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Effect of Inducers and Aging on Rabbit Liver Microsomal Drug-Metabolizing Enzymes

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SUMMARY

Rocket immunoelectrophoresis was used to quantitate the two major forms of rabbit liver cytochrome P-450, i.e., P-450$_{LM2}$ and P-450$_{LM4}$, in microsomes isolated from rabbit livers after treatment with inducers or during aging. In liver microsomes of untreated 100-day-old rabbits, P-450$_{LM4}$ is the predominant isoenzyme and accounts for 40% of the total cytochrome P-450 content. In contrast, P-450$_{LM2}$ is present in very low amounts in untreated rabbit liver microsomes. Treatment with phenobarbital induces P-450$_{LM2}$ to a level of about 33% of the total liver cytochrome P-450, whereas, β-naphthoflavone treatment increases the level of P-450$_{LM4}$ 4-fold, to a level of about 74% of total microsomal cytochrome P-450. Cholesterol or cholestyramine treatments also increase microsomal P-450$_{LM2}$ and P-450$_{LM4}$ levels. We also quantitated the levels of liver microsomal drug-metabolizing enzymes in rabbits of different age. Microsomes isolated from neonatal rabbit livers contain a very low level of (< 100 pmoles/mg of microsomal protein) cytochrome P-450. The specific content of either total cytochrome P-450 or P-450$_{LM4}$ is maximal in liver microsomes from 50- to 100-day-old rabbits, then decreases with increasing age. Treatment with phenobarbital increases the content of total microsomal cytochrome P-450 2-fold, and the induced P-450$_{LM2}$ becomes the major isoenzyme. NADPH-cytochrome P-450 reductase activity followed a similar age-dependent pattern. Phenobarbital treatment increased the specific activity of reductase for all age groups except 50-day-old rabbits. The level of cytochrome b_5 is low in neonatal liver, but increased to a maximal level at 50 days, and remained at this level for all age groups. Phenobarbital treatment did not significantly alter the concentration of cytochrome b_5. The change in P-450$_{LM2}$ content corresponds well with our previous report that the levels of P-450$_{LM2}$-mRNA and cytochrome P-450-associated drug-metabolizing activities change upon treatment with phenobarbital and during aging.

INTRODUCTION

The cytochrome P-450-containing mixed-function oxidase system is the major pathway for the detoxication of drugs and other xenobiotics, the activation of chemical carcinogens, and metabolism of endogenous compounds (1, 2). Multiple forms of cytochrome P-450 are present, depending on the species, tissue, type of inducer, age, and sex (2). The concentration and inducibility of multiple forms of cytochrome P-450 may have significant impact on drug toxicity and chemical carcinogenesis in target tissues of various animals.

Several forms of cytochrome P-450 have been purified from rabbit liver microsomes. P-450$_{LM2}$[1] and P-450$_{LM4}$ are the major forms induced by PB[2] and BNF, respectively (3), while P-450$_{LM4}$ is the predominant form present in untreated liver microcomes (3). The absolute quantities of P-450$_{LM2}$ and P-450$_{LM4}$ in microsomes are not known at present. We and others have shown that cholestyramine treatment increased cholesterol 7α-hydroxylase activity 2- to 3-fold in rats or rabbits (4–6). We have isolated P-450$_{LM4}$ from cholestyramine-treated rabbit liver microsomes which are able to catalyze the 7α-hydroxylation of cholesterol in the reconstituted enzyme system and has the same immunochemical properties and peptide map as P-450$_{LM4}$ isolated from liver microsomes of control, PB-treated, or BNF-treated rabbits (6). A high-choles-

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[1] The rabbit liver cytochrome P-450 isoenzymes are designated according to their relative electrophoretic mobilities in sodium dodecyl sulfate/polyacrylamide gel electrophoresis: P-450$_{LM2}$, the major phenobarbital-induced form (M_r = 49,000); P-450$_{LM4}$, the major β-naphthoflavone-induced form (M_r = 54,000).

[2] The abbreviations used are: PB, phenobarbital, BNF, β-naphthoflavone; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G.

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terol diet decreases or has no effect on cholesterol 7α-hydroxylase activity (4).

In neonatal animals, drug metabolism activity is low but increases with age until maturity. In senescent animals, liver drug metabolism activity is decreased (7). Recent reports also suggest that the levels of both cytochrome P-450 and NADPH-cytochrome P-450 reductase activity (7, 8) decrease with age in rats. Similar age-related changes in cytochrome P-450 levels and drug metabolism activities in liver were also observed in rabbits (9). Our previous results suggest that the alteration in cytochrome P-450 levels in liver microsomes parallels the changes in the levels of translatable mRNA specific for P-450$_{LM2}$ in different age groups of untreated and PB-treated rabbits (9). In the present study we used antibodies against purified P-450$_{LM2}$ and P-450$_{LM4}$ to quantitate two major forms of cytochrome P-450 in liver microsomes during induction and aging. Rocket immunoelectrophoresis provides a fast and convenient method for the quantitation of multiple forms of cytochrome P-450 in microsomes (10). The alteration of levels of P-450 reductase and cytochrome b_5 were determined using other methods.

