IN VITRO METABOLISM OF DRUG SUBSTRATES BY LIVER MICROSOMAL ENZYMES FROM MALE, PREGNANT AND NONPREGNANT FEMALE RATS

by

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ABBREVIATIONS

AMP	adenosine phosphate
ATP	adenosine triphosphate
cpm	counts per minute
dpm	disintegrations per minute
DPH	diphenylhydantoin
pHPPH	5 (p-hydroxyphenyl) 5 phenyl hydantoin
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
3MC	3 methylcholanthrene
mRNA	messenger ribonucleic acid
mμmole	millimicromole
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
SKF 525-A	2 diethylaminoethyl 2,2 diphenylvalerate hydrochloride
9000 xg S	9000 xg supernatant
SER	smooth endoplasmic reticulum
RER	rough endoplasmic reticulum
TLC	thin layer chromatography
GLC	gas-liquid chromatography

INTRODUCTION

A. INTRODUCTORY REMARKS

1. Drugs and Pregnancy

The thalidomide tragedy of the early 1960's awakened a new interest in the possibility that drugs, taken during pregnancy, could cause birth defects. In this case, a clear cut connection was shown between the ingestion of a seemingly harmless, non-toxic tranquilizer during the first trimester of pregnancy, and the ten thousand infants, world-wide, born with phocomelia (1).

Although maternal and fetal circulatory systems are not directly connected, transfer of materials between mother and fetus takes place within the placenta. Drugs administered to the mother do not affect the fetus unless they cross the placenta. Lipid solubility enhances transplacental passage (2).

Mirkin has shown that diphenylhydantoin (DPH), a highly lipid soluble anti-convulsant, can cross the placenta in humans, rats and mice (3). The offspring of epileptic mothers on anticonvulsant drugs are reported to have a higher incidence of cleft lip, cleft palate (3, 4) and hemorrhagic diseases (5) than the newborn of a similar population not taking drugs. Studies in mice implicated DPH itself, rather than its metabolites, as the probable cause of toxicity and teratogenicity (6). Since DPH is a drug whose use might be mandatory throughout pregnancy for the protection of both mother and unborn child, evidence of its possible harmful effects is of therapeutic significance.

The duration of action of lipid soluble drugs depends to a great extent on the rate at which these drugs can be biotransformed to more

polar compounds and conjugated, prior to their excretion from the body (7). The rate of biotransformation of drugs during pregnancy becomes a matter of central importance. One is led to ask the question: what, then, is the effect of pregnancy itself on drug metabolism?

2. The Effect of Pregnancy on Drug Metabolism

A search of the literature revealed a surprising lack of information on the effect of pregnancy on drug metabolism. Studies in humans and animals inferred a decreased in vivo drug metabolism on the basis of indirect evidence. The work of Migeon et al (8) with injected ^{14}C-cortisol in pregnant women in late term, showed a decreased rate of appearance of cortisol metabolites in plasma and urine. Crawford and Rudofsky (9) compared the urinary excretion patterns of pregnant and non-pregnant women patients who were administered meperidine and promazine. The pregnant women excreted more of the unchanged drug and less of the metabolites, suggesting a decreased or impaired drug biotransformation. King and Becker (10) found an increase in the sleeping time produced by pentobarbital when they compared the effect of that drug in rats in late pregnancy with nonpregnant controls. Gabler and Hubbard (11) reported an increase in the half-life of DPH disappearance from the plasma of near-term pregnant rhesus monkeys and a change in the ratio of 5 (p-hydroxy phenyl) 5 phenyl hydantoin (pHPPH), the monohydroxylated metabolite, to the dihydrodiol derivative of DPH in the urine.

These in vivo findings of a diminished or impaired drug metabolism during pregnancy seemed to be confirmed by the few in vitro studies that have been reported. Creaven and Parke (12) called attention to the diminished metabolism of coumarin and biphenyl in the 10,000 xg hepatic

supernatant from pregnant chinchilla rabbits and of biphenyl in the same liver fraction from pregnant rats. Neale and Parke (13) equated the decreased biphenyl hydroxylase activity of liver microsomal preparations from pregnant rats compared to nonpregnant controls with the decrease in their cytochrome P-450 content. Guarino et al (14) found a decrease in V_{max} for both aniline hydroxylase and ethylmorphine demethylase in hepatic microsomes from 20-day pregnant rats, compared to nonpregnant females. When these data were expressed in terms of the cytochrome P-450 concentration, the differences were equalized, leading the authors to conclude that the decrease in enzyme activity was related to the decrease in cytochrome P-450 concentration.

Our in vitro study will attempt to verify or refute the postulate of a decreased or impaired drug biotransformation during pregnancy. Before outlining our approach to the problem, we will sketch the background against which its solution is set. What follows is a brief historical survey of the discovery and characterization of the enzymes of the endoplasmic reticulum. The ways in which these drug metabolizing enzymes seem to be regulated or controlled form the central theme of this review. Factors known to modify drug metabolism will be discussed (7).

B. MICROSOMAL FRACTION OF LIVER HOMOGENATES

1. History

The microsomal fraction of liver homogenates contains the enzymes which are largely responsible for the biotransformation of drugs (15). Work began on the isolation and characterization of liver microsomes with

the pioneering experiments of Albert Claude (16) in the late 1930's. Using the then newly developed method of differential high speed centrifugation, he separated a slowly sedimenting fraction of hepatocytes. Claude named this fraction, composed of lipid (mostly phospholipids) and protein in association with ribonucleic acid and hemin, "microsomes" or little bodies.

Development of the electron microscope made possible the visualization of the network of membranes of the endoplasmic reticulum. The work of Palade and his associates, Siekevitz, Dallner, Omura (17, 18, 19) has done much to elucidate the fine morphology, chemistry and biosynthesis of this organelle.

2. Microsomal Enzymes

The enzymes of the electron transport chains (20) are bound to the membranes of the endoplasmic reticulum. These enzymes are involved in the biotransformation of drugs (21, 22), carcinogens (23), insecticides (24) and steroids (25, 26). Mason gave the name of xenobiotics to those compounds normally "foreign to the metabolic network of an organism" (27) and characterized their hydroxylation as a mixed-function oxidation (28). Mixed-function oxidases catalyze reactions of the general type $AH + 2e + O_2 \longrightarrow AOH + O_2^{--}$. These enzymes catalyze the consumption of one molecule of O_2 per molecule of substrate. They have two interdependent catalytic activities, the reduction of one atom of an oxygen molecule coupled to the specific oxygenation or hydroxylation of a substrate with the other atom. For this they require both oxygen acceptors and 2-electron donors.

In 1957, 1958, Gillette, Brodie and La Du (29, 30) demonstrated that the liver microsomal enzyme system requiring NADPH and molecular oxygen

for activity was a common pathway for the biotransformation or hydroxylation of a number of drugs. In 1963, Estabrook et al (31) detected a peculiar cytochrome in the microsomes of the adrenal cortex that was able to hydroxylate progesterone at position C-21. Subsequent work by Omura and Sato (32, 33) and Omura, Sato, Cooper, Rosenthal and Estabrook (34) revealed that this cytochrome was the same as the pigment found many years earlier in liver microsomes by both Klingenberg (35) and Garfinkel (36). Omura et al (34) named this pigment cytochrome P-450 because of the distinct peak formed at 450 mμ when it was treated with CO and sodium dithionite.

Microsomal enzymes have been the object of intensive study and the subject of several symposia (37, 38, 39). The split beam spectrophotometer and the techniques developed by Chance (40) using the difference spectrum, have proved to be invaluable tools in the study of the cytochrome pigments. Cytochrome P-450 has not been isolated in its native form since it is tightly bound to the membranes of the endoplasmic reticulum and denatures easily. It is recognized and usually measured by the magnitude of the spectral change between 450 mμ and 490 mμ in the difference spectrum upon treatment with CO and sodium dithionite (32). Another cytochrome pigment, b_5, has been isolated from the microsomal fraction and purified (41). It is characterized and measured by the magnitude of spectral change in the difference spectrum between 425 mμ and 410 mμ upon reduction with NADPH.

In addition to the cytochromes, the microsomes contain a variety of flavoproteins. The flavoprotein called NADH-cytochrome b_5 reductase has been isolated and purified by Strittmatter (42). A second flavo-

protein, NADPH-cytochrome c reductase was originally isolated from liver by Horecker (43) and has been studied in detail by Williams and Kamin (44).

3. Electron Transport Schemes

Several schemes have been proposed for the electron transport chains which participate in the mixed function oxidations of the endoplasmic reticulum. According to Remmer (15) and Schenkman (45), the reaction takes place as follows (Figure 1): NADPH functions exclusively as the electron donor. A FAD-containing flavoprotein, known either as NADPH-cytochrome c reductase or NADPH-cytochrome P-450 reductase, transfers the reducing equivalents to the terminal oxidase, cytochrome P-450. The reduced heme of the P-450 is now able to bind one molecule of oxygen, activated, presumably, by picking up one electron from the heme-iron. The substrate to be oxidized interacts with the ferric form of the hemoprotein. How the second electron comes into play and how one atom of oxygen interacts with the substrate is not yet definitely known. Cytochrome P-450 can be reduced enzymatically without binding substrate.

A scheme proposed by Hildebrandt and Estabrook (46), which is a modification of an earlier scheme by Estabrook and Cohen (47), differed from the above in one important respect. It proposed that cytochrome b_5 mediates the transfer of the second electron to the cytochrome P-450-substrate-0_2 complex and that this electron can come from either NADH or NADPH. Mason (recent seminar) finds FMN as well as FAD in the flavoprotein enzyme, NADPH-cytochrome P-450 reductase.

4. Distribution of Microsomal Enzymes

There is still some disagreement about the distribution of the

Figure 1: Scheme of electron transport from NADPH to cytochrome P-450 from Remmer (15), Schenkman (45).

Substrate (SH) to be oxidized interacts with the ferri form of cytochrome P-450. NADPH functions as an electron donor. Electrons are picked up by a reduced FAD containing flavoprotein known as NADPH-cytochrome c reductase. Electron flows from reduced flavin enzyme to cytochrome P-450. Reduced heme of cytochrome P-450 can now bind a molecule of oxygen which is "activated." The nature of this activated oxygen and the manner in which the second electron necessary for mixed-function oxidation comes into play are unknown. At the end of the cycle the hemoprotein is again in the oxidized (ferri) form, substrate has been hydroxylated to SOH.

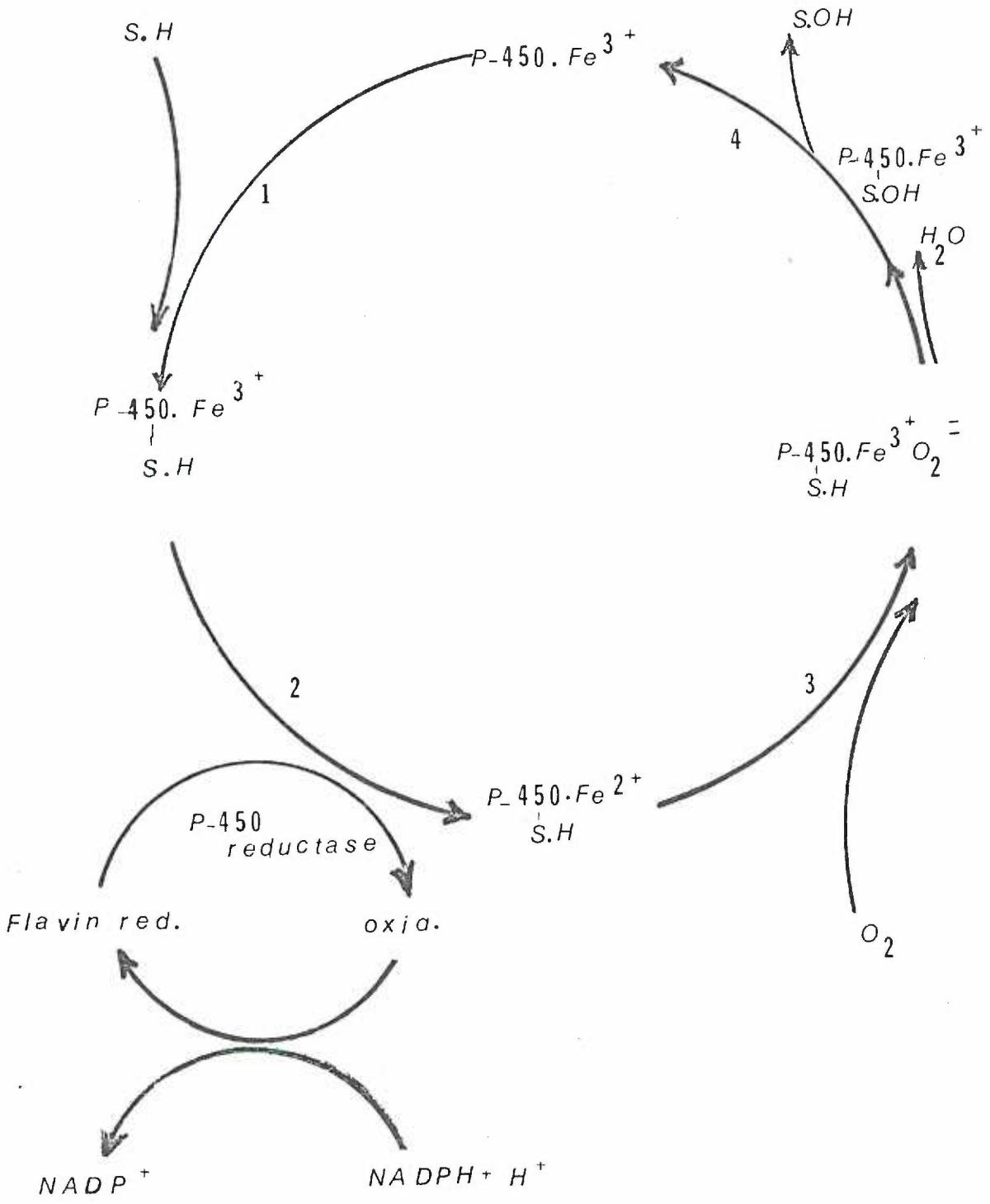
CYTOCHROME P-450
S.H
P-450. Fe 3+
1
P-450. Fe 3+
S.H
2
P-450·Fe 2+
S.H
3
O2
P-450.Fe 3+ O2
S.H
4
S.OH
P-450.Fe 3+
S.OH
H2O
P-450
reductase
Flavin red.
oxia.
NADP+
NADPH+ H+

FIGURE 1

microsomal enzymes in the endoplasmic reticulum. The membranes have been subfractionated, according to methods developed by Dallner (48) or Rothschild (49), into rough and smooth subfractions. The enzyme activities of both fractions have been tested with many substrates. Fouts and Gram (50) reported that the NADPH-requiring enzymes appear to be more concentrated in the smooth membranes than in the rough. They cautioned that such comparisons must take into account the pathway studied, the source of NADPH, the method of subfractionation and the species of animal tested.

Orrenius and Ernster (51) have studied the distribution of aminopyrine demethylase activity in rough and smooth subfractions of phenobarbital-induced rat liver microsomes. On the basis of equal aminopyrine demethylase activity in both subfractions twenty-four hours after phenobarbital injection, these authors concluded that this enzyme was homogeneously distributed in the endoplasmic reticulum.

Later induction experiments reported by Ernster and Orrenius (52) followed the time course of enzyme induction with phenobarbital in the rough endoplasmic reticulum (RER) and the smooth endoplasmic reticulum (SER), by measuring aminopyrine demethylase and NADPH-cytochrome c reductase activities and cytochrome P-450 content. Enzyme activities increased rapidly and reached a maximum in the RER at six hours after phenobarbital injection. At that time activities began to increase in the SER and they had exceeded the levels found in the RER after twelve to twenty-four hours.

These data corroborated the developmental studies of Dallner et al (18), where electron micrographs seemed to show that enzyme synthesis took place in the rough membranes and that these became smooth when

synthesis was completed. The biochemical data showing an overall increase in enzyme activity in the SER after several phenobarbital injections (52) were consistent with the morphological findings of Remmer and Merker (53). Their electron micrographs showed an increase in the membranes of the SER after phenobarbital treatment.

C. CONTROL OF THE DRUG METABOLIZING ENZYMES

1. Induction

Numerous studies have dealt with the enzyme systems of the endoplasmic reticulum because of their important role in the biotransformation of drugs and other foreign substances. According to Conney (54), these enzymes can be induced by a number of chemicals such as drugs, insecticides and carcinogens, compounds with little in common except their lipid solubility at physiological pH. Conney defines enzyme induction as "increased concentration of enzyme protein." This can be due to an increased rate of protein synthesis, to stabilization of the protein (decreased rate of protein degradation), or to a combination of these two. Unlike prokaryotic cells, control of protein synthesis in animal cells depends on more than the synthesis or nonsynthesis of specific m-RNA. Gene-amplification, rapid intracellular RNA turnover, a variably stable messenger RNA could all play a regulatory role (55). The mechanism of action of drugs, insecticides and carcinogens in their "induction" of microsomal enzymes is presently being defined.

As early as 1954, Brown, Miller and Miller (24) observed the stimulatory effect of foreign compounds on the activity of liver microsomal enzymes. Conney et al (56) showed that small amounts of polycyclic

hydrocarbons like 3 methylcholanthrene (3MC) induced a several-fold increase in the activities of liver microsomal enzymes and that the increase seemed to be due to an increase in the synthesis of enzyme protein. The stimulatory effect of barbiturates on the drug metabolizing enzymes was first reported by Remmer (57) while studying the mechanisms of barbiturate tolerance. Later he and Merker were able to show that these drugs caused an increase in smooth membranes of the endoplasmic reticulum (53).

a. Protein synthesis

Several workers have reported that increased drug metabolism after phenobarbital is related to increased protein synthesis. Kato et al (58) prevented this increase by treatment with dl-ethionine which blocks protein synthesis by decreasing ATP levels in the liver (59). They also demonstrated that the increase in drug metabolizing activity was in the sedimentable microsomal fraction and that it was not due to some soluble inhibitor or activator in the supernatant fraction. Hart et al (60) found that pretreatment with phenobarbital enabled newborn rabbits to metabolize hexobarbital, aminopyrine and p-nitrobenzoic acid, while untreated animals had very little or no drug metabolizing ability.

Elegant incorporation studies of Arias, Doyle and Schimke (61) showed that hepatic microsomes have a rapid and heterogeneous rate of enzyme turnover. In their studies they used ^{14}C-guanidino-arginine, which is not reutilized because of the high arginase activity in liver. They were able to show that increased steady state levels of drug metabolizing enzymes after phenobarbital pretreatment were due to increased enzyme synthesis rather than a decrease in the rate of turnover.

Omura et al (62) showed that phenobarbital exerted its effect on the turnover of microsomal enzymes in two ways. First, there was the specific stimulation of synthesis of NADPH-cytochrome c reductase and cytochrome P-450. Secondly, there was a relatively nonspecific reduction in degradation of many kinds of microsomal proteins.

b. Differences between phenobarbital and 3 methylcholanthrene induction

Recent studies have investigated the difference in induction between phenobarbital and 3 MC. Mannering et al (63) reported that a different pigment, which they named P_1-450, was synthesized upon induction with 3 MC rather than by phenobarbital. They argued that the induction process with 3 MC was different from that of phenobarbital since thioacetamide, which blocked phenobarbital induction, had little or no effect on 3 MC induction. Hildebrandt and Estabrook (64), on the basis of spectral studies, claimed that there were two types of CO-binding pigment in liver microsomes: P-450, induced by phenobarbital and P-446, induced by 3 MC. These cytochromes differed not only in their absorption maxima upon treatment with CO and sodium dithionite, but also in their extinction coefficients. Kuntzman et al (65) agreed that 3 MC induced a hemoprotein different from that induced by phenobarbital. Induction with 3 MC not only produced an increase in the rate of benzpyrene hydroxylation, but also altered the kinetics of the reaction. This implied a change in the enzyme itself as well as a change in the amount of enzyme.

