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COMPARISON OF THE FORM(S) OF CYTOCHROME P-450 INDUCED BY ETHANOL AND GLUTETHIMIDE IN CULTURED CHICK HEPATOCYTES

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Abstract—In this study, using a combination of immunological and enzymatic characterizations, we compared the forms of cytochrome P-450 induced by ethanol and glutethimide in primary cultures of chicken embryo hepatocytes. Recently we purified a cytochrome P-450 of 50K molecular weight from chicken embryo liver using glutethimide as a prototypic inducer. Antibodies to both this chicken cytochrome P-450 and to rabbit cytochrome P-450 form 3a from the IIE subfamily detected microsomal proteins of 50K induced by either ethanol or glutethimide in cultured chick embryo hepatocytes, indicating the antigenic homology of these subfamilies of cytochromes P-450 among different animal species. However, the antibody to glutethimide-induced chick cytochrome P-450 of 50K inhibited *p*-nitrophenol hydroxylase and benzphetamine demethylase activities 85–90% in microsomes from both ethanol- and glutethimide-treated cells, indicating similar epitopes whose integrity is required for catalytic activity. In contrast, antibodies to rabbit cytochrome P-450 form 3a had little to no effect on these same microsomal activities. Both ethanol and glutethimide induced microsomal *p*-nitrophenol and aniline hydroxylase activities in cultured chick embryo hepatocytes. In microsomes from ethanol-treated cells, the turnover of *p*-nitrophenol per cytochrome P-450 was 2-fold greater than that induced by glutethimide treatment, suggesting that ethanol is inducing a form of cytochrome P-450 that has greater catalytic activity with this substrate than glutethimide-induced forms. Thus, in cultured chick embryo hepatocytes, ethanol may induce cytochromes P-450 from both the IIB and IIE subfamilies.

Cytochrome P-450 comprises a family of hemoproteins that are involved in the oxidative metabolism of numerous endogenous and exogenous chemicals. Several forms of cytochrome P-450 have been identified in experimental animals and have been categorized into different families largely according to a combination of their amino acid and gene sequences (for review see Refs 1–4). Hepatic cytochromes P-450 have been classified into four different families [2–4]. Ethanol and phenobarbital are prototypic inducers of different forms of cytochrome P-450 that are classified into the same family (II), but different subfamilies, designated IIE and IIB respectively [2–4]. Cytochromes P-450 in the subfamily IIE have up to 50% amino acid sequence homology with cytochromes P-450 in the IIB subfamily [5], but have greater catalytic activity towards *p*-nitrophenol [6, 7], aniline, dimethylnitrosamine, and alcohols than cytochromes P-450 from the IIB subfamily (for review see Ref. 8).

The specific response to inducers of cytochrome $P\text{-}450_{IIE}$ differs depending on the experimental animal and the inducer used. In rats [9–14], as in hamsters [15] and mice [16], ethanol-mediated induction of cytochrome P-450 in the liver results in an increase in the content of microsomal cytochrome P-450, as measured spectrophotometrically. In contrast, in rabbits, ethanol-mediated increases in microsomal cytochrome P-450 cannot be detected by spectrophotometric measurement [17]. Cytochromes P-450 of the IIE subfamily, purified from two different animal species, rat (P-450j) and rabbit (P-450 form 3a), are identical in their catalytic activities and exhibit 80% homology in their primary structure [18].

Ethanol induces microsomal hexobarbital hydroxylation activity in rat liver [19], yet purified rat cytochrome P-450 from the IIE subfamily has no catalytic activity with hexobarbital as a substrate [18], suggesting that ethanol may induce another form of cytochrome P-450 in rats, responsible for the increased hexobarbital hydroxylase activity. Recently, three prototypic inducers of cytochrome P-450j from the IIE subfamily, acetone, isoniazid, and 3-methylpyrazole, as well as ethanol, were reported to also increase cytochrome P-450 from the IIB subfamily [20, 21].

We have reported previously that ethanol causes a phenobarbital-like induction of cytochrome P-450 in primary cultures of chicken embryo hepatocytes [22–24]. This conclusion was based on enzymatic activities measured in broken cell preparations [22] as well as metabolism of substrates by intact cells [23]. We have purified a major protein of 50,000

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molecular weight from 17-day-old chicken embryos treated with glutethimide and have demonstrated by immunoblotting that ethanol increases a protein of similar antigenicity and molecular weight in cultured chick embryo hepatocytes [24]. In the present study, we compared by enzymatic and immunological techniques the forms of cytochrome P-450 induced by ethanol and glutethimide in cultured chick embryo hepatocytes.

