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Rabbit Lung Flavin-containing Monooxygenase

Purification, Characterization, and Induction during Pregnancy

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SUMMARY

A flavin-containing monooxygenase has been purified to apparent homogeneity from lung microsomes of pregnant rabbits and characterized with respect to a number of physical and catalytic parameters. The apparent molecular weight, as determined on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was 59,000, and the lung microsomal flavoprotein was shown to contain 14 nmol of FAD/mg of protein. Addition of $NADP^+$ to the oxidized flavoprotein produced a shift in the spectrum characteristic of the flavin-containing monooxygenase from porcine liver, and addition of small amounts of NADPH to the oxidized rabbit lung enzyme produced a stable spectral intermediate consistent with that of a 4a-peroxyflavin. Rabbit lung flavin-containing monooxygenase differed markedly from the porcine liver enzyme in exhibiting a broader pH optimum from 8.5–10.5, by not being inhibited by concentrations of sodium cholate as high as 1% and by withstanding, in the absence of NADPH, incubation at 45° for at least 10 min with no significant loss of activity. Unlike the pig liver enzyme, purified rabbit lung enzyme was not activated by *n*-octylamine and, in fact, *n*-octylamine stimulated NADPH oxidation. A number of compounds known to be substrates of the pig liver enzyme, including benzphetamine, chlorpromazine, and imipramine, are not substrates for the rabbit lung enzyme, whereas prochlorperazine and trifluoperazine are excellent substrates. Antibodies to rabbit lung flavin-containing monooxygenase were raised in guinea pig and utilized for the immunoquantitation of this enzyme throughout gestation. The activity (as determined by *N*,*N*-dimethylaniline-*N*-oxidation) and amount of rabbit lung flavin-containing monooxygenase were maximally induced (5-fold) on the 28th day of gestation. Liver microsomes from rabbit did not contain any of the lung form of flavin-containing monooxygenase at any time during gestation, as evidenced by results from Western blotting. These results demonstrate that, at least in rabbit, flavin-containing monooxygenase can exist as more than a single form. The physiological significance of the induction of this enzyme during pregnancy is not known.

INTRODUCTION

Flavin-containing monooxygenase (EC 1.14.13.8) is a mammalian flavoprotein, found in many tissues, which oxidizes a wide variety of nitrogen- and sulfur-containing drugs and xenobiotics (1–3). Although originally characterized as a mixed-function amine oxidase, substrates containing both an oxidizable nitrogen and sulfur are preferentially oxidized at the sulfur atom (2, 3).

Tertiary amines are metabolized to the *N*-oxide, secondary amines yield *N*-hydroxy metabolites, and primary amines, with few exceptions (4), are not substrates. Sulfur-containing substrates include thioureylenes, thiocarbamides, thioamides, thiols, and sulfides (1). The only known endogenous substrate of flavin-containing monooxygenase is cysteamine, which is oxidized to the disulfide, cystamine. Ziegler and Poulsen (5) have hypothesized a model in which the flavin-containing monooxygenase plays a crucial role in the synthesis of polypeptide disulfide bonds through an intermediate consisting of a protein-cysteamine mixed disulfide.

The great majority of work concerning characterization of flavin-containing monooxygenase has been performed by Ziegler *et al.* with purified porcine liver enzyme. Porcine flavin-containing monooxygenase is sensitive to inhibition by anionic detergent, is activated by primary alkylamines, has a pH optimum of 8.5, is inac-

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tivated rapidly above 40° in the absence of cofactor, and exhibits a reaction mechanism (Ordered Ter-Bi) unlike any other known flavoprotein monooxygenase requiring an external reductant (6).

Few reports have appeared concerning purified hepatic flavin-containing monooxygenase from other mammals. Recently, our laboratory (7) and that of Hodgson and associates (8) have isolated flavin-containing monooxygenase from lung microsomes of rabbits and found this enzyme to be immunochemically and catalytically distinct from the form present in rabbit liver.

Rabbit microsomal flavin-containing monooxygenase activity (as measured by DMA[1]-*N*-oxidation) is higher in lung than in liver (9) and is induced almost 2-fold during the final stages of pregnancy or by the administration of glucocorticoids (10). Devereux *et al.* (11) have partially purified flavin-containing monooxygenases from rabbit liver and lung and suggested that they may be different enzymes. The activity of flavin-containing monooxygenase also appears to be under the control of sex steroids in other systems (12–17). Progesterone is a positive effector, whereas testosterone is inhibitory, but the effects are tissue specific (14). The activity of the rat liver enzyme is under developmental regulation and increases with age (15). In female mouse liver, the enzyme has also been shown to be under nutritional and diurnal control (16) and is induced 5-fold in placental microsomes during late gestation but not in liver, lung, kidney, or uterus (17).

This laboratory has been involved in studying the induction of lung microsomal cytochrome P-450-mediated ω-hydroxylation of prostaglandins (18). In the course of purifying cytochromes P-450 from pregnant rabbit lung, we discovered a flavoprotein fraction which co-eluted with P-450 from *n*-octylamino-Sepharose 4B. The present study describes the further purification of this fraction, presents evidence that this flavoprotein is a type of flavin-containing monooxygenase, establishes its nonidentity with either the porcine or rabbit liver enzymes, and follows the time course of its induction during gestation.

EXPERIMENTAL PROCEDURES

Materials

Methimazole, PMSF, Lubrol PX, DTT, and $NADP^+$ were purchased from Sigma. DMA, octylamine, and cyanogen bromide were purchased from Aldrich.

Pharmacia was the source of DEAE-Sepharose 4B and Sepharose 4B. Diaminooctane was obtained from Pfaltz and Bauer Inc., Stamford, CT, and sodium cholate was from Calbiochem-Behring. NADPH and NADH were from P-L Biochemicals. All reagents and equipment used for SDS-PAGE were from Bio-Rad except the Coomassie Brilliant Blue R-250 (Eastman). *N*-Octylamino-Sepharose 4B (19) and hydroxylapatite (20) were prepared as described previously. The apparatus used for Western blotting was from E-C Apparatus Corp., and the nitrocellulose was purchased from Schleicher & Schuell. The hybrid dot manifold and nitrocellulose filters were obtained from Bethesda Research Laboratories. ^{125}I-Protein A was from ICN and the x-ray film (XAR-5) from Eastman Kodak.

Methods

Adult white New Zealand female rabbits were obtained between the 5th and 10th day of gestation from Rabbit Hill, Madison, WI or Howard Smith Farms, Seymour, WI. Rabbits were killed by injecting KCl into the heart. Lungs were immediately removed and perfused with ice-cold saline.

Purification of rabbit lung flavin-containing monooxygenase. The lungs from 8–12 pregnant rabbits (28th day of gestation) were pooled, weighed, and minced in 3 volumes of 10 mM potassium phosphate, pH 7.5, 0.15 M KCl, 1 mM EDTA, 0.1 mM PMSF. Tissues were homogenized first with a Polytron homogenizer (6 periods of 10 sec with 1 min between sonications to allow for cooling) and then with a glass homogenizer and Teflon pestle. Pulmonary microsomes were prepared, and the rabbit lung flavin-containing monooxygenase was purified along with cytochromes P-450 using a modification of the procedure of Guengerich for the purification of rabbit lung cytochromes P-450 (21).

