibodies in rabbits

The method was essentially that of Fasco *et al.* (1981). *P*-450_{GLUT} (200 µg) in Freund's complete adjuvant was injected intradermally. After 6 weeks the procedure was repeated with 40 µg of antigen in Freund's complete adjuvant. Then 4 weeks later, 40 µg of antigen in 0.25 ml of 0.9 % NaCl was injected into an ear vein, and, 7 days later, blood was collected from a lower ear vein.

Antiserum against the *P*-450_{MC} was prepared by the same procedure except that 100 µg of purified *P*-450_{MC} was used for the initial immunization.

Immunoabsorption of anti-*P*-450_{GLUT} serum

Anti-*P*-450_{GLUT} serum was immunoabsorbed against microsomes from untreated chick-embryo liver immobilized on CNBr-activated Sepharose 4B as follows. The microsomes were solubilized in 0.05 M-potassium phosphate/20 % glycerol/0.5 mM-EDTA/1 % cholate/0.2 % Lubrol PX, pH 7.4, and then centrifuged at 100 000 *g* for 60 min. The supernatant was then dialysed overnight against 10 vol. of 0.1 M-NaHCO₃ (pH 8.5)/0.2 % Lubrol PX. CNBr-activated Sepharose 4B was washed according to the manufacturer's instructions, and incubated with solubilized microsomes at the ratio of 15 mg of protein to 1 ml of gel. For the adsorption, 7.5 ml of serum was added to 3.5 ml of the gel and the mixture incubated overnight at 4 °C with rocking. After centrifugation, the serum was decanted and stored frozen.

Preparation of IgG

IgG was prepared from sera using Protein A-Sepharose as described by Ey *et al.* (1978).

Detection of *P*-450 isoenzymes by immunoblotting

Immunochemical detection was performed using modifications of the method of Towbin *et al.* (1979). Briefly, samples were solubilized by boiling for 2 min in buffer containing 0.2 % (w/v) SDS/5 % (v/v) mercapto-ethanol. Proteins were then separated at 200 V for 1 h by SDS/PAGE in a 10 % (w/v) acrylamide gel. The resolved proteins were transferred electrophoretically to nitrocellulose at 100 V for 1 h. The nitrocellulose sheets were blocked with 5 % non-fat powdered milk in 0.02 M-Tris/saline, pH 7.4, containing 0.5 % Tween 20 for 1 h (Johnson *et al.*, 1984). Then the sheets were rinsed and treated sequentially (with intermediate washing in saline) with specific rabbit anti-serum, with a 1:2500 dilution of alkaline-phosphatase-conjugated goat anti-rabbit IgG, and then with 5-bromo-4-chloro-3-indolyl phosphate toluidine salt and *p*-Nitro Blue Tetrazolium chloride (Bio-Rad, Richmond, CA, U.S.A.) as in the manufacturer's instructions.

Hepatocyte cultures

Primary cultures from livers of 16-day-old White-Leghorn-chicken embryos were prepared and maintained in Williams E medium as described previously (Sinclair *et al.*, 1982). Chemicals were added during the second day of culture after rinsing cells twice with Williams E medium containing dexamethasone and tri-iodothyronine, but not insulin. Compounds of poor water solubility were added in DMSO (maximum concn. 4 µl/ml of culture medium). When alcohols were added to the cells, 20 mM-Hepes, pH 7.4, was added to the medium and the plates were wrapped in plastic films as described by Sinclair *et al.* (1981). Microsomes were prepared from homogenates of cultured cells as described previously (Sinclair *et al.*, 1979).

Assays

Spectral determinations of cytochrome *P*-450 were performed by the method of Omura & Sato (1964), an absorption coefficient of 91 mM⁻¹·cm⁻¹ for the difference in absorption between 450 and 490 nm being used. SDS/PAGE was by the method of Laemmli (1970), with lactate dehydrogenase (36 kDa), aldolase (41 kDa), fumarase (49 kDa), catalase (57 kDa) and bovine serum albumin (66 kDa) as molecular-mass standards. Ethoxyresorufin de-ethylase (EROD) was measured by a modification of the direct fluorimetric method of Burke *et al.* (1985) as described by Sundstrom *et al.* (1988). Benzphetamine demethylase (BPDM) activity was measured as described by Sinclair *et al.* (1981). Protein determinations were by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Antibody inhibition of enzyme activities

