PGE_2. This fact may explain its efficiency even in pathological conditions of hyperaggregability with decreased sensitivity of platelets to PGI_2 or PGE_1. Aspirin, dipyridamole and sulfinpyrazone treatments were without effect. The mechanism of action of ticlopidine was discussed.

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Hydroxylation of hexobarbital and benzo[*a*]pyrene by hepatic microsomes isolated from the fetal stumptailed monkey (*Macaca arctoides*) A developmental study

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It is now well documented [1–3] that liver obtained from human fetuses as well as from fetuses of nonhuman primates possesses relatively well developed oxidative, hydrolytic and conjugative drug-metabolizing enzyme systems early in gestation. However, only one study has been published which follows the development of human fetal hepatic drug metabolism as a function of gestational age [4]. Due to legal and ethical restrictions, this study was limited to the first 20 weeks of gestation. To gain insight into the development of fetal hepatic drug metabolism and its significance to the developing human, we proposed the nonhuman primate, specifically the stumptailed macaque (*Macaca arctoides*), as an appropriate animal model for such studies [5–9]. The purpose of this investigation was to follow the development of benzo[*a*]pyrene and hexobarbital hydroxylase in microsomes isolated from the fetal stumptailed mon-

key at various gestational ages and to compare the data with that obtained from microsomes of adult rat and human fetal liver.

Experimental

Microsomes from liver of fetal and newborn stumptailed monkeys and adult male Sprague–Dawley rats (225–275 g) were prepared as described previously [9] with the exception that livers were first perfused, *in situ*, with 0.1 M potassium phosphate buffer (pH 7.4) instead of 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer. Perfusion of livers minimized the hemoglobin contamination of the isolated microsomes, facilitating cytochrome P-450 determination [7]. Hexobarbital hydroxylase activity was assayed according to the method of Kupfer and Rosenfeld [10] as described by Marietta *et al.* [11] with the following modifications. The reaction (final volume, 1.0 ml) was initiated by addition of cofactors and terminated by 1.0 ml of chilled 1.0 M citrate–15% NaCl buffer (pH 5.5). The incubation mixture contained 1.5 mg microsomal protein (fetal/newborn) or 0.5 mg microsomal protein (rat). Incubation times were 20 min for fetal/newborn enzyme or 4 min for rat enzyme preparations. Under these conditions, the reaction was linear with respect to both time and protein concentration. Following extraction of the hydroxylated metabolites into ethyl acetate [10], the organic phase was transferred to clean scintillation vials and evaporated to dryness (60°, under N_2). The residue was dissolved in 45 μl methanol, and the resulting solution was applied to the preabsorptive zone of a LK6D silica gel plate (Whatman, Inc., Clifton, NJ). The vial was rinsed once with an additional 45 μl methanol, and the resulting solution was again applied to the TLC plate. After drying in air, the plates were developed in chloroform–acetone (9:1, v/v). The solvent front was allowed to travel 15 cm from the top of the preabsorptive zone [10]. Pilot experiments indicated that the metabolite of hexobarbital, 3-hydroxyhexobarbital (3-OH-HEX), was localized in the area 0–1 cm from the origin while hexobarbital was localized in an area 7.5–9.5 cm from the origin, in agreement with the work of Kupfer and Rosenfeld [10]; other sections did not contain activity greater than 1.5 times background. Thus, for all studies, only the section of the plates from 0 to 1 cm above the origin was scraped and the radioactivity was quantified.

Because of the sensitivity required to study fetal hepatic drug metabolism, it is critical that the [^{14}C]hexobarbital be repurified within 24 hr prior to use. This was accomplished by chromatography as described above except that a PLK5 preparative TLC plate (Whatman, Inc.) was utilized. The area 7.0–9.0 cm from the origin was scraped into a test tube and eluted three times with 5.0 ml chloroform–acetate (9:1, v/v). The resulting solutions were pooled, evaporated to dryness (60°) under nitrogen, and reconstituted with ethanol to a volume containing 10^5 dpm/10 μl.