MATERIALS AND METHODS

Animals. All rabbits (New Zealand White) were purchased from a local rabbitry. Young male rabbits about 100 days old and weighing approximately 11 kg were used for induction and purification of cytochrome P-450. They were maintained on PB-containing drinking water (0.1%, w/v, pH 7.4) for 5 days or given one single i.p. injection of BNF (80 mg/kg of body weight) 20 hr before sacrifice (3). Cholesterol and cholestyramine treatments were carried out by feeding rabbits a diet containing 1% cholesterol (J. T. Baker, analytical grade) or 4% cholestyramine (Mead Johnson) in 5% corn oil for 10 days (5). A regular light/dark cycle (2 p.m. to 2 a.m. light) was maintained (5). For the aging study, male rabbits of different ages were given two injections of PB (80 mg/kg of body weight per 24 hours), then killed 12 hr after the last injection (9). Pregnant rabbits were maintained on PB-containing drinking water for 6 days prior to delivery. Neonates, which were not sexed, were used within 6 hr after birth. Rabbits were killed by KCl (2 M) injection or decapitation (newborn).

Microsomes. Five or more rabbit livers were pooled in each group. Microsomes were prepared by the method of van der Hoeven and Coon (11) and suspended in 50 mM Tris buffer (pH 7.4) containing 0.1 mM EDTA and 20% glycerol.

Purification of cytochrome P-450. P-450$_{LM2}$ and P-450$_{LM4}$ were purified from liver microsomes isolated from PB- or BNF-treated rabbits according to the method of Haugen and Coon (3). The purified cytochrome P-450 showed only one major band when analyzed by SDS/polyacrylamide gel electrophoresis and had a specific content of 14–16 nmoles of heme per milligram of protein.

Preparation of antibodies against purified cytochrome P-450. Antibodies against P-450$_{LM2}$ and P-450$_{LM4}$ were raised in goats according to standard procedures (12). IgG fractions were prepared from antisera by ammonium sulfate fractionation and DEAE-cellulose chromatography (12). Antibodies against P-450$_{LM2}$ or P-450$_{LM4}$ cross-react only with the homologous antigen as characterized by Ouchterlony double-diffusion (13).

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis was performed according to the method of Pickett *et al.* (10). Agarose slabs (1% agarose, Bio-Rad, standard low electroendosmosis) contained 0.075 M Tris/barbital/sodium barbital buffer (pH 8.8), 0.5% sodium cholate, and 0.2% Emulgen 911. Goat anti-P-450$_{LM2}$ serum was added to the warm agarose gel mixture at a final concentration of 1 mg/ml. For the quantitation of P-450$_{LM4}$, goat anti-P-450$_{LM4}$ IgG was used at a concentration of 0.6 mg/ml. Microsomes or antigens were mixed in 0.25 M sucrose, 0.01 M Tris-HCl (pH 7.4), 0.2% Emulgen 911, and 0.5% sodium cholate. Plastic plates (Gel Bond, FMC, 85 × 100 mm) were used to cast 15-ml agarose gels. Antigen (15 μl) was applied in each well, and electrophoresis was performed at 120 V for 20 hr at 10°, using an LKB multiphore apparatus. Samples of purified antigen were always run as standards. Gels were washed, dried, and then stained with 0.5% Coomassie blue. After destaining, the area under each rocket-shaped precipitin peak was determined. A standard plot of antigen concentration versus area of the precipitin peak was used to quantify the antigen concentration in microsomes (10). All quantitations were done at least three times with a reproducibility of ±5%.

Other Methods. The concentration of total cytochrome P-450 in microsomes was determined from the CO difference spectra of the reduced protein using an extinction coefficient of 91 mM^{-1} cm^{-1} (14) for difference between A_{max} (in the 450-nm region) and $A_{490\ nm}$. NADPH-cytochrome P-450 reductase activity was assayed by its ability to catalyze the reduction of cytochrome *c* (15). One unit of reductase activity is defined as the amount of enzyme required to reduce 1 μmole of cytochrome *c* per minute at 30°. The concentration of cytochrome b_5 in the microsomes was determined from the NADH-reduced minus oxidized difference spectra using an extinction coefficient of 185 mM^{-1} cm^{-1} for $A_{424\ nm} - A_{409\ nm}$ (14). Protein concentrations were determined by the method of Lowry *et al.* (16), with bovine serum albumin as the standard. SDS/polyacrylamide gel electrophoresis followed the discontinuous pH buffer system of Laemmli (17).