Microsomes (50 pmol of *P*-450 in 0.1 ml of 0.1 M-sodium phosphate, pH 7.4) were preincubated with preimmune or immune IgG at room temperature so that total IgG was 20 µg/pmol of *P*-450. The preincubation was 20 min for anti-*P*-450_{GLUT} and 10 min for anti-*P*-450_{MC}. Then the assay reagents (substrate, NADPH-generating system and MgCl₂) were added and the mixture incubated at 37 °C for 30 min for BPDM and 10 min for EROD. Preimmune IgG had no effect on BPDM or EROD.

RESULTS

Purification of *P*-450_{GLUT} from chick-embryo liver

A 48 h exposure to the drug glutethimide increased microsomal hepatic proteins of 50 and 53 kDa as compared with untreated chick-embryo liver (Fig. 1, lanes 4 and 6). The purification of the 50 kDa protein, *P*-450_{GLUT}, is summarized in Table 1. During chromatography on DE-52 DEAE-cellulose, haemoproteins were separated into three fractions (Fig. 1): (i) an unbound fraction, (ii) a fraction eluted with 20 mM-potassium phosphate and (iii) a fraction eluted by the KCl gradient. This last fraction was not further purified. The second fraction from the DE-52 column was further purified by hydroxyapatite chromatography, yielding a protein of 50 kDa (Fig. 1, lane 3), although the specific content was not increased, suggesting some haem was lost. The reduced CO difference spectrum had a maximum at 452 nm. This preparation was used to immunize rabbits. The unbound fraction contained a major protein of

Fig. 1. Electrophoretic analysis by SDS/PAGE of hepatic microsomes from treated chicken embryos and purified cytochrome *P*-450s

Lanes 1 and 7, markers (29, 36, 40, 49, 57 and 68 kDa); lane 2, purified *P*-450_{MC} (1 µg of protein); lane 3, purified *P*-450_{GLUT} (1 µg of protein); lane 4, control liver microsomes (25 µg of protein); lane 5, MC-induced liver microsomes (25 µg of protein), lane 6, GLUT-induced liver microsomes (25 µg of protein). A 1.2 mm gel with 5–10 % acrylamide gradient was used. Proteins were stained with Coomassie Blue.

approx. 53 kDa (results not shown). This protein may be the 56 kDa form of *P*-450 reported by Oron & Bar-Nun (1984a) or it could be the 53 kDa phenobarbital-inducible glucuronyltransferase isolated from chick-embryo liver by Burchell *et al.* (1983).

Purification of *P*-450_{MC}

A 48 h exposure of chick embryos to methylcholanthrene increased a microsomal protein of 57 kDa (Fig. 1, lane 5) not present in untreated liver (lane 4). The purification of *P*-450_{MC} is summarized in Table 1. The major *P*-450 fraction that was not retained by the

DEAE-Sephacel column was bound by CM-52 cellulose. The haemoprotein was eluted from the column at 75 mM-potassium phosphate. The combined fractions had an average specific content of 8.1 nmol of *P*-450/mg of protein (Fig. 1, lane 2). This *P*-450 had a reduced CO difference maximum at 450 nm. Further chromatography on hydroxyapatite resulted in almost complete loss of the cytochrome. Thus the purest fractions from the CM-52 column, as determined by SDS/PAGE, were used to immunize rabbits.

Specificity of antibodies as determined by inhibition of mixed-function-oxidase activities

Treatment of chick embryos with glutethimide induces BPDM, whereas treatment with methylcholanthrene (MC) induces EROD activity (J. Bement, J. Sinclair & P. Sinclair, unpublished work). To test the specificities of the antibodies raised to each *P*-450 species, chick-embryo liver microsomes from both glutethimide- and methylcholanthrene-treated chicken embryos were preincubated with IgG prepared from antisera to *P*-450_{GLUT} and *P*-450_{MC} before measuring the enzyme activities. Anti-*P*-450_{GLUT} inhibited BPDM activity by 75 % in GLUT-induced microsomes (Fig. 2a), but did not inhibit EROD activity in MC-induced microsomes (Fig. 2b). Anti-*P*-450_{MC} completely inhibited EROD activity in MC-induced microsomes (Fig. 2b), but also inhibited BPDM activity in GLUT-induced microsomes to some extent (Fig. 2a).