METHODS

Preparation and treatment of the cultured hepatocytes

Primary cultures of chicken embryo hepatocytes were prepared in Williams E medium containing insulin, dexamethasone and 3,3',5-triiodothyronine, as described previously [25]. After 20 hr in culture, the medium was changed, with 20 mM HEPES* included and insulin deleted from the fresh medium. GLUT or ethanol was added directly after the medium change. GLUT was dissolved in dimethyl sulfoxide (DMSO) and added to the medium to final amounts of 10 μg GLUT/ml and 2 μl DMSO/ml. Cultured hepatocytes were exposed to GLUT for 24 hr, a time period determined previously to cause maximal induction of cytochrome P-450 in these cells (J. Sinclair, P. Sinclair, W. Bement, and S. Shedlofsky, unpublished results). Cells were exposed to ethanol at a final concentration of 200 mM for 48 hr, as described previously [22].

Treatment of intact chicken embryos

Fifteen-day-old chicken embryos were either untreated or exposed to GLUT at 6 mg in 0.1 ml DMSO per egg for 48 hr (injected into the fluid surrounding the embryo).

SDS polyacrylamide gel electrophoresis and immunoblots

Microsomes were analyzed by a discontinuous SDS polyacrylamide electrophoresis system in a Biorad Minigel apparatus, using a 10% separating gel and a 4% stacking gel. Samples were electrophoresed at 200 V for 1 hr to separate the proteins. Gels were then either stained with Coomassie blue to visualize the proteins, or the proteins were transferred to nitrocellulose sheets at 100 V for 1 hr and immunoblotted by a modification of the procedure of Towbin *et al.* described in Ref. 24. The nitrocellulose sheets containing the transferred microsomal proteins were rinsed in TBS for 5 min, blocked in 5% powdered non-fat milk in TBS containing 0.5% Tween 20 for 1 hr at room temperature (25°), rerinsed in TBS for 5 min, and then incubated with a 1:1000 dilution of serum containing antibodies to either chick cytochrome P-450$_{GLUT50K}$ (rabbit sera) or to rabbit cytochrome P-450 form 3a (sheep sera).

After 1 hr at room temperature (25°), the sheets were washed three times in TBS, and the rabbit sera-blotted sheet was incubated with goat anti-rabbit IgG (1:2500 dilution) and the sheep sera-blotted sheet was incubated with donkey anti-sheep IgG (1:1000 dilution). Both IgG preparations were conjugated with alkaline phosphatase, and the color developed with a Biorad BCIP/NBT solution.

Assays

Microsomes were prepared from homogenates of livers and cultured hepatocytes as described previously [26], overlaid with 0.1 M sodium phosphate buffer, pH 7.4, and stored at −20°. Microsomes could be stored for several weeks with no loss of PNPH, aniline hydroxylase, or BPHET activity.

Proteins were measured by the procedure of Lowry *et al.* [27] using BSA as a standard.

Microsomal cytochrome P-450 was measured spectrophotometrically, as described previously [26].

Microsomal PNPH activity was measured by the following modification of the procedure used by Reinke and Moyer [6]: the reaction mixture contained 0.5 mg microsomal protein, unless indicated otherwise, in a final volume of 0.5 ml containing 0.05 M Tris–Cl, pH 7.4, 1 mM ascorbate, 5 mM $MgCl_2$, 0.8 mM $NADP^+$, 0.10 units isocitrate dehydrogenase, 30 mM isocitrate, and 0.4 mM *p*-nitrophenol, unless indicated otherwise. The reaction was initiated by addition of the complete cofactor mix containing *p*-nitrophenol. A blank for each reaction contained an equivalent amount of denatured microsomes from untreated embryos, incubated with the complete cofactor mix. The absorption of the product, 4-nitrocatechol, was scanned between 500 and 700 nm and an E_{mM} (Δ 525 − 675 nm) of 4-nitrocatechol was determined to be 11.2 under these conditions. Ascorbate was included during the reaction to stabilize the catechol as it was generated. Omission of ascorbate led to a 40% loss of product (results not shown).

Aniline hydroxylase was measured in the same buffer system as that for PNPH, except that ascorbate was omitted from the reaction mixture. The reaction was initiated by the addition of microsomes and incubation was for 30 min at 37°. The product was measured as described by McCoy [28]. The reaction was measured initially over a substrate concentration range of 0.025 to 20.0 mM aniline, and an increase in reaction rate as a function of substrate concentration was found to be biphasic, confirming the findings of Burnet and Darby [29] for chicken embryo liver. Forms I and II of aniline hydroxylase [28] were determined by measuring the activities at 0.09 and 20 mM aniline, two concentrations which, by the Michaelis–Menten plot, corresponded to separate phases of the reaction in microsomes from untreated embryos (results not shown). BPHET activity was measured as described previously [22], using 0.1 to 0.2 mg of microsomal protein per reaction volume of 0.14 ml.

Antibody inhibition of enzyme activity

PNPH. Microsomes were preincubated in Tris buffer with preimmune IgG alone or with a combination of preimmune and immune IgG at a con-

* Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; GLUT, glutethimide; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; BPHET, benzphetamine demethylase; PNPH, *p*-nitrophenol hydroxylase; BSA, bovine serum albumin; BCIP, 5-bromo-, 4-chloro-, 3-indole toluidine phosphate; NBT, *p*-nitrobluetetrazolium chloride; cytochrome P-450$_{GLUT50K}$, a major cytochrome P-450 of 50K molecular weight purified from the livers of glutethimide-treated chick embryos.

stant amount of IgG of 4 mg per reaction, in the presence of substrate, for 3 min at room temperature. The reaction was then initiated by the addition of cofactor mix. An additional control was run without IgG and with no preincubation. The preimmune sera were not inhibitory for either BPHET or PNPH.

