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Tissue- and Species-Dependent Expression of Multiple Forms of Mammalian Microsomal Flavin-Containing Monooxygenase

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SUMMARY

The immunochemical relatedness, multiplicity, and expression of microsomal flavin-containing monooxygenases were assessed in hepatic, pulmonary, and renal microsomal preparations from adult, male rabbits, mice, rats, guinea pigs, and hamsters. Preparations from adult female (pregnant and nonpregnant) and immature male and female rabbits were also examined. Microsomes were analyzed by immunoblotting with polyclonal antibodies to flavin-containing monooxygenases purified from pig, mouse, and rabbit liver, and rabbit lung. Pulmonary flavin-containing monooxygenases, which differ from the major forms of the enzyme present in liver, were detected in pulmonary samples from all species. The major hepatic enzymes, or very closely related proteins, were also detected in pulmonary samples from every species except the rabbit. Three forms of pulmonary flavin-containing monooxygenase are found in rabbit and guinea pig. Differences in the expression of these three forms were observed with rabbits; all three were detected with some individuals and only two with others. The hepatic form of the flavin-containing monooxygenase is present in kidney of all species examined, and the pulmonary form is detected in kidney of rabbits, mice, and hamsters, but not rats or guinea pigs. Kidneys and lungs of individual rabbits were of the same phenotype with respect to the expression of the pulmonary forms of the enzyme. The findings show that the expression of flavin monooxygenase isozymes is tissue and species dependent.

The oxidative metabolism of a variety of drugs, pesticides, and other chemicals can be catalyzed by the microsomal flavin-containing monooxygenase (flavin monooxygenase), EC 1.14.13.8 (1, 2). Most of the metabolites formed by this enzyme are biologically inactive and can be excreted; however, some are particularly reactive and potentially carcinogenic or mutagenic (1). The physiologial role of the flavin monooxygenase is uncertain but it may be related to the formation of disulfide bonds during protein synthesis (3).

A great deal is known about the flavin monooxygenase purified from pig liver (1, 2), but the properties of the enzyme from other species and from extrahepatic tissues are not well defined. It is clear, however, that significant differences between hepatic and pulmonary flavin monooxygenases do exist. The first indication of this was provided by Devereux *et al.* (4) who reported marked differences between the effects of Hg^{2+} on the activities of flavin monooxygenases partially purified from rabbit liver and lung. As it turns out, the flavin monooxygenases from liver and lung are isozymes with different apparent monomeric molecular weights and immunochemical properties (5–7). In addition, catalytic differences exist; primary alkylamines appear to be substrates only for the pulmonary isozyme (8), and some tricyclic antidepressants only for the hepatic isozyme (5). With most other sulfur-, nitrogen-, or phosphorous-containing substrates, the activities of the two isozymes appear to be fairly similar (9). Marked species differences among pulmonary flavin monooxygenases may also exist. Pulmonary flavin monooxygenase activity with chlorpromazine or imipramine ranges from reasonably high in the mouse to undetectable in the rabbit (10, 11). In the same species, however, pulmonary activities with other flavin monooxygenase substrates are very similar (9, 12).

We have now used antibodies to a pulmonary and several hepatic flavin monooxygenases to examine the isozyme composition present in microsomal preparations from liver, lung, and kidney of several species. The findings provide an explanation for the large differences among species in pulmonary flavin monooxygenase activities and indicate that expression of flavin monooxygenase isozymes in different tissues and species is highly complex.

Materials and Methods

Animals. New Zealand White rabbits (4 or 25 weeks old) were purchased from Dutchland Farms (Denver, PA); Fisher and Sprague-Dawley rats (9–12 weeks old), Hartley guinea pigs (8–10 weeks old), and Syrian Golden hamsters (13 weeks old) were from Charles River

ABBREVIATIONS: EDTA, ethylenediaminetetraacetate; anti-P, antibody to pig liver enzyme; anti-M, antibody to mouse liver enzyme; anti-lung, antibody to rabbit pulmonary enzyme.

