MOLECULAR PHARMACOLOGY, 13, 1092-1104

Characteristics of the Liver Microsomal Drug-Metabolizing Enzyme System of Newborn Rats

MICHAEL M. IBA,[1] LESTER F. SOYKA,[2] AND MARTIN P. SCHULMAN

Department of Pharmacology, University of Illinois College of Medicine, Chicago, Illinois 60612

(Received January 31, 1977)
(Accepted June 15, 1977)

SUMMARY

IBA, MICHAEL M., SOYKA, LESTER F. & SCHULMAN, MARTIN, P. (1977) Characteristics of the liver microsomal drug-metabolizing enzyme system of newborn rats. *Mol. Pharmacol.*, 13, 1092-1104.

Sodium dithionite-reduced hepatic microsomes from neonatal (5-7-day-old) rats displayed (a) an absorption maximum at 452 nm in the presence of carbon monoxide and (b) an equilibrium for the ethyl isocyanide-induced absorption peaks at 430 and 455 nm at pH 7.9. These preparations elicited little or no spectral change with ethylmorphine but elicited the type R-I spectral change with other type I substrates. Carbon monoxide formation in the presence of NADPH and the rate of conversion of cytochrome P-450 to cytochrome P-420 by mersalyl (sodium [(3-hydroxymercuri-2-methoxypropyl)-carbamoyl]phenoxyacetate) were greater in microsomes from neonates than in those from adults. In the presence of NADPH, lipid peroxidatic activity was high, ethylmorphine *N*-demethylation was low, and aniline hydroxylation was the same in microsomes from neonates compared with the same activities in microsomes from adults. In the presence of NADH, ethylmorphine *N*-demethylase activities in both the adult and neonatal preparations were identical, but more cytochrome P-450 was reducible by this nucleotide in the latter preparation. These observations demonstrate that the drug-metabolizing system of the neonate differs from that of the adult.

INTRODUCTION

Numerous studies on the hepatic mixed-function oxidase system indicate that it differs both qualitatively and quantitatively between adults and neonates (1-5). Such differences are thought to account for the low capacity of newborns to metabolize many lipophilic drugs. Since there is little or no correlation between levels of individual components of the system and its over-all capacity, quantitative differences may not wholly explain the low capacity of the newborn to metabolize drugs. Current research has focused on qualitative aspects: for example, differences were observed in the K_m and V_{max} (1, 5), sensitivity to inhibitors (4), and pH optimum (3) for the aminopyrine *N*-demethylation of microsomes from the neonate and those of the adult; atypical spectral changes were induced by various compounds in microsomes from neonates of various species (6-9); and the half-life and stability (10, 11) of cytochrome P-450 in neonates were found to be less than those of the cytochrome in adults. The locus of these anomalies within the monooxygen-

This work was supported in part by United States Public Health Service Grant HD 05362.

[1] Portions of this study have been submitted to the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Present address, Department of Pharmacology, University of Vermont College of Medicine, Burlington, Vermont 05401.

ISSN 0026-895X

ase system, which consists of cytochromes P-450 and b_5, NADPH- and NADH-dependent reductases, and lipids, has not been established. The present studies are concerned with spectral and enzymatic observations on liver microsomes of adult and neonatal rats, which are offered as evidence for the existence of neonatal cytochrome P-450[3] with different properties from its adult counterpart.

MATERIALS AND METHODS

Handling of animals and isolation of microsomes. Sprague-Dawley rats were raised in bedding of hardwood shavings and fed standard Purina laboratory chow. Neonates were caged six to a mother and were 5, 7, or 14 days old at death. Weaning was carried out at 22 days of age, after which the animals were kept three to a cage. Adult males weighed 150–200 g. Except for preweanlings, rats were fasted overnight prior to decapitation. Hemoglobin-free liver microsomes were prepared as previously described (12). At least two serial washings with 1.15% KCl were necessary to eliminate hemoglobin from microsomes of neonates. At the end of each 105,000 × *g* centrifugation the ultracentrifuge tubes, containing packed microsomal pellets, were warmed to approximately 10° and gently agitated. The resulting slurry was decanted from the tubes after gentle shaking with 1-ml aliquots of 1.15% KCl. This procedure minimized contamination of the microsomal fraction with the glycogen pellet at the bottom of the tube, which contained visible traces of hemoglobin.

Spectral studies. Assays of cytochrome P-450 and P-420 were performed according to a modification of the procedure of Omura and Sato (13, 14), in which the reducing agent (sodium dithionite, NADPH, or NADH) was added after bubbling the microsomal suspension with CO. When pyridine nucleotides were the reducing agents, the suspension was kept anaerobic in Thunberg cuvettes. A recorder speed of 4 cm/min (40 nm/min) was used for all spectral recordings. The contents of cytochrome P-450 and P-420 were calculated using extinction coefficients of 91 mM^{-1} cm^{-1} and 111 mM^{-1} cm^{-1}, respectively (13, 14). The formation of CO as an index of destruction of microsomal macromolecules (15) was estimated as described by Nishibayashi *et al.* (16). The ethyl isocyanide spectra of reduced P-450 and the crossover pH were determined by the procedure of Imai and Sato (17), except that microsomes were suspended in Tris-KCl buffers (0.5 M) prepared according to Sigma Chemical Company (18). The final pH values of the buffer solutions (containing sodium dithionite, EtNC,[4] and microsomes) were determined directly in the cuvette at room temperature, and these were the values used in the determination of the EtNC crossover pH.

