THE INFLUENCE OF CIGARETTE SMOKE EXPOSURE ON DIAZEPAM METABOLISM IN THE GUINEA PIG MATERNAL-FETAL UNIT

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Diazepam metabolism in the guinea pig maternal-fetal unit

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ABSTRACT

The placental transfer , disposition and biotransformation of diazepam (DZ) was examined in the materno-placento-fetal unit of term guinea pigs. Pharmacokinetic data was compiled by quantitating plasma and tissue levels of DZ and metabolites by gas chromatography. N-desmethyldiazepam (NDZ) was the sole metabolite detected in guinea pig plasma. The $\beta t1/2$ values of DZ and NDZ were 1.4 and 5.3 hr. Both DZ and NDZ were distributed to the maternal and fetal liver, adipose tissue and brain, and the placenta, 60 min after an oral dose of DZ (10 mg/kg). Levels in fetal tissues exceeded those of dam plasma.

The *in vitro* N-demethylation of diazepam and aminopyrine (AP) and the hydroxylation of benzo(a)pyrene (BP) were deficient in hepatic microsomes of the fetus compared to the adult. The placenta was capable of N-demethylating DZ and AP. *In vitro* biotransformation was studied using compounds known to modify cytochrome P448 activity - an effect that has been reported following exposure to cigarette smoke. Fetal liver was responsive neither to monooxygenase induction by β-naphthoflavone administration nor to inhibition by incubation with α-naphthoflavone, whereas the liver of the dam did respond. Following exposure to cigarette smoke for the last 10 days of pregnancy, N-demethylation of DZ and AP in the fetal liver alone displayed induction. BP hydroxylation was markedly enhanced in microsomes from both the fetal liver and the placenta.

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CONDENSE

Le transfert placentaire, la disposition et la biotransformation de diazépam (DZ) ont été examinés à la fin de la gestation dans l'unité maternel-placentaire-foetal des cochons d'Inde. Les données pharmacocinétiques étaient compilées en quantifiant par chromatographie gazeuse les taux plasmatiques et tissulaires de diazépam et de ses métabolites. Le N-desméthyldiazépam (NDZ) était le seul métabolite détecté dans le plasma des cochons d'Inde. Les valeurs $t_{1/2}$ de DZ et de NDZ étaient 1,4 h et 5,3 h. Une heure après l'administration orale de diazépam (10 mg/kg), le DZ et le NDZ étaient distribués aux foies, aux tissus adipeux, aux cervaux maternels et foetaux, et aux placentas. Les taux relevés dans les tissus foetaux ont excédé ceux du plasma maternel.

La N-déméthylation du DZ et de l'aminopyrine (AP) et l'hydroxylation du benzo(a)pyrene (BP) *in vitro* étaient déficientes dans les microsomes hépatiques du foetus par rapport à celles de l'adulte. Le placenta était capable de N-déméthyler le DZ et l'AP. La biotransformation *in vitro* était étudiée en utilisant des substances qui modifient l'activité du cytochrome P448 - un effet signalé à la suite d'exposition à la fumée de cigarette. Le foie foetal n'était sensible ni à l'induction de la monooxygénase par l'administration de β-naphthoflavone, ni à l'inhibition par incubation avec le α-naphthoflavone, tandis que le foie de la mère a réagi. Suivant l'exposition à la fumée de cigarette pendant les dix derniers jours de la grossesse, la

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N-déméthylation de DZ et de l'AP dans le foie foetal seulement présentaient l'induction. L'hydroxylation du BP a grandement augmenté dans les microsomes du foie foetal et du placenta.