Breeding Laboratories (Wilmington, MA); Long Evans rats (9–12 weeks old) were from Blue Spruce Farms (Altamont, NY); C57BL/J mice were from The Jackson Laboratory (Bar Harbor, ME); and Dub:ICR mice (9–12 weeks old) were from Dominion Laboratories (Dublin, VA). Pig livers were obtained from the Jesse Jones Company (Garner, NC). Experiments were carried out with males except when noted otherwise.

Preparation of microsomes. Tissues pooled from 10 rabbits, 40 mice, 12 rats, 6 guinea pigs, or 6 hamsters were used for the preparation of renal, pulmonary, and hepatic microsomes by standard procedures (13). Microsomes were also prepared from the tissues of individual rabbits. Microsomal preparations were stored suspended in sucrose (0.25 M) at −20°.

Enzyme purification and raising of antibodies. Mouse (ICR) and pig hepatic flavin monooxygenases were purified by the method of Sabourin *et al.* (14). Hepatic and pulmonary flavin monooxygenases were purified from adult male rabbits by the method of Tynes *et al.* (8). The purified proteins were injected intradermally into goats, antisera were obtained, and IgG was isolated by ammonium sulfate precipitation and anion chromatography (15). Antibodies to cytochrome P-450 isozyme 2 have been described previously (16).

Electrophoresis, immunoblotting, and immunostaining. Electrophoresis of microsomal proteins was on polyacrylamide gels (7.5%) in the presence of sodium dodecyl sulfate according to the method of Laemmli and Favre (17). Samples were treated with buffer containing β-mercaptoethanol and were heated at 95° for 3 min prior to electrophoresis unless otherwise specified. Following electrophoresis, the separated proteins were transferred to nitrocellulose sheets and stained by a modification (18) of the method of Towbin *et al.* (19). The nitrocellulose sheets were then treated sequentially with bovine serum albumin (3%) for 15 min at 40°, flavin monooxygenase antibodies for 60 min at room temperature, rabbit anti-goat IgG (1:100), and goat peroxidase-antiperoxidase (1:3000). Quantitation of immunostaining was accomplished with a soft laser densitometer (Zeineh) by comparison of sample staining intensities with those of standards. Apparent monomeric molecular weights on polyacrylamide gels were determined by comparison with bovine serum albumin (66,000), catalase (60,000), fumarase (50,000), cytochrome P-450 isozyme 2 (52,000), and cytochrome P-450 isozyme 5 (58,000).

Analytical methods. Rates of flavin monooxygenase-catalyzed oxidation of NADPH were determined using a UV-vis spectrophotometer (Aminco DW-2a) in the split-beam mode. Measurements were made at 340 nm with NADPH and enzyme in the sample cuvette and NADPH in the reference cuvette (9). Standard incubations contained Tricine buffer (100 mM, pH 8.4), NADPH (0.125 mM), EDTA (1.0 mM), Emulgen 911 (1.0%), *n*-octylamine (3 mM), and microsomal protein (0.2–0.6 mg/ml). Incubations were run at 37° with thiourea (1 mM) as the substrate. Protein concentrations were determined by the method of Lowry *et al.* (20).

Materials. All immunochemical reagents were purchased from Cappel Laboratories (Cochranville, PA); Tricine, NADPH, and *n*-octylamine were from Sigma Chemical Co. (St. Louis, MO); and thiourea was from Fisher Scientific Co. (Fairlawn, NJ). Emulgen 911 was a gift from the Kao-Atlas Co. (Tokyo, Japan). All other chemicals used were reagent grade or better and were purchased from commercial sources.

