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Immunochemical Comparison and Quantitation of Microsomal Flavin-Containing Monooxygenases in Various Hog, Mouse, Rat, Rabbit, Dog, and Human Tissues

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SUMMARY

The distribution of microsomal flavin-containing monooxygenase (EC 1.14.13.8, *N*,*N*-dimethylaniline *N*-oxidizing) was examined using a technique involving separation of proteins by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; electrophoretic transfer to nitrocellulose sheets; staining of the sheets for the enzyme by sequential treatment with rabbit anti-porcine liver monooxygenase, goat anti-rabbit immunoglobulin G, and horseradish peroxidase/rabbit anti-peroxidase complex; and densitometry of the visualized bands. The absolute levels of the enzyme were estimated in homogenates and microsomal fractions of porcine liver, lung, kidney medulla and cortex, aorta, thyroid, lymph nodes, pancreas, jejunum, and skin. The enzyme had a lower apparent monomeric molecular weight than the porcine liver enzyme (56,500) in all of the other species examined. Because of the lack of immunochemical identity of the enzyme among the various species examined and the inavailability of purified enzyme from species other than hog, only relative estimates could be made for the levels of enzyme found in the various tissues examined from these species. Enzyme levels were estimated in liver, lung, and kidney of rats and mice and in liver and lung of rabbits. In all cases the highest enzyme concentration was found in liver. However, in male (but not female) mice the enzyme concentration was nearly as high in lung and kidney as in liver. All of these observations are consonant with previous reports of the distribution of *N*,*N*-dimethylaniline oxidase activity. However, the lability of enzyme activity and the presence of potential endogenous substrates and effectors are not factors in estimates of enzyme distribution made with this technique. The qualitative and quantitative data obtained using these immunochemical techniques may be of further use in understanding the role of this enzyme in various processes involving both endogenous and xenobiotic compounds.

INTRODUCTION

A microsomal flavin-containing monooxygenase (EC 1.14.13.8, *N*,*N*-dimethylaniline *N*-oxidizing) catalyzes the oxidation of a variety of nitrogen- and sulfur-containing compounds in liver and other tissues (1). This enzyme has been suggested to have a role in the oxidation of endogenous cysteamine (1). Many xenobiotics, such as phenothiazines, are hydroxylated to more polar derivatives which are presumably less toxic than the parent compounds (2). On the other hand, oxidation by this enzyme can play a role in the bioactivation of other compounds, such as aminoazobenzenes (3) and alkylhydrazines (4), to potentially toxic derivatives. This enzyme complements another microsomal mixed-function oxidase system consisting of cytochrome P-450 and NADPH-cytochrome P-450 reductase (5). The flavin-containing monooxygenase appears to be inherently less stable than cytochrome P-450 or its reductase and has been purified only from porcine liver to date. Distinction of roles for cytochrome P-450 and the flavin-containing monooxygenase in the metabolism of certain compounds can be difficult and is most commonly done with inhibition studies (6). Because of its lability and the existence of potential endogenous substrates and effectors (7), the activity of the flavin-containing monooxygenase can be difficult to estimate and the enzyme is often overlooked (1).

We have recently adapted an immunoelectrophoretic method for the estimation of concentrations of enzymes and applied this technique to measure levels of certain cytochrome P-450 hemoproteins in various tissues of rats, rabbits, and humans (8). The method entails identification of proteins by monomeric M_r as well as immunolog-

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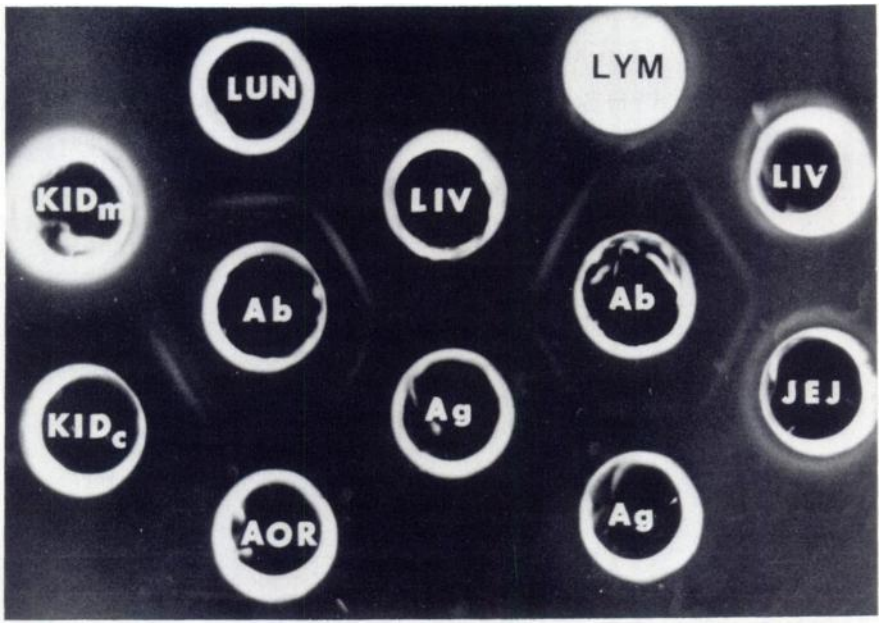