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Abbreviations and Symbols

α-NF	α-Naphthoflavone
AP	aminopyrine N-demethylase
β-NF	β-Naphthoflavone
BP	benzo(a)pyrene hydroxylase
Cl	plasma clearance
DPPC	dipalmitoyl phosphatidylcholine
DZ	diazepam
g	accelerations of gravity
gm	gram
HCl	hydrochloric acid
hr	hour
i.v.	intravenous
KCl	potassium chloride
L	litre
m	meter
M	Molar
min	minute
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NaOH	sodium hydroxide
NDZ	N-desmethyldiazepam
OX	oxazepam
PAH	polycyclic aromatic hydrocarbon
S.D.	standard deviation

S.E.	standard error of the mean
TEM	temazepam
t1/2	half-life
Vd	volume of distribution
w/v	weight by volume

Prefixes

n	nano
μ	micro
m	milli
c	centi
k	kilo

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INTRODUCTION

Introduction

A growing concern among clinicians today is how xenobiotics encountered by a mother will affect the developing fetus. The consensus is that very few agents are 100% 'safe' and all will inevitably exert some effect on a rapidly growing embryo. Drugs that are considered 'safe' for adult usage are finding increased frequencies of obstetrical use (Kanto, 1982; Mandelli et al, 1975). One such medication, diazepam (Valium), is administered to pregnant women to treat a variety of conditions and toxemias. Although not commonly known as a teratogen when administered during pregnancy, the safety of diazepam as an obstetric medication has yet to be established. Administration during late pregnancy has been associated with a number of complications in the newborn, known collectively as "floppy infant syndrome" (Cree et al, 1973; Gillberg 1977; Rementeria and Bhatt, 1977; Rowlatt, 1978; Speight, 1977).

A fetus' or newborn's ability to eliminate a maternally-administered compound is compromised by the immaturity of its hepatic biotransformation and renal clearance systems. In addition, a mother may consume or be exposed to an agent which will influence her own as well as her fetus' enzymatic functions. As one example, the widespread prevalence of cigarette smoking among pregnant women has provoked the need to examine the development of a fetus' drug-metabolizing abilities in the context of this environmental stress.

Diazepam: Activity and Pharmacokinetic Properties

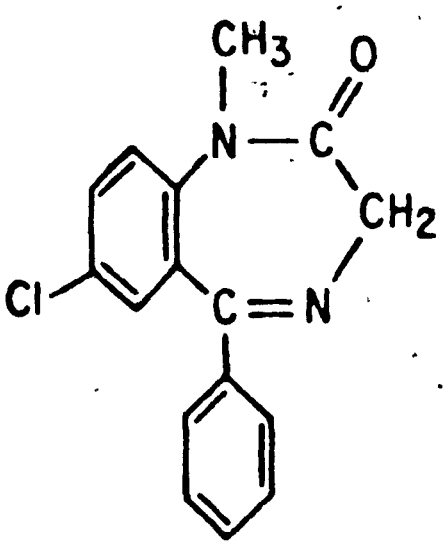
7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, diazepam (Figure 1), is a member of a group of compounds known as the 1,4-benzodiazepines, so called because of the characteristic diazepin ring bound to two benzene rings structure that is shared by the congeners.

First available in the sixties, diazepam and its congeners soon found enormous popularity as anxiolytic and hypnotic agents. In North America, the benzodiazepines account for almost 70% of all prescriptions for anxiolytics (Simon and Boutelier, 1983). Psychotherapeutic drugs themselves account for 15% of all prescriptions written annually (Rickels, 1981). This group of compounds function by exerting a depressive effect upon the central nervous system when bound to specific benzodiazepine binding sites associated with receptors for the neurotransmitter, γ-aminobutyric acid (GABA). Once bound to the supramolecular complex, benzodiazepines potentiate the inhibitory effect of GABA released at neuronal synapses (Costa and Guidotti, 1979). Diazepam is also an effective myorelaxant and anti-convulsant, especially when administered during status epilepticus (Baldessarini, 1980).

Studies of the pharmacokinetics of diazepam (DZ) reveal considerable inter-individual variation for all parameters. After oral administration of an average therapeutic dose of 10 mg, DZ is rapidly absorbed, reaching peak plasma concentrations in 1 hour in adults and only 15-30 minutes in children. The drug exhibits an extensive concentration-independent plasma

Figure 1

The structure and metabolic pathway of diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2- one). The major route of biotransformation of diazepam is N-demethylation to nordiazepam (N-desmethyldiazepam). A minor route is hydroxylation to temazepam (3-hydroxydiazepam). Through other minor pathways, nordiazepam is hydroxylated and temazepam is N-demethylated to form oxazepam. Temazepam and oxazepam are conjugated with glucuronic acid to form water-soluble excretable metabolites (Nicholson, 1981).