Results

Immunoreactivities of antibodies with purified flavin monooxygenase isozymes. The reactivities of antibodies to hepatic or pulmonary flavin monooxygenases with purified flavin monooxygenase are tissue, but not species, specific. For example, anti-P detects purified pig or mouse liver flavin monooxygenase on immunoblots but shows little recognition of the purified rabbit lung enzyme (Fig. 1). Similar results were obtained with anti-M. The anti-lung antibody showed the opposite specificity: recognition of the purified lung enzyme, but

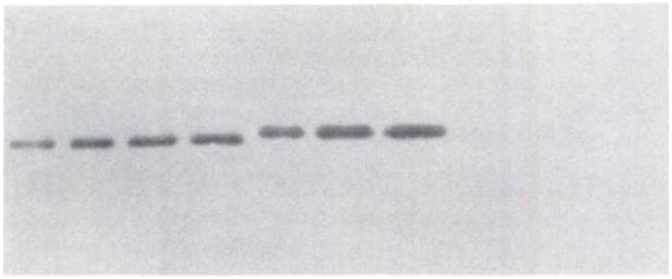

Fig. 1. Reactivity of anti-P with purified hepatic and pulmonary flavin monooxygenases. Purified pig liver flavin monooxygenase (lanes *1–4;* 0.025, 0.05, 0.1, and 0.2 μg of protein, respectively), purified mouse liver flavin monooxygenase (lanes *5–7;* 0.1, 0.2, and 0.3 μg of protein, respectively), and purified rabbit lung flavin monooxygenase (lanes *8–10;* 0.1, 0.2, and 0.3 μg of protein, respectively) were immunoblotted.

little or no reactivity with the pig or mouse hepatic enzymes. The relative staining intensities obtained with the three purified enzymes and three antibody preparations are shown in Fig. 2. Results obtained with anti-P and anti-M were nearly identical: maximum intensity with the primary antigen, about 50% of maximum with the hepatic enzyme from the other species, and less than 5% of maximum with the rabbit lung enzyme. The reactivity of anti-lung with the two hepatic enzymes was also less than 5% of the reactivity obtained with the rabbit lung enzyme.

Reactivity of anti-P, anti-M, and anti-lung with microsomal proteins. The reactivity of anti-P with proteins in hepatic microsomal preparations from mouse, rabbit, rat, hamster, pig, and guinea pig examined by immunoblotting is shown in Fig. 3. A single band of staining was observed with all species, results that were also obtained with anti-M and antibodies to the rabbit liver enzyme. The apparent monomeric molecular weights of the proteins detected were between 56,000 and 59,000. Reactivity of anti-lung with pulmonary microsomal proteins was also observed across species lines; results for rabbit, hamster, guinea pig, and mouse are shown in Fig. 4. Three bands of staining, all having mobilities less than that of the protein detected in liver by anti-P or anti-M, were evident with pulmonary microsomes from guinea pig. Although less apparent, multiple bands were also seen with pulmonary microsomes from rabbit. With the rabbit, however, the mobilities of the pulmonary proteins were all greater than those of the hepatic protein. (For purposes of identification, we will refer to the proteins detected by anti-P and anti-M as "hepatic isozymes" and the proteins detected by anti-lung as "pulmonary isozymes.")

Expression of hepatic and pulmonary isozymes in liver, lung, and kidney of several species. Although reactivity between anti-lung and rabbit hepatic microsomal proteins could not be detected, anti-lung produced the same staining pattern with rabbit renal microsomes as it did with rabbit pulmonary microsomes (Fig. 5A). Anti-M, on the other hand, reacted strongly with rabbit hepatic microsomes, weakly with rabbit renal microsomes, and not at all with rabbit pulmonary microsomes (Fig. 5B). Immunostaining with either anti-P or the antibody to rabbit liver enzyme gave the same results obtained with anti-M (not shown). When microsomal preparations from mouse (ICR) liver, lung, and kidney were examined, results with anti-lung were the same as those with rabbit samples: positive with lung and kidney, but not with liver (Fig.