Hydroxylation of benzo[*a*]pyrene was assayed using a modification of the method of Nebert and Gelboin [12]. Glucose-6-phosphate (2.35 μmoles), $MgCl_2$ (2 μmoles), NADP (0.22 μmole), glucose-6-phosphate dehydrogenase (0.2 I.E.U.), bovine serum albumin (10 mg), microsomal protein (0.2 mg fetal, 0.1 mg rat), and 0.1 M HEPES buffer (pH 7.55) were added to the incubation. The reaction was initiated by addition of the cofactor solution which had been preincubated at 37° for 5 min. Incubation (20 min fetal enzyme, 5 min rat enzyme), extraction of metabolites, and analysis by spectrofluorometry were as previously described [11] in a room darkened except for a yellow light source. Sensitivity and accuracy of the spectrophotometer were checked using 3-hydroxybenzo[*a*]pyrene and quinine sulfate as standards.

On two occasions we were able to obtain (with appropriate written consent) liver from human fetuses (age 17–19 weeks gestation) following termination of pregnancy by dilatation, evacuation, and curettage. In both cases, the mothers were nonsmokers and had not taken medications for at least 2 weeks prior to surgery as determined from patient histories. Microsomes were isolated from these livers as described above with the exception that these livers were not perfused prior to homogenization. All reactions containing microsomes isolated from human fetal liver were as described above for fetal/newborn enzyme.

Michaelis constants and maximum velocities were determined from a least squares fit of the data using the method of Hofstee [13]. Substrate concentrations were: hexobarbital, 2.4×10^{-4} to 7.68×10^{-3} M (fetal enzyme) or 6×10^{-5} to 1.92×10^{-3} M (rat enzyme); benzo[*a*]pyrene, 2×10^{-6} to 8×10^{-5} M. Cytochrome P-450 content and protein concentration were determined as previously described [9].

Results and discussion

Hepatic microsomes isolated from liver of monkey fetuses at midterm, threequarter term, nearterm, and 2 weeks after birth, as well as from adult male Sprague–Dawley rats, were studied with respect to the ability to catalyze the hydroxylation of benzo[*a*]pyrene and hexobarbital. With respect to hexobarbital, the amount of metabolite present in the zone 0–1 cm from the origin was too low to allow us to verify conclusively that it was 3-OH-HEX. Our assumption that the radioactivity present in this zone was 3-OH-HEX is based on the following: (a) the R_f of the polar compound formed on incubation of hexobarbital with the complete incubation mixture was in agreement with that of Kupfer and Rosenfeld [10], and (b) the amount of metabolite formed when hexobarbital was incubated in a mixture lacking either microsomes or cofactors, or containing heat-inactivated microsomes (100°, 10 min) was 6- to 8-fold lower than when the complete incubation mixture was used (Table 1). The kinetic data for the hydroxylation of benzo[*a*]pyrene and hexobarbital are presented in Table 2. Since each value for V_m and the apparent K_m is the mean of three to four genetically different fetuses, a 2-fold difference in apparent K_m or V_m was not considered to be biologically significant. The apparent K_m values for benzo[*a*]pyrene and hexobarbital hydroxylation were significantly different for each substrate and did not change from midgestation through 2 weeks after birth. The apparent K_m for the hydroxylation of benzo[*a*]pyrene by microsomes from fetal monkey liver is in general agreement with that obtained with hepatic microsomes isolated from adult male rats. However, there is an order of magnitude difference between the apparent K_m obtained with microsomes isolated from primate liver as compared to rodent liver. The apparent K_m values for the hydroxylation of benzo[*a*]pyrene and hexobarbital by microsomes isolated from two human fetal livers were: 15 and 16 μM, and 1.44 and 0.98 mM respectively. These data are in agreement with those obtained from monkey fetal liver (Table 2).

