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Development of basal and induced testosterone hydroxylase activity in the chicken embryo *in ovo*

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1 The sensitivity of the developing embryo to xenobiotics is highly dependent on the expression of metabolizing enzymes including cytochromes P450 (CYP). In the present study, therefore, the ontogeny of the CYP-dependent system in the chick was investigated with testosterone hydroxylase activity as a marker of CYP expression.

2 Chicken embryo livers were assayed for basal and phenobarbitone (PB)-induced regio- and stereoselective testosterone hydroxylase activity, from the first appearance of the liver as a discrete organ at 5 days of incubation through day 10 posthatching. In addition, whole embryo preparations were assayed at 3 and 4 days of incubation.

3 Whereas testosterone 16β-hydroxylase and androst-4-ene-3,17-dione-linked activities were expressed during all stages of embryonic development, testosterone 6α-, 6β-, 7α- and 16α-hydroxylase activities were observed only in basal embryos from 8 days of incubation. Furthermore, testosterone 2α- and 2β-hydroxylase activities were detected exclusively from 10 days of incubation onward. All activities increased steadily throughout development as did the responsiveness of the embryonic liver to PB induction.

4 A typical pattern of development with a higher activity from 10 to 14 days of incubation (testosterone 16α-, 7α-, 6α- and 2β-hydroxylase activities; up to 4.1 ± 0.3 pmol mg^{-1} protein min^{-1} at 13 days of incubation for testosterone 7α-hydroxylase) or shifted to 14 to 18 days of incubation (testosterone 6β-, 2α- and 16β-hydroxylase activities: up to 56.6 ± 1.4 pmol mg^{-1} protein min^{-1} at 16 days of incubation for testosterone 6β-hydroxylase) was observed. There was a tendency towards an increased activity for all activities around hatching, specifically from 19 days of incubation to 4 days posthatching (up to $1{,}759.3 \pm 179.4$ pmol mg^{-1} protein min^{-1} at 1 day posthatching for androst-4-ene-3,17-dione-linked activity).

5 The highest level of PB-induced enzyme activity was observed for testosterone 2α-hydroxylase activity (95.14 ± 7.35 and 660.19 ± 45.27 pmol mg^{-1} protein min^{-1}) at 12 days of incubation and day 3 posthatching, respectively. Except for testosterone 2α- and 2β-hydroxylase activities at 3 to 4 days of incubation, all metabolites were detectable during the first period of organogenesis in the presence of PB.

6 The use of highly specific substrates, studies on the immunoinhibition of metabolism by polyclonal antibodies raised against highly purified rat CYPs, and the use of selective inhibitors seemed to reveal a wide pleiotropic response with the posssible presence in liver of PB-treated chickens of CYP1A together with CYP2H1/H2, CYP2E and CYP3A.

Keywords: Cytochrome P450; chicken embryo liver; testosterone hydroxylase; phenobarbitone induction

Introduction

The development of the cytochrome P450 (CYP) mono-oxygenase system can affect the sensitivity of a developing organism to toxins and drugs (Todd & Bloom, 1980; Juchau *et al*., 1985; Hamilton & Bloom, 1986; Lorr *et al*., 1989). CYP comprises a superfamily of haemoproteins that metabolize a large number of endogenous, as well as exogenous, chemicals giving rise to reactive intermediates or inactive water-soluble products through oxidative, reductive, peroxidative and desaturative mechanisms (Gonzalez, 1989; Guengerich, 1993). It is, thus, relevant to investigate CYP expression during embryonic and neonatal development when cells are growing rapidly and hence more vulnerable to toxins. Of particular importance is the study of the role of specific CYPs in mediating selective tissue injury and transformation during development (Eskola & Toivanen, 1974).

Fourteen families of CYPs are found in mammals (Nelson *et al*., 1996), eight of them contain a single enzyme that plays a critical role in biotransformation or synthesis of steroid hormones, fatty acids and cholesterol. Endogenous metabolism by these enzymes is usually considered crucial for physiological homeostasis and development. Based on differences in substrate specificity between species, genetic polymorphisms and the divergence of these enzymes during evolution, it was argued that CYP evolved to metabolize xenobiotics with a crucial selective role in neutralizing phytotoxins (Nelson & Strobel, 1987; Gonzalez & Nebert, 1990). The CYP family probably evolved from a common ancestor, based on a high sequence similarity between all species (Nebert & Gonzalez, 1987). Three CYP families (designated CYP1, CYP2 and CYP3) metabolize primarily foreign compounds. The CYP3 family (pregnenolone-16α-carbonitrile-(PCN)-inducible) is estimated to have diverged from pre-existing forms approximately 1,100 million years ago. The estimated divergence time for the major 3-methylcholanthrene-(MC)-(CYP1) and phenobarbitone-(CYP2)-inducible families is 800 million years ago

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(Thomas & Capecchi, 1987). Since the avian-mammal divergence was around 300 million years ago (Dickerson, 1971), the avian genome contains representative members of all three of these families of CYP genes (Sinclair & Sinclair, 1993). Thus, the chicken is a good species to study CYP evolution because of its evolutionary position between mammals and lower orders. Such an experimental model is widely used in the field of genetic toxicology, as the early chicken embryo is capable of activating a wide spectrum of xenobiotics to produce DNA damaging metabolites (Bloom, 1983; Brunstrom, 1986). In addition, this model can be a sensitive indicator of chemical toxicity and/or teratogenicity (Hamilton *et al*., 1983).