Microsomes were resuspended in 0.1 M potassium phosphate, pH 7.25, 30% glycerol, 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF to a protein concentration of 2 mg ml^{-1} and solubilized by adding sodium cholate to a final concentration of 0.6% with stirring at 4° for 30 min. The solubilized microsomes were centrifuged at 106,000 × *g* for 90 min and then applied to a column of *n*-octylamino-Sepharose 4B, previously equilibrated with 0.1 M potassium phosphate, pH 7.25, 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 0.7% cholate (buffer A). The column was washed with three column volumes of buffer A containing 0.46% cholate and the flavin-containing monooxygenase (and cytochrome P-450) eluted with buffer A containing 0.33% cholate and 0.08% Lubrol PX. The *n*-octylamino-Sepharose 4B eluate was concentrated (PM 30), dialyzed for 2 hr against 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.1 mM PMSF, followed by overnight dialysis against 10 mM potassium phosphate, pH 7.7, 20% glycerol, 0.1 mM EDTA, 0.1 mM PMSF, 0.1 mM DTT, 0.2% cholate, and 0.1% Lubrol PX (buffer B). This fraction was then loaded onto a column of DEAE-Sepharose, previously equilibrated with buffer B. The flavin-containing monooxygenase and $P\text{-}450_I$ were eluted by washing with buffer B, and the flavin-containing monooxygenase eluted slightly ahead of $P\text{-}450_I$ (18). Fractions with flavin-containing monooxygenase and $P\text{-}450_I$ were adjusted to pH 7.25 and loaded onto hydroxylapatite columns previously equilibrated with buffer B at pH 7.25. $P\text{-}450_I$ and flavin-containing monooxygenase were resolved by elution with a linear gradient of 10–50 mM potassium phosphate, pH 7.25, in buffer B. Fractions containing flavoprotein (free of any traces of heme protein) were combined, diluted 3-fold, and loaded onto small hydroxylapatite columns equilibrated with 10 mM potassium phosphate, pH 7.25, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.1 mM PMSF. The column was washed overnight with this same buffer, and the detergent-free flavin-containing monooxygenase eluted with 0.3 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA.

The enzyme was concentrated, if necessary, and dialyzed overnight with 50 mM potassium phosphate, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.1 mM PMSF, 0.1 mM reduced glutathione, and frozen in small aliquots at −80°.

Assay of flavin-containing monooxygenase. The activity of purified flavin-containing monooxygenase toward various substrates was measured by following the substrate-dependent rate of NADPH oxidation at 340 nm (3) using a Hewlett-Packard 8450A diode array spectrophotometer. DMA-*N*-oxidase was determined by the method of Ziegler and Pettit (22).

Antibody to rabbit lung flavin-containing monooxygenase was raised in guinea pig as described previously (7). The IgG fraction was isolated from antisera by ammonium sulfate precipitation and DEAE-Sepharose chromatography (23). Western blots of SDS-PAGE gels were analyzed by immunostaining with the guinea pig IgG followed by ^{125}I-Protein A (24, 25). Following development of autoradiograms, quanti-

[1] The abbreviations used are: DMA, *N*,*N*-dimethylaniline; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

TABLE 1

Specific activity and yield of flavin-containing monooxygenase during purification from pregnant rabbit lung microsomes

Fraction	Activity[a]	Protein[b]	Specific activity	Yield
	nmol min^{-1}	*mg*	*nmol min*$^{-1}$ *mg*$^{-1}$	%
Microsomes	5200	290	18	100
Solubilized microsomes	3400	190	18	66
N-Octylamino-Sepharose 4B	2600	40	65	51
DEAE-Sepharose	2400	28	86	46
Hydroxylapatite[c]	1700	3.8	450	33

[a] Activity was calculated as nmol *N*,*N*-dimethylaniline-*N*-oxide formed min^{-1} at 37° and pH 8.4 according to the method of Ziegler and Pettit (22). Assays were performed on samples which had been frozen and thawed at least once.

[b] Protein was determined by the method of Lowry *et al.* (30) with human serum albumin as standard.

[c] This final fraction was assayed following detergent removal, concentration, and dialysis.

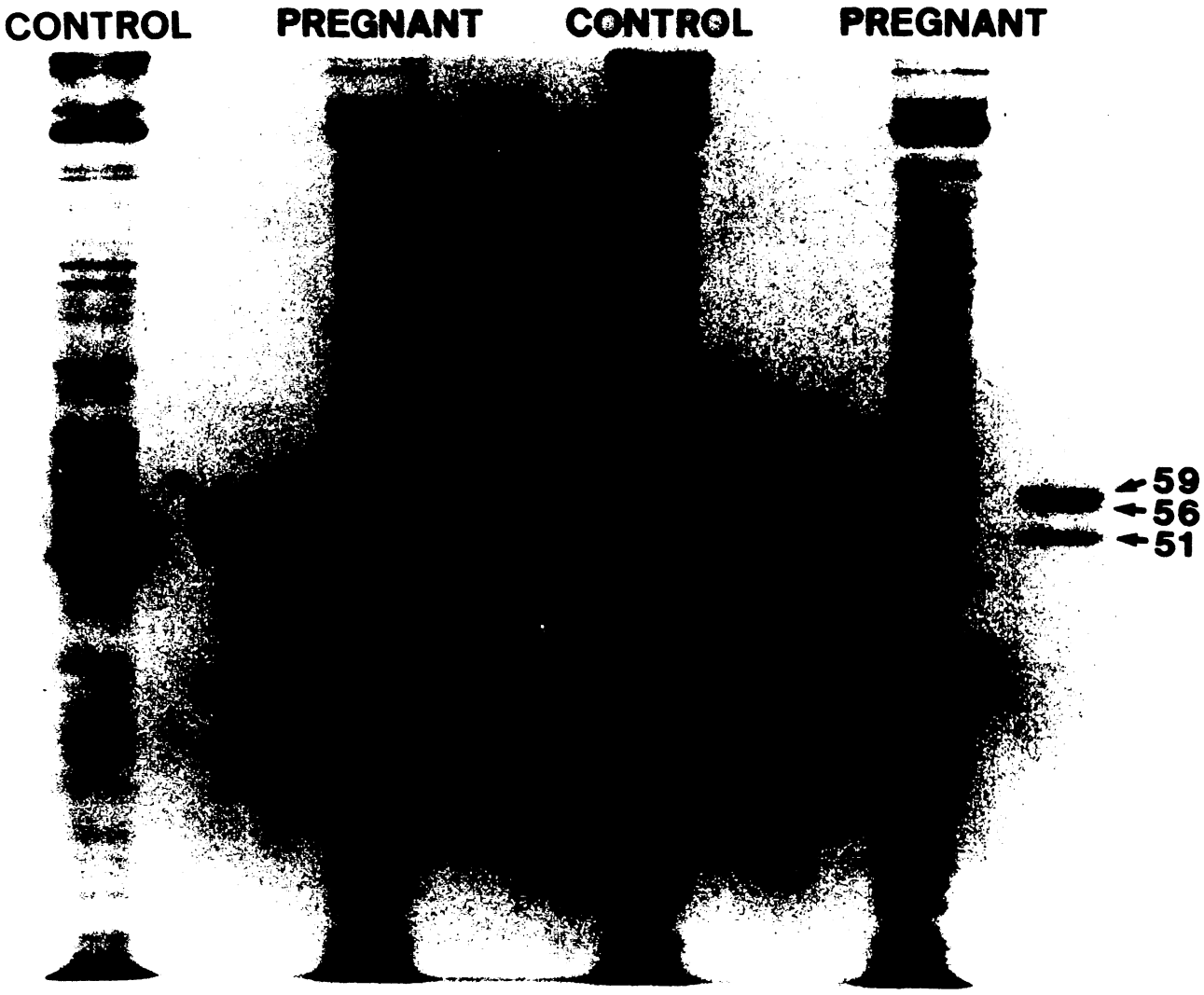

FIG. 1. *SDS-PAGE of control (nonpregnant female) and pregnant rabbit lung microsomes and purified cytochromes P-450 and flavin-containing monooxygenase*