For studies on the conversion of P-450 to P-420 by mersalyl, microsomes (1–2 mg/ml of 0.05 M Tris-HCl in 0.92% KCl, pH 7.5—buffer A) were placed in sample and reference cuvettes. Anaerobiosis was achieved in the sample cuvette (a modified Thunberg cuvette with a plunger assembly, containing 2 mg of solid sodium dithionite in its side arm reservoir) by bubbling with CO for 2 min. The CO had previously been passed through a deoxygenating solution consisting of 0.5% sodium dithionite and 0.5% anthraquinonesulfonic acid in 0.1 N sodium hydroxide. After a baseline had been established, the microsomes in the reference cuvette were reduced with 2 mg of sodium dithionite and those in the sample cuvette were reduced by mixing the dithionite in the side arm reservoir with the CO-treated microsomal suspensions. Following the recording of the absorption spectrum between 400 and 490 nm, 30–90 nmoles of mersalyl in 10-μl aliquots were added to the reference cuvette as well as the sample cuvette through the plunger assembly; the recording was immediately repeated. Subsequent recordings were made at 3-min intervals for a total of 90 min. The contents of P-450 and P-420 at different time intervals were calculated according to Nishibayashi and Sato (19).

[3] Cytochrome P-450 as used in this paper does not distinguish between the different species of the hemoprotein unless explicitly stated otherwise.

[4] The abbreviation used is: EtNC, ethyl isocyanide.

Total heme was estimated by the pyridine hemochromogen method of Falk (20). The absolute, reduced, and CO-reduced spectra of hemoglobin were also recorded, and hemoglobin content was estimated from the CO difference spectrum using an extinction coefficient of 154 mM^{-1} cm^{-1}.

For spectral studies on the interaction of drugs with P-450, aniline hydrochloride (resublimed), ethylmorphine hydrochloride, hexobarbital (recrystalized from hot 5 N sodium hydroxide), sodium phenobarbital, and aminopyrine were dissolved in buffer A and titrated appropriately with either dilute KOH or HCl (approximately 1 N) to a final pH of 7.5. The difference spectra of the interaction of the substrates with microsomes were recorded as described by Schenkman *et al.* (21) following successive additions of 10-μl aliquots of the respective substrate solutions to the sample cuvette and equivalent amounts of buffer to the reference. All spectra were recorded at room temperature on a Shimadzu split-beam recording spectrophotometer, model MPS-50L. The accuracy of the wavelength of the instrument was calibrated against the emission lines at 435.8 and 546.1 nm of a mercury vapor lamp.

Assay of drug metabolism and lipid peroxidation. Aniline hydroxylase activity was assayed by *p*-aminophenol production (22). Ethylmorphine *N*-demethylase activity was measured by formaldehyde production (22), but without semicarbazide in the reaction mixtures. No differences were found in apparent enzyme activity when semicarbazide was added. Generally semicarbazide was omitted after preliminary experiments revealed that the concentration of semicarbazide used by other investigators caused a precipitate in incubation mixtures containing microsomes from the neonate. In addition, semicarbazide lowered lipid peroxidatic activities. Lipid peroxidation was measured by the thiobarbituric acid assay (23).

RESULTS

CO difference spectra. The absorption spectra of the CO complex of dithionite-reduced cytochrome P-450 from neonatal (5-day-old) or adult (55–60-day-old) rats are shown in Fig. 1. While the spectrum from the adult exhibited the characteristic absorption peak at 450 nm, that from the neonate appeared at approximately 452 nm. A slight shoulder around 421 nm, possibly due to hemoglobin and/or cytochrome P-420 contamination, was always seen in the spectra from neonatal preparations.

To see whether the shift in absorption peak of the reduced P-450 complex with CO in neonatal microsomes was caused by contamination with non-P-450 heme compounds, CO difference spectra of reduced microsomes were recorded in the presence of added hemoglobin. Figure 2 shows that the addition of adult or neonatal hemoglobin from dialyzed blood to microsomes

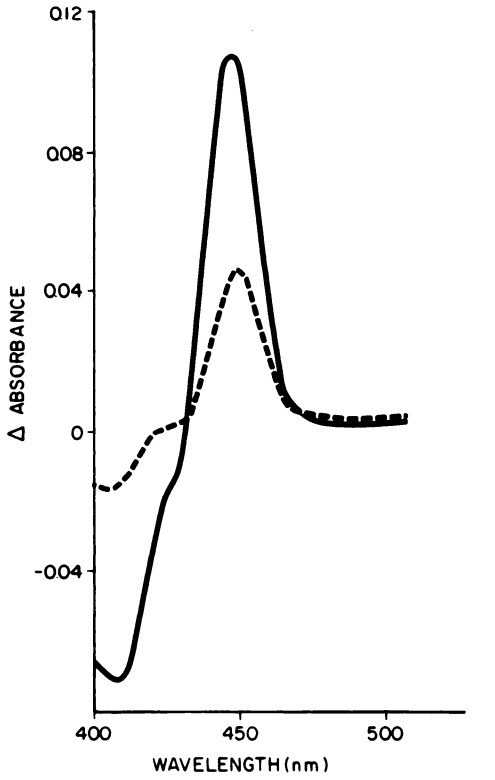

FIG. 1. *Carbon monoxide difference spectra of dithionite-treated liver microsomes*

Microsomal protein concentrations were 1.0 mg/ml. ——, microsomes from adult male (50-day-old) rats (absorption maximum, 450 nm); - - -, microsomes from neonatal (5-day-old) rats (absorption maximum, 452 nm).

