AN ABSTRACT OF THE THESIS OF

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One well known characteristic of the cytochrome P-450 system is its inducibility by exogenous chemicals but flavin-containing monooxygenase (FMO) seems to be refractory to such treatment. Previous studies have shown that FMO could be regulated developmentally, presumably through alterations in hormonal status. FMO activity is influenced by some nutritional factors like starvation and ascorbic acid deficiency and also by diabetic disease. The regulation of rabbit FMO throughout gestation and post-partum has been followed in maternal liver, lung, kidney and bladder utilizing immunoquantitation with antibodies, enzyme activity assays, northern and slot blotting using ^{32}P-labelled rabbit FMO cDNAs. Rabbit liver and lung FMO significantly increased throughout gestation. Two peaks were observed at mid- and late-gestation and those peaks correspond to the peak of plasma progesterone (mid-gestation) and plasma glucocorticoids (late-gestation) during pregnancy, respectively. FMO in rabbit kidney was

induced during late-gestation but not at mid-gestation and the peak during late-gestation is consistent with plasma glucocorticoid levels. Hormone implantation or injection studies support the hypothesis that FMOs in liver and lung are inducible by both progesterone and glucocorticoids, whereas, FMO in kidney is under the control of glucocorticoids. Estradiol induced FMO in female rabbit lung but not in males. FMO in fetal tissue dramatically increased before birth possibly by the regulation of glucocorticoids and continuously increased after birth. Diurnal variations of FMO were observed in rabbit lung with highest levels at 12 noon and lowest levels at 8 PM. The results of these studies indicate that FMO is under long-term developmental and hormonal control and short-term diurnal regulation at the level of gene expression but the effects observed appear to be sex- and tissue-dependent.

Flavin-Containing Monooxygenase, Tissue Specific Regulation in Rabbit

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FLAVIN-CONTAINING MONOOXYGENASE, TISSUE SPECIFIC REGULATION IN RABBIT

INTRODUCTION

Mammalian Flavin-Containing Monooxygenase (FMO, EC 1.14.13.8) is a flavoprotein, which oxygenates a wide variety of structurally diverse xenobiotics containing soft-nucleophiles, predominantly nitrogen and sulfur (1). Most metabolites formed by this enzyme are biologically inactive and can be easily excreted, however, some are potentially carcinogenic or mutagenic (1). The catalytic cycle of FMO involves reduction of FAD by NADPH. After reduction by NADPH, $NADP^+$ remains bound to the enzyme providing it with a certain amount of stabilization. Dioxygen is then bound at the 4-alpha position of the flavin creating a hydroperoxy moiety at this site (1, 2 and Fig. A). Therefore, subtrate is not required for flavin reduction or oxygen binding and compounds prone to oxidation via a peracid are extremely good substrates for the enzyme (1). The ability of FMO to oxygenate such a wide variety of compounds is because any soft nucleophile with access to the active site is oxygenated with chemistry similar to oxidation by organic peroxides (3). Consequently, secondary and tertiary amines, as well as many sulfur-containing chemicals, have been shown to be substrates for the enzyme (1). However, there do appear to be some protein-mediated constraints influencing access to the active site (4). For example, chemicals which possess more than one charged atom,

example, chemicals which possess more than one charged atom, such as glutathione, are not substrates. The specificity of FMO results from exlusion of non-substrate (endogenous amines and thiols) from the active site (2, 5). The only known endogenous substrate of FMO is cysteamine, which is oxidized to the disufide, cystamine. The physiological role of FMO is uncertain but it may be related to the formation of disufide bonds during protein synthesis (6).

The FMO and P-450-dependent monooxygenases share some common features, such as localization in microsomes, requirements for oxygen and NADPH and activity toward the same substrates (1). These similarities led to early skepticism concerning the existence of FMO. Subsequent studies have demonstrated a number of interesting differences between these monooxygenase systems. Various P-450 gene families are inducible by xenobiotics such as 3-methylcholanthrene (1A family), phenobarbital (2B family), ethanol (2E family), peroxisome proliferators (4B family) and steroids (3A family) (7). With the possible exception of steroids, FMO has been shown not to be inducible by exposure to any agent. In addition, compared to P-450, FMOs are notoriously thermolabile (1). Liver FMOs from a number of species are stimulated by primary alkyamines, such as n-octylamine, which is an inhibitor of P-450 (1). Another interesting difference relates to the unusual kinetics of FMO, in which the binding of substrate has no effect on turnover, making all substrates

have equivalent V_{max} values. These differences have been exploited in studies of the relative role of these two systems in the metabolism of a particular xenobiotic (8-10).

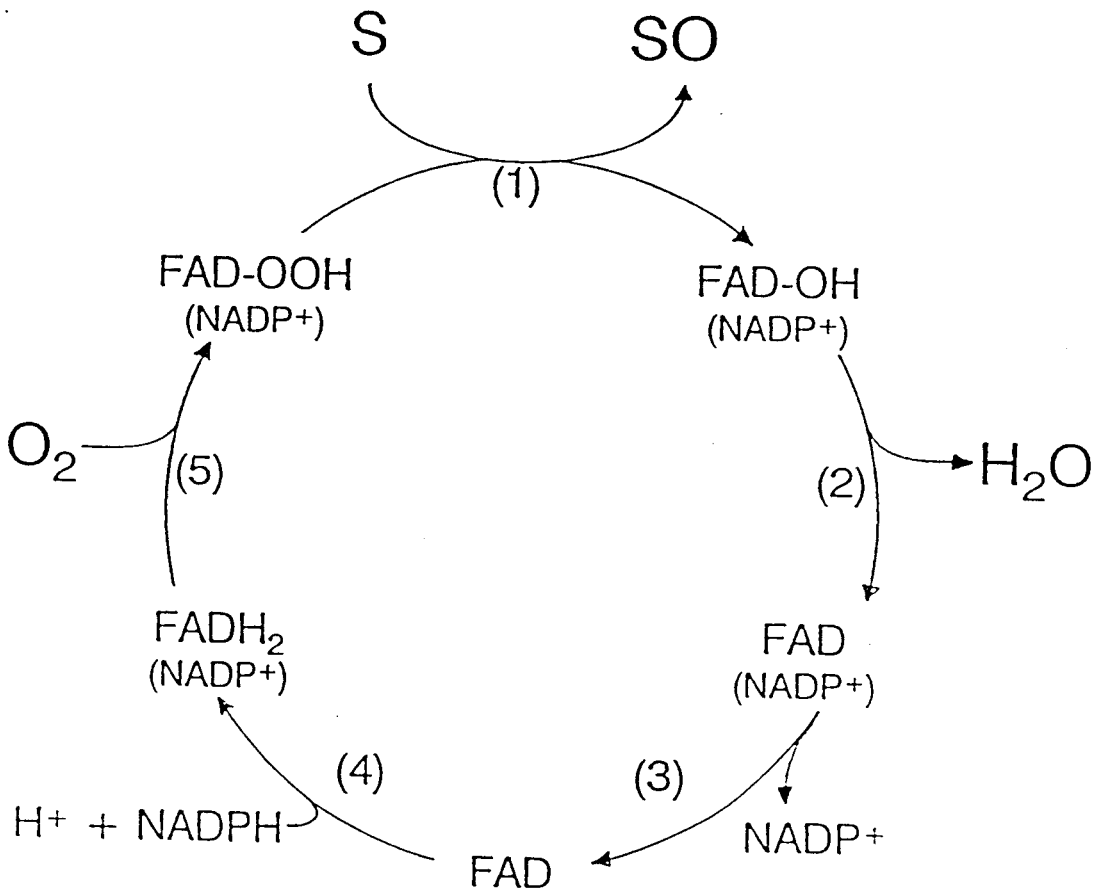
Recently, the existence of multiple forms of FMO was documented when the first extrahepatic FMO was purified and charaterized from rabbit lung (11-13). It was found that lung and liver FMO were markedly distinct with respect to physiochemical properties such as thermostability, pH optima and sensitivity to anionic detergents. Lung and liver FMO also were found to be immunochemically and catalytically distinct (14-16). The cDNA sequence and deduced protein sequence showed that rabbit lung and liver FMO are different gene products. Interestingly, rabbit liver FMO displayed much greater similarity to pig liver FMO (87%) than to rabbit lung FMO (56%) (17, 18), indicating that distinct lung and liver FMOs evolved prior to speciation. In addition to rabbit, other species which have been shown to have distinct lung FMOs include mouse, rat, guinea pig, hamster, sheep and monkey (19, 20). A recent study demonstrated four different forms of FMO (IA1, IB1, IC1 and ID1) in human (21). The human IB FMO present in small amounts in lung, liver and kidney, is 85% identical to the rabbit lung enzyme. The "lung" FMO is also present in other rabbit tissues including kidney and bladder. Rabbit FMOs in different tissues may be regulated independently and have different substrate specificities and evidence exists for sex- and developmental-related differences

(1). Unlike the well known characteristic of the cytochrome P-450 dependent monooxygenase systems, FMO seems to be refractory to induction by exogenous chemicals. Previous studies have shown that FMO could be regulated developmentally, presumably through alterations in hormonal status (1, 22).

These studies, utilizing rabbit tissues, were designed to investigate for the mechanisms of developmental and hormonal regulation of FMO.

Figure A. Major steps in the catalytic cycle of mammalian microsomal flavin-containing monooxygenases. The cycle is from Ziegler (1990) (2).

Figure A.


S
SO
(1)
FAD-OOH
(NADP+)
FAD-OH
(NADP+)
O_2
(5)
(2)
H_2O
$FADH_2$
(NADP+)
FAD
(NADP+)
(4)
(3)
H^+ + NADPH
FAD
NADP+

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Chapter 1

Tissue Specific Regulation of Flavin-Containing Monooxygenase (FMO) Throughout Gestation in Rabbits

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Tissue Specific Regulation of Flavin-Containing Monooxygenase (FMO) Throughout Gestation in Rabbits.

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ABSTRACT

Flavin-containing monooxygenase (FMO) is not induced by xenobiotics but is regulated by endogenous factors including the steroid hormones. During the 31 day gestational period of rabbit, the plasma levels of progesterone increase until mid-gestation and then decline to non-pregnant levels by parturition, whereas, glucocorticoids levels peak only at parturition. It has been shown that FMO is induced during gestation in rabbit. Rabbit tissues were collected throughout gestation (0, 10, 15, 20, 25, 28 and 31 day) and postpartum (1, 7, 21 and 29 day). Microsomes and total RNA were prepared from each tissue. ^{14}C-Dimethylaniline N-oxidase in lung increased with pregnancy and was highest on days 15 and 31 of gestation and declined after parturition. Immunoquantitation by western blotting was consistent with FMO activity; lung protein levels were highest on the 15th and 31st day of gestation. A rabbit lung FMO cDNA probe was used to quantify FMO mRNA by northern blotting and showed that mRNA levels were also highest on the 15th and 31st day of gestation. The protein level, FMO activity and FMO mRNA expression in kidney indicate that kidney FMO was induced at the time of parturition presumably by glucocorticoids, whereas, lung FMO may be regulated by both progesterone and glucocorticoids. No evidence was observed for a sex difference or change during pregnancy in the level of FMO in bladder. This suggests that the levels and activity of FMO are regulated by endocrine

factors and controlled by gene expression in a tissue-specific manner.

Introd.

INTRODUCTION

Mammalian flavin-containing monooxygenase (FMO, EC 1.14.13.8) is a flavoprotein, which catalyzes the oxygenation of a large variety of nitrogen- and sulfur-containing drugs and xenobiotics (1). Most of the metabolites formed by this enzyme are biologically inactive and easily excreted, however, some are potentially carcinogenic or mutagenic (1). The only known endogenous substrate of flavin-containing monooxygenase is cysteamine, which is oxidized to the disulfide, cystamine. The physiological role of FMO is uncertain but it may be related to the formation of disulfide bonds during protein synthesis (2). At least three different forms of FMO exist in rabbit (3-5). The cDNA sequence of rabbit lung and liver confirm different gene products that are related with 56% identity in amino acid sequence (6). The extrahepatic FMO is also present in rabbit kidney and bladder and evidence exists for sex- and developmental-related differences. A recent study demonstrated four different forms of FMO, (IA1, IB1, IC1 and ID1)[1] in human (7,8). The human IB FMO, present in small amounts in lung, liver and kidney, is 85% identical to the rabbit lung enzyme (8). Rabbit FMOs in different tissues may be regulated independently and have different substrate specificities. FMO is not inducible by exogenous compounds but is regulated by endogenous factors like sex steroids and

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[1] This nomenclature has been suggested by R.M. Philpot and U.A. Meyer.

glucocorticoids (9). The purpose of the present study is to examine the developmental regulation of the "lung" form of FMO in maternal rabbit tissues.

MATERIALS AND METHODS

ANIMALS

Timed-pregnant New Zealand white rabbits were obtained from the Rabbit Research Institute at Oregon State University and housed in the Laboratory Animal Resource Center. Animals were maintained on a 12-hour day-night cycle, and feeding was ad libitum. In order to follow the developmental regulation of the levels of FMO activity, protein levels and mRNA, a total of 20 pregnant, 2 non-pregnant female and 2 male rabbits were used. Two pregnant females were killed on days 10, 15, 20, 25, 28 and 31 of gestation. Following parturition, two rabbits were killed on days 1, 7, 21 and 28 post-partum. Animals were killed by CO_2 asphxiation. The tissues were immediately removed and frozen in liquid nitrogen and stored at -90°C until assayed.

MICROSOME PREPARATION

Microsomes were prepared using a modification of the procedure of Guengerich (10). The levels of microsomal protein were assayed by the method of Lowry et al., (11) using BSA[2] as the standard.

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[2]Abbreviations used: BSA, bovine serum albumin; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PBS, 20 mM potassium phosphate, pH 7.4, 0.8% NaCl; IgG, immunoglobulin G; DMA, N,N'-dimethylaniline; PCR, polymerase chain reaction; 20 X SSC, 3 M sodium chloride, pH 7.0, 0.3 M sodium citrate.

IMMUNOQUANTITATION

The microsomal proteins were resolved by SDS-PAGE and then electrophoretically transferred to nitrocellulose. Following 1 hour incubation at room temperature with 2% BSA in PBS, the blots were stained with guinea pig anti-rabbit lung FMO IgG (20 µg IgG per ml in 2% BSA-PBS buffer with 0.05% Triton X-100) for 1.5 hour at room temperature. The FMO-IgG complexes were visualized by incubating with [^{125}I]-protein A for 1 hour followed by autoradiography (12). Immunoquantitation was performed by laser densitometry and relative amounts of FMO were calculated utilizing the purified rabbit lung FMO as standard for lung and relative densities of blots were compared for kidney and bladder.

ASSAY OF FMO ACTIVITY

The metabolism of [^{14}C]-DMA by microsomal FMO was performed by HPLC to resolve the N-oxide and N-demethylated metabolites. Microsomes (0.1 mg) or purified FMO (0.02 mg) were incubated in 0.1 M tricine, pH 8.5 containing 1 mM EDTA, and an NADPH generating system [1 mM NADPH, 0.02 M isocitrate and 1 Unit isocitrate dehydrogenase]. [^{14}C]-DMA was added to a final concentration of 0.5 mM (1-2 µCi/µmol). All components, except the microsomes or purified FMO, were preincubated for 2 min and the reaction initiated by the addition of enzyme. The reaction was terminated by the addition of 0.1 ml of methanol. HPLC analysis was performed with a Whatman RAC II

Partisil 5 ODS-3 (4.6 mm x 10 cm) column eluted with 62% MeOH in water at 0.5 ml/min. Metabolite quantitation was performed with an on-line radiochemical detector (Beckman Model 171, equipped with a solid cell).

RNA ISOLATION

Total RNA were prepared by the modified method of acid guanidinium thiocyanate-phenol-chloroform extraction (13).

RABBIT LUNG cDNA (1643 bp) PREPARATION

To obtain a cDNA, 1 μg of the female (28 day gestation) lung mRNA was used as a template to make a lung library. Oligo (dT) was used as a primer along with superscript reverse transcriptase enzyme under the conditions recommended by Gibco/BRL. One-fifth of the total cDNA was then used in a PCR reaction. Utilizing the rabbit lung FMO cDNA sequence published by Lawton et al. (6), we employed primer sequences from 67 to 94 and from 1683 to 1710, respectively. The entire coding region was amplified in a fragment of 1643 bp. The DNA in the PCR reaction was treated with T4 DNA polymerase to ensure the presence of blunt ends, then fractionated on an agarose gel, the 1643 bp band excised and eluted using glass milk. The DNA was then ligated with SmaI-digested, CIAP-treated pUC19 which was then used to transform E. coli DH5a. The fragment was oriented in the plasmid opposite to β-galactosidase. The intact cDNA fragments were excised from

pRNH135 utilizing EcoRI and BamHI digestion.

ELECTROPHORESIS AND TRANSFER

Electrophoresis of RNA through agarose-containing formaldehyde was performed by published procedures (14). The amount of RNA in each sample was measured by optical density at 260 and 280 nm; 20 µg of each sample was analyzed through agarose gel containing formaldehyde in duplicate. RNA in the gel was transferred to nylon membrane by capillary movement overnight using 20 X SSC buffer and the transferred blot was exposed to UV light (U.V. Crosslinker, XL-1000, Fisher Biotech) to fix the RNA on the membrane (15).

HYBRIDIZATION

A probe used to quantify rabbit lung FMO mRNA on northern blots was obtained by random primer-labeling of rabbit lung FMO cDNA (1643 bp) with α^{32}P-dCTP (16). After prehybridization for 4 h in 6 X SSC, 50% formaldehyde, 0.1% SDS, 5 X Denhardt's solution, 100 µg/ml salmon sperm DNA and 100 µg/ml tRNA, hydbidization was performed at 42°C overnight in the same medium in the presence of cDNA labeled with [α-^{32}P]dCTP (ICN) with a random primer-labeling kit from Boehringer Mannheim. The filter was washed in 2 X SSC buffer containing 0.1% SDS twice at room temperature for 15 min and 0.1 X SSC buffer with 0.1% SDS at room temperature for 15 min. The membrane was then exposed to Kodak X-OMAT AR film (Kodak, Rochester, NY) for 3

days with an amplifying screen at -90°C. Integrity of RNA was routinely assessed with a β-actin cDNA probe to demonstrate that each lane contained approximately equal amounts of mRNA.

RESULTS AND DISCUSSION

Little work has been published on the hormonal regulation of FMO. Sex-related differences in the concentration or enzyme activity of FMO have been observed in rats, mice, and rabbits (17). During late gestation, changes in the hormonal milieu appear to be responsible for FMO induction in rabbit lung (3,18).

The regulation of rabbit FMO throughout gestation and postpartum has been followed in maternal lung, kidney and bladder utilizing immunoquantitation with antibody to lung FMO. In a previous report, it was found that rabbit lung FMO is maximumally induced on day 28 of gestation and decreased post-partum (3,19). In this study, the peak levels of lung protein were observed at mid- and late-gestation, whereas, the protein peak in kidney was at the time of parturition (Figs. 1.1A, 1.1B, 1.2). No evidence was observed for a sex difference or change during pregnancy in the level of protein in rabbit bladder (Figs. 1.2, 1.9A, 1.9B). The measurement of enzymatic activity in lung, kidney and bladder was consistent with protein levels during pregnancy (Fig.1.3). Rabbit lung FMO is higher in adult female rabbits compared to males (Fig.1.4) and this is consistent with the results of previous studies (3,18-20). The kidney FMO expression was slightly higher in adult females than in adult males (Fig.1.4). This is in contrast to a previous study where kidney FMO expression in males was higher than that of females (3). To examine the

levels of FMO mRNA expressed in maternal lung during gestation, total RNA was isolated from lungs of rabbits killed at various times of gestation and postpartum. The levels of mRNA quantitated by northern and slot blotting using ^{32}P-labeled rabbit lung FMO cDNA were significantly increased throughout gestation and two peaks were observed at mid-gestation and at the time of parturition (Figs. 1.5A, 1.5B). The levels of FMO mRNA decline after parturition to levels at or below those in virgin females. Peaks of lung FMO mRNA during mid- and late-gestation are consistent with protein levels (Figs. 1.1, 1.5). The mid-gestation peak corresponds to the peak of progesterone levels in the rabbit, and the peak during late-gestation is consistent with glucocorticoid levels (21, Fig. 1.6) and the serum levels of corticosteroid-binding globulin during pregnancy (22). Therefore, suggestive evidence exists for hormonal regulation of rabbit lung FMO transcription during pregnancy and post-partum although other mechanisms such as increased mRNA stability can not be ruled out. FMO mRNA levels in rabbit kidney were significantly increased during late-gestation, but not at mid-gestation (Figs. 1.8A, 1.8B). The pattern of kidney FMO mRNA expression is consistent with that of protein levels and activity during gestation (Figs. 1.2, 1.3, 1.7, 1.8). This result suggests that FMO in kidney is not influenced by progesterone but may be regulated by glucocorticoids which reach a peak at parturition (21, Fig.1.6). Another current study in our

laboratory involving hormone implantation or injection to rabbits, supports these results. Both progesterone and dexamethasone induced FMO in male rabbit lung, whereas, only dexamethasone induced FMO in male rabbit kidney. Estradiol could induce FMO in female rabbit lung but not in male (M.-Y. Lee et.al., unpublished).

The results presented here confirm the dominant role of hormonal regulation in the control of FMO, but the effects observed appear to be sex- and tissue-dependent.

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We wish to thank our colleagues, Jack Kelly, David Stresser, and Gayle Orner for help in preparation of rabbit tissues. This work was supported by USPHS grant HL 38650. Portions of this work were presented at the 1991 International Congress of Biochemistry, Jerusalem, Israel.

Figure 1.1. Western blots of rabbit lung FMO throughout gestation probed with guinea pig anti-rabbit lung FMO IgG (A) and quantitation of rabbit lung FMO throughout gestation (B).

(A) Rabbit lung microsomes (each sample contained 10 µg of microsomal protein) were electrophoretically transferred from SDS-PAGE to nitrocellulose. The nitrocellulose was immunostained with guinea pig anti-rabbit lung FMO IgG (20 µg/ml) followed by ^{125}I-protein A as decribed by Burnette (12). (B) The values shown were obtained using densitometry from autoradiograms of western blots. * * - Statistically significant compared to non-pregnant female (Student's t test, $p<0.05$). Each value represents the mean of two rabbits.

Figure 1.1A.

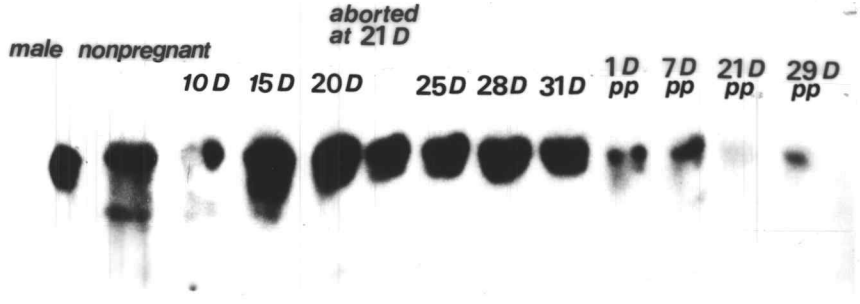

male
nonpregnant
10D
15D
aborted at 21D
20D
25D
28D
31D
1D pp
7D pp
21D pp
29D pp

Figure 1.1B

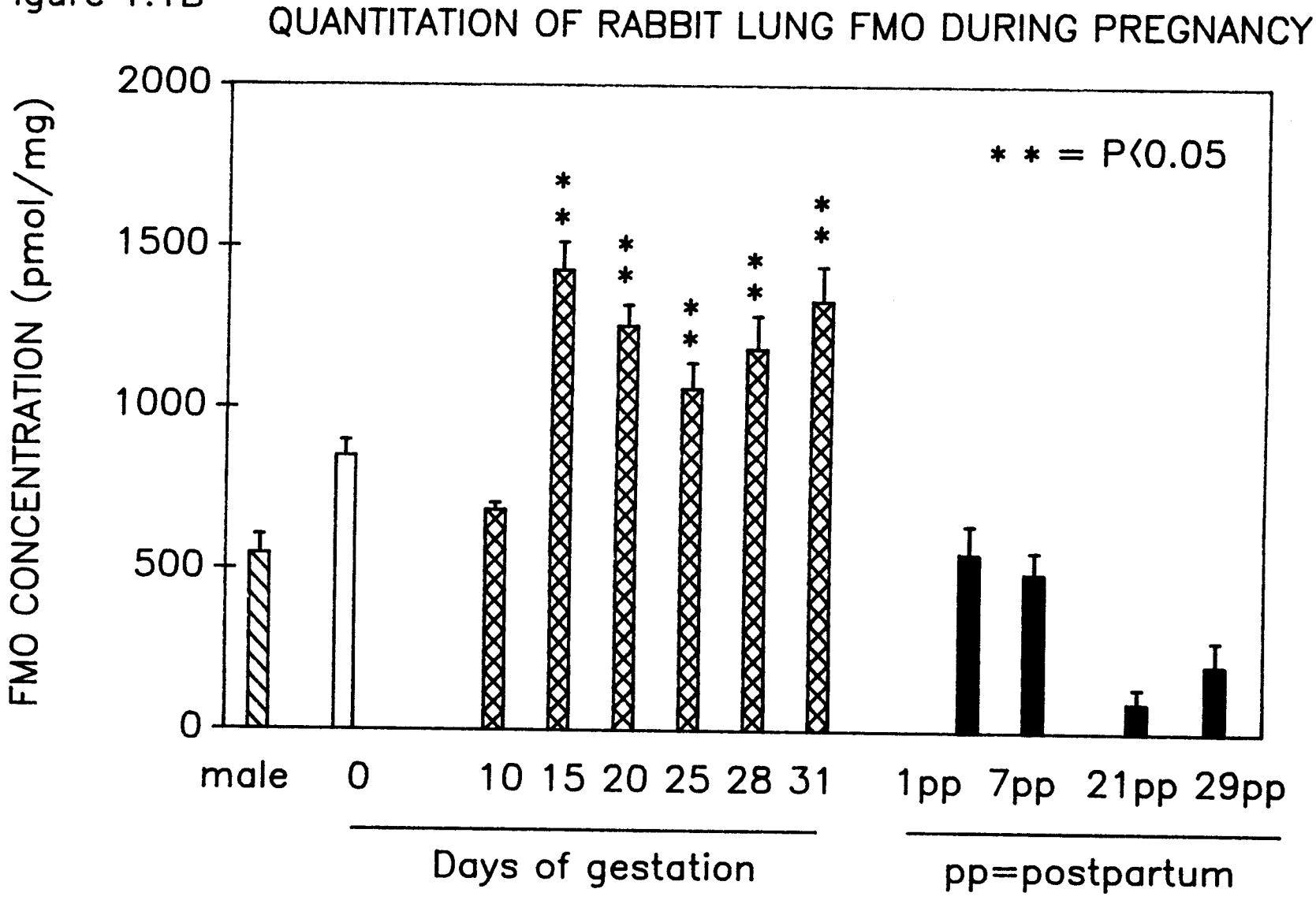

QUANTITATION OF RABBIT LUNG FMO DURING PREGNANCY
FMO CONCENTRATION (pmol/mg)
2000
1500
1000
500
0
* * = P<0.05
male
0
10 15 20 25 28 31
1pp 7pp 21pp 29pp
Days of gestation
pp=postpartum

Figure 1.2. FMO levels in different tissues of pregnant rabbits throughout gestation. The relative amount of FMO in lung, kidney and bladder during pregnancy and post-partum were compared, with densitometry of western blots using the antibody to rabbit lung FMO. Values represent the mean of 2 rabbits.

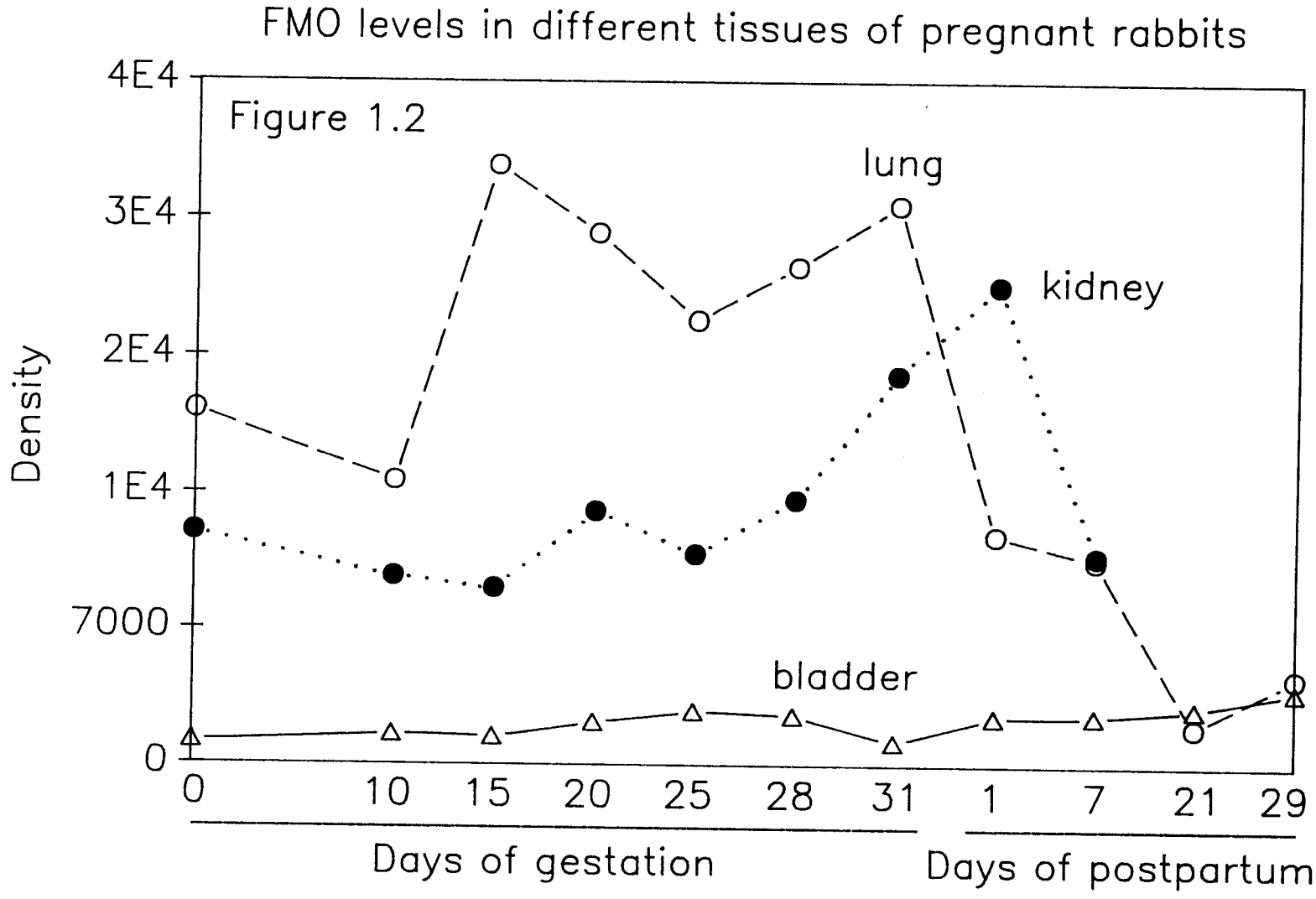

FMO levels in different tissues of pregnant rabbits
Figure 1.2
lung
kidney
bladder
Density
4E4
3E4
2E4
1E4
7000
0
0
10
15
20
25
28
31
1
7
21
29
Days of gestation
Days of postpartum

Figure 1.3. **^{14}C-N,N-Dimethylaniline N-oxidation by microsomes in different tissues of rabbits during pregnancy and post-partum.** The relative activities of FMO in lung, kidney and bladder during pregnancy and post-partum were compared. Each value represents the mean of duplicate assays of 2 different animals.