Lanes marked control, 50 μg of lung microsomal protein from nonpregnant rabbits; lanes labeled pregnant, 50 μg of lung microsomal protein from pregnant (28th day) rabbits. Lanes labeled P-450$_I$, P-450$_M$, and FMO (flavin-containing monooxygenase) contained 2 μg of the respective proteins. Electrophoresis was performed by the method of Laemmli (29) as described under "Experimental Procedures." The minimum molecular weights were calculated by comparing relative mobility to a set of protein standards (not shown).

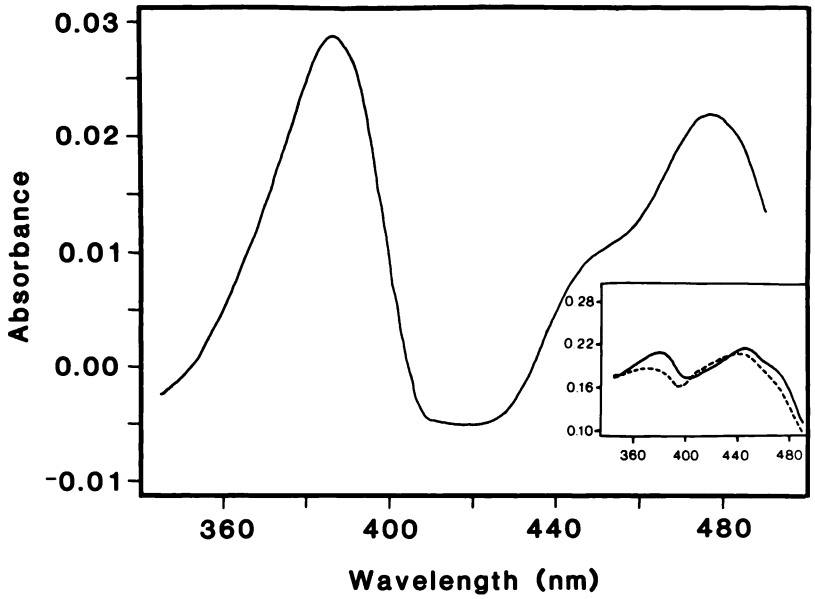

FIG. 2. *The NADP⁺-difference spectrum of oxidized rabbit lung flavin-containing monooxygenase*

Inset, the absolute oxidized spectrum of purified rabbit lung flavin-containing monooxygenase (10 μM) was recorded using a Hewlett-Packard 8450A diode array recording spectrophotometer (solid line). $NADP^+$ was then added to a final concentration of 37.5 μM and the spectrum repeated (dashed line). The difference spectrum shown is the calculated difference between the two recorded spectra shown in the inset. Repetition of the above with purified pig liver enzyme produced identical spectra (data not shown).

tation was performed by densitometry. In order to analyze a large number of samples, a modification of a recently developed hybrid dot technique was employed (26). The guinea pig IgG was first made monospecific by immunoadsorption by passing the IgG fraction through a Sepharose 4B column to which solubilized liver microsomal protein from pregnant rabbit had been covalently attached (27). Immunoquantitation of the hybrid dots was performed by cutting out the individual wells and counting on a γ counter.

Other assays. The flavin content of purified rabbit lung flavin-containing monooxygenase was determined by the method of Faeder and Siegel (28).

SDS-PAGE was performed as described by Laemmli (29) using an 8% separating gel. Protein was assayed according to Lowry *et al.* (30) with human serum albumin as standard.

RESULTS

Purification. Treatment of lung microsomes from pregnant rabbit with 0.6% cholate solubilized 66% of the microsomal flavin-containing monooxygenase (Table 1). Solubilized flavin-containing monooxygenase was bound to *n*-octylamino-Sepharose 4B and coeluted with cytochrome P-450 upon washing with 0.08% Lubrol PX. When this fraction was loaded onto a DEAE-Sepharose column, flavin-containing monooxygenase and $P\text{-}450_I$ eluted with the wash buffer, the flavoprotein eluting slightly ahead of $P\text{-}450_I$. The fractions containing flavoprotein and $P\text{-}450_I$ were combined and loaded onto a hydroxylapatite column. Elution with a linear gradient of potassium phosphate successfully resolved flavin-containing monooxygenase and $P\text{-}450_I$. Following detergent removal, the final yield was 33% with a 25-fold increase in purity relative to microsomes (Table 1).

Purified rabbit lung flavin-containing monooxygenase appears homogeneous on SDS-PAGE (Fig. 1) and can be resolved from two major isozymes of cytochrome P-450 previously purified from lung microsomes of pregnant rabbits (18). The increase in relative intensity of a band corresponding in molecular weight to the flavin-containing monooxygenase in pregnant (28th day) rabbits, compared to control nonpregnant females, suggests an induction of the protein during pregnancy. However, one must be cautious of such an interpretation as other lung microsomal proteins of comparable molecular weight may be present. For instance, the third P-450 isozyme purified from lung microsomes of pregnant rabbit (18), $P\text{-}450_{II}$ (LM_5), exhibits a molecular weight of 58,000 under these conditions. Analysis of the flavin content of rabbit lung flavin-containing monooxygenase demonstrated that the enzyme contains only FAD (14 nmol/mg).

In order to characterize purified rabbit lung flavin-containing monooxygenase, we compared it to the purified pig liver enzyme with respect to a number of its well known properties.

Spectral properties and pH optimum. Addition of $NADP^+$ to oxidized rabbit lung flavin-containing monooxygenase produced an identical shift in the flavoprotein spectrum (Fig. 2) to that previously demonstrated with the porcine liver enzyme. Furthermore, addition of equimolar NADPH to chilled oxidized rabbit lung enzyme produced a spectrum resembling a 4a-peroxyflavin intermediate (Fig. 3, top), similar to that obtained with the pig liver flavin-containing monooxygenase (Ref. 6 and Fig. 3, bottom).

Reactions catalyzed by the liver enzyme characteristically exhibit a pH optimum of about 8.5. The pH optimum of purified rabbit lung flavin-containing monooxygenase activity toward methimazole was much broader and somewhat higher (8.5–10.5) than the pig enzyme (Fig. 4). This higher pH optimum for the rabbit lung enzyme has also been observed with *N,N*-dimethylaniline or thiobenzamide as substrates (8, 9).