RESULTS

Rocket immunoelectrophoresis of P-450$_{LM2}$ and P-450$_{LM4}$. Normal rocket-shaped precipitin patterns were obtained from the immunoelectrophoresis of purified antigen P-450$_{LM2}$ or P-450$_{LM4}$ into gels containing the homologous antibody. No cross-reactivity was observed when antigens were immunoelectrophoresed into the heterologous antibody. As can be seen from Fig. 1, the peak area is proportional to the concentration of P-450$_{LM2}$ or P-450$_{LM4}$ in the range from 0.02 to 0.10 mg/ml of antigen. The slope of the standard curve for P-450$_{LM4}$ is smaller than that for P-450$_{LM2}$, indicating that the titer of antibody against P-450$_{LM4}$ is lower than that of LM$_2$ (3, 18). This is also the reason why P-450$_{LM4}$ IgG was used for

immunoelectrophoresis. Similar to the report on the quantitation of rat cytochrome P-450 (10), the standard curves for rabbit cytochrome P-450 do not cross the origin. Rocket immunoelectrophoresis of liver microsomes from PB- and BNF-treated rabbits also generates single rocket-shaped precipitin peaks when allowed to react with antibodies against P-450$_{LM2}$ or P-450$_{LM4}$, respectively. A linear relationship between peak area and microsomal protein concentration was also obtained.

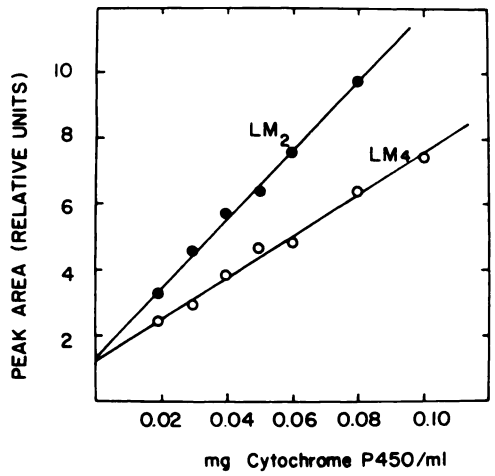

FIG. 1. *Rocket immunoelectrophoretic assays of purified cytochrome P-450*

Assays were carried out using agarose gel containing antiserum (1 mg/ml) against P-450$_{LM2}$ or IgG (0.6 mg/ml) against P-450$_{LM4}$ as described under Materials and Methods. ●, Areas of the precipitation peaks are plotted versus P-450$_{LM2}$ concentrations; ○, areas of the precipitation peaks are plotted versus P-450$_{LM4}$ concentrations.

Effects of inducers on the quantity of P-450$_{LM2}$ and P-450$_{LM4}$ in rabbit liver microsomes. Figure 2 shows an SDS/polyacrylamide gel of liver microsomal preparations from control, PB-, BNF-, cholestyramine- and cholesterol-treated rabbits. P-450$_{LM4}$ was present in all microsomes; in contrast, P-450$_{LM2}$ was present in significant amounts only in liver microsomes of PB-treated rabbits. Cholestyramine treatment clearly induced P-450$_{LM2}$; in addition, two bands with molecular weight between P-450$_{LM2}$ and P-450$_{LM4}$ are also visible in this microsomal preparation, possibly LM$_{3b}$ and LM$_{3C}$ (19). However, cholesterol treatment did not change the microsomal polypeptide pattern. The rocket immunoelectrophoretic patterns of P-450$_{LM2}$ and P-450$_{LM4}$ in various microsomal preparations are shown in Fig. 3. P-450$_{LM4}$ is present in liver microsomes isolated from untreated, PB-, BNF-, cholestyramine-, or cholesterol-treated rabbits. BNF treatment clearly increased the level of P-450$_{LM4}$ in the microsomes. In contrast, P-450$_{LM2}$ was present in significant quantities in liver microsomes isolated from PB-treated rabbits. In these experiments, large amounts of microsomal protein (3 mg/ml) were used in order to detect the low level of P-450$_{LM2}$ present in liver micro-