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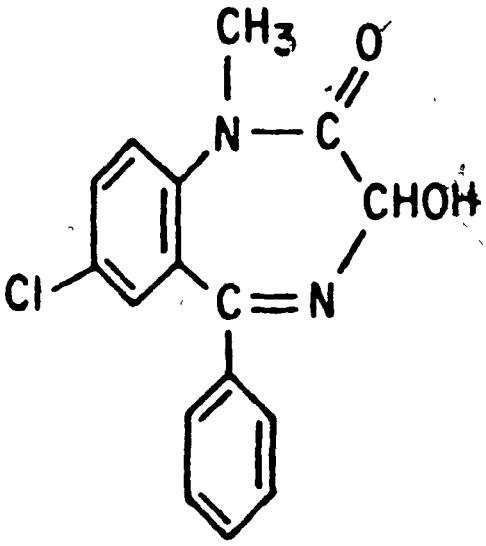

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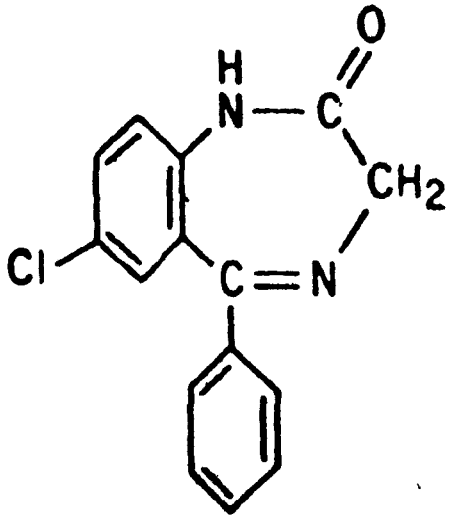
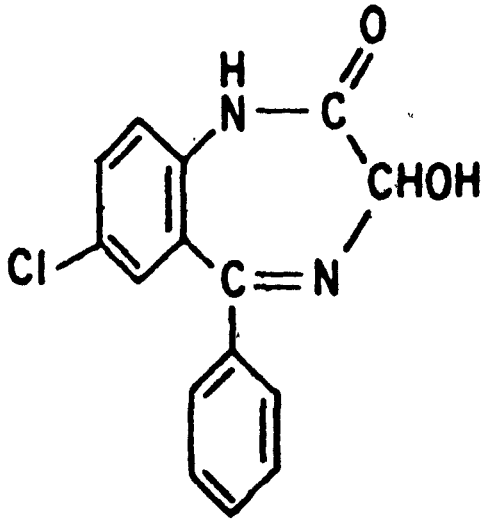


NORDIAZEPAM



OXAZEPAM

protein binding with more than 95% bound (Johnson et al, 1979; Moschito and Greenblatt, 1983).

An open two-compartment model illustrates the half-life (t1/2) of an acute dose of DZ. The first component comprises the α or distributive t1/2, averaging 2.5 hours. The β or elimination phase may range from 1.5-3 days, or longer if the drug is administered repeatedly. Several researchers have observed age and sex-related differences in a number of kinetic parameters of diazepam metabolism. Klotz reported a linear increase of the βt1/2 from 20 hours at 20 years of age to about 90 hours at 80 years. Plasma clearance ranged between 20-32 ml/min with no age dependence. He therefore attributed the prolonged βt1/2 in elderly subjects to an increase in the initial volume of distribution (Vd) of the drug (Klotz et al, 1975). Macleod and colleagues (1979) found smaller age-related differences in the βt1/2: 32.0 hours versus 41.7 for young and old subjects respectively. Female βt1/2 values were larger than males'. Clearance values fell within the range cited above, although men cleared diazepam significantly more quickly than females: 33.2 ml/min versus 18.1 ml/min. The apparent Vd was similar for all, ranging from 76.1-103.2 litres. Greenblatt also observed longer t1/2s in females and elderly subjects. Elderly females also displayed the greatest Vd values. An examination of the smoking habits of these individuals revealed that those men and women with the highest clearance values were all heavy smokers (Greenblatt et al, 1980).