The chicken embryo and adult chickens are responsive to PB induction (Strittmeyer & Umberger, 1969) giving rise to a pleiotropic response (Gupta *et al*., 1990a). Full-length cDNA sequences have been isolated for two major CYP forms increased by PB-like inducers in chicken embryo livers. These enzymes are 93% similar in their amino acid sequence (Hobbs *et al*., 1986; Hansen & May, 1989) and are immunochemically related to CYP2B1/2B2 induced by PB in rats (Brooker & O'Connor, 1982). The chicken isoforms were named CYP2H1 and 2H2 (Hokama *et al*., 1988; Sinclair *et al*., 1990). More than one MC or β-naphthoflavone inducible CYP resembling the rodent forms (CYP1A1/1A2 and CYP2E1) have also been purified from chicken liver (Gupta *et al*., 1990b; Nakai *et al*., 1992).

Further investigations into the ontogeny of different CYP families are needed to define the metabolic role of CYP in mutagenicity and teratogenicity during development. Basal and PB induction of CYP and aminopyrine N-demethylase activity were found to be detectable in chicken liver by 7 days of incubation (Lorr & Bloom, 1987). However, the time-course of PB induction of CYPs in chicken liver throughout all embryonic stages of development has not been critically examined.

The aim of the present investigation was to study the expression pattern of hepatic CYP-associated catalytic activities in basal and PB-induced microsomes during embryonic and neonatal development, by use of the regioselectivity and stereoselectivity of testosterone as a polyfunctional substrate. *In vitro* experiments with specific substrates, as well as studies on the immunoinhibition of metabolism by polyclonal antibodies raised against purified rat CYP isozymes and the use of typical CYP inhibitors, were performed to characterize the PB-induced pleiotropic response.

Methods

Animals and treatments

Fertilized hen's eggs (White Leghorn, Cornell K-MFO line of chickens representing a subline of the Cornel K-strain) were purchased from a local breeder (Gagliardi E., Lugo, Ravenna, Italy). This line is very sensitive to MC and PB indution of CYP activities (Hamilton *et al*., 1983). Eggs were incubated at 37.5 ± 0.5°C and at 85% relative humidity and were rotated every 6 h. Experimental eggs were selected for fertility and normal embryonic development by candling (Hamilton *et al*., 1983). The first incubation day was called day 0 of embryonic incubation. The material for enzymatic analysis was collected from 3 to 20 days of incubation and through day 10 after hatching. A 1.5 mm diameter hole was made in the shell directly over the air cell and PB or vehicle (sterilized physiological saline) was injected through the inner shell membrane into the fluids surrounding the embryo (Spencer & Rifkind, 1990) with a Gilson micropipette fixed-volume (100 μl $\pm$ 1%). The hole was sealed with a 10 mm^2 piece of adhesive tape. The eggs were returned to the incubator and the embryos were assayed after 24 h, a period shown to be sufficient for CYP induction (Lorr & Bloom, 1987). Newly hatched chickens were kept in chicken brooders with heat lamps and provided with chicken feed and water *ad libitum*. Embryos and hatched chicks were injected with PB or vehicle at 50% of the LD_{50} calculated each day by the method of Litchfield & Wilcoxon (1987). This was necessary because of the different chemical susceptibility (i.e. different LD_{50}) during embryogenesis (Spencer & Rifkind, 1990). Representative doses were: embryos at 5 days of incubation received 4 mg of PB (2.2 mmol kg^{-1}, b.w.); embryos at 14 days of incubation received 7.0 mg of PB (3.9 mmol kg^{-1}, b.w.); embryos at 18 days of incubation received 11.6 mg (6.5 mmol kg^{-1}, b.w.); and hatched chickens received 100 mg kg^{-1}, b.w. PB (0.4 mmol kg^{-1}, b.w.) (Lorr & Bloom 1987). Samples of pooled livers were used: the number of individuals per treatment varied with embryo age: 90 per treatment from 3 to 5 days of incubation, 70 embryos from 6 to 9 days of incubation, 60 embryos from 10 to 13 days of incubation, 50 embryos from 14 to 15 days of incubation, 30 embryos from 16 to 17 days of incubation, 20 embryos at 18 days of incubation and over, 10 birds at 1–5 days posthatching and 6 birds at 6–10 days posthatching. The pooled livers were statistically considered as a single individual.