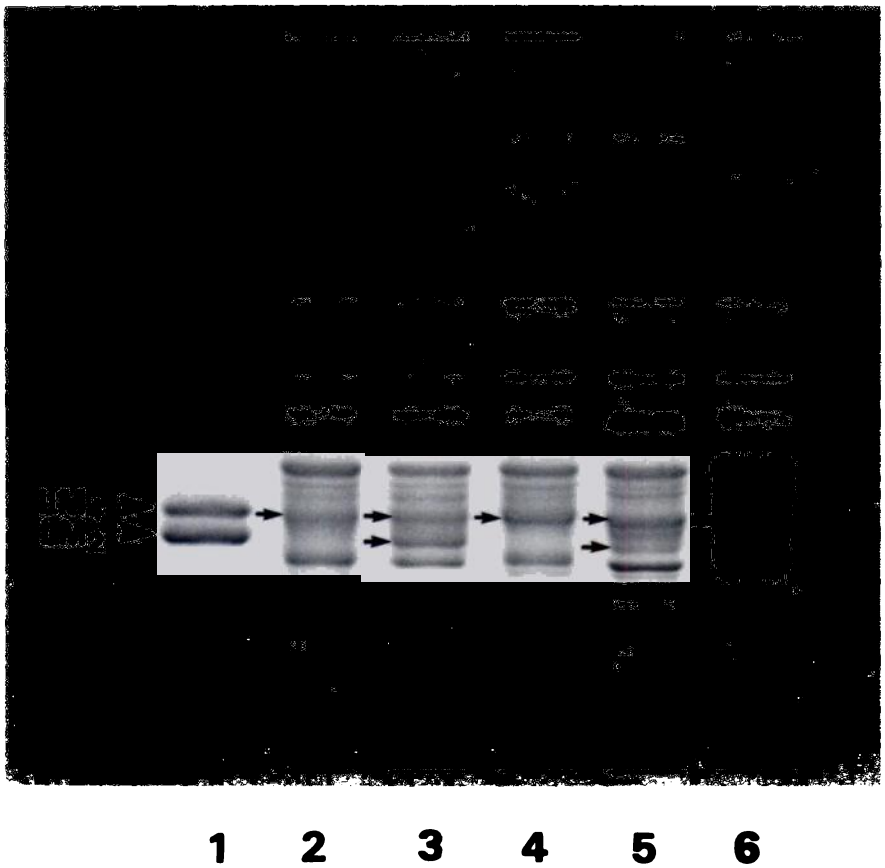

FIG. 2. *SDS/polyacrylamide gel electrophoresis of rabbit liver microsomes*

Slab gels (1.5 mm thick) were used as described under Materials and Methods. Microsomal protein (60 μg) was applied to each well. Samples analyzed were as follows: Well *1*, purified P-450$_{LM2}$ and P-450$_{LM4}$, 6 μg each; Wells *2–6* represent liver microsomes from control (*2*), PB-treated (*3*), BNF-treated (*4*), cholestyramine-treated (*5*), and cholesterol-treated (*6*) rabbits.

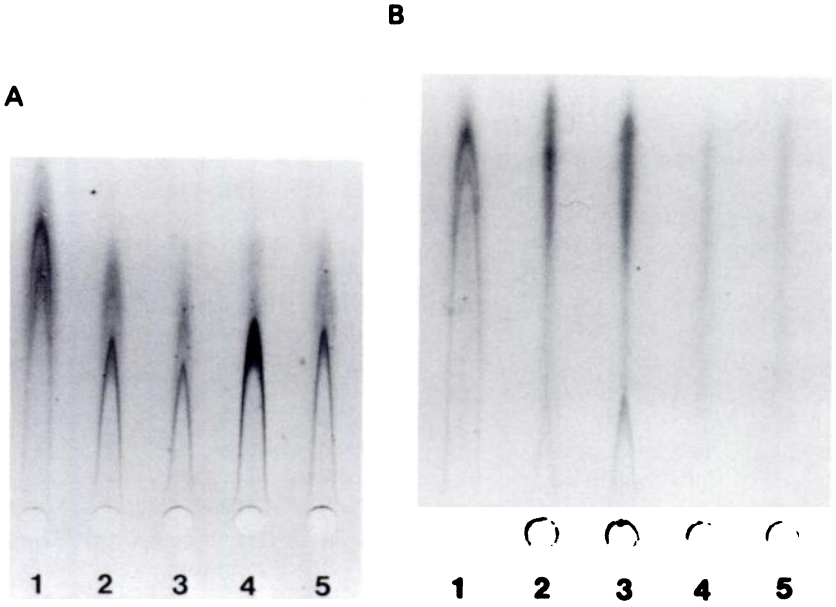

FIG. 3. *Rocket inmunoelectrophoretic profiles of cytochrome P-450*

A. Solubilized microsomes (1 mg/ml) were immunoelectrophoresed in gels containing the P-450$_{LM4}$ IgG fraction. Wells *1-5* represent liver microsomes isolated from rabbits pretreated with BNF (*1*), PB (*2*), untreated (*3*), cholestyramine (*4*), and cholesterol (*5*).

B. Solubilized microsomes (3.0 mg/ml) were immunoelectrophoresed in gels containing P-450$_{LM2}$ antiserum. Wells *1-5* represent liver microsomes isolated from rabbits pretreated with PB (*1*), BNF (*2*), cholestyramine (*3*), cholesterol (*4*), or untreated (*5*).

somes from untreated, BNF-, cholestyramine-, or cholesterol-treated rabbits. A very faint precipitin peak could be detected in these liver microsomes, especially in cholestyramine- and BNF-treated rabbits. Except for PB and cholestyramine treatment, the quantitation of the precipitation peaks in microsomes could not be made with accuracy. Streaks were also seen in these gels, possibly due to the low antigen and high protein concentrations. Some of these gels contained a faint peak above or below the major peaks. This may be the characteristic of the antigen (20), especially when high protein concentrations were used in immunoelectrophoresis.