Immunochemical analysis of isoenzymes of cytochrome *P*-450 in chick hepatocyte cultures after treatment with various inducing chemicals

In the past we have characterized the induction of *P*-450 isoenzymes in the cultured cells by the following criteria: shift of absorption peak, appearance of induced proteins on SDS/PAGE and changes in specific enzyme activities (Althaus *et al.*, 1979; Sinclair *et al.*, 1981, 1982). Here we have compared these criteria with immunoblot analysis.

Fig. 3 and Table 2 present results with microsomes from chick hepatocyte cultures treated with chemicals that are known to induce various isoenzymes of hepatic *P*-450 in intact animals. The concentrations of these chemicals were previously established to be optimal in the culture for induction of *P*-450 determined spectrally

Table 1. Purifications of *P*-450_{GLUT} and *P*-450_{MC}

Abbreviations: PEG, poly(ethylene glycol); N.D., not determined.

Cytochrome isoenzyme	Step	Total <i>P</i> -450 (nmol)	Yield (%)	Specific content (nmol/mg of protein)
<i>P</i> -450 _{GLUT}	Microsomes	687	[100]	2.75
	PEG (6–20 %) fractionation	440	64	N.D.
	DE-52 column (fractions eluted with 20 mM-potassium phosphate)	96	14	8.25
	First hydroxyapatite column	54	9	7.9
	Second hydroxyapatite column	59	9	9.9
<i>P</i> -450 _{MC}	Microsomes	332	[100]	0.8
	PEG (6–20 %) fractionation	212	64	1.2
	DEAE-Sephacel	99	30	2.5
	Cellulose CM-52 column	20	9	8.1

Fig. 2. Effects of anti-*P*-450_{GLUT} and anti-*P*-450_{MC} on the BPDM activity of GLUT-induced microsomes (a) and on the EROD activity of MC-induced microsomes (b) from chick-embryo liver

BPDM activity in the absence of specific IgG, but in the presence of pre-immune IgG, was 3.4 nmol of formaldehyde/min per mg of protein. EROD activity in the absence of IgG, but in the presence of preimmune IgG, was 0.71 nmol of resorufin/min per mg of protein. The indicated amounts of anti-*P*-450_{GLUT} (○) or anti-*P*-450_{MC} (△) IgG were added. The total amount of IgG in each reaction was kept constant by the addition of pre-immune IgG. Microsomes were preincubated with IgG for 20 min (BPDM) and 10 min (EROD) at room temperature before the addition of other assay components.

or enzymically. In the immunoblots shown in Fig. 3, the 50 kDa protein detected by anti-*P*-450_{GLUT} was detected in untreated as well as treated cells. Chemicals which induced BPDM and total *P*-450 (Table 2) also gave increased intensity of staining. Purified *P*-450_{GLUT} (Figs. 3c and 3g, lanes 16 and 30) reacted with anti-*P*-450_{GLUT}, whereas purified *P*-450_{MC} (Fig. 3c, lane 17) did not. MC (Figs. 3b, 3d and 3f, lanes 3, 10 and 20) increased a 57 kDa protein that reacted strongly with anti-*P*-450_{MC}, but only slightly increased the 50 kDa protein that reacted with anti-*P*-450_{GLUT} (Figs. 3a, 3c, 3e and 3g, lanes 3, 10, 20 and 25). The purified *P*-450_{MC} (lane 17) reacted with anti-*P*-450_{MC}, but purified *P*-450_{GLUT} (lane 18) did not. The intensities of the blot varied somewhat from experiment to experiment, as seen by variation in the microsomes from untreated cells (Fig. 3, lanes 1, 18 and 24). The immunoblots shown in Figs. 3(e), 3(g), and 3(f) are more intense, owing to a longer development time. Increases in the 57 kDa protein corresponded with increased EROD activity (Table 2).