FMO activities in different tissues of pregnant rabbits

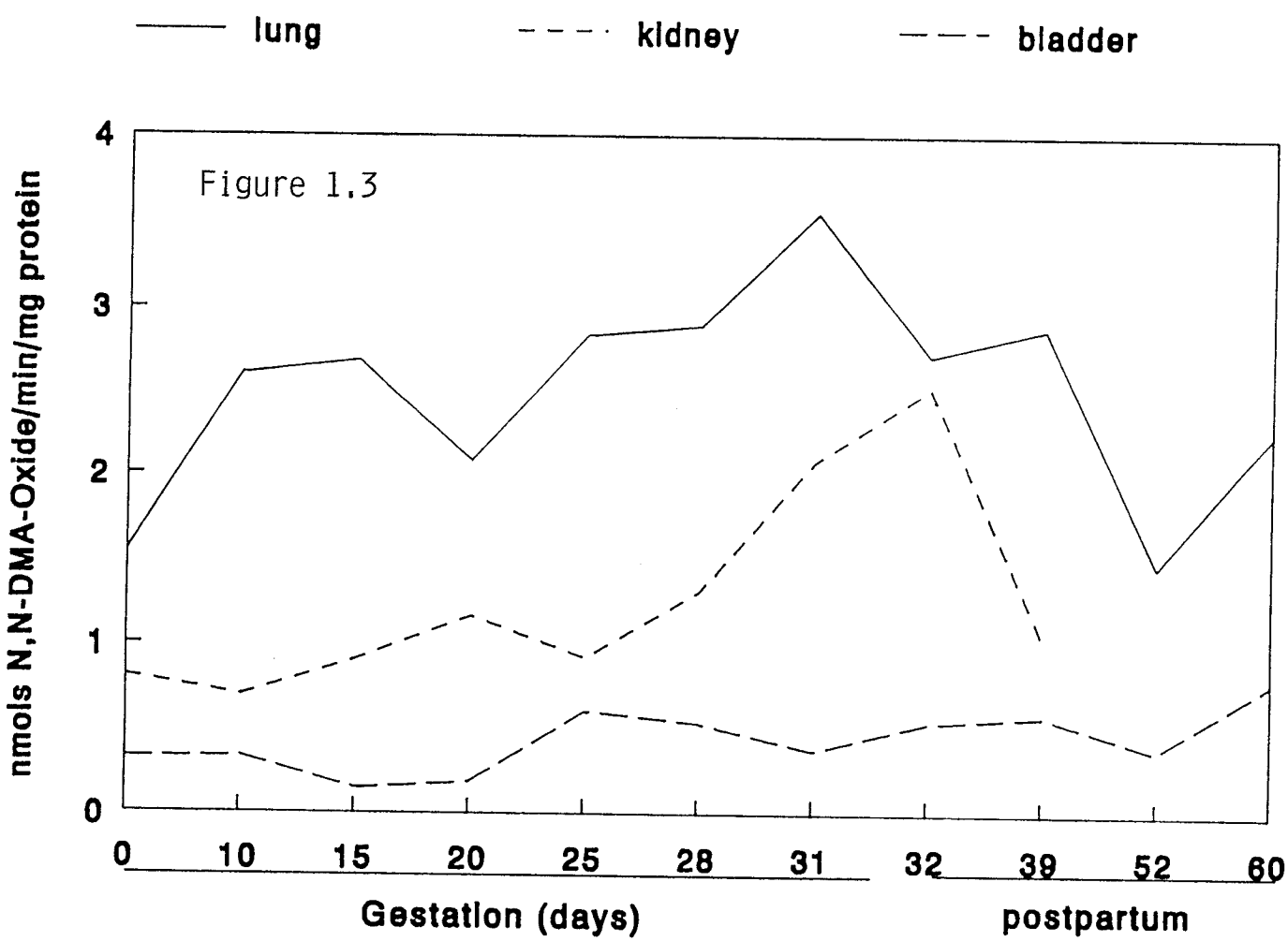


Figure 1.4. FMO levels in tissues of male and non-pregnant female. The relative amount of immunoreactive FMO was determined using antibody to lung FMO and densitometry from the western blots. Protein concentrations of male lung, kidney and bladder were compared to those of non-pregnant females. Values represent the mean of 2 rabbits.

FMO levels in tissues of male and non-pregnant female

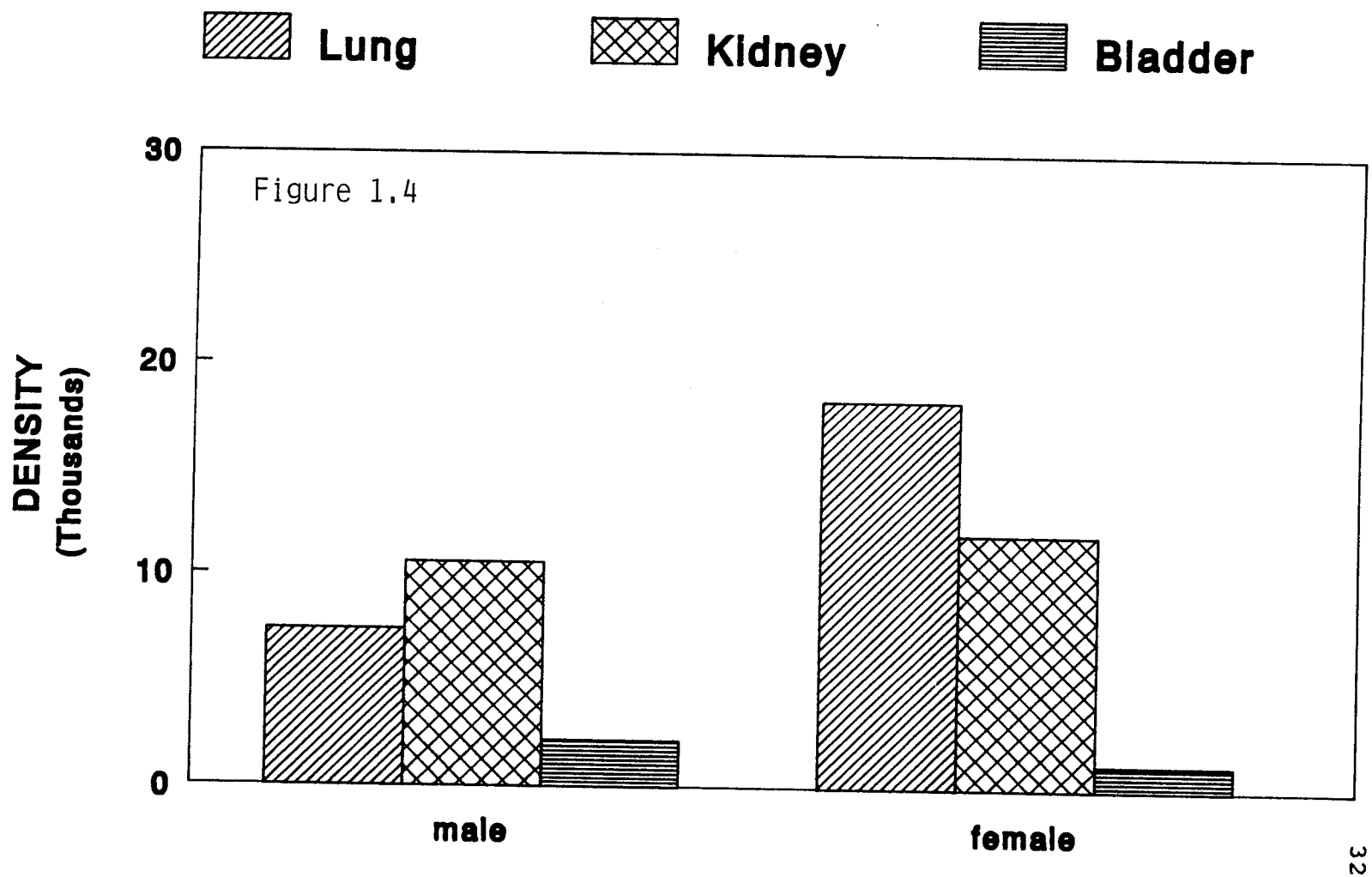
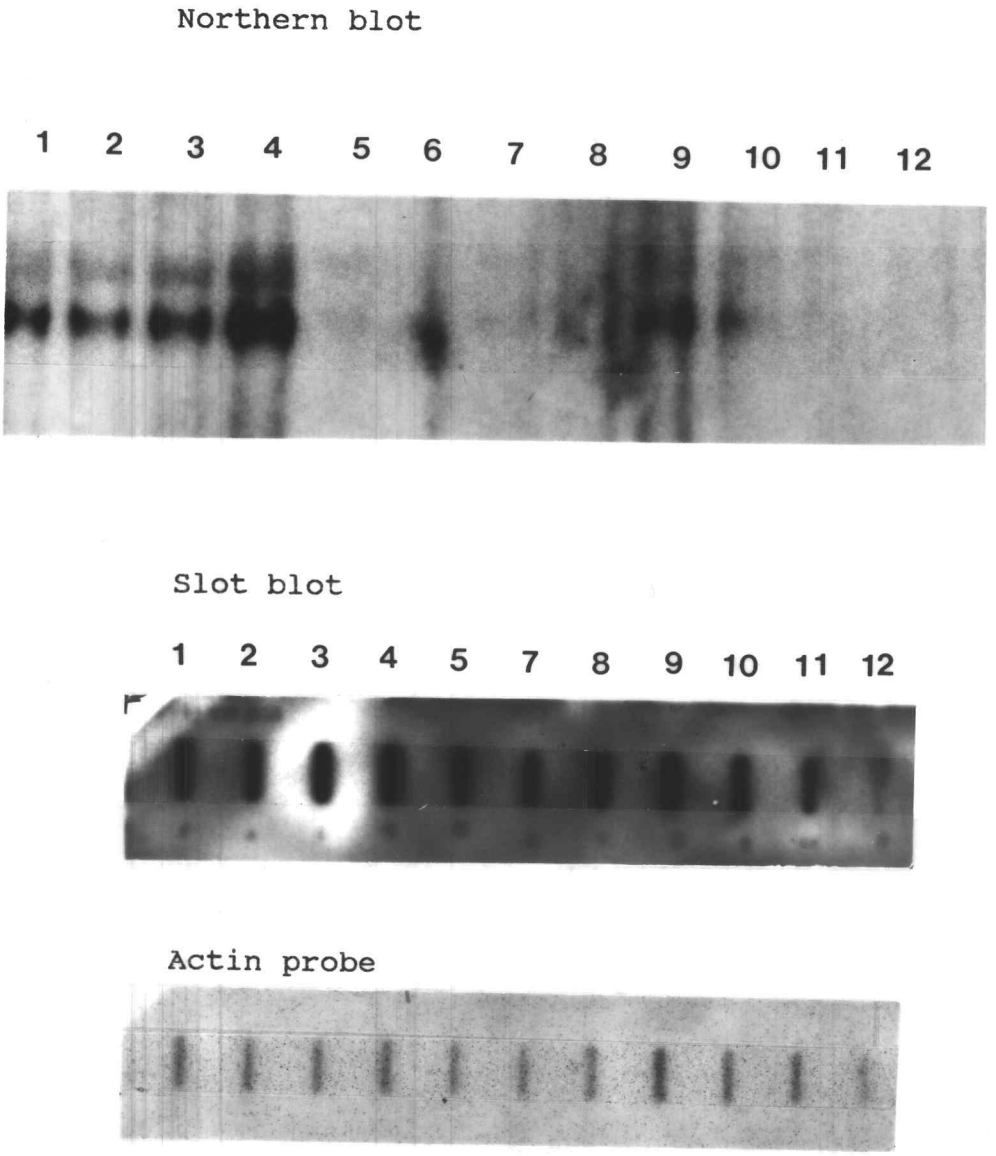


Figure 1.5. Northern blots and slot blots of rabbit lung FMO throughout gestation (A) and quantitation of rabbit lung FMO mRNA expression (B). (A) Total RNA isolated from rabbit lung was analyzed by northern and slot blotting with ^{32}P-labeled rabbit lung FMO cDNA. The slot blot was subsequently reprobed with a human fibroblast β-actin cDNA to assess the relative amounts of RNA analyzed in each sample. (B) The data are produced using the densitometry of autoradiograms of total RNA northern blots. Lane 1, male; lane 2, non-pregnant female; lane 3, 10 day; lane 4, 15 day; lane 5, 20 day; lane 6, aborted; lane 7, 25 day; lane 8, 28 day; lane 9, 31 day pregnancy; lane 10, 7 day; lane 11, 21 day; lane 12, 29 day post-partum. Each lane contained 20 µg total RNA. Values represent the mean of 2 rabbits.

Figure 1.5A.


Northern blot
1 2 3 4 5 6 7 8 9 10 11 12
Slot blot
1 2 3 4 5 7 8 9 10 11 12
Actin probe

RABBIT LUNG FMO mRNA EXPRESSION DURING PREGNANCY

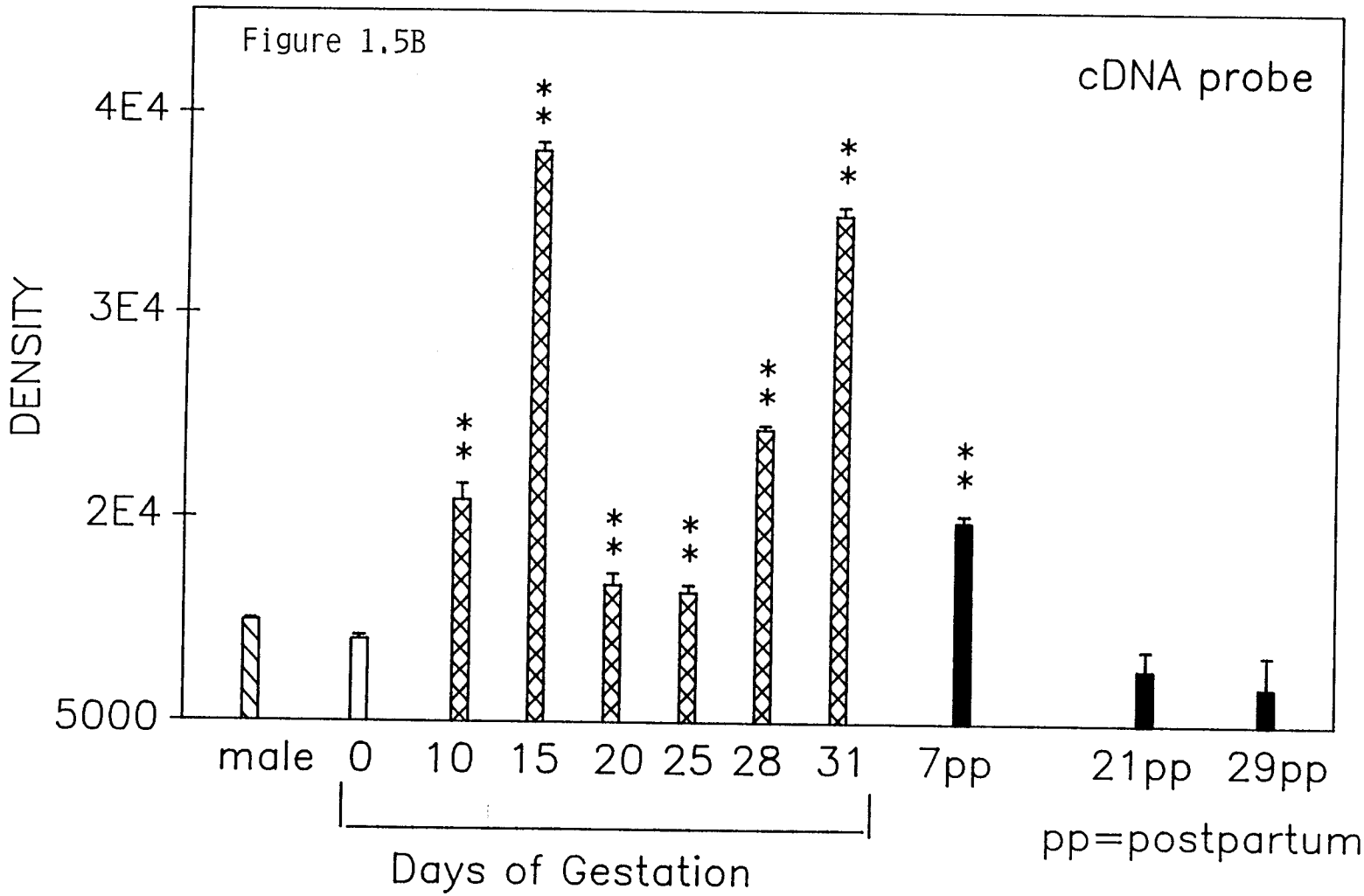
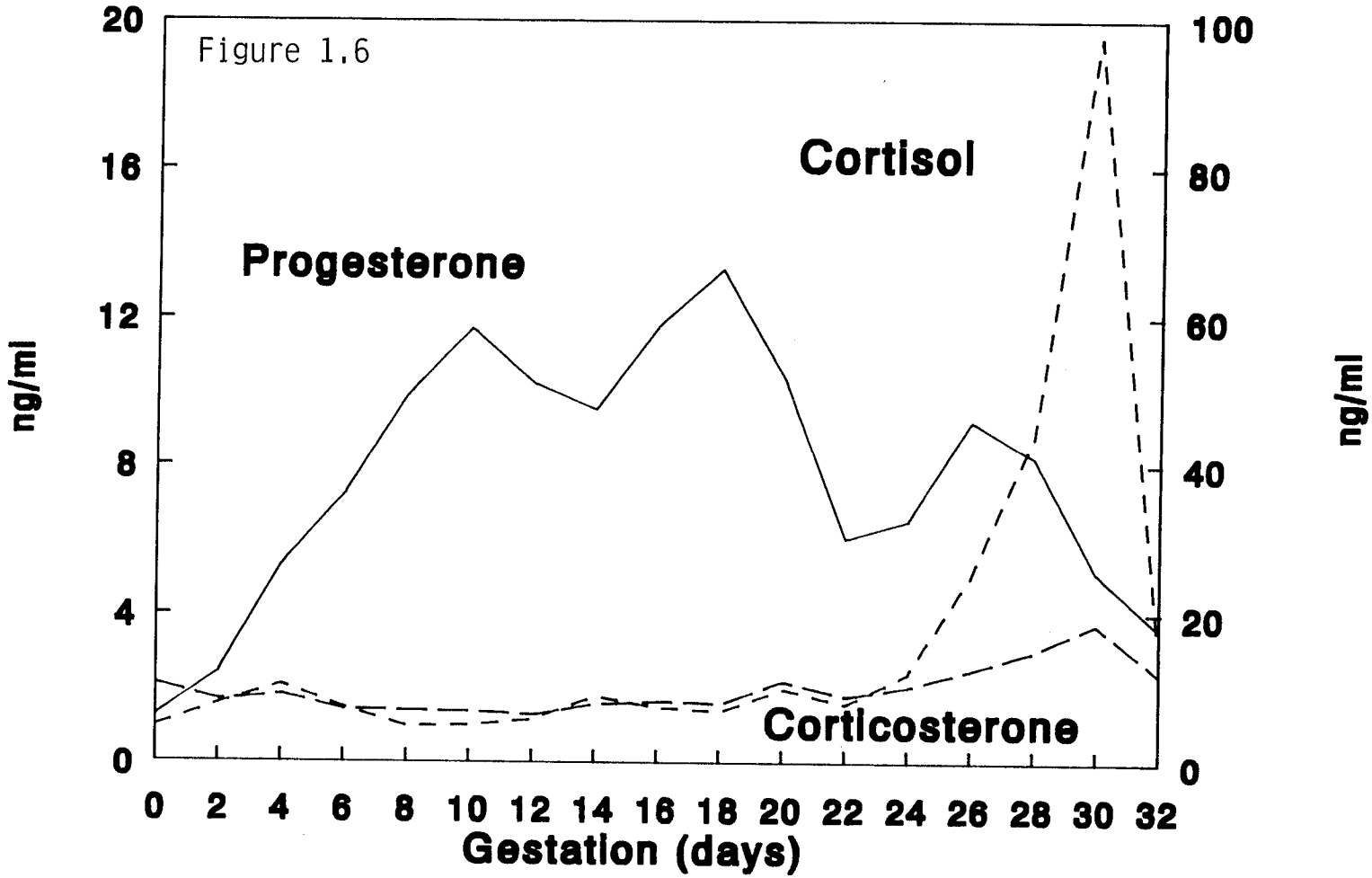


Figure 1.6. Peripheral plasma levels of progesterone, cortisol and coticosterone in rabbit during pregnancy. Adapted from Baldwin et al., (21).

Plasma hormone levels in pregnant rabbits

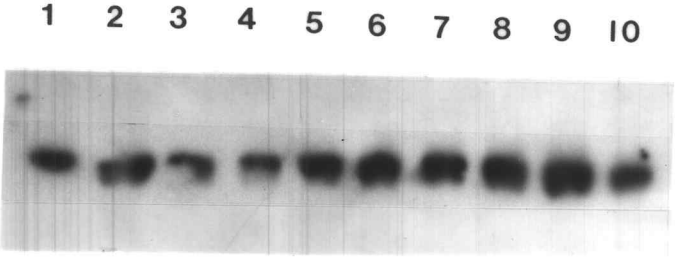


adapted from Baldwin and Stabenfeldt (1974)

Figure 1.7. Western blots of rabbit FMO in kidney throughout gestation probed with guinea pig anti-rabbit lung FMO IgG (A) and quantitation of FMO in kidney throughout gestation (B).

(A) Rabbit kidney microsomes (50 µg protein) were electrophoretically transferred from SDS-PAGE to nitrocellulose. The nitrocellulose was immunostained with guinea pig anti-rabbit lung FMO IgG (20µg/ml) followed by ^{125}I-protein A as described by Burnette (12). lane 1, male; lane 2, non-pregnant female; lane 3, 10 day; lane 4, 15 day; lane 5, 20 day; lane 6, 25 day; lane 7, 28 day; lane 8, 31 day of gestation; lane 9, 1 day; lane 10, 7 day postpartum. (B) Values shown were obtained using densitometry from autoradiograms of western blots. * * - Statistically significant compared to non-pregnant female (student's t test, $p<0.05$). Each value represents the mean of 2 rabbits.

Figure 1.7A.



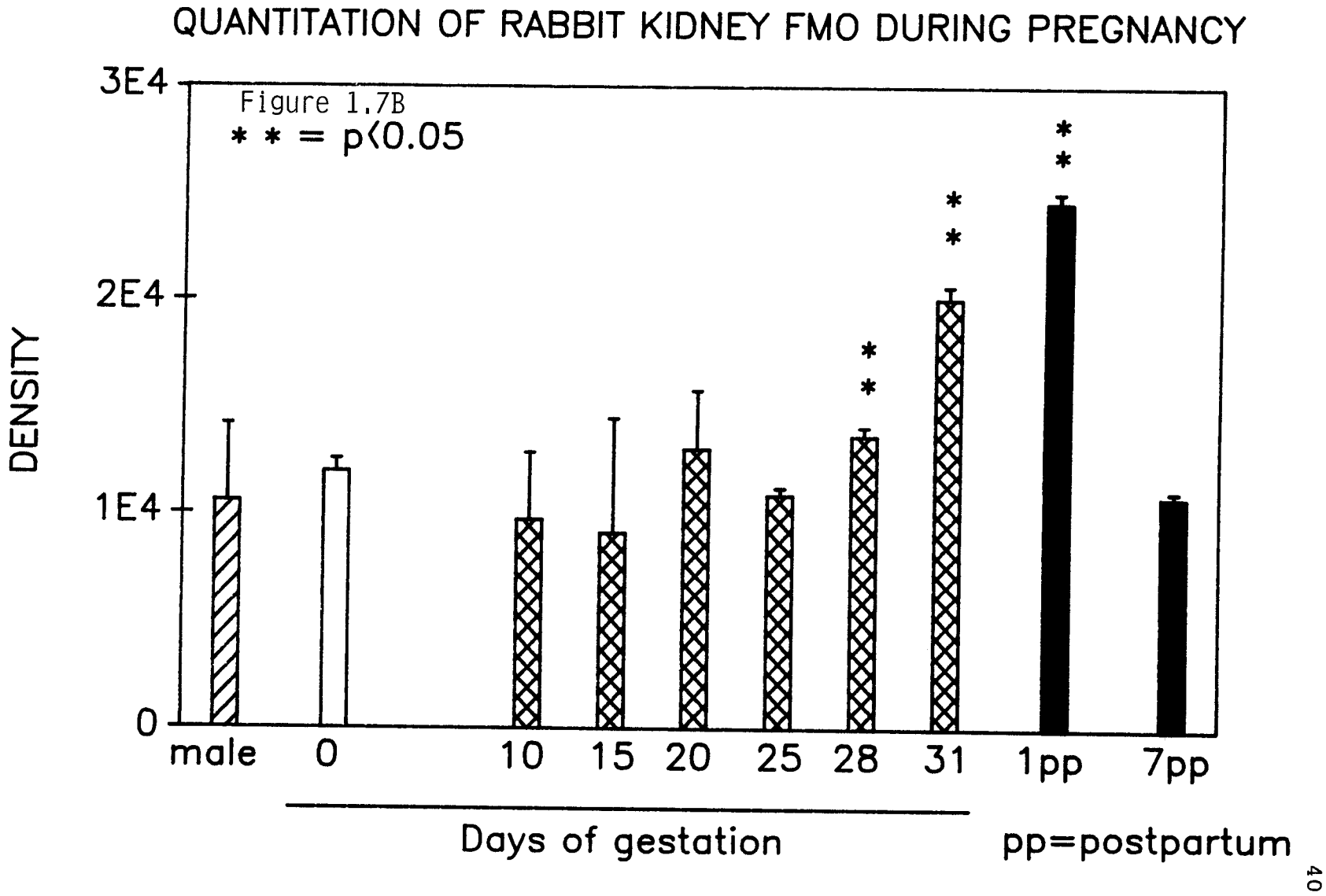
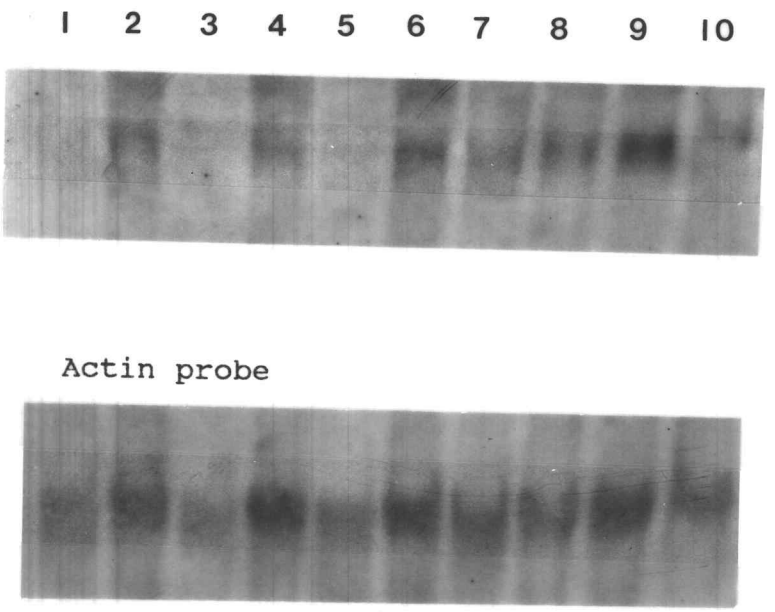

QUANTITATION OF RABBIT KIDNEY FMO DURING PREGNANCY
Figure 1.7B
* * = p<0.05
DENSITY
3E4
2E4
1E4
0
male
0
10
15
20
25
28
31
1pp
7pp
Days of gestation
pp=postpartum

Figure 1.8. Northern blots of rabbit FMO in kidney throughout gestation (A) and quantitation of rabbit mRNA expression in kidney (B). (A) Total RNA isolated from rabbit kidney was analyzed by northern blotting with ^{32}P-labeled rabbit lung FMO cDNA. Lane 1, male; lane 2, non-pregnant female; lane 3, 10 day; lane 4, 15 day; lane 5, 20 day; lane 6, 25 day; lane 7, 28 day; lane 8, 31 day pregnancy; lane 9, 1 day; lane 10, 7 day post-partum. Each lane contained combined 20 μg total RNA of 2 rabbits. The blot was subsequently reprobed with a human fibroblast β-actin cDNA (^{32}P-labelled) to assess the relative amounts of RNA analyzed in each sample. (B) The data are produced using densitometry of autoradiograms from northern blots.

Figure 1.8A.