The quantities of P-450$_{LM2}$ and P-450$_{LM4}$ in various liver microsomal preparations were calculated and are summarized in Table 1. In the untreated microsomes, P-450$_{LM4}$ was the predominant cytochrome P-450 isoenzyme and accounted for about 40% of total microsomal cytochrome P-450. The level of P-450$_{LM2}$ in the same microsomes was estimated to be less than 5%. PB treatment induced P-450$_{LM2}$ to be the predominant isoenzyme in microsomes. The specific content of P-450$_{LM4}$ was also increased significantly by PB, although it represents a smaller percentage of total cytochrome P-450 in PB-treated microsomes than in untreated microsomes. BNF treatment increased the P-450$_{LM4}$ level 4-fold, which represents about 74% of total microsomal cytochrome P-450. Treatment of rabbits with a diet containing 4% cholestyramine or 1% cholesterol significantly increased the P-450$_{LM4}$ content. In addition, the P-450$_{LM2}$ level was also increased in cholestyramine microsomes. It is interesting to note that the content of unidentified cytochrome P-450 (constitutive forms, other than P-450$_{LM2}$ and P-450$_{LM4}$) was about 1.0 nmoles/mg in all microsomal preparations except in microsomes of PB-treated rabbits, which contained higher levels (1.4 nmoles/mg) of unidentified cytochrome P-450. This fact suggests that PB may induce other form(s) of cytochrome P-450 beside P-450$_{LM2}$ in rabbit liver.

Effects of aging on the levels of rabbit liver microsomal cytochrome P-450, NADPH-cytochrome P-450 reductase, and cytochrome b_5. The specific content of total cytochrome P-450 in liver microsomes varied with the age of the rabbit. In our hands, cytochrome P-450 levels in neonatal liver microsomes were too low to be detected with accuracy by spectrophotometry. This was also evidenced by the lack of polypeptide bands in the molecular weight range 45,000–60,000 (9). Ouchterlony double-immunodiffusion experiments did not detect the presence of either P-450$_{LM2}$ or P-450$_{LM4}$ in neonatal liver micro-

TABLE 1

Quantitation of cytochrome P-450$_{LM2}$ and P-450$_{LM4}$ in liver microsomes of 100-day-old rabbits by rocket immunoelectrophoresis

Microsomal induction	Specific content of total cytochrome P-450 in microsomes[a]	P-450$_{LM2}$ in microsomes		P-450$_{LM4}$ in microsomes[a]	
	nmoles/mg	*nmoles/mg*[b]	*% total P-450*	*nmoles/mg*[b]	*% total P-450*
Control	1.92	0.09[c]	4.7	0.75	39
PB	3.68	1.22	33.0	1.02	28
BNF	3.90	0.07[c]	1.8	2.88	74
Cholestyramine	2.40	0.21	8.8	1.28	53
Cholesterol	1.98	0.07[c]	3.5	1.01	51

[a] Values determined by reduced CO difference spectra.

[b] Values represent mean of four determinations by rocket immunoelectrophoresis.

[c] Values are estimated. The area of the precipitation peaks could not be determined with complete accuracy.

somes (data not shown). Rocket immunoelectrophoresis also failed to reveal a cytochrome P-450-antibody precipitin line for the neonatal liver microsomes (data not shown). Upon treatment with PB, a band with molecular weight corresponding to P-450$_{LM2}$ was induced in the neonatal liver microsomes. Analysis by rocket immunoelectrophoresis showed a faint peak, and the level of P-450$_{LM2}$ was estimated to be about 0.026 nmole/mg, which is about 7% of total microsomal cytochrome P-450 in neonatal microsomes (Fig. 4A). P-450$_{LM2}$ levels were high in 50- and 100-day-old rabbit liver and clearly decreased with age. The level of P-450$_{LM4}$ was too low to be detected in neonatal liver microsomes of PB-treated rabbits (Fig. 4B), but was high in 50- and 100-day-old rabbits and decreased significantly in 300- and 830-day-old rabbits. The specific content of total liver microsomal cytochrome P-450 and P-450$_{LM4}$ was maximal in 50- to 100-day-old rabbits (Fig. 5A). In older rabbits, the total microsomal cytochrome P-450 content decreased significantly; however, the level of P-450$_{LM4}$ also decreased but to a lesser extent. PB treatment increased total microsomal cytochrome P-450 concentration about 2-fold for all age groups. However, the specific content of total cytochrome P-450 as well as P-450$_{LM2}$ and P-450$_{LM4}$ decreased with age (Fig. 5B). P-450$_{LM2}$ and P-450$_{LM4}$ represent about 30% and 22%, respectively, of total cytochrome P-450 in liver microsomes of PB-treated rabbits of all age groups except neonates. These results indicate that total and specific forms of cytochrome P-450 decrease with age, but the inducibility of P-450$_{LM2}$ by PB treatment does not alter with age.