3,3',4,4'-Tetrachlorobiphenyl at 0.1 μ M maximally induced EROD, with a slight increase of BPDM (Table 2). However, at 3.4 μ M-tetrachlorobiphenyl, EROD activity was decreased (Table 2), as found previously with high concentrations of this biphenyl (P. Sinclair *et al.*, 1984). The decrease in EROD activity appears to be reflected in decreased intensity of staining in the immunoblot with anti-*P*-450_{MC} (Fig. 3b, lanes 8 and 9), a finding reproduced in several experiments. It also appears that 3.4 μ M-tetrachlorobiphenyl decreased the 50 kDa protein (Fig. 3, lane 9). However, this latter result was found to be variable. Similar effects on enzymic activities were obtained with high and low concentrations of 2,3',4,4',5-pentabromobiphenyl (Table 2). The decrease in EROD activity at high pentabromobiphenyl concentration was associated with decreased staining with anti-*P*-450_{MC} of the 57 kDa protein. However, the higher concentration of this biphenyl increased both the 50 kDa protein and further increased BPDM activity (Fig. 3e, lanes 22 and 23; Table 2). This pattern of induction of both isoenzymes of cytochrome *P*-450 by mono- α -sub-

stituted biphenyls also occurs in rodents (Parkinson *et al.*, 1980). Aroclor 1254, a mixture of chlorinated biphenyls, also increased both proteins and both enzyme activities (Fig. 3, lane 21). Isosafrole caused a slight, but detectable, increase in the 57 kDa protein (Fig. 3, lane 5). In rats, isosafrole induces the *P*-450_d isoenzyme, one of the two isoenzymes induced by MC (Thomas *et al.* 1983).

Fig. 3(g) shows immunoblots of cells which had been treated with ethanol, 3-methylbutan-1-ol and 2-methylpropan-1-ol. We had previously concluded from assays of BPDM, EROD and biphenyl hydroxylation that the *P*-450 induced by these alcohols has properties similar to the cytochrome induced by propylisopropylacetamide treatment (Sinclair *et al.*, 1981, 1982; J. Sinclair *et al.*, 1984; J. Sinclair *et al.*, 1986). With the anti-*P*-450_{GLUT} serum, the immunoblot shows that the alcohols increased a protein of 50 kDa. However, unlike MC, the alcohols did not increase the *P*-450 of 57000 kDa detected by anti-*P*-450_{MC}.

Oron & Bar-Nun (1984a) have reported that phenobarbital treatment of chick-embryo hepatocyte cultures increased synthesis of a protein of 56 kDa that was detected with the antibody they prepared to a protein purified from phenobarbital-treated roosters. We found treatment of the cells with 1 mM-sodium phenobarbital, in experiments similar to those shown in Fig. 3, increased the same protein of 50 kDa that was increased by treatment of cultures with propylisopropylacetamide or glutethimide (results not shown; Lincoln *et al.*, 1988). Anti-*P*-450_{GLUT} did not detect a 56 kDa protein, nor did anti-*P*-450_{MC} detect a protein of 57 kDa in phenobarbital-treated cells. In the same experiment, 10 μ M-chlordane, like phenobarbital, was found to increase the 50 kDa, but not the 57 kDa, protein (results not shown).

DISCUSSION

We have purified two isoenzymes of *P*-450 from chick-embryo treated with either GLUT or MC and have used antibodies to demonstrate that cultures of chick

Fig. 3. Immunoblots of microsomes from induced hepatocyte cultures

Chick liver cell microsomes from the same experiment described in the legend of Table 2, or similar experiments, were electrophoresed, the proteins transferred to nitrocellulose and immunochemically detected with either anti-P-450_{GLUT} (a, c, e and g) at 50 kDa or anti-P-450_{MC} (b, d and f) (57 kDa) as described in the Materials and methods section. The second antibody was conjugated with alkaline phosphatase. Unless shown, the amounts of inducing chemical used are those given in Table 2. A 7 µg portion of microsomal or a 0.2–1.0 µg portion of purified cytochrome protein was added to each lane. Untreated, GLUT- and MC-treated samples were included on each sheet to compare separate treatments within separate experiments. Lane 1, untreated; lane 2, GLUT; lane 3, MC; lane 4, mephenytoin; lane 5, isosafrole; lane 6, metyrapone; lane 7, propylisopropylacetamide; lane 8, 0.1 µM-3,3',4,4'-tetrachlorobiphenyl; lane 9, 3.4 µM-3,3',4,4'-tetrachlorobiphenyl; lane 10, MC; lane 11, GLUT; lane 12, control; lane 13, lindane; lane 14, lysodren; lane 15, PCN; lane 16, 1.0 µg of purified cytochrome P-450_{GLUT}; lane 17, 0.2 µg of purified P-450_{MC}; lane 18, untreated; lane 19, propylisopropylacetamide; lane 20, MC; lane 21, Aroclor; lane 22, 0.2 µM-2,3',4,4',5-pentabromobiphenyl; lane 23, 5 µM-pentabromobiphenyl; lane 24, untreated; lane 25, 0.9 µM-MC; lane 26, 0.14 mM-propylisopropylacetamide; lane 27, 200 mM-ethanol; lane 28, 30 mM-2-methylpropan-1-ol; lane 29, 10 mM-3-methylbutan-1-ol; lane 30, 1 µg of purified P-450_{GLUT}.