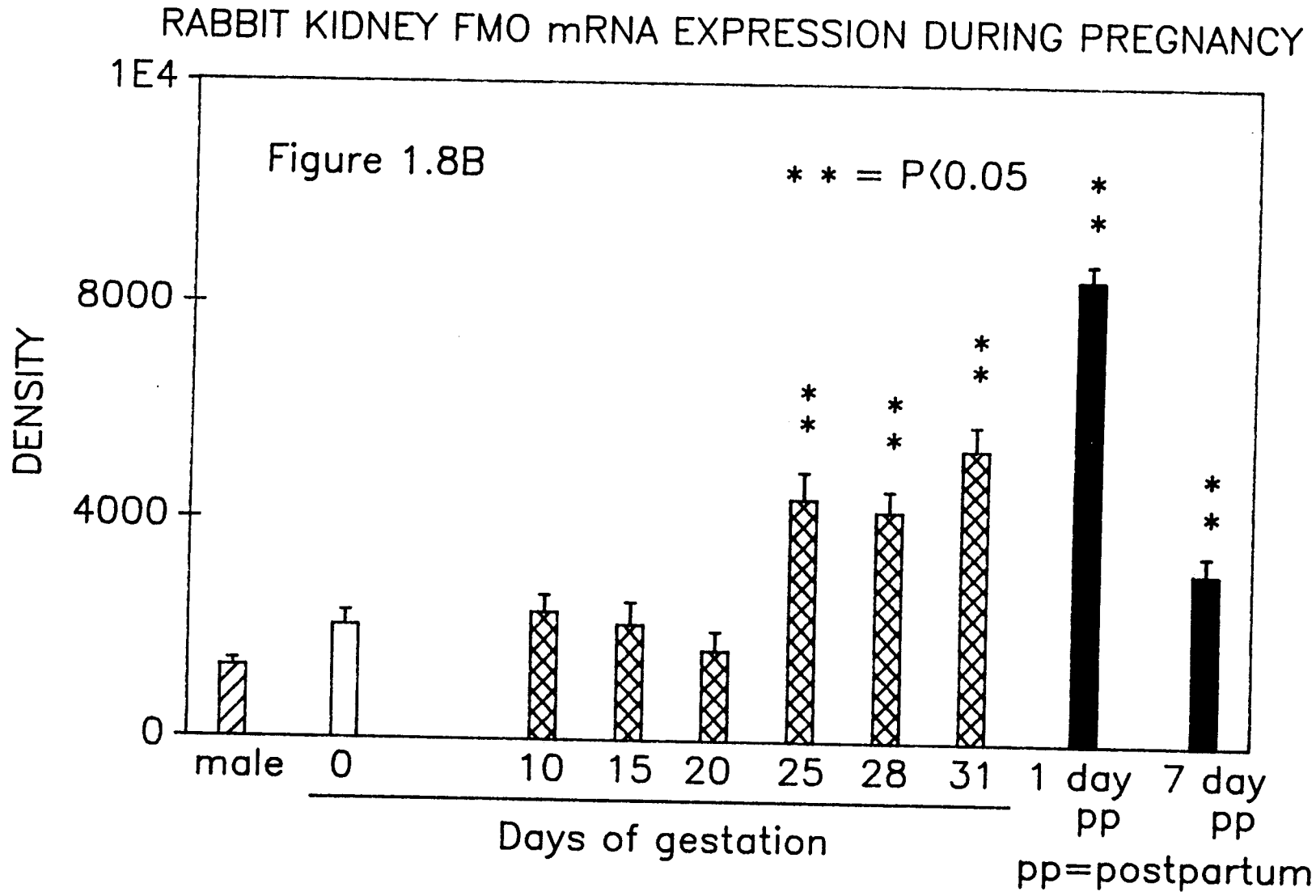
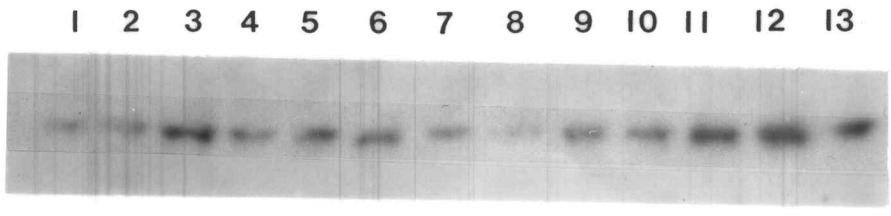
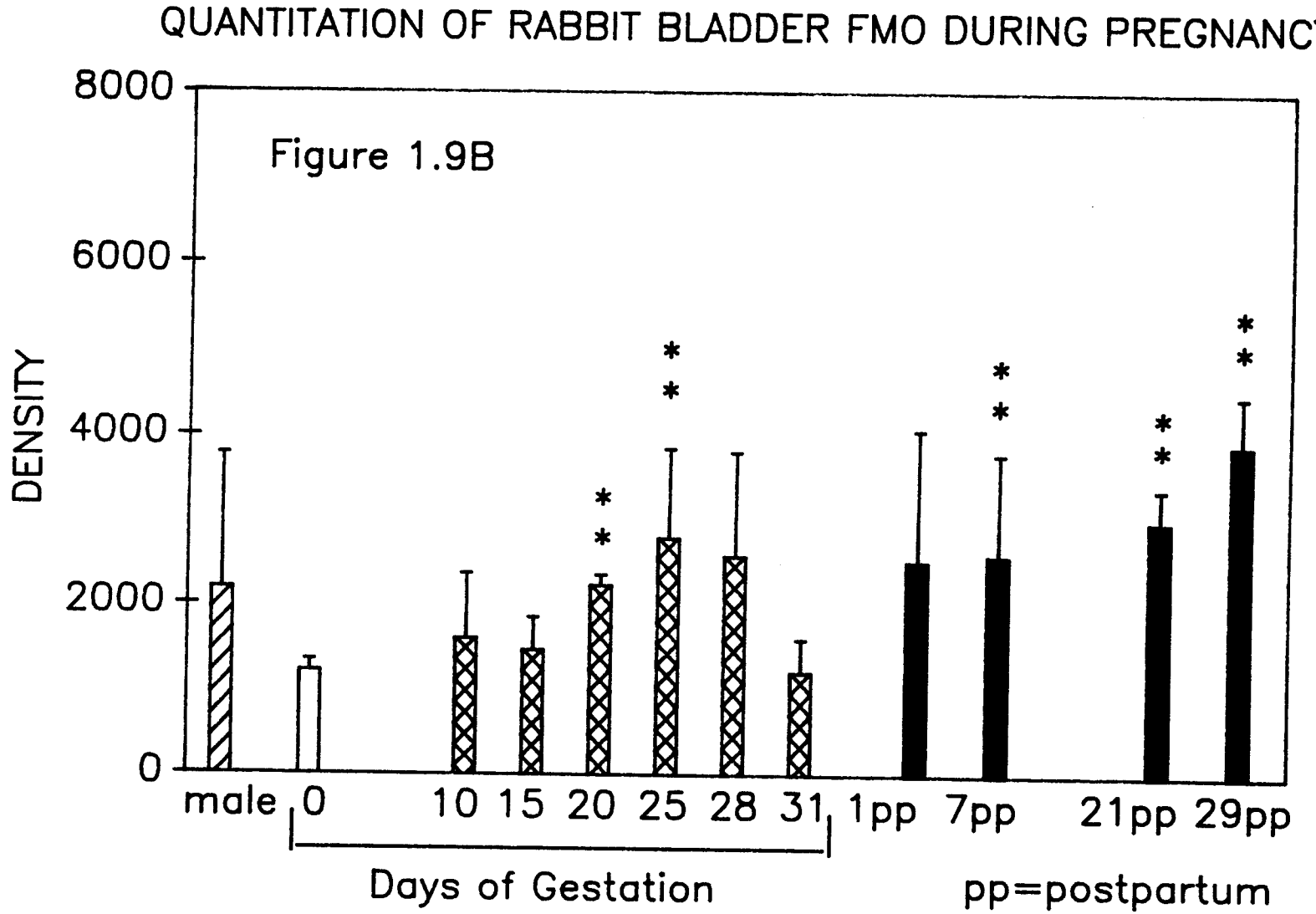

RABBIT KIDNEY FMO mRNA EXPRESSION DURING PREGNANCY
Figure 1.8B
* * = P<0.05
1E4
8000
4000
0
DENSITY
male
0
10
15
20
25
28
31
1 day pp
7 day pp
Days of gestation
pp=postpartum

Figure 1.9. Western blots of rabbit FMO in bladder throughout gestation probed with guinea pig anti-rabbit lung FMO IgG (A) and quantitation of FMO in bladder throughout gestation (B).

(A) Rabbit bladder microsomes (50 µg protein) were electrophoretically transferred from SDS-PAGE to nitrocellulose. The nitrocellulose was immunostained with guinea pig anti-rabbit lung FMO IgG (20µg/ml) followed by ^{125}I-protein A as described by Burnette (12). Lane 1, male; lane 2, non-pregnant female; lane 3, 10 day; lane 4, 15 day; lane 5, 20 day; lane 6, 25 day; lane 7, 28 day; lane 8, 31 day pregnancy; lane 9, 1 day; lane 10, 7 day; lane 11, 21 day; lane 12, 29 day post-partum; lane 13, aborted at 21 day. (B) The data are produced using densitometry of autoradiograms from western blots and each value represents the mean of 2 rabbits.

Figure 1.9A.




QUANTITATION OF RABBIT BLADDER FMO DURING PREGNANCY
Figure 1.9B
DENSITY
8000
6000
4000
2000
0
male
0
10 15 20 25 28 31
1pp 7pp 21pp 29pp
Days of Gestation
pp=postpartum

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Chapter 2

Developmental Regulation of Flavin-Containing Monooxygenase (FMO) in Fetal and Neonatal Tissues

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Developmental Regulation of Flavin-Containing Monooxygenase (FMO) in Fetal and Neonatal Rabbit Lung and Liver.

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ABSTRACT

Unlike the much-studied cytochrome P-450-dependent monooxygenase system, mammalian microsomal FMO is not inducible by xenobiotics, but does appear to be regulated developmentally, presumably through alterations in hormonal status. It is believed that enzyme induction by glucocorticoids is mediated by specific cell receptors. The number of glucocorticoid-specific nuclear binding sites in rabbit fetal lung increases during the last 10 days of gestation, a process which correlates with increased levels of pulmonary surfactant and rising levels of cortisol in the fetal circulation. Coricosteroids induce specific enzymes in many tissues such as the embryonic retina, fetal gut, and newborn liver. The levels of rabbit fetal and neonatal FMO have been followed from day 15 fetuses to day 29 neonates by assaying enzyme activity, protein levels (western blotting) and mRNA levels (northern blotting with ^{32}P-labeled rabbit lung cDNA). Fairly high levels of FMO are found in fetal rabbit lung and liver at 25 days and continue to rise until birth at which point the levels are about 60% that of the adult. The levels continue to rise after birth to adult levels by 3 weeks of age. The results of this study indicate that FMO is under developmental and hormonal control, possibly at the level of transcription. Alterations of FMO activity in pregnancy or early development are important for study given the role that FMO plays in the metabolism of foreign drugs.

INTRODUCTION

Flavin-containing monooxygenase (FMO) catalyzes the oxygenation of a great number of xenobiotics containing a soft nucleophilic substituent, usually a nitrogen or sulfur atom (1). Unlike the much-studied cytochrome P-450-dependent monooxygenase system, mammalian microsomal FMO is not inducible by xenobiotics, but does appear to be regulated developmentally, presumably through alterations in hormonal status (2). A current study in this laboratory indicates that the activity and level of protein and mRNA of rabbit FMO could be developmentally regulated throughout gestation and progesterone and glucocorticoids appear to be involved, but the mechanisms might be tissue-specific. It is believed that enzyme induction by glucocorticoids is mediated by specific cell receptors. A number of observations indicate that glucocorticoids may play an important role during lung maturation and biochemical differentiation. Administration of exogenous glucocorticoids to mammalian fetuses at appropriate stages of gestation accelerates morphological development of the lung and causes precocious appearance of pulmonary surfactant (3-6).

In addition, endocrine ablation, such as decapitation of rat fetuses, results in inhibition of normal lung maturation and decreased levels of surfactant in fetal lungs (7). A deficiency of surfactant in lung of human infants is considered to be the primary cause of respiratory distress

syndrome, a major cause of death in premature infants (8). It is generally believed that the first step in the cellular action of steroid hormones involves the interaction of the hormone with specific cytoplasmic receptors (9). The steroid receptor complex then migrates to the nucleus, where it binds to specific acceptor sites on the chromatin (9-11). As a result of this interaction, the transcriptional apparatus is activated to produce specific RNA and finally yield appropriate proteins. Rabbit fetal lung nuclei contain macromolecules which have the properties of physiological receptors for glucocorticoids which may exert their effects by inducing the synthesis of key enzymes involved in the biosynthesis of the phospholipid components of surfactant (12). The increase in concentration of nuclear binding sites between day 20 and day 28 of gestation correlates with reported changes in pulmonary epithelial cell maturation and surfactant concentrations in lung extracts and evidence for the presence of cytoplasmic cortisol-binding components in fetal lung was also presented (12). Another report also demonstrated specific binding of dexamethasone in fetal lung extracts. This report also describes the isolation and properties of macromolecular-glucocorticoid complexes extracted from the cytosol fractions of fetal lung and fetal liver (13). Glucocorticoids promote fetal maturation (14) and changes in plasma corticosteroid-binding globulin (CBG) levels undoubtedly influence glucocorticoid bioavailability during

critical stages of development (15).

Two forms of liver FMO has been isolated from guinea pig and rabbit (16, 17) and two human liver FMO cDNAs have been sequenced (18). The major form in rabbit liver (form I) is 87% identical to the pig liver form and form I in human. Interestingly, FMO form I is present in only very small amounts in adult human livers, but is present in fetal liver. The major form in adult human liver is 85% identical to rabbit liver form II. Little or nothing is known of the developmental regulation of rabbit forms I and II. The only antibody available for this study is the one raised against pig liver (form I).

The purpose of this study is to examine developmental regulation of FMO in fetal and neonatal rabbit lung and liver tissue which seems to be under developmental and hormonal control, possibly at the level of transcription.

MATERIALS AND METHODS

ANIMALS

Timed-pregnant New Zealand white rabbits were obtained from the Rabbit Research Institute at Oregon State University and housed in the Laboratory Animal Resource Center. Animals were maintained on a 12-hour day-night cycle (7 AM - 7 PM), and feeding was ad libitum. In order to follow the developmental regulation of the levels of FMO activity, protein and mRNA, fetal and neonatal rabbits were used. The fetuses were sacrificed on days 20, 25, 28 and 31 of gestation. Following parturition, two neonatal rabbits were euthanized on days 1, 15, 22 and 29 after birth. Animals were euthanized by CO_2 asphxiation. The tissues were immediately removed and frozen in liquid nitrogen and stored at -90°C until assayed.

MICROSOME PREPARATION

Microsomes were prepared using a modification of the procedure of Guengerich (19). The levels of microsomal protein were assayed by the method of Lowry et al., (20) using BSA[3] as the standard.

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[3]BSA, bovine serum albumin; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PBS, 20 mM potassium phosphate, pH 7.4, 0.8% NaCl; IgG, immunoglobulin G; DMA, N,N'-dimethylaniline.

IMMUNOQUANTITATION

The microsomal proteins were resolved by SDS-PAGE and then electrophoretically transferred to nitrocellulose. Following an 1 hour incubation at room temperature with 2% BSA in PBS, the blots were stained with either guinea pig anti-rabbit lung FMO IgG or rabbit anti-pig liver FMO IgG (20 µg IgG per ml in the 2% BSA-PBS buffer with 0.05% Triton X-100) for 1.5 hour at room temperature. The FMO-IgG complexes were visualized by incubating with [^{125}I]-protein A (0.1 µCi/ml in 2% BSA-PBS buffer) followed by autoradiography (21). Immunoquantitation was performed by laser densitometry. The relative amounts of FMO were calculated utilizing the purified rabbit lung FMO as standard for lung and relative densities of blots were compared for liver.

ASSAY OF FMO ACTIVITY

The metabolism of [^{14}C]-DMA by microsomal FMO was performed by HPLC to resolve the N-oxide and N-demethylated metabolites. Microsomes (0.1 mg) or purified FMO (0.02 mg) were incubated in 0.1 M tricine, pH 8.5 containing 1 mM EDTA, and an NADPH generating system [1 mM NADPH, 0.02 M isocitrate and 1 Unit isocitrate dehydrogenase]. [^{14}C]-DMA was added to a final concentration of 0.5 mM (1-2 µCi/µmol). All components except the microsomes or purified FMO were preincubated for 2 min and the reaction initiated by the addition of enzyme. The reaction was terminated by the addition of 0.1 ml methanol. HPLC

analysis was performed with a Whatman RAC II Partisil 5 ODS-3 (4.6 mm x 10 cm) column eluted with 62% MeOH in water at 0.5 ml/min. Metabolite quantitation was performed with an on-line radiochemical detector (Beckman, Model 171 equipped with a solid cell).

RNA ISOLATION

Total RNA was prepared by the modified method of acid guanidinium thiocyanate-phenol-chloroform extraction (22).

RABBIT LUNG cDNA (1643 bp) PREPARATION

To obtain a cDNA, 1 μg of the female (28 day gestation) lung mRNA was used as a template to make total cDNA. Oligo (dT) was used as a primer along with superscript reverse transcriptase enzyme under the conditions recommended by Gibco/BRL. One-fifth of the total cDNA was then used in a PCR reaction. Utilizing the rabbit lung cDNA sequence done by Lawton et al. (23), we selected the primers to be from 67 to 94 and from 1683 to 1710, respectively. Note that the entire coding region has been amplified in a fragment of 1643 bp. The DNA in the PCR reaction was treated with T4 DNA polymerase to ensure the presence of blunt ends, then fractionated on an agarose gel, the 1643 bp band excised and eluted using glass milk. The DNA was then ligated with SmaI-digested, CIAP-treated pUC19 which was then used to transform E. coli DH5a. The fragment was oriented in the plasmid opposite to β-galactosidase. The

intact cDNA fragments were excised from pRNH135 utilizing EcoRI and BamHI digestion.

ELECTROPHORESIS AND TRANSFER

Electrophoresis of RNA through agarose containing formaldehyde was performed by published procedures (24). The amount of RNA in each sample was measured by optical density at 260 and 280 nm; 20 µg was analyzed by electrophoresis in duplicate. RNA in the gel was transferred to nylon membrane by capillary movement overnight using 20 X SSC buffer and the transferred blot was exposed on UV light (UV Crosslinker, XL-1000, Fisher Biotech) to fix the RNA on the membrane (25).

HYBRIDIZATION

The probe, used to quantitate rabbit lung FMO mRNA on northern blots, was obtained by random primer-labeling of rabbit lung FMO cDNA (1643 bp) with α^{32}P-dCTP (26). After prehybridization for 4 hour in 6 X SSC, 50% formaldehyde, 0.1% SDS, 5 X Denhardt's solution, 100 µg/ml salmon sperm DNA and 100 µg/ml tRNA, hydbidization was performed at 42 °C overnight in the same medium in the presence of cDNA labeled with [α-^{32}P]dCTP (ICN) by a random primer-labeling kit from Boehringer Mannheim. The filter was washed in 2 X SSC buffer containing 0.1% SDS twice at room temperature for 15 min and 0.1 X SSC buffer with 0.1% SDS at room temperature for 15 min. The membrane was then exposed to Kodak X-OMAT AR film (Kodak,

Rochester, NY) for 6 days with an amplifying screen at -90°C. Integrity of RNA was routinely assessed with a ^{32}P-β-actin cDNA probe to demonstrate that each lane contained approximately equal amounts of RNA.

RESULTS AND DISCUSSION

In fetuses and neonatal rabbits, there was a significant development of FMO protein, activity and mRNA expression in lung (Figs.2.1, 2.2). A specific content of 0.8 nmol FMO per mg protein would represent 5% of total lung microsomal protein. The fetal (sex undetermined) lung FMO levels were readily detectable at day 25 and rose steadly until birth. The protein levels continued to increase until day 15 (about 0.51 nmol/mg) after birth at which point the levels are about 80% that of the adult and then decreased slightly, showing unexpectedly low values at day 22 after birth. Perhaps the ratio of males to females was higher at day 22 than other groups. The fetal lung FMO mRNA expression between day 20 and parturition increased by more than two-fold (Figs. 2.3, 2.4). DMA N-oxidase activity in lung microsomes began to increase at 3 days before birth in fetus and continued to increase after birth until activity at day 29 after birth was near the adult levels (Fig. 2.5).

The western blot shown in Figure 2.6 indicates that the antibody to pig liver FMO (form I) cross-reacted with more than a single band from rabbit liver, especially with microsomes from fetal and neonatal rabbits. It is not possible at this time to separately quantitate rabbit liver FMO forms I and II, as no antibodies are available. These enzymes differ in molecular weight by only about 500 daltons and are very difficult to resolve by SDS-PAGE. Therefore, for the purposes

of this study, the major band in fetal and noenatal liver microsomes was quantified with no conclusion as to the number of forms or their identities.

The hepatic FMO protein levels also started to increase before birth and continued to increase after birth and the level of protein at day 29 after birth was above the adult levels (Figs. 2.6, 2.7). DMA N-oxidase activities in fetal liver microsomes increased steeply from day 25-31 and continued to increase in neonates until day 29 (except on day 15). Activities of both 31 day fetuses and 29 day neonates were near those of the adults (Fig. 2.8).

A current study in this laboratory has shown that the activity and the level of protein and mRNA of rabbit FMO could be developmently regulated throughout gestation and progesterone and glucocorticoids were implicated, but the mechanisms might be tissue-specific (Lee et al., unpublished). Both the plasma level of glucocorticoids and corticosteroid-binding globulin during pregnancy reach a peak at the time of parturition (27, 28). According to the results with fetal lung shown above, the degree of FMO increase before birth is rapid and correlates to the peak of plasma glucocorticoid levels at parturition (Fig. 2.9). This result indicates that FMO induction in fetal lung before birth may be under developmental and hormonal control by glucocorticoids, perhaps at the level of transcription. This is supported by previous reports that glucocorticoids play an important role during

lung maturation and biochemical differentiation (27) and administration of exogenous glucocorticoids to mammalian fetuses at appropriate stages of gestation accelerates morphological development of the lung and causes precocious appearance of pulmonary surfactant (3-6).

A number of hormones and enzymes which are important in metabolism are subject to regulation by the ratio of cysteamine/cystamine (29). FMO is present in endoplasmic reticulum at the site of protein synthesis, and could provide oxidizing equivalents ideal for peptide disulfide bond formation and the oxidation of cysteamine to cystamine by FMO could be a potential source of cellular disulfide (29).

Rabbit fetal lung nuclei contain macromolecules which have the properties of physiological receptors for glucocorticoids which may exert their effects by inducing the synthesis of key enzymes involved in the biosynthesis of the phospholipid components of surfactant (12). The increase in concentration of nuclear binding sites during late gestation, resulting in pulmonary epithelial cell maturation and surfactant concentrations in lung extracts (12), correlates with fetal lung FMO induction before birth and suggests an involvement in protein-disulfide bond formation for the structural function of protein especially for fetal and neonatal animals.

ACKNOWLEDGEMENTS

We wish to thank our colleagues, Jack Kelly, David Stresser, and Gayle Orner for help in preparation of rabbit tissues. This work was supported by USPHS grant HL 38650. Portions of this work were presented at the 1991 International Congress of Biochemistry, Jerusalem, Israel.

Figure 2.1. Western blot analysis of fetal and neonatal rabbit lung microsomes probed with guinea pig anti-rabbit lung FMO IgG and comparison to adult levels. lane 1, adult male; lane 2, adult nonpregnant female; lane 3, 25 day fetus; lane 4, 28 day fetus; lane 5, 31 day fetus; lane 6, 1 day neonate; lane 7, 15 day neonate; lane 8, 22 day neonate; lane 9, 29 day neonate. Each lane contained 50 µg protein.

Figure 2.1.

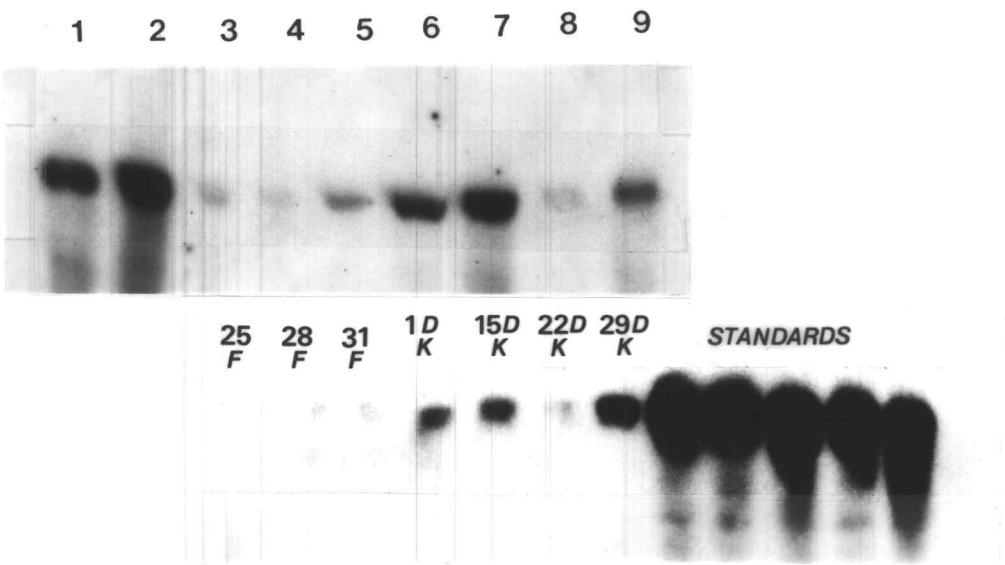


Figure 2.2. Quantitation of rabbit lung FMO in fetuses and neonates and comparison to adult levels. The values shown were obtained using densitometry of autoradiograms of western blots of 2 different gels (Fig.2.1) probed with guinea pig anti-rabbit lung FMO.

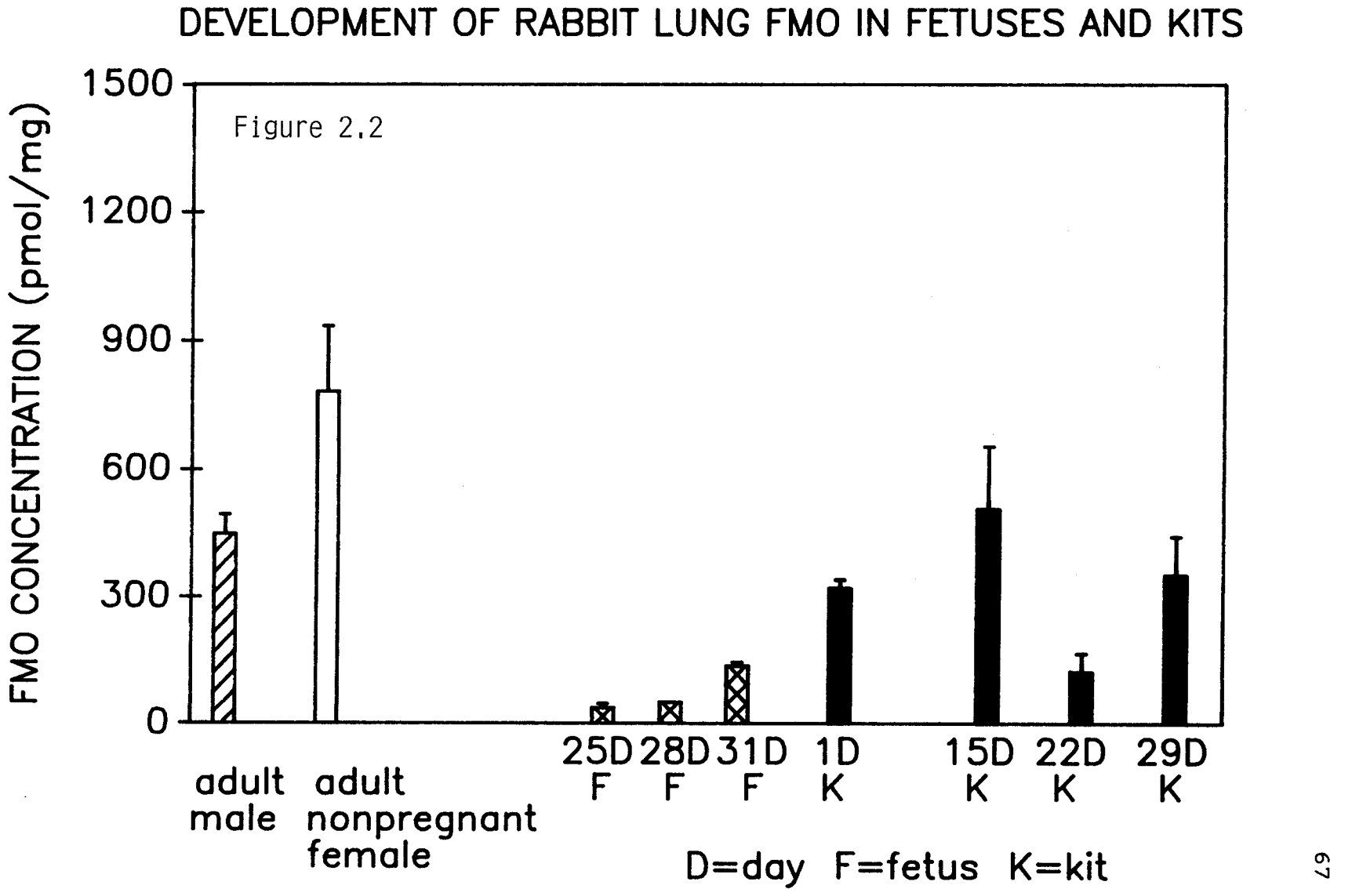

DEVELOPMENT OF RABBIT LUNG FMO IN FETUSES AND KITS
Figure 2.2
FMO CONCENTRATION (pmol/mg)
1500
1200
900
600
300
0
adult male
adult nonpregnant female
25D F
28D F
31D F
1D K
15D K
22D K
29D K
D=day F=fetus K=kit

Figure 2.3. Northern blot analysis of fetal and neonatal rabbit lung total RNA and comparison to adult levels. RNA was probed with ^{32}P-labelled rabbit lung FMO cDNA. lane 1, adult male; lane 2, adult nonpregnant female; lane 3, 20 day fetus; lane 4, 25 day fetus; lane 5, 1 day neonate. Each lane contained 20 µg total RNA.

Figure 2.3.

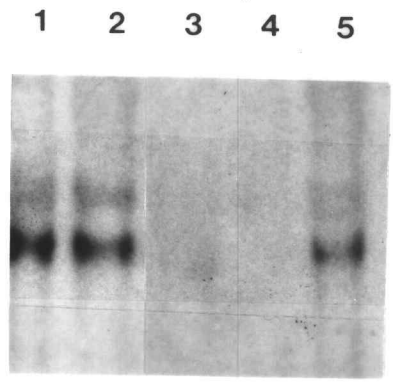


Figure 2.4. Fetal and neonatal rabbit lung FMO mRNA expression with rabbit lung FMO cDNA probe. The graph was produced using a densitometry from northern blots (Figure 2.3) of 2 different gels.

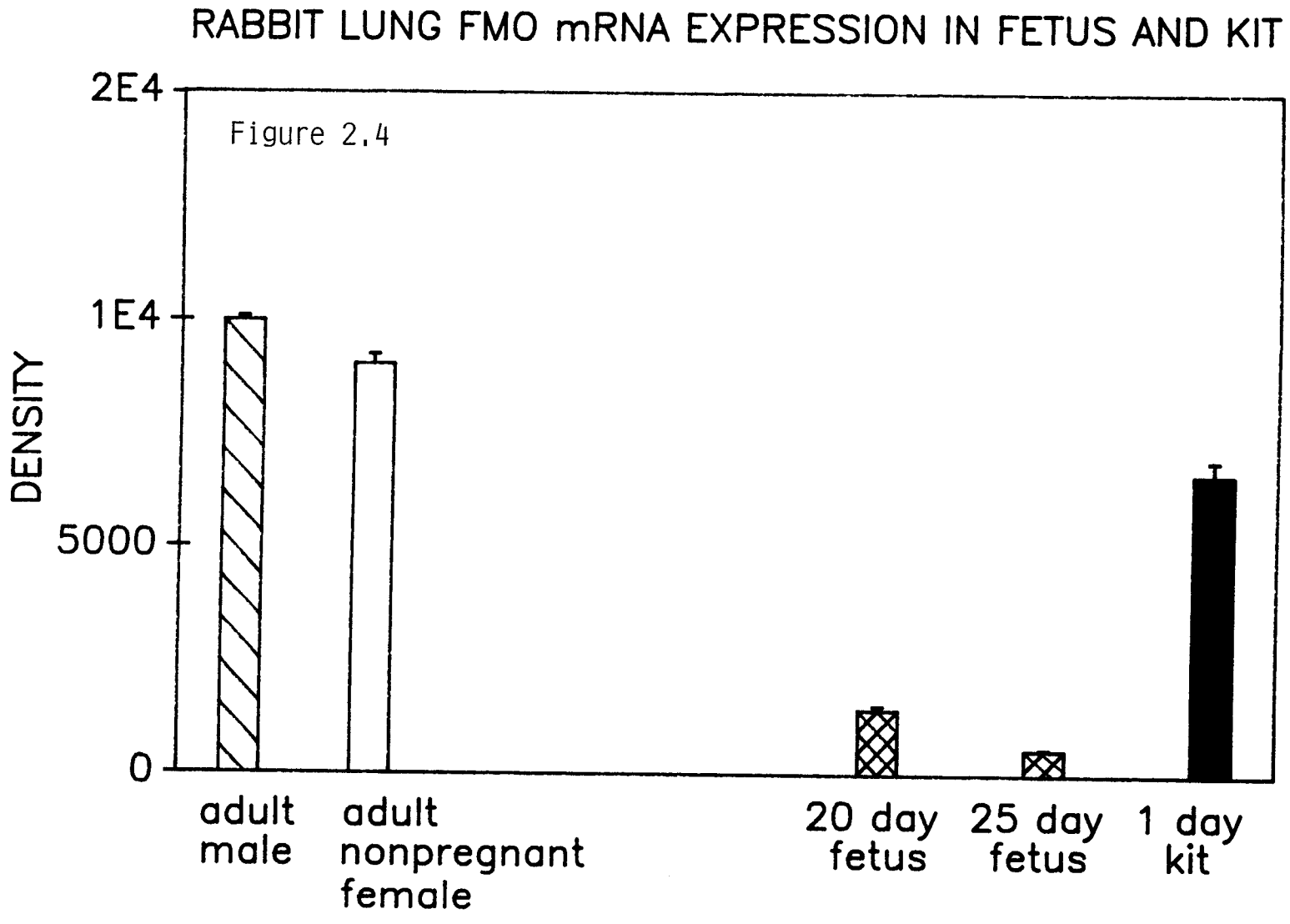

RABBIT LUNG FMO mRNA EXPRESSION IN FETUS AND KIT
Figure 2.4
2E4
1E4
5000
0
DENSITY
adult male
adult nonpregnant female
20 day fetus
25 day fetus
1 day kit

Figure 2.5. ^{14}C-N,N-dimethylaniline N-oxidation by fetal and neonatal rabbit lung microsomes and comparison to adult levels. Each value represents the mean of duplicates from 2 different animals.