2. Genetic Factors in Control

Gillette (7) has pointed out that the activity of the drug metabolizing enzymes of microsomes varies among animal species, strains and individuals. This implies that genetic factors influence drug metaboliz-

ing ability. Vessell and Page (66) compared the metabolism of antipyrine and phenylbutazone in identical and fraternal twins. The rates of antipyrine disappearance from the plasma of fraternal twins varied more than these rates in identical twins. Phenylbutazone disappearance rates showed no consistent variation. From this they inferred genetic control in the metabolism of antipyrine. Furner et al (67) found that the differences in metabolism of various drug substrates due to age, sex and drug pretreatment were not parallel in any of four strains of rats (Wistar, Sprague Dawley, Holtzman, Long Evans). This also implied a genetic component in addition to the other factors controlling drug metabolism. Conney et al (68) reported species differences in the induction of microsomal enzymes with phenobarbital. In the rat, phenobarbital had a small stimulatory effect, but in the rabbit, the stimulatory effect was much greater. Inhibition studies with 2-diethylaminoethyl 2,2 diphenylvalerate hydrochloride (SKF 525-A) reported by Kato et al (69) showed marked differences in inhibition kinetics in rats, mice and rabbits with aniline, aminopyrine, hexobarbital and p-nitroanisole.

3. Hormonal Control

Sex, age, stress and extirpation of various endocrine glands (7) affect the capacity of the liver to metabolize drugs, indicating that hormones may also influence drug metabolism. Hormones represent many classes of compounds (phenols, peptides, proteins and steroids), but their common physiologic role is the regulation and coordination of body functions. Two general biochemical mechanisms have recently emerged to explain how hormones regulate these processes (70). The first, illustrated by epinephrine and by many peptide hormones, involves action on

a pre-existing enzyme system. The hormone activates the membrane bound adenyl cyclase, which in turn converts ATP to cyclic AMP (71). Cyclic AMP then acts as a "second messenger", either activating or repressing the enzyme pathway in question. The second pattern of hormonal action seems to apply generally to the steroid hormones (72). The hormone enters the cell and binds to an extranuclear receptor protein, characteristic of the target cell. The steroid-protein complex migrates to the nucleus where it initiates or accelerates specific RNA synthesis. This sets in motion those events which lead eventually to the specific action of the hormone (e.g., estrogenic hormone stimulates uterine growth).

According to Conney (54) "androgens, estrogens, progestational steroids, glucocorticoids, anabolic steroids, norepinephrine, insulin and thyroid hormone influence drug action by altering the activity of the drug metabolizing enzymes." It is not known at present if hormones alter the activity of these enzymes at a primary level, as discussed above, or if they act on the enzymes themselves as inhibitors or effectors of enzyme action. There is evidence that the same microsomal enzyme systems are involved in the metabolism of both drugs and steroid hormones (73). In this case steroid hormones might compete for available drug metabolizing enzymes. In vitro data by Tephly and Mannering (74) and Juchau and Fouts (75) seem to confirm this premise.

a. Sex differences in drug metabolism

Although there is no substantial evidence of sex difference in the drug metabolizing ability of humans, a number of papers have reported such differences in rats and mice (76, 77). Several drug substrates are more actively metabolized in the adult male rat than the adult female,

while the opposite is often the case in mice. Relatively little work has been done with respect to other species. However, on the basis of existing data, there seem to be no sex-related differences in the drug metabolizing ability of guinea pigs, rabbits, cats and dogs (76). Murphy and DuBois (78) showed that the low rate of conversion of guthion to an anticholinesterase in adult female and young male rats was increased by administration of methyl testosterone. High conversion activity was decreased by castration. They attributed the stimulation and maintenance of high enzyme concentration to the action of androgens. Axelrod (79) found that methyl testosterone increased the metabolism of narcotic drugs by female rats. Quin et al (76) reported that testosterone increased hexobarbital metabolism in female rats, and estrogen decreased hexobarbital metabolism in male rats.

b. Anabolic steroids

Booth and Gillette (80) showed that anabolic and androgenic steroids enhanced the activity of certain drug metabolizing enzymes. Steroids such as 19-nortestosterone, having relatively low androgenic potency but high anabolic activity, are as effective as methyl testosterone in increasing the metabolism of hexobarbital and monomethyl-4 aminoantipyrine by liver microsomes from female rats. Kato and Gillette (81, 82) reported that a variety of treatments were able to impair the activity of the markedly sex-dependent microsomal enzymes from male rats. Starvation, adrenalectomy, hypoxia, administration of alloxan, thyroxin, epinephrine, ACTH, formalin and morphine all decreased the rates of aminopyrine and hexobarbital metabolism in male rats, but they had little effect in female rats. The metabolism of both aniline and zoxazolamine

which ordinarily shows no sex difference, was either not affected, or slightly increased in both male and female rats. Since these procedures also decreased the metabolism of hexobarbital and aminopyrine in castrated rats receiving methyl testosterone, their impairing effects could not be due to decreased synthesis or release of androgenic hormone. According to the authors, they were due, possibly, to decreased effectiveness of anabolic steroids in stimulating these microsomal enzymes.

c. Pituitary-adrenal control

Several findings suggested that the pituitary-adrenal system plays a regulatory role in drug metabolism. Adrenalectomy caused a decrease in the activity of rat liver microsomes which oxidize substrates such as hexobarbital, aminopyrine (82) and ethylmorphine (83) and the impairment was greater in males than females (82, 83). Castro et al (83) found that the decreased metabolism in males was associated with a reduction in the NADPH-cytochrome c reductase concentration, but little change was noted in the cytochrome P-450 concentration. Administration of cortisone reversed these effects. Stress, produced in the rat by hindleg ligation for 2½ hours, caused a shortened response to hexobarbital, pentobarbital, meprobamate and zoxazolamine (84). These effects did not occur in hypophysectomized or adrenalectomized animals.

D. SPECTRAL BINDING[1]

In 1963, Narasimhulu et al (85) observed that addition of substrate to adrenal cortex microsomal suspensions caused an alteration of the

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1. The term spectral binding refers to the interaction of substrate with microsomal suspensions, causing a change in the difference spectrum. An enzyme-substrate complex is formed, but it is not known at present if this interaction is the same as the one which takes place during enzymic activity.

difference spectrum. Remmer et al (86), using rat liver microsomes and Imai and Sato (87), using rabbit liver microsomes found that the addition of various substrates to the microsomal suspensions caused two types of spectral changes in the difference spectra. These changes were termed type I and type II (88).

1. Type I and Type II Compounds

Type I spectral change is characterized by a trough in the difference spectrum at 420 mμ and a peak at approximately 390 mμ. Type I compounds include such substrates as hexobarbital, aminopyrine, DPH, chlorpromazine, SKF 525-A, testosterone and estradiol. Type II spectral change is characterized by a trough in the difference spectrum at approximately 392 mμ and a peak between 425 mμ and 435 mμ. With the exception of aniline, compounds causing type II spectral change, e.g., nicotinamide, pyridine, nicotine, are not substrates of the mixed-function oxidases.

2. Spectral Binding and Sex Difference in Drug Metabolism

Studies of spectral change with different substrates have provided useful information about the microsomal enzymes. They have helped illucidate the nature of the sex difference noted in these enzymes in rats. Schenkman et al (89) concluded, on the basis of studies with hexobarbital, aminopyrine and aniline, that the higher rate of oxidation of hexobarbital (and aminopyrine) in male rats was due to the greater maximal binding of substrate and not to the higher content of cytochrome P-450.

Kato et al (90) found that adrenalectomized or morphine-treated male rats showed decreased hexobarbital hydroxylation and decreased

maximal binding of that substrate, while aniline binding and hydroxylation were not affected. Female rats, rabbits and mice of either sex did not show these effects. These authors concluded, with Schenkman, that the decrease in hexobarbital hydroxylation in treated male rats was related to the decrease in the maximal binding of that substrate to the microsomal suspensions. Furthermore, these treatments impaired the action of androgen and prevented the increase in hexobarbital binding in males.

3. Cytochrome P-450--One Enzyme or Many?

The question of whether there is one relatively non-specific cytochrome P-450, or a family of closely related, more specific enzymes, is still not definitely answered. The stereospecificity of certain hydroxylations and the varying inducibility of what seem to be different forms of cytochrome P-450, seem to confirm the latter view.

A case can still be made for one cytochrome P-450 with several binding sites. Schenkman et al (88) suggested that type II spectral change was due to interaction of substrate with iron at the CO-binding site of the heme. Schenkman and Sato (91) suggested that type I spectral change was caused by substrate interaction with the apoenzyme, causing an increase in the electronegativity or polarity of the sixth ligand[2] of the heme. Studies by Hildebrandt et al (92) showing that P-450 and P-446 were interconvertible by addition of substrate, have strengthened the hypothesis that there is only a single type of cytochrome, reacting differentially with a variety of substrates.

2. "The ligand of the microsomal heme which interacts with oxygen and CO has arbitrarily been designated as the sixth ligand for ease in referring to it." Schenkman et al (91).

E. CONCLUDING REMARKS

A great deal of interest has been shown recently in perinatal pharmacology, but little work has been done to determine whether or not pregnancy itself modifies drug metabolism. The reports that do exist infer a decreased in vivo metabolism and the in vitro data seem to corroborate these findings.

We have cited work showing that the liver microsomal enzyme system is the common pathway for the biotransformation of a number of drugs. These enzymes can be induced by a variety of lipid soluble substances, including some drugs. There is evidence that protein synthesis is somehow stimulated and the increased enzyme activity is due mainly to the increased concentration of enzyme.

Other factors seem to influence drug metabolism. Age, species, strain, sex (in rats and mice) all play a role in controlling the activity of these enzymes. The ability of male rats to metabolize certain drugs at a much higher rate than females has been related to the stimulatory action of androgen on the binding of substrate. Spectral binding studies provide a useful probe into the mechanisms involved in these enzymic reactions, but definitive answers await further purification of the components of the system.

With this literature review as background, the problem central to this project will now be outlined.

F. STATEMENT OF THE PROBLEM

The generalized question as to the effect of pregnancy on drug metabolism will be narrowed to focus on liver enzymes known to be related

to the body's capacity to biotransform drugs. The specific question asked is, does pregnancy inhibit the capacity of liver enzymes to metabolize drugs? And, if inhibition is found, what factors might contribute to this reduction in activity?

Three groups of animals will be used as test subjects in this study, male and pregnant and nonpregnant rats. A variety of measurements will be made on each group of animals, including body and liver weights, protein, and cytochrome P-450 and b_5 concentrations of hepatic tissues. Spectral binding constants for type I and II substrates, using microsomal suspensions derived from each group of rats, will be compared. The activities of liver homogenates prepared from the three groups of animals will be determined using several substrates and these activities contrasted. The data derived from the three sets of rats will be compared and statistically evaluated.

The results should either support or refute the original postulate that pregnancy inhibits the capacity of liver enzymes to metabolize drugs.

MATERIALS AND METHODS

A. EXPERIMENTAL ANIMALS AND PREPARATION OF MICROSOMES

Pregnant (primagravida), nonpregnant female and male Sprague Dawley rats, purchased from Simonsen Co., Gilroy, Calif., were used throughout this study. The animals were housed either individually in hanging wire cages, or in pairs in plastic cages with Sani-cel (ground corn cob) bedding. Water and Lab Blox Chow (Allied Mills, Chicago, Ill.) were presented ad libitum. All animals were allowed to acclimate to their new environment for at least seven days prior to use in the study. Pregnant rats were sacrificed in late term, between days 19 and 21 of pregnancy.

Animals were sacrificed by decapitation and the livers were rapidly removed, placed immediately in ice-cold 0.1 M, pH 7.4, phosphate buffer, washed with the buffer, blotted dry and weighed. The livers were minced in 3 volumes of the phosphate buffer and homogenized in an iced glass mortar with a motor driven teflon pestle ($\sim$ 600 rpm). The resulting homogenate (25% w/v) was then centrifuged at 9000 x g for 30 minutes in a Servall RC-2B centrifuge at 3°C, using an SS-34 rotor. The supernatant was removed carefully by aspiration (avoiding the pellet at the bottom and the lipid layer at the top), used the same day or stored at -90°C for use within one month. Preliminary work showed no significant loss of enzyme activity within one month. An aliquot of the 9000 xg supernatant (9000 xg S) was centrifuged at 105,000 xg for one hour at 3°C in a Beckman Model L3-40 ultracentrifuge using a number 40 rotor. The supernatant was poured off carefully and the microsomal pellet which

remained was resuspended by gentle homogenization in either the phosphate buffer, described above, or in 0.1 M Tris containing 0.15 M KCl, pH 7.36. The microsomes were used the same day.

The protein content of both the 9000 xg S and the resuspended microsomal pellet was determined by the method of Lowry et al (93), using bovine serum albumin as standard.

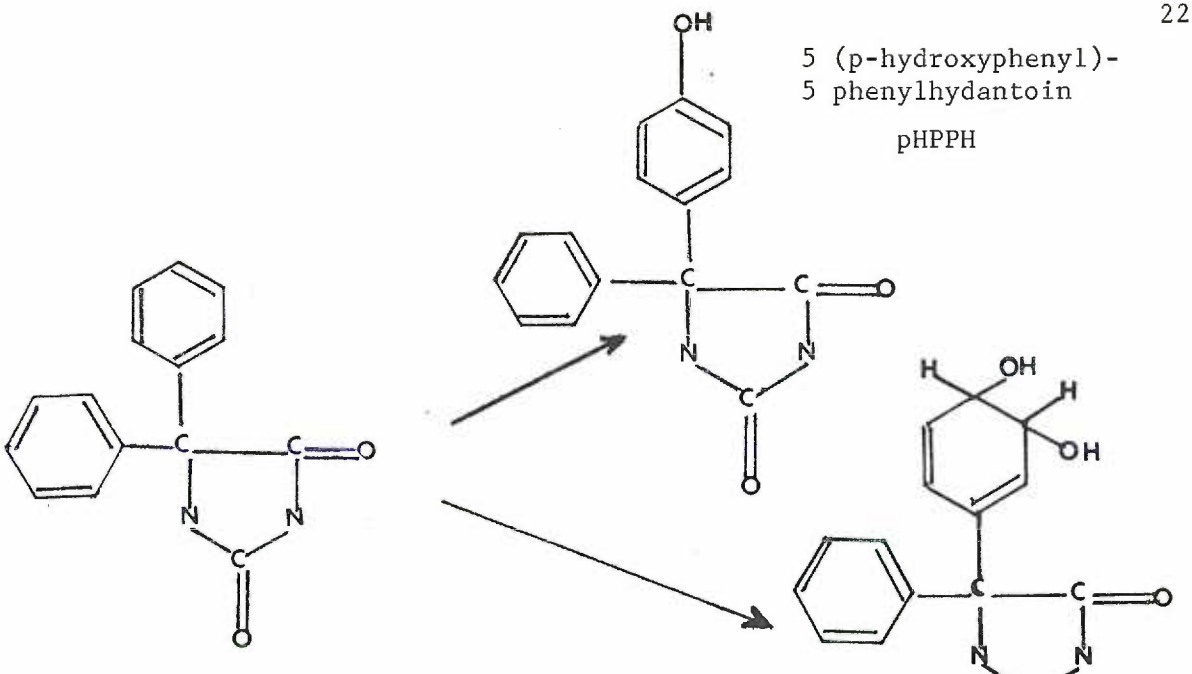
B. DPH METABOLISM

1. Introduction

When the study of DPH metabolism was initiated in our laboratory several years ago, the need for a method which measured the metabolites of DPH as well as the parent compound soon became apparent. The colorimetric method of Dill and co-workers (94) has been widely used for the assay of DPH itself in blood, urine and tissue samples. Their procedure involved a double extraction of DPH into $CHCl_3$, nitration of the phenyl group, reduction to an aromatic amine, diazotization, and coupling with the Bratton & Marshall reagent (95). Gerber and Arnold (96) used a rapid radioactive dilution method to measure the $t_{\frac{1}{2}}$ of DPH disappearance in mice. A similar method was used by Kutt and Verebely (97) in their in vitro studies which measured DPH metabolism by means of substrate disappearance.

2. In Vitro Assay of DPH Metabolism

The method developed in our laboratory (98) measured the two major products formed during the in vitro hydroxylation of DPH. These are the monohydroxylated metabolite (pHPPH) and the dihydrodiol, shown below:



5 (p-hydroxyphenyl)-
5 phenylhydantoin

pHPPH

5,5 diphenylhydantoin

(DPH)

5-(3,4-dihydroxy-
1,5-cyclohexadiene-1-
yl) 5 phenylhydantoin

(dihydrodiol)

from Chang et al (99)

In the DPH assay, 4-^{14}C diphenylhydantoin (New England Nuclear Corp., Boston, Mass., specific activity 4.65 mc/millimole) was used as radioactive substrate, shown to be at least 98% pure by thin layer chromatography (TLC). The 9000 xg S of rat liver homogenates was the source of DPH hydroxylase and an NADPH-generating system was included. Thin layer chromatography, autoradiography and liquid scintillation counting were used to separate, visualize and measure the products formed during the reaction.

When determining DPH hydroxylase activity or kinetic constants, the assay mixture usually contained the following components in a total volume of 3 ml 0.1 M pH 7.4 phosphate buffer: 0.5-1.0 ml 9000 xg S with a protein concentration of 5-12 mg/ml final solution; an NADPH-generating

system consisting of 2.67×10^{-3} M $MgCl_2$, 3×10^{-3} M ATP, 9.3×10^{-4} M NAD, 8.3×10^{-4} M NADP, 3.9×10^{-3} M glucose-6-phosphate and glucose-6-phosphate dehydrogenase at a final concentration of 0.33 units/ml. Radioactive DPH was mixed with cold substrate (kindly supplied by Parke, Davis and Co.) such that the specific activity of the substrate mixture was 2.3×10^5 dpm/μ mole. The concentration of this mixture was varied in the reaction flask between 0.7×10^{-4} M to 5×10^{-4} M. The range of substrate used was limited by its solubility in the reaction mixture.

The NADPH-generating system and the enzyme-substrate mixture (containing a marble for better mixing) were pre-incubated in separate flasks for 5 minutes at 37°C in a Dubnoff metabolic shaker under O_2. The reaction was initiated by the addition of the NADPH-generating system to the enzyme-substrate mixture. After brief mixing, 1 ml of the reaction system was removed for a zero-time sample and added to 3 ml of ice-cold ethyl acetate in a screw top test tube, terminating the reaction. The original reaction flask was stoppered with a one-holed rubber stopper to reduce evaporation, and the incubation continued with shaking and gassing. At the end of 15 minutes another 1 ml aliquot was removed and added to 3 ml of ethyl acetate. The tubes containing the zero-time and the 15-minute samples were shaken for 15 minutes and then centrifuged for 5 minutes to remove protein and break up micelles. The ethyl acetate layer was transferred to another test tube and evaporated to dryness in a hot water bath. This extraction was repeated 3 times, using 3 ml of ethyl acetate each time, and the dried extracts pooled. The combined dried extracts were carefully redissolved in 50 μl of methanol and a 10 μl aliquot of this was spotted on an F-254 silica gel thin layer chroma-

tography plate (Brinkman Co.), which had been activated at 80°C for 30 minutes. The TLC plate was chromatographed in a benzene-methanol-glacial acetic acid solvent system (45:8:4), dried, overlaid with Eastman Kodak BB-54 Medical X-ray film, and stored in a cassette for 2-5 days. The dark spots on the developed film indicated the location of DPH and its two main metabolites. The R_f values for each metabolite and the parent compound corresponded with those of standards. The areas containing the metabolites were marked, scraped and placed in scintillation vials along with 10 ml of Bray's solution (100) and counted in a Nuclear Chicago Liquid Scintillation Counter. Efficiency was calculated by the channels ratio method.

3. Preliminary Studies

a. NADPH-generating system

Several steps in the above procedure required preliminary testing before they were established as routine. The NADPH-generating system was patterned after the one described by Kutt and Verebely (97). The requirement for each of the various components was evaluated in the following experiment.

Fresh or frozen and thawed 9000 xg S was dialyzed at 3°C for 24 hours in 3 changes of 20 times its volume of 0.1 M pH 7.4 phosphate buffer. The small soluble molecules were thus dialyzed out, and whatever was essential for full enzyme activity had to be added back to the system. The DPH hydroxylating activity of the dialyzed enzyme preparation containing the complete NADPH-generating system was taken as 100% (Table 1). The assay was repeated, omitting one or other of the cofactors and the % of total activity calculated. Results showed that

Table 1:

This table shows the results of an experiment to determine the co-factors required for maximal activity in the assay of DPH metabolism. Activity was measured in dpm (disintegrations per minute) obtained from total metabolites formed in the reaction using ^{14}C-DPH as substrate and dialyzed 9000 xg S as enzyme source. Activity in system containing complete NADPH-generating system (97) was taken as 100%.