Throughout the time period studied, V_m, expressed as nmoles product $\cdot$ min^{-1} $\cdot$ (mg microsomal protein)$^{-1}$ (Table 2), increased for both substrates. However, the rate of increase for the two drugs differed. That for benzo[*a*]pyrene hydroxylation increased almost 100-fold between midterm and 2 weeks after birth whereas V_m for the hydroxylation of hexobarbital increased only 10-fold. V_m values for benzo[*a*]pyrene and hexobarbital hydroxylation obtained with microsomes isolated from the two human fetal livers were: 0.019 and 0.017, and 0.031 and 0.021 nmoles product $\cdot$ min^{-1} $\cdot$ (mg microsomal protein)$^{-1}$ respectively.

When V_m was expressed in terms of the amount of cytochrome P-450 [i.e. nmoles product $\cdot$ min^{-1} $\cdot$ (nmole cytochrome P-450)$^{-1}$, Table 3], two different patterns emerged. V_m for the hydroxylation of hexobarbital was invariant from midterm through 2 weeks after birth, whereas the V_m for benzo[*a*]pyrene hydroxylation increased more than 10-fold from midterm to threequarter and then

Table 1. Effects of the various components of the incubation mixture on hexobarbital hydroxylation by microsomes obtained from a fetal liver at nearterm*

Incubation	3-OH-Hexobarbital [nmoles·min^{-1}·(mg protein)$^{-1}$]
Complete (10-min incubation)	4.8
Complete (0-min incubation)	0.8
Minus cofactors	0.6
Minus microsomes	0.6
Heat-inactivated microsomes	0.7

* Protein (1.5 mg) and 3.84×10^{-3} M hexobarbital (containing 632,157 dpm) per incubation.

Table 2. Kinetics of the hydroxylation of benzo[*a*]pyrene and hexobarbital by microsomes isolated from liver of fetal and newborn stumptailed monkeys and adult rats*

	Benzo[*a*]pyrene hydroxylase			Hexobarbital hydroxylase		
Animal	N†	K_m (μM)	V_m‡	N†	K_m (mM)	V_m‡
Stumptailed monkey						
Midterm	4	3.52 ± 0.85	0.0056 ± 0.0012	3	1.49 ± 0.42	0.060 ± 0.012
Threequarter term	4	8.69 ± 1.43	0.046 ± 0.009	3	2.84 ± 1.21	0.254 ± 0.061
Nearterm	3	8.08 ± 1.48	0.069 ± 0.004	3	4.49 ± 1.69	0.357 ± 0.058
Newborn	3	6.34 ± 0.41	0.520 ± 0.143	3	1.62 ± 0.06	0.643 ± 0.116
Sprague–Dawley rat						
Adult	3	14.1 ± 2.8	1.66 ± 0.18	3	0.127 ± 0.002	9.57 ± 0.37

* Values are means ± S.E.
† Number of livers.
‡ nmoles product·min^{-}·(mg microsomal protein)$^{-1}$.

Table 3. Maximum velocity (V_m) for the hydroxylation of benzo[*a*]pyrene and hexobarbital by microsomes isolated from liver of fetal and neonatal stumptailed monkeys*

	N†	Benzo[*a*]pyrene hydroxylase‡	N†	Hexobarbital hydroxylase‡
Midterm	4	0.019 ± 0.010	3	0.95 ± 0.16
Threequarter term	4	0.32 ± 0.08	3	1.86 ± 0.52
Nearterm	3	0.17 ± 0.03	3	1.25 ± 0.26
Newborn	3	0.64 ± 0.09	3	1.15 ± 0.04

* V is expressed in nmoles product·min^{-1}·(nmole cytochrome P-450)$^{-1}$.
† Number of livers.
‡ Mean ± S.E.

remained constant from threequarter through 2 weeks after birth. In a previous communication [9], we documented similar patterns with respect to V_m for the *N*-demethylation of meperidine and methadone. The pattern of development of V_m for the hydroxylation of of hexobarbital (Table 3) resembles that for methadone *N*-demethylation while that for benzo[*a*]pyrene hydroxylation (Table 3) is similar to that observed for meperidine *N*-demethylation [9]. Although the total recovery of cytochrome P-450 was constant throughout the last half of gestation (27.5 ± 5.2%), it is possible that the recovery of discrete populations of this cytochrome was not constant. This could lead to the observed differences in the development of V_m. Another explanation for the two patterns observed in the development of V_m for these four substrates is the ontogenesis, at different rates, of various forms of cytochrome P-450 [1, 3, 14, 15]. Studies designed to test these hypotheses are currently underway in our laboratory.