As shown in Figure 1, DZ is N-demethylated by hepatic monooxygenases to form N-desmethyldiazepam (NDZ), a

pharmacologically active metabolite which may account for a good proportion of the CNS effects of DZ (Nicholson, 1981). The t1/2 of NDZ ranges from 51-120 hours. The metabolite is less highly bound to plasma proteins - 92.9%, and its free-fraction is twice that of DZ (Guérre-Milo et al, 1979; Nau et al, 1984).

NDZ is enzymatically hydroxylated at the C3 position to form oxazepam, also an active compound. The hydroxylated metabolite is then glucuronidated and excreted in the urine. Almost no unchanged free drug is excreted.

Because of the nature of the conditions diazepam is used to treat, i.e. anxiety and insomnia, the drug is often administered repeatedly for a long period of time and accumulation in the liver and brain may occur. Habituation and tolerance may also develop. Due to the persistence of the drug and its metabolites in the tissues as a result of its slow elimination, withdrawal symptoms may not appear for at least a week after cessation of therapy (Greenblatt et al, 1981; Rickels, 1981).

Diazepam Use During Pregnancy

Obstetrical uses for diazepam are diverse. It has been administered subchronically in doses of 5-40 mg/day in early pregnancy to treat psychiatric complications. These may be linked to pregnancy, e.g. threatened abortion, or may be an extension of pre-existing habitual use (Kanto, 1982). Diazepam

may be prescribed at 15 mg/day to treat toxemia of pregnancy, specifically eclampsia and severe pre-eclampsia. In treating pre-eclampsia, diazepam is used because of its anticonvulsant properties; it has no hypotensive effects (Mandelli et al, 1975; Moore and McBride, 1978; Shannon et al, 1972). The drug is commonly applied acutely, 10-30 mg at parturition, to induce relaxation of the pelvic musculature and also to prevent eclamptic episodes, as well as reduce anxiety (Erkkola et al, 1973; Idanpaan-Heikkila et al, 1971; Kanto et al, 1973). It is also administered during Caesarian section (Haram and Bakke, 1980).

The various administrations in late pregnancy have been associated with a variety of neonatal symptoms that have come to be known as "floppy infant syndrome". Early reports described hypotonia (Shannon et al, 1972), low Apgar scores (Flowers et al, 1969) and hypothermia (Cree et al, 1973; Owens et al, 1972). Others have also observed primary or secondary apnea, respiratory depression, aspiration of food, poor suckling, neurologic depression and sedation (Rowlatt, 1978; Speight, 1977). These effects remain visible for 1-3 days. Measurement of plasma levels of DZ and NDZ in these infants revealed that both were detectable in significant concentrations for as long as 8 days (Cree et al, 1973). There is concurrence that administration of a dose of 30 mg or more in the 15 hours prior to delivery may lead to neonatal complications (Cree et al, 1973; Kanto, 1982).

Other teratogenic effects of diazepam have not been clearly established. There is some indication of an increased

incidence of midline cleft deformities of the lip or palate, although this remains controversial (Rosenberg et al, 1983; Safra and Oakley, 1975; Saxen and Saxen, 1975).

The Pharmacokinetics of Diazepam in Pregnancy

Multiple facets of drug metabolism and distribution may be influenced by the physiological changes that accompany pregnancy.

1. Maternal Properties

A group of 18 pregnant women administered 10 mg diazepam i.v. exhibited a mean elimination t1/2 of 65 hours, twice the value of non-pregnant women. However plasma clearance, 28 ml/min, was the same in both groups, therefore the prolonged t1/2 was believed to be related to changes in the distribution of diazepam and not to a pregnancy-related reduction in hepatic function (Moore and McBride, 1978).