TABLE 1

Co-factor requirements for DPH metabolism by dialyzed 9000 xg supernatant

Systems	dpm[a]	% Activity
9000 xg S (dialyzed + complete NADPH-generating system)	5706	100
9000 xg S + CGS[b] -NADP	2882	50.5
9000 xg S + CGS -(G-6-P[c] & G-6-P DH[d])	630	11.1
9000 xg S + CGS -NAD	6031	105
9000 xg S + CGS -ATP	5632	98.5

[a]Disintegrations per minute of total metabolites of DPH

[b]Complete NADPH-generating system

[c]Glucose-6-phosphate

[d]Glucose-6-phosphate dehydrogenase

NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were essential for full activity, while NAD and ATP were not. Apparently endogenous glucose-6-phosphate dehydrogenase lost activity upon standing and the addition of an excess assured adequate reduction of NADP.

b. Extraction of metabolites

On the basis of a series of partition experiments, ethyl acetate was shown to be more effective than either $CHCl_3$ or benzene in extracting pHPPH and the dihydrodiol, Gabler er al (11). For this reason ethyl acetate was the solvent of choice for the extraction of DPH and its metabolites from the incubation mixture.

c. pH dependence

The activity of DPH hydroxylase was tested at a number of pH's to establish the optimum pH for this reaction. A series of buffers, 0.1 M phosphate buffers, pH 5.5 - 7.5 and Tris buffers of similar molarity, pH 7.8 - 8.8, were used and the DPH assay run as described previously. The pH of the reaction mixture was determined before and after incubation and enzyme activity plotted vs. pH. In no case did the pH vary by more than 0.2 pH units.

Figure 2 shows pH dependence curves for the metabolism of DPH and several of the substrates used in this study. In the case of DPH, total activity was expressed as dpm (disintegrations per minute)/15 minutes incubation/10 mg 9000 xg S protein. With aniline, aminopyrine and hexobarbital, enzyme activities were expressed in terms of absorbancy/time/10 mg protein. Wave lengths at which optical densities were measured and time period for incubations are described in following section on assays.

Figure 2: pH dependence curves for the various enzyme assays.

Enzyme activity of the 9000 xg S was assayed with aniline, aminopyrine, hexobarbital and diphenylhydantoin (as described in Materials and Methods) at a number of pH points in the 5.5 - 8.5 range. Curves show pH range at which maximal activity was obtained with each substrate.

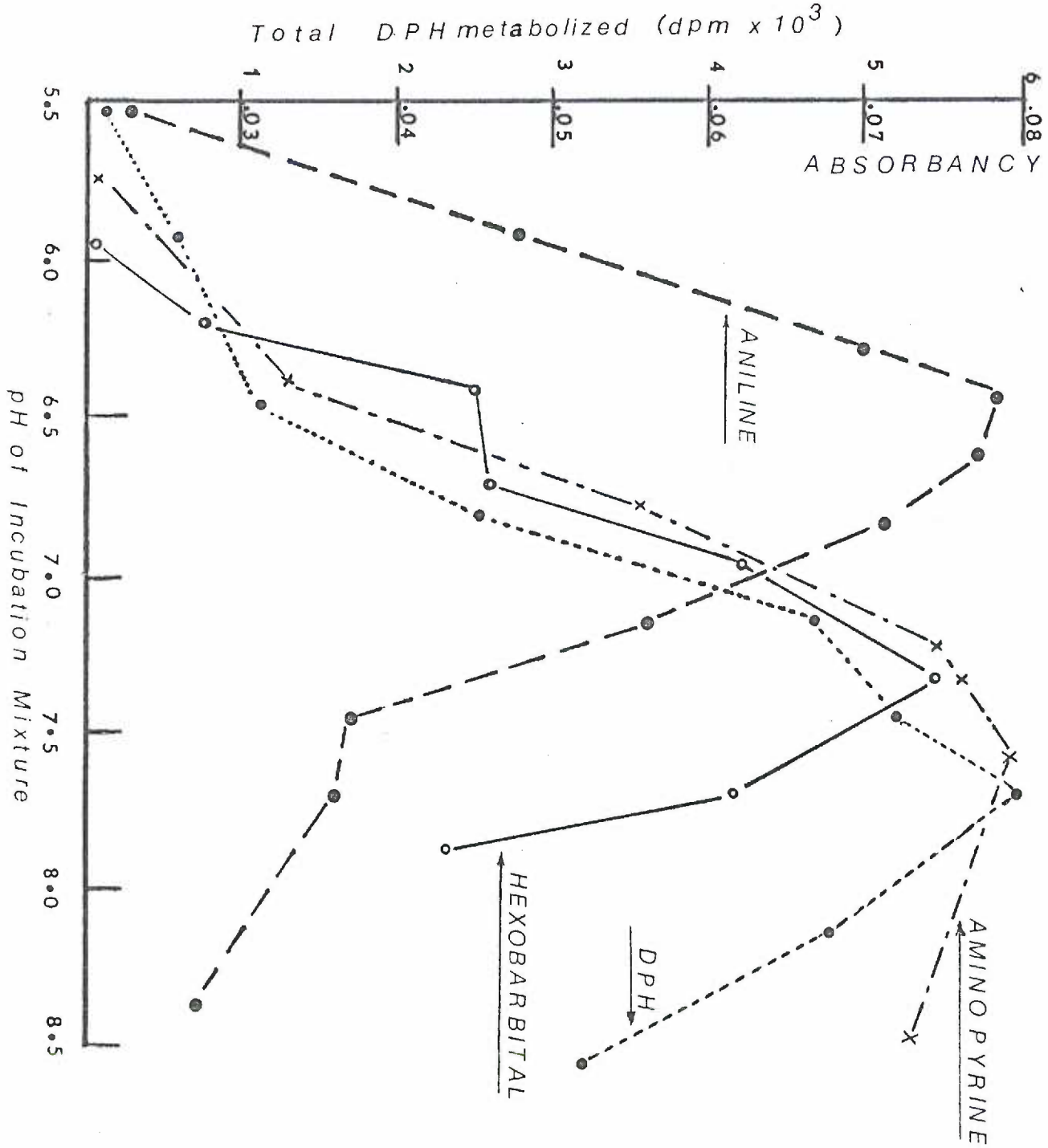

Total DPH metabolized (dpm x 10³)
1
2
3
4
5
6
.03
.04
.05
.06
.07
.08
ABSORBANCY
ANILINE
HEXOBARBITAL
DPH
AMINOPYRINE
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH of Incubation Mixture

FIGURE 2

Optimum pH range for the assay of DPH, aminopyrine and hexobarbital was approximately 7.4 - 7.8, while optimum pH for aniline assay was ~6.5.

d. Preparation of radioactive standards

Products and substrate were separated by TLC and visualized by autoradiography. Authentic m and pHPPH was supplied by A. J. Glazko (Parke, Davis & Co., Ann Arbor, Michigan).

In order to prepare radioactive standards of the monohydroxylated and the dihydrodiol derivates of DPH, ^{14}C-DPH was injected into either monkeys or rats, their urine collected and frozen. Since most of the HPPH formed in vivo is excreted in the conjugated form (~95%), which is insoluble in ethyl acetate, the urine was pretreated with β glucuronidase in order to hydrolyze some of the glucuronide before extraction. Acid hydrolysis was avoided initially because this would have resulted in a loss of some of the dihydrodiol. Chang et al (99) reported that acid and heat cause part of the dihydrodiol to form equal amounts of the 3 and 4 monohydroxylated derivates.

Total 24 hour urine samples were incubated overnight at 37°C with 2 mg/ml β glucuronidase in phosphate buffer (pH 6.8), and the system was then frozen until needed. Samples were thawed, pooled, filtered and lyophilized to reduce volume. The lyophilized urine was extracted several times with ethyl acetate and the extracts were dried, pooled and redissolved in a small quantity of absolute methanol. The methanol was streaked across a TLC silica gel plate, chromatographed using the solvent system previously described and overlaid with an x-ray plate. Each area containing a radioactive metabolite was scraped and re-extracted separately several times with ethyl acetate, as described above, and restreaked on a

separate TLC plate. This was repeated until only one streak appeared on a plate with an R_f value which corresponded to that of standard HPPH or to the values for the dihydrodiol found in the literature (99). These areas were scraped, extracted in ethyl acetate and dried.

Since the initial treatment with β glucuronidase did not completely hydrolyze all the conjugated pHPPH, some insoluble conjugate remained after ethyl acetate extraction of the lyophilized urine. This residue was heated with 6N HCl in a boiling water bath, the hydrolysate was then neutralized with 6N NaOH, buffered with 0.1 M pH 7.4 phosphate buffer, extracted with ethyl acetate, chromatographed and the metabolites were scraped and re-extracted until purified.

Figure 3 is an autoradiograph of a TLC plate containing 3 unknowns, A, B and C and 3 radioactive standard markers, D, E and F. R_f's of the standards are (D) dihydrodiol = .16, (E) pHPPH = .34 and (F) DPH = .57. Sample A is an aliquot of the dihydrodiol fraction from β glucuronidase-treated urine, B is an aliquot of the HPPH fraction from β glucuronidase treated urine. Areas on the plate corresponding to R_f = .16 from sample A, R_f = .34 from samples B and C, R_f = .37 from sample B were scraped and sent to another laboratory[3] for analysis by gas-liquid chromatography (GLC). The results of GLC analysis are presented in Table 2. Metabolite with R_f = .37 was found to be mHPPH, which is not a normal metabolite of DPH in rats or monkeys and was probably formed during acid hydrolysis of the urine. The metabolite with R_f = .34 was shown to be pHPPH. The analysis also showed that the dihydrodiol fraction apparently contained

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3. Analysis was performed by Dr. Gordon Conard, Dental Research Institute, Chicago, Ill.

Figure 3: Autoradiograph of TLC plate

This photograph of an autoradiograph shows the TLC separation of radioactive metabolites of DPH. Three unknown were streaked on plate at A, B, and C. Radioactive standard markers at D (dihydrodiol), E (pHPPH), and F (DPH) were used to identify the components of the unknowns.

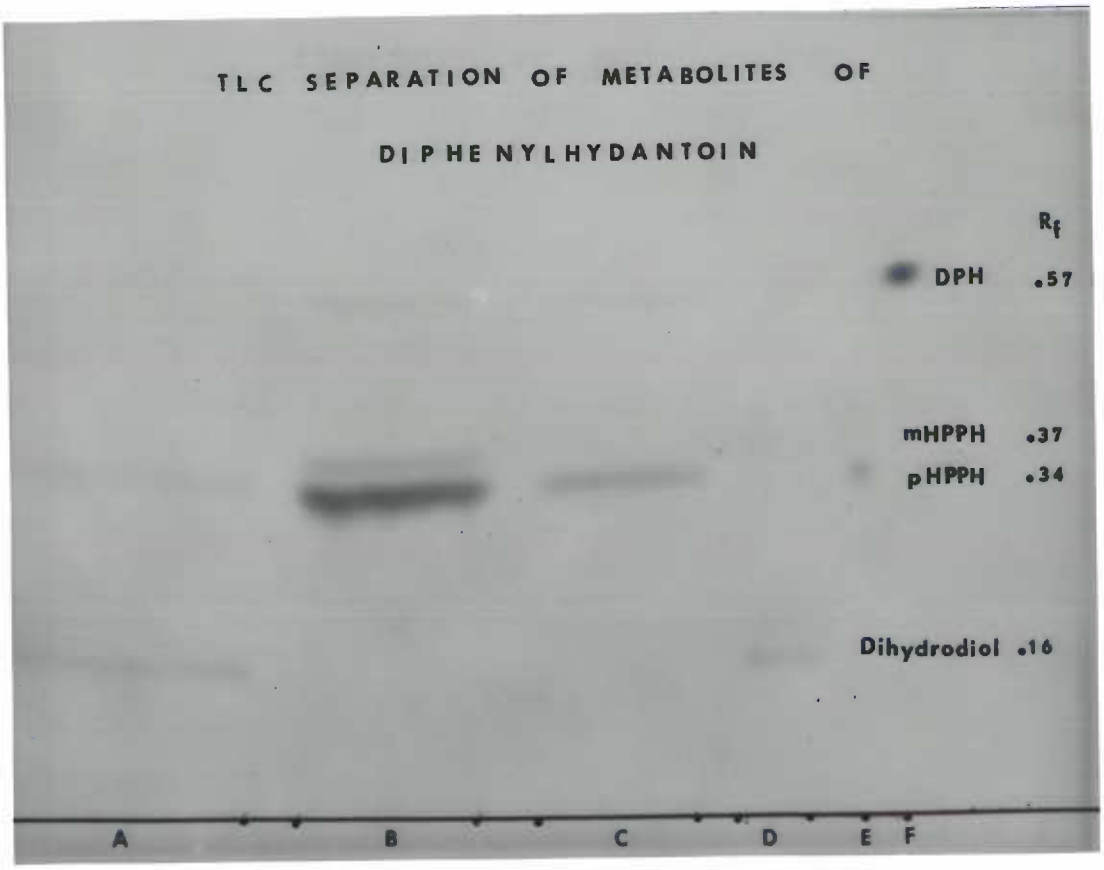
TLC SEPARATION OF METABOLITES OF
DIPHENYLHYDANTOIN
R_f
DPH .57
mHPPH .37
pHPPH .34
Dihydrodiol .16
A
B
C
D
E
F

FIGURE 3

Table 2:

This table shows the relative purity of metabolites of DPH separated by TLC. Areas corresponding to R_f values listed were scraped (see figure 3) and analyzed by gas-liquid chromatography. Data are discussed on pages 29 and 32.

TABLE 2

Gas-liquid chromatographic analysis of
fractions separated by thin layer chromatography

R_f	% mHPPH	% pHPPH	% dihydrodiol
.37	95.54	4.46	-
.34	7.7	92.3	-
.16	20.05	22.84	57.11

equal amounts of m and pHPPH (~25%). This is not surprising, since it has been noted that the preparation of volatile derivatives of the dihydrodiol for GLC results in the conversion of some of the dihydrodiol to roughly equivalent amounts of m and pHPPH (99). It is highly unlikely that the two monohydroxy derivatives were contaminants of the original dihydrodiol sample because the R_f values are so widely separated.

e. Channels ratio counting

Efficiency of counting was calculated by the channels ratio method. A quench correction curve was constructed according to the Nuclear Chicago Liquid Scintillation Manual (101). A set of quenched C-14 standards (Nuclear-Chicago Model 180060 C-14 Quenched Standards Set) of known dpm was counted and the % efficiency calculated by means of the formula:

$$\% \text{ efficiency} = \frac{\text{CPM Scaler A}}{\text{dpm}}$$

The ratio of counts in Scaler B/Scaler A was also calculated, and the % efficiency was plotted against the channels ratio. The channels ratio was then calculated for all unknowns and their efficiency read off the quench correction curve. Figure 4 is a reproduction of the curve used throughout these studies.

C. ASSAYS

All assays were checked initially to establish the substrate concentration, protein (enzyme) concentration and the time during which the reaction rates were linear. In all cases enzyme activities were assayed at a substrate concentration such that zero order kinetics were obeyed unless otherwise specified.

Figure 4: Quench correction curve

Curve was used to determine efficiency of counting ^{14}C in all assays of DPH metabolism using radioactive DPH. The % efficiency was first determined with a series of quenched C-14 standards as described on page 32 and these data plotted against the ratio of dpm in Scaler B/Scaler A.

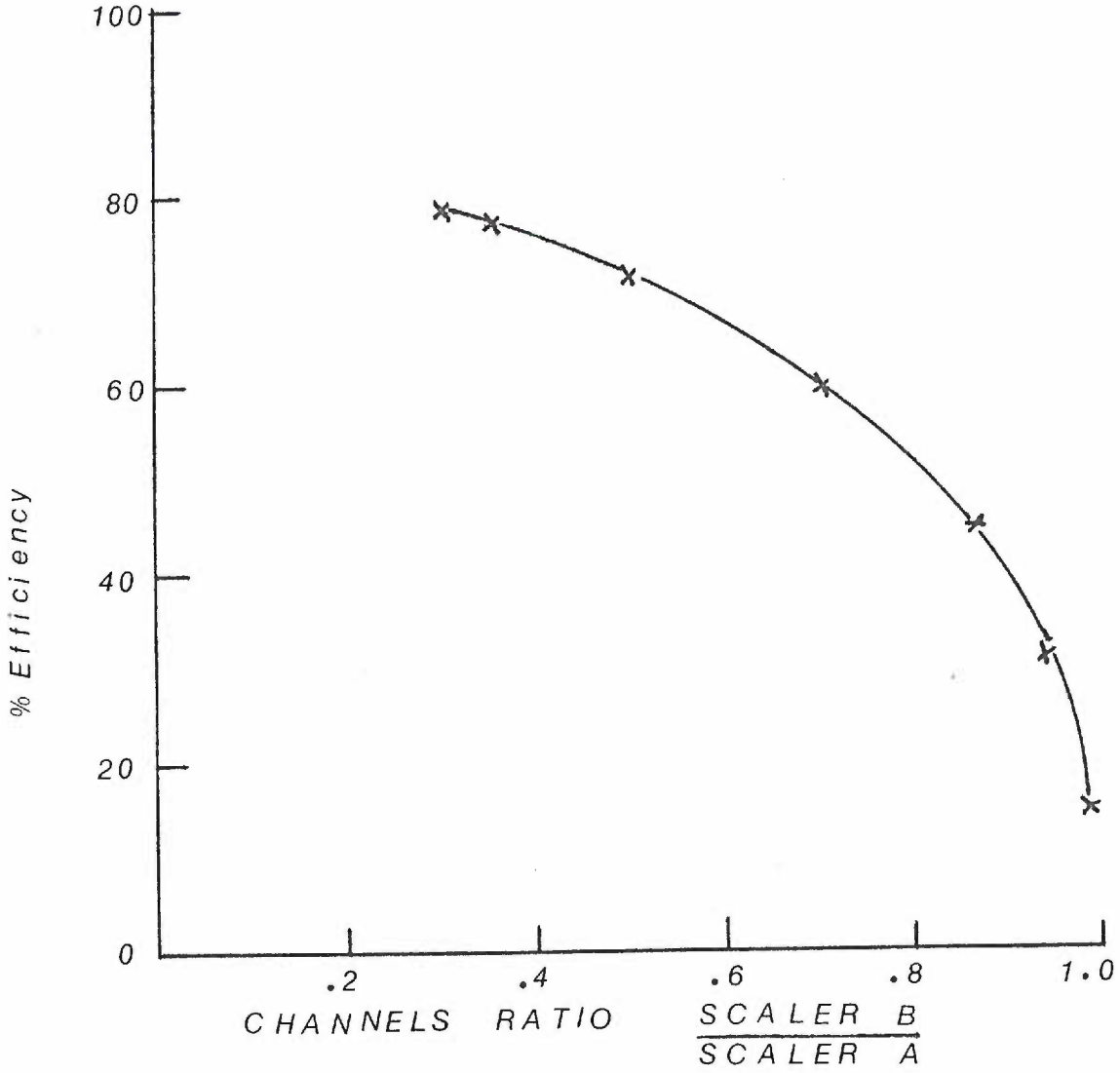
100
80
60
40
20
0
% Efficiency
.2
.4
.6
.8
1.0
CHANNELS RATIO
SCALER B
SCALER A

FIGURE 4

The procedure followed in the subsequent assays was the same as that described for the determination of DPH hydroxylase activity unless otherwise noted. The reaction mixture contained substrate, 9000 xg S as enzyme source and an NADPH-generating system. This generating system differed somewhat from the one used in the assay of DPH metabolism and contained the following substances: 2.67×10^{-3} M $MgCl_2$, 2.67×10^{-2} M KCl, 8×10^{-4} M ATP, 4.36×10^{-3} M nicotinamide, 7.44×10^{-4} M NAD, 6.6×10^{-4} M NADP, 3.9×10^{-3} M glucose-6-phosphate and 0.33 units glucose-6-phosphate dehydrogenase/ml final solution. The reaction was initiated and incubation carried out as described. The reaction was terminated by removal of a 1 ml aliquot of the incubation mixture and the proteins precipitated. Proteins were removed by centrifugation and color reaction run on an aliquot of the clear supernatant. Readings were made in a Coleman Jr. Spectrophotometer against a reagent blank. A standard was run with each set of determinations. Controls containing the complete reaction system without substrate were usually run with each assay and this blank value subtracted from the sample value. Activities are expressed as mμmoles of product formed/time/10 mg protein.