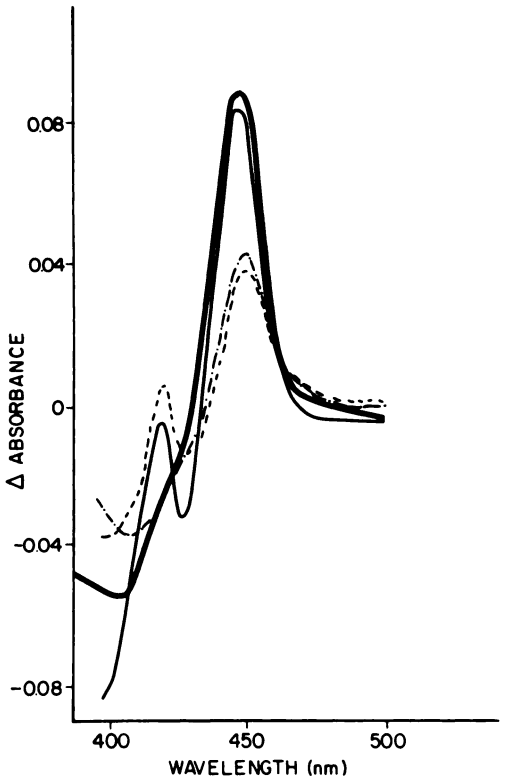

FIG. 2. *Effect of added hemoglobin on CO difference spectra of dithionite-treated liver microsomes*

Microsomal suspensions contained 1.0 mg of microsomal protein per milliliter. —— (curve *A*), microsomal suspensions from adult male (50-day-old) rats. Dithionite-treated sample cuvette containing CO was recorded against reference cuvette containing microsomes with CO (no dithionite); absorption maximum, 450 nm. -·-· (curve *B*), microsomal suspensions from neonatal (5-day-old) rats, treated as in *A*; absorption maximum, 452 nm. —— (curve *C*), microsomal suspensions from adult male (50-day-old) rats. Both sample and reference cuvettes contained 0.3 μM rat hemoglobin and dithionite, but only the sample cuvette was treated with CO; absorption maximum, 450 nm. - - - (curve *D*), microsomal suspensions from neonatal (5-day-old) rats, treated as in *C*; absorption maximum, 452 nm.

from adults or neonates did not alter the wavelengths of maximal absorption observed in Fig. 1. Prominent peaks around 421 nm were noted, which undoubtedly were contributed by reduced carboxyhemoglobin. Addition of CO, but not dithionite, to the reference cuvette did not alter the positions of the absorption peaks (Fig. 2), although the magnitudes of absorbance changes were slightly less than those seen in Fig. 1. These results show that neither contaminating hemoglobin nor the assay procedure was responsible for the 2 nm shift in absorption seen in microsomes from neonates. Since the shift in the absorption maximum of the reduced-CO spectra observed above in microsomes from the neonate may have been due to some effects of dithionite, the carbon monoxide spectrum of reduced cytochrome P-450 as well as the concentration of this hemoprotein was examined using NADPH as the reducing agent. With added NADPH (final concentration, 2 mM), the absorption maxima for the reduced-CO spectra of both microsomal preparations (data not shown) were no different from those seen in Fig. 1. This experiment further differentiated between the hemoproteins in the two microsomal preparations: adult microsomes required an average of 7 min longer than microsomes from neonates to elicit a maximum absorption peak around 450 nm. This probably reflected the differences in the rates of reduction of the two hemoproteins. This observation with NADPH was in contrast with the almost instantaneous formation of maximum absorption peaks when dithionite was the reducing agent. After the maximum absorption of the CO complex of the NADPH-reduced cytochrome P-450 was obtained, the content of the hemoprotein was calculated using the absorbance change between 450 and 490 nm. It was assumed that the maximum P-450 content in microsomes is achieved with dithionite as the reducing agent, and that the extinction of cytochrome P-450 is independent of the reducing agent. Based on these assumptions, 90% of the total content of the pigment in adult microsomes was reducible by NADPH; in neonatal microsomes, this value was 64% (Table 1). The maximum absorption peak of adult microsomes remained stable for at least 5 min while that of neonatal microsomes did not, rapidly decaying to a value of about 75% of the maximum within 25 sec and gradually diminishing thereafter. This diminution was accompanied by a decrease in total heme and an increase in P-420 content,

suggesting instability of the pigment in neonatal microsomes.

In contrast with the differences just described with NADPH, maximum peaks were elicited instantaneously in the presence of NADH in both preparations, and the peaks remained stable for over 10 min. The fractional content of cytochrome P-450 reducible by NADH was 30% in adult and 60% in neonatal microsomes (Table 1).

Ethyl isocyanide difference spectra. Differences in pH-dependent spectra of dithionite-reduced microsomes have been interpreted as evidence for the existence of a different species of cytochrome P-450 in microsomes from rats treated with polycyclic hydrocarbons (24). These spectra were measured in the present study to ascertain whether a different type of cytochrome P-450 was indeed present in microsomes from neonates. Figure 3 shows plots of the heights of the 430 and 455 nm peaks of reduced–ethyl isocyanide spectra at various pH values in microsomes from adults and neonates. For adult microsomes, the two peaks attained equal heights at pH 7.3, in agreement with the observations of other investigators (24–26). In contrast, in neonatal microsomes the equilibrium pH was 7.9.