BPHET. Microsomes were preincubated with cofactor mix and the reaction was initiated by the addition of substrate.

Source of chemicals

The sources of all chemicals used in this study have been described previously [22] except for the following chemicals: aniline, glutethimide, ascorbate, *p*-nitrophenol, 4-nitrocatechol, and alkaline phosphatase-conjugated anti-sheep IgG and anti-rabbit IgG were purchased from the Sigma Chemical Co. (St. Louis, MO); nitrocellulose sheets, BCIP, and NBT were purchased from Bio-Rad (Richmond, CA); and benzphetamine was a gift from Upjohn Pharmaceuticals (Kalamazoo, MI).

RESULTS

SDS polyacrylamide gel electrophoresis of hepatic microsomes

In cultured chick hepatocytes, ethanol and GLUT both induced two microsomal proteins of 50K and 53K (Fig. 1A). Although GLUT-mediated induction of the 53K protein was apparent on these gels, the ethanol-mediated induction was not, since the lane containing the ethanol sample was wider than that of the control microsomes. An ethanol-mediated increase in this protein was more dramatic in other experiments (results not shown).

Only the proteins of 50K cross-reacted with antibody prepared against a cytochrome P-450 of 50K purified from the livers of GLUT-treated embryos (Fig. 1, A and B, lanes 1–6). Both ethanol and GLUT increased the amount of immunoreactive 50K protein compared to untreated hepatocytes (Fig. 1B, lanes 2, 3 and 5), as has been found previously [24].

Antibody to rabbit cytochrome P-450 form 3a from the IIE subfamily cross-reacted with the chick embryo cytochrome P-450 of 50K purified from the livers of GLUT-treated embryos, indicating the presence of common antigenic sites between the rabbit and chick proteins (Fig. 1C). This antibody also cross-reacted with proteins of 50K in microsomes from cultured hepatocytes exposed to either ethanol or GLUT. In contrast, in microsomes from untreated hepatocytes, both from culture and embryo, there was no cross-reactivity detected at the 50K positions, using the antibody to rabbit cytochrome P-450 form 3a. Two proteins of 53K and 57K were also detected in all microsomal samples with this antibody; however, these proteins were not increased by treatment with ethanol or GLUT.

Enzymatic characterization of ethanol-induced cytochrome P-450 in chick hepatocytes

We measured two enzyme activities used to detect induction of cytochrome P-450 from the IIE subfamily in experimental animals, *p*-nitrophenol hydroxylase [6, 7] and aniline hydroxylase [28], and one activity, benzphetamine demethylase, previously found to be more specific for phenobarbital- than 20-methylcholanthrene-induced forms of cytochrome P-450 in these cells [22].

p-Nitrophenol hydroxylase

The rate of formation of *p*-nitrocatechol was linear for 40 min with protein concentrations up to 1 mg per reaction (results not shown). Ninety percent of exogenous *p*-nitrocatechol added to these incubation mixtures was recovered at the end of the reaction, indicating no additional metabolism of the product.

The effect of increasing substrate concentration on hepatic microsomal PNPH activity was examined in both chick embryo and in cultured chick hepatocytes treated with different inducers of cytochrome P-450 (Figs 2 and 3). In all samples the plot of the rate of product formation with increasing substrate concentration was hyperbolic. These results contrast to findings with ethanol-treated rabbits, in which the microsomal activity decreases with substrate concentrations greater than 100 μM [7]. The reaction rate with microsomes from chick hepatocytes was identical at pH 6.8 and 7.4, unlike the pH optimum of 6.8 found in rabbit microsomes [7] (results not shown).

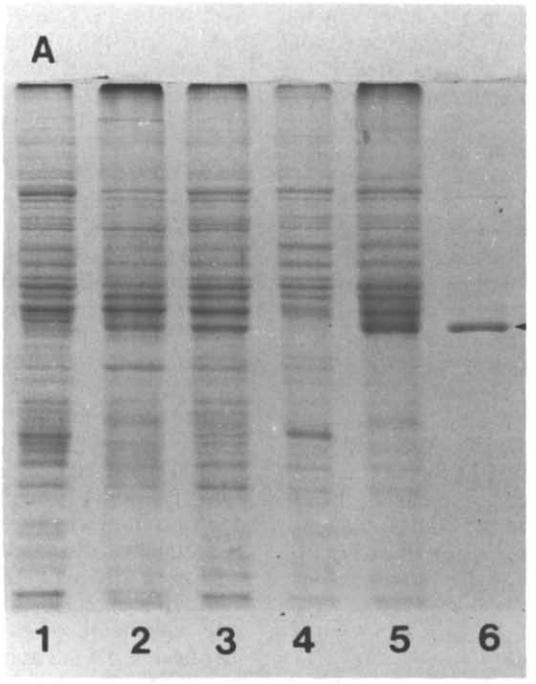
The kinetic parameters were determined from a double-reciprocal plot. The K_m values of the microsomal reaction were similar for the different treatments (0.12 ± 0.02 mM for five separate treatments). In contrast to the K_m values, the V_{max} values as nanomoles per minute per milligram protein, were increased approximately 7- and 3-fold by GLUT in both the embryo (untreated, 0.14; GLUT-treated, 0.99) and the cultured hepatocytes (untreated, 0.12; GLUT-treated, 0.4), respectively, and 2-fold by ethanol in the cultured hepatocytes (ethanol-treated, 0.23).