Development of N,N–DMA oxidation in fetal and neonatal lung

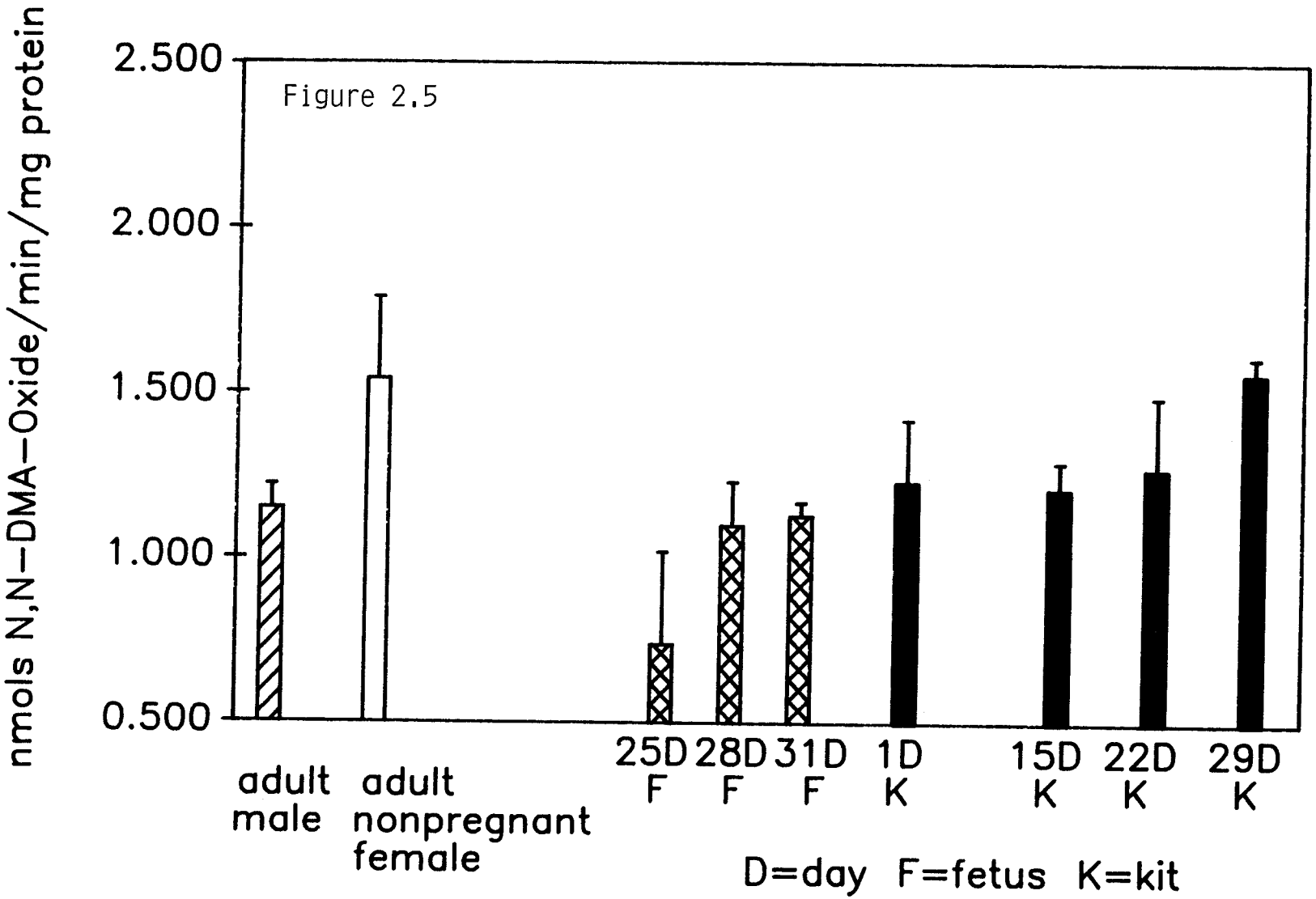


Figure 2.6. Western blot analysis of fetal and neonatal rabbit liver microsomes probed with rabbit polyclonal antibody to pig liver FMO and comparison to adult levels. lane 1, 20 µl standard of pig microsomes from OXYgene (Dallas) Western Blot Kit; lane 2, male; lane 3, nonpregnant female; lane 4, 25 day fetus; lane 5, 28 day fetus; lane 6, 31 day fetus; lane 7,8, 1 day neonate; lane 9,10, 15 day neonate; lane 11, 22 day neonate; lane 12, 29 day neonate. Each lane contained 50 µg protein except the standard.

Figure 2.6.

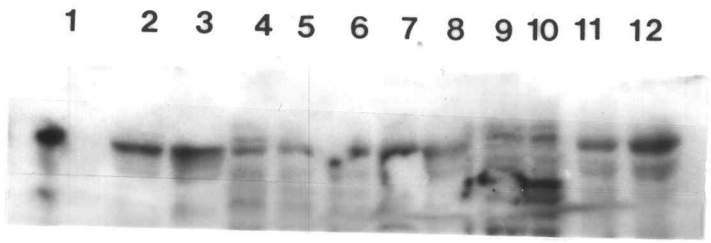


Figure 2.7. Quantitation of fetal and neonatal rabbit liver FMO and comparison to adult levels. The values shown were obtained using densitometry from autoradiograms of western blots (Fig.2.6) of 2 different gels probed with antibody to pig liver FMO.

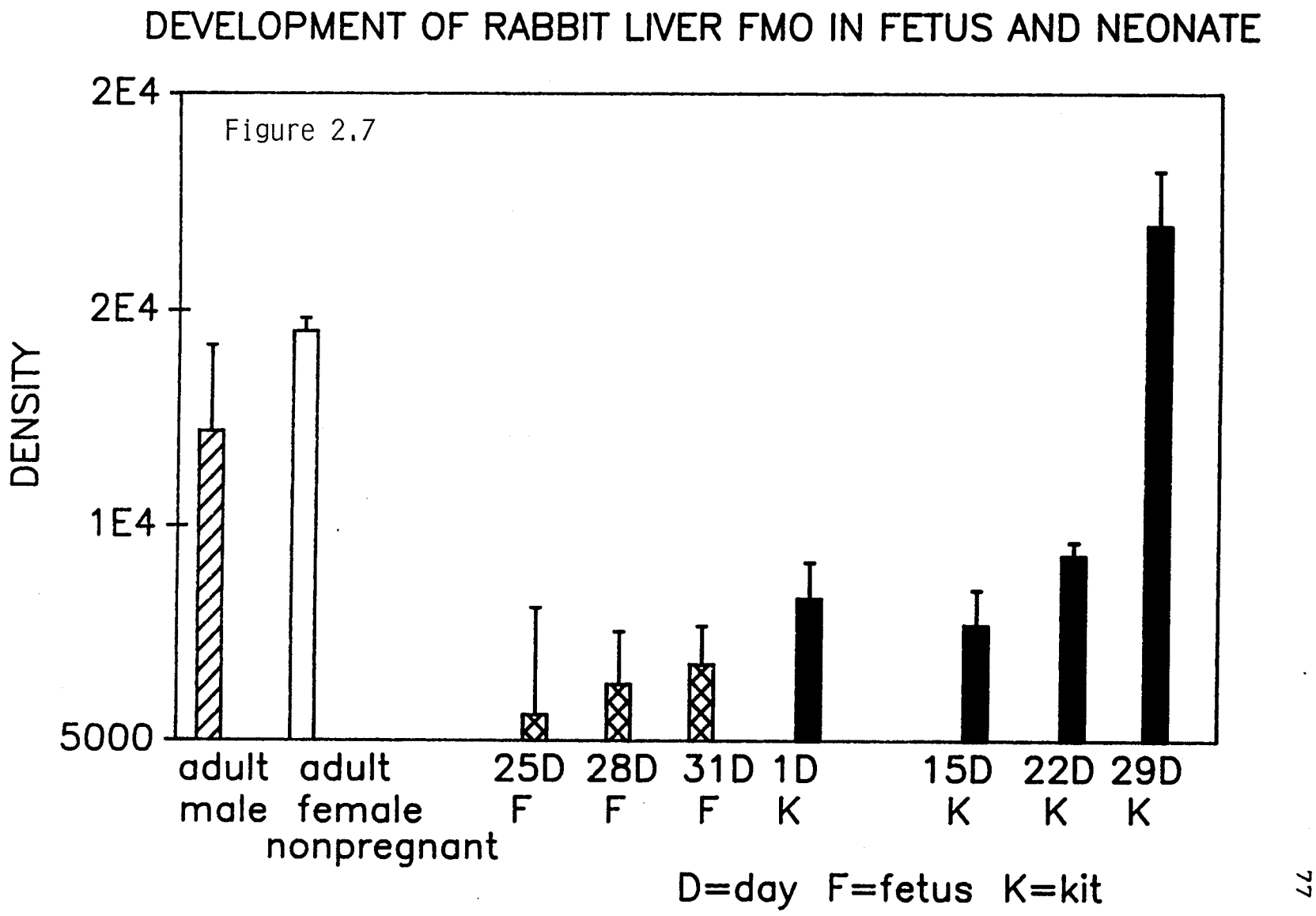

DEVELOPMENT OF RABBIT LIVER FMO IN FETUS AND NEONATE
Figure 2.7
DENSITY
2E4
2E4
1E4
5000
adult male
adult female nonpregnant
25D F
28D F
31D F
1D K
15D K
22D K
29D K
D=day F=fetus K=kit

Figure 2.8. ^{14}C-DMA N-oxidation by fetal and neonatal rabbit liver microsomes and comparison to adult levels. Each value represents the mean of duplicate assays from 2 different animals.

Development of N,N–DMA oxidation in fetal and neonatal liver

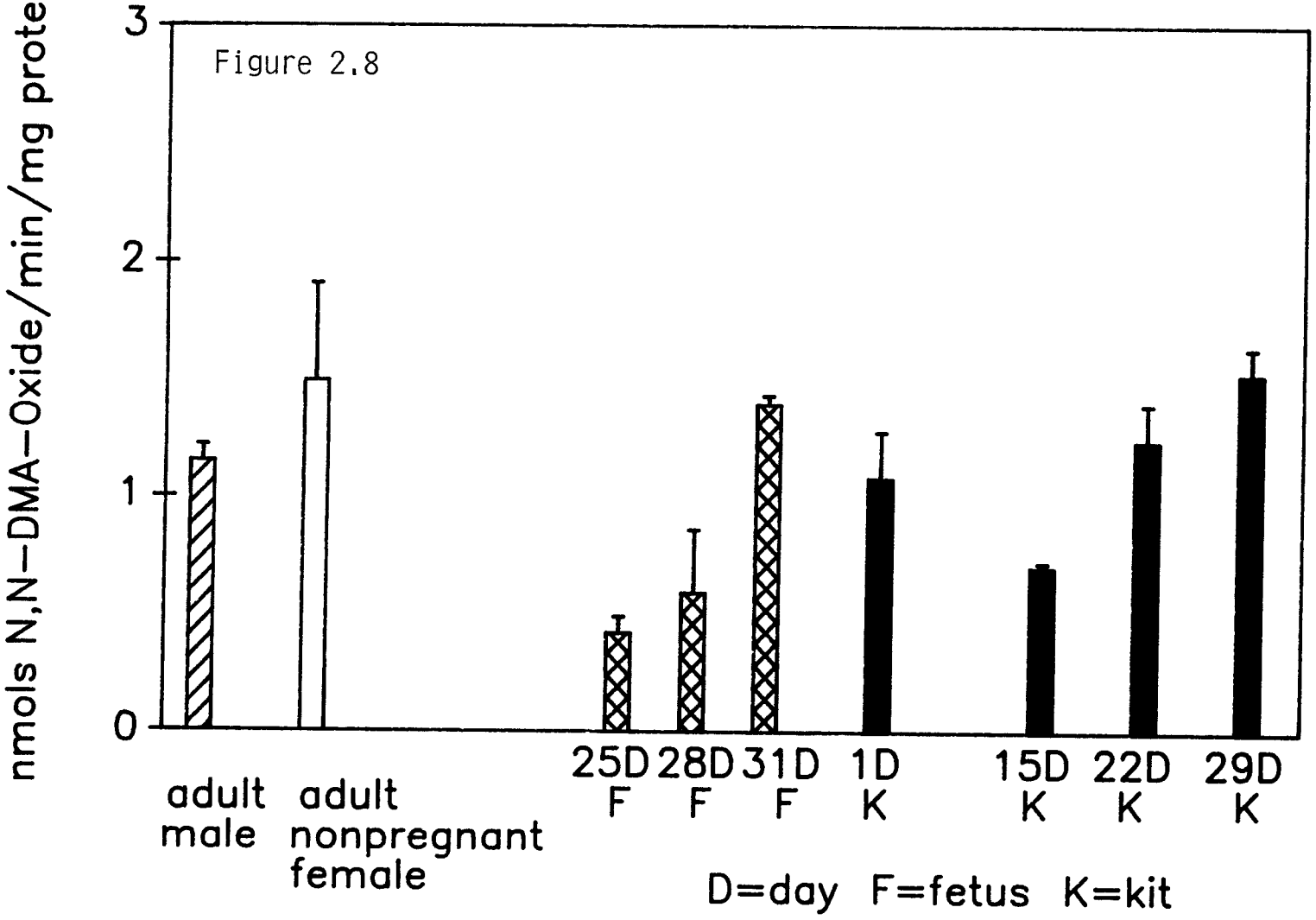
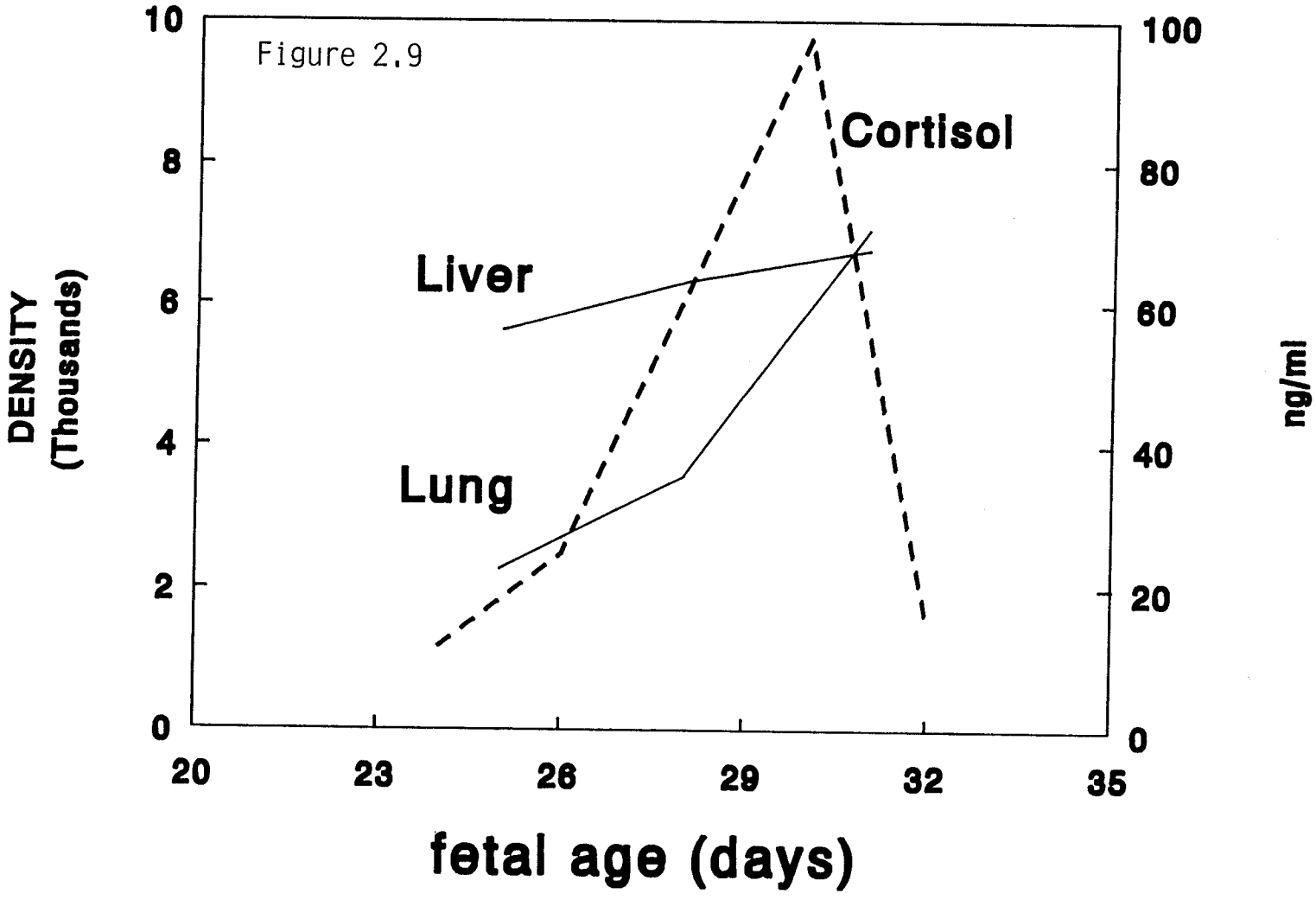


Figure 2.9. Peripheral plasma levels of progesterone, cortisol and corticosterone in rabbit during pregnancy and correlation of fetal lung and liver FMO with maternal plasma cortisol levels. Adapted from Baldwin et al., (1974) (27).

Correlation of fetal lung and liver FMO with maternal plasma Cortisol levels



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Chapter 3

Induction of Flavin-Containing Monooxygenase (FMO) by Steroid Hormones in Rabbit Tissues

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Induction of Flavin-Containing Monooxygenase (FMO) by Steroid Hormones in Rabbit Tissues.

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ABSTRACT

Unlike the cytochrome P-450-dependent monooxygenase system, mammalian microsomal FMO is not inducible by xenobiotics, but does appear to be regulated developmentally, presumably through alterations in hormonal status. FMO activity is influenced by some nutritioal factors such as starvation, ascorbic acid deficiency and diabetic disease. Previous study showed that the levels of rabbit lung FMO during pregnancy were induced at mid- and late-gestation which correlates to the peak of plasma levels of progesterone and glucocorticoids, respectively, throughout gestation. The FMO in kidney was significantly increased during late-, but not mid-gestation, suggesting that FMO in kidney is not influenced by progesterone but may be regulated by glucocorticoids which peak at parturition. Subcutaneous injection with dexamethasone, progesterone, estradiol, aldosterone and estradiol plus progesterone to male rabbits, resulted in progesterone and dexamethasone both significantly inducing lung FMO at the level of protein and mRNA. In kidney, only dexamethasone increased FMO. Intraperitoneal implantation of hormone capsules with estradiol and progesterone in female rabbits resulted in both estradiol and progesterone inducing lung FMO. These data support the hypothesis that rabbit "lung" FMO is developmentlly regulated and progesterone, estradiol and glucocorticoids are involved, but the mechanisms may be tissue-and sex-specific.

INTRODUCTION

Mammalian flavin-containing monooxygenase (FMO, EC 1.14.13.8) is a flavoprotein, which catalyzes the oxygenation of a large variety of nitrogen and sulfur-containing drugs and xenobiotics (1). Most of the metabolites formed by this enzyme are biologically inactive and can be easily excreted, however, some are potentially carcinogenic or mutagenic (1). The only known endogenous substrate of flavin-containing monooxygenase is cysteamine, which is oxidized to the disulfide, cystamine (2). The physiological role of FMO is uncertain but it may be related to the formation of disulfide bonds during protein synthesis (2). At least three different forms of FMO exist in rabbit (3-5). The cDNA sequence of rabbit lung and liver document that they are different gene products which are related with 56% identity in amino acid sequence (6). The extrahepatic FMO is also present in rabbit kidney and bladder and evidence exists for sex- and developmental-related differences. A recent study demonstrated four different forms of FMO, (IA1, IB1, IC1 and ID1) in human (7,8). The human IB FMO, present in small amounts in lung, liver and kidney, is 85% identical to the rabbit lung enzyme (8). Rabbit FMOs in different tissues may be regulated independently and have different substrate specificities. Unlike the much studied cytochrome P-450-dependent monooxygenase system, mammalian FMO is not inducible by exogenous compounds but does appear to be regulated developmently, presumably through alterations in

hormonal status (9). Current studies in our laboratory have shown that the levels of rabbit lung FMO during pregnancy increase at mid- and late-gestation, which correspond to the peak of progesterone and glucocorticoids, respectively, during gestation. The FMO in kidney was significantly increased during late-, but not mid-gestation, suggesting that FMO in kidney is not influenced by progesterone but may be regulated by glucocorticoids which peak at the time of parturition. The purpose of this study is to examine the effect of hormone implantation to female rabbits and hormone injection to male rabbits on FMO induction in rabbit tissues.

MATERIALS AND METHODS

ANIMALS

Six female New Zealand white rabbits were obtained from the Rabbit Research Institute at Oregon State University and housed in the Laboratory Animal Resource Center. Animals were maintained on a 12-hour day-night cycle (7 AM - 7 PM), and feeding was ad libitum. In order to follow the effect of hormone administration on FMO activity and protein levels, two ALZET osmotic pumps (model 2001, flow rate 1 µl/hr for 7 days) were filled with propylene glycol for controls, other two pumps were filled with 0.208 mg β-estradiol/ml of propylene glycol with 5% EtOH and 0.01% ascorbic acid (gives delivery rate of 5 µg β-estradiol/day), and the other two pumps were filled with 55 mg progesterone/ml of propylene glycol with 5% EtOH (delivery rate of 1.3 mg progesterone/day in 338 mOsm of intraperitoneal fluid). They were implanted intraperitoneally in rabbits, one per each rabbit (10-12). Blood samples were collected by heart puncture at 0, 18, 42, 60, 90, and 114 hour after implantation. Plasma was separated and kept at -20°C until analyzed by radioimmunoassay. After final collection of blood samples, animals were euthanized by CO_2 asphxiation. The tissues were immediately removed and frozen in liquid nitrogen and stored at -90°C until assayed. Hormones were subcutaneously injected to male rabbits (about 3 kg each) for 5 days. Sunflower oil was injected into 3 control rabbits, dexamethasone (2 mg/kg/day) was injected into 4 rabbits,

dexamethasone (2 mg/kg/day) was injected into 4 rabbits, progesterone (2 mg/kg/day) was injected into 3 rabbits, estradiol (20 µg/kg/day) was injected into 4 rabbits, aldosterone (20 µg/kg/day) was injected into 3 rabbits, and estradiol (20 µg/kg/day) for 5 days then progesterone (2 mg/kg/day) for 5 days was injected into 3 rabbits.

RADIOIMMUNOASSAY

The plasma levels of progesterone and estradiol from rabbits were measured by radioimmunoassay (RIA) as decribed by Sower and Schreck (13).

MICROSOME PREPARATION

Microsomes were prepared using a modification of the procedure of Guengerich (14). The levels of microsomal protein were assayed by the method of Lowry et al. (15), using BSA[4] as the standard.

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[4]BSA, bovine serum albumin; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dedocyl sulfate; PBS, 20 mM potassium phosphate, pH 7.4, 0.8% NaCl; IgG, immunoglobulin G; DMA, N,N'-dimethylaniline.

IMMUNOQUANTITATION

The microsomal proteins were resolved by SDS-PAGE and then electrophoretically transferred to nitrocellulose. Following 1 hour incubation at room temperature with 2% BSA in PBS, the blots were stained with guinea pig anti-rabbit lung FMO IgG (20 µg IgG per ml in the 2% BSA-PBS buffer with 0.05% Triton X-100) for 1.5 hour at room temperature. The FMO-IgG complex was visualized by incubating with [^{125}I]-protein A for 1 hour followed by autoradiography (16). Immunoquantitation was performed by laser densitometry and relative amounts of FMO were calculated utilizing the purified rabbit lung FMO as standard for lung and relative densities of blots were compared for kidney.

ASSAY OF FMO ACTIVITY

The metabolism of [^{14}C]-DMA by microsomal FMO was performed by HPLC to resolve the N-oxide and N-demethylated metabolites. Microsomes (0.1 mg) or purified FMO (0.02 mg) were incubated in 0.1 M tricine, pH 8.5 containing 1 mM EDTA, and a NADPH generating system [1 mM NADPH, 0.02 M isocitrate and 1 Unit isocitrate dehydrogenase]. [^{14}C]-DMA was added to a final concentration of 0.5 mM (1-2 µCi/µmol). All components except the microsomes or purified FMO were preincubated for 2 min and the reaction initiated by the addition of enzyme. The reaction was terminated by the addition of 0.1 ml methanol. HPLC analysis was performed with a Whatman RAC II Partisil 5 ODS-3

(4.6 mm x 10 cm) column eluted with 62% MeOH in water at 0.5 ml/min. Metabolite quantitation was performed with an on-line radiochemical detector (Beckman Model 171 with a solid cell).

RESULTS AND DISCUSSION

There are several reports describing induction of FMO by endogenous factors, including steroid hormones, rather than exogenous chemicals. During late gestation, changes in the hormonal milieu appear to be responsible for induction of FMO in rabbit lung (3, 17). Cortisol, through its diurnal secretion, may regulate the hepatic FMO activities in female mice (18). Diabetes can induce imipramine N-oxidation in mice, an activity mediated exclusively by FMO (19).

The results of the radioimmunoassays showed that both plasma progesterone and estradiol levels of hormone pump-implanted female rabbits began to increase at 60 hours after implantation and maintained the enhanced level until killed (Figs. 3.1 and 3.2). Western blot analysis of lung microsomes from the hormone pump-implanted female rabbits (114 hours post-implantation), stained with guinea pig anti-rabbit lung FMO IgG, shows that both progesterone and estradiol induce the lung FMO in female rabbits (Figs. 3.3 and 3.4). In the study employing subcutaneous injection with dexamethasone, progesterone, estradiol, aldosterone, or estradiol plus progesterone to male rabbits, dexamethasone and progesterone both significantly induced lung FMO protein (Figs. 3.5 and 3.6). DMA N-oxidase activities of both progesterone and dexamethasone injected male lung microsomes were also significantly higher than that of control (Fig. 3.7). Our report reveals that there are sex-related differences in

induction of FMO protein and activity in rabbit lung by hormone administration. Estradiol induced FMO in female but not in male, whereas progesterone induced FMO in both male and female rabbit lung. Although the route of administration, sex and source of rabbits are different, this result is supported by several previous reports which showed sex differences in the concentration or enzyme activity of FMO in rats, mice, and rabbits (20) and sex-related differences in mouse liver microsomal activity due to testosterone repression of the liver enzyme (21, 22). Rat liver FMO also appears to be positively regulated by testosterone, whereas estradiol exhibits repression of FMO levels (9).

Western blot analysis of rabbit kidney microsomes stained with guinea pig anti-rabbit lung FMO IgG has shown that only dexamethasone induced FMO in kidney (Figs. 3.8 and 3.9). DMA N-oxidase activity of microsomes from dexamethasone injected rabbit kidney was significantly higher than that of control (Fig. 3.10).

Another study in our laboratory correlated rabbit lung FMO induction with respect to protein, enzyme activity, and mRNA during mid- and late-gestation with plasma levels of progesterone and glucocorticoids while glucocorticoid (but not progesterone) levels correlated with FMO in kidney during late-gestation (M.-Y. Lee et. al.). The results of this study on hormone-implanted or injected rabbits in which both progesterone and dexamethasone induced FMO in lung while only

dexamethasone induced FMO in kidney correlate with the tissue-specific hormonal regulation phenomena during pregnancy in rabbits. Since we only examined four hormones, other hormones not used in our experiment may regulate FMO. Some previous reports support this different hormonal regulation pattern in different tissues. The hormonal regulation in lung and kidney from mice and rats was different from that in liver (22, 23). Gestation increases FMO activity in rabbit lung (3, 17, 24) and in mouse placenta microsomes (25) but not in mouse liver or lung.

The study discussed above indicates that FMO regulation is under hormonal control, but the effects observed appear to be sex-, tissue-, and species-dependent.

ACKNOWLEDGEMENT

We would like to thank our colleague, Jack Kelly for help in preparation of rabbit tissues. This research was supported by USPHS grant HL 38650.

Figure 3.1. Changes in plasma progesterone levels (mean ± SE) of hormone pump implanted female New Zealand white rabbits. Each value represents the mean of duplicates from 2 different animals.

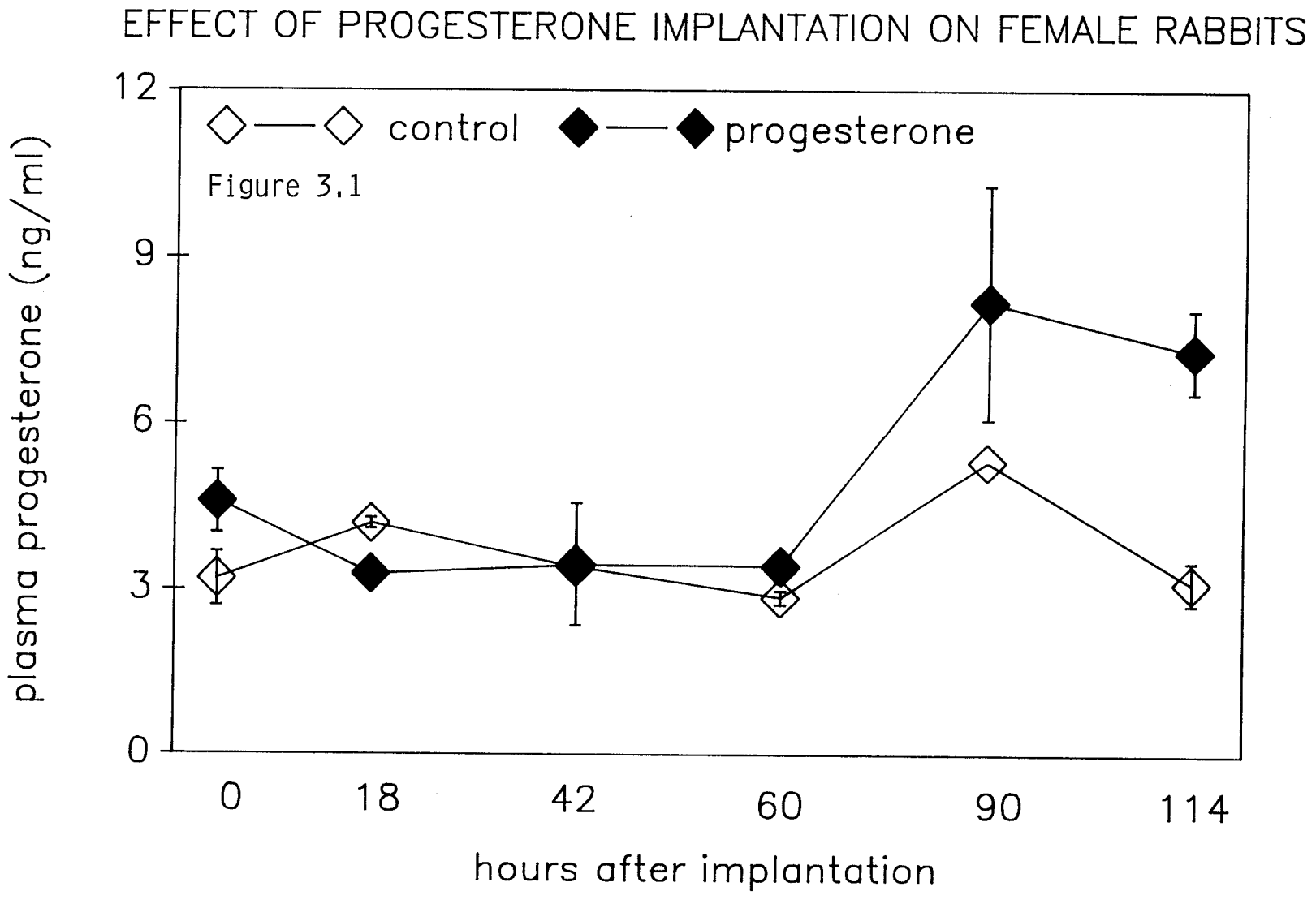

EFFECT OF PROGESTERONE IMPLANTATION ON FEMALE RABBITS
control
progesterone
Figure 3.1
plasma progesterone (ng/ml)
12
9
6
3
0
0
18
42
60
90
114
hours after implantation

Figure 3.2. Changes in plasma estradiol levels (mean ± SE) of hormone pump implanted female New Zealand white rabbits. Each value represents the mean of duplicates from 2 different animals.