Preparation of microsomal fraction

The embryo liver was placed in warm 0.05 M Tris-HCl buffer (pH 7.4) containing KCl 1.15% (w/v), 1 mM EDTA and 20 mM 2(3) *tert*-butyl-4-hydroxyanisole (BHT). Whole embryos were assayed from 3 to 4 days of incubation since the liver first appeared as a discrete organ at 5 days of incubation. The chickens were killed by cervical dislocation and their livers quickly and aseptically removed and rinsed in warm 0.05 M Tris-HCl (pH 7.4) buffer. All subsequent steps were carried out at 4°C. For the preparation of the S9 fraction, the livers were homogenized in 4 vol (w/v) of 0.05 M Tris-HCl buffer (pH 7.4) with a Teflon pestle (Potter-Elvehjem) and centrifuged at $9{,}000 \times g$ for 20 min. The post-mitochondrial supernatant obtained was centrifuged for 60 min at $105{,}000 \times g$, the pellet resuspended in 0.1 M $K_3P_2O_7$ (pH 7.4), 1 mM EDTA and 20 mM BHT, and centrifuged again for 60 min at $105{,}000 \times g$ to obtain the microsomal fraction (Paolini *et al*., 1995). The resultant washed microsomal pellets were then resuspended with a hand-driven homogenizer in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol (v/v). Subcellular preparations were immediately frozen in liquid nitrogen, stored at -80°C and used within a week.

Inhibition studies

Immunoinhibition experiments were performed with rabbit polyclonal antibodies anti-(rat CYP2B1/2), anti-(rat CYP 3A1/2), anti-(rat CYP2E1) and anti-(rat CYP1A1/2). Hepatic microsomes, taken from PB-treated chickens at 2 days posthatching, were preincubated in 0.1 M phosphate Na^+/K^+ buffer (pH 7.4) in the presence of antibodies (6 mg protein nmol^{-1} cytochrome P450) for 20 min, then incubated in the testosterone assay. The incubation of the microsomes with the specific rat CYP1A1 inhibitor α-naphthoflavone (0.5 mM) was performed for 10 min before the enzymatic assay.

Testosterone hydroxylase activity

Incubation and isolation Incubations contained either liver microsomes or whole embryos (equivalent to 1–2 mg protein), 0.6 mM $NADP^+$, 8 mM glucose 6-phosphate, 1.4 u glucose 6-phosphate dehydrogenase and 1 mM $MgCl_2$, in a final volume of 2 ml 0.1 M phosphate Na^+/K^+ buffer (pH 7.4). The mixture was preincubated for 5 min at 37°C. The reaction was performed at 37°C by shaking and started by the addition of 80 mM testosterone (dissolved in methanol). After 10 min, the reaction was stopped with 5 ml ice-cold dichloromethane and 12 nmol corticosterone (internal standard) in methanol. After 1 min vortexing, phases were se-

parated by centrifugation at $2{,}000 \times g$ for 10 min and the aqueous phase was extracted once more with 2 ml dichloromethane. The organic phase was extracted with 2 ml 0.02 N NaOH to remove lipid constituents, dried over anhydrous sodium sulphate and transferred to a small tube. Dichloromethane was evaporated at 37°C under nitrogen and the dried samples stored at −20°C. The samples were dissolved in 100 μl methanol and analysed by high performance liquid chromatography (h.p.l.c.) (Platt *et al*., 1989).

H.p.l.c. separation and quantification Chromatographic separations were performed with a system consisting of a high-pressure pump (Waters Model 600E, Multisolvent Delivery System), a sample injection valve (Rheodyne Model 7121, Cotati, CA, U.S.A.) with a 20 μl sample loop and an ultraviolet (u.v.) detector (254 nm, Waters Model 486, Tunable Absorbance Detector) connected to an integrator (Millennium 2010, Chromatography Manager). For reversed-phase separation of metabolites, NOVA-PAK C18 analytical column (60 Å, 4mm, 3.9×150 mm, Waters) was used as stationary phase. The mobile phase consisted of a mixture of solvent A (7.5% (v/v) tetrahydrofuran in water) and solvent B (7.5% (v/v) tetrahydrofuran and 60% (v/v) methanol in water) at a flow rate of 1 ml min^{-1}. Metabolite separation was performed by a gradient from 30% to 100% (v/v) of solvent B over 30 min. The eluent was monitored at 254 nm and the area under the absorption band was integrated. The concentration of metabolites was determined by the ratio between respective metabolite peak areas and corticosterone (internal standard) and the calibration curves obtained with synthetic testosterone derivatives (Van der Hoeven, 1984; Paolini *et al*., 1996a).

Ethoxyresorufin O-deethylase activity

The reaction mixture consisted of 0.025 μM $MgCl_2$, 1.7 μM ethoxyresorufin, 0.08 ml of sample (0.32 mg of protein) and 130 mM NADPH in 2.0 ml 0.05 M Tris-HCl buffer (pH 7.4). The rate of resorufin formation at 37°C was calculated by comparing the rate of increase in relative fluorescence to the fluorescence of a known amount of resorufin (excitation 522 nm, emission 586 nm) (Lubet *et al*., 1985).

Protein concentration

Protein was determined according to the method described by Lowry *et al*. (1951) and Bailey (1967), with bovine serum albumin as standard. Samples were diluted 200 times to provide a suitable protein concentration.