The turnover of *p*-nitrophenol per cytochrome P-450 molecule in the untreated embryo increased, upon culturing the hepatocytes, from 0.4 to 1.15 ($P < 0.005$, Table 1). Treatment of the cells with ethanol did not increase the turnover compared to untreated cells (1.16 vs 1.15). Even though GLUT increased the amount of PNPH per mg protein in both the embryo and the cultured hepatocytes, the turnover of *p*-nitrophenol per molecule of cytochrome P-450 was less than that in microsomes from untreated or ethanol-treated hepatocytes.

Unlike PNPH activity, the turnover of aniline hydroxylase activity per nmole cytochrome P-450, at both low and high substrate concentrations, was identical in the cultured hepatocytes regardless of the treatment, indicating that this assay could not be used to identify an ethanol-specific form of cytochrome P-450 in the chick hepatocytes (data not shown).

Benzphetamine demethylase

GLUT and ethanol both induced BPHET in the cultured hepatocytes. The turnover of substrate per cytochrome P-450 in microsomes from ethanol-treated cells was significantly greater than in microsomes from both untreated and GLUT-treated hepatocytes (Table 1).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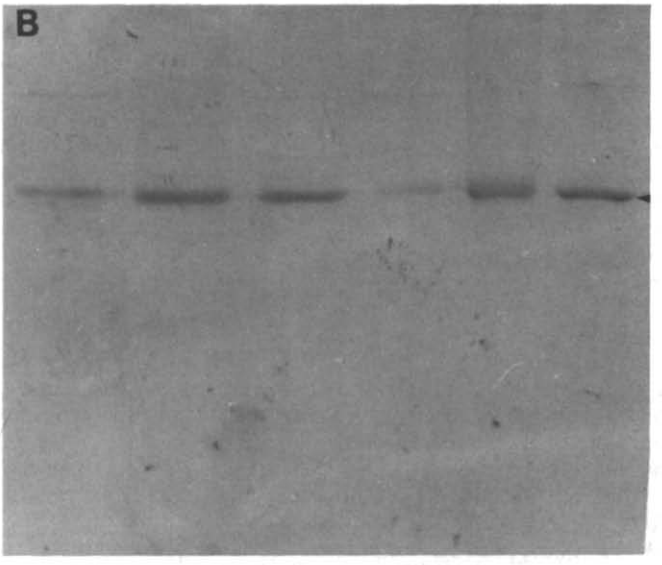
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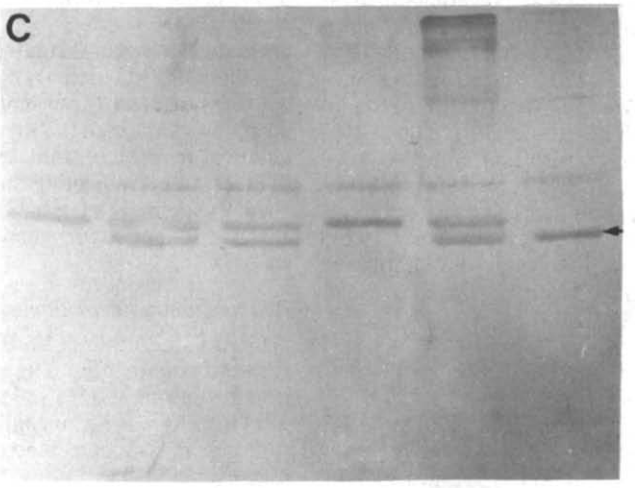
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Fig. 1. Characterization of basal and induced microsomal proteins on SDS polyacrylamide gels and immunoblots. Microsomes were electrophoresed on a Biorad minigel apparatus, using a 10% separating gel and a 4% stacking gel. The electrophoresis, transfer to nitrocellulose and staining techniques are described in Methods. The amount of microsomal protein applied to each well was 10 μg. Culture microsomes: (lane 1) control; (lane 2) ethanol; (lane 3) glutethimide. Embryo microsomes: (lane 4) control; (lane 5) glutethimide; and (lane 6) cytochrome P-$450_{GLUT50K}$, 0.5 μg. (A) Coomassie blue stain; (B) immunoblot with anti-cytochrome P-$450_{GLUT50K}$; (C) immunoblot with anti-rabbit cytochrome P-450 form 3a. The arrow head indicates the protein of 50K. The molecular weight markers used for this determination were: BSA, 66K; catalase, 57K; fumarase, 49K; aldolase, 40K; and lactic dehydrogenase, 36K.