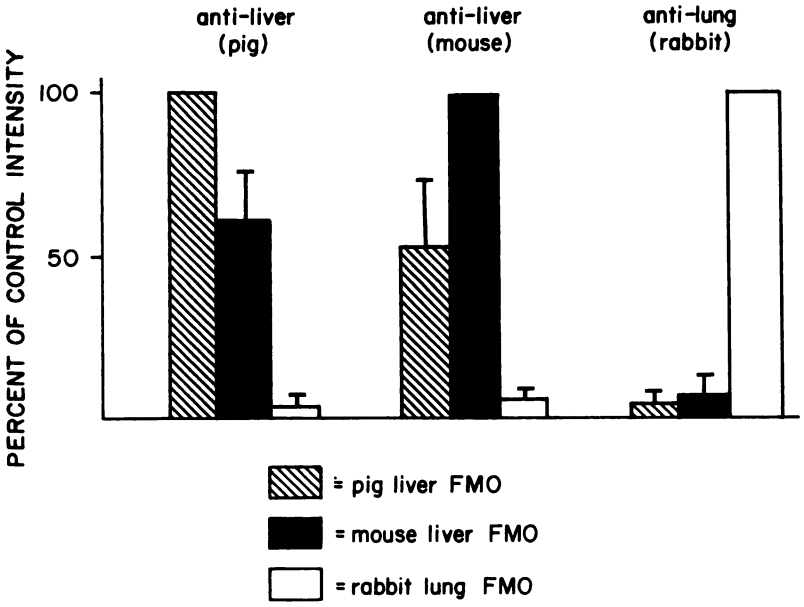

Fig. 2. Relative staining intensities of purified hepatic and pulmonary flavin monooxygenases (*FMO*) reacted with antibodies to the hepatic and pulmonary enzymes. Results obtained with purified pig liver enzyme (■), mouse liver enzyme (■), and rabbit lung enzyme (□) are shown. The staining intensities resulting from the reactivities between antibodies and the antigens against which they were raised were arbitrarily given a value of 100%. The data represent the mean and standard deviation of three determinations.

Fig. 3. Detection of homologous forms of flavin monooxygenase in hepatic microsomal preparations from several species. Hepatic microsomal proteins from mouse (lane *4*, 20 μg), pig (lane *8*, 5 μg), rabbit (lane *9*, 20 μg), rat (lane *10*, 40 μg), guinea pig (lane *11*, 10 μg), and hamster (lane *12*, 20 μg) were immunoblotted and stained using anti-P. Purified mouse liver enzyme (lanes *1–3;* 0.05, 0.1, and 0.2 μg of protein) and pig liver enzyme (lanes *5–7;* 0.05, 0.1, and 0.2 μg of protein) are shown for comparison.

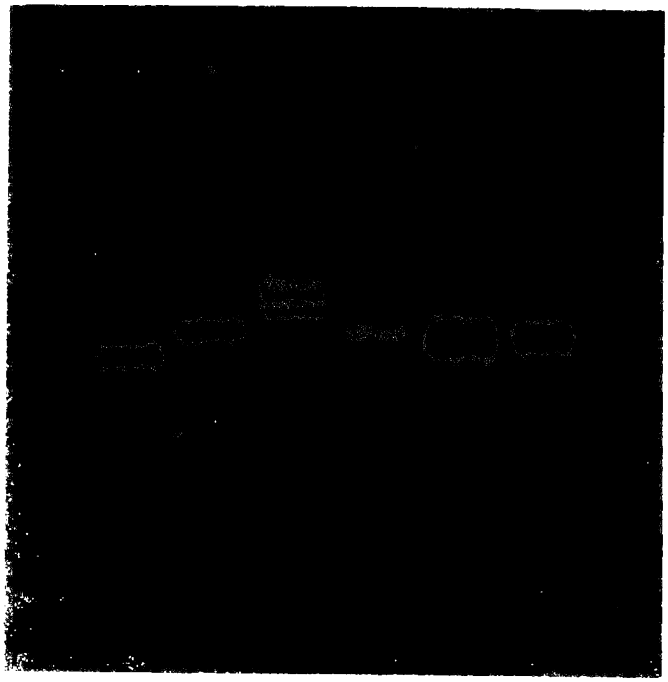

Fig. 4. Detection of homologous pulmonary flavin monooxygenases in pulmonary microsomal preparations from several species. Pulmonary microsomal protein from rabbit (lane *1*, 3 μg), hamster (lane *2*, 20 μg), guinea pig (lane *3*, 20 μg), and mouse (lane *4*, 20 μg) were immunoblotted and stained using anti-lung. Purified rabbit lung enzyme (lanes *5* and *6*, 0.05 and 0.02 μg of protein) is shown for comparison.