Table 2. Effect of chemical treatments on microsomal cytochrome *P*-450, benzphetamine demethylase and ethoxyresorufin de-ethylase

Chick liver cells were plated in 10-cm-diameter dishes and after 24 h were treated with the indicated chemicals for 22 h. The contents of four or five dishes were harvested together for each treatment and microsomes were prepared as described in the Materials and methods section. Portions of the same microsomal preparation were analysed for *P*-450 content, BPDM and EROD activities, and for immunoblotting as shown in Fig. 3 and described in the Materials and methods section. Data are presented as means, with individual values in parentheses or with s.d. values for triplicate determinations. Abbreviation: N.D., not detectable.

Treatment	Concn. (μ M)	<i>P</i> -450 (nmol/mg of protein)	BPDM (nmol/h per mg of protein)	EROD (nmol/min per mg of protein)
Untreated		0.075 (0.07, 0.08)	13 $\pm$ 2	N.D.
GLUT	45	0.66 (0.65, 0.67)	177 $\pm$ 8	N.D.
Lindane	20	0.92 (0.89, 0.94)	132 $\pm$ 8	N.D.
Lysodren	15	0.97 (0.97, 0.97)	118 $\pm$ 8	N.D.
PCN	30	0.67 (0.64, 0.70)	75 $\pm$ 2	N.D.
Mephénytoin	180	0.65 (0.63, 0.67)	49 $\pm$ 4	N.D.
Isosafrole	310	0.41 (0.40, 0.42)	71 $\pm$ 15	N.D.
Metirapone	500	0.84 (0.78, 0.89)	93 $\pm$ 20	N.D.
Propylisopropylacetamide	140	0.78 (0.77, 0.79)	130 $\pm$ 1	N.D.
MC	1	0.32 (0.30, 0.33)	45 $\pm$ 9	2.75 $\pm$ 0.13
3,3',4,4'-Tetrachlorobiphenyl	0.1	0.28 (0.27, 0.28)	28 $\pm$ 6	2.67 $\pm$ 0.29
3,3',4,4'-Tetrachlorobiphenyl	3.4	0.17 (0.17, 0.17)	40 $\pm$ 5	0.35 $\pm$ 0.03
2,3',4,4',5-Pentabromobiphenyl	0.2	0.26 (0.25, 0.28)	39 $\pm$ 2	2.25 $\pm$ 0.28
2,3',4,4',5-Pentabromobiphenyl	5.0	0.19 (0.18, 0.19)	58 $\pm$ 5	0.61 $\pm$ 0.18
Aroclor 1254	(6 μ g/ml)	0.34 (0.33, 0.35)	68 $\pm$ 3	0.68 $\pm$ 0.11