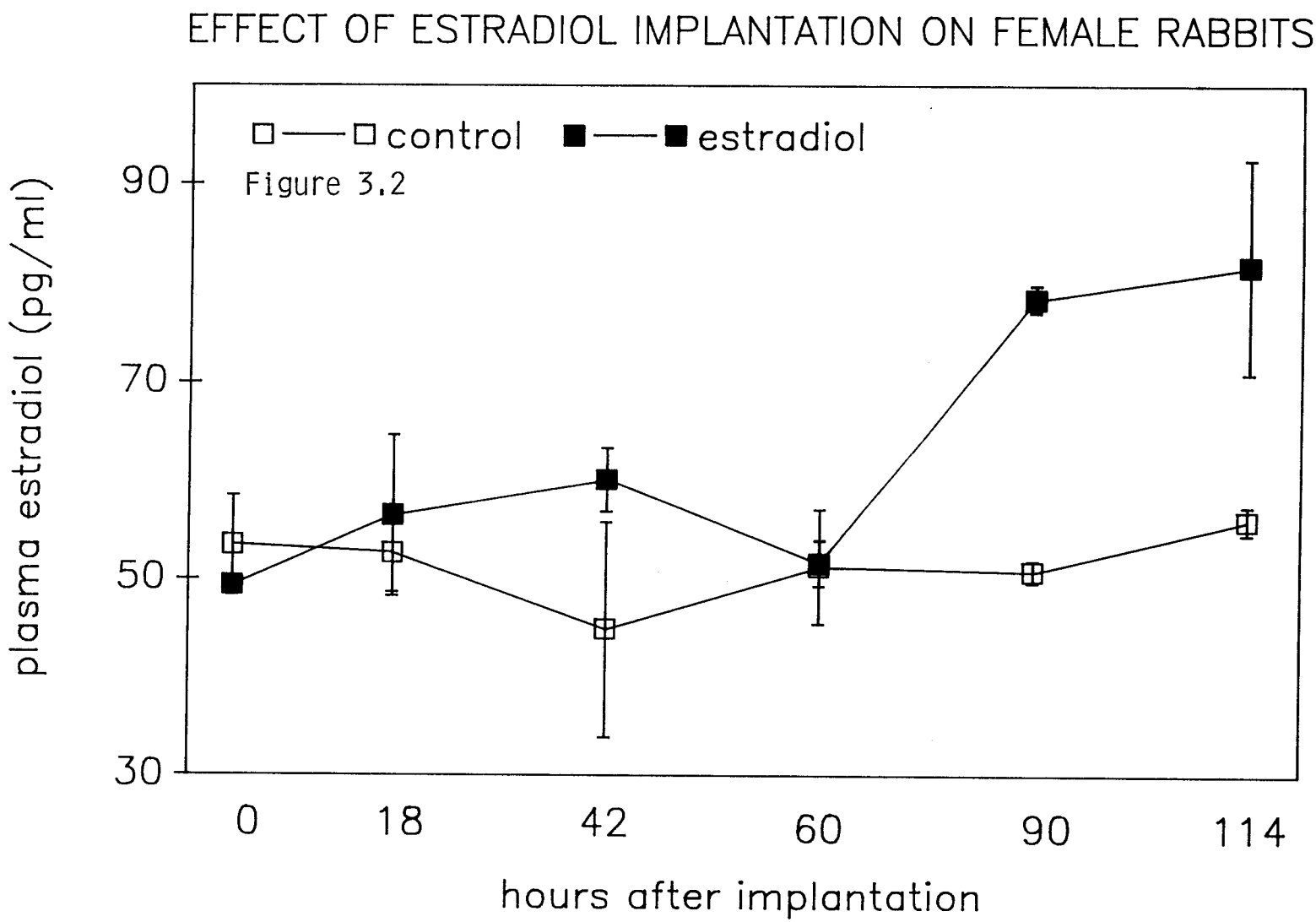

EFFECT OF ESTRADIOL IMPLANTATION ON FEMALE RABBITS
control
estradiol
Figure 3.2
plasma estradiol (pg/ml)
90
70
50
30
0
18
42
60
90
114
hours after implantation

Figure 3.3. Western blot analysis of lung microsomes from female rabbits 114 hours after hormone pump implantation (probed with antibody to rabbit lung FMO). Lane 1,2, control; lane 3,4, estradiol; lane 5,6, progesterone. Each lane contained 50 μg of protein.

Figure 3.3.

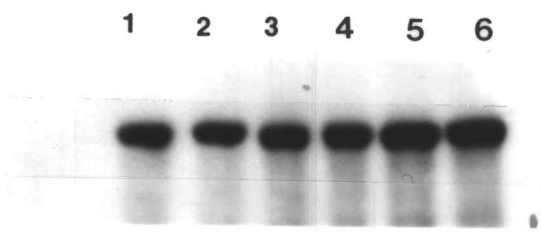


Figure 3.4. Quantitation of FMO in lung microsomes from progesterone or estradiol implanted female rabbits 114 hour post-implantation. The values shown were obtained using densitometry from autoradiograms of western blots of 2 different gels probed with antibody to rabbit lung FMO. Bars represent standard error of the mean.

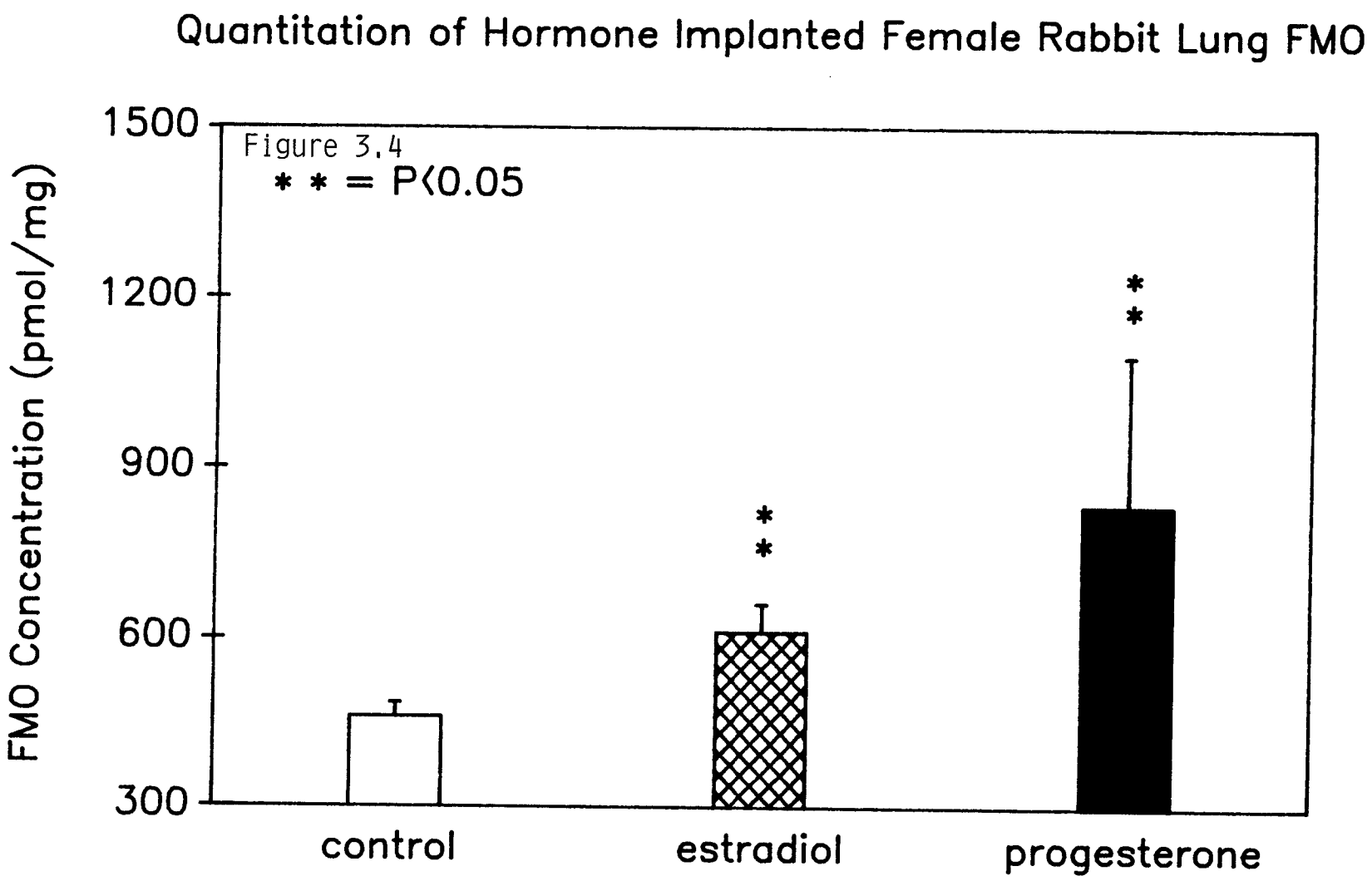

Quantitation of Hormone Implanted Female Rabbit Lung FMO
Figure 3.4
* * = P<0.05
FMO Concentration (pmol/mg)
1500
1200
900
600
300
control
estradiol
progesterone

Figure 3.5. Western blot analysis of lung microsomes from hormone injected male rabbits probed with antibody to rabbit lung FMO. lanes 1-3, 16-18, control; lanes 4,19 (5.75 pmol), 5,20 (8.63 pmol), 6,21 (11.5 pmol), purified rabbit lung FMO; lanes 7-10, dexamethasone; lanes 11-13, progesterone; lanes 14,15,22,23, estradiol; lanes 24-26, aldosterone; lanes 27-29, estradiol plus progesterone. Each lane contained 50 µg protein except the purified FMO standards.

Figure 3.5.

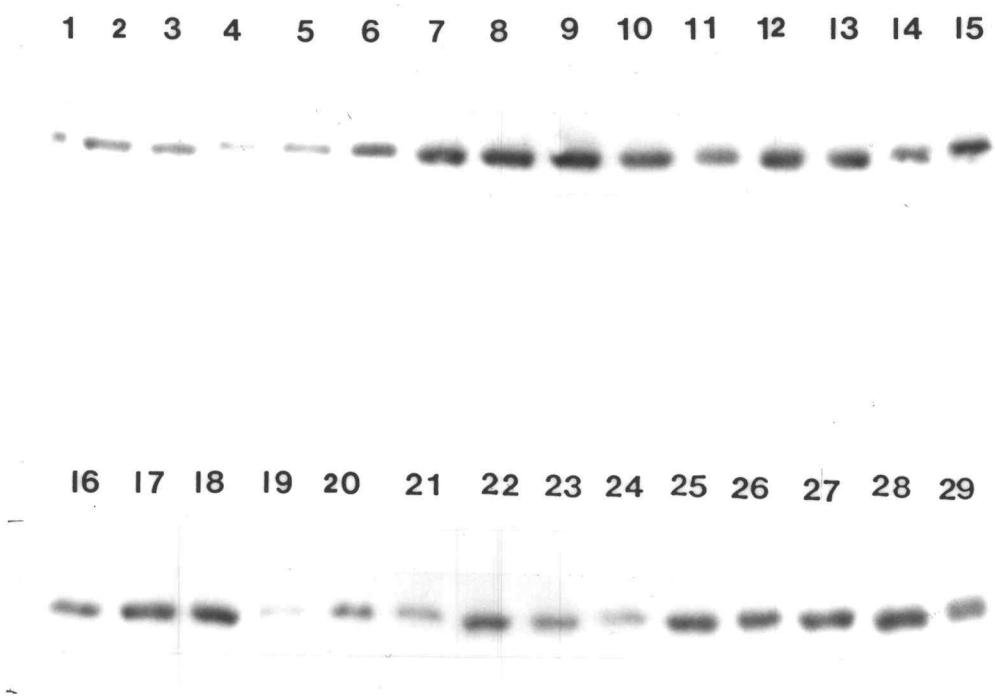


Figure 3.6. Quantitation of lung FMO from hormone injected male rabbits. The values shown were obtained using densitometry from autoradiograms of western blots (Fig.3.5) probed with antibody to rabbit lung FMO. Bars represent standard error of the mean. (* * = $P<0.05$)

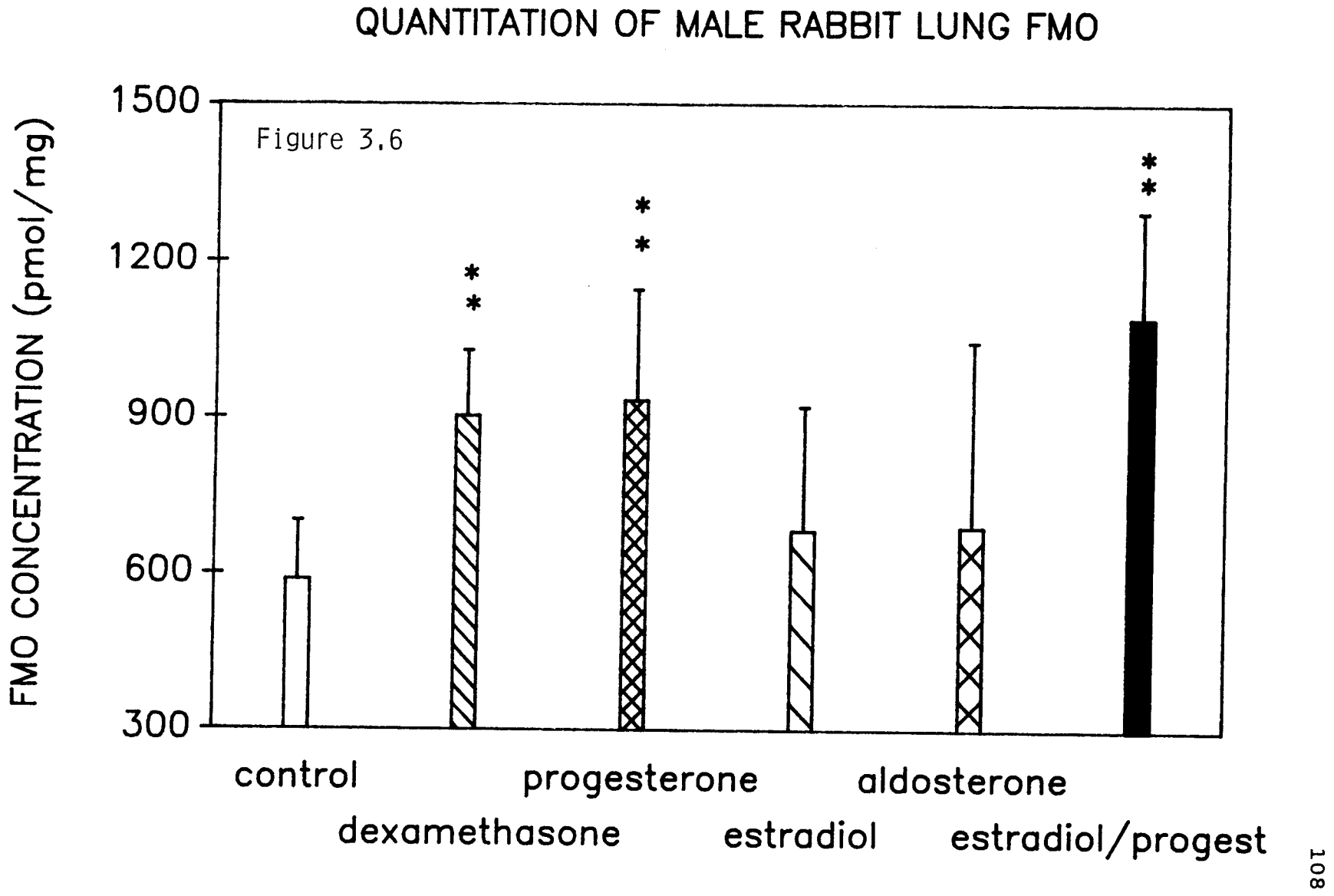

QUANTITATION OF MALE RABBIT LUNG FMO
Figure 3.6
FMO CONCENTRATION (pmol/mg)
1500
1200
900
600
300
control
dexamethasone
progesterone
estradiol
aldosterone
estradiol/progest

Figure 3.7. ^{14}C-Dimethylaniline N-oxidation by hormone injected male rabbit lung microsomes. Each value represents the mean of duplicate assays from 2 different animals. Bars represent standard error of the mean. (* * = $P<0.05$)

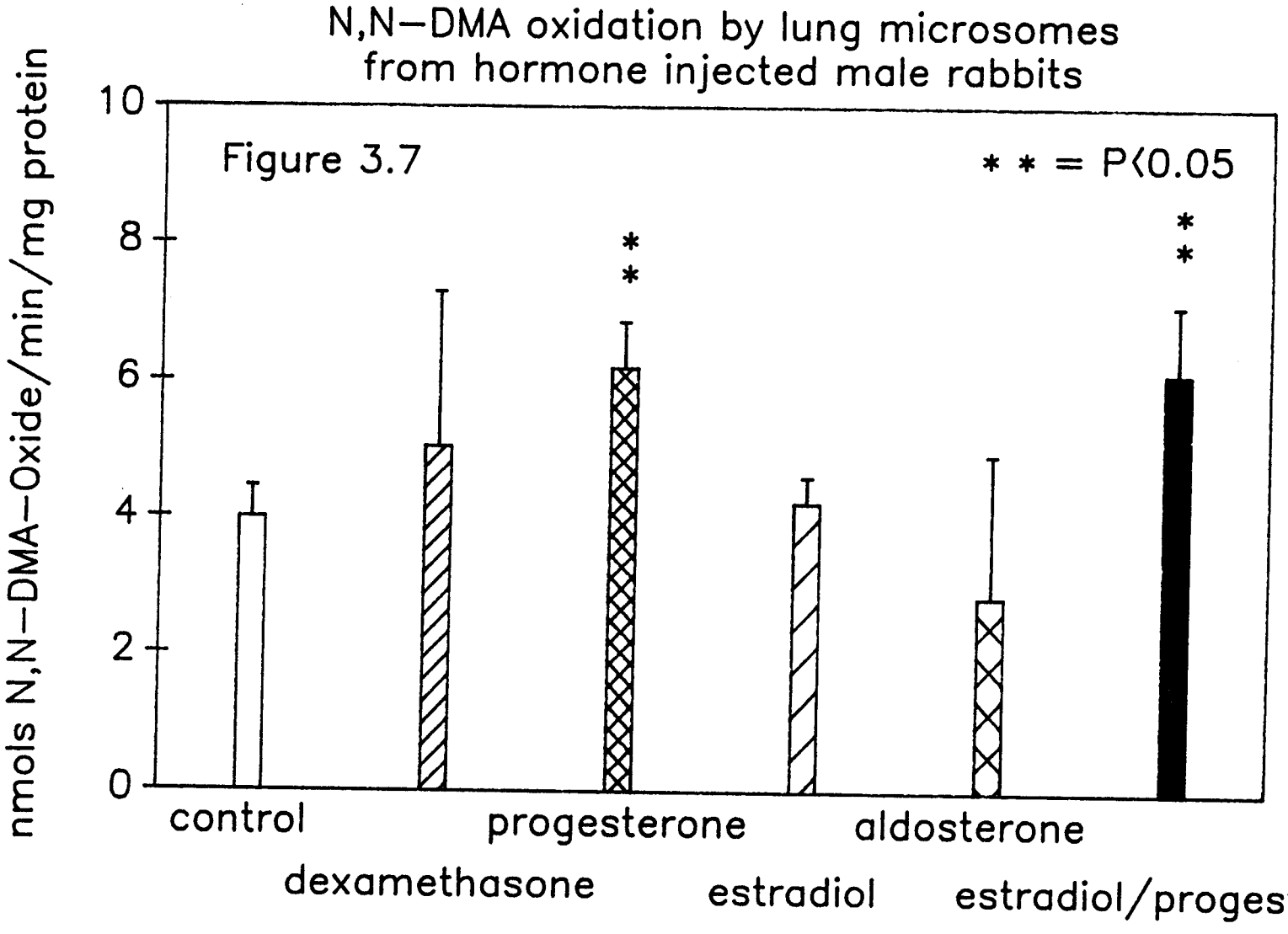

N,N–DMA oxidation by lung microsomes
from hormone injected male rabbits
Figure 3.7
* * = P<0.05
nmols N,N–DMA–Oxide/min/mg protein
10
8
6
4
2
0
control
progesterone
aldosterone
dexamethasone
estradiol
estradiol/progest.

Figure 3.8. Western blot analysis of hormone injected male rabbit kidney microsomes probed with antibody to rabbit lung FMO. lanes 1-3, 11-13, control; lanes 4-7, dexamethasone; lanes 8-10, progesterone; lanes 14-17, estradiol; lanes 18-20, aldosterone; lanes 21-23, estradiol plus progesterone. Each lane contained 50 μg protein.

Figure 3.8.

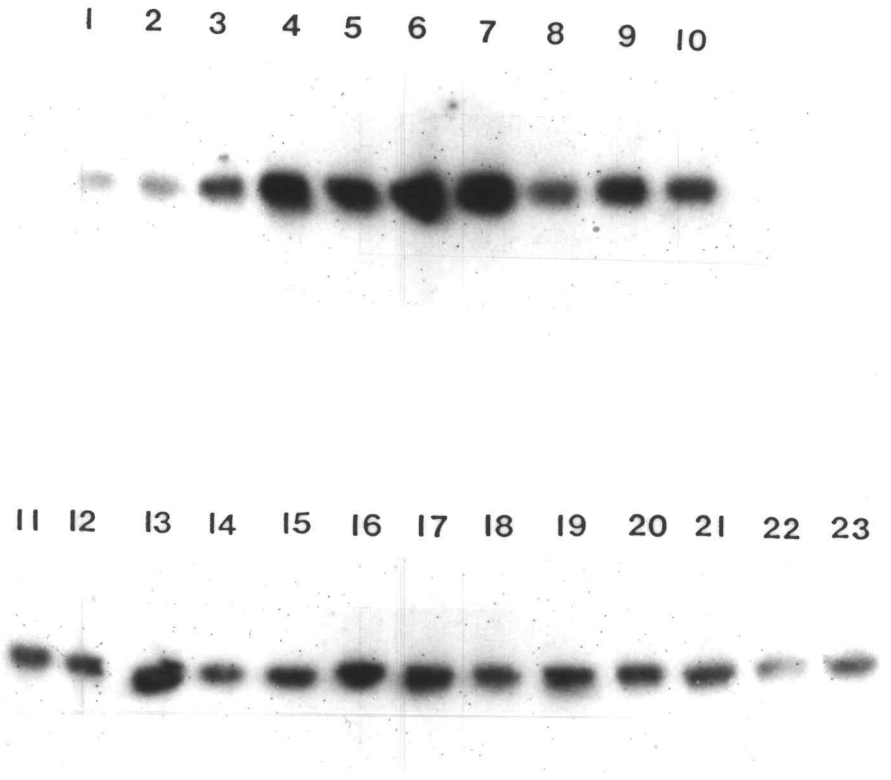


Figure 3.9. Quantitation of kidney FMO from hormone injected male rabbits. The values shown were obtained using densitometry from autoradiograms of western blots (Fig.3.8) probed with antibody to rabbit lung FMO. Bars represent standard error of the mean. (* * = $P<0.05$)

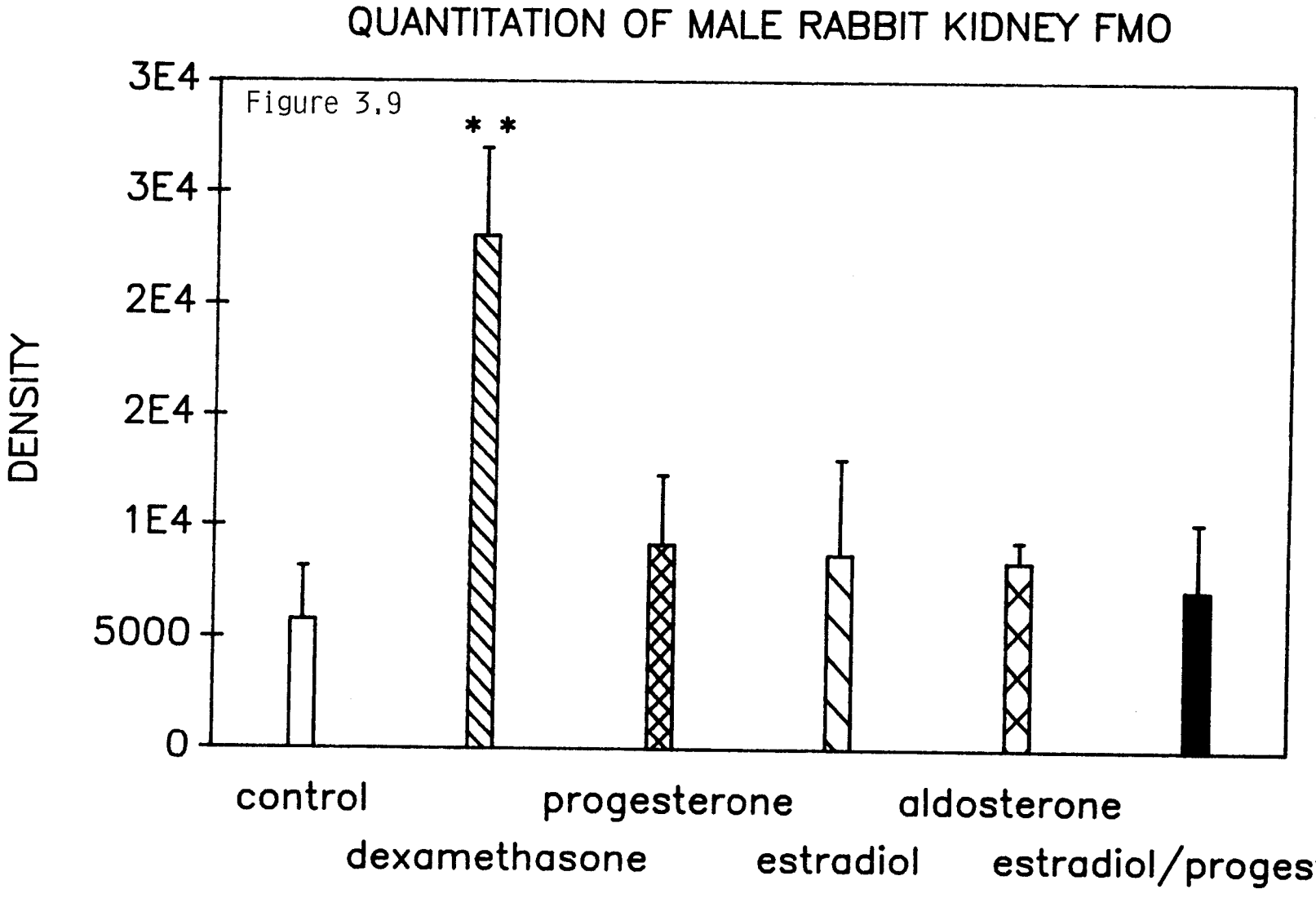
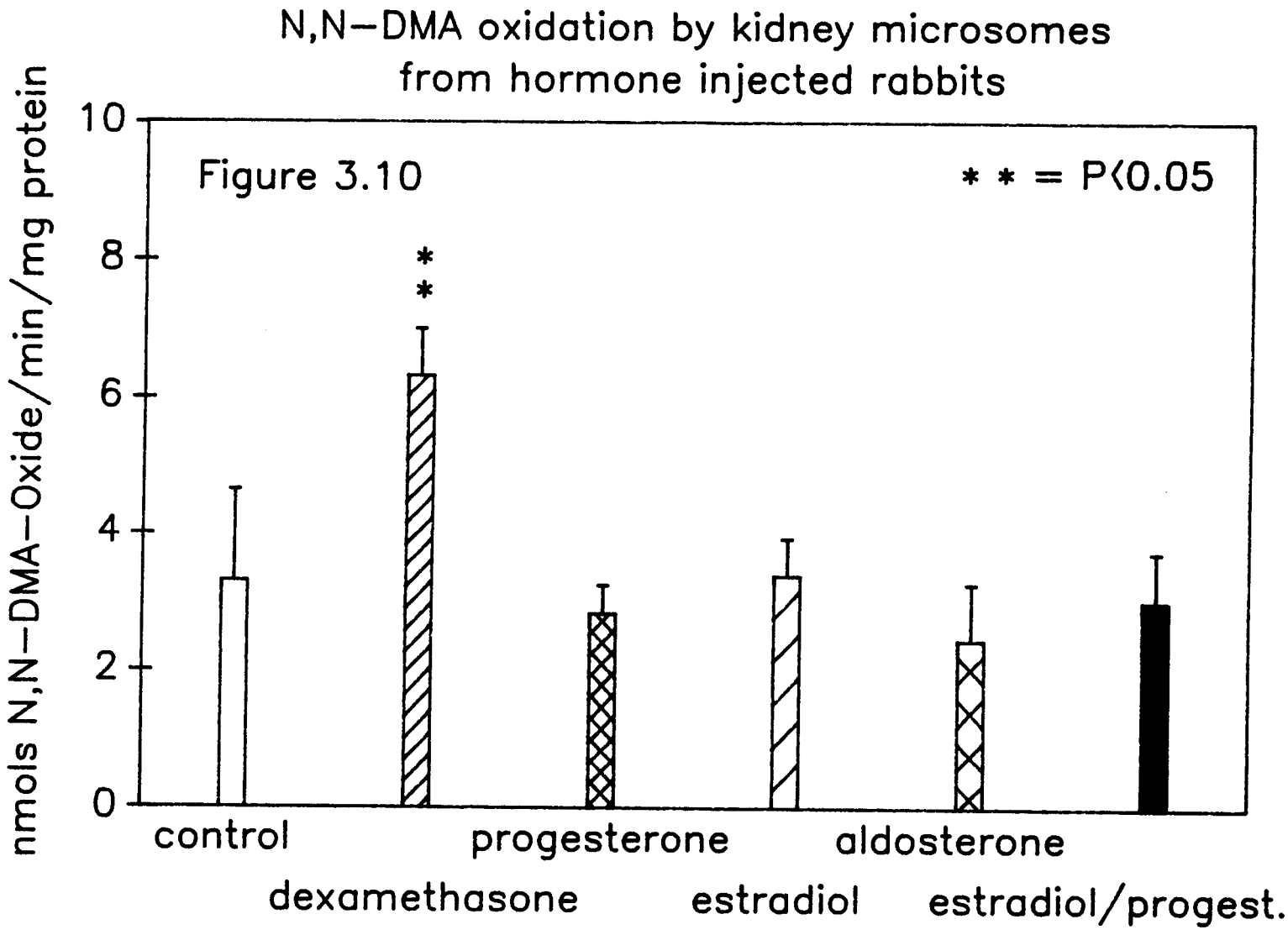

QUANTITATION OF MALE RABBIT KIDNEY FMO
Figure 3.9
DENSITY
3E4
3E4
2E4
2E4
1E4
5000
0
* *
control
dexamethasone
progesterone
estradiol
aldosterone
estradiol/progest

Figure 3.10. ^{14}C-Dimethylaniline N-oxidation by kidney microsomes from hormone injected male rabbits. Each value represents the mean of duplicate assays from 2 different animals. Bars represent standard error of the mean.