The levels of NADPH-cytochrome P-450 reductase also followed a similar age-dependent change in rabbit liver microsomes (Fig. 6). The specific activity of reductase as measured by its ability to reduce cytochrome *c*, although low, was still detectable in neonatal liver microsomes. Fifty-day-old rabbits had the highest liver microsomal reductase specific activity, which then decreased with an increase in rabbit age. PB treatment significantly increased reductase activity in liver microsomes of all age groups except 50-day-old rabbits. The induction of reductase activity was higher in neonates and older rabbits. Consequently, NADPH-cytochrome P-450 reductase activities were about the same in adult rabbit liver microsomes, irrespective of the aging process.

We also examined the level of rabbit liver microsomal cytochrome b_5 in rabbits of different age (Fig. 7). In neonatal liver microsomes, the level of cytochrome b_5 was low, and was increased significantly with age and PB treatment. In older rabbits, cytochrome b_5 concentration remained relatively constant throughout the age range studied. PB treatment did not significantly alter the specific content of cytochrome b_5 in older rabbit liver microsomes.

DISCUSSION

This is the first detailed report on the quantitation of P-450$_{LM2}$ and P-450$_{LM4}$ in rabbit liver microsomes. Our results indicate that P-450$_{LM4}$ is the predominant isoenzyme in microsomes from untreated rabbits, and other isoenzymes of cytochrome P-450 (60%) are constitutive forms, i.e., LM$_{3b}$ and LM$_{3c}$. BNF treatment induced the total cytochrome P-450 2-fold and enhanced the P-450$_{LM4}$ level to about 74% of the total cytochrome P-450, which actually represents a 4-fold induction relative to its specific content in untreated microsomes. In contrast, cytochrome P-448, the 3-methylcholanthrene-induced form in rats, is present in very low levels in liver microsomes of untreated rats (10, 13). PB treatment increased total cytochrome P-450 content 2-fold; however, P-450$_{LM2}$ became the predominant isoenzyme in PB-induced microsomes, and we estimate at least a 12-fold induction of the enzyme. Similarly, the major form of cytochrome P-450 in PB-treated rats also represents about 36% of the total cytochrome P-450 in liver microsomes (10). Recently, Guengerich *et al.* (21) reported the quantitation of cytochrome P-450 isoenzymes in rats, rabbits, and humans using immunochemical staining coupled with SDS/polyacrylamide gel electrophoresis. Their results showed lower levels of P-450$_{LM4}$ in BNF-induced microsomes and higher levels of P-450$_{LM2}$ in PB-induced microsomes than our results. The discrepancy between these two reports

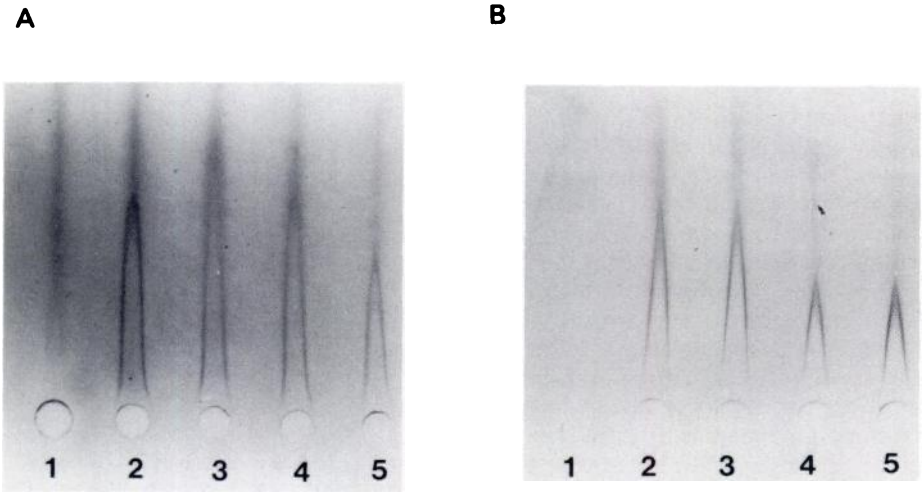

FIG. 4. *Rocket immunoelectrophoretic profiles of P-450$_{LM2}$ and P-450$_{LM4}$ in liver microsomes of PB-treated rabbits*

Solubilized microsomes (1.5 mg/ml) were immunoelectrophoresed in gels containing (A) P-450$_{LM2}$ antiserum or (B) P-450$_{LM4}$ IgG. Wells *1–5* represent liver microsomes isolated from neonatal, 50-day-old, 100-day-old, 300-day-old, and 830-day-old rabbits pretreated with PB.