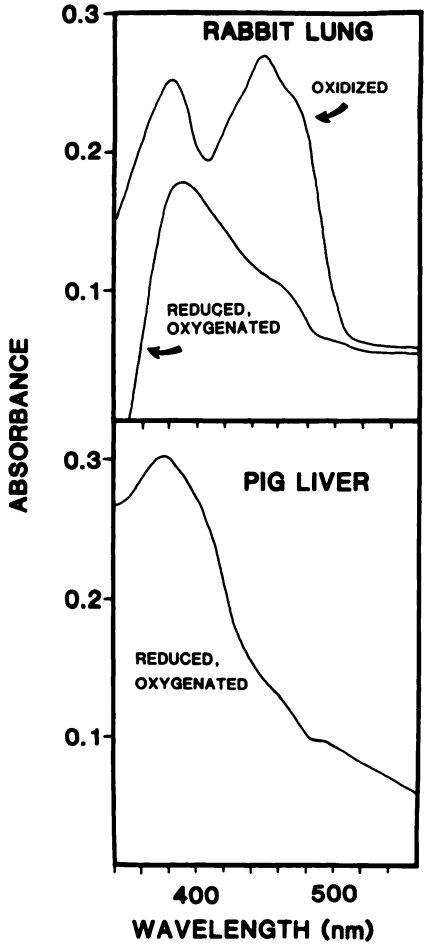

FIG. 3. *Spectra of the putative 4a-peroxyflavin intermediates of purified rabbit lung (top) and pig liver (bottom) flavin-containing monooxygenase*

Top panel, oxidized spectrum (top line, upper figure) of purified rabbit lung flavin-containing monooxygenase was obtained with 15 μM of purified enzyme in 50 mM potassium phosphate buffer, pH 7.2, at 4°. The putative 4a-peroxyflavin intermediate (bottom line, upper figure) was recorded following the addition of 50 μM $NADP^+$ and 15 μM NADPH. The spectrum shown was obtained 90 min following the addition of NADPH. Bottom panel, the putative 4a-peroxyflavin intermediate of the purified pig liver enzyme was formed as described above except that the concentrations of enzyme, $NADP^+$, and NADPH were 20 μM, 75 μM and 20 μM, respectively, and the spectrum was recorded 5 min following the addition of NADPH.

Sensitivity to detergent and thermolability. Pig liver flavin-containing monooxygenase has also been shown to be sensitive to inhibition by anionic detergents and to be very thermolabile (3). Sodium cholate produced a concentration-dependent inhibition of the pig enzyme with almost no detectable activity at 1% detergent (Fig. 5, lower curve). In contrast, the rabbit lung enzyme activity was slightly (10–25%) stimulated by sodium cholate (Fig. 5, upper curve). The insensitivity of rabbit lung flavin-containing monooxygenase to sodium cholate was a fortuitous finding and permitted the simultaneous purification of this enzyme and cytochrome P-450 from a single preparation of microsomes from pregnant rabbits.

Porcine liver flavin-containing monooxygenase is very thermolabile, especially in the absence of NADPH or $NADP^+$. Preincubation of pig liver enzyme at 45°, in the absence of NADPH, reduces the activity to less than 15% within 2 min (Fig. 6, lower curve). Inclusion of NADPH provides substantial protection against thermal inactivation (Fig. 6, middle curve). Purified rabbit lung enzyme was stable at 45° in the absence of NADPH for at least 10 min (Fig. 6, upper curve).

Substrate specificity and the effect of primary alkylamines. We have previously demonstrated (7) that, consistent with results from microsomal incubations and whole lung perfusion experiments (31, 32), the purified rabbit lung enzyme is not active toward chlorpromazine or imipramine. We now report that other substrates of the pig liver enzyme, which are poor substrates for the rabbit lung enzyme, include benzphetamine, pyrilamine, chlorpheniramine, thioridazine, and mesoridazine (data not shown). Conversely, prochlorperazine and trifluoperazine are excellent substrates for the purified rabbit lung enzyme with K_m values of 3 and 30 μM, respectively. The latter result is consistent with the work of Breyer (33) who found that rabbit lung microsomes had an extremely high capacity for the *N*-oxidation of perazine.

Stimulation of activity by primary alkylamines is diagnostic for pig liver flavin-containing monooxygenase (3); however, rabbit is one species in which such a stimulation is not observed. Instead, with purified rabbit lung enzyme, we found that *n*-octylamine could act as an inhibitor with other substrates and could, itself, stimulate NADPH oxidation at a substantial rate.

Preliminary results performed by Dr. Daniel Ziegler[2] and his associates indicate that the effects of primary alkylamines on the rabbit lung enzyme are quite complicated. Apparently, these compounds can act as substrates or uncouplers of the rabbit lung enzyme. In the latter case, there are both a one-electron reduction of O_2 to produce O_2^- and a two-electron reduction of O_2 to generate H_2O_2 (data not shown).

Activity and immunoquantitation during gestation. Western blots of rabbit lung and liver microsomes and purified rabbit lung flavin-containing monooxygenase, followed by immunostaining with guinea pig anti-rabbit lung flavin-containing monooxygenase IgG and ^{125}I-protein A and autoradiography, demonstrated that the antibody recognized at least two proteins in microsomes from rabbit lung (Fig. 7). The higher molecular weight band corresponds to the purified rabbit lung antigen. The antibody cross-reacts with a protein in rabbit lung of lower molecular weight which is also present in liver microsomes from rabbit. The identity of this lower molecular weight protein is uncertain. In any case, it is apparent that no immunochemically similar protein corresponding to rabbit lung flavin-containing monooxygenase is present in rabbit liver microsomes. Subsequently, guinea pig anti-rabbit lung flavin-containing monooxygenase IgG was purified by immunoadsorption using a column of solubilized rabbit liver microsomal protein linked to Sepharose 4B. The IgG fraction excluded by this column no longer recognizes the lower

[2] Dr. Daniel M. Ziegler, The University of Texas at Austin, personal communication.

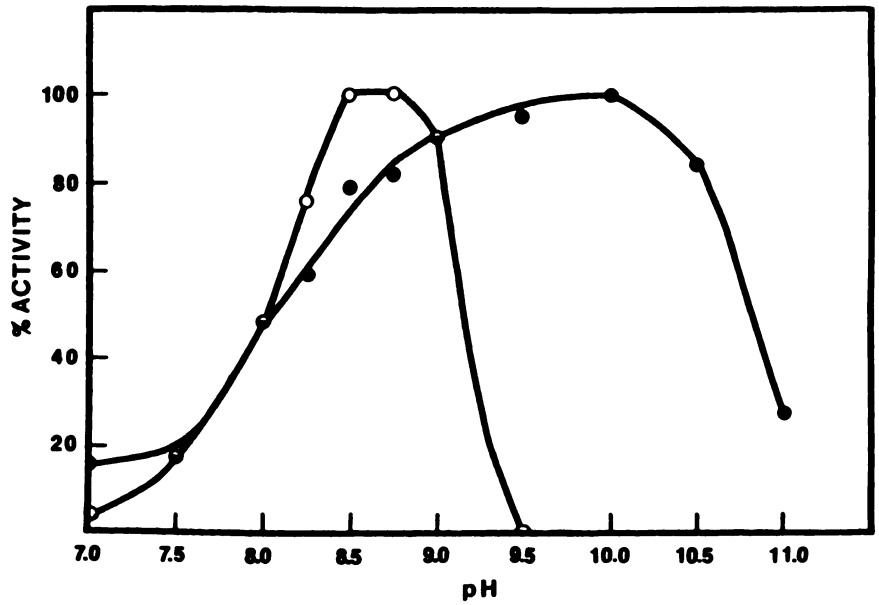

FIG. 4. *pH profile of purified pig liver and rabbit lung flavin-containing monooxygenase*

Activities were determined at 37° in 0.5 ml of 0.1 M Tricine. Background NADPH oxidation was determined by measuring Δ340 nm (1–2 min) with buffer and NADPH (0.25 mM) in both cuvettes and enzyme (50 μg) in the sample cuvette. Substrate (1 mM methimazole) was then added to both cuvettes and NADPH oxidation determined for another 1–2 min. Values shown are the means of duplicates and are corrected for background NADPH oxidation. The 100% activity for the pig liver enzyme (open circles) was 414 nmol min^{-1} mg^{-1} and 714 nmol min^{-1} mg^{-1} with the rabbit lung enzyme (closed circles).

