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BIOCHEMICAL CHARACTERIZATION OF COUMARIN 7-HYDROXYLASE ACTIVITY IN CHICK EMBRYO LIVER MICROSOMES

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Abstract—Coumarin occurs naturally in the diet and can induce and inhibit cytochrome P450 enzymes. Hepatic coumarin 7-hydroxylase activity is the major pathway for coumarin metabolism in humans but not in rats, most strains of mice, or other laboratory animals. Coumarin 7-hydroxylase activity and the effects of chemical inhibitors and inducers on this activity were studied in 19-day-old chick embryo liver microsomes. Activity was between 35 and 75 nmol/mg protein/hr which is approximately 2-fold higher than reported for human liver microsomes. The pH optimum was 7.8 and the K_m determined by both an ether extraction and a high performance liquid chromatography method was 7.3 ± 0.9 (±SD) μM. Substrate inhibition was evident at coumarin concentrations above 250 μM (activities at 1000 and 4000 μM coumarin were 84 and 40% of V_{max}, respectively). The K_i values (±SD) for inhibitors of microsomal coumarin 7-hydroxylase activity *in vitro* were: α-naphthoflavone, 46.9 ± 19.8 nM; metyrapone, 0.8 ± 0.9 μM; aniline, 12.3 ± 8.2 μM; cimetidine, 70.9 ± 27.9 μM; *N*-nitrosodimethylamine, 0.7 ± 0.9 mM; and dimethyl sulfoxide, 7.9 ± 1.9 mM. Treatment of chick embryos with pyrazole (40 μmol) increased coumarin 7-hydroxylase by 50% at 24 hr, but this activity was unaffected by treatment of embryos with 3-methylcholanthrene (2 μmol) or glutethimide (8 μmol). Thus, hepatic coumarin 7-hydroxylase activity in 19-day-old chick embryos is higher than in most laboratory animals and has similar biochemical properties as the enzyme in humans and mice. The chick embryo liver may be a useful system for studies on the biochemical effects of coumarin and the regulation of cytochrome P450-dependent coumarin 7-hydroxylase.

Coumarin (2*H*-1-benzopyran-2-one) is found in some edible plants and essential oils, is used as a fixative agent for odor in cosmetics, soaps and tobacco products, and was used previously as a food flavoring. In recent studies, coumarin in combination with cimetidine was investigated for the treatment of certain carcinomas [1–3]. Coumarin was banned as a food additive in 1954 because of its hepatotoxicity in rats [4, 5]. However, the importance of hepatotoxic and possible carcinogenic effects of coumarin in the rat has been questioned in part because the rat metabolizes coumarin differently from humans [6]. In humans, coumarin is hydroxylated primarily at position 7 by cytochrome P450 (P450)-dependent coumarin 7-hydroxylase, whereas in rats and most laboratory animals hydroxylation at position 3 predominates.

In addition to interspecies differences, hepatic coumarin 7-hydroxylase activity also displays considerable variation within species [7–12]. Studies using the DBA/2 and AKR/J mouse strains which have high and low hepatic coumarin 7-hydroxylase activity, respectively, have shown that genetic regulation of this enzyme is by the *Coh* locus, a single autosomal gene that displays an additive mode of inheritance [9, 12, 13]. In both mice and humans, one or more enzymes of the P450IIA subfamily are responsible, at least in part, for coumarin 7-hydroxylase activity [9, 10]. Polyclonal antibodies made to the purified mouse coumarin 7-hydroxylase P450 and to rat P450IIA inhibit coumarin 7-hydroxylase activity in human liver microsomes, indicating a common epitope in this P450(s) in these species [10, 14].

In previous studies on the effect of coumarin on hepatic heme biosynthesis and P450-dependent monooxygenase activities in chick embryo liver, coumarin 7-hydroxylase activity in this species was found to be comparable to that reported for humans [8, 10, 11, 14, 15]. In the present paper, we report on the optimal assay conditions and biochemical properties of coumarin 7-hydroxylase activity in the 19-day-old chick embryo liver, and the effects of chemical inducers and inhibitors on this activity.