FIG. 1. *Double-diffusion immunoprecipitin analysis of hog tissues using anti-hog liver flavin-containing monooxygenase*

The antibody (IgG fraction, 0.33 mg of protein) was placed in the two center wells (*Ab*), and the peripheral wells contained either purified hog liver flavin-containing monooxygenase (6 μg) (*Ag*) or microsomes derived from the indicated hog tissues. Microsomes were dissolved in 10 mM Tris-acetate buffer (pH 7.4) containing 1 mM EDTA, 20% glycerol, 1% (w/v) sodium cholate, and 1% (w/v) Lubrol PX; the amounts of microsomal protein applied were 0.22, 0.13, 0.14, 0.25, 0.34, 0.18, and 0.14 mg from hog liver (LIV), lung (*LUN*), kidney medulla (KID_m), kidney cortex (KID_c), aorta (*AOR*), jejunal mucosa (*JEJ*), and lymph nodes (*LYM*), respectively.

ical activity and permits quantitation at a level of less than 0.5 pmole. In this report, we describe the use of this technique to quantitate the absolute levels of flavin-containing monooxygenase in various porcine tissues. We also make species and sex comparisons in terms of qualitative and relative quantitative differences.

MATERIALS AND METHODS

Tissues and enzyme preparations. Tissues from female hogs were donated by Dr. D. M. Ziegler, University of Texas (Austin, Tex.) and were collected at a local slaughterhouse and shipped frozen in dry ice. Sprague-Dawley rats (200–250 g) and adult Swiss ICR mice were obtained from Harlan Industries (Indianapolis, Ind.). New Zealand White rabbits were purchased from Hilltop Farms (Columbia, Tenn.). The animals were killed as described elsewhere (9). A beagle dog liver was provided by Dr. F. F. Kadlubar, National Center for Toxicological Research (Jefferson, Ark.). The human liver samples used here were obtained from organ donors who had been maintained on artificial life-support systems; each liver was frozen within 30–60 min after death of the donor. All tissues were homogenized, and microsomal fractions were prepared as described elsewhere (10). Samples were frozen at −70° until use.

Purified porcine liver flavin-containing monooxygenase (11) and rabbit antisera raised to the purified enzyme were kindly provided by Dr. D. M. Ziegler. Rat liver cytochromes P-450 PB_{B_2}[2] and BNF_{B_2} and epoxide hydrolase were isolated as described elsewhere (12), and antisera were raised in rabbits (9).

[2] The abbreviations used are: PB_{B_2}, liver cytochromes P-450 induced by phenobarbital (12); BNF_{B_2}, liver cytochromes P-450 induced by β-naphthoflavone (12); SDS, sodium dodecyl sulfate; IgG, immunoglobulin G.

Assays. Protein concentrations were estimated using the method described by Lowry *et al.* (13).

Double-immunodiffusion precipitin plates were handled and photographed as described elsewhere (9).

The immunoelectrophoretic techniques are described elsewhere (9, 14, 15), and modifications used for quantitative measurements in this laboratory have also been described (8). Briefly stated, proteins were separated by SDS-polyacrylamide gel electrophoresis (16) and resolved proteins were electrophoretically transferred from polyacrylamide to nitrocellulose. The nitrocellulose sheets were stained for individual proteins by means of a sequential treatment with rabbit antiserum to each protein under consideration, goat anti-rabbit IgG, peroxidase/rabbit anti-peroxidase complex, and 3,3′-diaminobenzidine/H_2O_2. For each nitrocellulose sheet on which samples were quantitated, a series of concentrations (0.3–5.0 pmoles) of purified antigen was also carried through the electrophoresis and staining procedure. Stained bands were scanned using a Kontes fiber optic densitometer, and the areas of the resulting peaks were measured by triangulation and compared with a standard curve prepared with the samples of purified antigen. Sources of electrophoretic and staining reagents were as previously described (8).