Antibody inhibition of microsomal mixed-function oxidase activities

In microsomes from ethanol-treated hepatocytes, antibody to cytochrome P-$450_{GLUT50K}$ inhibited PNPH activity 90% and BPHET activity 80% (Fig. 4). Similar inhibitions were obtained in microsomes from GLUT-treated hepatocytes. In contrast, in microsomes from untreated hepatocytes, maximum inhibition of PNPH was only 50% and BPHET 40% with this antibody.

In contrast to the results obtained with antibody to chick cytochrome P-$450_{GLUT50K}$, antibody to rabbit cytochrome P-450 form 3a from the IIE subfamily caused little to no inhibition of microsomal PNPH or BPHET from the cultured cells regardless of the treatment (results not shown).

DISCUSSION

We have reported previously that ethanol induces a cytochrome P-450 from the IIB family in primary cultures of chicken embryo hepatocytes [22–24]. These conclusions were based on the spectral and enzymatic characterization [22, 23]. In the cultured chick hepatocytes, both ethanol and GLUT increased two microsomal proteins of 50K and 53K molecular weights. Recently, we purified a cytochrome P-450 of 50K molecular weight from the livers of chicken embryos treated with GLUT [24], that may be identical to a cytochrome P-450 of 50K purified by May and coworkers from phenobarbital-treated chick embryos [30]. Antibodies prepared against the GLUT-induced 50K protein cross-reacted with the microsomal protein of 50K molecular weight in the cultured chick hepatocytes. The amount of this immunoreactive protein increased after treatment of the cultured hepatocytes with either ethanol or GLUT, suggesting that this region of the gel contains a cytochrome P-450 that is induced by ethanol or GLUT (Fig. 1B). Furthermore, antibodies to cytochrome P-$450_{GLUT50K}$ almost completely inhibited PNPH and BPHET activities in microsomes from hepatocytes treated with ethanol or GLUT (Fig. 4), suggesting that ethanol- and GLUT-induced forms of cytochrome P-450 in these cells have similar epitopes whose integrity is required for catalytic activity. Although the antibody inhibition suggests that both ethanol- and GLUT-induced forms of cytochrome P-450 are identical, this antibody preparation may not be able to distinguish separate forms of P-450 induced by ethanol and GLUT.

Antibody against rabbit cytochrome P-450 form 3a from the IIE subfamily detected a microsomal protein of 50K from ethanol- and GLUT-treated cells (Fig. 1C), suggesting that this antibody recognizes a chick cytochrome P-450 from the II family. However, ethanol-induced PNPH and BPHET activities were not inhibited by this antibody (results not shown), making it difficult to ascertain whether

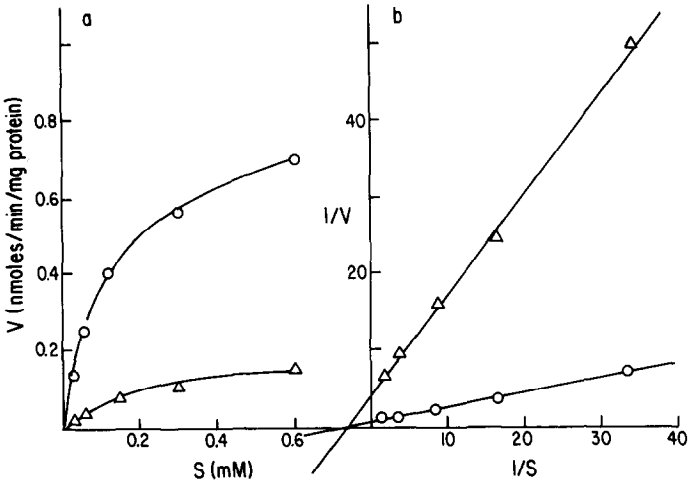

Fig. 2. Kinetics of PNPH in microsomes from chick embryo liver. Microsomes from either untreated (△) or GLUT-treated (○) embryos were analyzed for PNPH as described in Methods, using increasing substrate concentrations and 1 mg microsomal protein per 40-min reaction. Each symbol represents the average of duplicate determinations on the same microsomal preparation, with the range falling within the symbol. (a) Michaelis–Menten plot; (b) Lineweaver–Burk plot.