5C). However, anti-M showed similar (within 2-fold) reactivity with all three tissues from mouse (Fig. 5D).

A summary of the above results, and those obtained with samples from C57 mice, guinea pigs, hamsters, and three strains of rats, is given in Table 1. Anti-lung was positive with lung samples from all species, negative with all liver samples, and positive with kidney samples from rabbits, mice, and hamsters. The animals examined could be placed into one of four categories with respect to differences among kidney, lung, and liver reactivities with anti-M or anti-P: first, differences of less than 2-fold (ICR mice, C57 mice, and Sprague-Dawley rats); second, reactivity of kidney greater than half that of liver, and reactivity of lung less than half that of liver (Long-Evans rats); third, reactivity of liver 3- to 5-fold greater than that of kidney and 5- to 10-fold greater than that of lung (guinea pig, hamster, and Fisher rats); and fourth, reactivity of liver 10-fold greater than kidney, and no hepatic enzyme detected in lung (rabbit).

Effect of age and sex on flavin monooxygenases in rabbit. The concentration of the pulmonary flavin monooxygenase isozyme is about 600 pmol/mg of protein in microsomal

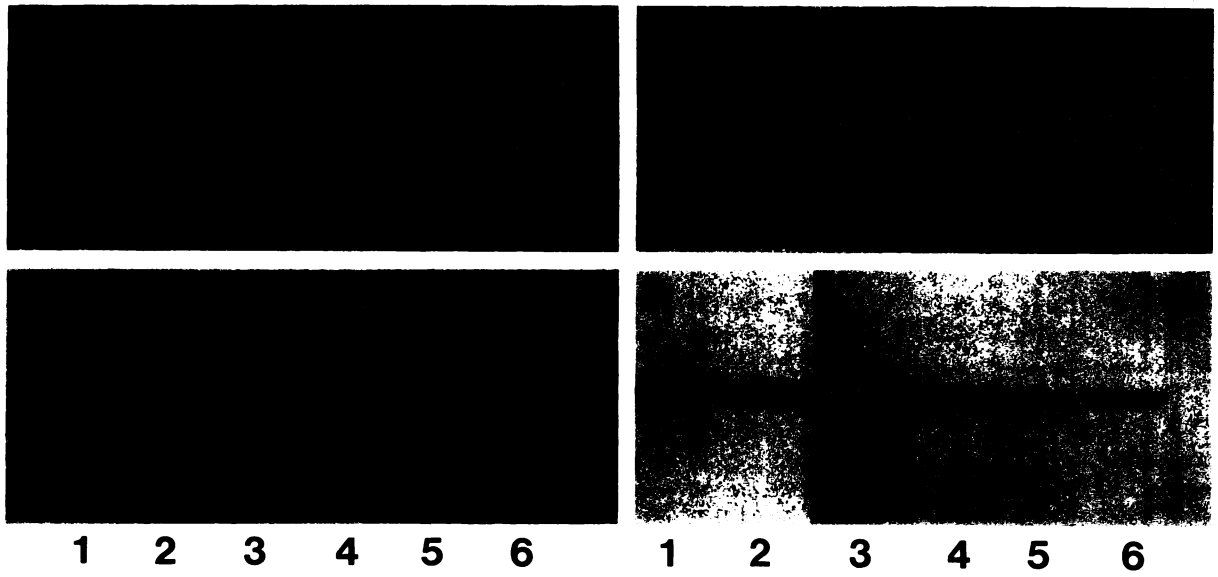

Fig. 5. Detection of hepatic and pulmonary isozymes of flavin monooxygenase in pulmonary, hepatic, and renal microsomal preparations from mouse and rabbit. Microsomal preparations from rabbit (A and B) and mouse (C and D) were stained using anti-lung (A and C) or anti-M (B and D). Lanes *1* and *2* are lung microsomes, lanes *3* and *4* are liver microsomes, and lanes *5* and *6* are kidney microsomes. Protein concentrations are as follows: A*1* (2 μg), A*2* (5 μg), A*3* (50 μg), A*4* (100 μg), A*5* (15 μg), A*6* (45 μg), B*1* (50 μg), B*2* (100 μg), B*3* (10 μg), B*4* (30 μg), B*5* (50 μg), B*6* (100 μg), C*1* (10 μg), C*2* (30 μg), C*3* (50 μg), C*4* (100 μg), C*5* (20 μg), C*6*, (60 μg), D*1* (10 μg), D*2* (30 μg), D*3* (10 μg), D*4* (30 μg), D*5* (10 μg), and D*6* (30 μg).

TABLE 1

Relative reactivities of lung, liver, and kidney microsomal preparations to anti-lung, anti-M, and anti-P

	Relative staining intensities[a]								
	Lung			Liver			Kidney		
	Anti-lung	Anti-M	Anti-P	Anti-lung	Anti-M	Anti-P	Anti-lung	Anti-M	Anti-P
Rabbit	100[b]	<0.3[c]	<0.3[c]	<0.3	27	27	5.7[d]	2.2	3.6
Mice (ICR)	2.3	37	16	<0.3	55[e]	32	0.5	31[f]	20
Mice (C57)	2.9	40	14	<0.3	48	21	0.5	46	24
Guinea pig	3.7	7.0	5.0	<0.3	100[g]	100[g]	<0.3	18	19