hepatocytes accumulated either or both of the two isoenzymes depending on the chemical treatments. The antibodies to the purified isoenzymes were highly effective in inhibiting the appropriate enzyme activities catalysed by *P*-450s in microsomes from induced chick embryos (Fig. 2) or from cultured hepatocytes (S. Sundstrom, J. Sinclair, L. Smith, P. Sinclair, unpublished work; Sinclair *et al.*, 1989). The results suggest that the isoenzymes purified from livers of GLUT- or MC-treated embryos are the major isoenzymes of *P*-450 induced by GLUT or MC which catalyse BPDM and EROD respectively. The antibodies did not cross-react with the inappropriate protein, as indicated by the immunoblotting (Figs. 3a–3g). We do not know why there was partial inhibition of BPDM in microsomes from GLUT-induced embryos by anti-*P*-450_{MC} (Fig. 2). This antibody did not detect the 50 kDa protein in immunoblots (Fig. 3). Further separation techniques will be needed to resolve whether more than one protein is present in the major molecular-mass regions of the purified proteins as detected by SDS/PAGE and immunoblots. Reverse-phase h.p.l.c. of the purified *P*-450_{GLUT} failed to detect more than one major peak (T. Ciardelli, J. Healey & P. Sinclair, unpublished work).

Other workers have used immunochemical techniques to detect induction of cytochrome *P*-450 of 50 kDa in liver of chicken embryos or chick hepatocyte cultures treated with phenobarbital-like inducers (Brooker *et al.*, 1983; Oron & Bar-Nun, 1984a,b; Bulgheroni, 1985). Brooker *et al.* (1983) found that mRNA for the 50 kDa protein was inducible in chick-embryo liver by phenobarbital but not by MC or PCN, known inducers of other *P*-450 isoenzymes in rats. We also found that the mRNA for the 50 kDa protein was induced in the cultured hepatocytes by propylisopropylacetamide or

GLUT (Hamilton *et al.*, 1988). Oron & Bar-Nun (1984a) reported that synthesis of a 56 kDa protein in cultured chick-embryo hepatocytes was increased in phenobarbital-treated, but not in untreated, cells. Subsequently, Oron & Bar-Nun (1984b) detected increased synthesis of both 50 and 54.5 kDa proteins induced by phenobarbital in the cultures using antibody to the major phenobarbital-induced *P*-450 of rat liver. This 54 kDa isoenzyme may be that purified by Ades (1983). Bulgheroni (1985) detected increased synthesis of a 50 kDa protein induced in chick hepatocyte cultures treated with mephénytoin. We conclude that the induced chick 50 kDa protein we have designated '*P*-450_{GLUT}' is probably the same as the 50 kDa *P*-450 described by these other workers. We found that antibody to the chicken 50 kDa protein sent to us by Dr. B. May (Department of Biochemistry, University of Adelaide, Adelaide, Australia) also detected, in immunoblots, a 50 kDa protein in induced microsomes from chick embryos treated with glutethimide (results not shown).

The antibody to the major MC-induced form of cytochrome *P*-450 isolated in the present study completely inhibited EROD activity (Fig. 2b), indicating that we have isolated the major form of *P*-450 that catalyses EROD in hepatic microsomes from MC-treated chick-embryo liver or hepatocytes. The antibody to this isoenzyme has also been used to study the mechanism of the uroporphyrin caused by many halogenated aromatic hydrocarbons [Sinclair *et al.*, 1987; Jacobs *et al.*, 1989 (the following paper)].

Our studies using immunoblots established that 50 and 57 kDa *P*-450 isoenzymes accumulated after treatments of chick-embryo hepatocytes with various chemicals. MC-like inducers increased both the 57 kDa protein and EROD activity. Those inducers, previously assumed to be

phenobarbital-like inducers in this culture system based on other criteria (Althaus *et al.*, 1979; Sinclair *et al.*, 1981; J. Sinclair *et al.*, 1984, 1986), all increased the major 50 kDa protein induced by GLUT.

The chemicals examined as inducers of *P*-450 isoenzymes in the chick culture were selected on the basis of their known inducing effects in both this system and in rodents. PCN, phenobarbital, chlordane, lysodren and the alcohols all increased the 50 kDa *P*-450_{GLUT} in our studies. In rats, PCN, phenobarbital and chlordane induce both *P*-450*p* and *P*-450*b* in intact animals, whereas only *P*-450*p* is induced in cultures of rat liver hepatocytes (Schuetz *et al.*, 1986). Metirapone was reported to be a potent inducer of total cytochrome *P*-450 in chick-embryo liver and liver cultures (Giger & Meyer, 1982) and in rat liver, but not in rat liver cultures (Lake & Paine, 1983). Lysodren has been shown to be a persistent phenobarbital-like inducer of *P*-450*b* in rat liver (Yoshioka *et al.*, 1984) and, in the chick liver culture, is the most potent phenobarbital-like *P*-450 inducer we have yet found (J. Bement & P. Sinclair, unpublished work). Ethanol induces both *P*-450*b* and *P*-450*j* in rats (Johanssen *et al.*, 1988).