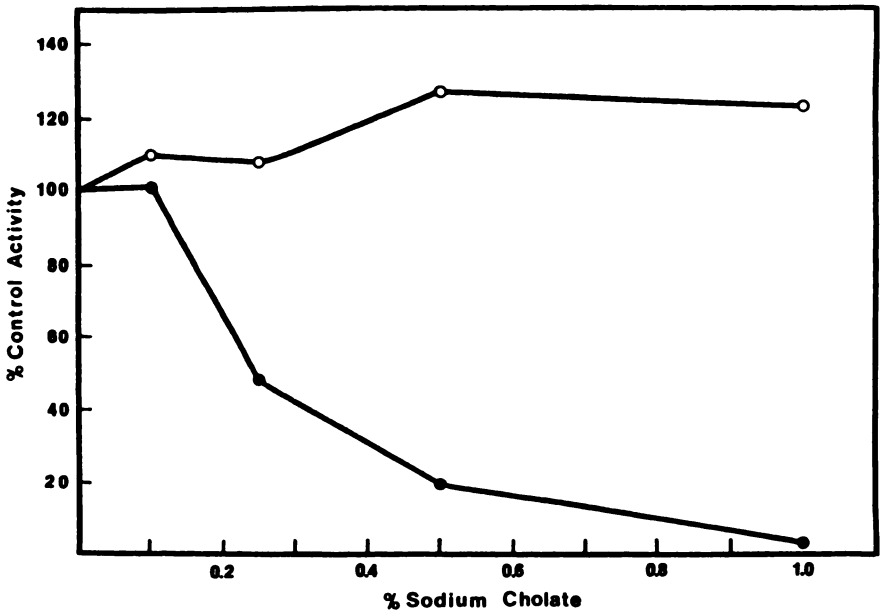

FIG. 5. *Inhibition of purified pig liver and rabbit lung flavin-containing monooxygenase by sodium cholate*

Activities were determined as described for Fig. 4 except that a pH of 8.4 was used and increasing amounts of sodium cholate were added. The 100% activity for the pig liver enzyme (closed circles) was 349 nmol min^{-1} mg^{-1} and for the rabbit lung enzyme (open circles) 503 nmol min^{-1} mg^{-1}.

molecular weight protein in Western blots of rabbit lung microsomes (Fig. 7, inset).

The relative amount of flavin-containing monooxygenase in rabbit liver and lung has been estimated previously by Western blotting and immunostaining with rabbit-anti-porcine liver enzyme IgG (24). It is unlikely that our immunopurified guinea pig-anti-rabbit lung enzyme IgG is cross-reacting with a similar protein as no liver microsomal protein in rabbit cross-reacts with our purified antibody. The identify of the protein cross-reacting with the rabbit anti-pig liver flavin-containing monooxygenase (24) is not known but could correspond to the lower molecular weight band seen in Western blots of rabbit liver and lung microsomes (Fig. 7) which are stained with nonimmunopurified guinea pig antibody.

This immunopurified guinea pig IgG provided us with an antibody that was essentially monospecific. Such monospecific antibodies can be utilized for immunoquan-

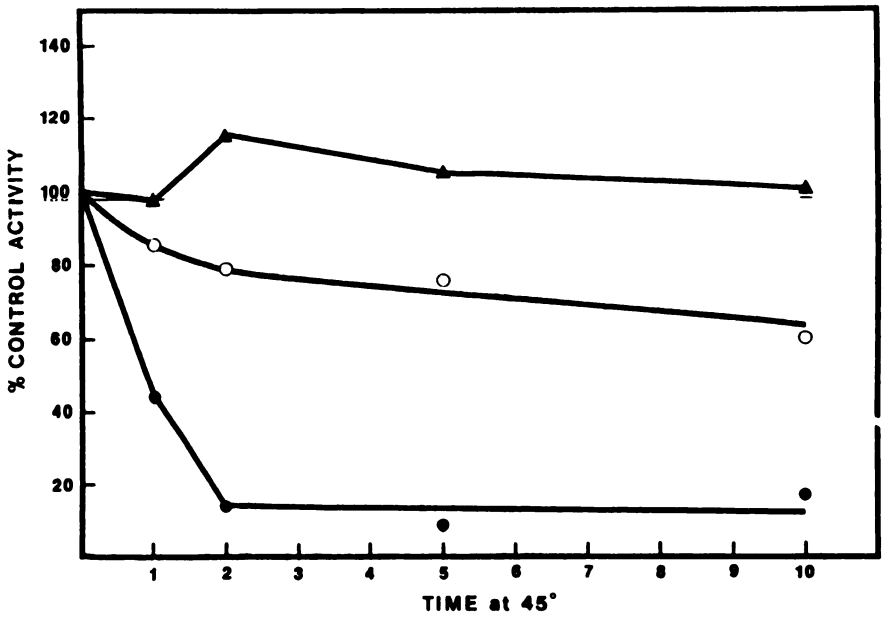

FIG. 6. *Thermolability of purified pig liver and rabbit lung flavin-containing monooxygenase*

Following a preincubation at 45° for the time period (in minutes) indicated, activities were determined with methimazole as substrate (pH 8.4) as described in the legend to Fig. 4. Closed circles, pig liver enzyme without NADPH during preincubation (100% activity = 372 nmol min^{-1} mg^{-1}); open circles, pig liver enzyme with NADPH during incubation (100% activity = 326 nmol min^{-1} mg^{-1}); closed triangles, rabbit lung enzyme without NADPH during preincubation (100% activity = 434 nmol min^{-1} mg^{-1}).

titation by a hybrid dot method (26). This procedure provides the advantages of an increase in sample number and sensitivity and a decrease in time and labor. We have utilized this procedure to immunoquantitate the rabbit lung enzyme throughout gestation in rabbit lung microsomes (Fig. 8, top). Comparison of immunoquantitation of rabbit lung flavin-containing monooxygenase with the time course, during gestation, of the induction of DMA-*N*-oxidase activity (Fig. 8, bottom) demonstrates that both parameters are maximally induced at 28 days of gestation. Guinea pig anti-rabbit lung flavin-containing monooxygenase IgG does not inhibit the enzymatic activity catalyzed either by the purified enzyme or rabbit lung microsomes. The antibody against the pig liver enzyme is also incapable of inhibiting the activity of its antigen (34).

