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EFFECTS OF PREGNANCY ON THE CYTOCHROME P-450 SYSTEM IN MICE

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Abstract—The effects of pregnancy on the hepatic cytochrome P-450-dependent mixed-function monooxygenase system (P-450) from day 6 to day 18 of gestation were examined in the C57BL/6J mouse. Pregnancy induced an initial increase and then a decrease in total P-450 content, a decrease in microsomal aminopyrine-*N*-demethylase activity, and had no effect on microsomal ethylmorphine-*N*-demethylase activity. Pregnancy also induced in the C57BL/6J and the DBA/2J mice a new major isozyme of P-450 ($P\text{-}450_{gest}$) as determined by high performance liquid chromatography and gel electrophoresis.

The cytochrome-P-450-dependent mixed-function-monooxygenase (P-450) system is an important family of genetically controlled isozymes responsible for the metabolism of many endogenous and exogenous substrates [1]. Select P-450 isozymes play a pivotal role in xenobiotic-induced cancer, mutation, and organ damage and also in developmental toxicology and pharmacology [2–4].

In the field of developmental toxicology of experimental animals, the P-450 system plays a role in chemically induced birth defects and maternal toxicities. Maternal and fetal genetic differences in the capacity to induce specific P-450 isozymes can alter the amounts of certain teratogens that covalently bind to fetal tissues and can alter the incidence of specific chemically induced birth defects [4, 5]. Maternal organ damage by some chemicals can also be altered by the activities of P-450 isozymes [5].

The possible importance of the P-450 system in human developmental toxicology has been demonstrated recently by a study of placental P-450 activity. In pregnant females who smoke, P-450 activity is less in placenta when the offspring have birth defects than when the offspring are without birth defects [6].

In developmental pharmacology, the P-450 system of the mother, placenta, and offspring is responsible for the metabolism of many endogenous and exogenous substrates, as described above. Many of the endogenous substrates such as steroids [7] and prostaglandins [8] play an important role in cellular growth and pregnancy itself. The function of P-450, if any, in regulating pregnancy and cellular growth has not been examined.

Although the P-450 system is important in developmental pharmacology and toxicology, relatively few studies have examined the effects of pregnancy on the P-450 system. These few studies have demonstrated that pregnancy has profound effects on the P-450 system. Pregnancy alters the metabolic clearance of many substrates that are metabolized by the P-450 system in both experimental animals [9–12] and humans [13–15]. Pregnancy similarly alters *in vitro* activity of some P-450 enzymes from hepatic microsomes of rodents [16–26] and lung microsomes of rabbits [27, 28]. The results of these studies sometimes conflict as to the exact effect of pregnancy on the metabolism of specific substrates in specific animal species, but the overwhelming final conclusion is that pregnancy significantly alters both the *in vitro* and the *in vivo* activities of many, but not all, of the P-450-dependent enzymes.

The mechanism by which pregnancy induces changes in the P-450 system appears to operate at three different levels: molecular, membrane, and cellular [29]. Pregnancy regulates P-450 activity at the molecular level by changes in the fraction of P-450 heme iron that occurs in the high spin state, and at the membrane level by changes in lipid and phospholipid content [30]. Pregnancy also regulates P-450 activity at the cellular level in at least the rabbit lung. Recently, Williams *et al.* [28] reported a new or modified lung microsomal P-450 isozyme in pregnant rabbits. Alteration of the P-450 isozyme profile during pregnancy has not been demonstrated directly in any other species.

In this study we examined the effects of pregnancy on hepatic P-450 isozymes in the C57BL/6J mouse throughout gestation and in the DBA/2J mouse at the end of gestation. The mouse was selected because it is a well established model system to study P-450 induction. In addition, unlike the rabbit where pregnancy alters lung but not liver P-450 function, in the rodent pregnancy alters liver [16–26] and possibly lung [23] P-450 function. Finally, there are only limited data on the effects of pregnancy on the P-450 system in the mouse [22–24, 26], since most pregnancy studies have used the rat [16–21, 25].