RESULTS AND DISCUSSION

Comparison of flavin-containing monooxygenases using double-immunodiffusion precipitin analysis. Diffusion of the purified porcine liver monooxygenase against antibody raised to the enzyme yielded a single line (Fig. 1). Detergent-solubilized porcine liver microsomes usually yielded a single precipitin line which fused with that formed by the purified enzyme. However, in some instances a second, minor line also appeared after pro-

longed diffusion. Lines forming patterns of fusion with the line formed with liver microsomes were observed with solubilized microsomes prepared from porcine lung, kidney, and jejunal mucosa. Lines could not be observed with solubilized microsomes prepared from porcine aorta or lymph nodes using indirect light or naphthol blue-black staining. Single immunoprecipitin lines were also observed when the antibody was tested against solubilized liver microsomes prepared from rat or dog, but the lines were generally weak and staining was required for visualization. Lines could not be definitely seen with solubilized human, mouse, or rabbit liver microsomes. The lines formed by the rat and dog samples were not continuous with the porcine liver precipitin line; i.e., a faint spur was formed in each case. The data are consistent with the view that the antibody raised to the porcine liver enzyme recognizes some antigenic sites on the flavin-containing monooxygenases of the other species but that only some of the sites contained in the porcine enzyme are present in the enzymes of the other tissues.

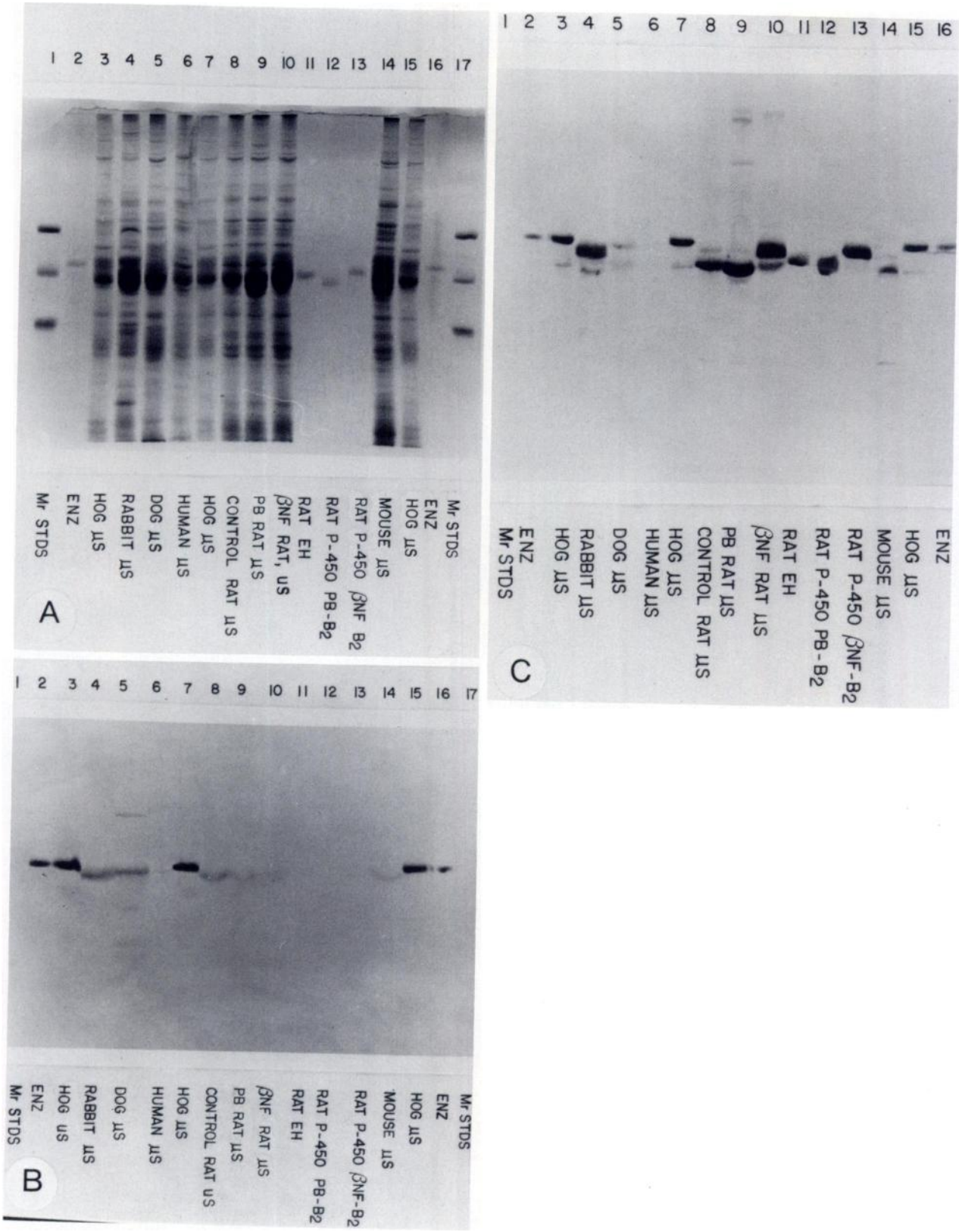
Qualitative comparison of flavin-containing monooxygenases using immunoelectrophoresis. The electrophoretic separation of the various samples is shown in Fig. 2A, where the polyacrylamide gel was stained for protein with Coomassie Brilliant Blue R-250. The purified porcine liver flavin-containing monooxygenase migrated as a single species of M_r 56,500.[3] Two purified rat liver cytochromes P-450 (i.e., PB_{B_2} and BNF_{B_2}) and epoxide hydrolase migrated with apparent monomeric M_r values of 51,000, 55,000, and 53,500, respectively, in this particular electrophoretic system. The profiles of microsomal samples from a number of species are also shown in this gel stained for protein. A duplicate gel was transferred to a sheet of nitrocellulose, which was stained for protein with Amido black. All of the purified proteins under consideration were transferred efficiently, and the patterns observed with the microsomal samples were similar to those observed before transfer (cf. refs. 8 and 15).