DISCUSSION

A flavin-containing monooxygenase has been purified to apparent homogeneity from lung microsomes of pregnant rabbits. Many of the properties of this enzyme, signalled from the beginning by its stability in the solubilizing agent, cholic acid, were shown to be unique from the well-characterized porcine liver enzyme. There exists a number of significant similarities between the two proteins, including molecular weight (Fig. 1), spectral properties (Figs. 2 and 3), and activity toward substrates such as DMA, methimazole, thioacetamide, and cysteamine. However, these enzymes also exhibit many important differences. Rabbit lung flavin-containing monooxygenase has a broader and higher pH optimum (Fig. 4), is not sensitive to inhibition by sodium cholate (Fig. 5), and is not as thermolabile as the porcine liver enzyme (Fig. 6). In addition, while the latter enzyme is activated approximately 2-fold by alkylamines such as *n*-octylamine, rabbit lung flavin-containing monooxygenase, with methimazole as substrate, was actually inhibited by *n*-octylamine. The addition of *n*-octylamine increased the rate of NADPH oxidation, thereby acting as an uncoupler. The uncoupling of the rabbit lung enzyme by primary alkylamines may have significant toxicological implications as basic amines are effectively bioconcentrated by lung. If these amines are bioconcentrated by the same cell types as those containing the enzyme, this could lead to localized production of H_2O_2 or O_2^- with subsequent peroxidation damage. Recently, Tynes *et al.* (8) have examined the activity of the rabbit lung enzyme with a number of primary alkylamines as substrates. In contrast to the initial findings of Ziegler,[2] they found no presumptive evidence for H_2O_2 production, although they could only account for 50% of the NADPH or O_2 consumed as the *N*-hydroxylamine product.

The activities with benzphetamine, chlorpromazine, and imipramine exhibited by the purified pig liver and rabbit lung enzymes were also quite different. The tricyclic antidepressants, chlorpromazine and imipramine, are bioconcentrated in lung, but in the case of rabbit, little *N*-oxidation by flavin-containing monooxygenase occurs in this tissue relative to liver (31, 32). Both of these compounds are excellent substrates for the pig liver enzyme, but the purified enzyme from rabbit lung is inactive toward chlorpromazine and imipramine (7).

In addition to benzphetamine, chlorpromazine, and imipramine, we have found rabbit lung flavin-containing monooxygenase to have little or no activity toward the phenothiazine derivatives, thioridazine and mesoridazine. However, phenothiazine derivatives containing piperazine side chains, such as prochlorperazine and trifluoperazine, are excellent substrates for the rabbit lung enzyme. In a previous study, rabbit lung microsomes

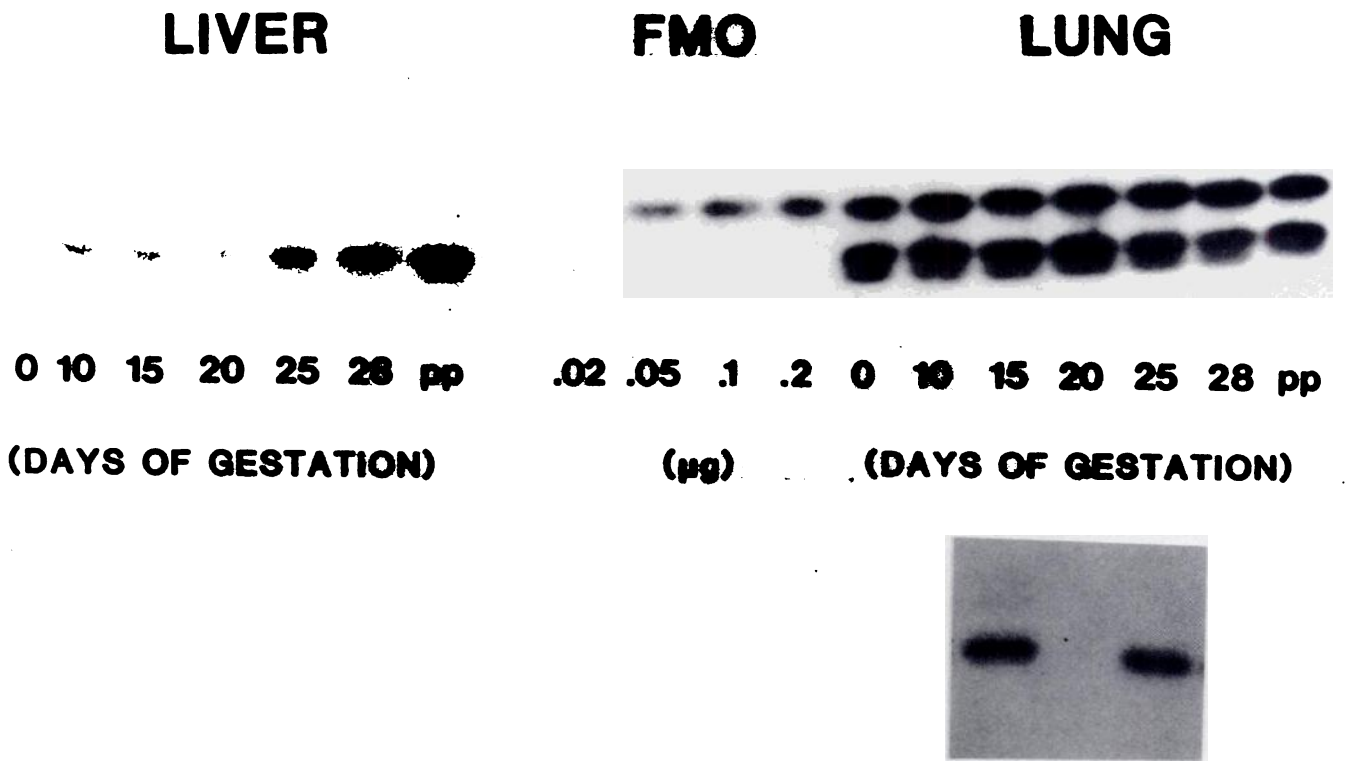

FIG. 7. *Autoradiograph of ^{125}I-labeled Western blot of purified rabbit lung flavin-containing monooxygenase (FMO) and rabbit liver and lung microsomes at various times of gestation*

SDS-PAGE was performed as described for Fig. 1 and proteins electrophoretically transferred to nitrocellulose sheets by Western blotting (200 mA, 18 hr). Nitrocellulose sheets were stained with guinea pig anti-rabbit lung flavin-containing monooxygenase IgG followed by ^{125}I-protein A as described under "Experimental Procedures." The amount of purified rabbit lung flavin-containing monooxygenase analyzed was 0.02–0.2 μg, and the microsomal sample wells contained 4 μg of total protein. Inset, the well on the left contained 0.1 μg of purified rabbit lung flavin-containing monooxygenase and the well on the right contained 4 μg of lung microsomal protein from a nonpregnant female rabbit. Visualization was as above except that the guinea pig IgG was first passed through a Sepharose column to which solubilized rabbit liver microsomal protein had been covalently attached.

exhibited a very high capacity for the *N*-oxidation of perazine (33).