Pelkonen [4] observed that the ability of human fetal liver to catalyze the biotransformation of a number of xenobiotics increased with fetal age up to about 13 weeks gestation and then levelled off with no subsequent changes in drug-metabolizing activity occurring up to 21 weeks gestation. This clearly is not the case with respect to fetal liver of the nonhuman primate. One possible explanation is that the biochemical development of the liver of the fetal monkey during the last half of gestation corresponds to that which is observed in the human during the first half of gestation. Alternatively, there could be at least two periods during which hepatic drug metabolism increases in human fetal liver. The first period, early in gestation, correlates with the initial development of smooth endoplasmic reticulum [1, 16]. It is during this stage of development that the capacity of human fetal liver to catalyze drug biotransformation is first identifiable. The second period, occurring during the latter half of gestation, would be related to an increase in the ontogenesis of various forms of cytochrome P-450. It is during this period that one might

expect changes in the activity of the fetal liver to catalyze the biotransformation of xenobiotics. This hypothesis must, however, remain untested since ethical and legal restrictions prohibit the use of fresh fetal tissue obtained by pregnancy termination at times past 20 weeks gestation.

In summary, we have demonstrated that liver obtained from the fetal stumptailed monkey is capable of catalyzing the hydroxylation of benzo[*a*]pyrene and hexobarbital as early as midterm and that the apparent K_m for these reactions is similar to that obtained with human fetal microsomes. Data have also been presented which suggest the possible ontogenesis of different forms of cytochrome P-450-mediated monooxygenases during fetal development. These data further substantiate the use of nonhuman primates as animal models for studying fetal hepatic drug metabolism and the possible role of drug metabolism in human fetal dysfunction, malformation, and transplacental carcinogenesis.

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Activity of various aldehyde-metabolizing enzymes in chemically-induced rat hepatomas*

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Chemically induced rat hepatomas have a unique aldehyde dehydrogenase (EC 1.2.1.3 and 1.2.1.5) phenotype characterized by a several-fold increase in total aldehyde dehydrogenase activity due to the appearance of several new cytosolic isozymes not detectable in normal liver [1–3]. In addition to other properties, the tumor isozymes differ from the normal liver aldehyde dehydrogenases by preferentially oxidizing aromatic aldehydes with NADP as coenzyme. The new isozymes are tumor-specific; they appear concomitant with the appearance of tumors grossly, several months after carcinogen exposure [4].† The phenotypic change is limited to the tumor. Neither morphologically and histologically normal liver directly adjacent to the tumor nor normal lobes of a tumor-bearing liver possess the tumor aldehyde dehydrogenase phenotype [4].†

We are interested in determining whether the tumor-specific aldehyde dehydrogenase phenotype is indicative of an overall alteration of aldehyde metabolism in hepatic tumors or whether the phenotype is due to transformation-associated, stable, genetic changes specific to the expression of the aldehyde dehydrogenases. To this end, we have examined the activities of aldehyde dehydrogenase, aldehyde oxidase, aldehyde reductase and alcohol dehydrogenase in normal rat liver, and in liver tumors induced by the aromatic amine, 2-acetylaminofluorene (2-AAF). In addition to aldehyde dehydrogenase, aldehyde reductase (EC 1.1.1.2) and aldehyde oxidase (EC 1.2.3.1) are directly involved in aldehyde metabolism in mammalian liver (for reviews, see Refs. 5 and 6). Alcohol dehydrogenase (EC 1.1.1.1) may provide a variety of aldehyde substrates for aldehyde dehydrogenase and, under certain conditions, can function in aldehyde reduction [5–7]. In

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