Formation of CO in microsomes. Since CO is produced in microsomes in the presence of oxygen and NADPH and competes with EtNC for binding to reduced P-450 (16), CO production was determined in neonatal and adult microsomes to ascertain whether this phenomenon was related to the observed age-dependent spectral differences. A broad peak at 450 nm, characteristic of the CO complex of reduced P-450, was observed with an NADPH-reduced suspension of microsomes under aerobic conditions (Fig. 4). CO formation was estimated as the ratio of absorbance changes between 450 and 490 nm in the absence as compared with the presence of added CO. This ratio was greater in neonatal (0.58) than in adult (0.16) microsomes, indicating greater CO production in the neonatal preparation. Under neither condition, however, was the wavelength for the absorption peak different from that observed in Fig. 1, showing that enhanced CO production was not responsible for the shift in absorption maximum of cytochrome P-450 in microsomes from

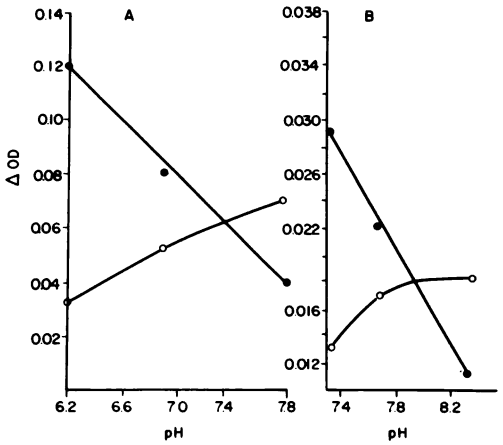

FIG. 3. *Dependence on pH of 455 and 430 nm peaks of ethyl isocyanide spectra of reduced microsomes*

●——●, disappearance of the 430 nm peak ($\Delta A_{430-490}$); ○——○, appearance of the 455 nm peak ($\Delta A_{455-490}$). A. Microsomes from adults; ethyl isocyanide equilibrium at pH 7.3. B. Microsomes from neonates; ethyl isocyanide equilibrium at ph 7.9.

TABLE 1

Effect of nucleotide or dithionite addition on cytochrome P-450 concentration of hepatic microsomes of adult and neonatal rats

Microsomal protein concentrations were 1.0 mg/ml. All values with NADPH or NADH as the reducing agent were obtained from spectra recorded under anaerobic conditions. Values are the means and standard deviations of three to five experiments.

Addition (final concentration)	Cytochrome P-450 concentration	
	Adults	Neonates
	nmoles/mg protein (% control)	
Dithionite (0.63 mg/ml)	1.25 ± 0.10	0.44 ± .07
NADPH (2 mM)	1.15 ± 0.05 (92)	0.29 ± .02 (66)
NADH (2 mM)	0.38 ± 0.03 (30)	0.26 ± .02 (60)

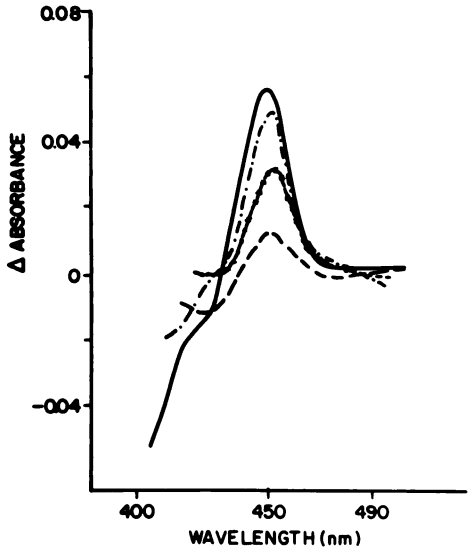

FIG. 4. *Carbon monoxide formation in NADPH-treated microsomes*

—— (curve *A*), microsomal suspensions (containing 0.7 mg of microsomal protein per milliliter in 0.05 M Tris-KCl buffer, pH 7.5) from adult rats were made anaerobic in both sample (Thunberg) and reference cuvettes by bubbling with nitrogen. The sample cuvette was then treated with NADPH and CO and allowed to stand for 10 min at room temperature. Contents of the sample cuvette were then recorded against those of the reference, which was treated with dithionite and NADPH. -·-· (curve *B*), microsomal suspensions (1.0 mg of protein per milliliter from neonates, treated as in *A*. ×——× (curve *C*), microsomal suspensions and treatment were the same as in *B*, except that the preparation was aerobic. Open cuvettes, containing the microsomal suspensions and NADPH, were left standing at room temperature for 10 min prior to spectral recording; no CO was added. - - - (curve *D*), microsomal suspensions (0.7 mg/ml) from adults, treated as described for *C*.

the neonate. The greater CO formation with concomitant diminution in the P-450 peak suggested greater instability of the neonatal hemoprotein. Perhaps the difference in peak heights of the spectra in the two preparations was related more to differences in autoxidizability of the P-450 in the two preparations. The effect of oxygen tension on the reduction of different species of P-450 has been reported (27).

Conversion of P-450 to P-420 by mersalyl. Further studies were conducted to establish whether enhanced lability was indeed characteristic of cytochrome P-450 from neonates, and whether a relationship existed between such lability and the anomalous spectra seen in microsomes from the neonate. The lability of microsomal P-450 was assessed as the rate of inactivation of the hemoprotein in the presence of mersalyl. Other investigators have shown that addition of mersalyl to microsomes causes quantitative but incomplete conversion of P-450 to P-420 (28, 29).