Hamster	4.0	13	6.1	<0.3	76	46	0.5	23	7.8
Rat (S-D)	T[h]	18	10	<0.3	28	17	<0.3	23	19
Rat (LE)	T	17	6.3	<0.3	49	17	<0.3	27	15
Rat (F)	T	12	6.5	<0.3	70	22	<0.3	19	8.5

[a] The highest staining intensities obtained with each antibody were arbitrarily set at 100 (anti-lung with rabbit lung, and anti-M and anti-P with guinea pig liver). The data represent the means of three experimental determinations.
[b] The amount of pulmonary isozyme in rabbit lung was 619 pmol/mg of microsomal protein as determined with the purified rabbit pulmonary isozyme as a standard.
[c] No reactivity was detected at the mobility of the rabbit hepatic isozyme.
[d] The amount of pulmonary isozyme in rabbit kidney was 35 pmol/mg of microsomal protein as determined with the purified rabbit pulmonary isozyme as a standard.
[e] The amount of hepatic isozyme in mouse liver was 229 pmol/mg of microsomal protein as determined with the purified mouse hepatic isozyme as a standard.
[f] The amount of hepatic isozyme in mouse kidney was 123 pmol/mg of microsomal protein as determined with the mouse hepatic isozyme as a standard.
[g] The staining intensities for anti-M and anti-P with guinea pig liver were equivalent to 416 and 603 pmol/mg of microsomal protein of mouse and pig hepatic enzyme, respectively.
[h] Trace amounts (less than 1% of the level in rabbit lung) were detected.

preparations from lungs of adult or immature (28 days old) male or female rabbits (Table 2). Pregnancy appears to increase the concentration of the pulmonary isozyme by more than 2-fold. No hepatic isozyme was detected by either anti-M or anti-P in any of the lung samples examined. The relative concentrations of the hepatic isozyme in microsomal preparations from livers of adult or immature male or female rabbits were also similar (Table 2). Pregnancy had little effect on the concentration of the hepatic isozyme in liver. The concentrations of the pulmonary isozyme in renal microsomal preparations were about 2-fold greater in samples from immature animals than in samples from adults (Table 2) and were from 5 to 15% of the concentrations found in samples from lung. Kidneys from immature rabbits also contained more hepatic isozyme (about 3-fold) than those from adult rabbits. Kidneys of male rabbits and pregnant rabbits contained about twice as much hepatic isozyme as those from non-pregnant females. Flavin monooxygenase activity obtained with pulmonary, hepatic, and renal microsomal preparations was in general agreement with total isozyme content (Table 2).