We examined the effects of pregnancy on hepatic

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P-450 function by three methods: (1) *in vitro* measurement of P-450 content and P-450-dependent aminopyrine and ethylmorphine *N*-demethylase specific activities, (2) the determination of isozyme profile by high performance liquid chromatography (HPLC), and (3) the determination of isozymes by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Our study demonstrated that pregnancy in C57BL/6J and DBA/2J mice had differential effects on the specific P-450 isozymes and induced a new major hepatic cytochrome P-450 isozyme, P-450$_{gest}$.

METHODS

Female C57BL/6J and DBA/2J mice (Jackson Laboratories, Bar Harbor, ME) were maintained five per cage on a 12-hr light/dark cycle in a temperature and humidity controlled environment and were provided Purina Lab Chow and water *ad lib.* Mice were mated by placing one male in the cage overnight and checking for vaginal plugs the following morning. The day after the vaginal plug was found was considered day 1 of gestation. On specific days of gestation, the animals were killed by cervical dislocation, and washed microsomes were prepared as previously described [31]. Virgin female mice of the same age as the pregnant mice were used as a control group. The microsomal pellet was resuspended in a 10 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 10 mM ethylene diamine tetraacetic acid (disodium salt; EDTA) and 1 mM dithiothreitol, and frozen at −70°. Microsomal protein was determined by the method of Lowry *et al.* [32], and P-450 content was measured by the method of Omura and Sato [33]. Aminopyrine-*N*-demethylase [34] and ethylmorphine-*N*-demethylase [35] activities were determined using microsomal protein diluted to 2 mg/ml in 1.15% KCl.

P-450 isozyme profiles were determined by the method of Kotake and Funae [31]. In brief, thawed microsomal suspensions were diluted with 1.15% KCl and centrifuged at 100,000 *g* for 60 min. The pellet was solubilized in 10 mM potassium phosphate buffer (pH 7.4) containing 0.5% recrystallized cholic acid, 0.1 mM EDTA, 0.2% Emulgen 911, and 20% glycerol (v/v). The final microsomal protein concentration was 5–10 mg/ml. Solubilized microsomes (0.5 mg protein) were applied to an ANPAC anion exchange column (ANSPEC Co., Warrenville, IL) previously equilibrated with buffer A which contained 20 mM Tris acetate (pH 7.4), 0.2% Emulgen 911, 1 mM EDTA, and 20% glycerol (v/v). The chromatogram was developed with a 40-min linear gradient of buffer B (buffer A with 0.4 M sodium trifluoroacetate). The elution profile was monitored at 417 nm.

The effect of pregnancy on the microsomal HPLC profile was compared with the effect of known inducing agents by treating pregnant and nonpregnant female mice with 3-methylcholanthrene (3MC), β-naphthoflavone (βNF), or phenobarbital. The treatment schedule for pregnant mice was designed so that microsomes were prepared on day 16 or 17 of gestation. Mice were treated either with four daily i.p. injections of phenobarbital (80 mg/kg dissolved in saline) and killed for preparation of microsomes 24 hr after the last injection, or with a single i.p. injection of either 3-MC or βNF (80 mg/kg of either chemical in corn oil) 48 hr before preparation of microsomes. Nonpregnant female mice were administered pregnenolone-16α-carbonitrile (PCN) (50 mg/kg body weight suspended in Tween 80–water, 1:4, v/v) via intraperitoneal injection daily for 5 days to induce P-450$_{pcn}$. These animals were killed, and microsomes were prepared 72 hr after the last injection.