N,N–DMA oxidation by kidney microsomes
from hormone injected rabbits
Figure 3.10
* * = P<0.05
nmols N,N–DMA–Oxide/min/mg protein
10
8
6
4
2
0
* *
control
dexamethasone
progesterone
estradiol
aldosterone
estradiol/progest.

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Chapter 4

Developmental Regulation of Hepatic Flavin-Containing Monooxygenase (FMO) Throughout Gestation in Rabbit and Effects of Injection of Steroid Hormones.

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Developmental Regulation of Hepatic Flavin-Containing Monooxygenase (FMO) Throughout Gestation in Rabbits and Effect of Injection of Steroid Hormones.

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ABSTRACT

Unlike the much studied cytochrome P-450-dependent monooxygenase system, mammlian microsomal FMO is not inducible by xenobiotics, but does appear to be regulated developmentally, presumably through alterations in hormonal status. During the 31 day gestational period of rabbit, the level of progesterone linearly increases until mid- gestation and then declines to original levels by parturition, while glucocorticoid levels peak only at parturition. It has been shown that FMO is induced during gestation in rabbit. In the present study, maternal hepatic levels of FMO have been followed throughout gestation in pregnant rabbits by assaying enzyme activity, protein levels (western blotting with antibody to pig liver FMO) and mRNA levels (northern blotting with ^{32}P-labeled rabbit liver FMO form 1 cDNA). The levels of FMO activity, protein and mRNA peak at both mid- and late-gestation. The early and late peaks coincide with peaks of plasma progesterone and glucocorticoids respectively, during pregnancy. Subcutaneous injection with dexamethasone, progesterone, estradiol, aldosterone or estradiol plus progesterone to male rabbits, resulted in dexamethasone and progesterone both significantly inducing liver FMO mRNA. Dexamethasone increased the yield of total RNA two-fold compared to controls. This suggests that the levels and activity of hepatic FMO are regulated by endocrine factors and controlled by gene expression.

INTRODUCTION

Mammalian flavin-containing monooxygenase (FMO, EC 1.14.13.8) is a flavoprotein, which catalyzes the oxygenation of a large variety of nitrogen and sulfur-containing drugs and xenobiotics (1). Most of the metabolites formed by this enzyme are biologically inactive and can be easily excreted, however, some are potentially carcinogenic or mutagenic (1). The only known endogenous substrate of flavin-containing monooxygenase is cysteamine, which is oxidized to the disulfide, cystamine. The physiological role of FMO is uncertain but it may be related to the formation of disulfide bonds during protein synthesis (2). At least three different forms of FMO exist in rabbit (3-5). Two liver FMO forms (I and II, which are 54% identical), have been characterized in rabbit (4). Rabbit liver form I is 87% identical to the pig liver enzyme, but only 56% identical to the rabbit lung enzyme, confirming that the rabbit liver and lung FMOs are distinct gene products which evolved prior to speciation. The lung form of rabbit FMO is also present in kidney and bladder and evidence exists for sex- and developmental-related differences. A recent study demonstrated four different forms of FMO in humans (designated by Philpot and Meyer as IA1, IB1, IC1 and ID1) (6, 7). Human IA1 is 87% identical to pig liver FMO and rabbit liver form I, but surprisingly, is absent from adult human liver, but rather is present in kidney. Human form IB1 is 85% identical to rabbit lung FMO and is present in small amounts in adult human

lung, liver and kidney (7). The major form in adult human liver is ID1, which shares 85% identity to rabbit form II. FMOs in different tissues may be regulated independently and have markedly different properties, including stability and substrate specificity. FMO is not inducible by exogenous compounds but is regulated by endogenous factors like steroid hormones (8). There are several reports decribing regulation of FMO by endogenous factors, including steroid hormones, rather than exogenous chemicals. During late gestation, changes in the hormonal milieu appear to be responsible for induction of FMO in rabbit lung (3, 9). Another current study in our laboratory, involving hormone implantation or injection to rabbits, has shown that both progesterone and dexamethasone induced FMO in male rabbit lung, whereas only dexamethasone induced FMO in kidney. Estradiol could induce FMO in female rabbit lung but not in male (M.-Y. Lee et.al., unpublished). The purpose of the present study is to examine the developmental regulation of FMO in maternal rabbit liver and the effect of subcutaneously injected hormones.

MATERIALS AND METHODS

ANIMALS

Timed-pregnant New Zealand white rabbits were obtained from the Rabbit Research Institute at Oregon State University and housed in the Laboratory Animal Resource Center. Animals were maintained on a 12-hour day-night cycle and feeding was ad libitum. In order to follow the developmental regulation of the levels of FMO activity, protein levels and mRNA, a total of 20 pregnant, 2 non-pregnant female and 2 male rabbits were used. Two pregnant females were euthanized on days 10, 15, 20, 25, 28 and 31 of gestation. Following parturition, two rabbits were euthanized on days 1, 7, 21 and 28 post-partum. Animals were sacrificed by CO_2 asphxiation. The tissues were immediately removed and frozen in liquid nitrogen and stored at -90°C until assayed. Hormones were subcutaneously injected into male rabbits (about 3 kg each) for 5 days as following: sunflower oil was injected into 3 control rabbits, dexamethasone (2 mg/kg/day) into 4 rabbits, progesterone (2 mg/kg/day) into 3 rabbits, estradiol (20 µg/kg/day) into 4 rabbits, aldosterone (20 µg/kg/day) into 3 rabbits, estradiol (20 µg/kg/day) for 5 days then progesterone (2 mg/kg/day) for 5 days was injected into 3 rabbits.

MICROSOME PREPARATION

Microsomes were prepared using a modification of the procedure of Guengerich (10). The levels of microsomal protein

were assayed by the method of Lowry et al. (11), using BSA[5] as the standard.

IMMUNOQUANTITATION

The microsomal proteins were resolved by SDS-PAGE and then electrophoretically transferred to nitrocellulose. Following an 1 hour incubation at room temperature with 2% BSA in PBS, the blots were stained with rabbit anti-pig liver FMO IgG (20 µg IgG per ml in the 2% BSA-PBS buffer with 0.05% Triton X-100) for 1.5 hour at room temperature. The FMO-IgG complexes were visualized by incubating with [^{125}I]-protein A (0.1 µCi/ml in 2% BSA-PBS buffer) for one hour followed by autoradiography (12). Immunoquantitation was performed by laser densitometry and relative densities of blots were compared.

---

[5]BSA, bovine serum albumin; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dedocyl sulfate; PBS, 20 mM potassium phosphate, pH 7.4, 0.8% NaCl; DMA, N,N'-dimethylaniline; IgG, immunoglobulin G; 20 X SSC, 3 M sodium chloride, pH 7.0, 0.3 M sodium citrate.

ASSAY OF FMO ACTIVITY

The metabolism of [^{14}C]-DMA by microsomal FMO was performed by HPLC to resolve the N-oxide and N-demethylated metabolites. Microsomes (0.1 mg) or purified FMO (0.02 mg) were incubated in 0.1 M tricine, pH 8.5 containing 1 mM EDTA, and an NADPH generating system [1 mM NADPH, 0.02 M isocitrate and 1 Unit isocitrate dehydrogenase]. [^{14}C]-DMA was added to a final concentration of 0.5 mM (1-2 μCi/μmol). All components, except the microsomes or purified FMO, were preincubated for 2 min and the reaction initiated by the addition of enzyme. The reaction was terminated by the addition of 0.1 ml methanol. HPLC analysis was performed with a Whatman RAC II Partisil 5 ODS-3 (4.6 mm x 10 cm) column eluted with 62% MeOH in water at 0.5 ml/min. Metabolite quantitation was performed with an on-line radiochemical detector (Beckman Model 171 equiped with a solid cell).

RNA ISOLATION

Total RNA were prepared by the modified method of acid guanidinium thiocyanate-phenol-chloroform extraction (13).

RABBIT LIVER cDNA (1641 bp) PREPARATION

According to the rabbit liver cDNA sequence done by Lawton et al. (14), the RBFMOH-N sense (5'-TGTACGTCGGAGAACATGGCCAAGCGA-3') and RBFMOH-C antisense (5'-AACTAGCAAATCTTTTACTTATAGG-3') primers were used to amplify the rabbit hepatic flavin-

containing monooxygenase cDNA produced from a library generated from mRNA from female rabbit liver at 28 days gestation. The resulting 1641 bp fragment, spanning from 34 to 1674 (14) was cloned into the SmaI site of pUC19 such that the transcriptional orientation of the cDNA was the same as that of the lacZ gene in the plasmid. None of the isolated clones exhibited the predicted AvaII site at 1486 bp, although they did demonstrate the expected pattern for all other enzymes examined (4.33 kbp).

ELECTROPHORESIS AND TRANSFER

Electrophoresis of RNA through agarose containing formaldehyde was performed by published procedures (15). The amount of RNA in each sample was measured by optical density at 260 and 280 nm; 20 µg was analyzed by electrophoresis in duplicate. RNA in the gel was transferred to nylon membrane by capillary movement overnight using 20 X SSC buffer and the transferred blot was exposed to UV (UV Crosslinker, XL-1000, Fisher Biotech) light to fix the RNA on the membrane (16).

HYBRIDIZATION

The probe, used to quantitate rabbit lung FMO mRNA on northern blots, was obtained by random primer-labeling of rabbit liver FMO form I cDNA (1641 bp) with α ^{32}P-dCTP (17). After prehybridization for 4 h in 6 X SSC, 50% formaldehyde, 0.1% SDS, 5 X Denhardt's solution, 100 µg/ml salmon sperm DNA and

100 µg/ml tRNA, hybridization was performed at 42 °C overnight in the same medium in the presence of cDNA labeled with [α-^{32}P]dCTP (ICN) by a random primer-labeling kit from Boehringer Mannheim. The filter was washed in 2 X SSC buffer containing 0.1% SDS twice at room temperature for 15 min and 0.1 X SSC buffer with 0.1% SDS at room temperature for 15 min. The membrane was then exposed to Kodak X-OMAT AR film (Kodak, Rochester, NY) for 3 days with an amplifying screen at -90°C. Integrity of RNA was routinely assessed with a ^{32}P β-actin cDNA probe to demonstrate that each lane contained approximately equal amounts of RNA.

RESULTS AND DISCUSSION

Little work has been published on the effects of the hormonal regulation of FMO. Sex-related differences in the concentration or enzyme activity of FMO have been observed in rats, mice, and rabbits (18). During late gestation, changes in the hormonal milieu appear to be responsible for FMO induction in rabbit lung (3, 9). A current study in our laboratory, involving regulation of FMO throughout gestation in rabbit lung and kidney, indicates that mid- and late-gestation peaks of FMO induction in lung correlate to the peaks of plasma concentrations of progesterone and glucocorticoids, respectively. The only peak of kidney FMO induction occurs in late gestation, consistent with plasma glucocorticoids level during pregnancy (19, Lee et.al., unpublished). Another current study in our laboratory, involving hormone implantation or injection supports these results; both progesterone and dexamethasone induced FMO in male rabbit lung, whereas, only dexamethasone induced FMO in male rabbit kidney. Estradiol could also induce FMO in female rabbit lung but not in males (Lee et.al. unpublished).

The regulation of rabbit FMO throughout gestation and postpartum has been followed in maternal liver utilizing immunoquantitation with antibody to pig liver FMO, DMA N-oxidation activity, and northern blotting using a ^{32}P-labelled rabbit liver FMO form I cDNA probe. The level of hepatic FMO was significantly elevated at the earliest gestational time

point examined (day 10) and the peak levels of protein from western blot analysis were observed at mid- and late-gestation (Figs. 4.1, 4.2). The levels of liver FMO form I mRNA, quantitated by northern blotting using ^{32}P-labelled rabbit liver FMO form I cDNA, significantly increased throughout gestation and two peaks were observed at mid- and late-gestation (Figs. 4.3, 4.4). DMA N-oxidase activities were elevated throughout pregnancy with peaks at mid- and late-gestation (Fig. 4.5). These mid- and late-gestation peaks in FMO protein, activity and mRNA expression correlate to levels of plasma progesterone and glucocorticoids during pregnancy (Fig. 4.6). Rabbit liver FMO is higher in non-pregnant female rabbits compared to males as determined by protein levels, activity, and mRNA expression (Figs. 4.2, 4.4, 4.5). A study of subcutaneous hormone injection with dexamethasone, progesterone, estradiol, aldosterone, or estradiol plus progesterone to male rabbits showed that dexamethasone and progesterone both significantly induced hepatic FMO mRNA and dexamethasone increased the yield of total RNA two-fold compared to controls (Figs. 4.7-4.9). The studies discussed above indicate that rabbit liver FMO, like lung and kidney FMO, is regulated by progesterone and glucocorticoids during gestation. These findings are not in agreement with a previous study which reported that FMO activity (as determined by DMA-N-oxide formation) did not increase during late gestation in rabbit liver microsomes (9). The results presented here

confirm the role of hormonal regulation in the control of hepatic FMO, but the effects appear to be sex- and tissue-specific.

ACKNOWLEDGEMENTS

We wish to thank our colleagues, Jack Kelly, David Stresser, and Gayle Orner for help in preparation of rabbit tissues. This work was supported by USPHS grant HL 38650.

Figure 4.1. Western blot analysis of rabbit liver microsomes throughout gestation probed with antibody to pig liver FMO. lane 1, male; lane 2, nonpregnant female; lane 3, 10 day; lane 4, 15 day; lane 5, 20 day; lane 6, 25 day; lane 7, 28 day; lane 8, 31 day of pregnancy; lane 9, 1 day postpartum; lane 10, 7 day postpartum; lane 11, 21 day postpartum; lane 12, 29 day postpartum. Each lane contained 50 µg protein.

Figure 4.1.

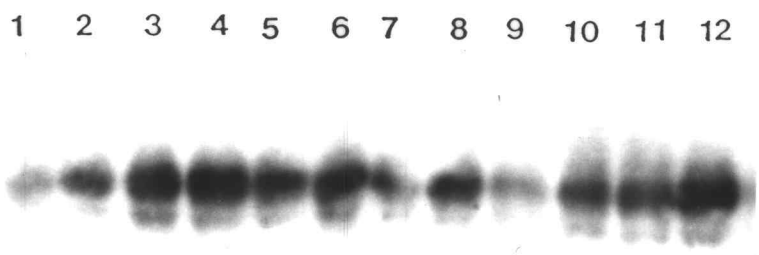


Figure 4.2. Quantitation of rabbit liver FMO during pregnancy. The values shown were obtained using densitometry from autoradiograms of western blots of 2 different gels (fig.4.1) probed with antibody to pig liver FMO. Bars represent standard error of the mean. (* * = $P<0.05$)

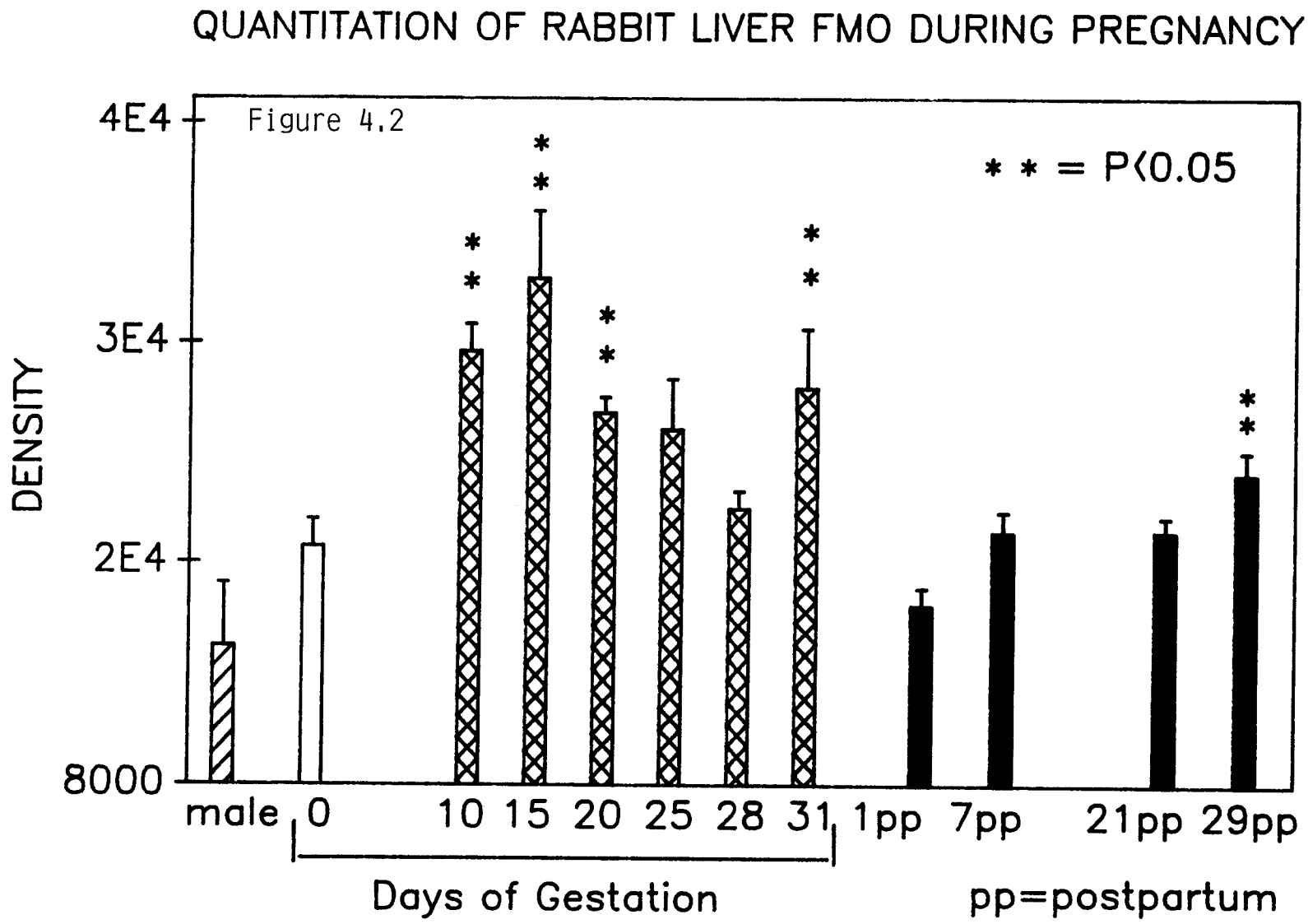

QUANTITATION OF RABBIT LIVER FMO DURING PREGNANCY
Figure 4.2
* * = P<0.05
DENSITY
4E4
3E4
2E4
8000
male
0
10
15
20
25
28
31
1pp
7pp
21pp
29pp
Days of Gestation
pp=postpartum

Figure 4.3. Northern blot analysis of rabbit liver total RNA throughout gestation probed with ^{32}P-labelled rabbit liver FMO form I cDNA. lane 1, male; lane 2, nonpregnant female; lane 3, 10 day; lane 4, 15 day; lane 5, 20 day; lane 6, 25 day; lane 7, 28 day; lane 8, 31 day of pregnancy; lane 9, 1 day postpartum; lane 10, 7 day postpartum; lane 11, 21 day postpartum; lane 12, 29 day postpartum. Each lane contained 50 μg RNA. The blot was subsequently reprobed with a ^{32}P-labeled human fibroblast β-actin cDNA to assess the relative amounts of RNA analyzed in each sample.

Figure 4.3.

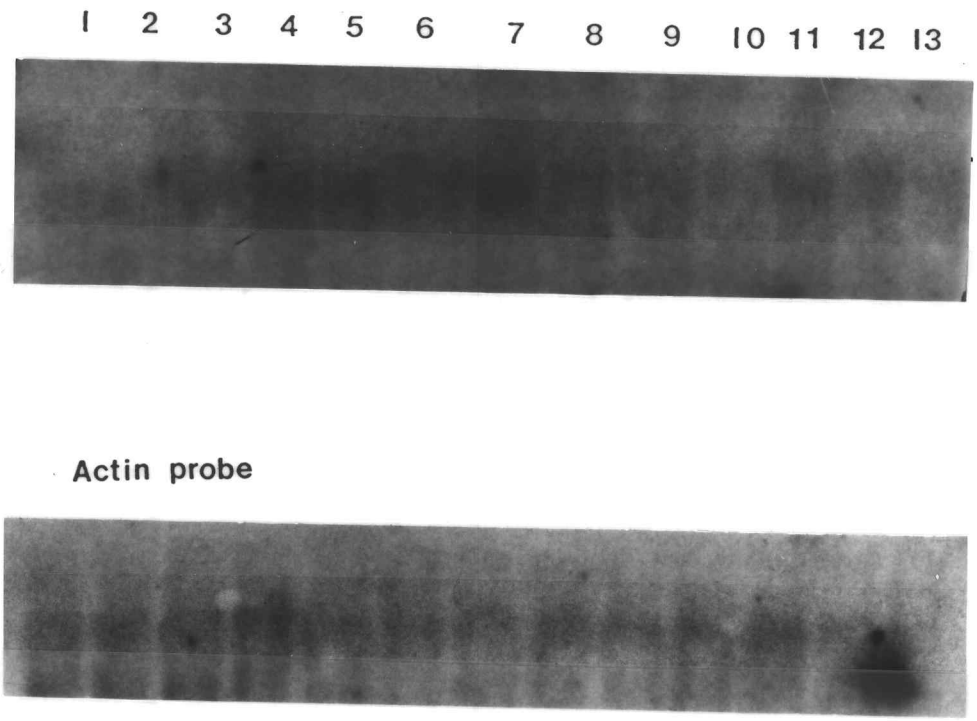


Figure 4.4. Rabbit liver FMO mRNA expression measured with a rabbit liver FMO cDNA probe throughout gestation. The graph was produced using densitometry from autoradiograms of northern blots of Figure 4.3.

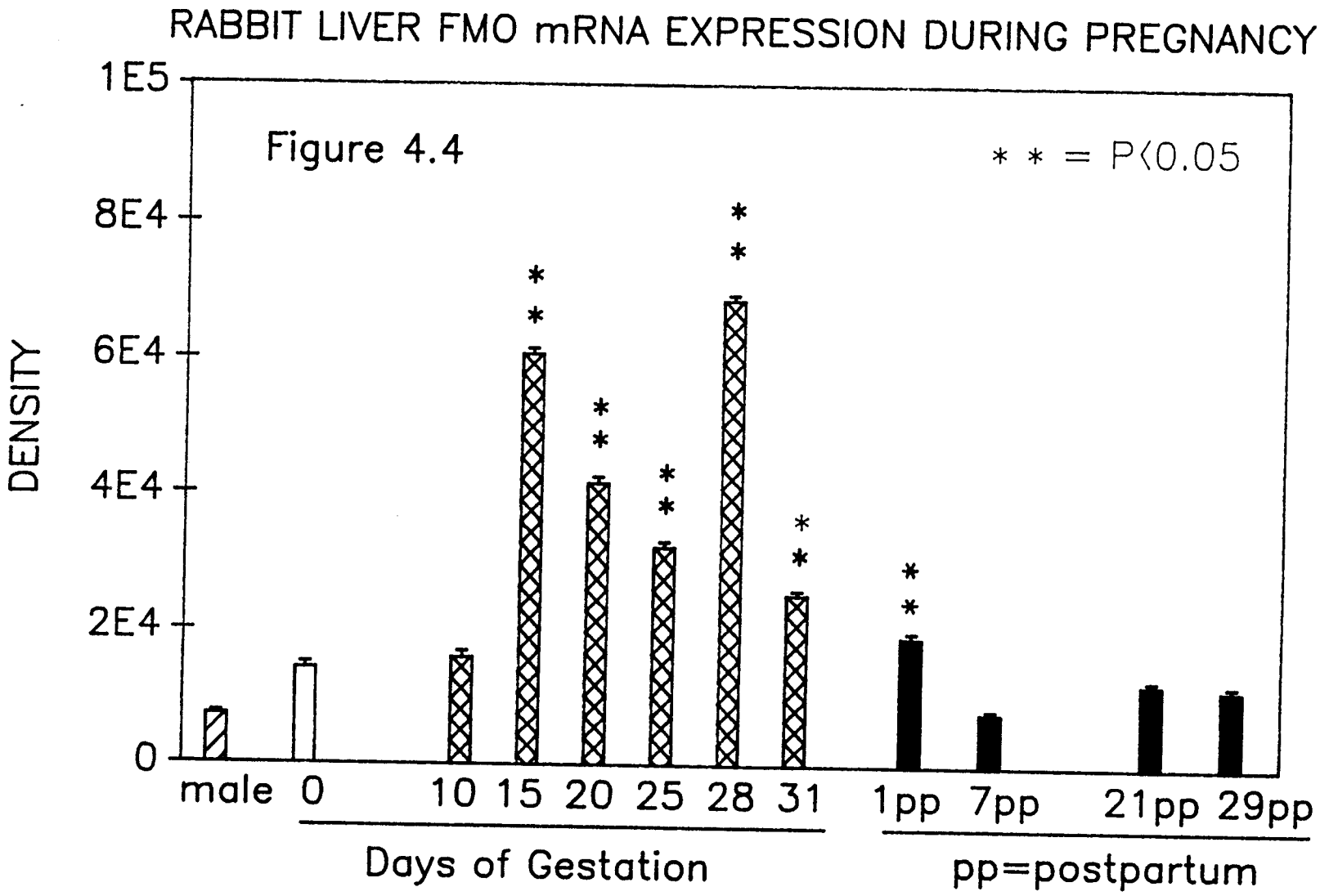

RABBIT LIVER FMO mRNA EXPRESSION DURING PREGNANCY
Figure 4.4
* * = P<0.05
DENSITY
1E5
8E4
6E4
4E4
2E4
0
male
0
10
15
20
25
28
31
1pp
7pp
21pp
29pp
Days of Gestation
pp=postpartum

Figure 4.5. ^{14}C-Dimethylaniline N-oxidation by rabbit liver microsomes throughout gestation. Each value represents the mean of duplicate assays of 2 different animals.

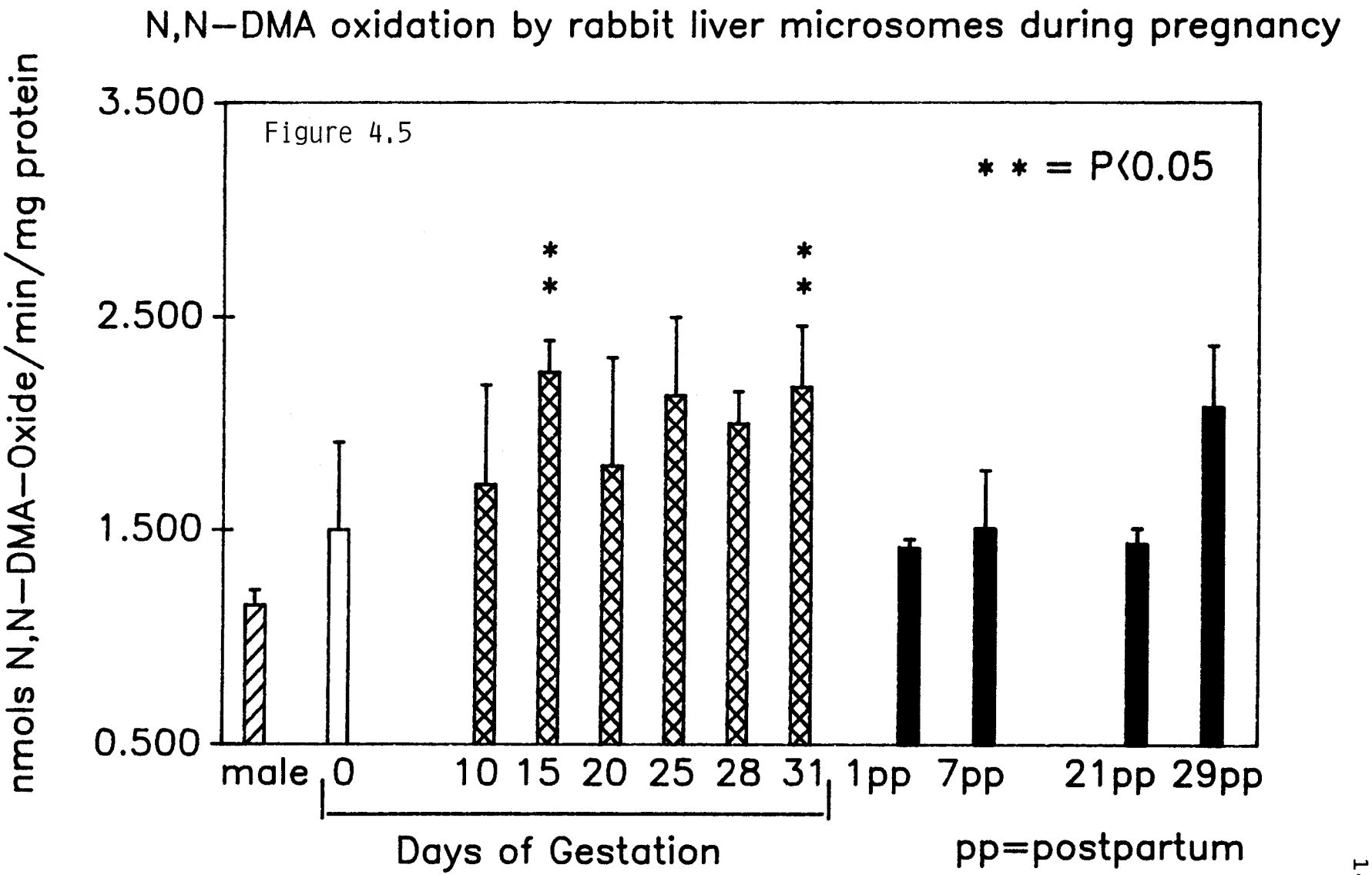
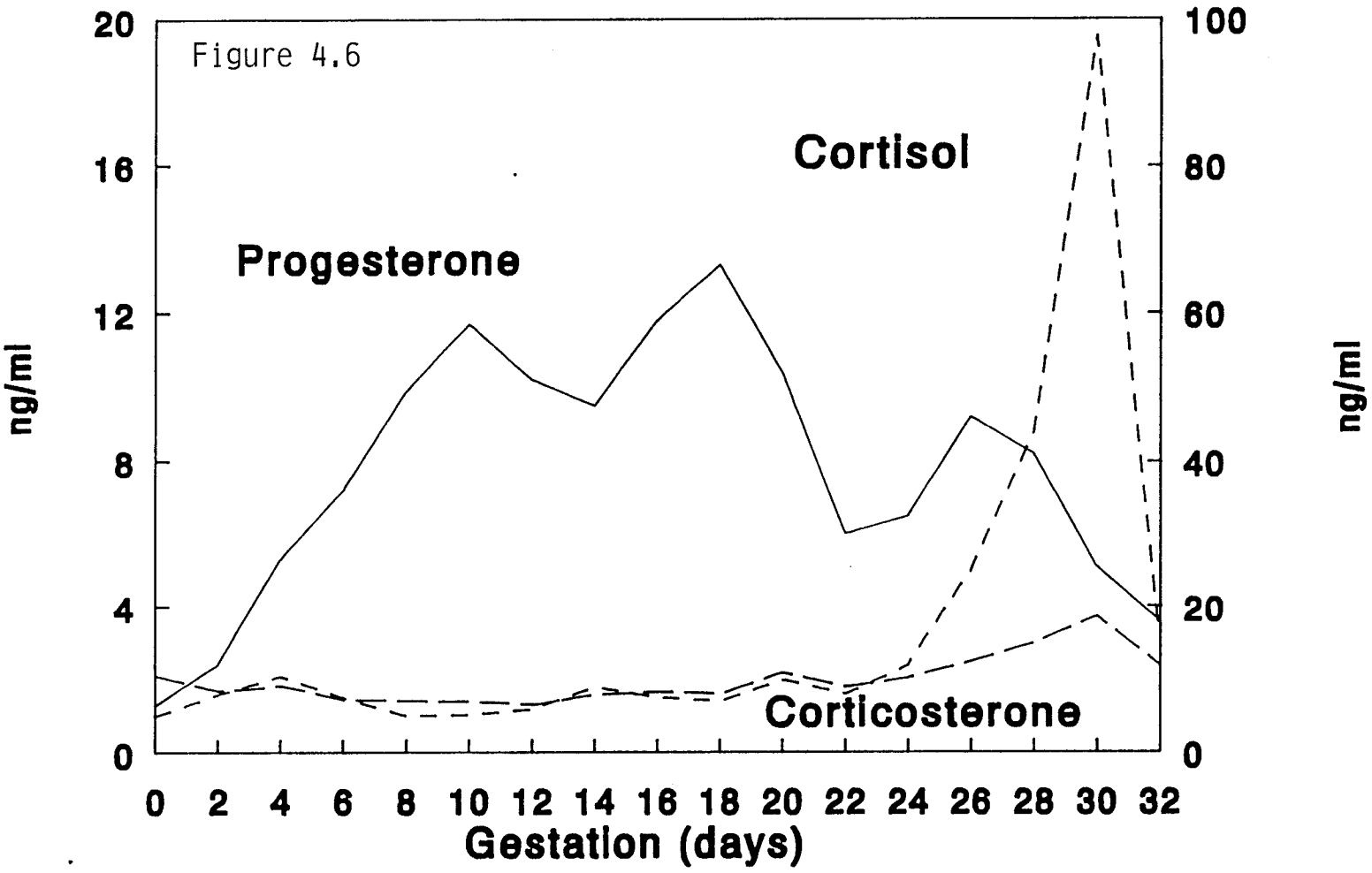

N,N–DMA oxidation by rabbit liver microsomes during pregnancy
Figure 4.5
* * = P<0.05
nmols N,N–DMA–Oxide/min/mg protein
3.500
2.500
1.500
0.500
male
0
10
15
20
25
28
31
1pp
7pp
21pp
29pp
Days of Gestation
pp=postpartum

Figure 4.6. Peripheral plasma levels of progesterone, cortisol and corticosterone in rabbit during pregnancy. This graph was adapted from Baldwin et al. (1974) (19).