Apparent multiple forms of rabbit pulmonary flavin monooxygenase. Preparations of flavin monooxygenase purified from rabbit lung could be resolved into three bands on immunoblots. These bands, referred to in order of increasing mobility as "a," "b," and "c," are compared to the single band of cytochrome P-450 isozyme 2 in Fig. 6A. High concentrations of purified pulmonary flavin monooxygenase isozyme (>2 μg) produced the same three bands when stained with anti-M (not

TABLE 2
Rabbit flavin monooxygenases: age and sex differences

Microsomal source[a]	Lung		Liver			Kidney			
	Pulmonary isozyme[b]	Activity	Hepatic isozyme (relative content)[c]		Activity	Pulmonary isozyme[b]	Hepatic isozyme (relative content)[c]		Activity
			anti-M	anti-P			anti-M	anti-P	
	pmol/mg					*pmol/mg*			
Male									
Adult	619 ± 54	14.9 ± 1.7	90 ± 17	100 ± 18	16.4 ± 3.6	35 ± 8	7 ± 4.0	13 ± 4	1.3 ± 0.5
Immature	592	16.3	74	80	15.9	81	16	30	3.7
Female									
Adult	563 ± 39	18.3 ± 2.2	52 ± 13	79 ± 12	7.9 ± 4.1	48 ± 11	4 ± 1	6 ± 2	0.6 ± 0.3
Pregnant	1388 ± 98	31.3 ± 0.6	100 ± 12	92 ± 7	18.0 ± 0.6	51 ± 20	9 ± 3	13 ± 5	1.5 ± 0.2
Immature	560	13.9	70	78	13.4	69	8	17	1.8

[a] Results for adult and pregnant animals are the mean ± standard deviation for values from four individual rabbits. Results for immature rabbits are for values from single pools of tissue from 10 animals.
[b] Quantitation of the pulmonary isozyme was done using the purified pulmonary isozyme as a standard.
[c] Relative amounts (100-maximum) of the hepatic isozyme are reported. The rabbit hepatic isozyme was not sufficiently pure for use as a standard. The maximum staining intensities observed with anti-M and anti-P were 124 ± 15 and 164 ± 30 pmol/mg of mouse hepatic and pig hepatic enzyme, respectively.

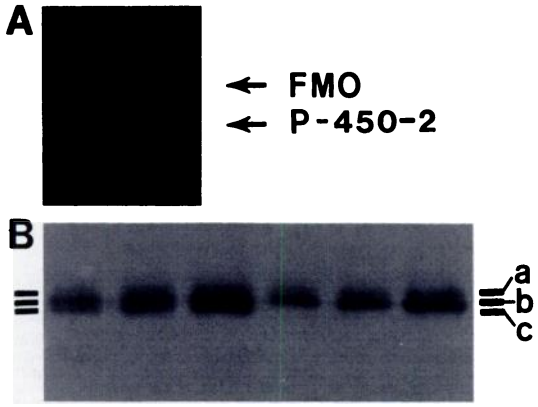

Fig. 6. Multiple pulmonary forms of rabbit flavin monooxygenase and individual variation. A. Comparison of bandwidths of flavin monooxygenase (*FMO*) and cytochrome P-450 isozyme 2 (*P-450-2*) in microsomes immunostained with both anti-lung and anti-isozyme 2. Lanes contain 3 μg (*left*) and 6 μg (*right*) of rabbit lung microsomal protein. B. Comparison of a type abc (*left three lanes*) and a type ab (*right three lanes*) rabbit. The three lanes of each individual rabbit contain 0.5, 1.0, and 2.0 μg of rabbit lung microsomal protein, respectively.