1. Aniline Hydroxylase

Aniline hydroxylase activity of 9000 xg S from rat liver homogenates was measured according to the method of Brodie and Axelrod (102), as modified by Imai et al (103).

Protein concentration was approximately 10 mg/ml, aniline concentration was 1×10^{-4} M. Incubation time was 15 minutes and proteins were precipitated with 10% TCA. One ml of 10% Na_2CO_3 and then 2 ml of a 2% phenol solution, in 0.2N NaOH, were added to 1 ml of the supernatant after

TCA precipitation, and the mixture incubated at 37°C for 30 minutes to develop the color. Readings were made at 630 mμ.

2. O-demethylation of p-nitroanisole

The o-demethylase activity of the 9000 xg S was assayed according to the method of Kato and Gillette (81), using p-nitroanisole as substrate at a concentration of 1×10^{-3} M and an enzyme protein concentration of approximately 10 mg/ml. The reaction was allowed to proceed for 30 minutes and proteins were precipitated with 10% TCA. Color was developed by addition of 4 ml of 0.2N NaOH to 1 ml of the supernatant after TCA precipitation and readings were made at 410 mμ.

3. Nitroreduction of p-nitrobenzoic Acid

P-nitroreductase activity of the 9000 xg S was assayed according to the method of Fouts and Brodie (105). Substrate concentration was 1×10^{-3} M p-nitrobenzoic acid and protein concentration approximately 10 mg/ml. The incubation was allowed to proceed for 30 minutes in an atmosphere of nitrogen. During the pre-incubation period and the first 5 minutes of the reaction, N_2 gas was bubbled slowly into the capped reaction vessel to insure removal of all O_2. After initial gassing, the flask was kept tightly capped to prevent entry of O_2, since earlier studies indicated that inadequate gassing significantly altered the reaction rates of this assay. Proteins were precipitated with 20% TCA at the end of the incubation period. The Bratton and Marshall reaction (94) was used to produce color, which was read at 550 mμ.

4. Aminopyrine Demethylase

A modification of the methods of La Du et al (105) and Cochin and Axelrod (106) was used for the assay of aminopyrine demethylase activity of the 9000 xg S fraction. Semi-carbazide, 5×10^{-3} M, was added to the reaction mixture to trap the formaldehyde formed (107). Aminopyrine concentration was 1×10^{-2} M and protein concentration was 2-3 mg/ml. A blank containing the enzyme protein and co-factors was run with each assay in order to measure intrinsic formaldehyde formation of the system in the absence of substrate. After a 15 minute incubation under O_2 the reaction was terminated and the proteins precipitated with 2 ml of 0.5 N perchloric acid (108). One ml of double strength Nash reagent (109) was added to 2 ml of the supernatant and color developed by incubation for 30 minutes at 60°C. Readings were made at 415 mμ. Formaldehyde was used as the standard for this assay.

5. Hexobarbital Hydroxylase

Hexobarbital hydroxylase activity of the 9000 xg S was assayed by measuring substrate disappearance. The method used was similar to the one described by Brodie et al (110) for pentobarbital and modified by Cooper and Brodie (22) for hexobarbital.

The reaction mixture contained the following substances in a final volume of 3 ml of 0.1 M pH 7.4 phosphate buffer: 5-12 mg protein/ml; an NADPH-generating system consisting of 6.7×10^{-3} M $Mgcl_2$, 6.5×10^{-3} M nicotinamide, 3.3×10^{-4} M NADP, 2×10^{-3} M glucose-6-phosphate and 0.33 units glucose-6-phosphate dehydrogenase/ml. In order to determine V_{max} and K_m for this reaction the substrate concentration (hexobarbital,

Winthrop Laboratories), was varied from 0.5 - 2.0 x 10^{-3} M.

The reaction was initiated by the addition of co-factors to the substrate-enzyme mixture. After brief mixing, a zero-time aliquot was removed, acidified and the hexobarbital extracted with heptane. The incubation was then continued for 30 or 60 minutes under O_2. At the end of this period another aliquot was removed, acidified with 0.1 M pH 5.5 phosphate buffer and the hexobarbital was extracted in heptane. The hexobarbital was then re-extracted from heptane into alkaline 0.8 M pH 11 phosphate buffer. The buffer was transferred to a quartz cuvette and read at 244 mμ against a reagent blank in a double beam Perkin-Elmer Spectrophotometer. The amount of hexobarbital hydroxylated during the assay was obtained by difference between the hexobarbital content at zero-time and at 30 or 60 minutes.

It should be noted that hexobarbital is relatively insoluble in water and as a consequence was dissolved in 0.1 N NaOH. Preliminary tests showed that hexobarbital was unstable in the NaOH solution and that the $t_{\frac{1}{2}}$ of its deterioration was 2.2 days. Therefore, a fresh 75 mM standard was made fresh daily and diluted to the desired concentrations.

6. Cytochrome P-450

The cytochrome P-450 content of the resuspended microsomal pellet was measured at room temperature in a recording double beam Perkin-Elmer Spectrophotometer, using the method described by Omura and Sato (32). The microsomal suspension was diluted with 0.1 M, pH 7.4 phosphate buffer to contain 1 - 1.5 mg protein/ml. Two 3 ml aliquots were treated with a few milligrams of $Na_2S_2O_4$. A carbon monoxide generator was made by mixing formic acid and concentrated H_2SO_4 in a flask having an outlet port.

The gas formed was bubbled slowly for 1 minute into the microsomal suspension in the sample cuvette, but the aliquot in the reference cuvette was left ungassed. The sample cuvette was scanned against the reference cuvette and the difference spectrum recorded. The change in absorbance was measured between 450 mμ and 490 mμ. The cytochrome P-450 concentration was calculated from the formula Δ O.D. = ε C, where ε, the extinction coefficient, was taken to be 91 mM^{-1} cm^{-1} (32). Figure 5a shows a typical difference spectrum obtained in the determination of cytochrome P-450.

7. Cytochrome b_5

Cytochrome b_5 was measured at room temperature by means of the spectral change resulting from its reduction with NADPH (36). A 1.5 ml aliquot of the microsomal suspension in the sample cuvette was diluted with 1.5 ml of the NADPH-generating system so that the protein concentration was 1 - 1.5 mg/ml. An equal volume of 0.1 M pH 7.4 phosphate buffer was added to another 1.5 ml aliquot of the microsomal suspension in a reference cuvette. The sample cuvette was scanned against the reference cuvette in a double beam spectrophotometer, and the difference spectrum was recorded. The change in absorbance was measured between 425 mμ and 410 mμ. The extinction coefficient was taken to be 165 mM^{-1} cm^{-1} (36) and the cytochrome content calculated as above. Figure 5b shows a typical difference spectrum between 425 mμ and 410 mμ.

8. Spectral Binding

Spectral binding constants for aniline and hexobarbital were determined, using the method described by Schenkman (88, 89). For the determination of aniline binding constants, two 3 ml aliquots of the micro-

Figure 5: Difference spectra obtained in the determinations of cytochrome P-450 and cytochrome b_5 concentrations.

(a) Cytochrome P-450 difference spectrum obtained by scanning reference cuvette against sample cuvette. Both cuvettes contained 3 ml of microsomal suspension (1 - 1.5 mg protein/ml) and a few mg $Na_2S_2O_4$. CO was bubbled into sample cuvette for 1 minute and Δ O.D. measured between 450 and 490 mμ. (b) Cytochrome b_5 difference spectrum obtained by scanning reference cuvette against sample cuvette. Sample cuvette contained microsomal suspension and equal volume of NADPH-generating system (1 - 1.5 mg/ml protein in final concentration). Reference cuvette contained microsomal suspension at same final protein concentration as sample. Δ O.D. was measured between 425 and 410 mμ.

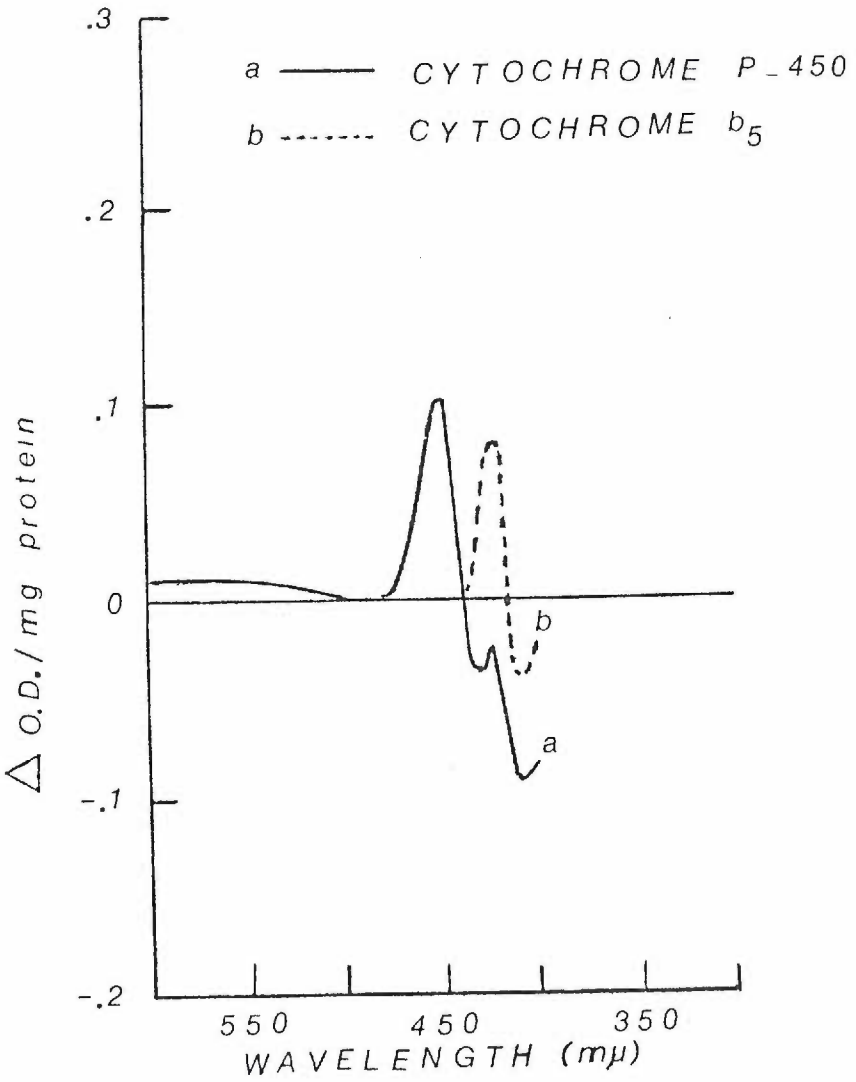
a —— CYTOCHROME P-450
b ------ CYTOCHROME b_5
.3
.2
.1
0
-.1
-.2
Δ O.D./mg protein
b
a
550
450
350
WAVELENGTH (mμ)

FIGURE 5

somal suspension (1 - 1.5 mg protein/ml) were placed in cuvettes, and aniline (75 mM) was added in μl increments to give a series of concentrations between 0.25 and 4.0 mM. An equal volume of phosphate buffer was added to the reference cuvette. After each addition of substrate and buffer, the sample cuvette was scanned against the reference cuvette and the difference spectrum recorded. The change in absorbance was measured between the peak at 430 mμ and the trough at 390 mμ. A Lineweaver-Burke plot of Δ O.D.$^{-1}$ vs. aniline concentration^{-1} gave values for Ks the spectral binding constant and Δ O.D.$_{max}$.

Determination of hexobarbital spectral binding constants differed from the procedure for aniline in only a few technical details. Microsomal protein concentration was 1.5 - 2.0 mg/ml and the hexobarbital (75 mM) was dissolved in 0.1 N NaOH. Consequently 0.1 N NaOH was added to the reference cuvette instead of phosphate buffer. Substrate was added in μl increments to give a series of concentrations between 0.125 and 2.0 mM and the change in absorbance was measured in the difference spectrum between the peak at 390 mμ and the trough at 420 mμ. Figure 6 shows a series of difference spectra obtained in these determinations.

Figure 6: Difference spectra obtained in determination of spectral binding constants with hexobarbital.

Reference cuvette was scanned against sample cuvette and $\triangle$O.D. measured between 420 and 390 mμ. Both cuvettes contained 3 ml microsomal suspension with a protein concentration of 1.5 - 2.0 mg/ml. Hexobarbital in 0.1N NaOH was added to sample cuvette to give series of concentrations from 0.125 - 2.0 mM. An equal volume of 0.1N NaOH was added to reference cuvette. Figure shows difference spectra with series of different concentrations of hexobarbital.

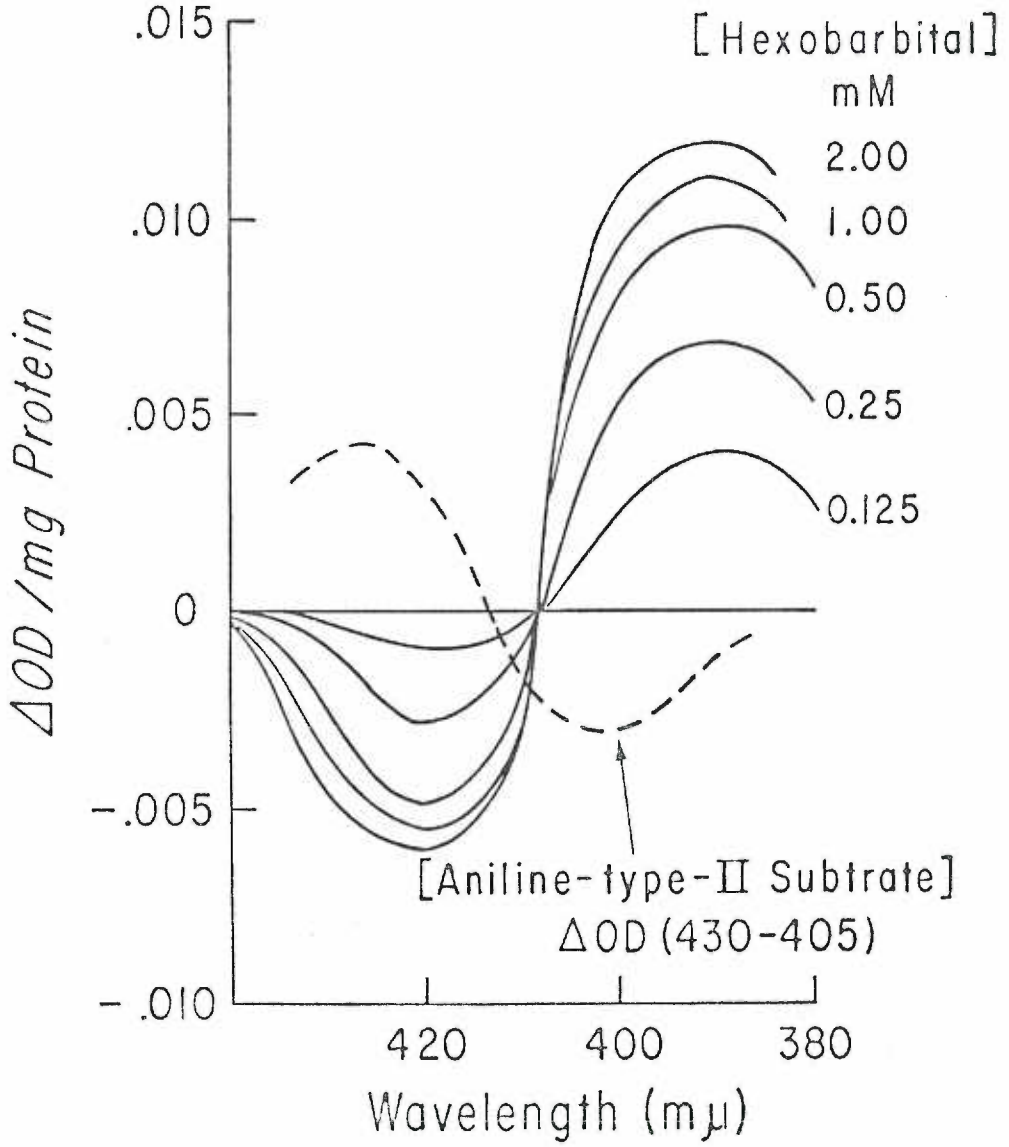
.015
.010
.005
0
-.005
-.010
ΔOD /mg Protein
[Hexobarbital]
mM
2.00
1.00
0.50
0.25
0.125
[Aniline-type-II Subtrate]
ΔOD (430-405)
420
400
380
Wavelength (mμ)

FIGURE 6

RESULTS

A. INTRODUCTION: Experimental design and statistical analysis

Several parameters of the hepatic drug metabolizing enzyme system from male, pregnant and nonpregnant female rats were studied and compared. Since a number of investigators had shown that male rats metabolize many drug substrates at a more rapid rate than nonpregnant females, claiming that this difference was related to the effect of androgenic (76, 78, 89, 90) and anabolic steroids (80, 81, 82) in males, we included in our experimental design a male-nonpregnant female comparison of drug metabolism and related measurements. This comparison served both as a broad check on the consistency of our results with those of others, and as an indicator of tendencies in the pregnant female to mimic the activity of males.

Raw data were collated according to the group to which an animal belonged (i.e., male, pregnant or nonpregnant female) and the mean and standard deviation for each group of animals were calculated and compared. The data obtained from the nonpregnant female group were compared to both the male and pregnant female groups. Statistical significance of the difference between means was estimated on the basis of the Student's t-test with the lower limit of acceptability set at the 95% confidence level.

Double reciprocal plots were used to calculate both the kinetic constants, apparent K_m and V_{max}, and the spectral binding constants, apparent K_s and $\triangle O.D._{max}$. In determining these constants, data for each group of animals were pooled and the regression slope calculated by a computerized least squares method. The regression line was plotted

from values for intercept and slope.

B. COMPARISON OF CERTAIN BODY MEASUREMENTS AMONG THE THREE GROUPS OF ANIMALS

1. Body Weight and Liver Weight

As can be seen in Table 3, male and nonpregnant female rats used in this study had approximately the same mean body weight and on the basis of this weight would be classed as adult animals.

The mean body weight of the 20-day pregnant female was significantly greater than that of the nonpregnant female control (385 g vs. 241 g). Approximately 74 g of this could be attributed to the weight of the uterus and its contents.[4] If this amount was subtracted from the maternal body weight, the corrected body weight was approximately 311 g. Some, but not all, of this additional weight of the pregnant rat probably resulted from water and salt retention, due to an aldosterone-like action of estrogen and progesterone on the kidney.

Average liver weight for each group of animals could be ranked, pregnant female > male > nonpregnant female, but when liver weight was calculated per 100 g body weight, the ranking was different: male > nonpregnant female > pregnant female. If, however, the corrected body weight of 311 g for the pregnant rat, instead of 385 g, was used to calculate g% liver, a value of 4.12 was obtained and the ranking was similar to that of total liver weight.

---

4. The 74 g figure was arrived at in the following manner. The uterus and its horns at term weighed ~ 4.5 g compared to 0.5 g in the cycling female, or a gain of 4.0 g. Average weight of the 20-21 day fetus with its enveloping membranes, fluid and attached placenta was ~ 7 g and the average litter was 10, in our series. Total average weight gain due to the uterus and its contents was therefore 74 g.

Table 3:

Body and liver weight and liver weight per 100 g body weight are compared in this table. Each value listed is the mean of a number of determinations. The significance of the difference between means is determined on the basis of the Student's t-test in two sets of comparisons. Pregnant females are compared to nonpregnant females. Males are compared to nonpregnant females.