Chemicals

Phenobarbitone (PB) was purchased from Merck and Co. (Rahway, NJ). Nicotinamide adenine dinucleotide phosphate, oxidized form ($NADP^+$), 16α-hydroxytestosterone, corticosterone, testosterone and androst-4-ene-3,17-dione (17-OT) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); glucose 6-phosphate and glucose 6-phosphate dehydrogenase from Boehringer-Mannheim (Germany); h.p.l.c. grade methanol, tetrahydrofuran and dichloromethane from Labscan Ltd. (Co. Dublin, Ireland); 7α-, 6β- and 16β-hydroxytestosterone from Steraloids (Wilton, NH); 6α-, 2α- and 2β-hydroxytestosterone were a generous gift from Dr P.G. Gervasi (CNR Pisa, Italy); rabbit polyclonal antibodies against purified rat liver CYPs for CYP1A1/A2, 2B1/B2, 2E1 and

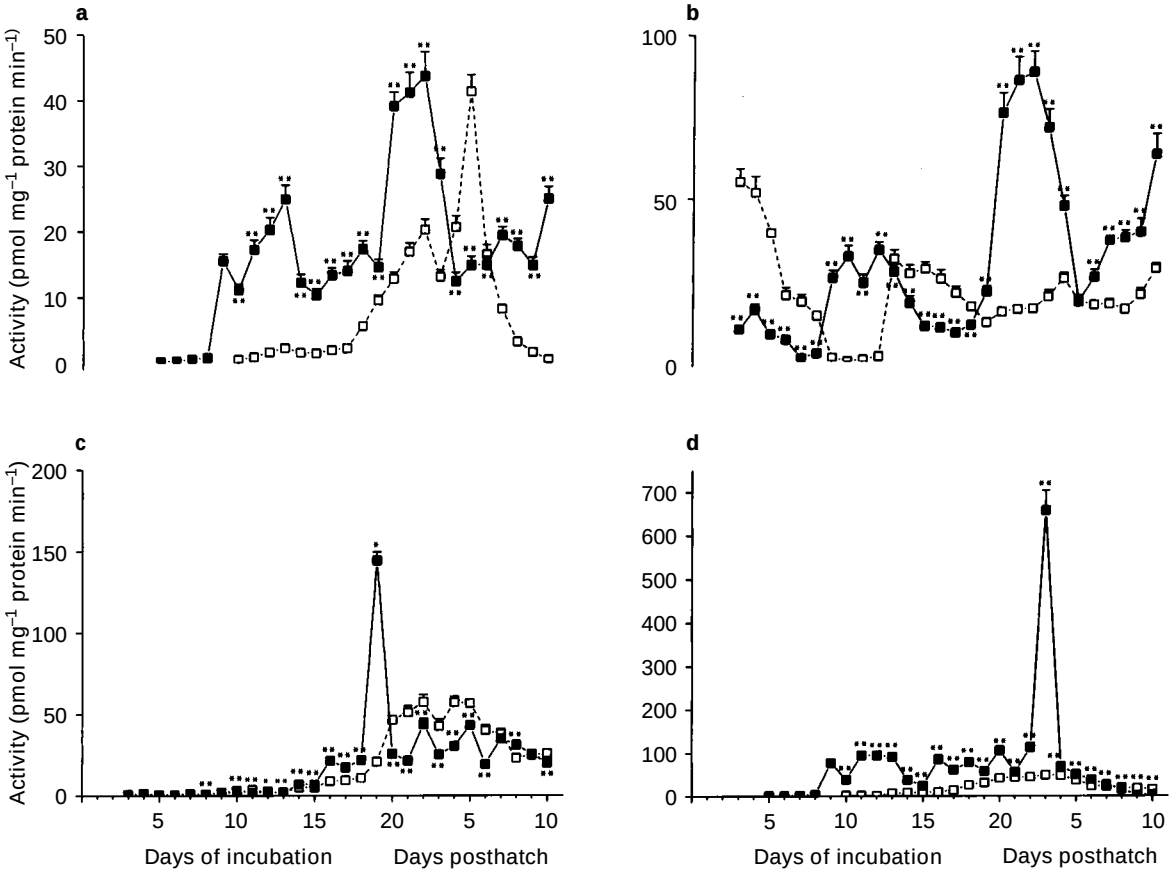

Figure 1 Developmental profile of the testosterone hydroxylases, (a) 2β-hydroxytestosterone, (b) 16β-hydroxytestosterone, (c) 16α-hydroxytestosterone and (d) 2α-hydroxytestosterone, in chicken from 3 days of incubation through 10 days posthatch. (□) Control and (■) sodium phenobarbitone-induced avians. Values are means of six independent experiments; vertical lines show s.d. $*P<0.05$, $**P<0.01$ vs control, by Wilcoxon's rank method.

3A1/A2 (Edwards *et al*., 1995) were purchased from Chemicon International Inc. (Temecula, CA, U.S.A.) and CYP-inhibitors (4-methylpyrazole, 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), α-naphthoflavone and troleandomycin) purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All chemicals and solvents used were of the highest purity commercially available.