METHODS

Materials. Coumarin, 7-hydroxycoumarin, pyrazole, 3-methylcholanthrene, dimethyl sulfoxide, *N*-nitrosodimethylamine, cimetidine and α-naphthoflavone were purchased from the Sigma Chemical Co. (St. Louis, MO), glutethimide from the GSV Pharmaceutical Corp. (Tuckahoe, NY), aniline hydrochloride from the Eastman Kodak Co. (Rochester, NY) and metyrapone from the Ciba Pharmaceutical Co. (Summit, NJ). All other chemicals were reagent grade. Fertile white leghorn chicken eggs of the Hyline strain were obtained from Texas A&M University (College Station, TX).

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Livers were obtained from adult male Wistar rats, male Hartley albino guinea pigs and female Syrian golden hamsters which were killed by an overdose of pentobarbital. Male 7- to 8-week-old C57Bl/6 mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) and killed by decapitation following light ether anesthesia. All animals were maintained on standard laboratory diets prior to being killed. Washed microsomes were prepared as described below.

Embryo treatment. Eggs were incubated and embryos treated as previously described [15]. For some experiments, chemicals were dissolved in dimethyl sulfoxide and 0.1 mL was injected into the fluid surrounding the embryo on day 18 of incubation; the embryos were killed by decapitation 24 hr later. In other experiments, untreated chick embryos were used and killed on day 19 of incubation. The livers were removed, separated from the gallbladder, and placed in ice-cold saline in groups of 2–5 livers for each treatment. The livers were homogenized in 3 vol. (w/v) of 0.25 M sucrose, 20 mM Tris–HCl buffer, pH 7.4, using a Teflon–glass homogenizer. Microsomes were prepared as previously described [15], suspended in 0.1 M potassium phosphate buffer, pH 7.4, 1.0 mM ethylenediaminetetraacetic acid and stored in liquid nitrogen until used. For some assays, the microsomal pellet was washed by suspending it in 0.15 M Tris, pH 8.0, and repelleting before storage as described above.

Microsomal assays. P450 was determined in microsomes diluted to 1–2 mg protein/mL with 0.1 M potassium phosphate buffer, pH 7.4, by measuring the CO-P450 versus reduced-CO-P450 difference spectrum [16].

Microsomal P450-dependent activities were assayed using modifications of the indicated procedures and substrate concentrations, which are described in detail elsewhere [15]. These included 7-ethoxyresorufin *O*-deethylase, 0.75 μM [17]; benzo[*a*]pyrene hydroxylase, 100 μM [18]; aniline 4-hydroxylase, 5 mM [19]; benzphetamine *N*-demethylase, 1.2 mM [20]; and *N*-nitrosodimethylamine *N*-demethylase, 5 mM [21].

Coumarin 7-hydroxylase activity was determined by measuring the production of 7-hydroxycoumarin using a modified procedure of Wood [22]. Unless stated otherwise, incubations consisted of 50 mM Tris, pH 7.8, 0.25 M sucrose, 3 mM $MgCl_2$, 125 μM coumarin and 0.3 to 0.4 mg microsomal protein, in a final volume of 0.5 mL. pH was measured at 23° with a Beckman φ44 pH meter (Beckman Instruments, Inc., Fullerton, CA) and a calomel combination pH electrode. Assays were conducted in screw-capped tubes, initiated by addition of an NADPH-generating system (1 mM NADP, 10 mM glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase/mL, final concentrations), and incubated for 10 min at 37°. The reactions were terminated and the 7-hydroxycoumarin product was quantitated by one of the following methods. An ether extraction method consisted of addition of 1.0 mL of ice-cold ethyl ether and immediately capping the tubes. After vigorous mixing, 0.4 mL of the ether layer was removed, dried under a stream of N_2 with gentle warming and the residue dissolved in 0.5 mL of 0.1 M potassium phosphate, pH 7.4. 7-Hydroxycoumarin was measured fluorometrically with an SLM 8000c spectrofluorometer (SLM Instruments, Inc., Urbana, IL) with excitation and emission wavelengths of 340 and 460 nm, respectively. The product was quantitated against 7-hydroxycoumarin in 0.1 M potassium phosphate, pH 7.4; a linear relationship in fluorescence and 7-hydroxycoumarin concentration between 0.02 and 9 μM was found.