TABLE 3

Body weight, liver weight and liver weight
% body weight in three groups of rats

Measurement	Pregnant Females (22 animals)	Nonpregnant Females (44 animals)	Males (28 animals)
Body weight-g	385[c] ± 74.1[1]	241 ± 46.0	252 ± 55.7
Liver weight-g	12.8[c] ± 2.46	8.94 ± 2.83	10.4[a] ± 2.73
Liver weight-g%	3.34[b] ± 0.31 (4.12)[2]	3.67 ± 0.59	4.08[b] ± 0.70

[a]Significantly different from nonpregnant female $P < .05$

[b]Significantly different from nonpregnant female $P < .01$

[c]Significantly different from nonpregnant female $P < .001$

[1]Mean ± standard deviation

[2]g% liver using corrected maternal weight which is gross maternal weight minus weight of uterus and contents

Although human fetal liver and near term placenta demonstrated a limited capacity to biotransform drugs (111), studies in our laboratory (unpublished data) showed that rat placenta and fetal liver had no similar metabolic activity, at least for the substrates studied in this project. Consequently, the liver of the pregnant rat processes many of the metabolites produced by the fetus and also appears to bear the main burden of exogenous drug metabolism. For these reasons there seemed to be little justification in treating the fetal compartment as an inanimate object and substracting its weight from the gross maternal weight. It would be more reasonable to compute g% liver on the basis of gross maternal weight and to use this figure (3.34 g%) as a measure of the amount of hepatic tissue available to monitor 100 grams of body weight in the pregnant female. Our data indicated that although there was compensatory hypertrophy of the liver during pregnancy (increased total liver weight) it did not match the increased total body weight, and that, on the basis of g% liver, the pregnant female had less hepatic tissue for coping with its metabolic burden than either the nonpregnant female or the male rat.

Data presented in Table 3 on g% liver paralleled similar data for males and nonpregnant females reported by other workers (81, 89, 112). However, studies done by two groups of investigators reported a slight increase in g% liver in pregnant females compared to nonpregnant females, even when gross maternal weight was used (14, 113).

2. Protein Concentrations of the Hepatic 9000 xg S and Microsomal Fractions

The 9000 xg S fraction represents the hepatic homogenate minus a layer of lipid floating on top of the supernate and a sediment of whole cells,

plasma membrane, mitochondria and nuclei, while the microsomal pellet is predominantly smooth and rough endoplasmic reticulum, glycogen and a collection of microbodies. These fractions are admittedly heterogeneous, but are probably representative of the relative composition of liver in each of the three groups of animals.

The protein concentrations of these fractions are presented in Table 4 as mg per g liver and mg per 100 g body weight.

a. Pregnant-nonpregnant female comparison

The protein concentration of the hepatic 9000 xg S, calculated per g liver was not significantly different in pregnant and nonpregnant females, while microsomal protein concentration (mg/g liver) was significantly higher ($P < .05$) in the livers of pregnant rats (Table 4). However, when these data were presented as mg% body weight, the 9000 xg S was found to be significantly lower ($P < .05$) in the pregnant female, and the difference in microsomal protein concentration was eliminated. These changes in comparative protein concentrations reflected the lower g% liver in the pregnant female.

b. Male-nonpregnant female comparison

When compared on a per gram liver basis, the protein concentrations of the hepatic 9000 xg S fractions in males and nonpregnant females were almost identical, while microsomal protein concentration was very significantly higher ($P < .001$) in males. Since (as has already been pointed out) the g% liver was higher in males than in nonpregnant females, the protein concentrations associated with both these fractions were also substantially higher in males when based on 100 g body weight.

Schenkman et al (89) reported higher microsomal protein concentration

Table 4:

Protein concentration was determined as described in Materials and Methods in the 9000 xg S and microsomal fractions of liver from male, pregnant and nonpregnant female rats. Data were collated and compared as described in Results, page 42. Protein concentrations are expressed as mg per g liver and per g% liver.

TABLE 4

Protein concentrations of the hepatic 9000 xg S and microsomal fractions from the three groups of animals

Measurement	Pregnant Females (22 animals)	Nonpregnant Females (43 animals)	Males (26 animals)
9000 xg S protein mg/g liver	137 ± 12.2[1]	140 ± 16.8	140 ± 17.5
9000 xg S protein mg/100 g body weight	459[a] ± 71.9	509 ± 76.5	572[b] ± 86.7
Microsomal protein mg/g liver	31.1[a] ± 3.80	28.4 ± 4.70	32.9[c] ± 4.80
Microsomal protein mg/100 g body weight	104 ± 18.0	103 ± 16.3	133[c] ± 26.9

[a] Significantly different from nonpregnant female $P < .05$

[b] Significantly different from nonpregnant female $P < .01$

[c] Significantly different from nonpregnant female $P < .001$

[1] Mean ± standard deviation

per gram liver in male rats compared to females, as well as greater relative liver weight in males. Kato and Gillette (81) showed almost identical hepatic microsomal protein concentrations (mg/g liver) in livers of males and females, but a somewhat greater g% liver in male rats. In general the data seemed to show a greater protein concentration associated with these fractions in the livers of male compared to female rats, possibly related to the known anabolic effect of androgenic hormone.

3. Cytochrome P-450 and Cytochrome b_5 Concentrations in Hepatic Microsomes

Hepatic microsomes contain two types of hemoprotein, cytochrome P-450 and cytochrome b_5 (47). Orrenius and Ernster (51) have shown that the drug hydroxylating activity of the microsomal fraction often parallels its cytochrome P-450 concentration. Cytochrome b_5 seems to function in the oxidative desaturation of fatty acids (114), although all the components of the system have not yet been isolated. Its role in the cytochrome P-450 system (46) remains speculative.

Cytochrome P-450 and cytochrome b_5 concentrations in hepatic microsomes were determined as described in the section on methods and are presented in Table 5.

a. Pregnant-nonpregnant female comparison

It made no difference whether cytochrome P-450 concentration was expressed in terms of mμmoles per mg microsomal protein, per gram liver or per 100 g "uncorrected" body weight, its concentration in the liver of pregnant females was significantly less ($P < .001$) than that of the nonpregnant rat. These results agreed with the findings of Guarino et al (14) who showed that hepatic microsomal cytochrome P-450 concentration was higher in the 6-day pregnant rat than in the nonpregnant

Table 5:

Cytochromes P-450 and b_5 concentrations were determined in liver microsomal suspensions from male, pregnant and nonpregnant female rats as described in Materials and Methods. Data were collated and compared as described in Results, page 42. Concentrations are expressed as mμmoles per mg microsomal protein, per g liver per g% liver. Total cytochromes P-450 and b_5 in whole liver is calculated by multiplying mean liver weight by mean value for concentration of cytochromes P-450 and b_5/g liver.

TABLE 5

Cytochrome P-450 and cytochrome b_5 concentrations in hepatic microsomes from the three groups of animals

	Pregnant Females (16 animals)	Nonpregnant Females (38 animals)	Males (21 animals)
Measurement			
Cytochrome P-450 mμmoles/mg m.p.*	.444[c] ± .062[1]	.612 ± .087	.738[c] ± .108
Cytochrome P-450 mμmoles/g liver	13.9[c] ± 2.67	17.1 ± 2.95	24.4[c] ± 5.30
Cytochrome P-450 mμmoles/g% liver	47.4 ± 9.50	62.6 ± 12.2	95.5[c] ± 21.4
Cytochrome P-450 mμmoles/whole liver	178	153	253
	(11 animals)	(39 animals)	(21 animals)
Cytochrome b_5 mμmoles/mg m.p.*	.478[a] ± .070	.589 ± .140	.569 ± .180
Cytochrome b_5 mμmoles/g liver	15.9 ± 2.40	16.4 ± 4.00	18.6[a] ± 3.86
Cytochrome b_5 mμmoles/g% liver	54.7 ± 10.2	58.1 ± 12.3	73.2 ± 16.9
Cytochrome b_5 mμmoles/whole liver	203	147	193

[a] Significantly different from nonpregnant female $P < .05$

[c] Significantly different from nonpregnant female $P < .001$

* Millimicromoles/mg microsomal protein

[1] Mean ± standard deviation

female controls, but significantly decreased in the 20-day pregnant animal.

It has already been argued that the most suitable basis for comparing drug metabolizing abilities should take into account the weight of liver available to monitor a standard weight of extrahepatic tissue, especially since drug dosing is based on total body weight. Our results showed that while the total cytochrome P-450 content of liver was increased in late pregnancy (178 mμmoles compared to 153 mμmoles in livers of nonpregnant females), the cytochrome P-450 concentration per 100 g body weight was markedly reduced in the pregnant female. If, as Orrenius and Ernster suggested, drug metabolism paralleled cytochrome P-450 concentration, one would expect the drug biotransforming activity to be reduced in the 20-day pregnant animal (51).

As mentioned, the role of cytochrome b_5 in drug metabolism is still questionable. In any case, the hepatic concentration of this hemoprotein was reduced during late pregnancy, but the differences in concentration between pregnant and nonpregnant rats were not as marked as those shown in cytochrome P-450 concentrations (Table 5).

b. Male-nonpregnant female comparison

Livers of male rats contained a significantly higher concentration ($P < .001$) of cytochrome P-450 than those of nonpregnant female rats, when this concentration was expressed as mμmoles per mg microsomal protein, per gram liver or per 100 grams body weight. Our data agreed with those of a number of investigators who also showed that hepatic microsomes of male rats contained higher concentrations of cytochrome P-450 than those of nonpregnant females (89, 90, 115).

A comparison of cytochrome b_5 concentrations in liver microsomes of male and nonpregnant female rats also revealed a general agreement with the data shown by other workers (89, 115).

C. COMPARISON OF DRUG BIOTRANSFORMATION ACTIVITIES OF THE HEPATIC 9000 xg S FROM THE THREE GROUPS OF ANIMALS

1. Introduction: Substrate Selection

The substrates tested in this study were selected because they represented a spectrum of biotransformation reactions. DPH and aniline are representatives of aromatic hydroxylations and hexobarbital undergoes side chain hydroxylation. Aminopyrine and p-nitroanisole are metabolized by N- and O-demethylation reactions, respectively, while p-nitrobenzoic acid[5] undergoes nitroreduction. Since pregnancy might not alter biotransformation in toto, but might selectively modify one or more enzyme pathways, the inclusion of a number of classes of substrate would enhance the possibility of finding such modification, if it occurred.

2. Kinetic Constants for DPH and Hexobarbital Hydroxylases

V_{max} for DPH and hexobarbital hydroxylation are expressed in terms of product formed or substrate used/30 minutes/10 mg 9000 xg S protein or mumole cytochrome P-450. These data, along with values for apparent K_m for these enzymes are presented in Table 6. Figures 7 and 8 are double

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5. According to Remmer (15) the reduction of nitro groups in drugs is mediated by the flavin enzymes of the endoplasmic reticulum. The sensitivity of nitroreduction to oxygen leads Gillette (116) to reason that cytochrome P-450 might be a component of microsomal nitroreductase as well as the oxidative enzymes and that oxygen may exert its action by keeping P-450 in the oxidized state. In an atmosphere of nitrogen, the reduced cytochrome P-450 (reduced by NADPH via NADPH-cytochrome c reductase) may in turn reduce p-nitrobenzoic acid to p-aminobenzoic acid.

Table 6:

Kinetic constants for DPH and hexobarbital hydroxylases were determined at a number of substrate concentrations using the 9000 xg S fraction of liver from male, pregnant and nonpregnant females as enzyme source. Assays are described in Materials and Methods. Data were calculated and compared as described in Results, page 42. V_{max} is expressed as mμmoles of product formed or substrate used/30 minutes incubation/ 10 mg protein or mμmole cytochrome P-450. K_m is in mM.

TABLE 6

Kinetic constants for DPH and hexobarbital hydroxylases in 9000 xg S fraction from the three groups of animals

		ANIMAL GROUP		
		Pregnant Female	Nonpregnant Female	Male
	# of Determinations	(9)	(17)	(13)
Substrate	Measurement			
DPH	K_m - mM	0.15	0.17	0.15
	V_{max}-mμmoles/10mg protein/30 minutes	34.2	40.0	46.1
	V_{max}-mμmoles/mμmole P-450/30 minutes	36.3	33.8	28.4
	# of Determinations	(10)	(7)	(10)
Hexobarbital	K_m-mM	0.37	1.07	0.68
	V_{max}-mμmoles/10 mg protein	94.5[a]	162	586[c]
	V_{max}-mμmoles/mμmole P-450/30 minutes	100	136	357[b]

[a] Significantly different from nonpregnant female $P < .05$

[b] Significantly different from nonpregnant female $P < .01$

[c] Significantly different from nonpregnant female $P < .001$

Figure 7: Comparison by double reciprocal plots of DPH dependence of DPH metabolism in the hepatic 9000 xg S from the three groups of animals.

Enzyme activity was determined as described in Materials and Methods using 5 - 12 mg protein/ml final solution and 4 concentrations of DPH (substrate), 0.7×10^{-4} M, 1.4×10^{-4} M, 2.8×10^{-4} M and 4.2×10^{-4} M. Activity (v) was calculated for each substrate concentration (S) in terms of millimicromoles of product formed/30 minutes/10 mg protein. The reciprocals of v and S were calculated and these values collated for all determinations within an animal group. Values for intercept and slope were obtained by means of a computerized least squares method, and regression line was plotted using these values. Each point represents mean $\pm$ standard deviation of number of determinations shown in Table 6, page 52. Intercept on ordinate gives value for $1/V_{max}$ and intercept on abscissa gives value for $1/K_m$. Males (.——.), Females (o----o), Pregnant (x—— --- ——x).

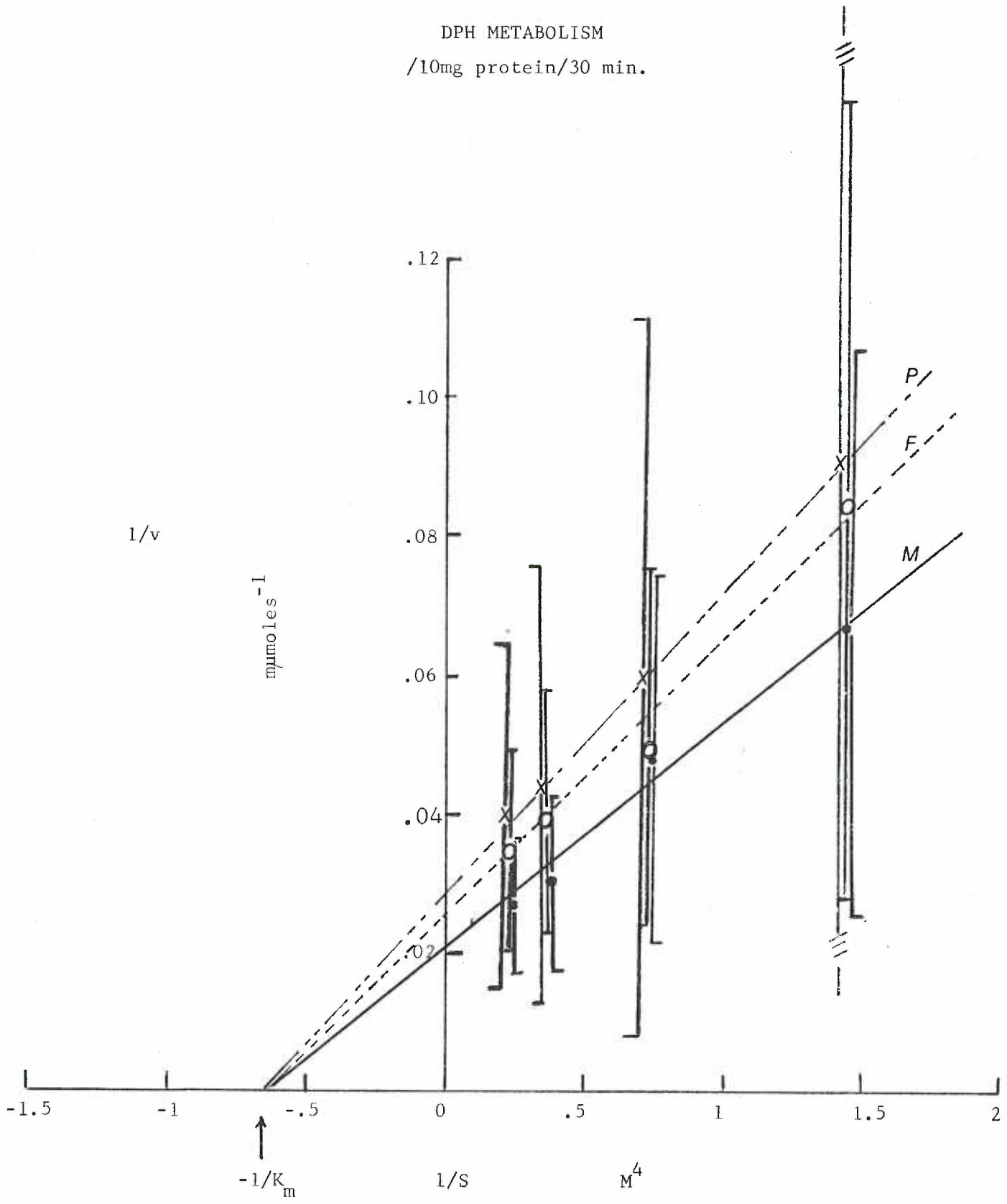
DPH METABOLISM
/10mg protein/30 min.
1/v
mμmoles-1
.12
.10
.08
.06
.04
.02
P
F
M
-1.5
-1
-.5
0
.5
1
1.5
2
-1/Km
1/S
M4

FIGURE 7

Figure 8: Comparison by double reciprocal plot of hexobarbital dependence of hexobarbital metabolism in the hepatic 9000 xg S from the three groups of animals.

Enzyme activities were determined as described in Materials and Methods using 5 - 12 mg protein/ml final solution and 4 or 5 concentrations of hexobarbital (substrate), 0.25 mM, 0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM. Data was calculated and regression line plotted as described for Figure 7. Each point represents mean $\pm$ standard deviation of number of determinations shown in Table 6, page 52. Intercept on ordinate gives value for $1/V_{max}$ and intercept on abscissa gives value for $1/K_m$. Males (. ____ .), Females (o----o), Pregnant (x— ---- —x).

HEXOBARBITAL METABOLISM

/10mg protein/30 min.

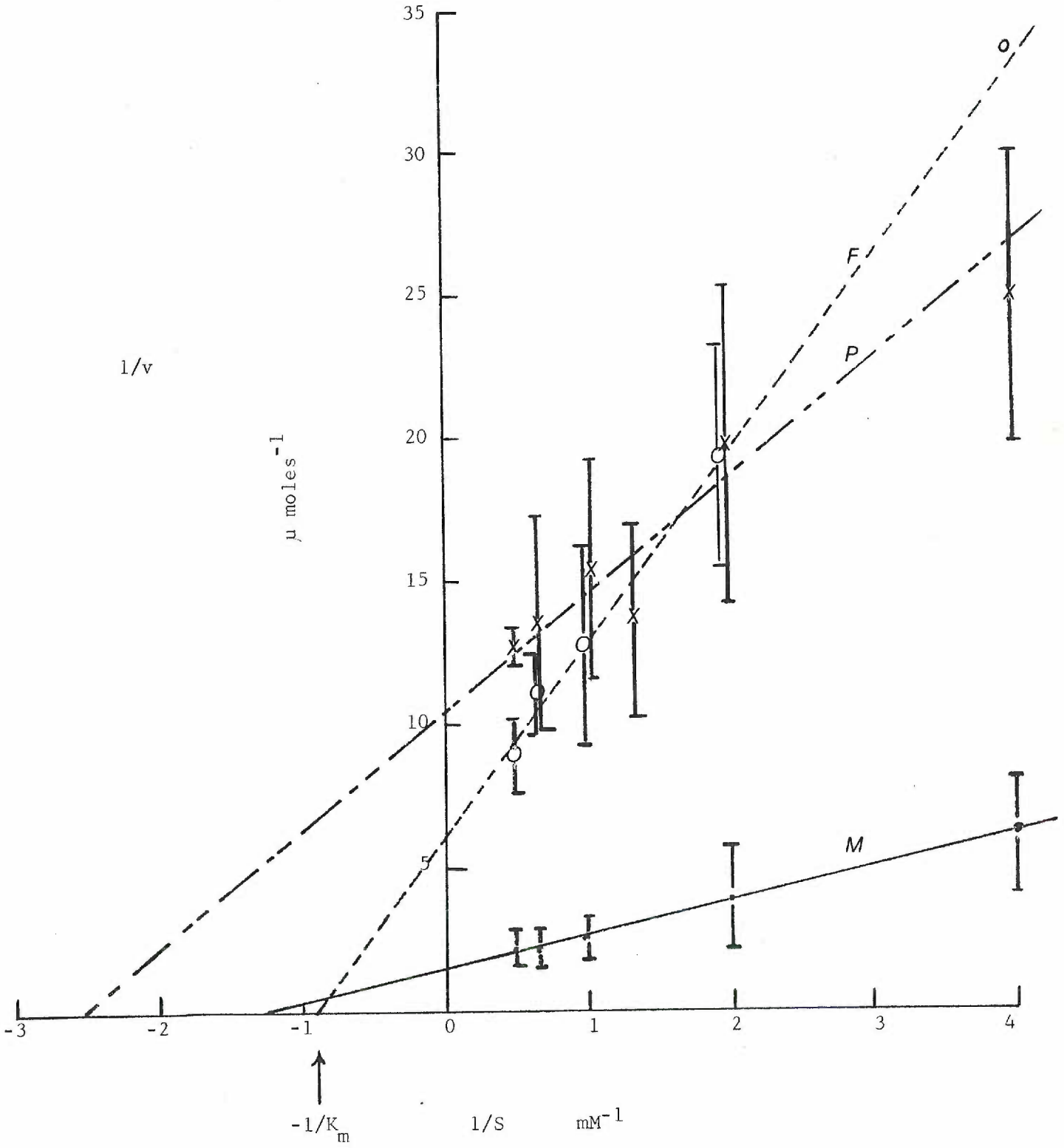

FIGURE 8

reciprocal plots of the kinetic data derived from each of the three groups of animals for DPH and hexobarbital hydroxylation.

a. Comparison of DPH hydroxylase among the three groups of animals

Since there was some indication from in vivo studies run in our laboratory (11) that DPH metabolism was altered in late pregnancy, it was of particular interest to determine if these changes might be reflected in either the apparent K_m or V_{max} for the in vitro hydroxylation of DPH.