In preliminary studies using microsomes from the adult or neonate, it was observed that (a) 30 μM was the concentration of mersalyl needed to convert maximum amounts of P-450 to P-420 within any given time period, (b) higher concentrations (up to 60 μM) neither enhanced the conversion nor caused destruction of the hemoproteins, and (c) conversion of P-450 was incomplete even when microsomes were incubated overnight in the presence of 100 μM mersalyl. Incubation of adult or neonatal microsomal suspensions with 40 μM mersalyl caused a time-dependent conversion of P-450 to P-420 (Fig. 5). In both adult and neonatal microsomes conversion was biphasic, with a very rapid first phase followed by a slow second phase. Table 2 shows the rates of conversion during the fast and slow phases, the half-lives of the fast and slow phases, and the time at which the content of P-450 equaled that of P-420 ("transition time"). The results show that the half-life of the initial phase depended on the age of the animal, being 1–2 min in neonates and 10–14 min in adults. The rate of conversion during the initial phase was higher in neonates (0.16 nmole/min) than in adults (0.04 nmole/min). The half-life of the fast phase, as well as the transition time, was shorter in neonates than in adults. However, the slow phase displayed characteristics that were similar in microsomes from both adults and neonates. Semilogarithmic plots of these conversions changed neither the biphasic pattern nor the values of the parameters examined.

Substrate-induced difference spectra. Substrate-elicited spectral changes in microsomes are indicative of the interactions of substrates with cytochrome P-450 (21, 30, 31). Different spectral changes have

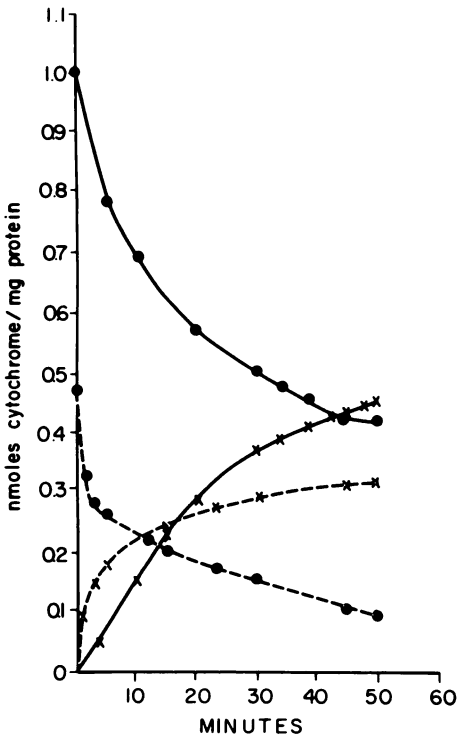

FIG. 5. *Conversion of cytochrome P-450 to P-420 by mersalyl as a function of time*

The sample and reference cuvettes contained microsomal protein (1 mg/ml), 40 μM mersalyl, and 2 mg of sodium dithionite, as described in MATERIALS AND METHODS. CO was added to the sample cuvette only. ●, decrease in P-450; ×, increase in P-420; ——, microsomes from adult (50-day-old) rats; - - -, microsomes from neonatal (5-day-old) rats. Each point is the mean of a minimum of three experiments on pooled livers.

been elicited by the same compound in microsomes from control rats and rats treated with polycylic hydrocarbons (32). Such treatment causes the induction of a distinct species of cytochrome P-450, which may be the cause of the differences (24, 25, 32). It was hoped that the type of spectral changes induced by substrates would indicate the absence or presence of a different type of P-450 in microsomal preparations from neonates. Figure 6 shows the spectral changes induced by the addition of various concentrations of aniline, ethylmorphine, or aminopyrine to liver microsomal suspensions form 7-, 14-, 21-, 30-, and 50-day-old rats. Accurate determination of K_s values for the substrates in microsomal suspensions from immature rats was hampered by the feeble spectral changes elicited. Aniline elicited type II difference spectra in microsomes from rats of all ages studied. However, in rats 30 days old or younger, the trough of the aniline-elicited spectra was asymmetrical between 390 and 410 nm at low concentrations of the drug; at higher concentrations of the drug, the spectral pattern showed only a single trough around 390 nm.

Ethylmorphine, at all concentrations studied, elicited only the typical type I spectral change in microsomes from rats of all ages studied. Other type I drugs, such as aminopyrine (Fig. 6A–D), hexobarbital, and phenobarbital (data not shown), elicited the type R-I difference spectral change in microsomes from rats 30 days old or younger. In microsomes from rats younger than 20 days, type I spectral changes were elicited only at very low concentrations (0.5 mM) of these substrates, above which the type R-I spectral change became prominent (data not shown). Between 20 and 30 days of age, intense type I spectral changes, which increased in magnitude with increasing substrate concentrations, were elicited by the substrates. At much higher concentrations, however, type R-I spectral changes, instead of type I, were seen. In microsomes from rats aged 50 days or more, only the typical type I spectral changes were elicited by these substrates at all concentrations. Results similar to those obtained with aminopyrine were observed with hexobarbital (data not shown).

Microsomal drug metabolism. Ethylmorphine was chosen as a prototype of type I drugs because of its ability to elicit only the type I spectral change in microsomes. Aniline was selected as the type II drug because it elicits only the type II spectral change. Livers from 7-day-old rats were chosen as the source of neonatal microsomes, since they were the youngest group of rats whose microsomes were obtained free of hemoglobin (as evidenced by the absence of a Soret peak in the oxidized-CO difference spectrum) and cytochrome P-420. This precaution was taken because these contaminants, com-

TABLE 2

Comparison of conversion of cytochrome P-450 to P-420 by mersalyl in microsomes from adult and neonatal rats

The contents of the cuvettes were the same as described for Fig. 5. Each value is the average of three experiments, with ranges of values in parentheses.