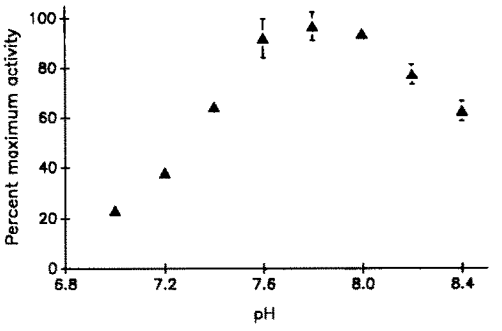

Fig. 1. Effect of pH on hepatic microsomal coumarin 7-hydroxylase activity of untreated 19-day-old chick embryos. Assays were conducted as described in Methods but the pH of the reaction buffer was varied as indicated. Values are means ± SD (N = 3) and are expressed as percent maximum activity for each microsomal preparation. The mean maximum activity for all microsomal preparations was 38.9 ± 8.0 nmol/mg protein/hr.

In separate assays, the coumarin 7-hydroxylase reactions were terminated by addition of 1.0 mL of high performance liquid chromatography (HPLC) grade methanol, the tubes were capped and the precipitated protein was removed by centrifugation. An aliquot of the supernatant was diluted 1:10 with methanol and 7-hydroxycoumarin quantitated by reverse-phase HPLC [23]. The chromatographic system (Waters Associates, Milford, MA) consisted of a 10 μm particle size μBondapak C_{18} radial compressed column (8 × 100 mm), a 600E system controller with an isocratic mobile phase of methanol–water–acetic acid (200:300:1) at a flow rate of 2.0 mL/min. 7-Hydroxycoumarin was monitored with a 470 scanning fluorescence detector with excitation and emission wavelengths at 338 and 452 nm, respectively. Coumarin was monitored by absorbance at 280 nm with a 484 tunable absorbance detector. Peaks were identified by retention time using known standards and quantitated by integration with a 745B data module.

RESULTS

The pH optimum for coumarin 7-hydroxylase activity in washed microsomes from 19-day-old chick embryo livers was 7.8 (Fig. 1). Activity quickly decreased at pH levels below 7.6 and above 8.0. Recovery of 7-hydroxycoumarin by ether extraction and stability of coumarin are not affected by pH in the range studied [22].

Coumarin 7-hydroxylase activity in washed chick

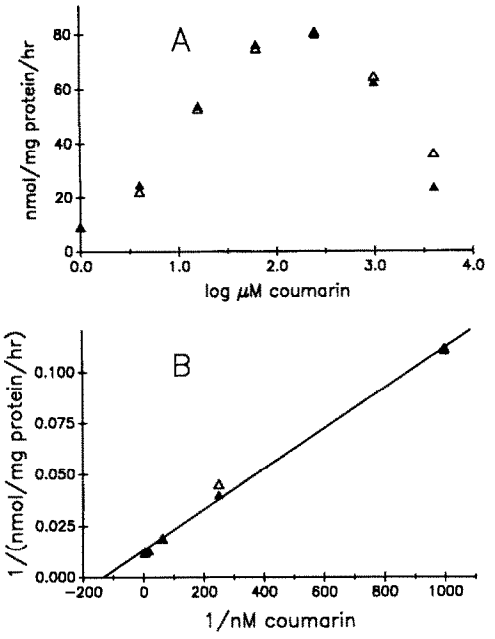

Fig. 2. (A) Coumarin 7-hydroxylase activity in untreated 19-day-old chick embryo liver microsomes at different coumarin concentrations, and (B) double-reciprocal plots of coumarin 7-hydroxylase activity at coumarin concentrations between 1 and 250 μM. K_m and V_{max} values were 7.3 ± 0.9 (±SD) μM and 74.7 ± 16 nmol/mg protein/hr, respectively. 7-Hydroxycoumarin was quantitated by either ether extraction (solid symbols) or HPLC analysis (open symbols). Values are means of two different experiments.