As can be noted in Table 6, there was little difference in either of these kinetic constants among the three groups of animals. Comparison of V_{max} per 10 mg protein gave the following ranking: males > nonpregnant females > pregnant females. If, however, the base was changed to maximal activity per mμmole cytochrome P-450, the rank order was reversed. There were no statistically significant differences among the three groups of animals in either case and on the basis of these data, it appeared that pregnancy did not significantly modify the ability of the liver to biotransform DPH.

The apparent K_m values for the three groups of animals were almost identical, ranging from 0.15 mM - 0.17 mM (Figure 7). Kutt and Verebely reported (97) a much lower Michaelis constant (0.03 mM) for DPH hydroxylase in both the 9000 xg S and microsomal fractions from male rats, as well as a maximal velocity which was approximately 1½ times that shown in our determinations.

b. Hexobarbital hydroxylase

i. Pregnant-nonpregnant female comparison

When expressed in mμmoles per 10 mg protein, the V_{max} of

hepatic hexobarbital hydroxylase from pregnant females was significantly less ($P < .05$) than that of nonpregnant females (Table 6). If these data were expressed per mμmole cytochrome P-450, thereby eliminating the difference in cytochrome P-450 concentration in the enzyme preparations from the two groups of animals (Table 5), the difference in maximal activity still remained, but it was no longer statistically significant. Similar normalization of data for pregnant and nonpregnant females was reported by Guarino et al (14) when V_{max} for aniline hydroxylase and ethylmorphine demethylase were expressed per mμmole cytochrome P-450.

A comparison of apparent K_m values for hexobarbital hydroxylase revealed an appreciably lower Michaelis constant in the enzyme preparations from the pregnant group (Table 6, Figure 8), indicating a greater affinity of substrate for enzyme in the pregnant group or the possible presence of an inhibitor in these preparations from the nonpregnant group. Guarino et al (14) found no significant differences between the apparent K_m values for either aniline hydroxylase or ethylmorphine demethylase in microsomes from nonpregnant and pregnant female rats.

ii. Male-nonpregnant female comparison

Hepatic preparations from male and nonpregnant female rats showed a striking difference in maximal hexobarbital activity (Table 6). V_{max} per 10 mg protein was 3.6 times higher in males than females. When these values were expressed per mμmole cytochrome P-450, the maximum velocity in males was still approximately two and a half times that of nonpregnant females, indicating that the magnitude of the sex difference in the rate of hexobarbital metabolism was due to more than the difference between the cytochrome P-450 concentrations in microsomes from male and

female rats (Table 5). A number of investigators have also reported a two to three times higher rate of hexobarbital oxidation in hepatic microsomes from male rats (76, 80, 81, 82, 89, 90).

The apparent K_m for hexobarbital hydroxylase was lower in enzyme preparations from male rats (0.68 mM) than in those of females (1.07 mM), indicating that a lower concentration of substrate was needed to reach half maximal velocity in males. Schenkman et al (89), also reported apparent K_m values which indicated a greater affinity for hexobarbital in the microsomes of male rats compared to those of females.

3. Enzyme Activities with Several Drug Substrates

As previously noted, enzyme activities of hepatic 9000 xg S fraction from each of the three groups of animals were determined using aminopyrine, aniline, p-nitroanisole and p-nitrobenzoic acid as substrates. Enzyme activities are expressed as μmoles of product formed/30 minutes incubation/10 mg protein or per μmole cytochrome P-450 or per 100 g body weight and are recorded in Tables 7, 8 and 9, respectively.

a. Pregnant-nonpregnant female comparison

With the exception of the N-demethylation of aminopyrine, the rate of metabolism (per 10 mg 9000 xg S protein) of all substrates tested was significantly less ($P < .05$ to $P < .01$) in the hepatic systems from pregnant rats compared to those of nonpregnant females (Table 7). However, when these activities were expressed per μmole cytochrome P-450, the statistical significance of the differences was lost (Table 8).

In Table 9 enzyme activities are expressed on the basis of 100 g body weight, thereby taking into consideration the relation of body weight to liver weight and reflecting the metabolic load placed on the liver in vivo.

Table 7:

Enzyme activities were determined with aniline, p-nitroanisole, aminopyrine and p-nitrobenzoic acid, using the 9000 xg S fraction of livers from males, pregnant and nonpregnant females as enzyme source. Assays are described in Materials and Methods, section C. Data were collated and compared as described in Results, page 42. Activities are expressed as mμmoles of product formed/30 minutes incubation/10 mg protein.

TABLE 7

Comparison of activities of the NADPH-dependent enzymes from the three groups of animals

SUBSTRATE	PATHWAY	ENZYME ACTIVITY mμMOLES PRODUCT/30MIN/10MG PROTEIN*		
		Pregnant Female	Nonpregnant Female	Male
Aniline	Aromatic hydroxylation	35.1[b] ± 7.56 (16)**	55.8 ± 23.9/ (38)	71.5[a] ± 22.8 (21)
p-nitro anisole	o-demethylation	73.5[b] ± 17.0 (5)	109 ± 25.1 (17)	126 ± 12.7 (6)
amino-pyrine	n-demethylation	311 ± 48.0 (7)	293 ± 44.3 (11)	646[c] ± 89.0 (6)
p-nitro-benzoic acid	nitro-reduction	19.1[a] ± 9.43 (15)	30.2 ± 16.8 (37)	47.8 ± 24.3 (18)

* 9000 xg S fraction used

** Bracketed number is number of determinations in each case

/ Mean ± standard deviation

[a] Significantly different from nonpregnant female $P < .05$

[b] Significantly different from nonpregnant female $P < .01$

[c] Significantly different from nonpregnant female $P < .001$

Table 8:

The data in Table 7 were recalculated in terms of mμmoles of product formed/30 minutes incubation/mμmole cytochrome P-450. Each determination was recalculated individually and the data collated and compared as described for Table 7.

TABLE 8

Comparison of activities[1] of the NADPH-dependent enzymes from the three groups of animals

SUBSTRATE	PATHWAY	ENZYME ACTIVITY mμMOLES PRODUCT/30MIN/mμMOLE CYTOCHROME P-450		
		Pregnant Female	Nonpregnant Female	Male
Aniline	Aromatic hydroxylation	36.1 ± 8.14* (16)**	45.1 ± 17.8 (41)	43.2 ± 12.6 (21)
p-nitro anisole	o-demethylation	97.3 ± 24.1 (5)	88.8 ± 20.6 (19)	88.2 ± 19.5 (6)
amino-pyrine	n-demethylation	292 ± 78.0 (7)	261 ± 47.8 (11)	403[c] ± 35.1 (6)
p-nitro-benzoic acid	nitro-reduction	18.8 ± 7.06 (15)	25.8 ± 13.6 (39)	29.1 ± 14.4 (18)

[c] Significantly different from nonpregnant female $P < .001$

* Mean ± standard deviation

** Bracketed number is number of determinations in each case

[1] Activity recalculated per mμmole cytochrome P-450

Table 9:

Enzyme activities with aniline, p-nitroanisole, aminopyrine and p-nitrobenzoic acid as well as V_{max} for DPH and hexobarbital hydroxylases were recalculated in terms of their activity per 100 g body weight, as described at the foot of Table 9. Mean values were used in the recalculation.

TABLE 9

Comparison of enzyme activities or V_{max} of the NADPH-dependent enzymes, calculated on the basis of 100 g body weight in the three groups of animals

Substrate	Enzyme Activity μmoles/30min/100 g body weight*			% Difference	
	Pregnant Female	Nonpregnant Female	Male	Pregnant-nonpregnant	Male-nonpregnant
Aniline	1.71	2.82	4.12	-39.4	+46.1
p-nitro-anisole	4.61	5.56	8.48	-17.1	+52.5
amino-pyrine	13.8	16.3	38.5	-15.2	+136
p-nitro-benzoic acid	.89	1.62	2.78	-45.1	+71.6
	V_{max} μmoles/30 min/100 g body weight**				
DPH	1.72	2.12	2.71	-18.9	+27.8
hexo-barbital	4.74	8.51	34.1	-44.3	+300

* Values are calculated by multiplying μmoles product formed/μmole cytochrome P-450 (Table 8) by μmoles cytochrome P-450/100 g body weight (Table 5).

** Values are calculated by multiplying V_{max}/μmole cytochrome P-450 (Table 6) by μmoles cytochrome P-450/100 g body weight (Table 5).

It is noteworthy that on this basis the rate of metabolism of every substrate studied was lower in the pregnant group compared to the non-pregnant group. The two demethylation pathways, along with DPH hydroxylation were depressed approximately 15-19%, while the reduction of p-nitrobenzoic acid and the hydroxylation of both aniline and hexobarbital were approximately 40-45% less. These findings agreed with the report of Neale and Parke (13), who related the decreased biphenyl hydroxylase activity of liver microsomal preparations from full-term pregnant rats to the decrease in cytochrome P-450 content of liver per unit body weight.

b. Male-nonpregnant female comparison

Rates of product formation, calculated on the basis of 10 mg 9000 xg S protein (Table 7), showed, that for all substrates tested, the hepatic preparations from males were metabolically more active than those from nonpregnant females. This difference was statistically significant for the biotransformation of aniline ($P < .05$), p-nitrobenzoic acid ($P < .01$), aminopyrine ($P < .001$), but not significant in the case of p-nitroanisole.

When enzyme activities were calculated per mμmole cytochrome P-450 (Table 8), the data for the metabolism of aniline, p-nitroanisole and p-nitrobenzoic acid in males and nonpregnant female rats underwent a remarkable normalization. This normalization was consistent with the concept that cytochrome P-450 may be the rate limiting factor in the conversion of these substrates. The rate of aminopyrine demethylation in male rats was still one and a half times that of nonpregnant females, indicating that the greater activity was probably due to factors other than cytochrome P-450 concentration. Sex differences in the metabolism

of hexobarbital have already been discussed (section 2.b.ii) and the supportive work of several investigators cited. Our findings showing a sex difference in the metabolism of aminopyrine were also similar to the reports of a number of these authors (76, 81, 82, 89).

In Table 9, where enzyme activities are presented on the basis of 100 g body weight, enzyme preparations from males showed a greater activity with all drug substrates tested than those from nonpregnant females. The approximately 50% greater rate of aniline and p-nitroanisole metabolism were presumably related to the similar increase in the quantity of cytochrome P-450 available to males compared to females on the basis of 100 g body weight. The increases of 28% in DPH metabolism and 72% in nitroreduction by enzymes from male rats seemed to be less directly related to cytochrome P-450 concentrations. The metabolism of aminopyrine was 136% greater and that of hexobarbital 300% greater in male compared to nonpregnant female rats, differences which were several times the difference in concentration of cytochrome P-450 (per g% liver) available to the two groups of animals.

4. Summary of Data in Section C

Figures 9 and 10 are graphical representations of much of the data presented in section C. The normalization of the activities (or V_{max}) of the enzymes involved in the biotransformation of aniline, p-nitroanisole, p-nitrobenzoic acid and DPH when calculated per mμmole cytochrome P-450 is visually apparent (compare Figures 9 and 10). It is also apparent in Figure 10 that the metabolism of aminopyrine and hexobarbital by enzymes from male rats remains one and a half to two and a half times that of females even when expressed per unit cytochrome P-450.

Figure 9: Bar graph comparing enzyme activities in the three groups of animals on the basis of 10 mg protein.

Two ordinate scales are used because of the differences in rates of enzyme activity. Activities are plotted in terms of μmoles of product formed or substrate used per 30 minutes incubation per 10 mg protein (Tables 6 and 7). Values obtained with each substrate using the 9000 xg S fraction from livers of males, pregnant and nonpregnant female rats are juxtaposed to show the relative activities in each group of animals.

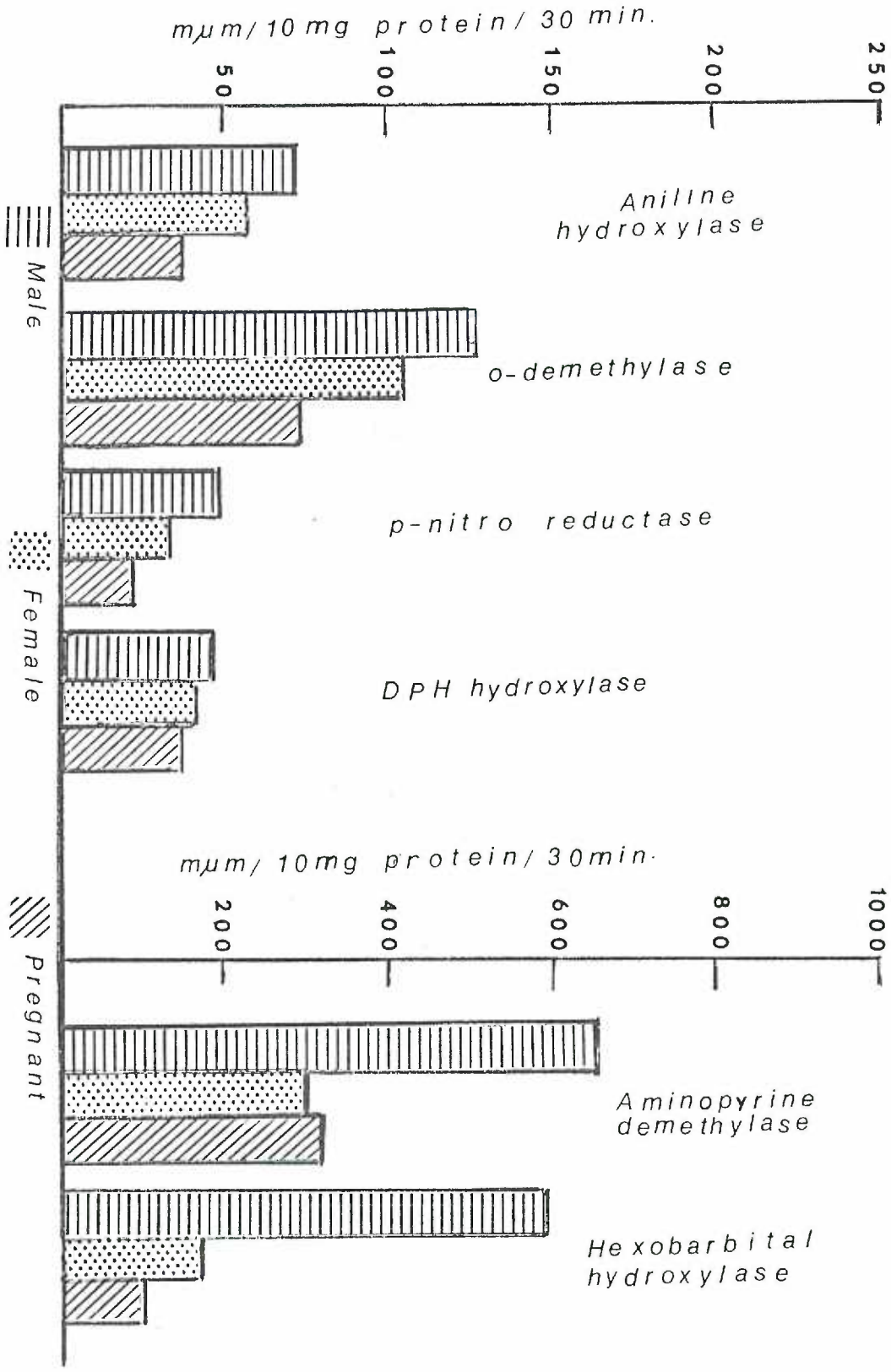
mμm/ 10 mg protein/ 30 min.
50
100
150
200
250
Aniline hydroxylase
o-demethylase
p-nitro reductase
DPH hydroxylase
Male
Female
mμm/ 10mg protein/ 30min.
200
400
600
800
1000
Aminopyrine demethylase
Hexobarbital hydroxylase
Pregnant

FIGURE 9

Figure 10: Bar graph comparing enzyme activities in the three groups of animals on the basis of a mμmole of cytochrome P-450.

Two ordinate scales are used because of the differences in rates of enzyme activity. Activities are plotted in terms of mμmoles of product formed or substrate used per 30 minutes incubation per mμmole cytochrome P-450 (Tables 6 and 8). Values obtained with each substrate using the 9000 xg S fraction from livers of male, pregnant and nonpregnant female rats are juxtaposed to show the relative activities in each group of animals.

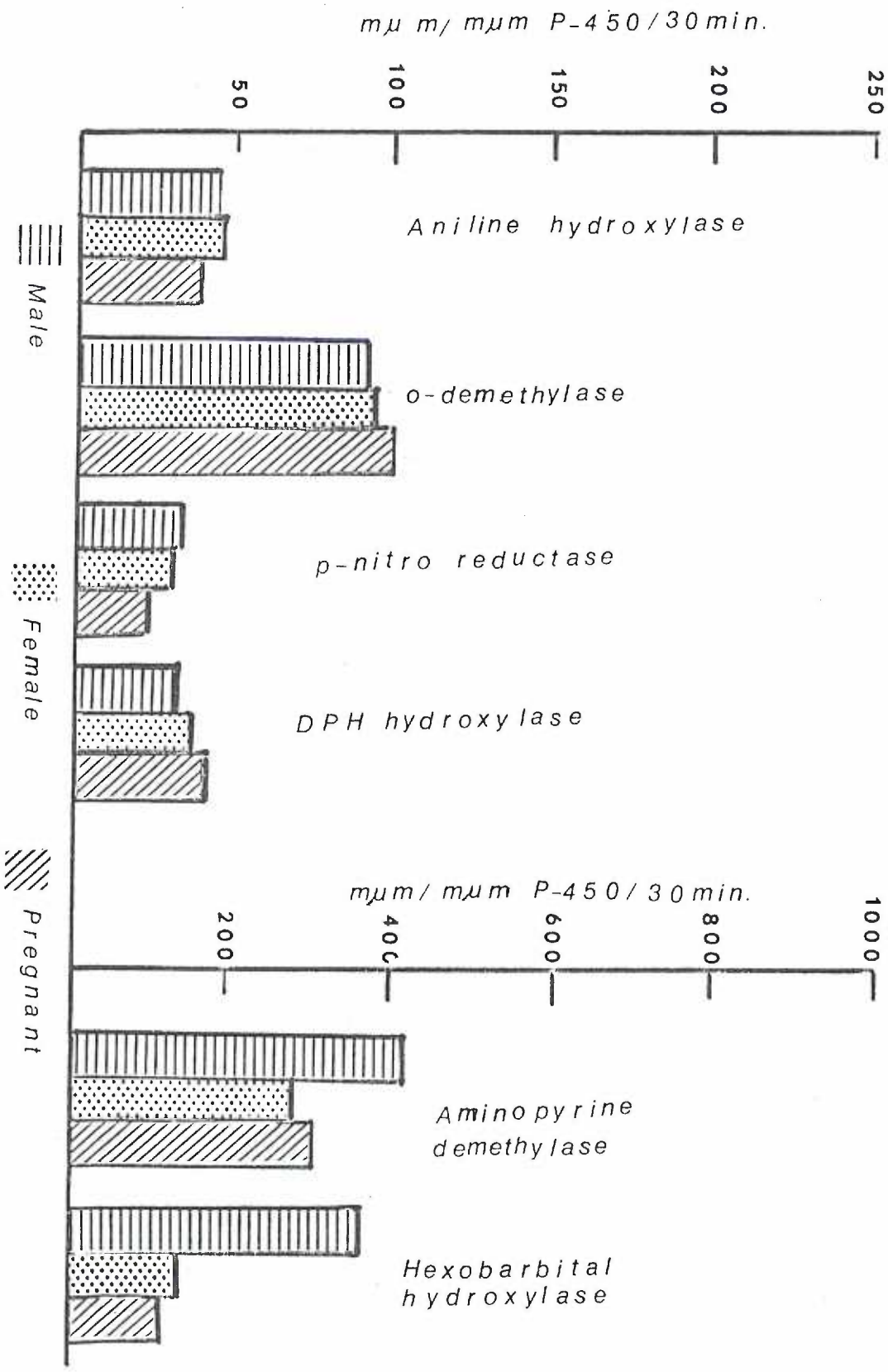
mμ m/ mμm P-450/30min.
50
100
150
200
250
Aniline hydroxylase
Male
o-demethylase
p-nitro reductase
Female
DPH hydroxylase
mμm/ mμm P-450/30min.
200
400
600
800
1000
Pregnant
Aminopyrine demethylase
Hexobarbital hydroxylase

FIGURE 10

D. COMPARISON OF SPECTRAL BINDING CONSTANTS WITH TYPE I AND II COMPOUNDS AMONG THE THREE GROUPS OF ANIMALS

1. Introduction

K_s, designated by Schenkman et al (88) as a "spectral dissociation constant", is operationally analagous to K_m, in that it is the concentration of substrate required for half-maximal spectral change, i.e., half $\triangle$ O.D.$_{max}$.