The rabbit lung enzyme also appears to be inactive toward the tertiary amine antihistaminics, chlorpheniramine and pyrilamine. These results would suggest an unusual substrate specificity for the rabbit lung enzyme; however, the structurally unrelated compounds, DMA, methimazole, thioacetamide, and cysteamine, are all good substrates for the rabbit lung enzyme as they are also for other forms of flavin-containing monooxygenase. Nevertheless, the lack of activity of the rabbit lung enzyme toward a number of other potential substrates is of interest and is currently being investigated.

Recently, increasing attention is being focused on the role of flavin-containing monooxygenase in oxidizing numerous nitrogen- and sulfur-containing drugs and xenobiotics. The highest amounts of this enzyme are found in the endoplasmic reticulum of liver, but it has been located also in the nuclear membrane (35) and is present in every nucleated mammalian cell examined.[2] The physiological function of flavin-containing monooxygenase is not known. Ziegler and Poulsen have postulated a role for this eynzme in the synthesis of protein disulfide bonds through the formation of cysteamine-protein mixed disulfide intermediates. Cysteamine is the only known endogenous substrate for flavin-containing monooxygenase.

Devereux and Fouts (10) reported in 1975 that the flavin-containing monooxygenase (as determined by DMA-*N*-oxide formation) increased 1.5–2-fold during the 25th–28th day of gestation in lung, but not liver, microsomes of pregnant rabbit.

The results presented here indicate a larger degree of induction (500%) than this previous study (67%) and a later time point during gestation at which this maximum is reached (28 days as opposed to 20). The present study includes results from activity measurements as well as immunoblotting techniques. These results may reflect strain differences.

The physiological inducer is uncertain. Devereux and Fouts (10) were able to achieve equivalent induction of rabbit lung DMA-*N*-oxidase by administration of de-

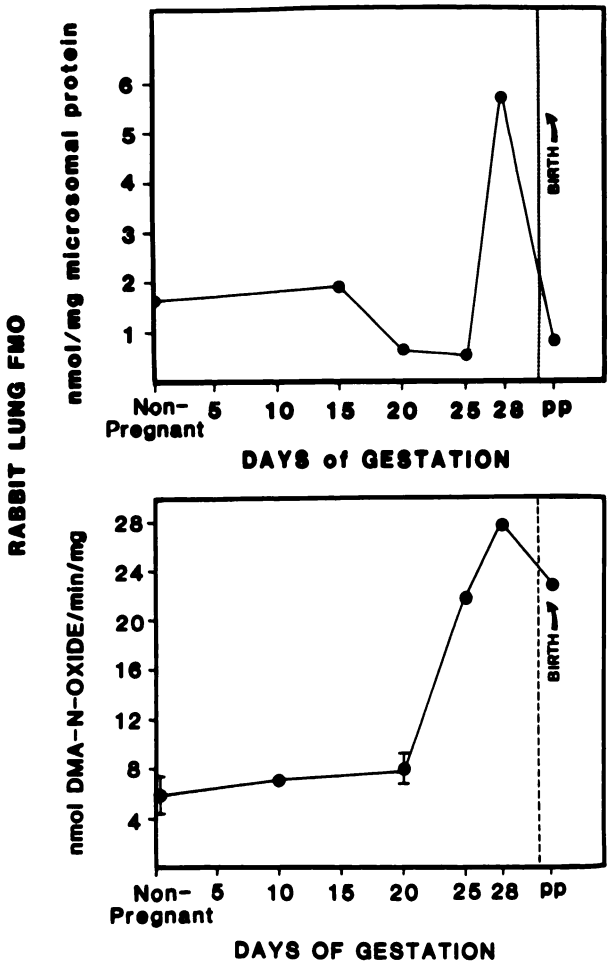

FIG. 8. Top. Immunoquantitation of rabbit lung flavin-containing monooxygenase from microsomes of rabbits during various stages of gestation. Microsomes from lungs of nonpregnant, pregnant (15, 20, 25, and 28 days), and postpartum (1 day) rabbits were applied to nitrocellulose sheets with the hybrid dot apparatus and then immunostained with antibody and ^{125}I-protein A as described under "Experimental Procedures." Immunoquantitation was determined by counting the ^{125}I spot and calculating the result as pmol of rabbit lung flavin-containing monooxygenase per mg of microsomal protein by a standard curve utilizing the purified antigen. Each value was determined in triplicate. Bottom. Time course of DMA-*N*-oxidase induction during gestation, in lung microsomes of pregnant rabbits. Lung microsomal *N*-oxidation of DMA was assayed, from rabbits killed at various time points throughout gestation, as described under "Experimental Procedures." The values shown represent the mean of duplicates except at 0 days (n = 4), 20 days (n = 3), and 1 day postpartum (n = 1).

oxycorticosterone. During pregnancy in rabbit, plasma levels of progesterone increase steeply between 2 and 10 days of pregnancy, remain at this peak until about day 20, and then gradually decline to original values at parturition (36). Deoxycorticosterone levels peak later in gestation. This observation is consistent with the fact that deoxycorticosterone is formed from progesterone by hydroxylation at carbon atom 21. There are other examples in the literature of the activity of mammalian flavin-containing monooxygenase being regulated by age, sex, pregnancy, and diurnal and estrous cycles, suggesting hormonal involvment (12–17).

The gestational time course of rabbit lung flavin-containing monooxygenase induction, as determined by immunoquantitation, demonstrated maximal induction (5-fold) on the 28th day of gestation, similar to the time and degree of induction of DMA-*N*-oxidase. The reason for the nonidentify of the time courses between days 15 and 25 (shown in Fig. 8) is not known.

Cysteamine is an excellent substrate for the rabbit lung enzyme. The oxidation of cysteamine by this enzyme is thought to be involved in the synthesis of protein disulfide bonds. In addition, there are a number of hormones and enzymes, important in metabolism, which are subject to regulation by the ratio of cysteamine/cystamine (37), and there is increasing evidence for implicating this ratio as a "third messenger" in regulation. The physiological significance of the induction of rabbit lung flavin-containing monooxygenase during pregnancy is unknown but could be related to its role in protein synthesis or its determination of the cysteamine/cystamine ratio in cells.