Changes in concentrations of plasma proteins during pregnancy may affect the plasma protein binding of all drugs. Maternal plasma albumin levels decline gradually during gestation, reaching their lowest levels prior to parturition. Levels return to normal within 5-7 weeks post-partum (Dean et al, 1980). An increased serum free fraction of diazepam has been significantly correlated ($p<0.001$) with the decrease in albumin levels (Dean et al, 1980). Perucca found that the free fraction of maternal diazepam began to deviate from non-pregnant control values at mid-pregnancy (Perucca et al,

1981).

Another factor contributing to the increase in the free fraction of diazepam in the serum of pregnant women is the presence of endogenous substrates which can displace it from its albumin binding site. These substrates may result from hormone production and lipid mobilization. Kuhnz and Nau (1983) demonstrated free fatty acid levels in maternal plasma to be almost twice those of non-pregnant controls (while bilirubin levels were lower). A positive correlation existed between free fatty acid levels in pregnant women and the free fractions of DZ and NDZ. The % unbound of diazepam and its major metabolite were 4.0 and 4.3 in women immediately after parturition compared to 2.3 and 2.9 for non-pregnant controls. Competition from bilirubin did not contribute to an increased free fraction since it was found to be slightly decreased in the plasma of mothers at term. Moreover, bilirubin does not displace diazepam from its albumin binding site (Broderson et al, 1983).

The enzymatic activity of the liver is a prime determinant of diazepam oxidation in the body. Because diazepam must be enzymatically biotransformed to be excreted, its rate of elimination will depend on the diffusion of the free drug into hepatocytes.

The presence of an increased free fraction of diazepam will eventually result in increased oxidative metabolism to maintain the same free concentration of drug. Consequently, the total drug concentration in the plasma will be reduced. Thus,

despite the fact that there is a higher unbound fraction of circulating DZ and NDZ, the pharmacological response to the administered dose would be expected to be the same as in the non-pregnant individual.

After rapid administration of the drug, e.g. intravenously, the increased free fraction of diazepam will initially allow elevated free concentrations to reach all well-perfused tissues and diffuse across endothelial barriers. The placenta is a highly perfused endothelial tissue that allows for rapid transfer of diazepam. Undoubtedly, the disposition of diazepam and metabolites in the pregnant woman will be influenced by the rapid placental transfer into the additional 'compartment' that is the fetus.

2. Placental Transfer

The human placenta is a highly villous hemomonochorial cellular membrane that separates the maternal and fetal circulations. From embryonic tissue develops the trophoblast, a layer of cells which quickly differentiates into the cytotrophoblast and the syncytiotrophoblast. As pregnancy proceeds, the cytotrophoblastic layer recedes and the cells of the syncytiotrophoblast form villi that invade the maternal blood vessels in the uterine wall. In the latest stages of development, only the cellular epithelium of the syncytiotrophoblast remains to separate maternal from fetal blood, thus the barrier is truly 'hemomonochorial' (Biggers, 1980).

Transport across this layer resembles epithelial transcellular transport. Diffusion of glucose and exchange of

oxygen and carbon dioxide occur. Many nutrients such as amino acids, essential ions and vitamins may cross by facilitated diffusion. Some amino acids and hormones will be actively transported across the placenta. Pinocytosis and filtration of small molecules through cellular channels also occurs (Mirkin and Singh, 1976).

In addition to transport, the placenta is involved in synthesis of hormones including estrogen, progesterone, human chorionic gonadotropin and human placental prolactin. It is active in glycogen synthesis, storage and breakdown (Juchau, 1982). Several groups have discovered that prostaglandins are produced and released by human term placentas (Harper et al, 1983; Kawano and Mori, 1983; Ylikorkala et al, 1983).

Functional placental monooxygenases are believed to participate in hormone metabolism, especially conversion of androgens to estrogens. The presence of monooxygenases in the placenta may modify transfer of drugs to the fetus. Some believe these enzymes provide a metabolic barrier to protect the fetus from pharmacologically active agents (Burba, 1979; Gillette, 1973; Levitz et al, 1978). They may also protect the fetus from corticosteroids and catecholamines present in the maternal circulation. These placental enzymes exhibit significantly higher activities toward selected substrates in mothers who are cigarette smokers (Nebert et al, 1969; Pelkonen et al, 1972; Vaught et al, 1979; Welch et al, 1968).