Measurement	Adult	Neonate
Concentration of P-450 at zero time (nmole/mg)	1.00	0.48
Half-life of first phase[a] (min)	12.0 (10–14)	1.5 (1.1–2.0)
Rate of conversion during first phase (nmole P-450/min)	0.04	0.16 (0.14–0.18)
Transition time[b] (min)	45.0 (39–51)	12.0 (10–14)
Half-life of second phase[c] (min)	62.0 (55–67)	35.0 (27–33)
Rate of conversion during second phase (nmole P-450/min)	0.005 (0.004–0.006)	0.006
Concentration of P-420 at 50 min (nmole/mg)	0.48	0.32
Concentration of P-420 at 50 min (nmole/mg)	0.48	0.32

[a] Initial, fast phase of conversion of P-450 to P-420.
[b] Time at which P-420 equaled P-450.
[c] Second, slow phase of conversion of P-450 to P-420.

monly abundant in neonatal microsomes, are known to catalyze the oxidation of various drug substrates (33).

Aniline hydroxylase activity was almost the same in microsomes from neonates as in those from adults (Table 3). In contrast, ethylmorphine *N*-demethylase activity in neonatal microsomes was only one-third the activity in adult microsomes. The values for ethylmorphine *N*-demethylase activity were lower than those reported by others (2, 34).

As observed earlier (Table 1), NADPH was 3 times more effective than NADH in reducing P-450 in microsomes from the adult. Contrary to the much greater activity of NADPH in reducing the P-450 in adult microsomes, NADH was as effective

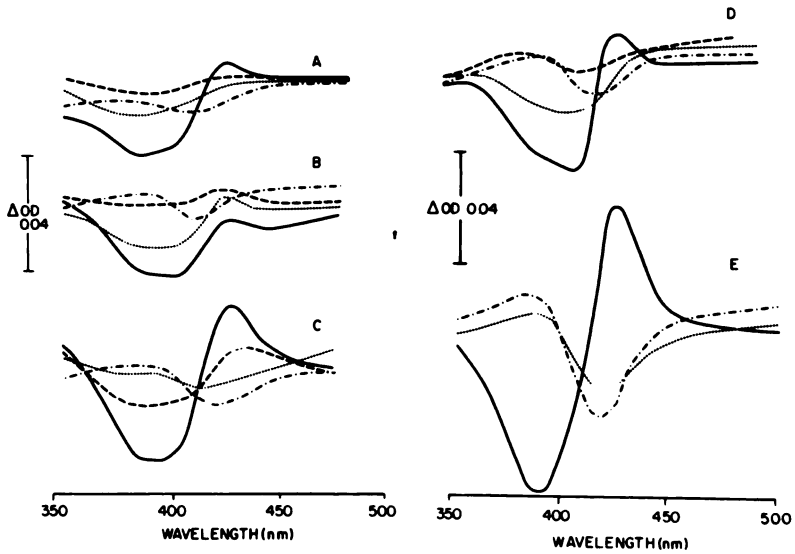

FIG. 6. *Spectral changes induced by aniline, aminopyrine, or ethylmorphine in microsomes from rats of various ages*

All microsomal suspensions contained 2 nmoles of cytochrome P-450 per milliliter. Ages were as follows: A, 7 days; B, 14 days; C, 21 days; D, 30 days; E, 50 days. ——, 5.0 mM aniline; - - -, 0.8 mM aminopyrine; · · · ·, 6.0 mM aminopyrine; -·-·, 3.0 mM ethylmorphine.

as NADPH in reducing the P-450 in neonatal microsomes. It was of interest, therefore, to investigate the effects of these nucleotides on enzymatic activity in microsomes from the neonate and the adult. Ethylmorphine *N*-demethylase was selected for this study because its behavior was different in microsomes from the adult and the neonate.

The effect of NADPH and/or NADH on ethylmorphine *N*-demethylase activity is also shown in Table 3. Taking the values obtained in the presence of NADPH as representative control activities, it can be seen that while NADH supported only 25% of the control activity in adult microsomes, the same nucleotide supported 67% of the control activity in microsomes from the neonate. When both nucleotides were present, ethylmorphine *N*-demethylase activity was 128% of the control activity of microsomes from the adult and 189% of the control value in the neonate (Table 3). The percentages of cytochrome P-450 in neonatal and adult microsomes that were reducible by either NADPH or NADH are shown in Table 1. It can be seen that the abilities of the individual nucleotides to reduce P-450 indeed paralleled their abilities to support ethylmorphine *N*-demethylase activity in the different microsomal preparations.

Microsomal lipid peroxidation. Carbon monoxide formation was higher in microsomes from neonates than in those from adults in the present study (see Fig. 4). Although this process was previously thought to result from the destruction of the heme of cytochrome P-450 (16) and reported to be related to microsomal lipid peroxidation (16, 35, 36), a more recent report has shown that microsomal lipids, and not heme, are the source of CO formed during lipid peroxidation (15). Microsomal lipid peroxidation causes a decrease in the drug-metabolizing activity of microsomes (16, 35–38). It seemed relevant, therefore, to examine the possible relationship of lipid peroxidation to the low drug-metabolizing activity in microsomes from the neonate.