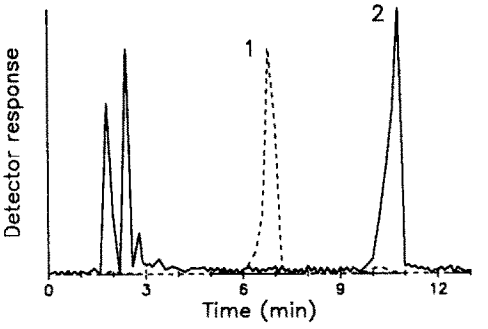

Fig. 3. Reverse-phase HPLC profile of chick embryo liver microsomes after incubation with coumarin using a μBondapak C_{18} column and an isocratic mobile phase of methanol–water–acetic acid (200:300:1) at 2 mL/min. 7-Hydroxycoumarin (peak 1) was monitored by fluorescence emission (dashed line) with excitation and emission wavelengths at 338 and 452 nm, respectively. Coumarin (peak 2) was monitored by absorbance at 280 nm (solid line). Detector responses are in arbitrary units.

embryo liver microsomes was determined using substrate concentrations between 1 and 4000 μM (Fig. 2A). Coumarin 7-hydroxylase activity increased with increasing coumarin concentration up to 250 μM but decreased at concentrations of 1000 and 4000 μM (Fig. 2A). Although a decrease in ether extraction of 7-hydroxycoumarin from the reaction mixtures and/or fluorescence quenching was evident at coumarin concentrations above 500 μM (data not shown), measurement of coumarin 7-hydroxylase activity by HPLC, which completely separated 7-hydroxycoumarin from the substrate prior to fluorescence measurement (Fig. 3), confirmed the decrease in coumarin 7-hydroxylase activity at coumarin concentrations of 1000 and 4000 μM (Fig. 2A). The K_m and V_{max} determined from double-reciprocal plots of enzyme activity at coumarin concentrations between 1 and 250 μM using both quantitation procedures was 7.3 ± 0.9 (±SD) μM and 74.7 ± 16.0 nmol/mg protein/hr, respectively (Fig. 2B).

Microsomal coumarin 7-hydroxylase activity, total P450 and selected monooxygenase activities were compared in 19-day-old chick embryo liver and adult rat, guinea pig, hamster and mouse liver (Table 1). Chick embryo coumarin 7-hydroxylase activity was 3-fold higher than that in the guinea pig, 5-fold higher than in the hamster, and 8-fold higher than in the mouse. Coumarin 7-hydroxylase activity was essentially absent in rat liver microsomes under the assay conditions used. The difference in coumarin 7-hydroxylase activities in the chick embryo compared to the other species was even greater if activity was expressed per nmol P450 (Table 1). Chick embryo aniline 4-hydroxylase, *N*-nitrosodimethylamine *N*-demethylase and benzphetamine *N*-demethylase activities corresponded more closely to these activities in the rodent species when expressed per mg protein but were greater in the chick embryo when expressed per nmol P450. Whether expressed on a protein or P450 basis, 7-ethoxyresorufin *O*-deethylase and benzo[*a*]pyrene hydroxylase activities were lower in the chick embryo than in the other species studied.