A DEAE chromatography procedure was developed to facilitate the rapid resolution of P-450$_{gest}$ from other P-450 isozymes. Washed hepatic microsomes (130 mg protein) prepared from gestational day 16 mice (N = 10) were solubilized in 13 ml of buffer C which contained 10 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, 0.5% recrystallized sodium cholate, 0.2% Emulgen 911, and 0.1 mM dithiothreitol. The solubilized microsomes were centrifuged at 100,000 *g* for 1 hr, and the clear supernatant fraction (120 mg protein, 0.44 nmol P-450/mg protein) was applied to a Whatman DE-52 column (1 × 30 cm) equilibrated with buffer C. The column was eluted with 100 ml of buffer C and subsequently with 100 ml of buffer C containing 50 mM potassium phosphate (pH 7.4). This procedure eluted most of the applied P-450. The remaining P-450s were eluted with 100 ml of buffer C containing 100 mM potassium phosphate and collected in 3-ml fractions. Fractions containing cytochrome P-450 were identified by absorbance at 417 nM. Those fractions that contained P-450$_{gest}$ were identified by HPLC. Those fractions in which P-450$_{gest}$ constituted more than 80% of the total P-450 were pooled (DEAE P-450 fraction) and subjected to additional studies.

The pooled DEAE chromatography fraction was purified further by HPLC as described above. Cytochrome P-450$_{gest}$, as resolved by HPLC (HPLC-1), was collected and subjected to two additional HPLC rerun steps (HPLC-2 and HPLC-3). An aliquot from each purification step was stored at −70° for later SDS–PAGE analysis on a Hoeffer Scientific water-cooled system using a 10.0% running gel and a 3.5% stacking gel as described by Laemmli [36]. Protein bands were visualized by a silver staining procedure [37].

Differences in enzyme activity during gestation were determined by analysis of variance using SAS (Carey, NC) procedures. Differences were considered significant for a $P < 0.05$.

RESULTS

Aminopyrine-*N*-demethylase activity (APD), ethylmorphine-*N*-demethylase (EMD) activity, and P-450 content of hepatic microsomes prepared from mice on days 6, 8, 10, 14, 17, and 18 of gestation are listed in Table 1. APD activity per mg protein decreased on each gestational day tested in comparison to values obtained from virgin female mice. Since P-450 content initially increased and then decreased only in late gestation, the ratio of APD activity to P-450 content was decreased throughout gestation (Fig. 1). In contrast, changes in EMD

Table 1. Effects of gestation on P-450 content and APD and EMD activities

Day of gestation	N*	Aminopyrine N-demethylase activity (nmol HCHO produced/ min/mg microsomal protein)	Ethylmorphine N-demethylase activity (nmole HCHO produced/ min/mg microsomal protein)	Cytochrome P-450 content (nmol P-450/mg microsomal protein)
VFM†	10	14.6 ± 0.6	7.89 ± 0.51	0.863 ± 0.043
6	9	11.9 ± 0.6‡	7.81 ± 0.54	0.990 ± 0.040‡
8	6	12.2 ± 0.8‡	8.12 ± 0.66	1.036 ± 0.049‡
10	7	12.1 ± 0.7‡	8.88 ± 0.61	0.979 ± 0.045
14	5	7.5 ± 0.8‡	5.87 ± 0.72‡	0.832 ± 0.054
17	17	8.4 ± 0.4‡	4.23 ± 0.43‡	0.749 ± 0.036‡
18	7	9.9 ± 0.7‡		0.700 ± 0.045‡

* Number of mice studied.
† Virgin female mice.
‡ $P < 0.05$ compared to nonpregnant controls.

activity per mg microsomal protein occurred in parallel with changes in cytochrome P-450 content.

When the effects of pregnancy on cytochrome P-450 isozymes were examined by HPLC, hepatic microsomes prepared from either pregnant or nonpregnant female mice resolved into numerous components (Fig. 2). The peaks that eluted between 9 and 17 min and at 30 min were described previously as the constitutive isozymes of P-450 and cytochrome b_5 respectively [31]. Pregnancy resulted in a reduction in the area under the curve of the constitutive isozymes and an alteration of the HPLC profile of the major constitutive isozymes of cytochrome P-450. Pregnancy also induced a new major peak in the cytochrome P-450 isozyme region that eluted between 18 and 19.2 min. This peak is hereafter referred to as $P\text{-}450_{gest}$. Using the area under the curve as an estimate of P-450 content, this peak accounted for as much as 15% of the total P-450 that eluted from the HPLC column.