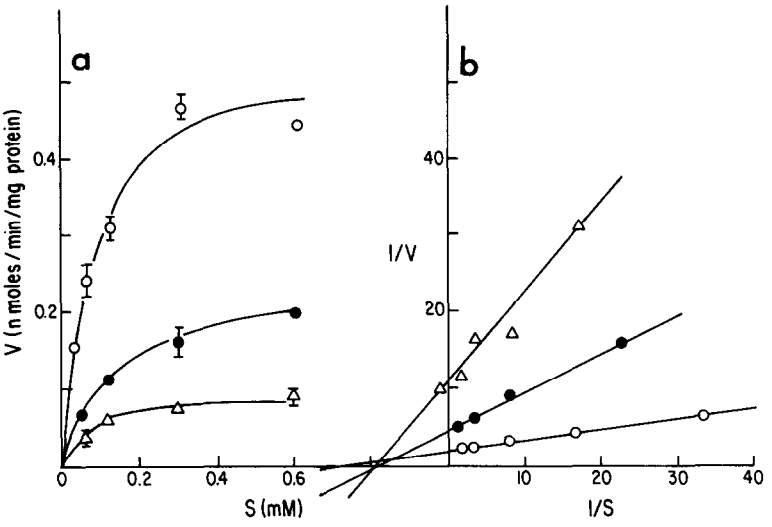

Fig. 3. Kinetics of PNPH in microsomes from cultured hepatocytes. Cells were either untreated (△), or exposed to ethanol (●) or GLUT (○) as described in Methods. Microsomes were prepared and analyzed for PNPH as described in Methods, using increasing concentrations of substrate and 1 mg protein per 40-min reaction. Each symbol represents the average of duplicate determinations of the same microsomal preparation with the range indicated by the vertical bar. (a) Michaelis–Menten plot; (b) Lineweaver–Burk plot.

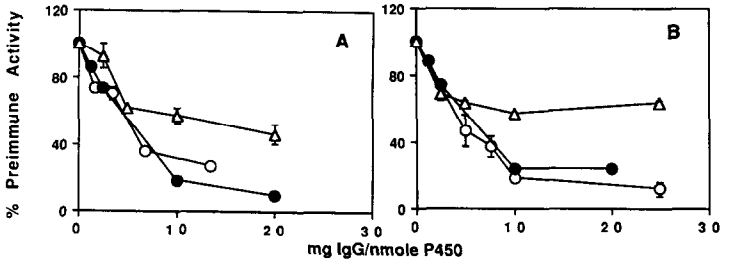

Fig. 4. Antibody inhibition of microsomal PNPH and BPHET: Anti-chick cytochrome P-450$_{GLUT50K}$. IgG prepared to cytochrome P-450$_{GLUT50K}$ was tested for inhibition of PNPH (A) and BPHET (B) activity, as described in Methods. Microsomes were from untreated (△), ethanol (●) and GLUT (○) treated cells. Each symbol represents the average of duplicate measurements on the same microsomal preparation with the range falling within the symbol, unless indicated otherwise.

Table 1. Comparison of mixed-function oxidase activities induced by ethanol and glutethimide in chick hepatocytes

	p-Nitrophenol hydroxylase			Benzphetamine demethylase		
		nmol per min per:			nmol per min per:	
Treatment	N	mg protein	nmol cytochrome P-450	N	mg protein	nmol cytochrome P-450
Cultured cells						
None	7	0.11 ± 0.03	1.15 ± 0.21	3	0.34 ± 0.05	2.73 ± 0.51
Ethanol	5	0.20 ± 0.04*	1.16 ± 0.38	3	1.00 ± 0.01*	4.61 ± 0.03*
GLUT	5	0.34 ± 0.12*	0.61 ± 0.11*†	3	2.32 ± 0.34*‡	3.41 ± 0.20*‡
Embryo						
None	9	0.10 ± 0.02	0.40 ± 0.09§	5	0.58 ± 0.16†§	2.50 ± 0.81‡
GLUT	6	0.64 ± 0.10*§	0.29 ± 0.03§‖	8	4.48 ± 2.39*§	2.40 ± 1.24‡

Cells and embryos were treated as described in Methods. PNPH and BPHET were analyzed in microsomes, at saturating substrate concentrations, 0.4 and 3 mM, respectively, using the assay procedures described in Methods. Each value represents the mean ± SD; N = number of separate experiments with separate batches of embryos. Statistical significance was assessed by Student's *t*-test.

* $P < 0.005$ compared to untreated hepatocytes.
† $P \leq 0.02$ compared to ethanol-treated hepatocytes.
‡ $P < 0.005$ compared to ethanol-treated hepatocytes.
§ $P \leq 0.005$ compared to cultured cells, regardless of treatment.
‖ $P \leq 0.02$ compared to untreated embryo.

the antibody to the rabbit form is reacting specifically with a chick cytochrome P-450 on the immunoblot. This antibody detected two additional chick microsomal proteins of 53K and 57K (Fig. 1C). It is uncertain whether these proteins are cytochromes P-450, since their amounts were not altered by any of the treatments. However, the 53K protein is probably a form of cytochrome P-450, since polyclonal antibody to rat cytochrome P-450b/e provided by Dr. D. Waxman also detected this protein. In addition, we have recently separated a form of cytochrome P-450 of 53K by preparative HPLC analysis of microsomes from GLUT-treated embryos that is also detected with the antibody to rat cytochrome P-450b/e (results not shown).