Statistics and computer analysis

Statistical analysis on biochemical data was performed by means of Wilcoxon's rank method as described by Box and Hunter (1978). The software used was Sigma Plot 5.0, Millennium 1.1 and Windows 3.1 run on an AT 486 IBM-compatible computer.

Results

The development of basal (control) and PB-induced testosterone hydroxylase activity in the liver from 5 days of incubation through to day 10 posthatching is shown in Figures 1 and 2. Because before 5 days of incubation the liver is not present as a discrete organ, at 3 and 4 days of incubation measurements were made on the whole embryo preparations. Basal testosterone hydroxylase activity increased steadily throughout development up to 10 days of incubation, with some exceptions. For example, both testosterone 16β-hydroxylase and androst-4-ene-3,17-dione-linked activities, highly expressed at the beginning of the embryonic period, decreased during the first organogenic period, reached a minimum at 10 and 8 days of incubation for testosterone 16β-hydroxylase and the atypical androst-4-ene-3,17-dione-linked mono-oxygenase (Waad *et al*., 1988; Bellucci *et al*., 1996) activities, respectively, and then started to increase following a comparable profile to that shown by the other mono-oxygenases measured. However, testosterone 6α-, 6β-, 7α- and 16α-hydroxylase activities were detected in control embryos from 8 days of incubation, testosterone 2α- and 2β-hydroxylase activities were detected from 10 days of incubation onward and testosterone 16β-hydroxylase and androst-4-ene-3,17-dione-linked activities were expressed throughout embryonic development. From 10 days of incubation onward, testosterone hydroxylase activity showed either a peak of specific activity from 10 to 14 days of incubation (testosterone 16α-, 7α-, 6α- and 2β-hydroxylase activities; for testosterone 7α-hydroxylase up to 4.1 ± 0.3 pmol mg^{-1} protein min^{-1} at 17 days of incubation) or from 14 to 18 days of incubation (testosterone 6β-, 2α- and 16β-hydroxylase activities; for testosterone 6β-hydroxylase up to 56.6 ± 1.4 pmol mg^{-1} protein min^{-1} at 16 days of incubation). After the first peak, activities increased again around hatching, specifically from 19 days of incubation to day 4 posthatching (up to $1{,}759.3 \pm 179.4$ pmol mg^{-1} protein min^{-1} at day 1 posthatching for androst-4-ene-3,17-dione-linked activity).

PB-induction allowed measurement of activity during the first organogenic period, with the exception of testosterone 2α- and 2β-hydroxylase activities at 3 and 4 days of incubation. Unexpectedly, the initial profile of the androst-4-ene-3,17-dione-linked activity as well as testosterone 16β-hydroxylase activity started from lower levels with respect to that occurring in untreated embryos, probably due to the toxic effect of the inducer itself towards the enzyme involved (Paolini *et al*., 1996b). In contrast, the formation of the other testosterone metabolites was increased in PB-induced preparations, particularly for testosterone 2α-hydroxylase activity which was 104.5 to 13.4 fold of the respective control value at 12 days of