The effects of different P450 inducers on coumarin 7-hydroxylase and other monooxygenase activities in the 18-day-old chick embryo liver microsomes were investigated (Table 2). Pyrazole, a selective inducer of hepatic coumarin 7-hydroxylase activity in mice [24, 25], was effective in increasing this activity by 50% in the chick embryo. Pyrazole also increased aniline 4-hydroxylase and *N*-nitrosodimethylamine *N*-demethylase activities. Glutethimide, a phenobarbital-type inducer of P450 in the chick embryo liver [26], did not induce coumarin 7-hydroxylase activity but did increase total P450, aniline 4-hydroxylase, benzo[*a*]pyrene hydroxylase and benzphetamine *N*-demethylase activities, and decreased *N*-nitrosodimethylamine *N*-demethylase activity. 3-Methylcholanthrene, a polycyclic aromatic hydrocarbon-type inducer, was also without effect on coumarin 7-hydroxylase activity but did increase total P450, 7-ethoxyresorufin *O*-deethylase and benzo[*a*]pyrene hydroxylase.

Using a substrate concentration of 125 μM coumarin, the inhibitory effects of six P450 inhibitors and substrates on coumarin 7-hydroxylase activity in chick embryo liver microsomes were determined (Fig. 4). The K_i values (±SD) for each chemical tested were determined using the activity of the

Table 1. Total P450 and monooxygenase activities in liver microsomes from 19-day-old chick embryos and selected laboratory rodents

Activity	Chick embryo	Rat	Guinea pig	Hamster	Mouse
Total P450	0.30 ± 0.01	0.71 ± 0.05	0.85 ± 0.13	1.00 ± 0.07	0.88 ± 0.04
Coumarin 7-hydroxylase, 125 μM	43.6 ± 4.1 (145.3)	0.06 ± 0.01 (0.08)	12.4 ± 3.4 (14.6)	8.6 ± 3.6 (8.6)	5.4 ± 1.3 (6.1)
Aniline 4-hydroxylase, 5 mM	23.8 ± 1.7 (79.3)	19.5 ± 1.8 (27.5)	22.3 ± 2.3 (26.2)	48.3 ± 5.5 (48.3)	41.0 ± 3.1 (46.6)
N-Nitrosodimethylamine *N*-demethylase, 5 mM	182 ± 11 (607)	63 ± 6 (89)	69 ± 10 (81)	180 ± 26 (180)	113 ± 10 (128)
7-Ethoxyresorufin *O*-deethylase, 0.75 μM	0.67 ± 0.10 (2.23)	2.51 ± 0.49 (3.53)	4.27 ± 0.24 (5.02)	5.94 ± 0.16 (5.94)	7.29 ± 0.48 (8.28)
Benzo[*a*]pyrene hydroxylase, 100 μM	6.6 ± 0.7 (22.0)	73 ± 6 (103)	104 ± 23 (122)	82 ± 12 (82)	99 ± 4 (113)
Benzphetamine *N*-demethylase, 1.2 mM	73 ± 4 (243)	63 ± 6 (89)	69 ± 10 (81)	180 ± 26 (180)	113 ± 10 (128)

Microsomes were prepared from livers and analyzed as described in Methods. The substrate concentration used follows the indicated enzyme activity. Values (means ± SD) are expressed as nmol/mg protein for P450 and nmol/mg protein/hr for all others (N = 3 groups of 2 or 3 pooled livers for chick embryos and 3 separate animals for rodents). Values in parentheses are average enzyme activities expressed as nmol metabolite/nmol P450/hr.

Table 2. Effects of pyrazole, glutethimide and 3-methylcholanthrene on hepatic microsomal P450 and monooxygenase activities in the 18-day-old chick embryo

Activity	Control (DMSO) N = 12	Pyrazole (40 μmol) N = 14	Glutethimide (8 μmol) N = 6	3-Methylcholanthrene (2 μmol) N = 5
Total P450	0.25 ± 0.05	0.30 ± 0.04	0.99 ± 0.13*	0.37 ± 0.04*
Coumarin 7-hydroxylase	42.8 ± 9.9	66.6 ± 14.0*	32.0 ± 5.7	46.4 ± 6.2
Aniline 4-hydroxylase	15.5 ± 5.8	31.5 ± 13.2*	40.3 ± 6.8*	20.0 ± 4.9
N-Nitrosodimethylamine *N*-demethylase	135 ± 33	202 ± 43*	91 ± 17†	117 ± 24
7-Ethoxyresorufin *O*-deethylase	0.52 ± 0.26	0.64 ± 0.30	1.09 ± 0.61	33.02 ± 13.12*
Benzo[*a*]pyrene hydroxylase	6.0 ± 1.3	6.4 ± 1.5	75.5 ± 7.2*	76.3 ± 32.1*
Benzphetamine *N*-demethylase	67 ± 17	83 ± 18	116 ± 42*	44 ± 2