Plasma hormone levels in pregnant rabbits



adapted from Baldwin and Stabenfeldt (1974)

Figure 4.7. Northern blot analysis of hormone injected male rabbit liver total RNA probed with ^{32}P-labelled rabbit liver FMO form I cDNA. lanes 1-3, control; lanes 4-7, dexamethasone; lanes 8,9, progesterone; lanes 10-13, estradiol; lanes 14-16, aldosterone; lanes 17,19, estradiol plus progesterone. Each lane contained 20 μg total RNA. The blot was subsequently reprobed with a ^{32}P-labeled human fibroblast β-actin cDNA to assess the relative amounts of RNA analyzed in each sample.

Figure 4.7.

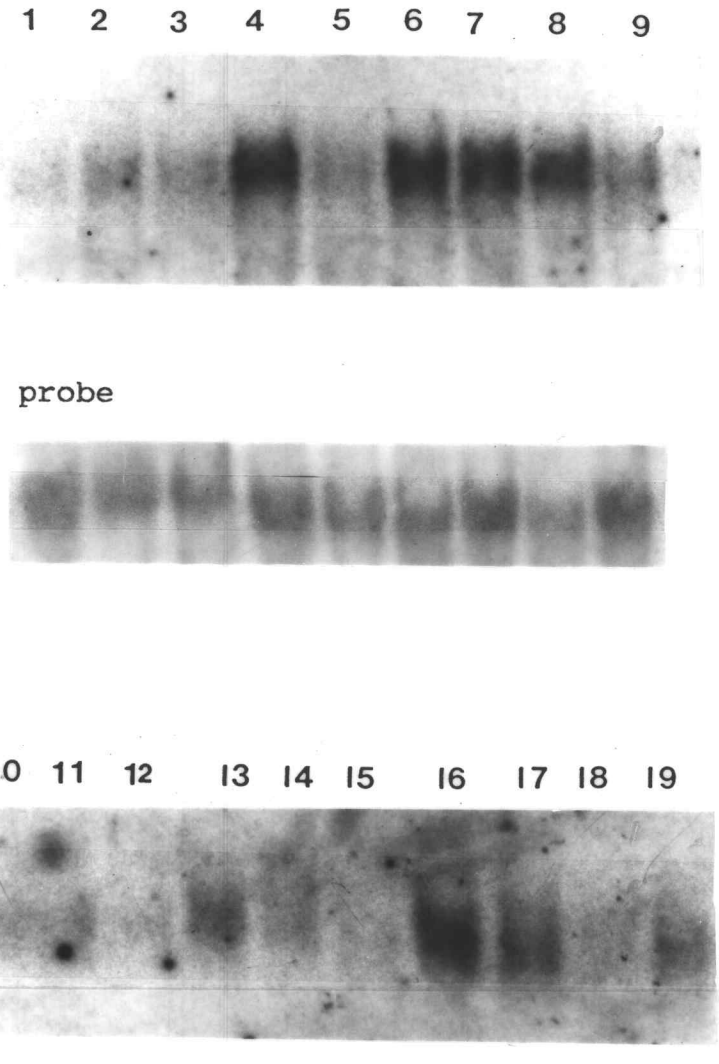


Figure 4.8. Hormone injected male rabbit liver FMO mRNA expression with rabbit liver FMO form I cDNA probe. The graph was produced using densitometry from autoradiograms of northern blots from figure 4.7. Bars represent standard error of the mean. (* * = $P<0.05$)

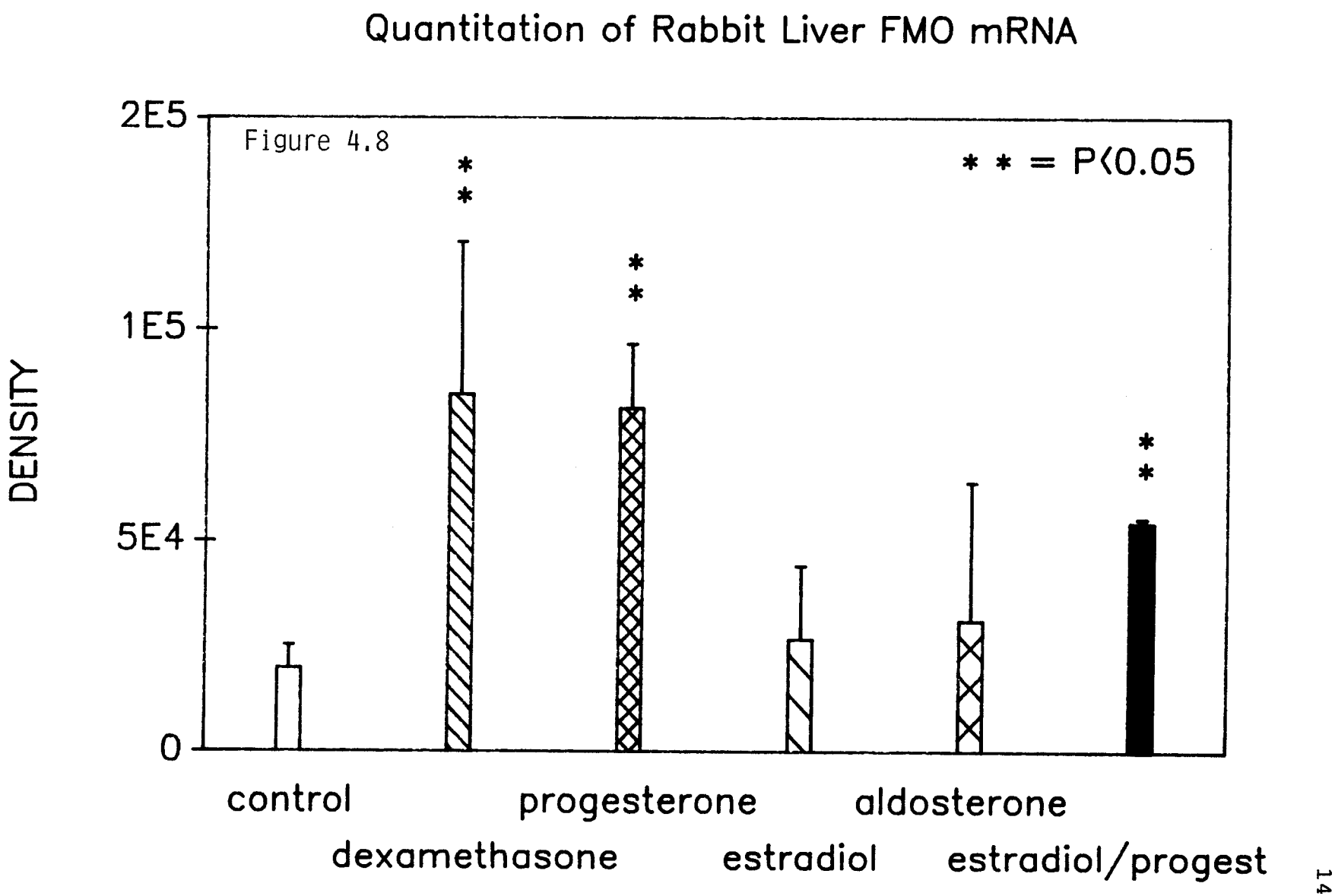
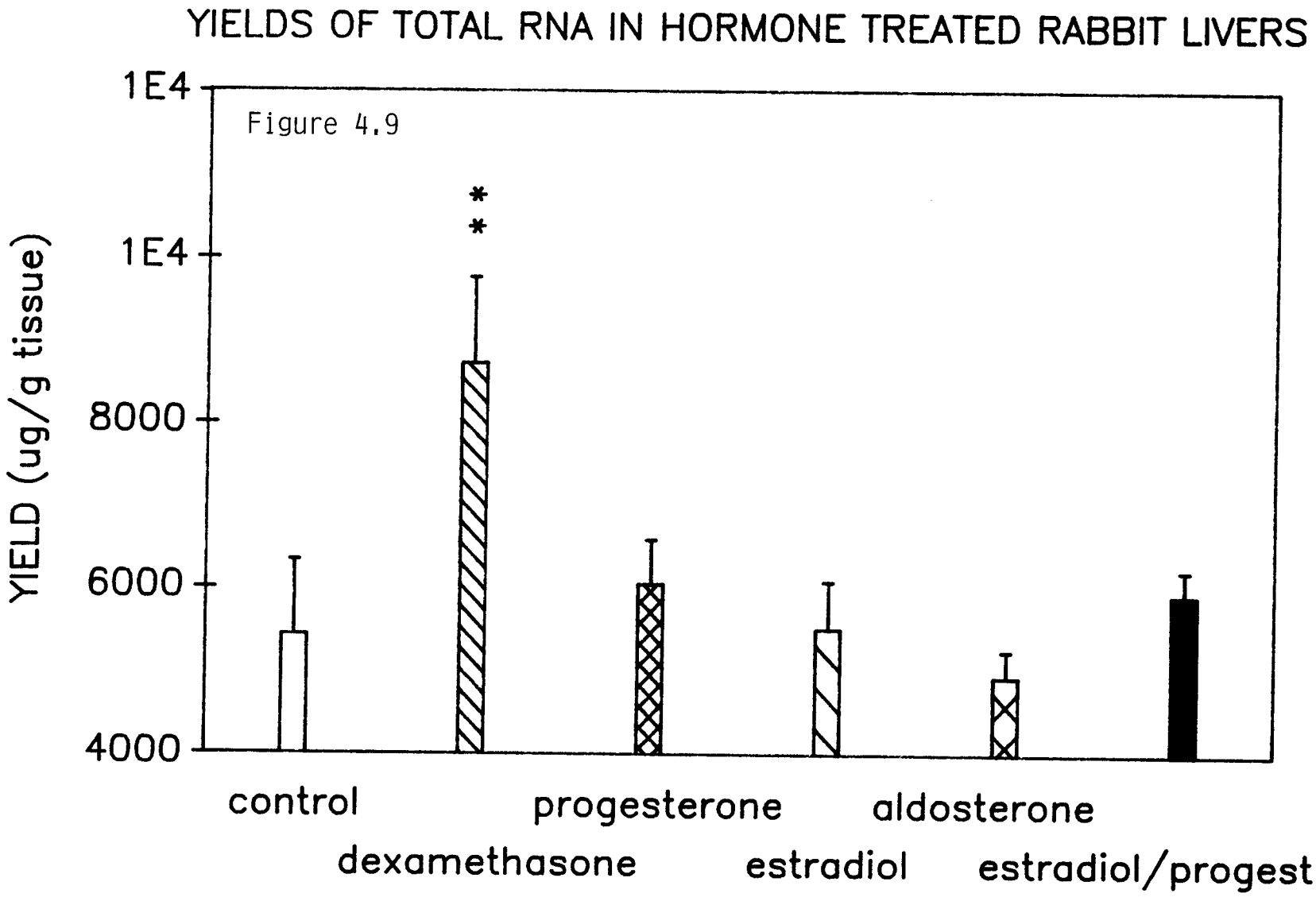

Quantitation of Rabbit Liver FMO mRNA
Figure 4.8
* * = P<0.05
2E5
1E5
5E4
0
DENSITY
control
dexamethasone
progesterone
estradiol
aldosterone
estradiol/progest

Figure 4.9. Yields of total RNA in hormone injected male rabbit livers as determined by A_{260} using spectrophotometer.


YIELDS OF TOTAL RNA IN HORMONE TREATED RABBIT LIVERS
Figure 4.9
YIELD (ug/g tissue)
1E4
1E4
8000
6000
4000
*
*
control
dexamethasone
progesterone
estradiol
aldosterone
estradiol/progest

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4. Ozols, J. (1989) Biochem. Biophys. Res. Commun. 163:49-55.

5. Ozols, J. (1990) J. Biol. Chem. 265:10289-10299.

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Chapter 5

Diurnal Variation of Flavin-Containing Monooxygenase (FMO) in Rabbit Tissues

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Diurnal Variation of Flavin-Containing Monooxygenase (FMO) in Rabbit Tissues.

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ABSTRACT

The diurnal variation of rabbit flavin-containing monooxygenase (FMO) was examined in lung and kidney. Levels of protein and mRNA as well as enzymatic activity in rabbit lung were quantified in groups of two female rabbits sacrificed at 4 AM, 8 AM, 12 noon, 4 PM, 8 PM and 12 midnight. In general, there was good agreement between FMO proteins, activities and mRNA levels at the various time points examined with minimum values at 8 PM and maximum values at 12 noon. A similar diurnal variation also exists in the kidney, but the pattern differs in degree. We speculate that the observed fluctuations in FMO levels with time of day may be correlated with protein regulation by the endogenous factors like hormones.

INTRODUCTION

Flavin-containing monooxygenase (FMO) catalyzes the oxygenation of a wide variety of foreign compounds bearing nucleophilic nitrogen or sulfur functional groups. While most of the metabolites formed by this enzyme represent detoxification reactions, some are potentially carcinogenic and mutagenic (1). Unlike the cytochrome P450 dependent mixed-function oxidase system, whose levels and associated enzyme activities can be regulated by exogenous factors such as drugs, environmental pollutants, synthetic steroids and food components, FMO levels appear to be governed solely by endogenous factors such as hormones.

FMO levels in rat and mouse liver appear to be regulated by steroid sex hormones (2-4). Sex differences have also been observed in rabbits (5,6). During late gestation in rabbit, FMO in lung increases in apparent correlation with plasma progesterone levels (M.-Y. Lee et. al. unpublished). Diabetes has been shown to increase FMO catalyzed imipramine N-oxidation in mice (7).

Hormonal changes in cortisol levels, which vary diurnally, have been postulated to regulate hepatic FMO levels in female mice (8). To our knowledge, diurnal changes in extrahepatic levels of FMO have not been examined.

Rabbit lung FMO has been purified by Williams et al., and Tynes et al. (9,10). The enzyme was found to be catalytically and immunologically distinct from that found in liver. Recent

sequencing of the corresponding cDNAs confirmed this finding (11). As part of ongoing efforts in this laboratory to further characterize the pulmonary form of rabbit FMO, we have examined the diurnal variation of the enzyme in lung and kidney of female New Zealand White rabbits.

MATERIALS AND METHODS

ANIMALS

Twelve New Zealand white female rabbits were obtained from the Rabbit Research Institute at Oregon State University and housed in the Laboratory Animal Resource Center. Animals were maintained on a 12-hour day-night cycle (7 AM-7 PM), and feeding was ad libitum. In order to follow the short term diurnal variation of the levels of FMO activity, protein and mRNA, two animals were killed at 4 AM, 8 AM, 12 noon, 4 PM, 8 PM and 12 midnight. Animals were killed by CO_2 asphxiation. The tissues were immediately removed and frozen in liquid nitrogen and stored at -90°C until assayed.

MICROSOME PREPARATION

Microsomes were prepared using a modification of the procedure of Guengerich (12). The levels of microsomal protein were assayed by the method of Lowry et al. (13) using BSA[6] as the standard.

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[6]BSA, bovine serum albumin; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PBS, 20 mM potassium phosphate, pH 7.4, 0.8% NaCl; IgG, immunoglobulin G; DMA, N,N'-dimethylaniline; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoic acid.

IMMUNOQUANTITATION

The microsomal proteins were resolved by SDS-PAGE and then electrophoretically transferred to nitrocellulose. Following an 1 hour incubation at room temperature with 2% BSA in PBS, the blots were stained with either guinea pig anti-rabbit lung FMO IgG or rabbit anti-pig liver FMO IgG (20 μg IgG per ml in the 2% BSA-PBS buffer with 0.05% Triton X-100) for 1.5 hour at room temperature. The FMO-IgG complexs were visualized by incubating with [^{125}I]-protein A (0.1 μCi/ml in 2% BSA-PBS buffer) for 1 hour followed by autoradiography (14). Immunoquantitation was performed by laser densitometry. The relative amounts of FMO were calculated utilizing the purified rabbit lung FMO as standard for lung and relative densities of blots were compared for liver.

ASSAY OF FMO ACTIVITY

1. ^{14}C-DMA metabolism assay.

The metabolism of [^{14}C]-DMA by microsomal FMO was performed by HPLC to resolve the N-oxide and N-demethylated metabolites. Microsomes (0.1 mg) or purified FMO (0.02 mg) were incubated in 0.1 M tricine, pH 8.5 containing 1 mM EDTA, and a NADPH generating system [1 mM NADPH, 0.02 M isocitrate and 1 Unit isocitrate dehydrogenase]. [^{14}C]-N,N-DMA was added to a final concentration of 0.5 mM (1-2 μCi/μmol). All components, except the microsomes or purified FMO, were preincubated for 2 min and the reaction initiated by the addition of enzyme.

The reaction was terminated by the addition of 0.1 ml methanol. HPLC analysis was performed with a Whatman RAC II Partisil 5 ODS-3 (4.6 mm x 10 cm) column eluted with 62% MeOH in water at 0.5 ml/min. Metabolite quantitation was performed with an on-line radiochemical detector (Beckman Model 171 equiped with a solid cell).

2. Methimazole S-oxidation assay.

The assay was performed by the procedure of Dixit and Roche (8). Final assay concentrations were 0.1 M tricine, 1.0 mM EDTA, 60 μM DTNB, 6.0 mM KPi, 0.025 mM DTT, 0.1 mM NADPH, and 1.0 mM methimazole. 0.5 ml buffer, H_2O, microsomes (0.25 mg/ml), and NADPH were added to a 1ml cuvette. After 40 seconds, 10 μl DTT and 60 μl DTNB in KPi were added. About 60% of DTNB will react with residual DTT to yield TNB having an absorbance of about 0.6 at 412 nm. After at least 2 min to prewarm to 37°C, the reaction was initiated by addition of 10 μl methimazole. The rate of reaction was compared with that of an identical mixture in the second cuvette lacking methimazole. The absorbance was measured at 412 nm. Note: ϵ for TNB = 28,200/M/cm, two molecules of TNB (nitro-5-thiobenzoate) are consumed per molecule product formed.

RNA ISOLATION

Total RNA were prepared by the modified method of acid guanidinium thiocyanate-phenol-chloroform extraction (15).

ELECTROPHORESIS AND TRANSFER

Electrophoresis of RNA through agarose containing formaldehyde was performed by published procedures (16). The amount of RNA in each sample was measured by optical density at 260 and 280 nm; 20 μg was analyzed by electrophoresis in duplicate. RNA in the gel was transferred to nylon membrane by capillary movement overnight using 20 X SSC buffer and the transferred blot was exposed to UV (UV Crosslinker, XL-1000, Fisher Biotech) light to fix the RNA on the membrane (17).

HYBRIDIZATION

The probe, used to quantitate rabbit lung FMO mRNA on northern blots, was obtained by random primer-labeling of rabbit lung FMO cDNA (1643 bp) (generously provided by Dr. R. N. Hines) with α ^{32}P-dCTP (18). After prehybridization for 4 hour in 6 X SSC, 50% formaldehyde, 0.1% SDS, 5 X Denhardt's solution, 100 μg/ml salmon sperm DNA and 100 μg/ml tRNA, hydbidization was performed at 42 °C overnight in the same medium in the presence of cDNA labeled with [α-^{32}P]dCTP (ICN) by a random primer-labeling kit from Boehringer Mannheim. The filter was washed in 2 X SSC buffer containing 0.1% SDS twice at room temperature for 15 min and 0.1 X SSC buffer with 0.1% SDS at room temperature for 15 min. The membrane was then exposed to Kodak X-OMAT AR film (Kodak, Rochester, NY) for 6 days with an amplifying screen at -90°C. Integrity of RNA was routinely assessed with a ^{32}P-labeled β-actin cDNA probe to demonstrate

that each lane contained approximately equal amount of RNA.

RESULTS

The diurnal variation of FMO in lung and kidney of female New Zealand White rabbits has been determined by 1) assaying the microsomes for enzymatic activity (^{14}C-DMA N-oxidation, S-methimazole oxidation), 2) immunoquantitation of levels of FMO by western blotting and 3) quantitation of mRNA by northern blot analysis.

Immunoquantified lung FMO protein levels rose from 4 AM to highest levels of 1203 ± 2.0 and 1265 ± 155 pmols/mg protein at 8 AM and 12 noon, respectively. This specific contents correspond to about 8% (by weight) of the total microsomal protein. Protein levels decreased to their lowest values at 8 PM (Figs. 5.1 and 5.2) with a total variation between highest and lowest values 2.5 fold. The level of pulmonary FMO mRNA quantitated by northern blotting using ^{32}P-labeled rabbit lung FMO cDNA similarly increased from 4 AM but did not reach a maximum level until 4 PM. An apparent rapid decline in mRNA levels occurred so that lowest levels were reached at 8 PM, similar to western blot results (Figs. 5.3 and 5.4). DMA N-oxidase activity in lung microsomes (Fig. 5.5) correlated reasonably well with western blot results showing peak values at noon (7.0 ± 0.75 nmols N,N-DMA-Oxide produced/min/mg protein) and exhibited lowest activity at 8 PM (3.28 ± 0.31 nmol/min/mg). Methimazole S-oxidation activity (Fig. 5.6) of lung microsome reflected DMA N-oxidase activity with the highest activity observed at 12 noon (1.16 ± 0.21 nmols

methimazole oxidized/min/mg protein) and lowest at 8 PM (0.61 ± 0.11 nmol/min/mg).

Because of variability in the data and the low levels of protein observed, no significant diurnal variation in FMO levels could be ascertained in kidney (Figs. 5.7-5.10).

DISCUSSION

Results presented here provide evidence for a diurnal variation in rabbit lung FMO protein. Maximum values are reached at approximately 12 noon and minimum values are found at 8 PM. A diurnal variation in hepatic FMO has been documented in fed and starved female mice with highest activity observed in animals sacrificed betweem 4 and 5 PM compared to 8 to 9 AM (8). This is the reverse of our observations (i.e. levels at 8 AM were higher than levels observed at 4 PM) in rabbit lung and kidney. This is not surprising since the presumed hormonal regulation of FMO activity observed during the estrous cycle and pregnancy has been found to be species- and tissue-dependent (5,6,20). Rabbit liver FMO does not vary substantially with time of day (unpublished results, this laboratory). Levels of mRNA were largely consistent with FMO levels and enzymatic activity, but a noticeable disparity exists at the 4 PM time point where levels and activity are definitely declining while mRNA levels are peaking. The reason for this is unknown, but it is likely that a lag in degradation is not occurring.

The magnitude in variation of FMO levels observed diurnally is small (approximately 0.8 nmol/mg protein versus approximately 5 nmol/mg) relative to that observed during pregnancy (6). It is unknown whether the large increase in FMO levels during late gestation overrides diurnal variation or levels continue to fluctuate at this stage with time of day.

Data on diurnal variation of hormones in rabbit are scant and it would be difficult to speculate on specific hormones responsible for inducing or repressing effects observed. Testosterone is apparently responsible for sex related differences in the liver enzyme in mice acting to repress microsomal activity (2,3). Rat liver FMO is repressed by estradiol and appears to be positively regulated by testosterone (19). Gestation is known to alter FMO levels. Specifically rabbit lung FMO and mouse placental FMO is strongly induced during pregnancy (5,6,20). A 20-fold induction of DMA N-oxidation is observed in the pig corpora lutea during the estrous cycle (21). Further evidence for hormone regulation of FMO was recently provided in experiments with hypophysectomized male and female rats (22). FMO activity was reduced in male liver yet showed no effect or was even stimulated in female liver. Tissue dependence was exhibited by the fact that FMO enzymatic activity increased 3-fold in kidney of males, yet decreased in lung in both sexes.

The disulfide-sulfhydryl ratio of rat hepatic tissue has been found to vary diurnally. This altering ratio has been speculated to represent a regulatory mechanism in that many enzymatic activities are either sulfhydryl or disulfide dependent (23). Cysteamine is the only known endogenous substrate for FMO and oxidation takes place to form the disulfide cystamine (24). Ziegler has proposed that FMO catalyzed formation of cystamine may represent a significant

source of cellular disulfides (24) which theoretically could alter this ratio and therefore be involved in enzyme regulation. However, FMO levels in all tissues examined have been found to be in much higher concentrations than the substrate cysteamine. The reaction would therefore likely be limited by the availabilty of substrate. Further studies on the mechanisms regulating the rate of cysteamine synthesis are needed before further speculations on the possbile involvement of FMO on enzyme regulation are made.

It was confirmed that FMO is under long-term control of hormones throughout gestation (Lee et. al., unpublished). Progesterone, dexamethasone, and estradiol were involved in the induction of FMO at the level of gene expression during gestation. While short-term diurnal variation of liver FMO is related to the variation of gluconeogenesis status (8), the mechanism of diurnal variation of extrahepatic FMO would be different. Dixit and Roche (1984) have shown that cortisol controls hepatic FMO activities in female mice through its diurnal variation. Glucocorticoids which involve in the glucose metabolism in hepatic tissue, may involve in short-term regulation of FMO in extrahepatic tissue. The data from this study indicate that there is short-term bimodel regulation of FMO in rabbit lung whereas the diurnal variation in kidney is negligible.

ACKNOWLEDGEMENT

We wish to thank Dr. Ronald N. Hines for providing the rabbit lung cDNA probe and Jack Kelly for help in preparation of rabbit tissues. This work was supported by USPHS grant HL 38650.

Figure 5.1. Western blot analysis of rabbit lung microsomes from 4 am to 12 midnight. Tissues were probed with antibody to rabbit lung FMO. lanes 1-2, 4 am; lanes 3-4, 8 am; lanes 5-6, 12 noon; lanes 7-8, 4 pm; lanes 9-10, 8 pm; lanes 11-12, 12 midnight; lanes 13-15, purified rabbit lung FMO, 5.75 pmol, 8.63 pmol, 11.5 pmol. Each lane contained 50 µg protein except FMO standards.

Figure 5.1.

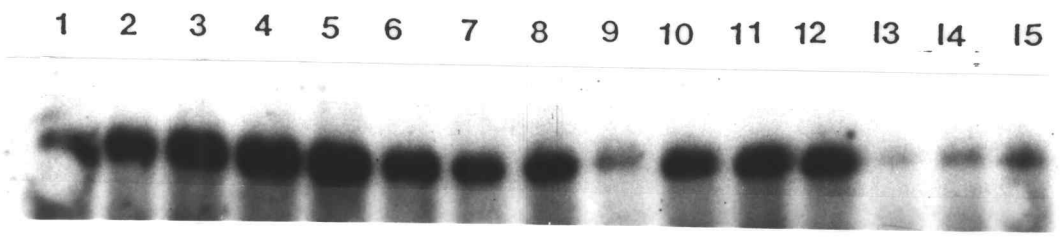


Figure 5.2. Quantitation of rabbit lung FMO from 4 am to 12 midnight. The values shown were obtained using densitometry from autoradiograms of western blots probed with antibody to rabbit lung FMO.
* * represents the statistical difference ($P<0.05$) when comparing the value of 8 pm with others.