The absolute spectral properties of the partially purified $P\text{-}450_{gest}$ peak are shown in Fig. 3. Absorption maxima for the oxidized, reduced, and reduced CO-bound $P\text{-}450_{gest}$ occurred at 416, 411 and 446 nm respectively. These spectral properties confirm that $P\text{-}450_{gest}$ is a cytochrome P-450 isozyme [38].

Cytochrome $P\text{-}450_{gest}$ was purified by DEAE column chromatography and identified by HPLC. Cytochrome $P\text{-}450_{gest}$ eluted from the DEAE column as one major band with the same HPLC retention time and peak shape as the pregnancy-induced component of the hepatic microsomal preparations (data

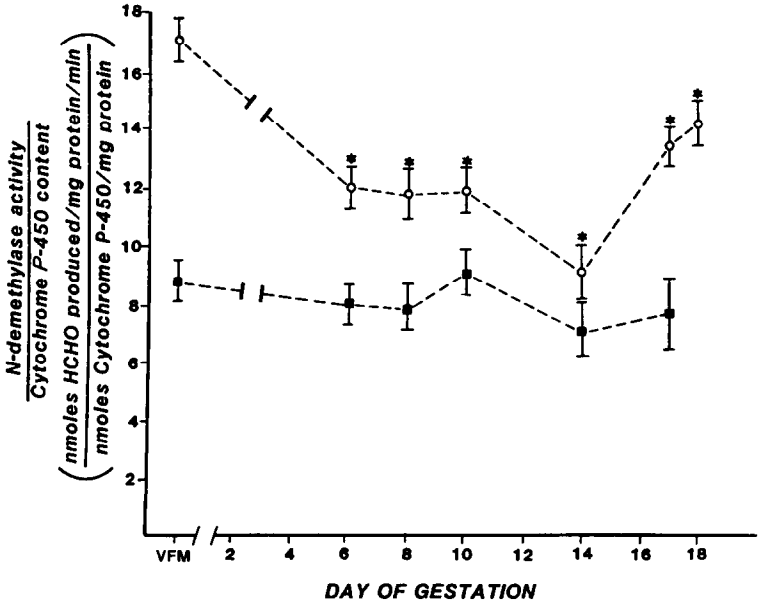

Fig. 1. Effect of gestational age on the ratios of aminopyrine-N-demethylase (○) and ethylmorphine-N-demethylase (■) activity per cytochrome P-450 content plotted versus gestational age. VFM represents virgin female mice. Error bars are the standard error of the mean; N = 5–17. Astericks indicate values that are significantly different from those for virgin female mice ($P < 0.05$).