Chick embryos were treated on day 18 of incubation with the indicated chemical and dose dissolved in 0.1 mL of dimethyl sulfoxide (DMSO). After 24 hr, the embryos were killed and 2–5 livers combined within each treatment group and analyzed as described in Methods using the same substrate concentrations as in Table 1. Values (means ± SD; N = 5–14 as indicated) are expressed as nmol/mg protein for P450 and nmol/mg protein/hr for all others.

*,† Significantly different from controls using analysis of variance and Dunnett's procedure: * $P < 0.01$, and † $P < 0.05$.

untreated, control, microsomes as V_{max}, a K_m of 7.3 (Fig. 2B), and assuming competitive inhibition for each inhibitor [11]. α-Naphthoflavone was the most effective inhibitor tested with a K_i of 46.9 ± 19.8 nM. The other chemicals tested and their K_i values were metyrapone, 0.8 ± 0.9 μM; aniline, 12.3 ± 8.2 μM; cimetidine, 70.9 ± 27.9 μM; *N*-nitrosodimethylamine, 0.7 ± 0.9 mM; and dimethyl sulfoxide, 7.9 ± 1.9 mM.

DISCUSSION

Reported here are the optimal assay conditions for coumarin 7-hydroxylase activity in 19-day-old chick embryo liver microsomes, and the effects of various inducers and inhibitors on this activity. These studies were instigated from previous findings that coumarin has both inducing and inhibitory effects on P450 enzyme activities in the chick embryo liver and that this species has a high level of coumarin 7-hydroxylase activity [15].

Using a substrate concentration of 125 μM, coumarin 7-hydroxylase activity ranged between 35 and 75 nmol/mg protein/hr in over twenty different chick embryo liver microsomal preparations. This is greater than the 10 to 45 nmol/mg protein/hr range reported for human liver microsomes [8, 10, 14]. Coumarin 7-hydroxylase activity in chick embryo liver was considerably higher than that found in the mouse, rat, hamster and guinea pig (Table 1) [7, 11, 14].

Coumarin 7-hydroxylase activity in chick embryo

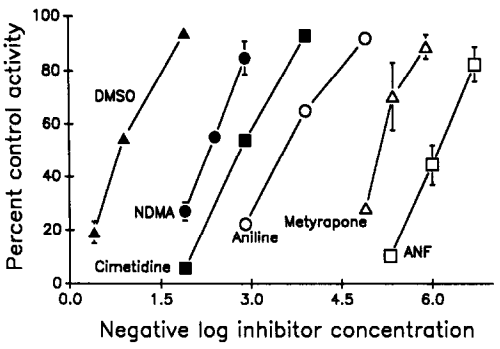

Fig. 4. Effects of P450 substrates and inhibitors on coumarin 7-hydroxylase activity when added to liver microsomes from untreated 19-day-old chick embryos. Chemicals were added to the reaction mixtures immediately before initiation of reactions. α-Naphthoflavone was dissolved in dimethyl sulfoxide and added at less than 0.1% dimethyl sulfoxide (v/v, final dimethyl sulfoxide concentration was 10 mM). All other chemicals were dissolved directly in the assay buffer. Values are means ± SD of three or four microsomal preparations expressed as percent of untreated microsomal activity. For incubations containing α-naphthoflavone, comparisons were made to microsomes in which an equal amount of dimethyl sulfoxide vehicle was added. The K_i (±SD) for each chemical was: dimethyl sulfoxide (DMSO), 7.9 ± 1.9 mM; *N*-nitrosodimethylamine (NDMA), 0.7 ± 0.9 mM; cimetidine, 70.9 ± 27.9 μM; aniline, 12.3 ± 8.2 μM; metyrapone, 0.8 ± 0.9 μM; and α-naphthoflavone (ANF), 46.9 ± 19.8 nM. The activity of all untreated microsomes was 65.0 ± 9.1 nmol/mg protein/hr (mean ± SD); the coumarin concentration was 125 μM for all preparations. Error bars are absent, where occluded by data points.