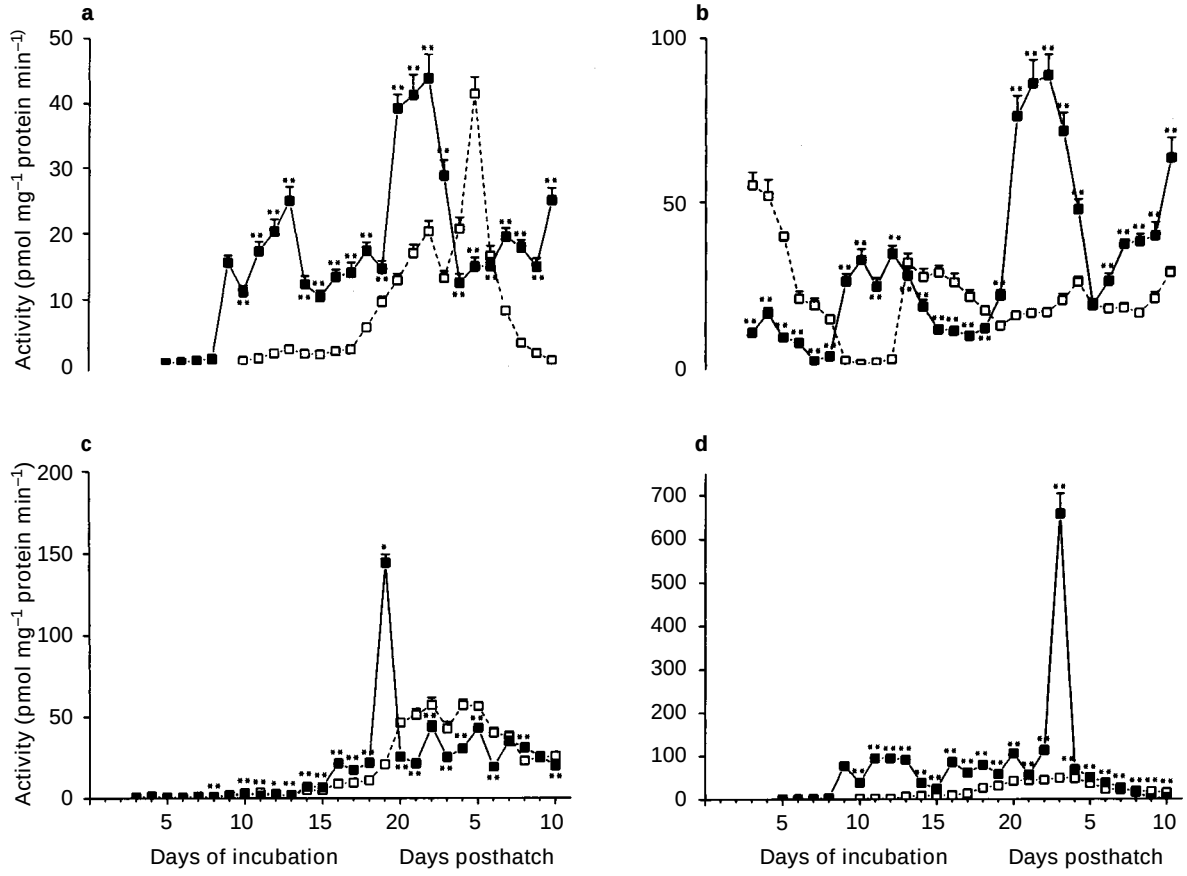

Figure 2 Developmental profile of the testosterone hydroxylases, (a) 2β-hydroxytestosterone, (b) 16β-hydroxytestosterone, (c) 16α-hydroxytestosterone and (d) 2α-hydroxytestosterone, in chicken from 3 days of incubation through 10 days posthatch. (□) control and (■) sodium phenobarbitone-induced avians. Values are means of six independent experiments; vertical lines show s.d. $*P<0.05$, $**P<0.01$ vs control, by Wilcoxon's rank method.

incubation (up to 95.1 ± 7.4 pmol mg^{-1} protein min^{-1}) and day 3 posthatching (up to 660.2 ± 45.3 pmol mg^{-1} protein min^{-1}), respectively.