Spectral binding constants, K_s and $\triangle$ O.D. max for the binding of aniline (type II) and hexobarbital (type I) to hepatic microsomes of rats were determined and calculated as previously outlined and are presented in Table 10. Figures 11 and 12 are double reciprocal plots of the spectral binding data derived from each of the three groups of animals with aniline and hexobarbital, respectively.

2. Spectral Binding with Aniline

a. Pregnant-nonpregnant female comparison

The maximal change in absorbance per mg microsomal protein (Table 10), when aniline was added to microsomes of pregnant and nonpregnant females was 0.012 and 0.018, respectively, or a ratio of 2/3. When calculated per mμmole cytochrome P-450, this ratio approached 1 (0.028/0.030), indicating that the original differences in $\triangle$ O.D.$_{max}$ were related to the difference in cytochrome P-450 concentration of the microsomal suspensions from the two groups of animals.

A similar trend in aniline hydroxylase activity has already been cited (C.3.a.). The ratio of aniline metabolism in pregnant to nonpregnant female rats was 2/3 (35.14 mμmoles/55.80 mμmoles) when compared on the basis of 10 mg 9000 xg S protein (Table 7). This ratio became

Table 10:

Spectral binding constants for binding of aniline and hexobarbital to hepatic microsomal suspensions from male, pregnant and nonpregnant female rats were determined as described in Materials and Methods. Data were calculated and compared as described in Results, page 42. Δ O.D.$_{max}$ is expressed per mg microsomal protein and per mμmole cytochrome P-450. K_s is in mM.

TABLE 10

Spectral binding constants for the binding of Type I (hexobarbital) and Type II (aniline) substrates to the microsomes of the three groups of animals

SUBSTRATE	MEASUREMENT	ANIMAL GROUP		
		Pregnant Female	Nonpregnant Female	Male
	# of Determinations	(11)	(29)	(23)
Aniline	K_s - mM	0.49	0.45	0.46
	$\Delta O.D._{max}$ per mg m.p.[d]	0.012[b]	0.018	0.029[c]
	$\Delta O.D._{max}$ per mμmole P-450	0.028	0.030	0.040[a]
	# of Determinations	(6)	(5)	(3)
Hexobarbital	K_s - mM	0.20	0.20	0.26
	$\Delta O.D._{max}$ per mg m.p.	0.006	0.009	0.022[a]
	$\Delta O.D._{max}$ per mμmole P-450	0.015	0.015	0.030

[a] Significantly different from nonpregnant female $P < .05$

[b] Significantly different from nonpregnant female $P < .01$

[c] Significantly different from nonpregnant female $P < .001$

[d] m.p. refers to microsomal protein

Figure 11: Comparison by double reciprocal plots of aniline dependence of type II spectral change using microsomal suspensions from the three groups of animals.

Spectral change with aniline was determined as outlined in Materials and Methods using 1 - 1.5 mg microsomal protein/ml final solution and 5 concentrations of aniline (substrate), 0.25 mM, 0.5 mM, 1.0 mM, 2.0 mM and 4 mM. $\triangle$ O.D. was calculated for each substrate concentration (S) in terms of $\triangle$ O.D./mg protein. Reciprocals, $1/\triangle$ O.D. and 1/S were calculated, data collated and regression lines plotted as described for Figure 7. Each point represents mean $\pm$ standard deviation of number of determinations shown in Table 10, page 66. Intercept on ordinate gives value for $1/\triangle$ O.D.$_{max}$ and intercept on abscissa gives value for $1/K_s$. Males (. ——.), Females (o----o), Pregnant (x— ---- —x).

ANILINE BINDING

per mg protein

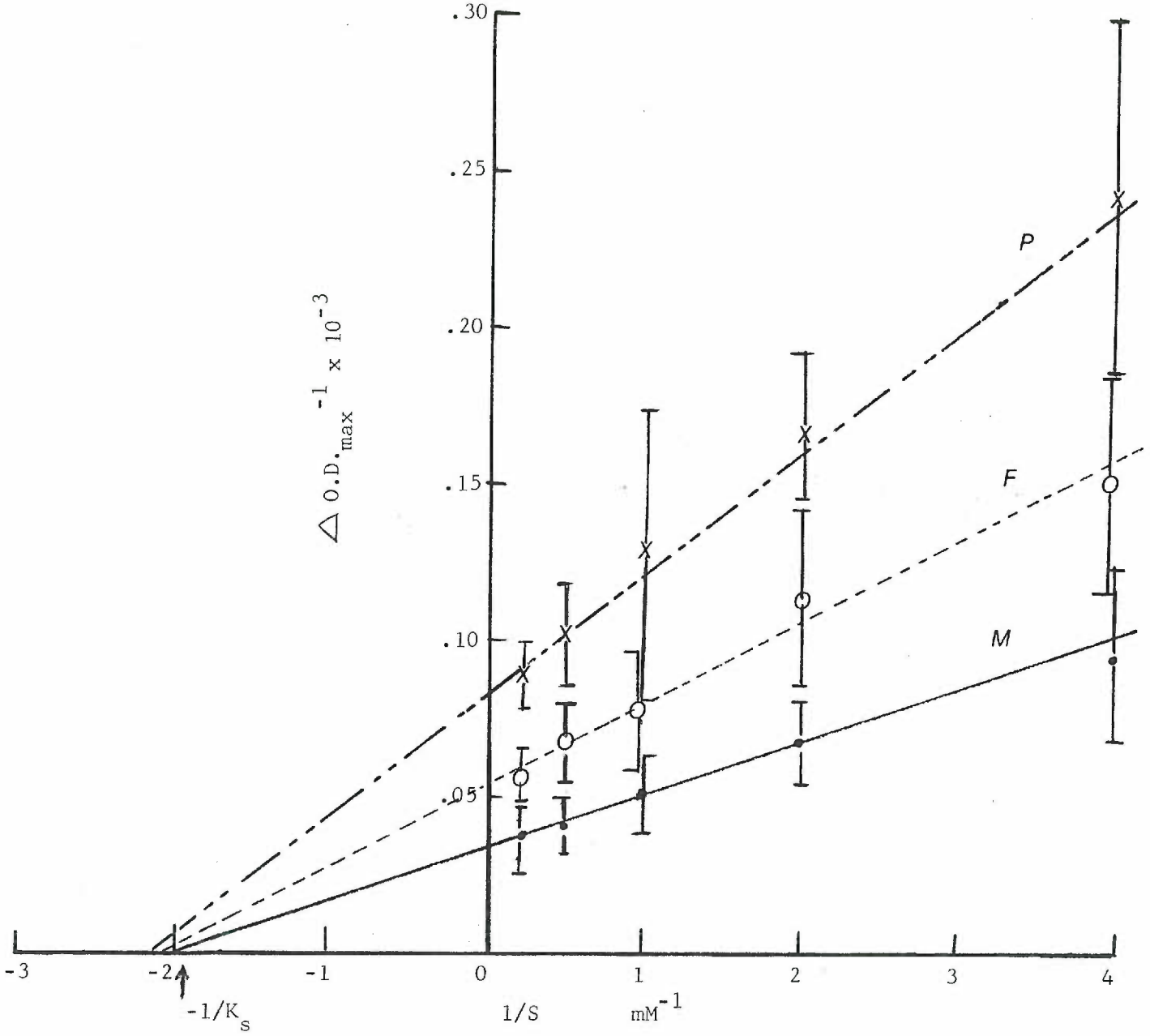

FIGURE 11

Figure 12: Comparison by double reciprocal plots of hexobarbital dependence of type I spectral change using microsomal suspensions from the three groups of animals.

Spectral change with hexobarbital was determined as outlined in Materials and Methods using 1.5 - 2.0 mg microsomal protein/ml final solution and 5 concentrations of hexobarbital, 0.125 mM, 0.25 mM, 0.5 mM, 1.0 mM and 2.0 mM. $\triangle$ O.D. was calculated for each substrate concentration (S) in terms of $\triangle$ O.D./mg protein. Reciprocals $1/\triangle$ O.D. and 1/S were calculated, data collated and regression lines plotted as described for Figure 7. Each point represents mean $\pm$ standard deviation of number of determinations shown in Table 10, page 66. Intercept on ordinate gives value for $1/\triangle$ O.D.$_{max}$ and intercept on abscissa gives value for $1/K_s$. Males (.____.), Females (o----o), Pregnant (x— ---- —x).

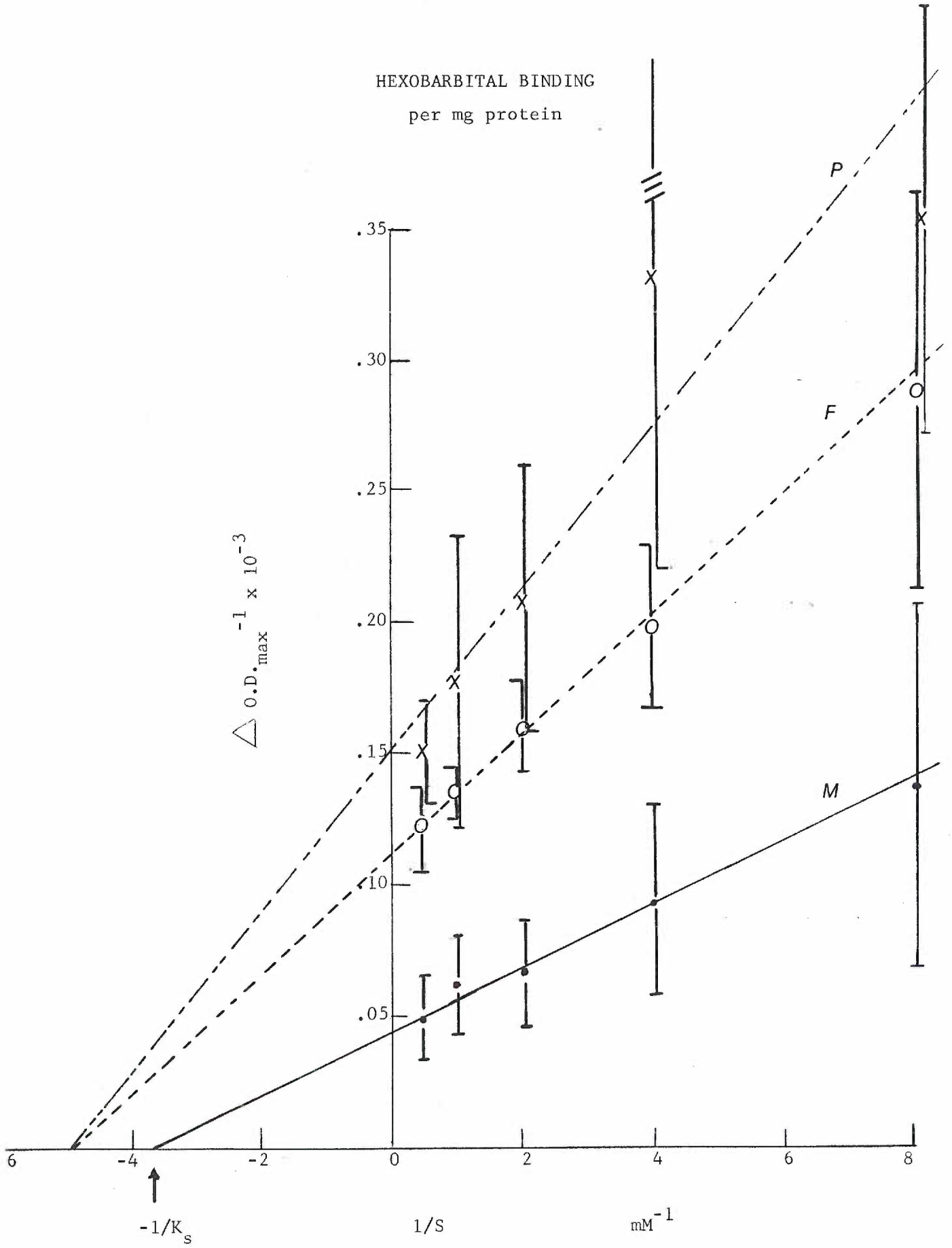
HEXOBARBITAL BINDING
per mg protein
P
F
M
.35
.30
.25
.20
.15
.10
.05
△ O.D.max -1 x 10-3
6
-4
-2
0
2
4
6
8
-1/Ks
1/S
mM-1

FIGURE 12

4/5 (36.08 mμmoles/45.10 mμmoles) when calculated per mμmole cytochrome P-450, but the statistical significance of the difference between the two groups of animals was eliminated (Table 8).

K_s values for the binding of aniline to microsomal suspensions from pregnant (0.49 mM) and nonpregnant females (0.45 mM) were almost the same (Figure 11), indicating that there were probably no qualitative differences in the binding capabilities of these enzyme preparations as measured spectrophotometrically.

b. Male-nonpregnant female comparison

Addition of aniline to microsomes from male rats produced a maximal spectral change of 0.029/mg microsomal protein, approximately one and a half times the Δ O.D.$_{max}$ of 0.018/mg microsomal protein generated with microsomes from nonpregnant females (Table 10). When calculated per mμmole cytochrome P-450, Δ O.D.$_{max}$ for males remained one third greater than that of females (0.040/0.030), indicating that the difference in the values for Δ O.D.$_{max}$ between the two groups of animals was greater than the difference in the cytochrome P-450 concentration of their respective microsomal suspensions.

Unlike these spectral binding data, our previous comparison of aniline hydroxylation rates in male and female rats (C.3.b.) did reveal a parallel between these activities and cytochrome P-450 concentration. The difference in rates of aniline metabolism per 10 mg 9000 xg S protein, between males and females (71.5 mμmoles vs. 55.5 mμmoles, respectively, Table 7) was eliminated when enzyme activities were calculated per mμmole cytochrome P-450 (43.2 mμmoles vs. 45.1 mμmoles, respectively, Table 8).

Guarino et al (117) found a similar relationship when comparing V_{max}

and $\triangle$ O.D.$_{max}$ for aniline hydroxylation and spectral binding, respectively, in the hepatic microsomal suspensions from phenobarbital treated rats. The increases in V_{max} and cytochrome P-450 concentration in microsomes from livers of treated rats were of the same magnitude, while the increase in $\triangle$ O.D.$_{max}$ was almost twice the elevation in cytochrome P-450 concentration. This suggested to the authors that phenobarbital may have induced synthesis of hemoprotein capable of binding aniline, as measured spectrophotometrically, but not necessarily directly involved in the metabolism of aniline.

Schenkman et al (89), on the other hand, reported that the sex difference in their values for V_{max} and $\triangle$ O.D.$_{max}$ for aniline was, in each case, of the same magnitude as the difference in cytochrome P-450 concentration between microsomes of male and female rats.

K_s values for aniline binding to microsomes of males (0.46 mM) and nonpregnant females (0.45 mM) were almost identical (Figure 11). Schenkman et al (89) were also unable to show any differences in the binding capacity of their enzyme preparations from male and female rat livers (K_s = 0.5 mM).

3. Spectral Binding with Hexobarbital

a. Pregnant-nonpregnant female comparison

Hexobarbital, added to hepatic microsomal suspensions from pregnant and nonpregnant female rats produced a maximal spectral change of 0.006 and 0.009 O.D. units per mg microsomal protein, respectively. When converted to $\triangle$ O.D.$_{max}$ per mμmole cytochrome P-450, the values for the two groups of animals became identical (0.015/mμmole cytochrome P-450, Table 10), indicating that the original difference in maximal binding was

probably due to the difference in cytochrome P-450 concentration of the microsomal suspensions. Similarly, the difference in V_{max} for hexobarbital hydroxylase between microsomes from pregnant and nonpregnant females lost its statistical significance when expressed per mμmole cytochrome P-450 (C.2.b.i. and Table 6).

K_s for the binding of hexobarbital to microsomal suspensions from both groups of animals were identical (0.20 mM), showing that there were no qualitative differences in the binding proteins from pregnant and nonpregnant females (Figure 12). However, variation in apparent K_m from pregnant (0.37 mM) and nonpregnant females (1.07 mM) seemed to indicate a qualitative difference in their hexobarbital hydroxylases. We are unable to explain this inconsistency between the two substrate constants (K_s and K_m) for hexobarbital.

b. Male-nonpregnant female comparison

Addition of hexobarbital to hepatic microsomal suspensions from male and female rats resulted in a $\triangle$ O.D.$_{max}$/mg microsomal protein of 0.022 and 0.009 optical density absorbance units, respectively, a ratio of 2.5/1 (Table 10). When these values were calculated per mμmole cytochrome P-450, thereby compensating for the difference in hemoprotein concentration in microsomes from these two animal groups, the difference in $\triangle$O.D.$_{max}$ was not eliminated and the ratio became 2/1 (0.030/0.015, Table 10). This sex difference was also apparent in the hydroxylation of hexobarbital (C.2.b.ii.). V_{max} per 10 mg 9000 xg S protein in males was 3.6 times that of female rats (Table 6). When maximal activities were expressed per mμmole cytochrome P-450, V_{max} for males remained 2.6 times that of females, indicating that the higher activity with microsomal

preparations from males was related to factors other than cytochrome P-450 concentration. These striking differences in V_{max} and $\Delta O.D._{max}$ between male and nonpregnant females were of similar magnitude and probably related to a common factor. No attempt was made to identify that factor.

Schenkman et al (89) also reported that microsomes from male rat livers oxidized hexobarbital at approximately three times the rate of microsomes from livers of females. This correlated with the maximal spectral change with hexobarbital, which was 2.5 times higher in microsomes from male liver. Their K_s values (males, 0.03 mM; females, 0.17 mM) and K_m values (males, 0.1 mM; females 0.17 mM) were both lower in microsomes from males, indicating a greater affinity of drug to enzyme in males. Although our determinations of apparent K_m (males, 0.68 mM; females, 1.07 mM, Table 6) were six times as high as those of Schenkman et al (89), they showed the same variation. Our K_s values (males, 0.26 mM; females, 0.20 mM, Table 10) varied only 25% while those of Schenkman et al (89) varied six fold between males and females.

DISCUSSION

Our data show that the concentration of hepatic microsomal cytochrome P-450 is significantly reduced ($P < .001$) in late pregnancy, when calculated in terms of mg microsomal protein, g liver and g% liver (Table 5). The hypertrophy of liver associated with pregnancy apparently does not cause general dilution of hepatic protein, since a comparison of protein concentration of the 9000 xg S from livers of pregnant and nonpregnant females shows no difference on a per g liver basis. A similar comparison of microsomal protein concentration (per g liver) reveals a significant increase ($P < .05$) in that fraction from pregnant females. Total cytochrome P-450 content expressed as a function of whole liver is also greater in the pregnant female (Table 4). However, the increase in liver weight during gestation does not keep pace with gross maternal body weight. The near term pregnant female has approximately 10% less hepatic tissue with which to monitor a standard unit of body weight (3.34 g% liver vs. 3.67 g% liver, Table 3).