liver microsomes decreased at coumarin concentrations above 250 μM (Fig. 2A). Although this was attributable in part to interference by coumarin on the extraction of 7-hydroxycoumarin into ethyl ether and/or quenching of 7-hydroxycoumarin fluorescence by coumarin at concentrations greater than 500 μM (data not shown), substrate inhibition of coumarin 7-hydroxylase was clearly involved. This was shown by HPLC separation of 7-hydroxycoumarin and coumarin in microsomal incubations (Fig. 3), which eliminated any interference. With this method of analysis, decreased enzyme activities were still apparent at coumarin concentrations above 250 μM (Fig. 2A). In contrast, only slight decreases in coumarin 7-hydroxylase activity occur in mouse liver microsomes at 10 mM coumarin [14].

Other biochemical parameters of hepatic coumarin 7-hydroxylase activity determined in the chick embryo were a pH optimum of 7.8 (Fig. 1) and a K_m of 7.3 μM (Fig. 2B). The pH optimum is similar to the 7.6 optimum reported for mouse liver microsomes [22]. The K_m is slightly higher than the K_m of 2 μM for mouse and human liver microsomes [11, 14].

Treatment of chick embryos with pyrazole increased hepatic coumarin 7-hydroxylase activity by approximately 50% (Table 2). In the mouse, pyrazole is a selective inducer of coumarin 7-hydroxylase activity although the magnitude of induction is dependent on the strain of mouse [9, 24, 27–29]. For example, in the DBA/2 mouse, pyrazole induces an 8-fold increase in coumarin 7-hydroxylase whereas 2-fold increases are common in other strains. In the DBA/2J mouse, pyrazole induction of coumarin 7-hydroxylase appears to be due to post-transcriptional stabilization of the mRNA for this P450 [30]. The smaller inductive response of liver coumarin 7-hydroxylase to pyrazole in the chick embryo than in the mouse suggests that coumarin 7-hydroxylase activity is already partially induced at this stage of development in chick embryo liver. Alternatively, differences in dose and time allowed for induction may be responsible. A dose of 200 mg/kg daily for 3 days gives maximum induction of coumarin 7-hydroxylase activity in the DBA/2N mouse, whereas in the present study chick embryos were treated with a single dose of 40 μmol (approximately 50 mg/kg embryo) 24 hr before being killed. However, a 3-fold higher dose (120 μmol pyrazole) did not induce a larger increase in coumarin 7-hydroxylase activity (data not shown), suggesting that differences in pyrazole dosage alone are not responsible for differences in induction of coumarin 7-hydroxylase activity in the chick embryo and mouse.

Pyrazole increased aniline 4-hydroxylase and *N*-nitrosodimethylamine *N*-demethylase as well as coumarin 7-hydroxylase in chick embryo liver microsomes, but did not increase total P450 (Table 2). It is not known whether these pyrazole-induced increases are due to induction of more than one species or families of P450 enzymes. In the mouse, pyrazole decreases total hepatic P450 and has no effect on aniline 4-hydroxylase or on *N*-nitrosodimethylamine *N*-demethylase activity [24, 28, 29]. In the rat, pyrazole induces a P450 isozyme in the ethanol-inducible P450IIE subfamily which has both aniline 4-hydroxylase and *N*-nitrosodimethylamine *N*-demethylase activities [31–33], but does not induce coumarin 7-hydroxylase [14]. An inducible P450 isozyme which is enzymatically similar to P450IIE has been identified in chicken liver and in primary cultures of chick embryo hepatocytes [34, 35]. It is possible that pyrazole induces one or more P450 enzymes in chick embryo liver that are related to the pyrazole-inducible P450IIE of the rat as well as the P450IIA in mice. Alternatively, pyrazole may induce an unrelated P450 isozyme that has catalytic activities in common with these pyrazole-inducible P450s in rodents. Immunochemical studies using antibodies to specific P450 enzymes should clarify this question.