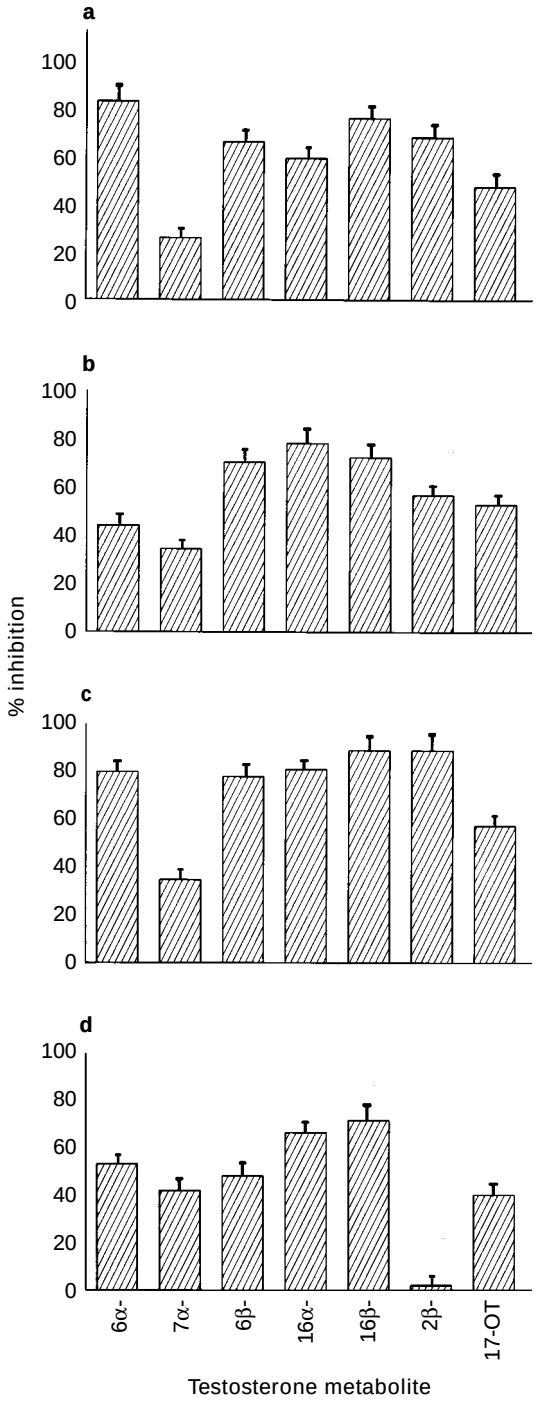

Figure 3 Effect of anti- (rat cytochrome P450) antibodies on the rates of formation of testosterone metabolites catalysed by hepatic microsomes from phenobarbitone-induced pups at 2 days posthatching. (a) Anti- (rat CYP 2B1/2), (b) anti- (rat CYP 3A1/2), (c) anti- (rat CYP 2E1) and (d) anti- (rat CYP 1A1/2). Microsomes were incubated in 0.1 M phosphate Na^+/K^+ buffer (pH 7.4) in the presence of antibodies (6 mg protein nmol^{-1} cytochrome P450) for 20 min before the enzymatic assay. The results are expressed as % inhibition of control activity which was 4.28 ± 0.21 (6α-), 4.69 ± 0.19 (7α-), 141 ± 6 (6β-), 18.21 ± 0.98 (16α-), 46.62 ± 2.43 (16β-), 16.28 ± 0.88 (2β-) and 460 ± 14, androst-4-ene-3,17-dione formation (17-OT) pmol mg^{-1} protein min^{-1}. With the exception of 2β-hydroxytestosterone in the presence of anti- (rat CYP 1A1/2), all inhibitory effects were statistically significant ($P<0.01$). Values represent the means ± s.d. of duplicate analyses of duplicate incubations.

Rat antibodies to CYP1A1/A2, CYP2B1/B2, CYP2E1 and CYP3A1/A2 were investigated for their ability to inhibit testosterone metabolism by chicken embryo hepatic microsomes; subcellular preparations were taken from PB-treated chickens at 2 days posthatching. Antibody concentration was selected to give the highest inhibition of the formation of the specific metabolites (Kaminsky *et al*., 1984). In this work attention was focused on major changes (>35/40%) in rates of formation of the major products in each case (Figure 3). CYP2B1/B2 is induced by PB and PCN in rats (Sinclair & Sinclair, 1993) as is the homologue CYP2H1/H2 in chicken (Lorr *et al*., 1989). CYP 2H1/H2 are immunochemically related to the major forms of CYP induced by PB in the rat (Brooker & O'Connor, 1982). Anti-CYP2B1/B2 inhibited mainly 6α- and 16β-hydroxylations (~80%) and, to a lesser extent, 6β-, 2β- and 16α-hydroxylations (~65%). Anti-CYP3A1/A2 exhibited a similar pattern of inhibition especially with 16α-hydroxylation. Anti-CYP2E1 antibodies inhibited testosterone 16β- and 2β-hydroxylase activities (~90%), as well as testosterone 6α-, 6β- and 16α-hydroxylase activities (~80%). Interestingly, the pleiotropic response induced by PB (which includes CYP2H1/H2, 3A and 2E1) could also involve a CYP form belonging to the CYP1A family as anti-CYP1A1/A2 strongly inhibited 16β- and 16α- (~65/70%) and, to a lesser extent, 6α-hydroxylation (~52%). These antibodies are also able to inhibit (~90%) the *O*-deethylation of ethoxyresorufin (a preferential CYP1A1 substrate) and α-naphthoflavone (0.5 mM), a selective inhibitor of rat CYP1A1 (Murray & Residy, 1990), was able to inhibit markedly testosterone 16α- (~-80%) and 16β-hydroxylase activities (~50%), whereas, in the other testosterone position the inhibition was less than 30% (data not shown). Once again, high levels of ethoxyresorufin *O*-deethylase activity were found in both basal and PB-induced chicken embryos, exhibiting a profile with two peaks (Figure 4) occurring in different periods to those exhibited by testosterone hydroxylase activities. The first one occurred at 9 days of incubation (~7 fold increase, up to 25.0 ± 2.0 pmol mg^{-1} protein min^{-1}) and the second one at day 8 posthatching (~4 fold increase up to 67.0 ± 6.3 pmol mg^{-1} protein min^{-1}).

Discussion

The chicken embryo exhibited testosterone hydroxylase activity from 3–4 days of incubation, the earliest point in development at which it is technically possible to assay activity and a full day before the liver becomes a discrete organ. Induction responsiveness to PB increased during development, reaching adult levels of induction by 8–9 days of incubation. The

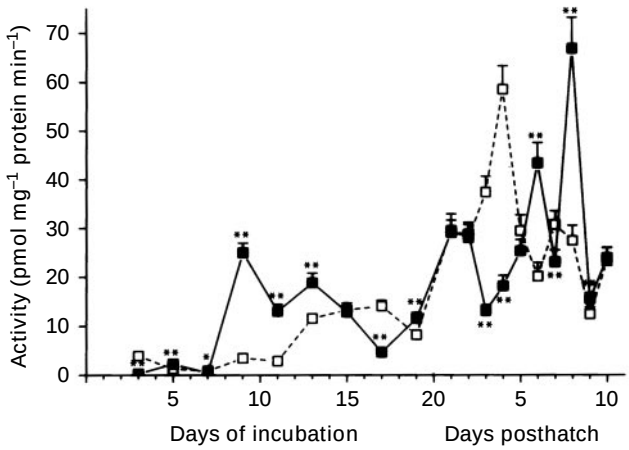

Figure 4 (□) Control and (■) sodium phenobarbitone-induced ethoxyresorufin *O*-deethylase activities in the chicken during embryonic and neonatal development. Values are means of six independent experiments; vertical lines show s.d. $^*P<0.05$, $^{**}P<0.01$ vs control, by Wilcoxon's rank method.

highest induced levels (testosterone 2α-hydroxylase) were recorded at 12 days of incubation and day 3 posthatching. Such behaviour is similar to that observed for aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity in basal and 3,4,3′,4′-tetrachlorobiphenyl-(TCB)-induced chicken embryos (Hamilton *et al*., 1983), even though, in contrast to TCB induction, the PB-dependent induction increased during development (Lorr & Bloom, 1987).