shown). Two phenotypes were discovered initially when pulmonary microsomes from 12 adult rabbits (4 males, 4 females, and 4 pregnant females) were examined: expression of all three proteins (type abc) with relative intensities of about 1:2:2 (a:b:c) for 7 individuals, and expression of a and b only (type ab) in a ratio of about 1:2 for the other 5 individuals. Examples of these phenotypes are shown in Fig. 6B. Matching results, with respect to band specificity and relative intensities, were obtained with renal microsomal preparations from the same individuals (not shown). The staining patterns seen with microsomal preparations from either tissue were not altered by changes in the temperature treatment of the samples or by elimination of β-mercaptoethanol from the procedure. Subsequently, eight additional pulmonary preparations from adult male rabbits were examined; one was type abc, five were type ab, and two appeared to be of a third phenotype, type bc. Therefore, of the 20 individual pulmonary preparations examined, 8 were type abc, 10 were type ab, and 2 were type bc. The activities of the pulmonary microsomal fractions (Table 2) were similar with respect to total isozyme content regardless of the phenotype, and ratios of staining intensities were similar for a given phenotype in males or females. Increases in isozyme content associated with pregnancy did not alter these ratios.

Discussion

The discovery of distinct pulmonary and hepatic forms of flavin monooxygenase demonstrated that the capacity of different tissues to carry out flavin monooxygenation reactions is not simply a function of different concentrations of identical enzymes (5, 6). Our finding of tissue and species differences in the expression of the pulmonary and hepatic isozymes serves to further complicate the picture.

Lungs of guinea pigs, hamsters, rats, rabbits, and mice contain an isozyme of flavin monooxygenase that is distinct from the major form present in liver. The pulmonary isozyme is not detected in liver of any species, but some hepatic isozyme is detected in the lungs of all species examined except rabbit. In addition, three forms of pulmonary flavin monooxygenase are detected in lungs of guinea pigs and rabbits. Examination of individual rabbits shows differences in the expression of these pulmonary forms of enzyme. The relative electrophoretic mobilities of hepatic and pulmonary isozymes of flavin monooxygenase are shown in Fig. 7.

Species differences in pulmonary flavin monooxygenase-catalyzed *N*-oxidation of chlorpromazine and imipramine (10, 11) are likely due to variability in expression of the hepatic isozyme in lung. The lack of these activities in rabbit lung is consistent with a lack of detectable hepatic isozyme and the inability of purified pulmonary isozyme to metabolize these substrates (5). In contrast, the pulmonary activities exhibited by the rat coincide with the expression of the hepatic isozyme in lung and the catalytic properties of purified hepatic isozymes (1, 14).

In addition to lung and liver, species variation in the expression of flavin monooxygenase isozymes is also evident in kidney. Pulmonary isozyme is detected in kidney microsomes from rabbit, mouse, and hamster, but not guinea pig or rat; hepatic isozyme is present in kidney microsomes from all species. These differences, as well as those in liver and lung, are especially evident with rabbit and guinea pig because of differences in the mobilities of the pulmonary and hepatic isozymes. The patterns of expression of the three forms of the pulmonary isozyme in rabbit kidney are identical to those observed in lung from the same individuals.

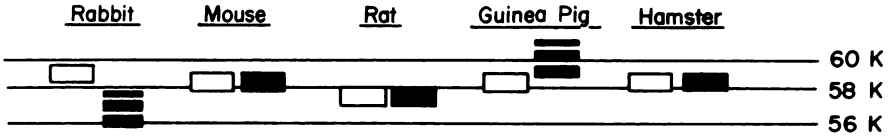

Fig. 7. Mobilities of hepatic (□) and pulmonary (■) isozymes of flavin monooxygenase on polyacrylamide gels in the presence of sodium dodecyl sulfate.

Although a great deal is known about the properties of the flavin monooxygenase from liver (1, 2, 9, 14), we are just beginning to understand the complex nature of this enzyme with respect to tissue and species differences. Dannan and Guengerich (21) first discovered that kidney and lung of several species contain protein related immunochemically to the pig hepatic flavin monooxygenase. Later, it was shown that lung and liver contain distinct flavin monooxygenase isozymes, and now it is clear that there are marked species and tissue differences in the expression of multiple forms of this enzyme.

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