In a previous study, the abilities of aliphatic alcohols and 5-substituted pyrazoles to induce *P*-450 were shown by structure-activity relationships to be correlated with the lipophilicity of the compounds (J. Sinclair *et al.*, 1986). This correlation may also hold for all the otherwise structurally unrelated compounds which were found in the present study to increase the 50 kDa isoenzyme of *P*-450. We found that even MC and 3,3',4,4'-tetrachlorobiphenyl, both highly lipophilic compounds, increased the 50 kDa isoenzyme and BPDm to some extent (Table 2, Fig. 3).

In summary, in the present study, we have shown by immunochemical criteria that the major *P*-450s induced by MC and GLUT in the intact chick-embryo liver are also inducible in cultures of chick-embryo hepatocytes. This is in contrast with many studies of cultures of rat hepatocytes in which the *P*-450*b* isoenzyme, the major phenobarbital-inducible form, is poorly inducible (Forster *et al.*, 1986; Newman & Guzelian, 1982), thus limiting usefulness of these cultures. Induction of mRNA and apoprotein of *P*-450*b* in rat hepatocyte cultures has been reported recently to be markedly improved by maintaining the cultures on an extracellular biomatrix isolated from a murine sarcoma (Bissell *et al.*, 1987; Schuetz *et al.*, 1988). In contrast with the rat hepatocytes, the chick hepatocytes in culture, as described in the present paper, require no special conditions for maintenance and inducibility of different cytochrome *P*-450s.

Note added in proof (Received 3 January 1989)

Hokama *et al.* (1988) recently reported the isolation of two forms of cytochrome *P*-450 from 15-day-old chickens treated with 3,3',4,4',5-pentachlorobiphenyl.

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REFERENCES

- Ades, I. (1983). *IRCS Med. Sci.* **11**, 302-303
- Althaus, F., Sinclair, J., Sinclair, P. & Meyer, U. (1979) *J. Biol. Chem.* **254**, 2148-2153
- Beaune, P., Kremers, P., Kaminsky, L., De Graeve, J., Albert, A. & Guengerich, F. (1986) *Drug Metab. Dispos.* **14**, 437-442
- Bissell, M., Arenson, D., Maher, J. & Roll, F. (1987) *J. Clin. Inv.* **79**, 801-812
- Black, S. & Coon, M. (1986) in *Cytochrome P450* (Ortiz de Montellano, P., ed.), pp. 161-216, Plenum, New York
- Brooker, J., Srivastava, G., Borthwick, I., May, B. & Elliott, W. (1983) *Eur. J. Biochem.* **136**, 327-332
- Bulgheroni, A. (1985) Ph.D. Thesis, Eidgenossischen Technischen Hochschule, Zurich
- Burchell, B., Pratt, G., Duffy, I. & West, L. (1983) *Biochem. J.* **214**, 517-523
- Burke, M., Thompson, S., Elcombe, C., Halpert, J., Haaparanta, T. & Mayer, R. (1985) *Biochem. Pharmacol.* **34**, 3337-3345
- Ey, P., Prowse S. & Jenkin, C. (1978) *Immunochemistry* **15**, 429-436
- Fasco, M., Kaminsky, L. & Guengerich, F. (1981) *Methods Enzymol.* **74**, 262-272
- Forster, U., Luippold, G. & Schwarz, L. (1986) *Drug Metab. Dispos.* **14**, 343-360
- Giger, U. & Meyer, U. (1982) *Biochem. Pharmacol.* **31**, 1735-1741
- Guengerich, F. (1988) *Cancer Res.* **48**, 2946-2954
- Hamilton, J., Bement, W., Sinclair, P., Sinclair, J. & Wetterhahn, K. (1988) *Biochem. J.* **255**, 267-275
- Hokama, Y., Koga, N. & Yoshimura, H. (1988) *J. Biochem. (Tokyo)* **104**, 355-361
- Jacobs, J., Sinclair, P., Bement, W., Lambrecht, R., Sinclair, J. & Goldstein, J. (1989) *Biochem. J.* **258**, 247-253
- Johanssen, I., Ekstrom, G., Scholte, B., Puzychi, D., Jornvall, H. & Ingelman-Sundberg, M. (1988) *Biochemistry* **27**, 1925-1934
- Johnson, D., Gautsch, J., Sportsman, J. & Elder, J. (1984) *Gene Anal. Technol.* **1**, 3-8
- Klotz, A., Stegeman, J. & Walsh, C. (1983) *Arch. Biochem. Biophys.* **226**, 578-592
- Laemmli, U. (1970) *Nature (London)* **227**, 680
- Lake, B. & Paine, A. (1983) *Xenobiotics* **13**, 725-730
- Lincoln, B., Healey, J. & Bonkovsky, H. (1988) *Biochem. J.* **250**, 189-196
- Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951) *J. Biol. Chem.* **193**, 265-275
- Newman, S. & Guzelian, P. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2922-2925
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370-2385
- Oron, L. & Bar-Nun, S. (1984a) *Biochem. Biophys. Acta* **805**, 291-299
- Oron, L. & Bar-Nun, S. (1984b) *Biochem. Biophys. Res. Commun.* **125**, 1096-1102
- Parkinson, A., Safe, L. & Safe, S. (1980) *Chem.-Biol. Interact.* **30**, 271-285
- Reid, L. & Jefferson, D. (1984) *Hepatology* **4**, 548-559
- Rifkind, A., Gillette, P., Song, C. & Kappas, A. (1973) *J. Pharmacol. Exp. Ther.* **185**, 214-225
- Schuetz, E., Wrighton, S., Safe, S. & Guzelian, P. (1986) *Biochemistry* **25**, 1124-1133
- Schuetz, E., Li, D., Omiecinski, C., Muller-Eberhard, U., Kleinman, H., Elswick, B. & Guzelian, P. (1988) *J. Cell Physiol.* **134**, 309-323
- Sinclair, J., Sinclair, P. & Bonkovsky, H. (1979) *Biochem. Biophys. Res. Commun.* **86**, 710-717