Preliminary experiments to establish optimal conditions for microsomal lipid peroxidatic activity showed that its rate was inversely related to the concentration of either microsomal protein or P-450 (below 1 mg/ml or 1 nmole/ml, respectively). Above these low concentrations, the rate was inversely related to time of incubation for about 10 min, after which it remained constant. The diminution in rate was not due to product destruction, since added malondialdehyde was recovered quantitatively from incubation mixtures containing various concentrations of microsomal protein or cytochrome P-450 incubated for

TABLE 3

Effect of nucleotide addition on activities of ethylmorphine N-demethylase and aniline hydroxylase of hepatic microsomes of adult and neonatal rats

Microsomal protein (2 mg) from adult or neonatal rat liver, in 1 ml of 50 mM Tris-KCl buffer, pH 7.5, was incubated at 37° with constant shaking for 10 min. The incubation mixture also contained an NADPH-generating system (1.2 mM NADP, 6.0 mM $MgCl_2$, 9.0 mM trisodium isocitrate, and 9.1 units of isocitrate dehydrogenase) and/or 2 μmoles of NADH added at the beginning and after 5 min of incubation. The final concentrations of ethylmorphine and aniline were 4 and 8 mM, respectively. Values in parentheses are percentages of values obtained with NADPH only. Results are the means and standard deviations of three to five experiments.

Addition	Microsomes from adults		Microsomes from neonates	
	Ethylmorphine *N*-demethylase activity[a]	Aniline hydroxylase activity[b]	Ethylmorphine *N*-demethylase activity[a]	Aniline hydroxylase activity[b]
NADPH	67.6 ± 7.1 (100)	4.5 ± 2.6	23.4 ± 3.1 (100)	5.0 ± 0.6
NADH	17.2 ± 2.0 (25)	ND[c]	15.7 ± 2.2 (67)	ND
NADPH + NADH	86.6 ± 6.5 (128)	ND	44.3 ± 4.5 (189)	ND

[a] Expressed as nanomoles of formaldehyde per milligram of protein per 10 min.

[b] Expressed as nanomoles of *p*-aminophenol per milligram of protein per 10 min.

[c] Not determined.

different times. At the end of the incubation period there were extensive losses of P-450, which were greater in neonatal than in adult microsomes. Owing to the diminution of rate, 1 min, which was the time interval during which minimal amounts of P-450 were destroyed, was chosen as the incubation period.

Lipid peroxidatic activity was 3 times higher in neonatal than in adult microsomes (Table 4). The activity pattern also seemed inversely related to content of microsomal cytochrome P-450 (Table 4). The results agree with those of preliminary experiments in which rates of malondialdehyde formation were higher at low rather than high P-450 concentrations, suggesting that the neonatal system might be a more efficient peroxidatic system than that of the adult. As already pointed out, neonatal liver microsomes are characterized by low P-450 concentrations. A similar age-related pattern in lipid peroxidation has been reported in rat liver mitochondria (39).

Results from early studies indicated that in microsomes, drug-metabolizing activities were inversely related to lipid peroxidatic activities and were inhibited by drug substrates (40). In the present study, another method of comparing lipid peroxidatic activity in the neonate with that in the adult was to study the phenomenon in the presence of various drug substrates. The effects of certain drugs on lipid peroxidatic activity were different in neonatal than in adult microsomes (Fig. 7). Lipid peroxidatic activity in microsomes from 50-day-old male rats was inhibited 68% in the presence of aniline, 54% in the presence of aminopyrine, and 25% in the pres-

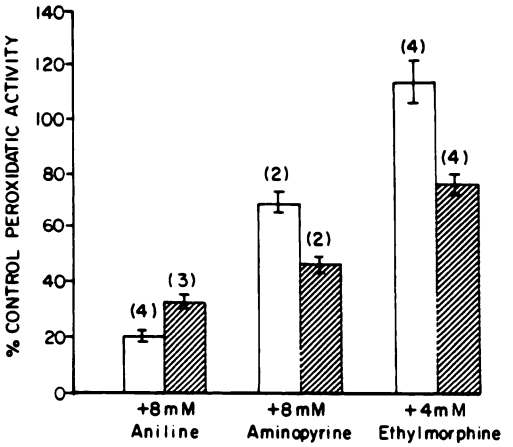

FIG. 7. *Effects of various drugs on microsomal lipid peroxidation in microsomes from adult and neonatal rat liver*

Hatched bars, adults; unshaded bars, neonates. Microsomal protein (2 mg) from adult or neonatal liver, in 1 ml of 50 mM Tris-KCl buffer, pH 7.5, was incubated at 37° with constant shaking for 10 min. The incubation mixture also contained an NADPH-generating system (1.2 mM NADP, 6.0 mM $MgCl_2$, 9.0 mM trisodium citrate, and 9.1 units of isocitrate dehydrogenase), 6 mM ADP, 0.3 mM $Fe(NH_4)_2(SO_4)_2$, and either 8 mM aniline, 8 mM aminopyrine, or 4 mM ethylmorphine. Malondialdehyde production was determined as described under MATERIALS AND METHODS. The numbers of experiments are shown in parentheses. Each experiment was performed with microsomes from either one adult liver or 5–10 pooled neonatal livers.

TABLE 4

Lipid peroxidatic activity in microsomes from rats of various ages

Microsomal protein (2 mg) from adult or neonatal rat liver, in 1 ml of 50 mM Tris-KCl buffer, pH 7.5, was incubated at 37° with constant shaking. The incubation mixture also contained an NADPH-generating system (1.2 mM NADP, 6.0 mM $MgCl_2$, 9.0 mM trisodium citrate, and 9.1 units of isocitrate dehydrogenase), 6 mM ADP, and 0.3 mM $Fe(NH_4)_2(SO_4)_2$. Malondialdehyde production was determined as described under MATERIALS AND METHODS. Values are the means and standard deviations of two to five experiments.