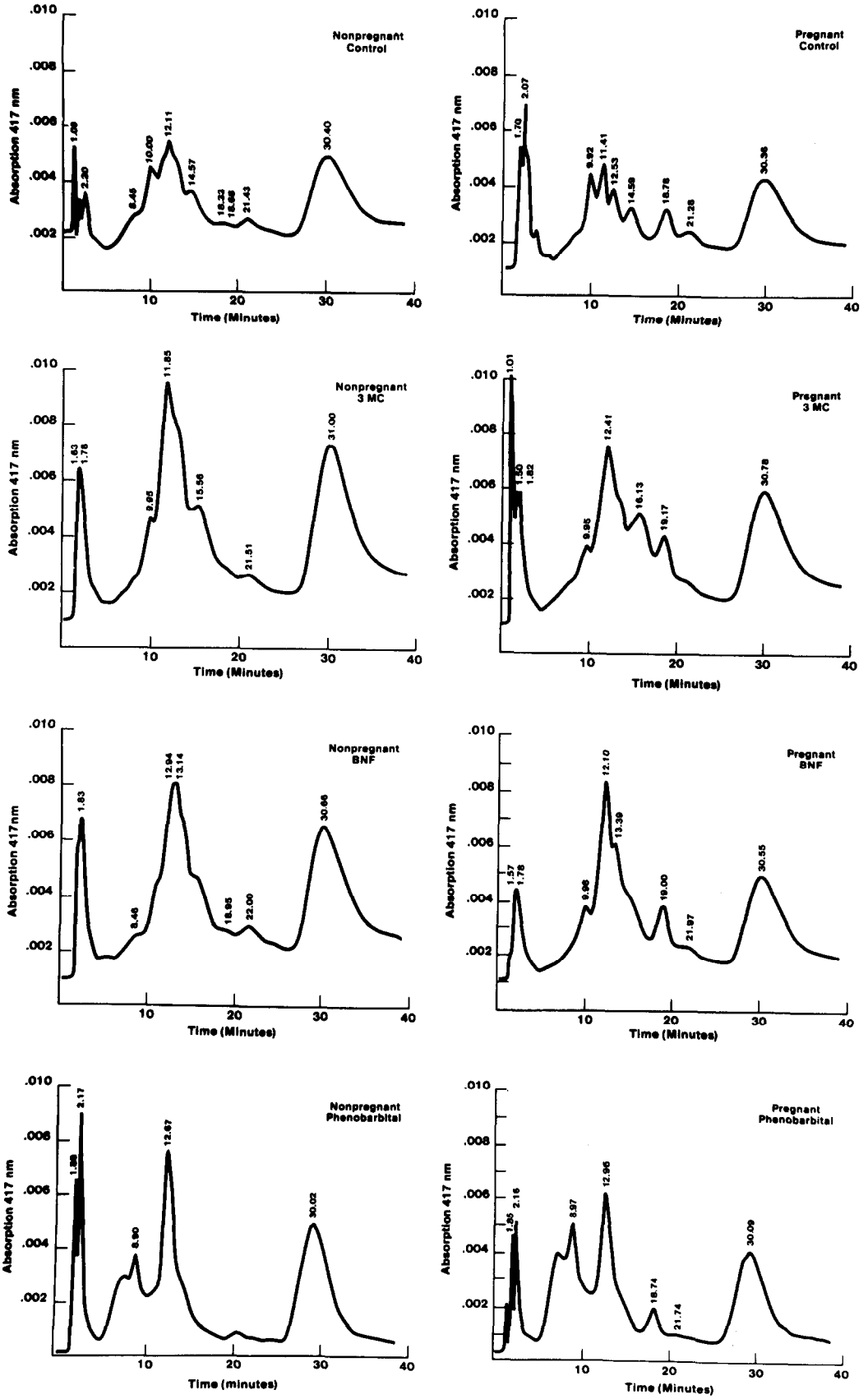

Fig. 2. HPLC elution profiles of solubilized hepatic microsomes prepared from nonpregnant and pregnant mice. Mice were treated with the classical inducers 3-methylcholanthrene (3MC), β-naphthoflavone (βNF), and phenobarbital. Constitutive P-450 isozymes eluted between 9 and 17 min and $P\text{-}450_{gcst}$ between 18 and 19.2 min.