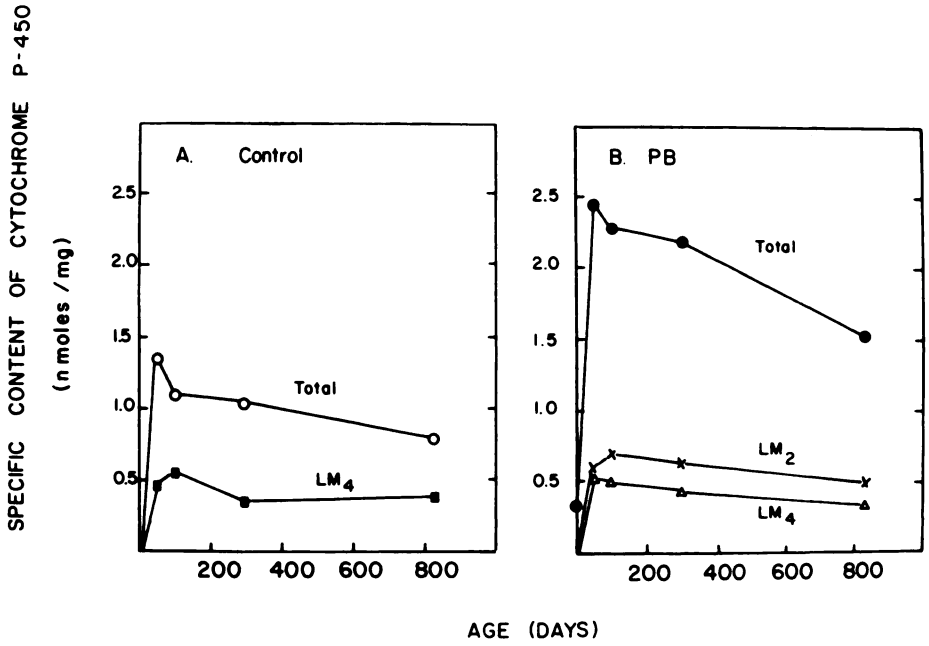

FIG. 5. *Alterations in liver microsomal cytochrome P-450 content with age*

A. Cytochrome P-450 content in liver microsomes of untreated rabbits. ○, Specific contents of total cytochrome P-450 as determined by reduced CO-complex difference spectra; ■, specific contents of P-450$_{LM4}$ as determined by rocket immunoelectrophoretic assays.

B. Cytochrome P-450 content in liver microsomes of PB-treated rabbits. ●, Specific contents of total cytochrome P-450 as determined by difference spectra of the reduced CO-complex. Specific contents of P-450$_{LM2}$ (×) and P-450$_{LM4}$ (△) were determined by rocket immunoelectrophoresis.

may be due to the different techniques employed or the antibodies they used were prepared using rat liver cytochrome P-450s.

All treatments with inducers increased microsomal P-450$_{LM4}$ content. We also found from our isolation and purification procedures that P-450$_{LM4}$ isolated from PB-induced microsomes always has higher specific content and over-all yield than P-450$_{LM4}$ isolated from control livers. In addition, P-450$_{LM4}$ preparations isolated from liver microsomes of control, PB-, BNF-, and cholestyramine-treated animals are apparently identical with respect to their catalytic activity (except P-450$_{LM4}$ from microsomes of cholestyramine-treated rabbits), immunochemical properties, and peptide maps (6). Cholestyramine treatment increased cholesterol 7α-hydroxylase activity by 3-fold in rabbit liver microsomes (6) and also increased the P-450$_{LM4}$ content (Table 1). We have previously reported that P-450$_{LM4}$ isolated from liver microsomes of cholestyramine-treated rabbits is unique in that it is active in reconstitution of cholesterol 7α-hydroxylase

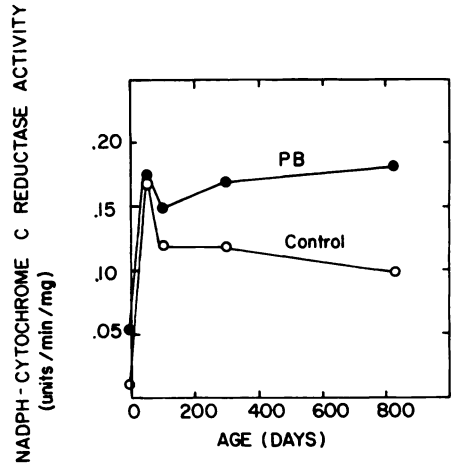

FIG. 6. *Alterations in microsomal NADPH-cytochrome P-450 reductase activity with age*

Specific activity was measured as described under Materials and Methods. Specific activity in liver microsomes from PB-treated (●) or untreated (○) rabbits.