Another gel was run in the same way as in Fig. 2A, and the resulting nitrocellulose sheet was stained for flavin-containing monooxygenase with the use of the antibody raised to the purified porcine liver enzyme and the described peroxidase staining procedure (Fig. 2B). In the mixture of porcine liver microsomal proteins, the antibody recognized a single protein which had the same apparent monomeric M_r (56,500) as the antigen used to prepare the antibody (lanes *3*, *7*, and *15*). The antibody recognized a protein with a slightly lower M_r (approximately 55,000) in rabbit and mouse liver microsomes (lanes *4* and *14*) and in liver microsomes prepared from untreated rats or rats treated with phenobarbital or β-naphthoflavone (5,6-benzoflavone) (lanes *8–10*). The M_r of the dog liver enzyme (55,500) was slightly greater than that of the rabbit, rat, and mouse enzymes but less than that of the porcine enzyme. In the case of the dog (lane *5*) minor bands of lower M_r were observed and are presumed to be the result of proteolysis. We feel that these bands represent fragments of the flavin-containing monooxygenase in this tissue, in view of the specificity of the antibody demonstrated in porcine liver microsomes. The reaction with the human liver microsomal sample was very faint (lane *6*), but the apparent M_r was 55,000 as in the case of most of the animals other than the hog. The antibody raised to porcine liver flavin-containing monooxygenase was shown not to react with rat liver epoxide hydrolase or either of two different purified rat liver microsomal cytochromes P-450 (lanes *11–13*).