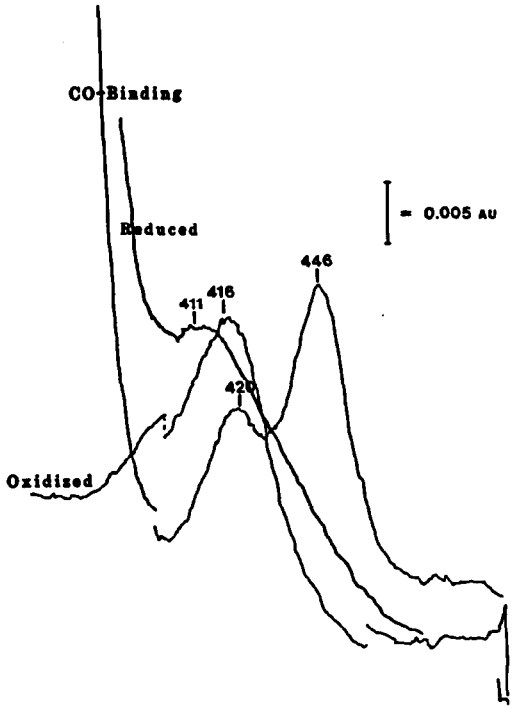

Fig. 3. Absolute spectra of oxidized, reduced, and reduced CO-bound cytochrome P-450$_{gest}$.

not shown). When the HPLC fractions that contained P-450$_{gest}$ were concentrated and repeatedly chromatographed by HPLC, cytochrome P-450$_{gest}$ continued to elute as a single component that retained its original peak symmetry and retention time.

Electrophoresis by SDS-PAGE of the hepatic microsomal protein prepared from nonpregnant and pregnant mice demonstrated an increase in at least one protein band in the microsomes prepared from pregnant mice (Fig. 4). The major protein in the DEAE and HPLC purified preparations occurred at the same location as the increased protein band seen in the microsomal preparation from pregnant mice. The apparent molecular weight of P-450$_{gest}$ was 51,000 daltons, as calculated by comparing the relative mobility to a set of protein molecular weight standards. P-450$_{gest}$ fractions collected from HPLC runs 2 and 3 contained P-450$_{gest}$ as one major protein band that was essentially free of proteins of other molecular weights.

As shown in Fig. 2, the classical P-450 isozyme inducers, phenobarbital, 3-MC, and βNF, did not induce P-450$_{gest}$ in hepatic microsomes of either pregnant or nonpregnant mice. In addition, PCN treatment did not induce P-450$_{gest}$ in hepatic microsomes prepared from nonpregnant female mice (data not shown). Each of the above inducers had a unique effect on cytochrome P-450 isozyme distribution, but