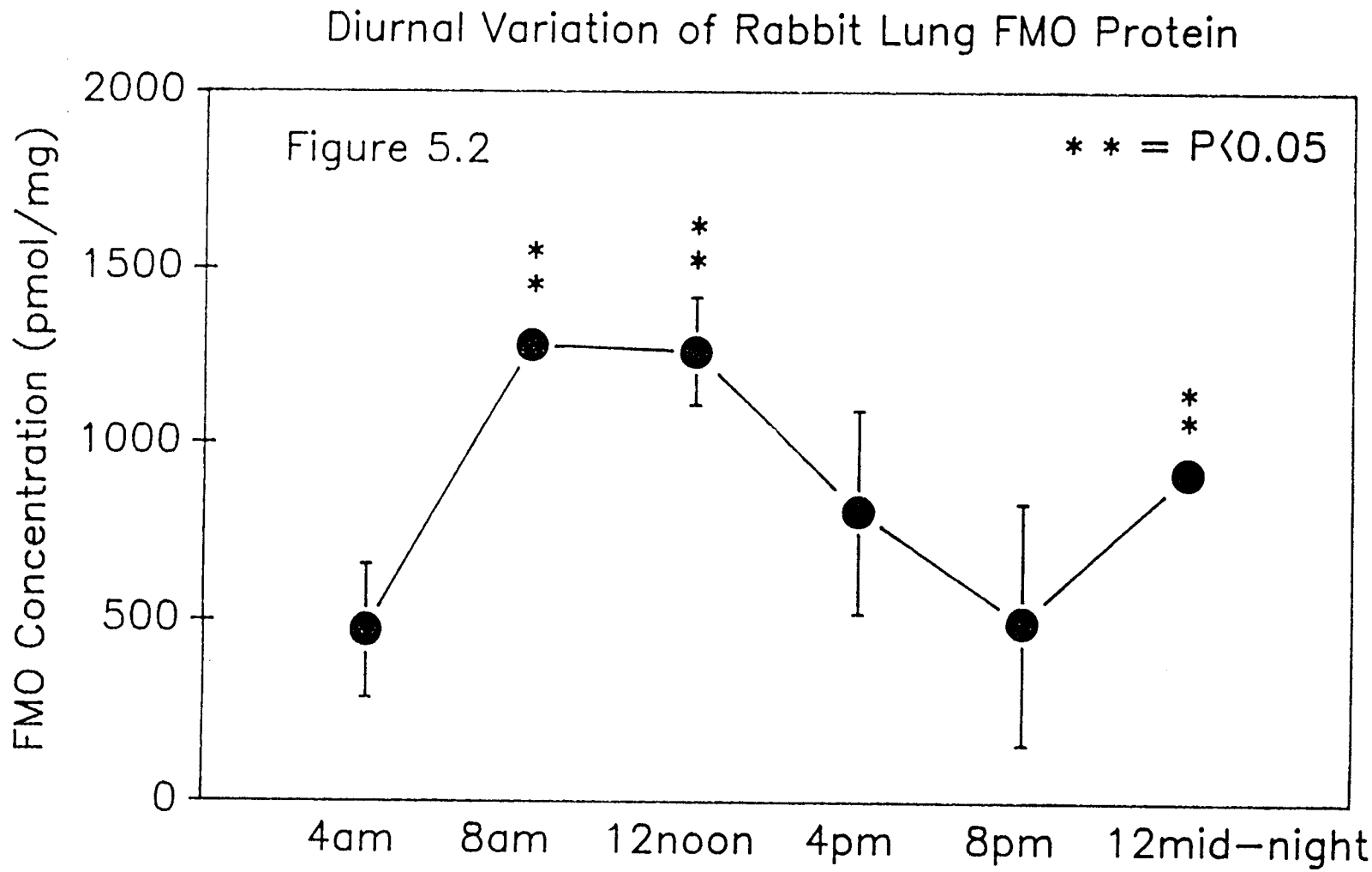

Diurnal Variation of Rabbit Lung FMO Protein
Figure 5.2
* * = P<0.05
FMO Concentration (pmol/mg)
2000
1500
1000
500
0
4am
8am
12noon
4pm
8pm
12mid-night

Figure 5.3. Northern blot analysis of rabbit lung total RNA from 4 am to 12 midnight. RNA was probed with ^{32}P-labelled rabbit lung FMO cDNA. lane 1, 4 am; lane 2, 8 am; lane 3, 12 noon; lane 4, 4 pm; lane 5, 8 pm; lane 6, 12 midnight. Each lane contained 20 µg total RNA. The blot was subsequently reprobed with a ^{32}P-labeled human fibroblast β-actin cDNA to assess the relative amounts of RNA analyzed in each sample.

Figure 5.3.

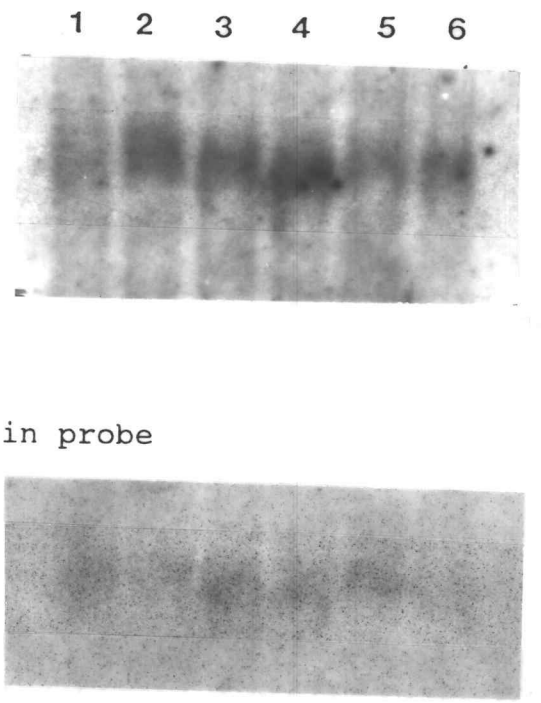


Figure 5.4. Rabbit lung FMO mRNA expression determined with rabbit lung FMO cDNA from 4 am to 12 midnight. The graph was produced using densitometry from northern blots in figure 5.3.

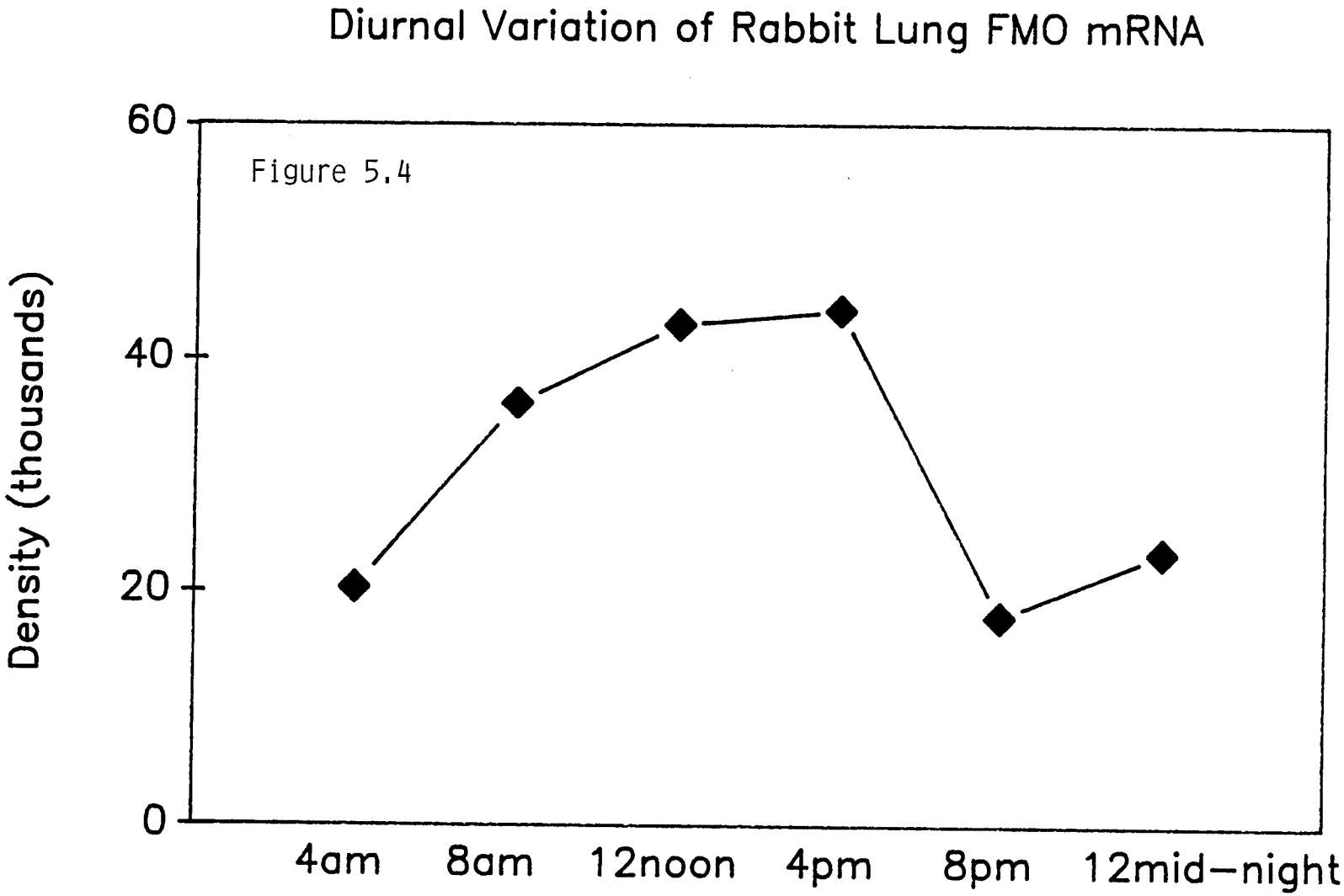

Diurnal Variation of Rabbit Lung FMO mRNA
Figure 5.4
Density (thousands)
60
40
20
0
4am
8am
12noon
4pm
8pm
12mid-night

Figure 5.5. ^{14}C-N,N-Dimethylaniline N-oxidation by rabbit lung microsomes from 4 am to 12 midnight. Each value represents the mean of 2 assay replications from the same preparations of 2 different animals. * * represents the statistical difference ($P<0.05$) when comparing the value of 8 pm with others.

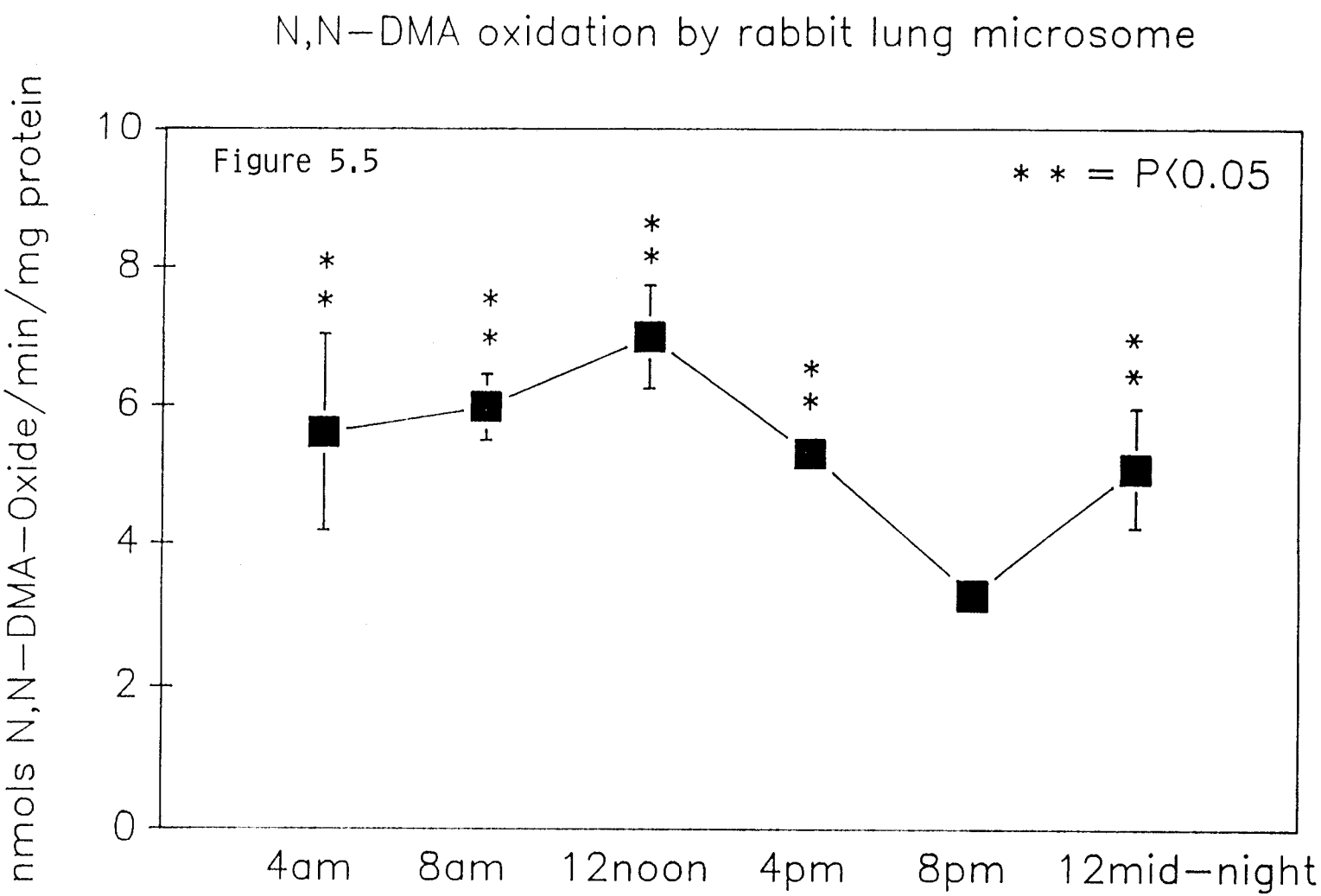

N,N−DMA oxidation by rabbit lung microsome
Figure 5.5
* * = P<0.05
nmols N,N−DMA−Oxide/min/mg protein
10
8
6
4
2
0
4am
8am
12noon
4pm
8pm
12mid−night

Figure 5.6. Methimazole S-oxidation by rabbit lung microsomes from 4 am to 12 midnight. Each value represents the mean of 2 assay replications from the same preparations of 2 different animals.
* * represents the statistical difference ($P<0.05$) when comparing the value of 8 pm with others.

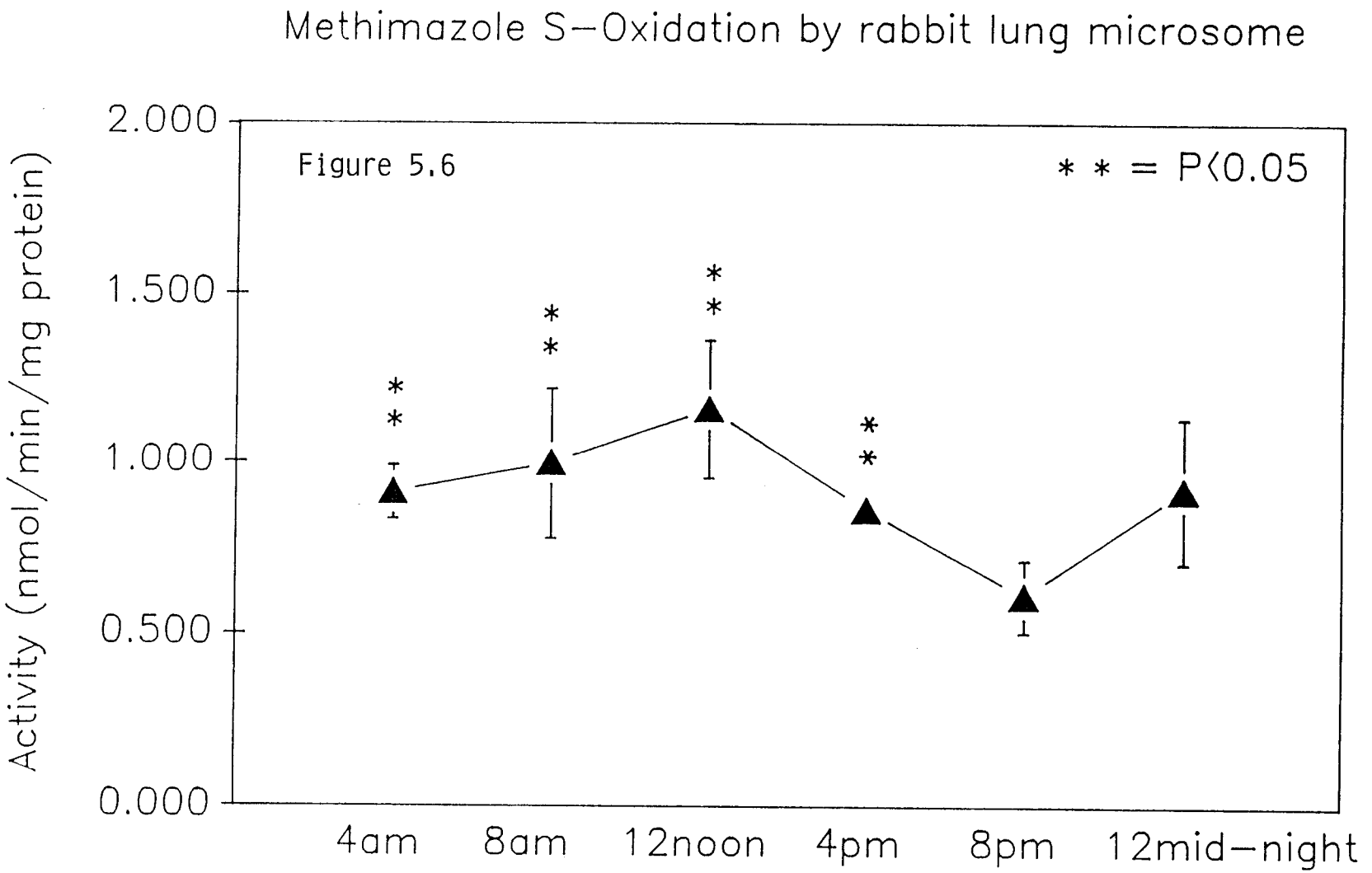

Methimazole S-Oxidation by rabbit lung microsome
Figure 5.6
* * = P<0.05
Activity (nmol/min/mg protein)
2.000
1.500
1.000
0.500
0.000
4am
8am
12noon
4pm
8pm
12mid-night

Figure 5.7. Western blot analysis of rabbit kidney microsomes from 4 am to 12 midnight. Tissues were probed with antibody to rabbit lung FMO. lanes 1-2, 4 am; lanes 3-4, 8 am; lanes 5-6, 12 noon; lanes 7-8, 4 pm; lanes 9-10, 8 pm; lanes 11-12, 12 midnight. Each lane contained 50 µg protein.

Figure 5.7.

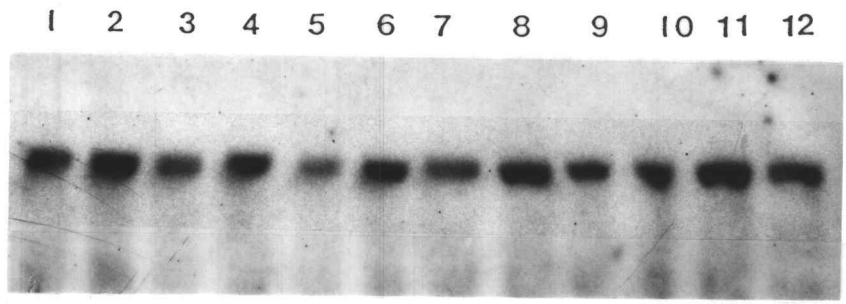


Figure 5.8. Quantitation of FMO in rabbit kidney from 4 am to 12 midnight. The values shown were obtained using densitometry from autoradiograms of western blots probed with antibody to rabbit lung FMO.

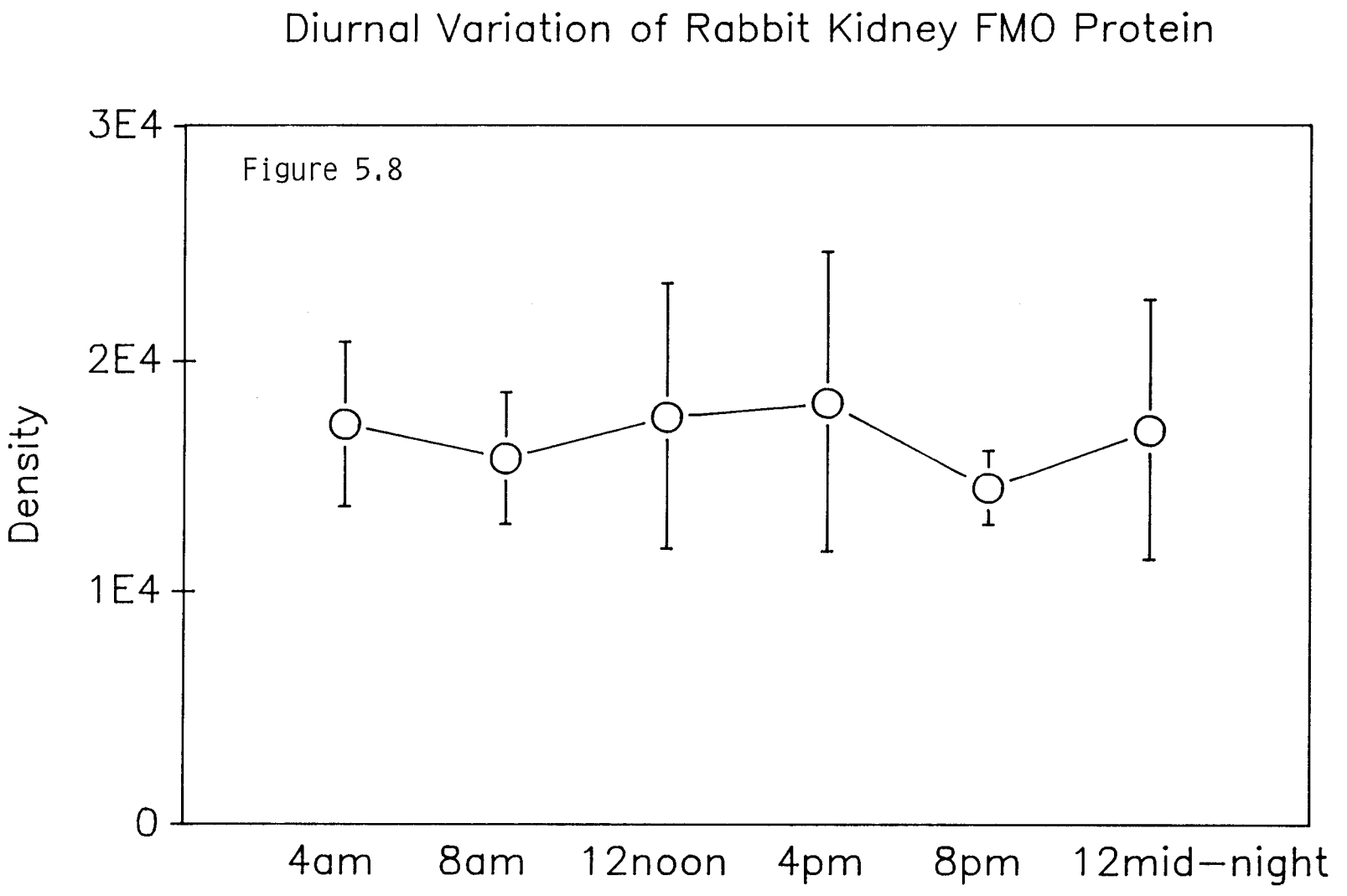

Diurnal Variation of Rabbit Kidney FMO Protein
Figure 5.8
3E4
2E4
1E4
0
Density
4am
8am
12noon
4pm
8pm
12mid-night

Figure 5.9. ^{14}C-N,N-Dimethylaniline N-oxidation by rabbit kidney microsomes from 4 am to 12 midnight. Each value represents the mean of 2 assays of different animals.

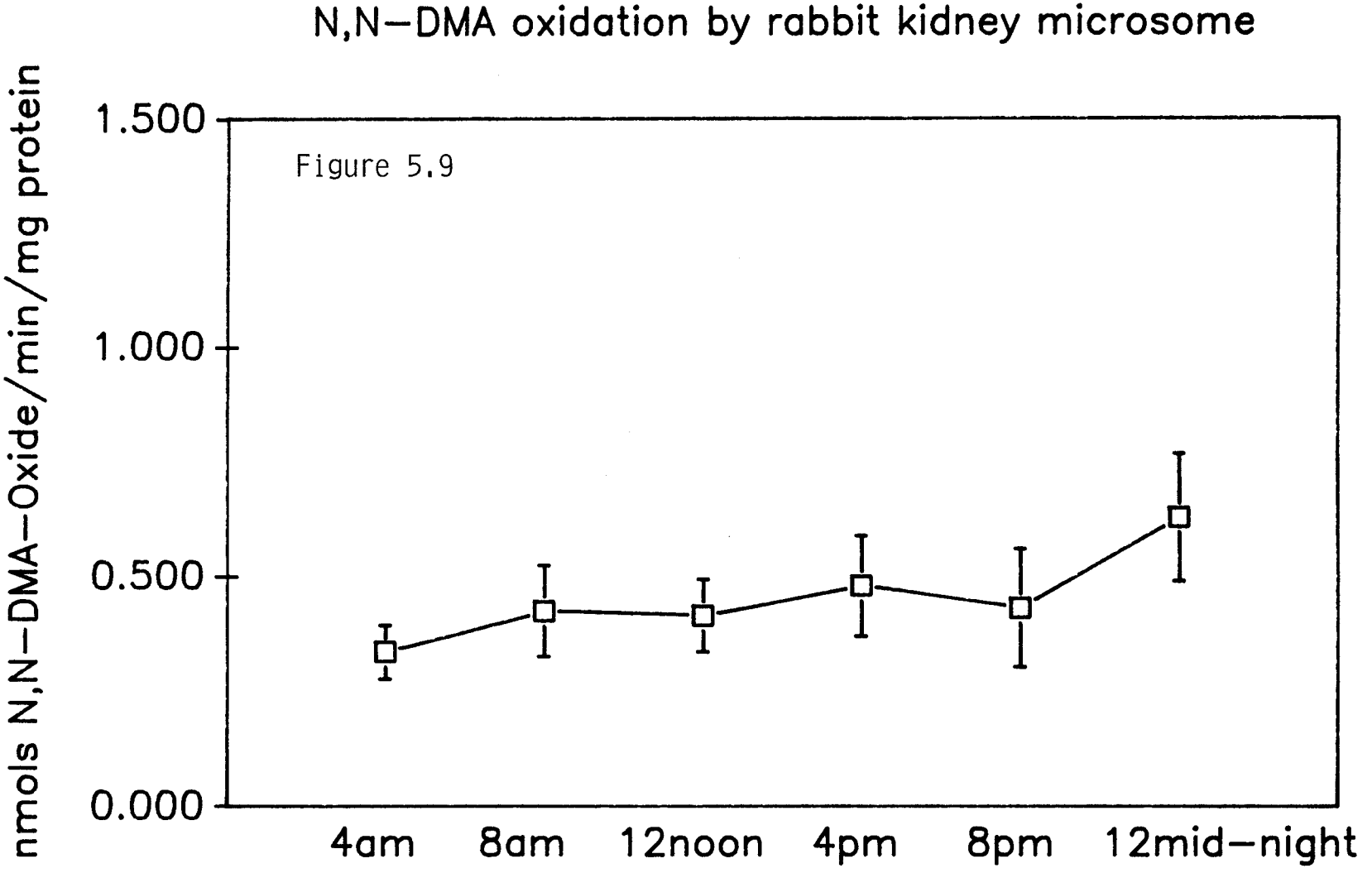
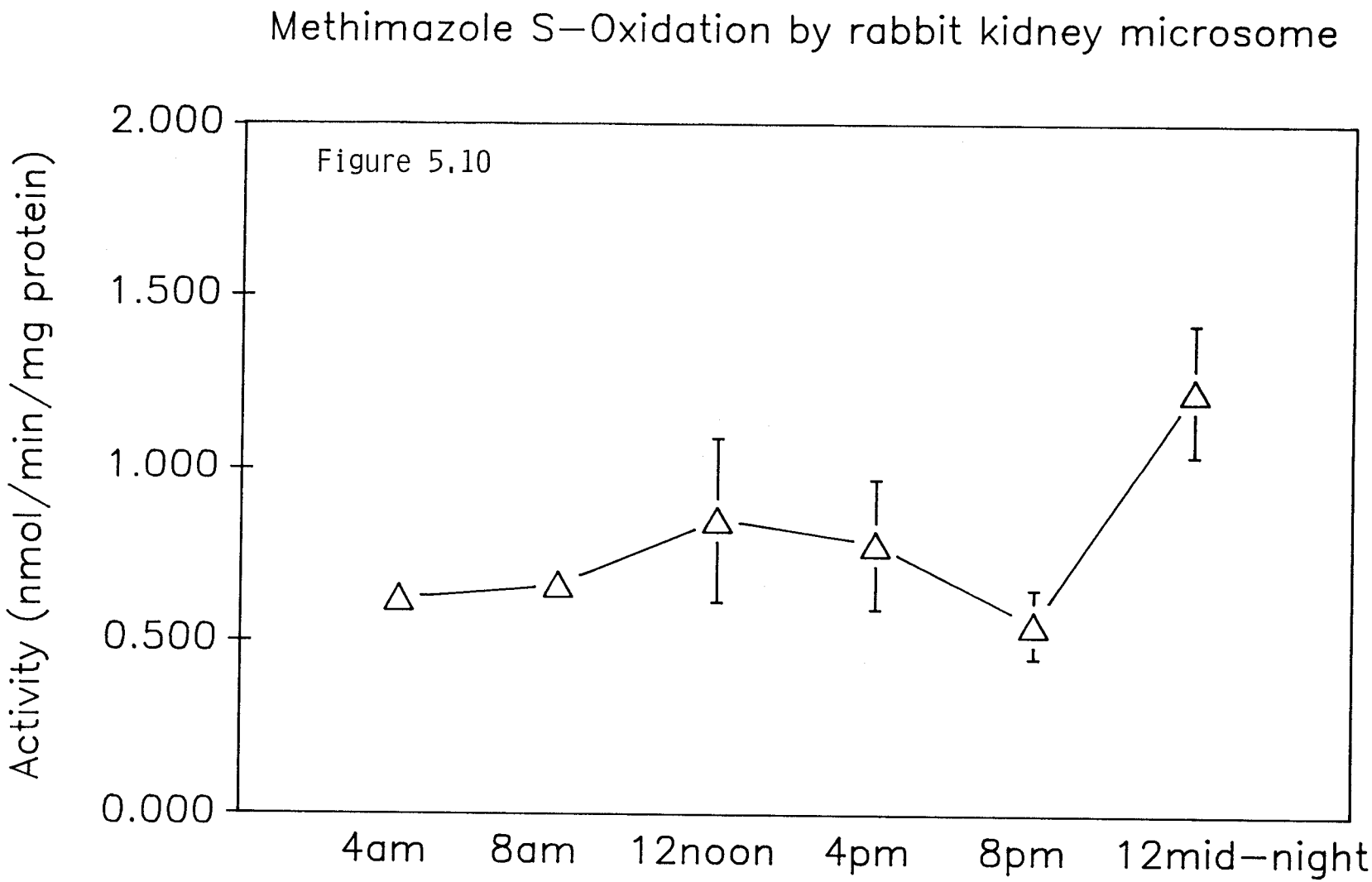

N,N−DMA oxidation by rabbit kidney microsome
Figure 5.9
nmols N,N−DMA−Oxide/min/mg protein
1.500
1.000
0.500
0.000
4am
8am
12noon
4pm
8pm
12mid−night

Figure 5.10. Methimazole S-oxidation by rabbit kidney microsomes from 4am to 12 midnight. Each value represents the mean of 2 assays of different animals.


Methimazole S—Oxidation by rabbit kidney microsome
Figure 5.10
Activity (nmol/min/mg protein)
2.000
1.500
1.000
0.500
0.000
4am
8am
12noon
4pm
8pm
12mid—night

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CONCLUSIONS

Rabbit lung FMO is catalytically and immunochemically distinct from rabbit liver FMO. Distinct hepatic and pulmonary forms of FMO are also present in a number of other species including the rat, mouse, hamster, guinea pig, sheep and human (17, 54, 56, 59). This extrahepatic form of FMO is also present in rabbit kidney and bladder. Rabbit FMOs in liver, lung, and kidney are regulated developmentally by age, sex and pregnancy. The relatively high levels of rabbit FMO in fetal and neonatal lung and liver are unusual for monooxygenases and may play a significant role in metabolism of drugs or exogenous chemicals crossing the placenta or administered through lactation. In addition to developmental regulation, rabbit FMO may be modulated rapidly, for example diurnally or by hormonal stimulation. The studies of the hormone receptor binding and the molecular mechanism of the developmental regulation of FMO in the rabbit and other species would be necessary for better understanding of hormones that regulate certain gene(s) involved.

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