In order to relate the migration of the flavin-containing monooxygenase in these electrophoretic systems to those of other known microsomal enzymes in rats, rabbits, and mice, a gel was prepared as in Fig. 2B and stained with a mixture of antibodies raised individually to porcine liver flavin-containing monooxygenase, rat liver epoxide hydrolase, and rat liver cytochromes P-450 PB_{B_2} and BNF_{B_2} (Fig. 2C). The latter two enzymes appear to be the major forms of microsomal cytochrome P-450 induced in rat liver by phenobarbital and β-naphthoflavone (or 3-methylcholanthrene), respectively (8, 9, 12, 19, 20). However, the levels of these particular isozymes of cytochrome P-450 are quite low in untreated animals (8, 19, 20). We have previously shown that the cytochromes P-450 recognized by these particular antibodies recognize proteins of the same M_r in mouse liver (8). In rabbit liver microsomes, anti-rat cytochrome P-450 PB_{B_2} recognizes cytochrome $P\text{-}450_{LM_2}$ (M_r 50,000) and anti-rat cytochrome BNF_{B_2} recognizes cytochrome $P\text{-}450_{LM_4}$ (M_r 53,000) (8). The data shown in Fig. 2B and C indicate that a number of known microsomal proteins are highly similar in M_r as judged by electrophoretic techniques such as those used here and suggest caution in the assignment of enzymes to protein-stained bands in this region. In rats, the flavin-containing monooxygenase migrates with the cytochrome P-450 designated BNF_{B_2}, which is the major form induced by β-naphthoflavone, 3-methylcholanthrene, or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (8). In rabbits, the flavin-containing monooxygenase migrates along with the cytochrome P-450 designated $P\text{-}450_{LM_4}$ (8). The characteristics of cytochromes P-450 and epoxide hydrolase in dog and porcine liver have not been described in sufficient detail to permit a useful comparison at this time.

Immunochemical quantitation of flavin-containing monooxygenase in various porcine tissues. Previous double-immunodiffusion precipitin data suggested that the flavin-containing monooxygenase is similar or identical in the various porcine tissues examined (Fig. 1). This view was strengthened by the observation that the same apparent M_r was found for the enzyme in all of the tissues (in which the enzyme was found) when immunoelectrophoresis was carried out as described (data not shown). Densitometry of the stained bands indicated that levels of less than 0.5 pmole of the monooxygenase could readily be quantified (Fig. 3). Experiments were carried out with both tissue homogenates and microsomal preparations to estimate the concentrations of enzyme in various tissues (Table 1). The data on crude homogenates were obtained in order to circumvent problems associated with variable recoveries of microsomes and the monooxygenase recovered in microsomal fractions from extrahepatic tissues. Livers from three different hogs yielded specific contents of the flavin-containing monooxygenase that

[3] This value is essentially identical with the 56,000 estimated by Poulsen and Ziegler (18). The discrepancy with the value of 64,000 based on flavin analysis is probably due to flavin loss or to abnormal binding of SDS.



were within experimental error. Two of these samples had been found to differ in specific catalytic activity toward *N,N*-dimethylaniline by 8-fold.[4] The absolute specific contents are in line with porcine liver microsomal flavin concentrations (approximately 0.6 nmole/mg of protein)[5] after correction for the contribution of NADPH-cytochrome P-450 reductase and NADH-cytochrome b_5 reductase (based upon fold purification needed for preparation of homogeneous enzyme) (*vide infra*) (21). The data of Ziegler and Mitchell (21) can be used to estimate a rough turnover number of 67 min^{-1} for the purified porcine liver flavin-containing monooxygenase (1040 nmoles/min/mg of protein ÷ 15.6 nmoles of flavin per milligram of protein). When the microsomal specific activity given in that same reference (23 nmoles/min/mg of protein) is divided by the average of three specific contents estimated in Table 1 (0.47 nmole/mg of protein), a turnover number of 49 min^{-1} is obtained, consonant with the value found for the purified enzyme. The data indicate that the flavin-containing monooxygenase accounts for about 1% of total porcine liver protein and about 3% of porcine liver microsomal protein.

Varying levels of flavin-containing monooxygenase were also detected and quantitated in porcine lung, kidney (medulla and cortex), aorta, thyroid, lymph nodes, pancreas, jejunum, and skin and are presented in Table 1. In general, the relative amounts of the enzyme in the various tissues reflect the relative *N,N*-dimethylaniline *N*-oxidase activities reported elsewhere (1, 22). Although previous work indicates that the flavin-containing monooxygenase is localized primarily in microsomal fractions, the data presented in Table 1 indicate that, with many extrahepatic tissues, the levels of the enzyme estimated in homogenates were as high as, or higher than, the levels in microsomal fractions. Such observations are explained by the difficulties in homogenizing cells derived from these tissues and the relative difficulties in subcellular fractionation of tissues other than liver. Furthermore, some of the enzymes have been localized in nuclei (23) and mitochondria[5] even in liver, particularly at certain stages of development.