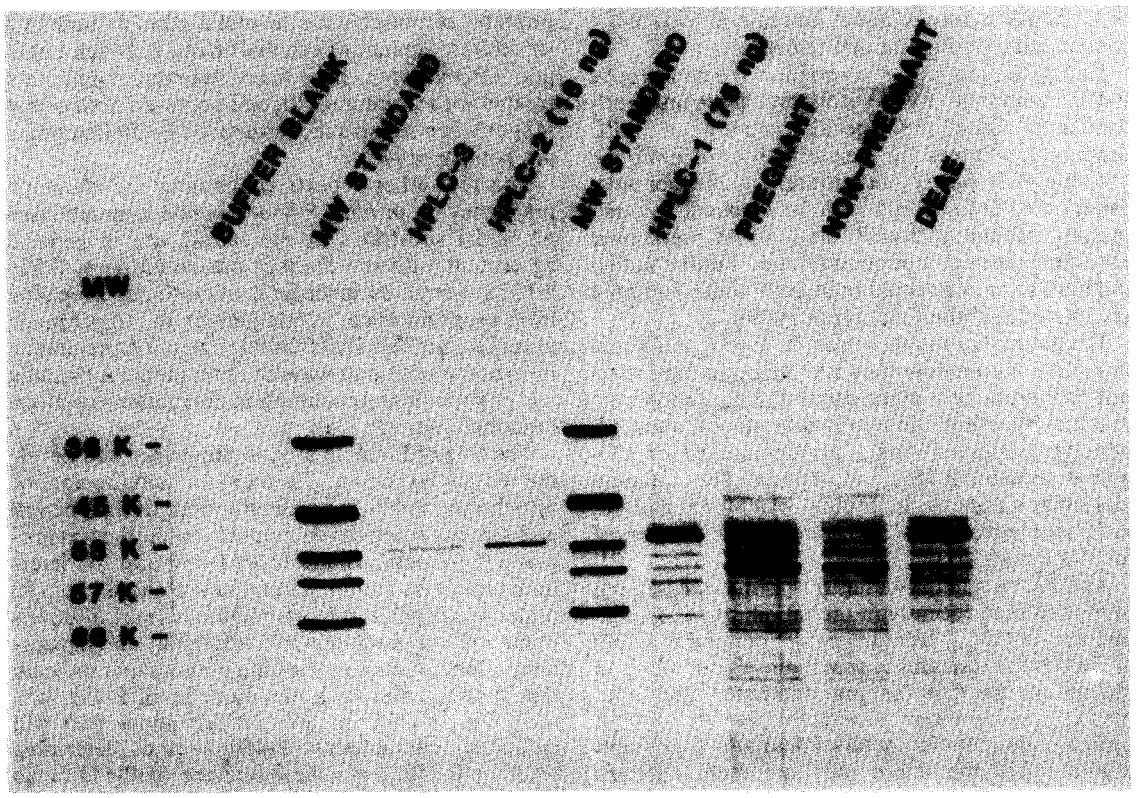

Fig. 4. Polyacrylamide slab gel electrophoresis of P-450$_{gest}$. Migaration was from bottom to top. Molecular weights of the protein standards are listed on the figure. Column headings refer to the following samples: Non-pregnant; Pregnant, pooled microsomes prepared from ten mice on day 16 of gestation; DEAE, microsomes from pregnant mice after partial purification using DEAE cellulose column chromatography; HPLC-1, HPLC-2, and HPLC-3, microsomes partially purified by DEAE column separation and the 1, 2, or 3 successive runs on the HPLC column.

none of the treatments induced the expression of cytochrome P-450$_{gest}$. Pregnancy reduced the degree of P-450 induction by the classical chemical inducers and altered the induced P-450 profiles.

Hepatic microsomes prepared from C57BL/6J mice on gestational days 6, 8, 10, 14, 16, and 18 were examined by HPLC (data not shown); cytochrome P-450$_{gest}$ was apparent on each day. The HPLC profile for hepatic microsomes prepared from DBA/2J mice on day 16 of gestation was nearly identical to the profiles for C57BL/6J pregnant mice. The P-450$_{gest}$ retention times and area under the curve were similar in both strains of inbred mice.

DISCUSSION

This study demonstrated that pregnancy had profound effects on hepatic P-450 isozymes in C57BL/6J and DBA/2J mice. As determined by HPLC, pregnancy decreased the overall content of the constitutive P-450 isozymes and induced a new major isozyme, P-450$_{gest}$. The SDS–PAGE analysis provided further evidence that pregnancy induced a new major isozyme of P-450 since the P-450$_{gest}$ protein (obtained by HPLC separation) migrated with an apparent new band on the electrophoretograms of hepatic microsomes prepared from pregnant mice. The induction of P-450$_{gest}$, which was maximal by day 6 of gestation, was observed before there was a decrease in the constitutive P-450 isozymes (chromatograms not shown). These two factors appear to be responsible for the initial increase in total P-450 content seen during the first trimester.

During the last half of pregnancy, the constitutive P-450 isozymes were probably not uniformly decreased since the specific activity of APD decreased, whereas the specific activity of EMD did not change. However, this differential effect of pregnancy on APD and EMD could be explained by other reasons, including that P-450$_{gest}$ could metabolize ethylmorphine but not aminopyrine. Future studies will have to be conducted to identify which isozymes are altered and the function of P-450$_{gest}$.

The pregnancy-induced protein (P-450$_{gest}$) isolated by DEAE chromatography and HPLC had the spectral properties of a cytochrome P-450. There were absolute spectrophotometric maxima of oxidized, partially purified P-450$_{gest}$ at 416 nm and of CO-bound reduced P-450$_{gest}$ at 446 nm. The apparent molecular weight of the protein was 51,000 daltons, within the molecular weight range usually observed for P-450 isozymes.