- Sinclair, J., Sinclair, P., Smith, E., Bement, W., Pomeroy, J. & Bonkovsky, H. (1981) *Biochem. Pharmacol.* **30**, 2805–2809
- Sinclair, J., Smith, E., Bement, W., Sinclair, P. & Bonkovsky, H. (1982) *Biochem. Pharmacol.* **31**, 2811–2815
- Sinclair, J., Wiebkin, P., Zaitlin, L., Smith, E. & Sinclair, P. (1984) *Biochem. Pharmacol.* **33**, 187–190
- Sinclair, J., Cornell, N., Zaitlin, L. & Hansch, C. (1986) *Biochem. Pharmacol.* **35**, 707–710
- Sinclair, J., Wood, S., Smith, L., Sinclair, P. & Koop, D. (1989) *Biochem. Pharmacol.*, in the press
- Sinclair, P., Bement J., Bonkovsky, H. & Sinclair, J. (1984) *Biochem. J.* **222**, 737–748
- Sinclair, P., Frezza, J., Sinclair, J., Bement, J., Healey, J. & Bonkovsky, H. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 1663
- Sinclair, P., Lambrecht, R. & Sinclair, J. (1987) *Biochem. Biophys. Res. Commun.* **146**, 1324–1329
- Steward, A., Dannan, G., Guzelian, P. & Guengerich, F. (1985) *Mol. Pharmacol.* **27**, 125–132
- Sundstrom, S., Sinclair, J., Smith, E. & Sinclair, P. (1988) *Biochem. Pharmacol.* **37**, 1003–1008
- Thomas, P., Reik, L., Ryan, D. & Levin, W. (1983) *J. Biol. Chem.* **258**, 4590–4598
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Wrighton, S., Campanile, C., Thomas, P., Maines, S., Watkins, P., Parker, G., Mendez-Picon, G., Haniu, M., Shively, J., Levin, W. & Guzelian, P. (1986) *Mol. Pharmacol.* **29**, 405–410
- Yoshioka, H., Miyata, T. & Omura, T. (1984) *J. Biochem. (Tokyo)* **95**, 937–947

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