The sharp increase in testosterone hydroxylase inducibility at hatching, closely paralleled the increase in control activity peaking around hatching for both control and PB-treated animals. Highest testosterone hydroxylase induction by PB was observed after 3 days posthatching for 2α-hydroxylase activity (13.4 fold versus control), whereas the highest specific activity was achieved for androst-4-ene-3,17-dione-linked activity (i.e., $3{,}403.4 \pm 233.8$ pmol mg^{-1} protein min^{-1}). Several authors have shown such a 'precipitous' increase of metabolizing enzymes just before hatching followed by a decline within the first week (Rifkind *et al*., 1973; Hamilton *et al*., 1983; Lorr & Bloom, 1987). One proposed mechanism for this increase is a change in isozyme composition post-hatching (Lorr *et al*., 1989). It seems that the pattern of activity closely follows the progression of absorption of the contents of the yolk sac. It has indeed been shown that lipid soluble yolk extracts induce metabolism in chick embryo liver microsomes (Powis *et al*., 1976). Thus, the highly inducible mono-oxygenase system during the hatching period could have evolved as a specific mechanism for the efficient metabolism of the lipid constituents of the yolk (Lorr & Bloom, 1987). PB treatment of embryos and chickens results in the induction of several hepatic CYPs, such as CYP2B1/B2 (2H1/H2), CYP2E1 and CYP3A (Lorr *et al*., 1989; Sinclair & Sinclair, 1993), giving rise to a pleiotropic response similar to that observed in mammals (Lubet *et al*., 1992).

Polyclonal antibodies against rat liver CYPs have been shown to inhibit rat and mouse liver microsomal activities and may be useful in supporting the presence and function of related forms in other species (Kaminsky *et al*., 1984; Waxman *et al*., 1985). Chicken CYPs are immunochemically related to the respective mammalian counterpart but cross reactions are possible and must be considered in the interpretation of our data. Therefore, the results obtained here, with anti-CYP2B1/B2, 3A1/A2, 2E1 and 1A1/A2 rat antibodies, are indicative and reveal the difficulty in differentiating the various CYPs and correlating them to specific testosterone metabolites. They show a high degree of inhibition by anti-CYP1A1/A2 of 16α-, 16β- and 6α-hydroxylase activities. This suggests the possible presence of isoforms belonging to the CYP1A family (or very similar isozymes) in PB-induced liver microsomes. Consistent with this hypothesis, the inhibitory effects obtained from microsomes in the testosterone hydroxylase assay in the presence of α-naphthoflavone, a CYP1A1 inhibitor (Murray & Residy, 1990), were able to reduce markedly 16α-hydroxylase actitivy by up to 80% (data not shown). Indeed, although no direct correlation between rat and chicken isozymes has been described, the other testosterone positions, with the exception of 16β-hydroxylase ($\sim 50\%$), were only slightly inhibited ($< 30\%$). Furthermore, ethoxyresorufin *O*-deethylase activity (a preferential CYP1A1/A2 probe in mammals) was induced up to 7 fold at 9 days of incubation in PB-treated samples. Peaks of activity occur early compared to that observed for testosterone hydroxylase activities during embryonic development, indicating the possible presence of a different isoenzyme. However, the contribution to this activity by other CYPs could not be ruled out. Our study lends support to the hypothesis that representative(s) of the CYP1A1/A2 family are present in the overall pleiotropic response induced by PB in avians. However, further studies are required to support our claims.

Information regarding the period of maximal PB induction (i.e. 11–12 days of incubation) are useful in *in vivo* quantitative structure-activity relationship investigations, to substantiate a direct correlation between inducing ability and hydrophobicity of chemicals found *in vitro* (Sinclair *et al*., 1986; Hansch *et al*., 1990). It would be possible to determine exactly the day at which the induction of a particular CYP is highest to examine a series of chemicals metabolized by such an isoform. Chick embryos have been shown to be an important and valuable system in physiology (Hansch *et al*., 1990), biochemistry (Van Mierop, 1967), toxicology (Speake *et al*., 1992) and teratology (Vesely *et al*., 1992; Bruyere & Stith, 1993). A unique feature of the avian embryonic system is their independence from maternal influences, providing a very useful model for studying developmental aspects of microsomal metabolism. The substantial capacity of the chicken embryo for CYP-mediated metabolic activation of a wide spectrum of xenobiotics commends its continued use as a model to investigate the role of such an enzymatic family in the effects of chemicals (including damage and transformation during early development).

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