We also find a reduced rate of biotransformation of aniline, p-nitroanisole and p-nitrobenzoic acid and a lower V_{max} for DPH and hexobarbital hydroxylases in late pregnancy when comparing hepatic 9000 xg S from pregnant and nonpregnant rats (per 10 mg protein, Tables 6 and 7). When these rates of drug metabolism are normalized by expressing them in terms of mμmole cytochrome P-450, no significant differences remain between pregnant and nonpregnant females in the biotransformation of these substrates, including aminopyrine (Tables 6 and 8). Consequently our experiments show a reduced in vitro drug metabolizing activity in late pregnancy that may be causally related to the reduction in cytochrome

P-450 concentration in hepatic microsomes from pregnant rats. This suggests, as did the experiments of Guarino et al (14), that the rate of drug metabolism in pregnant as well as nonpregnant rats is limited by the concentration of cytochrome P-450 in their hepatic microsomes.

Neale and Parke (13) reported a 22% decrease in biphenyl hydroxylation and cytochrome P-450 concentration in microsomal preparations from full-term pregnant rats when expressed in terms of a unit of liver weight. Both measurements were significantly increased in the pregnant animals "when the results were expressed as a function of total liver weight." "The total capacity of the pregnant animal to metabolize foreign compounds was increased." However, since the pregnant animal also increased in weight, "the hydroxylating activity and cytochrome P-450 content per unit body weight decreased." According to the authors this was "confirmed by the fact that sleeping time in rats dosed with 100 mg hexobarbital/kg of total body weight was increased by 32% in full-term pregnant rats." When we calculate enzyme activity in a similar manner, in terms of g% liver (Table 9), we find a reduction of 15-45% in the rate of biotransformation of all test substrates, which correlates reasonably well with the 25% lower cytochrome P-450 concentration in the near term pregnant rat. This evidence, along with the other in vitro studies showing a reduction in activity in the microsomal drug metabolizing enzymes from pregnant females (12, 13, 14) seems to justify the inference that pregnancy impairs the in vivo metabolism of drugs (8, 9, 10, 11).

It may be simply fortuitous that these in vitro and in vivo data agreed. According to Gillette et al (118), "even when the investigator is interested only in relating the activity of the drug metabolizing

enzymes in liver to the biological half-life of the drug in the animal, a number of problems may arise which are difficult or even impossible to evaluate with liver preparations alone." Gram et al (119) reported that starvation altered both K_m and V_{max} of microsomal hexobarbital hydroxylase, suggesting that these changes were reflected in the prolonged hexobarbital sleeping time shown by fasted rats. Gillette, one of the co-authors of this study, converted their data to show rate of drug clearance from the liver (7) and on this basis found that the half-life of hexobarbital would not have been significantly different in the fasted and control animals. He concluded that this alteration of kinetic properties of the microsomal enzyme would not alone have accounted for the increased sleeping time. In an experiment using a liver-perfusion system, Thurman and Scholz (120) obtained evidence that starvation may decrease the metabolism of drugs by decreasing the NADPH level of hepatocytes. Such data is unobtainable with the standard NADPH-dependent test system, since an excess of NADPH is added to microsomes to maintain maximal enzyme activity.

The activity of the microsomal drug metabolizing enzymes can be altered by a number of other treatments, including removal of endocrine glands, administration of hormones and repeated administration of a number of drugs such as phenobarbital. Initial studies of variation in enzyme activity were often limited to a determination of cytochrome P-450 concentration, but it soon became apparent that the activity of microsomal enzymes was not always proportional to the concentration of this hemoprotein. Castro et al (83) showed that the decrease in ethylmorphine demethylase activity in microsomes from adrenalectomized male rats

paralleled the decrease in NADPH-cytochrome c reductase and cytochrome P-450 reductase, rather than the decrease in cytochrome P-450 concentration per se, or its ability to bind substrate. Remmer et al (121) found a correspondence between the rate of hydroxylation and the magnitude of spectral change with hexobarbital using microsomes prepared from livers of phenobarbital induced rats. Increases in both parameters were several times greater than the increase in cytochrome P-450 concentration. Sex difference in the metabolism of a number of drug substrates by rats, unrelated to the differences in cytochrome P-450 concentration in their hepatic microsomes, has been shown by several investigators (89, 90). These studies reported a correlation between the increased metabolism of hexobarbital (89, 90) and aminopyrine (89) and the increased magnitude of spectral change with these type I substrates in microsomes from male compared to female rats.

Our data indicate that the sex difference in V_{max} for hexobarbital is more closely related to this difference in Δ O.D.$_{max}$ for hexobarbital (Tables 6 and 10) than to differences in cytochrome P-450 concentration in microsomes from male and female rats (Table 5). According to Kato et al (90), sex difference may be related to the action of androgen in male rats to alter the binding capacity of cytochrome P-450 for hexobarbital. Our K_s values for spectral binding with hexobarbital are fairly close, showing little difference in affinity of the binding protein for this substrate in microsomes from male and nonpregnant female rats (Table 10). On the other hand, apparent K_m values do indicate a greater affinity of the terminal oxidase for hexobarbital in microsomes from males (Table 6). Thus it would appear that K_m and K_s values cannot be related to each

other with impunity.

Studies showing the effect of androgenic and anabolic steroids on the activity of the drug metabolizing enzymes in male rats bring to the fore the whole question of steroid-drug interaction. Conney (54), in his review of microsomal enzyme induction, listed a number of similarities between drug and steroid hydroxylases in liver microsomes. Kuntzman et al (73) reported that agents which stimulated microsomal oxidation of drugs also stimulated microsomal hydroxylation of steroids. Conney et al (122) found that CO inhibited steroid hydroxylation by liver microsomes as well as many drug hydroxylation reactions. The low Michaelis constant for hydroxylation of testosterone, progesterone and estradiol by liver microsomes would suggest that these steroids are normal substrates for the oxidative drug metabolizing enzymes (123), and on the basis of apparent K_m's should be powerful competitors for these enzymes. Tephly and Mannering (74) have shown that steroids such as estradiol, testosterone and androsterone, added in vitro to microsomal suspensions, competitively inhibited the metabolism of a number of drugs. Juchau and Fouts (75) confirmed that very low concentrations of progesterone and norethynodrel added to microsomes inhibited the hydroxylation of hexobarbital, aniline and zoxazolamine. Kutt and Verebely (97) reported inhibition of DPH metabolism in vitro at relatively high concentrations of testosterone, estrone and progesterone (10^{-3} M to 10^{-2} M).

Although it has been confirmed that steroid hormones added in vitro competitively inhibit microsomal drug metabolism, the in vivo data are not unequivocal. Werk et al (26) attributed the stimulation of extra-adrenal metabolism of cortisol to 6 β cortisol during DPH therapy to the

possible inducing effect of DPH on drug metabolizing enzymes. Conney and coworkers (124) found a decrease in the anaesthetic action of deoxycorticosterone, androsterone and large doses of progesterone in rats treated with phenobarbital. While Remmer (15) conceded that endogenous steroids were hydroxylated by the microsomal drug hydroxylases to a very small extent, he contended that liver contains enzymes which are specific for the inactivation of these hormones prior to excretion (125). One set of enzymes forms the 17 ketosteroids and another set is responsible for A ring reduction to tetrahydro compounds which are then conjugated and excreted.

Even if steroid hormones are not the natural substrates of the hepatic drug metabolizing enzymes, it is still conceivable that high levels of circulating progesterone and estrogenic hormones during pregnancy might competitively inhibit drug metabolism by these microsomal enzymes in vivo. Since we did not test specifically for the presence of these endogenous steroids in our enzyme preparations, we cannot rule them out or implicate them directly in the impaired drug metabolizing capability shown in late pregnancy. Determinations of kinetic and spectral binding constants were undertaken, in part, to reveal the possible presence of endogenous inhibitors or activators in our system. We have already discussed the correlation between the decrease in V_{max} for DPH and hexobarbital hydroxylases in late pregnancy and the difference in the cytochrome P-450 concentration of liver microsomes from pregnant and nonpregnant females (Table 6). The values for Δ O.D.$_{max}$ with aniline (type II) and hexobarbital (type I) in microsomes from pregnant and nonpregnant females also show a direct correlation with cytochrome P-450

concentration (Table 10). K_s values for these substrates are almost identical in both groups of animals, indicating that there are no qualitative differences in the binding characteristics of their respective microsomal components (Table 10). Apparent K_m for DPH hydroxylase does not differ between pregnant and control animals but apparent K_m for hexobarbital hydroxylase in microsomal suspensions from pregnant females is one third that of nonpregnant controls (Table 6). This greater affinity of the terminal oxidase from pregnant rats for hexobarbital may be due to the presence of an endogenous activator carried into our in vitro test system. This is the only indication of possible qualitative difference between the enzyme preparations from pregnant and control animals shown in our study.

Guarino et al (14) found no differences between control and pregnant animals in apparent K_m for either aniline or ethylmorphine, thus ruling out the presence of an endogenous inhibitor in their in vitro study. They reasoned that the high levels of circulating steroids might competitively inhibit drug metabolism by the microsomal enzymes in the intact animal and their failure to observe such inhibition may have been due to the dilution or loss of the inhibitor (if indeed one was present) during preparation of microsomes.

This illustrates once again the difficulty of relating in vitro data to what occurs in the living animal. Interpretation of data from induction experiments may also give rise to such difficulty. The design of these experiments often involves repeated pretreatment with the inducing substance and verification of induction by demonstrating increased in vitro metabolism of a test drug. Gabler (unpublished data) found that DPH metabolism was inhibited while aniline metabolism was stimulated in

the 9000 xg S or microsomal fractions from livers of ^{14}C-phenobarbital treated rats compared to controls. A search for the cause of these contradictory data led to the discovery that ^{14}C-phenobarbital was carried into the enzyme preparations, inhibiting DPH metabolism and masking the induction of the microsomal enzymes.

Juchau and Fouts (75) also reported that the synthetic progestational steroid, norethynodrel, either stimulated or inhibited metabolism of hexobarbital or zoxazolamine depending upon the time interval between the last pretreatment with steroid and sacrifice of the animal. If the time interval was short, metabolism of hexobarbital and zoxazolamine was invariably less than the non-treated control. If, on the other hand, the time interval was greater than 20 hours, liver preparations displayed a significantly greater drug metabolizing activity than controls. Since norethynodrel had been shown by them to inhibit hexobarbital and zoxazolamine metabolism in vitro, it would be reasonable to assume that when the animals were sacrificed shortly after an inducing dose of the drug some of the steroid was carried into the liver preparations, thereby inhibiting drug metabolism.

Gerber and Arnold (96) studied DPH metabolism in mice, reporting that repeated pretreatment with DPH (1 mg for 4 days) induced synthesis of enzymes involved in the metabolism of DPH, as shown by a reduction in DPH $t_{\frac{1}{2}}$ from 16 hours to 7.5 hours. A parallel study of the effect of similar DPH pretreatment on hexobarbital sleeping time and zoxazolamine paralysis time showed increased sleeping and paralysis time in the treated rats when measurements of drug effects were made 1½ hours after the injection of DPH. When these measurements were made 40 hours after

final DPH injection, there was a significant decrease in the sleeping and paralysis time. These data were explained by the fact that approximately 90% of the DPH remained in the mouse 1½ hours after injection and may have competed for metabolism with hexobarbital and zoxazolamine. After 40 hours, less than 0.1% of the DPH injected remained and hexobarbital and zoxazolamine were metabolized at an increased rate due to the induction of the microsomal enzymes.

The investigations discussed above show clearly that modifiers of drug metabolizing enzymes may be carried over into an in vitro system. It is known that such modifiers can alter independently either the K_m or V_{max} of an enzyme catalyzed reaction. Therefore, the similarity shown in our K_m values in liver preparations from pregnant and nonpregnant animals does not completely rule out the possible presence of an endogenous modifier in our in vitro systems. Since we did not undertake a search for such a modifier, this question must remain open.

The duration of drug action in the body is seldom determined solely by its rate of biotransformation. Other factors such as absorption, binding to plasma proteins and tissue receptors, distribution to various tissues, conjugation and excretion all play a role (126). It is conceivable that pregnancy could modify any or all of these processes in the living animal.

An alteration in the activity of the conjugating enzymes could have an effect on the duration of action of a drug requiring conjugation prior to excretion. Recently Levi and Ashley (127) demonstrated that inhibition of glucuronyl conjugation of pHPPH by salicylamide, administered in vivo, led to a prolonged plasma half-life of DPH. They speculated that

an accumulation of pHPPH in the liver resulted in a feedback inhibition of the biotransformation of DPH to pHPPH. Creaven and Parke (12) reported a reduced rate of glucuronyl conjugation of dl-borneal in liver homogenates from rats during late pregnancy when compared to its conjugation rate in nonpregnant controls. Gabler (unpublished data) was unable to demonstrate any difference in either V_{max} or K_m of UDP-glucuronyltransferase prepared from livers of pregnant and control rats when pHPPH or p-nitrophenol were used as substrates. Recent evidence indicates that UDP-glucuronyltransferase is a family of enzymes, with each member having a rather limited specificity. Pregnancy may cause an alteration in activity of some conjugating enzymes and not affect others.

A change in the percentage of drug bound could have a profound effect on the half-life and pharmacologic activity of a drug. According to Gillette (7), an increase in the percent of bound drug from 90 to 95% (a reduction of 50% in free drug concentration) could increase its half-life by as much as 50%. Plasma drug binding has been correlated with plasma protein concentration, and in the case of many drugs binding is dependent specifically on albumin concentration in the plasma. A number of investigators (128-131) have shown that the total protein concentration in blood falls during pregnancy (7-10%), resulting primarily from a decrease in plasma albumin (16-32%). Decreases in serum protein and serum albumin begin in the first trimester of pregnancy and become progressively more marked as gestation advances. Brown (132) reported the binding of phenolsulfonphthalein to plasma protein decreased in a similar manner.

Steroids are known to be transported in the plasma, bound in part

to albumin. The increased steroid levels during pregnancy could alter the affinity of plasma proteins for the binding of exogenous substances, like drugs. Their potential role as competitors for plasma protein binding sites should be investigated. A change in the physiological status of an individual has already been shown to alter plasma protein-drug interaction. For example, uremia and hyperbilirubinemia decrease the capacity of blood to bind several drugs (133, 134).

Decreased drug-plasma protein interaction due to decreased plasma protein concentration or competition for binding sites by endogenous agents would imply a shorter biological half-life for the drug. It might also lead to a greater availability of the drug for entrance into other non-vascular compartments. Anton and Rodriquez (135) administered sulfamethoxypyridazine (SMP) to pregnant rats and measured the concentration of the drug in a number of maternal and fetal tissues. They then gave a second group of pregnant rats both SMP and sulfinpyrazone (SPZ), a drug that competes with SMP for available binding sites on albumin. They reported a highly significant difference in the distribution of SMP, with marked elevation in its concentration in the fetal compartment. By analogy, any factor reducing the number of available binding sites in the plasma during pregnancy, for example, a reduction in albumin concentration or competition with endogenous agents could alter drug distribution with potentially hazardous consequences to both maternal and fetal animal. Since the fetus has, at best, a limited capacity to metabolize foreign substances (111), a drug after entering the fetal compartment must re-enter the maternal compartment for catabolism. In this way decreased binding of drug by plasma proteins could conceivably

prolong its half-life in the body when the recipient is pregnant.

"Since pregnancy is associated with dramatic and profound physiologic and biochemical changes in the maternal organism it would not be surprising if the drug metabolizing capability of the mother was also altered" (2). We have pointed out a number of ways in which pregnancy can modify other factors involved in limiting drug action. The net effect of all these possible modifications could be the nullification or accentuation of the approximately 25% impairment in in vitro drug metabolism shown in this study. Consequently the conclusions drawn from our experiments should be applied with restraint to the intact animal. Further caution must be exerted when extrapolating data from animal experiments to the human species where the rate of drug metabolism is two to ten times slower than that found in the rat (15). Because of the importance of this area, substantial effort is needed if a more rational approach to drug therapy during the period of gestation is to be developed. However, we feel confident to assert that drug metabolism is altered by pregnancy and that drugs should be administered only for the protection of mother and unborn child and the recipient closely monitored.

SUMMARY

1. Both liver weight and total body weight increased in the pregnant female rat as compared to the nonpregnant female control. Maternal weight gain exceeded liver hypertrophy so that at term the pregnant female had approximately 10% less liver available to monitor 100 g body weight (10% less g% liver).
2. Net synthesis of protein seemed to keep pace with liver enlargement during pregnancy since the concentrations of protein in the 9000 xg S fractions from pregnant and nonpregnant females were almost the same. Interestingly, microsomal protein concentration per g liver increased during gestation, indicating that the increased liver weight was due, at least in part, to protein synthesis.
3. Microsomal cytochrome P-450 concentration, expressed in terms of mμmoles per mg microsomal protein, per g liver or per g% liver was very significantly decreased in late pregnancy. However, total cytochrome P-450 content, expressed as a function of whole liver was increased in the pregnant female near term. Cytochrome b_5 concentration followed similar trends but differences in its concentration were not as great as those shown with cytochrome P-450.
4. When expressed in terms of enzyme activity per 10 mg 9000 xg S protein, isolated from livers of pregnant and nonpregnant rats, the metabolism of aniline, p-nitroanisole and p-nitrobenzoic acid was significantly decreased ($P < .05$ or less) in systems prepared from pregnant animals. No significant difference in the biotransformation of aminopyrine was noted between the two groups of animals. If the activities were "normalized" with respect to the cytochrome P-450

concentrations in the enzyme preparations from the two groups of animals (expressed per mμmole cytochrome P-450), the differences in enzyme activities were eliminated.

If, on the other hand, these activities were compared on the basis of 100 g body weight (g% liver), there was a 15-45% reduction in the rates of metabolism of all test substrates in enzyme preparations from pregnant rats. This corresponded reasonably well to the approximately 25% decrease in cytochrome P-450 concentration per g% liver in late pregnancy.

5. Similarly, V_{max}'s for DPH and hexobarbital hydroxylases were also decreased in late pregnancy when expressed in terms of 10 mg 9000 xg S protein or g% liver. However, when these data were normalized as previously described, the differences shown in maximum velocities of these enzymes between the two groups of animals were lost.

The apparent K_m values for DPH hydroxylase were almost identical in both groups of animals, while apparent K_m for hexobarbital hydroxylase in the pregnant female was one third that of the nonpregnant control. The reason for the greater affinity of the terminal oxidase for hexobarbital in the pregnant female is not known at present.

6. The maximal spectral change (Δ O.D.$_{max}$) using hexobarbital (type I) and aniline (type II) as binding ligands was decreased in microsomal suspensions from pregnant as compared to nonpregnant females. These values were also equalized when expressed in terms of unit cytochrome P-450. K_s values with either substrate were almost identical in the two groups of animals, indicating that there were probably no qualitative differences in the binding capabilities of their microsomal

suspensions, as measured spectrophotometrically.

7. The equalization which occurred when the various measurements were normalized in terms of a mumole of cytochrome P-450, indicated that the activity of this hemoprotein was possibly rate limiting. Therefore the reduction found in the biotransformation capabilities of the microsomal drug metabolizing enzymes in late pregnancy may be causally related to the reduction in cytochrome P-450 concentration in liver of pregnant rats.

8. A parallel series of comparisons was made between nonpregnant females and a group of male rats. Our data showed substantial agreement with the published reports of several similar studies comparing the two groups of rats. Most of the values for parameters measured were greater in livers of male rats compared to those of females. Cytochrome P-450 concentration was significantly greater in liver from male rats when expressed either in terms of mg microsomal protein or g liver, and was approximately 50% greater on the basis of g% liver. All enzyme pathways showed a greater activity in males compared to females paralleling or exceeding the increased cytochrome P-450 concentration. The sex differences in V_{max} and $\triangle O.D._{max}$ for hexobarbital were of the same magnitude and each was several fold greater than the difference in cytochrome P-450 concentration. These differences indicated that something other than cytochrome P-450 concentration plays a role in controlling enzyme activity and spectral binding in the case of certain type I substrates in male rats.

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