3-Methylcholanthrene treatment had no effect on coumarin 7-hydroxylase activity in the chick embryo (Table 2), which is consistent with observations in the mouse [14, 25], and suggests that the P450I family is not involved in this enzyme activity. Glutethimide, a phenobarbital-type inducer of P450 enzymes in the chick embryo liver [26, 34], produced a decrease in this activity while inducing benzphetamine *N*-demethylase and total P450 (Table 2) [15]. This is in contrast to mice in which phenobarbital increases coumarin 7-hydroxylase

activity several-fold [12, 22, 24, 25]. The refractoriness of chick embryo coumarin 7-hydroxylase to induction by a phenobarbital-like inducer may be due to different control mechanisms for expression of this P450(s). Alternatively, there may be some biochemical feature of phenobarbital responsible for induction of coumarin 7-hydroxylase activity which is lacking in glutethimide. Structure–activity studies of different barbiturate analogues should clarify this question and provide additional information on control of coumarin 7-hydroxylase expression.

Comparison of our data with regard to effects of known inhibitors and substrates of P450 enzymes on coumarin 7-hydroxylase activities suggest similarities in the chick embryo liver and other species. In chick embryo liver microsomes, the most effective inhibitor tested was α-naphthoflavone followed by metyrapone, both producing a 50% decrease in activity at concentrations of 0.9 and 6.7 μM, respectively (Fig. 4). Coumarin 7-hydroxylase activity in human liver biopsy homogenates is inhibited approximately 55% by 10 μM α-naphthoflavone and 45% by 100 μM metyrapone [11], and in mouse liver microsomes, metyrapone inhibited this activity by more than 50% at 50 μM [22]. Although human coumarin 7-hydroxylase activity required inhibitor concentrations 10-fold greater than those producing comparable inhibition of the chick embryo enzyme, this may be partly due to the differences in liver preparations assayed (i.e. chick embryo microsomes versus human homogenates), as well as differences in substrate concentration. In studies of the human enzyme, coumarin was used at 1 mM whereas 0.125 mM was used in the present studies. Because these chemicals are competitive inhibitors of coumarin 7-hydroxylase [11], higher substrate concentrations would require higher inhibitor concentrations for comparable inhibition. Aniline is also an inhibitor of coumarin 7-hydroxylase activity in the chick embryo (Fig. 4) and mouse liver microsomes [22], as well as human liver homogenates [11].

In summary, the chick embryo liver has coumarin 7-hydroxylase activity at levels comparable to that reported for human liver and higher than most laboratory rodents. In addition, the chick embryo enzyme has biochemical and enzymatic features in common with the enzyme in both humans and mice. Purification of chick embryo P450(s) with this activity and further studies with antibodies against P450s from other species will help to determine the relationship of the chick embryo P450-dependent coumarin 7-hydroxylase with other species. The chick embryo would be a convenient and supplementary model for studies on the expression of P450-dependent coumarin 7-hydroxylase.

The chick embryo liver is a convenient non-mammalian system in which hepatic heme biosynthesis and P450-associated activities are readily induced by drugs, hormones and other chemicals [15, 36–38]. As shown here, coumarin 7-hydroxylase activity in the chick embryo liver is higher than in several rodent species and comparable to that reported for human liver [8, 10, 14]. Therefore, the chick embryo may be a suitable model for study of the regulation of this as well as other P450-dependent enzyme activities.

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