Hydroxylations of *p*-nitrophenol and aniline have proven relatively specific for forms of cytochrome P-450 in the IIE subfamily [6–8]. Using these assays as probes, we investigated whether ethanol also increases a cytochrome P-450 from the IIE subfamily in the cultured chicken hepatocytes. Both ethanol and GLUT induced PNPH activity in these cells (Table 1). In microsomes from ethanol-treated cells, the turnover number for *p*-nitrophenol was greater than that detected with GLUT treatment, although the difference in substrate turnover was less than that observed in rats [6] and rabbits [7] treated with ethanol versus phenobarbital-like inducers of cytochrome P-450. Ethanol and ethanol-like inducers of cytochrome P-450 have been found recently to induce forms of cytochrome P-450 from both the IIB and IIE subfamilies in rats [20, 21]. Perhaps, in cultured chick hepatocytes, ethanol may be inducing two different forms as well, but the form responsible for the greater PNPH turnover may be a minor component of the total induced cytochrome P-450.

Although ethanol increased the specific activity of PNPH in the cultured hepatocytes, the turnover number of substrate was similar to that measured in microsomes from untreated cells (Table 1). In rats, cytochrome P-450j is not present in the embryo but appears shortly after birth, and increases dramatically within the first 3 weeks [20, 31]. In the chicken embryo, although microsomal PNPH activity was present in the embryo, the turnover of PNPH increased merely by culturing the hepatocytes (Table 1). This increase may be due to the expression of cytochrome P-450 from the IIE subfamily that is triggered by culturing the cells, a suggestion consistent with the expression of other adult functions in chick embryo hepatocytes in culture that are not present in the embryonic state, such as UDP-glucuronyl transferase [32], hemopexin [33], and adult plasma proteins [34].

In summary, in cultured chick hepatocytes, ethanol- and GLUT-induced forms of cytochrome P-450 appear to be similar, as shown by immunological studies. However, the enzymatic data suggest that ethanol increased a form of cytochrome P-450 that is either absent or present in lower proportions in GLUT-treated hepatocytes. Efforts are being undertaken to isolate the forms of hepatic cytochrome P-450 induced by ethanol in chickens.

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REFERENCES

1. Black SD and Coon MJ, P-450 Cytochromes: Structure and function. *Adv Enzomol* **60**: 35–87, 1987.
2. Adesnik M and Atchison M, Genes for cytochrome P-450 and their regulation. *CRC Crit Rev Biochem* **19**: 247–305, 1986.
3. Nebert D and Gonzalez F, P-450 Genes: Structure, evolution and regulation. *Annu Rev Biochem* **56**: 945–993, 1987.
4. Nebert D, Adesnik M, Coon M, Estabrook R, Gonzalez F, Guengerich P, Gunsalus I, Johnson E, Kemper B, Levin W, Phillips I, Sato R and Waterman M, The P-450 gene superfamily: Recommended nomenclature. *DNA* **6**: 1–14, 1987.
5. Khani SC, Zaphiropoulos PG, Fujita VS, Porter TD, Koop DR and Coon MJ, cDNA and derived amino acid sequence of ethanol-inducible rabbit liver cytochrome P-450 isozyme 3a ($P\text{-}450_{ALC}$). *Proc Natl Acad Sci USA* **84**: 638–642, 1987.
6. Reinke L and Moyer M, *p*-Nitrophenol hydroxylation: A microsomal oxidation which is highly inducible by ethanol. *Drug Metab Dispos* **13**: 548–552, 1985.
7. Koop DR, Hydroxylation of *p*-nitrophenol by rabbit ethanol-inducible cytochrome P-450 isozyme 3a. *Mol Pharmacol* **29**: 399–404, 1986.
8. Koop D and Coon M, Ethanol oxidation and toxicity: role of alcohol P-450 oxygenase. *Alcoholism Clin Exp Res* **10**: 44S–49S, 1987.
9. Plourde V, Hetu C and Joly J-G, Aniline hydroxylation and 7-ethoxycoumarin-*O*-deethylation activities in solubilized and partially resolved cytochromes P-450 from ethanol-fed rats. *Can J Physiol Pharmacol* **62**: 266–271, 1984.
10. Hetu C and Joly J, Differences in the duration of the enhancement of liver mixed function oxidase activities in ethanol-fed rats after withdrawal. *Biochem Pharmacol* **34**: 211–1216, 1986.
11. Joly J-G, Ishii H, Teschke R, Hasumura Y and Lieber CS, Effect of chronic ethanol feeding on the activities and submicrosomal distribution of NADPH cytochrome P-450 reductase and the demethylases for aminopyrine and ethylmorphine. *Biochem Pharmacol* **22**: 1532–1535, 1973.
12. Comai K and Gaylor JL, Existence and separation of three forms of cytochrome P-450 from rat liver microsomes. *J Biol Chem* **248**: 4947–4955, 1973.
13. Joly JG, Villeneuve JR and Mavier P, Chronic ethanol administration induces a form of cytochrome P-450 with specific spectral and catalytic activities. *Alcoholism* **1**: 17–19, 1977.
14. Morgan E, Devine M and Skett P, Changes in the rat hepatic mixed-function oxidase system associated with chronic ethanol vapor inhalation. *Biochem Pharmacol* **30**: 595–600, 1981.
15. McCoy G, Influence of chronic ethanol consumption on hamster liver microsomal *O*-dealkylase activities and cytochrome b_5 content. *Biochem Pharmacol* **34**: 4263–4267, 1985.
16. Schnellmann RG, Wiersma DA, Randall DJ, Smith TL and Sipes IG, Hepatic mixed function oxygenase activity and glutathione-*S*-transferase activity in mice following ethanol consumption and withdrawal. *Toxicology* **32**: 105–116, 1984.
17. Koop D, Morgan E, Tarr G and Coon M, Purification and characterization of a unique isozyme of cytochrome P-450 from liver microsomes of ethanol-treated rabbits. *J Biol Chem* **257**: 8472–8480, 1982.
18. Ryan D, Koop D, Thomas D, Coon M and Levin W, Evidence that isoniazid and ethanol induce the same