Age	Malondialdehyde, 1-min incubation, with respect to:		Malondialdehyde, 5-min incubation, with respect to:	
	Protein	P-450	Protein	P-450
days	*nmoles/mg*	*nmoles/nmole*	*nmoles/mg/min*	*nmoles/nmole/min*
5	14.6 ± 2.1	23.0 ± 2.9	6.3 ± 0.56	13.1 ± 1.00
15	10.8[a]	18.1[a]	4.8 ± 0.20	8.4 ± 0.40
50	4.4 ± 0.2	4.4 ± 0.3	2.6 ± 0.38	4.8 ± 0.32

[a] Single determination.

ence of ethylmorphine. In neonatal microsomes, in contrast, inhibition was 80% in the presence of aniline and 31% in the presence of aminopyrine; ethylmorphine exerted no inhibitory effect, and may even have been reponsible for a slight (10%) stimulation.

DISCUSSION

The results presented here demonstrate that certain spectral and enzymatic characteristics of neonatal rat liver microsomes differ from those of adults. Short and Smith (41) reported CO and EtNC spectra of reduced liver microsomes from fetal and neonatal swine similar to those in the present studies. These investigators, however, attributed their spectra not to different forms of cytochrome P-450 but to some unidentified hemoprotein in their preparations. Their observations (41) may be peculiar to the porcine system and suggestive of the presence of similar pools of cytochrome P-450 at all ages in this species. The observation that the decay of radioactive P-450 from the porcine liver microsomes was monophasic at all ages (11), in contrast with the biphasic pattern of decay in the rat (10), would support this point of view.

The pH intercept for the 430 and 455 nm absorption peaks of reduced-EtNC spectra of liver microsomes is about 7.4 in untreated adult rats, but shifts to 6.9 in microsomes from rats treated with polycyclic hydrocarbons. The significance of this shift is not clear, but it has been associated with the presence of a specific class of P-450 hemoproteins designated P_1-450 or P-448 (24, 42), each having distinct spectral and catalytic properties. These observations suggest that the shifts in the reduced-CO spectra and the EtNC equilibrium pH observed in liver microsomes from neonates may be taken as evidence for the presence of different hemoproteins. However, other factors may influence this shift, such as a different composition of the endoplasmic reticulum and/or a different configuration of the hemoprotein within the membrane matrix, and cannot be ruled out at present. Our studies with nucleotides indicated that the contribution of NADH to, or its reactivity with, mixed-function oxidase activity may be higher perinatally than in adult life. The higher fraction of P-450 reducible by NADH as well as the higher rate of *N*-demethylation of ethylmorphine in the presence of this nucleotide in neonates than in adults may also indicate differences in nucleotide specificity of the hemoprotein in the neonate.

The type R-I spectral changes observed with typical type I substrates in microsomes from neonates in the present studies have also been reported for liver microsomes from fetuses and neonates (7, 43) and animals treated with polycyclic hydrocarbons (32). It has been suggested that the type R-I spectral change is indicative of the presence of endogenous ligands at the type I binding sites of P-450. From this point of view, the preponderance of the R-I spectral change in the presence of typical type I compounds would indicate the presence of large amounts of endogenous ligands in microsomes from neonates. Alternatively, the type R-I spectral change may be additional evidence for the presence of P-450 in neonates differing from that predominant in adults. Relevant to this view is the idea that the type R-I spectral change may indicate the binding of substrates to the apoprotein at sites other than the type I binding site (44).

Our results on lipid peroxidation showed higher activity in microsomes from newborns than in those from adults. This may be accounted for, in part, by the greater amounts of unsaturated lipids in microsomes from neonates (12). Contrary to our results, Renton and Eade (45) observed lipid peroxidation to be greater in 25-day-old rats than in newborns. Perhaps accelerated destruction of the structure of membranes in microsomes from neonates could account for the lower rates of lipid peroxidation obtained by Renton and Eade (45). Their incubations were carried out for longer periods, and we have shown that prolonged incubation leads to a decrease in rates of lipid peroxidation. We have no immediate explanation for the diminished rates of this process with increasing microsomal protein or time. Since we had elim-

inated product destruction as a possible cause, other circumstances, such as product inhibition and/or the destruction of cofactors and enzymes supporting lipid peroxidation, can be considered.

Wolff and Bidlack (15) have speculated that the loss of P-450 during lipid peroxidation may be associated with the action of the hemoprotein as a peroxidase. Such action could account for the loss of P-450 observed in the present studies. The greater loss of the hemoprotein in microsomes from neonates than in microsomes from adults then would suggest, among other things, that the hemoprotein from neonates has greater peroxidase activity.

A clearer picture of the relationship of lipid peroxidation to drug metabolism was evident when lipid peroxidation was assayed in the presence of drug substrates. A differential effect of ethylmorphine on the lipid peroxidatic activities of adult and neonatal microsomal preparations was observed: 25% inhibition in contrast with a slight (10%) stimulation in neonates. This suggested that the mechanism of ethylmorphine metabolism may be different in the adult and neonate. The corollary is quite in agreement with the differential rates of *N*-demethylation of this substrate in adult and neonatal microsomal preparations. The inhibitory effects of aniline on lipid peroxidation, which were similar in adult and neonatal microsomal preparations, also agree with our observation that aniline hydroxylation proceeded at equal rates in adult and neonatal microsomal preparations. Such differential metabolism of two substrates by microsomal preparations from the neonate suggests substrate specificity, which is probably conferred by an atypical cytochrome P-450. We have presented evidence in the present study for the presence of such a hemoprotein in the liver of the neonatal rat.

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