The transfer of a compound across the placenta will depend on a number of factors, including lipid solubility, degree of ionization, molecular weight, plasma free fraction as well as

placental blood flow, placental monooxygenase activity, placental aging and possibly fetal circulation. The time constant for placental transfer of a highly lipid-soluble, small (<500 daltons), unionized compound is such that it would take only 3 times longer to cross than if fetal tissues were directly supplied by maternal blood! (Dawes, 1973)

Diazepam has a molecular weight of 284.7 and a pKa of 3.4. Small, unionized at physiological pH and highly lipid-soluble, diazepam is assured of extremely rapid transfer. The drug crosses *in vivo* and *in vitro* by diffusion. But unlike water-soluble agents like glucose, urea, and salicylate that diffuse across by entering water-filled extracellular channels, lipophilic compounds such as diazepam diffuse both through these channels and directly through the cells of the syncytiotrophoblast (Guerre-Mile et al, 1979). No active transport has been observed.

3. Fetal Properties

The earliest studies of placental transfer of diazepam reported a drug concentration in the fetal circulation very close to maternal levels (Cavanagh and Condo, 1964; De Silva et al, 1964). With more investigations into the placental transfer of this drug, it became apparent that fetal levels of DZ and metabolites greatly depend on 1) the dose administered, e.g. acute or repeated, and 2) the interval of time between dosing and delivery, i.e. the period of exposure of the neonate to the drug.

Acute doses of 5-30 mg diazepam to the mother have resulted in almost immediate transfer to the fetus. Many

researchers have observed fetal blood concentrations to rapidly increase, reporting ratios of fetal:maternal plasma diazepam levels of 1.5-2.0 for dose-delivery intervals of up to 7 hours (Erkkola et al, 1973, Haram et al, 1978, Idanpaan-Heikkila et al, 1971). A recent study (Bakke and Haram, 1982) suggested that the concentration of diazepam in mixed (arterial and venous) umbilical cord blood is negatively correlated with the dose-delivery interval for the first 10 minutes of exposure. At very short intervals, the distribution equilibrium between mother and fetus will be achieved rapidly and the fetal plasma will see its highest levels of drug. Measurement of neonatal plasma levels during this time will give a good reflection of total concentrations of drug to which the infant may be exposed.

As the interval increases, net transfer of diazepam is prolonged and the attainment of steady-state is delayed. A significant positive correlation exists ($r=0.62$) between the dose-delivery interval and the total (plasma + tissue) fetal concentration between 2-24 hours. Because of the high lipid-solubility of the drug, it will diffuse rapidly to fetal tissues. Immature biotransformation and elimination capabilities may result in a substantial volume of distribution. Therefore, subsequent measurements of diazepam in fetal plasma may not accurately reflect exposure of the fetal unit as a whole (Bakke and Haram, 1982). Repeated dosing of diazepam during late pregnancy has resulted in feto-maternal plasma drug ratios close to unity, however the equilibrium in this situation has caused very high concentrations of DZ and

NDZ to be found in the heart, lungs and brain (Kanto, 1982). This tissue accumulation may be related to the adverse cardiovascular, respiratory and central effects reported in infants of women given diazepam at term (Kanto, 1982; Mandelli, 1975). Adverse effects to the fetus when diazepam must be administered in doses exceeding 30 mg may be avoided by limiting the dose-delivery interval to a period of less than 10 minutes (Bakke and Haram, 1982).

When Erkkola observed high ratios of fetal:maternal DZ plasma levels he suggested that the 'trapping' in fetal blood may be due to better binding of diazepam to fetal plasma proteins (Erkkola et al, 1973). Kanto and colleagues (1973) showed that no drug was bound to fetal red blood cells and reported 86% plasma protein binding, a value lower than that of normal adults (97%). More recently, Nau reported the % unbound of DZ and NDZ in perinatal plasma to be 2.4 and 2.9 respectively, compared to 4.0 and 4.3 in maternal plasma. Although albumin levels were decreased in the perinate, 26.6 gm/L compared to 40.2 gm/L for control adults, fetal free fractions were believed to resemble control adult values because of the low free fatty acid levels in the plasma: 334μM compared to 555μM in control adults and 993 μM in mothers.