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REFERENCES

1. Ziegler, D. M. Microsomal flavin-containing monooxygenase: oxygenation of nucleophilic nitrogen and sulfur compounds, in *Enzymatic Basis of Detoxication* (W. B. Jakoby, ed). Academic Press, New York, Vol. 1, pp. 201–227, (1980).
2. Poulsen, L. L. Organic sulfur substrates for the microsomal flavin-containing monooxygenase, in *Reviews in Biochemical Toxicology* (E. Hodgson, J. R. Bend, and R. M. Philpot, eds). Elsevier/North-Holland, New York, Vol. 3, pp. 33–40, (1981).
3. Ziegler, D. M., and L. L. Poulsen. Hepatic microsomal mixed-function amine oxidase. *Methods Enzymol.* **52**:142–151 (1978).
4. Poulsen, L. L., B. S. S. Masters, and D. M. Ziegler. Mechanism of 2-naphthylamine oxidation catalyzed by pig liver microsomes. *Xenobiotica* **6**:481–498 (1976).
5. Ziegler, D. M., and L. L. Poulsen. Protein disulfide bond synthesis: a possible intracellular mechanism. *Trends Biochem. Sci.* **2**:79–81 (1977).
6. Poulsen, L. L., and D. M. Ziegler. The microsomal FAD-containing monooxygenase. Spectral characterization and kinetic studies. *J. Biol. Chem.* **254**:6449–6455 (1979).
7. Williams, D. E., D. M. Ziegler, D. J. Nordin, S. E. Hale, and B. S. S. Masters. Rabbit lung flavin-containing monooxygenase is immunochemically and catalytically distinct from the liver enzyme. *Biochem. Biophys. Res. Commun.* **125**:116–122 (1984).
8. Tynes, R. E., P. J. Sabourin, and E. Hodgson. Identification of distinct hepatic and pulmonary forms of microsomal flavin-containing monooxygenase in the mouse and rabbit. *Biochem. Biophys. Res. Commun.* **126**:1069–1075 (1985).
9. Devereux, T. R., and J. R. Fouts. *N*-Oxidation and demethylation of *N,N*-dimethylaniline by rabbit liver and lung microsomes. Effect of age and metals. *Chem.-Biol. Interact.* **8**:91–105 (1974).
10. Devereux, T. R., and J. R. Fouts. Effect of pregnancy or treatment with certain steroids on *N,N*-dimethylaniline demethylation and *N*-oxidation by rabbit liver or lung microsomes. *Drug Metab. Dispos.* **3**:254–258 (1975).
11. Devereux, T. R., R. M. Philpot, and J. R. Fouts. The effect of Hg^{2+} on rabbit hepatic and pulmonary solubilized partially purified *N,N*-dimethylaniline *N*-oxidases. *Chem.-Biol. Interact.* **18**:277–287 (1977).
12. Heinze, E., P. Hlavica, M. Kiese, and G. Lipowsky. *N*-Oxygenation of arylamines in microsomes prepared from corpora lutea of the cycle and other tissues of the pig. *Biochem. Pharmacol.* **19**:641–649 (1970).
13. Wirth, P. J., and S. S. Thorgeirsson. Amine oxidase in mice—sex differences and developmental aspects. *Biochem. Pharmacol.* **27**:601–603 (1978).
14. Duffel, M. W., J. M. Graham, and D. M. Ziegler. Changes in dimethylaniline *N*-oxidase activity of mouse liver and kidney induced by steroid sex hormones. *Mol. Pharmacol.* **19**:134–139 (1981).
15. Das, M. L., and D. M. Ziegler. Rat liver oxidative *N*-dealkylase and *N*-oxidase activities as a function of animal age. *Arch. Biochem. Biophys.* **140**:300–306 (1970).
16. Dixit, A., and T. E. Roche. Spectrophotometric assay of the flavin-containing monooxygenase and changes in its activity in female mouse liver with

nutritional and diurnal conditions. *Arch. Biochem. Biophys.* **233**:50–63 (1984).
17. Osmitz, T. G., and A. P. Kulkarni. Oxidative metabolism of xenobiotics during pregnancy: significance of microsomal flavin-containing monooxygenase. *Biochem. Biophys. Res. Commun.* **109**:1164–1171 (1982).
18. Williams, D. E., S. E. Hale, R. T. Okita, and B. S. S. Masters. A prostaglandin ω-hydroxylase cytochrome P-450 (P-450$_{PG-\omega}$) purified from lungs of pregnant rabbits. *J. Biol. Chem.* **259**:14600–14608 (1984).
19. Imai, Y., and R. Sato. An affinity column method for partial purification of cytochrome P-450 from phenobarbital-induced rabbit liver microsomes. *J. Biochem.* (*Tokyo*) **75**:689–697 (1974).
20. Tiselius, A., S. Hjerten, and O. Levin. Protein chromatography on calcium phosphate columns. *Arch. Biochem. Biophys.* **65**:132–155 (1956).
21. Guengerich, F. P. Preparation and properties of highly purified cytochrome P-450 and NADPH-cytochrome P-450 reductase from pulmonary microsomes of untreated rabbits. *Mol. Pharmacol.* **13**:911–923 (1977).
22. Ziegler, D. M., and F. H. Pettit. Formation of an intermediate *N*-oxide in the oxidative demethylation of *N,N*-dimethylaniline catalyzed by liver microsomes. *Biochem. Biophys. Res. Commun.* **15**:188–193 (1964).
23. Masters, B. S. S., J. Baron, W. E. Taylor, E. L. Isaacson, and J. Lospalluto. Immunochemical studies on electron transport chains involving cytochrome P-450. I. Effects of antibodies to pig liver microsomal reduced triphosphopyridine nucleotide-cytochrome *c* reductase and the nonheme iron protein from bovine adrenocortical mitochrondria. *J. Biol. Chem.* **246**:4143–4150 (1971).
24. Dannan, G. A., and F. P. Guengerich. Immunochemical comparison and quantitation of microsomal flavin-containing monooxygenase in various hog, mouse, rat, rabbit, dog, and human tissues. *Mol. Pharmacol.* **22**:787–794 (1982).
25. Burnette, W. N. "Western Blotting." Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and detection with antibody and radioiodinated Protein A. *Anal. Biochem.* **112**:195–203 (1981).
26. Domin, B. A., C. J. Serabjit-Singh, and R. M. Philpot. Quantitation of rabbit cytochrome P-450, form 2, in microsomal preparations bound directly to nitrocellulose paper using a modified peroxidase-immunostaining procedure. *Anal. Biochem.* **136**:390–396 (1984).
27. Thomas, P. E., D. Korzeniowski, D. Ryan, and W. Levin. Preparation of monospecific antibodies against two forms of rat liver cytochrome P-450 and quantitation of these antigens in microsomes. *Arch. Biochem. Biophys.* **192**:524–532 (1979).
28. Faeder, E. J., and L. M. Siegel. A rapid micromethod for determination of FMN and FAD in mixtures. *Anal. Biochem.* **53**:332–336 (1973).
29. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**:680–685 (1970).
30. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
31. Ohmiya, Y., and H. M. Mehendale. Metabolism of chlorpromazine by pulmonary microsomal enzymes in the rat and rabbit. *Biochem. Pharmacol.* **31**:157–162 (1982).
32. Ohmiya, Y., and H. M. Mehendale. *N*-Oxidation of *N,N*-dimethylaniline in the rabbit and rat lung. *Biochem. Pharmacol.* **32**:1281–1285 (1983).
33. Breyer, U. Metabolism of the phenothiazine drug perazine by liver and lung microsomes from various species. *Biochem. Pharmacol.* **20**:3341–3351 (1971).
34. Masters, B. S. S., and D. M. Ziegler. The distinct nature and function of NADPH-cytochrome *c* reductase and the NADPH-dependent mixed-function amine oxidase of porcine liver microsomes. *Arch. Biochem. Biophys.* **145**:358–364 (1971).
35. Patton, S. E., G. M. Rosen, E. J. Raukman, D. G. Graham, B. Small, and D. M. Ziegler. Hamster hepatic nuclear mixed-function amine oxidase: location and specific activity. *Mol. Pharmacol.* **18**:151–156 (1980).
36. Harrington, F. E., and J. D. Rothermel. Daily changes in peripheral plasma progesterone concentrations in pregnant and pseudopregnant rabbits. *Life Sci* **20**:1333–1340 (1977).
37. Ziegler, D. M. Role of reversible oxidation-reduction of enzyme thiols—disulfides in metabolic regulation. *Annu. Rev. Biochem.* **54**:305–329 (1985).

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