Relative levels of flavin-containing monooxygenase detected in various tissues of other animals. Since all of the porcine liver flavin-containing monooxygenase antigenic determinants do not appear to be present in the enzyme(s) found in other animal species (*vide supra*) and the purified enzymes from these species were not available, we were unable to estimate the absolute levels of flavin-containing monooxygenase present in tissues of animals other than hogs. With the assumption that intertissue variation does not occur within these species, we compared the relative levels of flavin-containing monooxygenase in the tissues of a number of species (Table 2).

The absolute amount of staining was greatest with rabbit liver and lung and dog liver microsomes. This observation may be the result of greater immunochemical similarity of the enzymes in these species with the porcine liver enzyme, as the differences in specific enzyme activity are not so great (1). The variation in levels of the human liver enzyme among three individuals was less than 2-fold. The mouse data are of interest in comparison to reported activities. In males, the level of hepatic enzyme was lower than that in females (Table 2). However, the concentrations of the enzyme were quite similar in male mouse liver, lung, and kidney. In female mice, the concentration of enzyme was substantially less in lung and kidney than in liver. These data are consonant with reported activity measurements (24). The levels found for male rats in the three tissues were similar to those seen in female mice and are consistent with activity data on homogenates reported elsewhere (1). However, levels of the enzyme were lower in female rat liver and lung in repeated experiments. Another series of rodent experiments is of interest. Preliminary qualitative data (Fig. 2B, lanes *8–10*) suggested that the specific content of the flavin-containing monooxygenase was lowered in liver microsomes of rats treated with phenobarbital or β-naphthoflavone. We found that treatment of

[4] The liver with the higher activity (Hog 2) was removed and packed in dry ice 5 min after the carotid artery was severed. The liver with the lower activity (Hog 1) was collected at the end of the slaughtering run (about 20 min after bleeding) (D. M. Ziegler, personal communication).

[5] D. M. Ziegler, personal communication.

FIG. 2. *Electrophoresis and immunoelectrophoresis of liver microsomes and purified microsomal proteins*

In all parts of the figure (A–C), the individual wells contained the same samples. Electrophoresis was carried out according to the method of Laemmli (16), with the anode at the bottom of the gel. Lanes *1* and *17*, M_r standards (*M_r STDS*): 1.0 μg each of bovine serum albumin (accepted M_r, 68,000), *Escherichia coli* L-glutamate dehydrogenase (M_r 53,000), and rabbit muscle aldolase (M_r 40,000); lanes *2* and *16*, 0.5 μg of purified hog liver microsomal flavin-containing monooxygenase (*ENZ*); lanes *3*, *7*, and *15*, 10 μg of porcine liver microsomal protein (*μS*); lane *4*, 20 μg of rabbit liver microsomal protein; lane *5*, 20 μg of dog liver microsomal protein; lane *6*, 20 μg of human liver microsomal protein (patient 22); lane *8*, 20 μg of microsomal protein prepared from untreated rats; lane *9*, 20 μg of liver microsomal protein prepared from phenobarbital (*PB*)-treated rats; lane *10*, 20 μg of liver microsomal protein prepared from β-naphthoflavone (*βNF*)-treated rats; lane *11*, 0.5 μg of purified rat liver microsomal epoxide hydrolase (*EH*); lane *12*, 0.5 μg of purified rat liver microsomal cytochrome P-450 PB_{B_2}; lane *13*, 0.5 μg of purified rat liver microsomal cytochrome P-450 BNF_{B_2}; and lane *14*, 20 μg of mouse liver microsomal protein. Unless noted otherwise, all microsomes were prepared from untreated male animals (human microsomes were from a female patient). A, The gel was stained for protein using Coomassie brilliant blue R-250 (17). B, Proteins were transferred from the gel to a sheet of nitrocellulose, and the sheet was stained for flavin-containing monooxygenase in the described manner (8). The rabbit anti-hog liver flavin-containing monooxygenase antisera was diluted 1:100 in this work. C, Proteins were transferred from the gel to a sheet of nitrocellulose, and the sheet was treated with a mixture of rabbit antisera prepared to hog liver flavin-containing monooxygenase (diluted 1:100), rat liver cytochrome P-450 PB_{B_2} (diluted 1:100), rat liver cytochrome P-450 BNF_{B_2} (diluted 1:100), and rat liver epoxide hydrolase (diluted 1:100) prior to treatment with goat anti-rabbit IgG, peroxidase/rabbit anti-peroxidase complex, and 3,3′-diaminobenzidine/H_2O_2 in the usual manner (8). In B and C, protein concentrations were similar to those used for Coomassie blue staining (A) except that 0.2 μg of purified porcine liver microsomal flavin-containing monooxygenase was used.