Another pregnancy-induced isozyme of P-450 has been identified recently in the rabbit lung [28]. This isozyme, P-450$_{pg\text{-}\omega}$, has an apparent molecular weight of 56,000 daltons and a maximum Soret peak of 450 nm in the CO binding difference spectrum. P-450$_{pg\text{-}\omega}$ metabolizes prostaglandins by omega-hydroxylation. P-450$_{pg\text{-}\omega}$ and P-450$_{gest}$ are probably different isozymes since they are from different organs and species, and they have different molecular weights. In addition, the absorbance spectra of P-450$_{pg\text{-}\omega}$ has a maximum absorbance at a different wavelength than that of P-450$_{gest}$.

The pregnancy-induced isozymes of P-450 may be excellent models for the study of the mechanism of induction [28]. The process of induction of P-450 isozymes by xenobiotics has been studied extensively [39], but the process of induction by natural phenomena such as pregnancy or development has not been studied. Pregnancy-related induction may offer an excellent opportunity to examine the molecular mechanism of the induction of a specific P-450 isozyme and the subsequent decrease of the same enzyme after delivery.

The same HPLC isozyme profile that was found in liver from pregnant C57BL/6J mice was also found in liver from the DBA/2J pregnant mice. Since a major difference between these two inbred strains is the amount of Ah receptor available for the induction of P_1-450 and P_3-450, the Ah locus would not appear to be related to the induction of P-450$_{gest}$. In addition, cytochrome P-450$_{gest}$ was not affected by the classical P-450 inducers, phenobarbital, 3-MC, βNF, or PCN. These findings would indicate that the processes for the chemical-related versus the pregnancy-related induction of P-450 isozymes are different.

The observation that the specific activity of APD decreased in the pregnant mouse whereas that of EMD did not is similar to that observed in the pregnant rat. In the pregnant rat, hepatic microsomal EMD activity decreases only when P-450 content decreases [16, 20], whereas APD specific activity increases in early gestation and falls below nonpregnant controls in late gestation [21, 25]. The differential effects of pregnancy on EMD and APD demonstrate that different isozymes of P-450 are probably responsible for the metabolism of these two substrates. Similar differential effects between EMD and APD activities have been observed in male rats treated with vitamin A [40] or 2-acetylaminofluorene [41] or when sex specific rat hepatic P-450 issozymes are compared [42].

The few other reports concerning the effect of pregnancy on *in vitro* P-450-dependent metabolism of model substrates in the mouse are in general agreement with the findings presented here. APD activity decreases in hepatic microsomes prepared from pregnant mice, in comparison to nonpregnant mice [22, 24, 26]. Differential effects of pregnancy on deacetylation pathways of other model substrates and environmental chemicals in the mouse have been described recently [26].

The role of P-450, if any, in pregnancy and development is unknown. Further characterization of cytochrome P-450$_{gest}$ by identification of its function and its cross-reactivity to monoclonal antibodies prepared from other isozymes of P-450 and by determination of its amino acid sequence should help in understanding the role of P-450 isozymes in developmental pharmacology and toxicology and in pregnancy itself. Although the data in this paper indicate that P-450$_{gest}$ may be a new isozyme of P-450, it is these future experiments outlined above that will definitely characterize P-450$_{gest}$ and determine whether P-450$_{gest}$ is a new isozyme of P-450 or an altered form of another isozyme.

In summary, we have identified major pregnancy-induced changes in P-450 isozyme profiles in C57BL/6J and DBA/2J mice. How these pregnancy-induced changes in P-450 isozymes in the mouse are related to other pregnancy-induced changes, such as altered

specific phospholipid content of the microsomal membrane [21, 30], altered spin state of the heme ferric ion [30], and altered polyamine levels [43], and to what degree they affect the overall *in vivo* P-450 function in various species, remain to be determined.

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