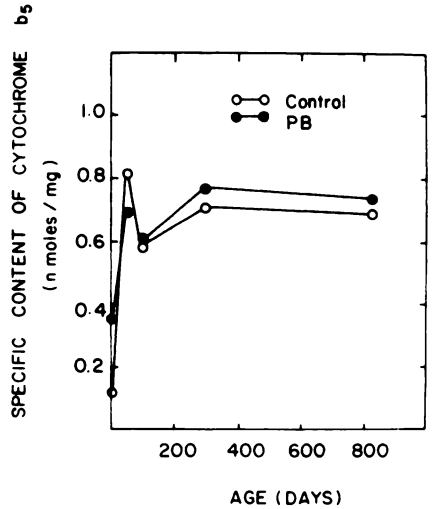

FIG. 7. *Alteration of microsomal cytochrome b_5 content with age*

Cytochrome b_5 was determined by reduced minus oxidized difference spectra as described under Materials and Methods. Specific content of cytochrome b_5 in liver microsomes from PB-treated (●) or untreated (○) rabbits.

(6). Cholestyramine treatment also increased the microsomal P-450$_{LM2}$ content. Our quantitative results also suggest that PB may induce other isoenzymes in microsomes—this is in contrast to BNF, which appears to induce only P-450$_{LM4}$. It is not known at present whether any of the constitutive forms, such as LM$_{3b}$ or LM$_{3c}$, are induced by PB or other inducers. However, our results suggest that LM$_{3b}$ and LM$_{3c}$ levels may be increased by cholestyramine treatment. These constitutive forms of cytochrome P-450 remain to be quantitated.

Previously we reported that the changes in drug metabolism activity for different age groups of rabbits corresponds to the changes in the total microsomal cytochrome P-450 and to the level of mRNA coding for P-450$_{LM2}$ (9). With the quantitation method established in this report, we were able to determine the levels of P-450$_{LM2}$ and P-450$_{LM4}$ in liver microsomes of rabbits of different ages. Our results suggested that P-450$_{LM2}$ and P-450$_{LM4}$ levels also follow an age-dependent change. This would explain observed changes in benzphetamine *N*-demethylase and 7-ethoxycoumarin *O*-deethylase activities (9). It should be mentioned that P-450$_{LM2}$ is 4- to 5-fold more active toward these two substrates than P-450$_{LM4}$ in the reconstituted enzyme system (22). The P-450$_{LM2}$ level also parallels the level of P-450$_{LM2}$-mRNA in the livers of rabbits of different age groups (9). Schwab *et al.* (23) reported that P-450$_{LM2}$ was induced by PB in neonatal rabbit livers. They also reported that benzphetamine *N*-demethylase activity in neonatal liver microsomes was 3-fold higher than that in adult liver microsomes. In contrast, our results revealed a much lower induction in P-450$_{LM2}$ and benzphetamine *N*-demethylase activity in neonatal liver microsomes upon treatment with PB (9). This is in accord with our previous report that mRNA for P-450$_{LM2}$ is present in very low levels in neonatal liver microsomes even after induction with PB (9). The reason for the discrepancy between our results and those of Schwab *et al.* (23) is probably due to different PB treatment and ages or sources of rabbits.

The induction of NADPH-cytochrome P-450 reductase in rat livers by PB has been reported (24). Recent experiments on the *in vitro* and the *in vivo* synthesis of reductase suggested that the level of translatable mRNA coding for NADPH-cytochrome P-450 reductase was increased by PB treatment (24–26). Also Schmucker and Wang (8) recently reported an age-dependent decline in NADPH-cytochrome P-450 reductase activity in rats which was thought to be due to the age-dependent alteration of the properties of the reductase. This study also revealed that the rate-limiting enzyme in microsomal cytochrome P-450-dependent drug metabolism, NADPH-cytochrome P-450 reductase, followed an age-dependent change in activity. However, we do not know whether the change in microsomal reductase activity is due to a decreased turnover number of existing reductase or a decreased enzyme level. This study will be carried out when an antibody against reductase is obtained. It is likely that the decrease in drug metabolism activity in older rabbit liver microsomes reflects both the alteration in levels of cytochrome P-450 isoenzymes and in NADPH-cytochrome P-450 reductase activity.

Cytochrome b_5 has been suggested to play a role in cytochrome P-450-dependent drug metabolism (22, 27). Our results indicate an age-dependent change in cytochrome b_5 concentrations in rabbit liver microsomes; however, cytochrome b_5 levels are relatively constant in older rabbit liver microsomes. PB treatment increased the cytochrome b_5 level in neonatal microsomes but had no significant effect on older rabbits. PB treatment has been shown to have no effect on the level of translatable mRNA specific for cytochrome b_5 in rat livers (26).

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