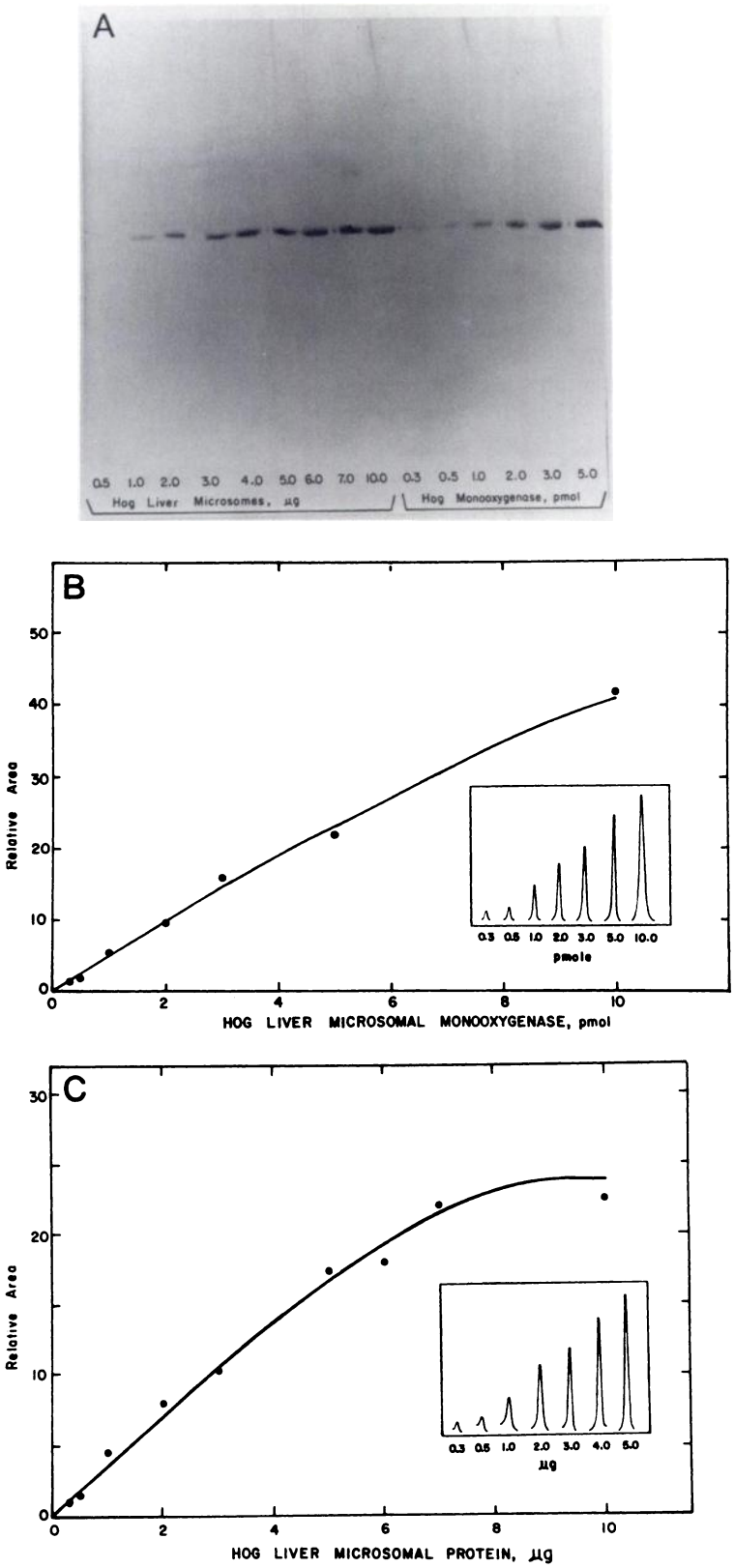


FIG. 3. *Immunoelectrophoresis and densitometry of flavin-containing monooxygenase in purified samples and porcine liver microsomes*

A, Electrophoretogram of resolved samples, with the indicated amounts of porcine liver microsomal protein or purified porcine liver microsomal flavin-containing monooxygenase that were electrophoresed in each well. B, Area under the densitometric peak as a function of the amount of purified porcine liver flavin-containing monooxygenase electrophoresed. The *inset* shows the actual densitometric traces. C, Area under the densitometric peak as a function of the amount of porcine liver microsomal protein electrophoresed. The *inset* shows the actual densitometric traces.