microsomal cytochrome P-450 in rat liver, an isozyme homologous to rabbit liver cytochrome P-450 isozyme 3a. *Arch Biochem Biophys* **246**: 633–644, 1986.
19. Mallov S and Baesl T, Effect of ethanol on rates of elimination and metabolism of zoxazolamine, hexobarbital, and warfarin sodium in the rat. *Biochem Pharmacol* **21**: 1667–1678, 1972.
20. Song B-J, Gelboin HV, Park S-S, Yang CS and Gonzalez FJ, Complementary DNA and protein sequences of ethanol-inducible rat and human cytochrome P-450s: Transcriptional and post-transcriptional regulation of the rat enzyme. *J Biol Chem* **261**: 16689–16697, 1987.
21. Johansson I, Ekstrom G, Scholte B, Puzycki D, Jornvall H and Ingelman-Sundberg M, Ethanol, fasting, and acetone inducible cytochromes P-450 in rat liver: Regulation and characterization of enzymes belonging to the IIB and IIE gene families. *Biochemistry* **27**: 1925–1934, 1988.
22. Sinclair JF, Sinclair PR, Smith EL, Bement WJ, Pomeroy J and Bonkowsky H, Ethanol-mediated increase in cytochrome P-450 in cultured hepatocytes. *Biochem Pharmacol* **30**: 2805–2809, 1981.
23. Sinclair J, Wiebkin P, Zaitlin L, Smith E and Sinclair P, Aminopyrine and biphenyl metabolism in cultured hepatocytes: induction by alcohols. *Biochem Pharmacol* **33**: 187–190, 1984.
24. Sinclair P, Frezza J, Sinclair J, Bement J, Healey J and Bonkovsky H, Immunochemical detection of induction of different isozymes of cytochrome P-450 in chick hepatocyte cultures. *Fed Proc* **45**: 1663, 1986.
25. Sinclair JF, Smith L, Bement WJ, Sinclair PR and Bonkovsky HL, Increases in cytochrome P-450 in cultured hepatocytes mediated by 3- and 4-carbon alcohols. *Biochem Pharmacol* **31**: 2811–2815, 1982.
26. Sinclair JF, Sinclair PR and Bonkowsky HL, Hormonal requirements for the induction of cytochrome P450 in hepatocytes cultured in a serum-free medium. *Biochem Biophys Res Commun* **86**: 710–717, 1979.
27. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
28. McCoy G, Differential effects of ethanol and other inducers of drug metabolism on the two forms of hamster liver aniline hydroxylase. *Biochem Pharmacol* **29**: 685–688, 1985.
29. Burnet F and Darby N, Ethanol induces an aniline hydroxylase in chick embryo liver without inducing cytochrome P-450. *Biochem Soc Trans* **12**: 686, 1984.
30. Brooker JD, Srivastava G, Borthwick IA, May BK and Elliott WH, Evidence that 2-allyl-2-isopropylacetamide, phenobarbital, and 3,5-diethoxycarbonyl-1,14-dihydrocollidine induce the same cytochrome P_{450} mRNA in chick embryo liver. *Eur J Biochem* **136**: 327–332, 1983.
31. Thomas P, Bandiera S, Maines S, Ryan D and Levin W, Regulation of cytochrome P-450j, a high affinity *N*-nitrosodimethylamine demethylase, in rat liver microsomes. *Biochemistry* **26**: 2280–2289, 1987.
32. Dutton G and Leakey J, The perinatal development of drug-metabolizing enzymes: What factors trigger their onset. *Prog Drug Res* **25**: 189–273, 1981.
33. Greininger G, Liang T, Beauring G, Goldfarb V, Metcalf S and Muller-Eberhard U, Hemopexin is a developmentally regulated, acute phase plasma protein in the chicken. *J Biol Chem* **261**: 15719–15724, 1986.
34. Grieninger G, Hertzberg K and Pindyck J, Fibrinogen synthesis in serum-free hepatocyte cultures: Stimulation by glucocorticoids. *Proc Natl Acad Sci USA* **11**: 5506–5510, 1978.