TABLE 1

Immunochemical estimation of flavin-containing monooxygense in porcine tissues

The procedures were carried out using 1–50 μg of liver protein as described in the text. All values represent the means of at least two determinations using at lest two concentrations of protein. A standard curve was prepared on each nitrocellulose sheet (cf. Fig. 3).

Tissue	Concentration	
	Microsomes	Homogenate
	nmoles/mg protein	
Liver		
Hog 1	0.45	0.17
Hog 2	0.50	0.18
Hog 3	0.46	—[a]
Lung	0.054	0.018
Kidney		
Cortex	0.012	0.012
Medulla	0.016	0.008
Aorta	0.005	0.015
Thyroid	0.005	0.006
Lymph nodes	0.002	0.002
Pancreas	0.006	0.003
Jejunum	0.001	0.004
Skin	—	0.020

[a] —, Not determined.

TABLE 2

Relative immunochemical staining for flavin-containing monooxygenase in microsomes prepared from tissues of various species

The techniques are presented under Materials and Methods and in the legend to Table 1.

Species and tissue	Relative staining	
	Male	Female
	/mg protein	
Hog		
Liver	—[a]	100
Lung	—	14
Kidney	—	4
Mouse		
Liver	2.3	3.4
Lung	1.4	0.7
Kidney	1.9	0.6
Rat		
Liver	3.5	0.7
Lung	0.6	0.1
Kidney	1.5	0.8
Rabbit		
Liver	10	6.7
Lung	6.9	2.8
Dog		
Liver	6.0	—
Human liver		
Patient 22 (age 32)	—	0.9
Patient 21 (age 50)	—	1.1
Patient 17S (age 24)	0.6	—

[a] —, Not determined.

male rats with phenobarbital reduced the apparent specific content of the flavin-containing monooxygenase to 45% ± 13% of the level observed in microsomes in untreated animals.[6] Treatment of male rats with β-naphthoflavone decreased the specific content of the monooxygenase to 31% ± 12%. These results may be explained in part, but not completely, by the known ability of these compounds to increase levels of total hepatic protein. The compounds seem actually to repress the flavin-containing monooxygenase.

CONCLUSION

The data show the species variation of flavin-containing monooxygenase. A sensitive immunoelectrophoretic technique, which does not rely on precipitating antibodies, was used to compare the subunit M_r values of the enzyme present in different species and to quantify the levels of the enzyme present in different porcine tissues. The data presented in Fig. 2 show the potential pitfalls inherent in using only levels of dye-stained protein bands in the crowded 40,000–60,000 M_r region of electrophoretograms of microsomal proteins to draw conclusions about the identity or levels of individual proteins. The technique also minimizes the risk of false conclusions drawn only on the basis of immunological reaction, as subunit M_r was used to confirm immunological similarity and to rule out reaction due to antibodies raised to any impurities. The catalytic activity of the microsomal flavin-containing monooxygenase is rather labile, and the techniques used here circumvent the need to maintain enzyme structure necessary for functional activity. However, the data presented here support the conclusions reached by others about relative levels of the enzyme found in various tissues of hogs, rats, and mice (1, 24).

The results are limited in the sense that the absolute levels of the enzyme cannot be determined in all cases because the enzyme has not been purified from species other than hog. However, the partial immunological similarity of the enzyme among the various species suggests that immunoadsorption techniques may be used to purify the enzyme from other species to raise more appropriate antibodies and to quantify absolute levels of the antigen in these species. Furthermore, the demonstration of the specificity of the antibody justifies the use of such preparations in carrying out localization experiments in various tissues to determine the location of the enzymes among various cells and subcellular organelles.

[6] In line with this result, another group found that treatment of Fischer 344 rats with phenobarbital lowered the specific microsomal activity from 1.61 (± 0.22) to 0.71 (± 0.08) nmoles of NADPH oxidized per minute per milligram of protein, a 56% decrease (E. J. Rauckman, personal communication).

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