That the human fetus possesses active but low hepatic monooxygenase activity has been demonstrated *in vitro* (Pelkonen and Karki, 1971). Reduction of nitro groups, O- and N-dealkylation, aromatic hydroxylation, epoxide formation and N-hydroxylation of foreign and endogenous substrates have all been shown to occur (Gillette et al, 1973; Pelkonen, 1980;

Pelkonen and Karki, 1973a, 1973b; Pelkonen et al, 1973: Waddell, 1972; Yaffe et al, 1970).

Kanto examined DZ and NDZ levels in separated arterial and venous umbilical cord blood in order to obtain a more accurate picture of placental transfer and, by examining differences between the two, possibly detect fetal biotransformation of the drug. Although he found higher levels of the metabolite in the umbilical vein than in maternal blood, 7 ng/ml vs. 3 ng/ml, the levels of NDZ in venous and arterial blood were approximately equal. Kanto did not consider the dose-delivery interval in his measurements (Kanto et al, 1973). Kanto suggested that the NDZ occurring in fetal blood was primarily a consequence of maternal biotranformation, as NDZ inherits enough of the physico-chemical properties of the parent compound to permit a comparable rate of transfer across the placenta. He did not exclude however, the possibility of placental and fetal metabolism of diazepam based on observations of oxidation of other substrates by these tissues (Pelkonen and Karki, 1971).

Mandelli (1975) examined metabolite levels in venous and arterial umbilical plasma with respect to the dose-delivery interval and found higher NDZ levels in venous plasma only at an interval of 6 hours. In addition, some free and conjugated methyl oxazepam, the hydroxylation product of diazepam, was observed in umbilical cord plasma. It is most likely that all or most of these metabolites are of maternal origin. Methyl oxazepam was also detected in very small amounts in the urine of infants within 48 hours after birth. It is possible that multiple oxidative pathways are functional in the term fetus or

newborn, however this activity may result from enzymatic induction occurring as a result of maternal exposure to an appropriate inducing agent, e.g. cigarette smoke. In premature infants there was no evidence of hydroxylase activity; neither methyl oxazepam nor oxazepam were detected in the plasma. NDZ did not appear in the plasma until 8 hours after dosing, and plasma levels of this metabolite continued to rise after 18 hours (Morselli et al, 1974).

Ackermann and Richter (1977) have demonstrated metabolism of diazepam in microsomal preparations from the liver of a 13-week-old fetus. At concentrations below 0.1mM, diazepam was primarily N-demethylated. Above this concentration, C-3 hydroxylation to methyl oxazepam was the main route of oxidation. Enzymatic activity was inhibited by 0.1 mM SKF-525A, pointing to involvement of cytochrome P450. Although this study confirmed the capacity of the fetal liver to both N-demethylate and hydroxylate diazepam, this does not prove that the metabolites found in the fetal umbilical circulation as reported above, resulted from fetal biotransformation. Concentrations employed in the *in vitro* incubations were considerably higher than therapeutic dose levels.

The disposition of diazepam and metabolites in fetal tissues will depend directly on placental transfer, fetal plasma protein levels and binding, plasma clearance, and biotransformation and excretion capabilities as discussed above. In addition, there are several indirect determinants, reflecting the physiological status of the maturing fetus, that will influence the distribution of a lipophilic agent (Danish,

1980). In the fetus at term, liver and brain size are greater relative to total body weight than are measured in older individuals. Adipose tissue is found in least amounts and total body water and the ratio of extracellular to intracellular water is at its highest. All these factors may influence the apparent Vd of a lipophilic drug such as diazepam (Morselli, 1976; Werry, 1978). Immature hepatic and renal functions may contribute to the prolonged t1/2 for diazepam observed in perinates. It is estimated that a newborn will not reach adult hydroxylating and N-demethylating activity until at least 5 months of age. Glucuronidation reactions are extremely low or undetected in the fetus throughout gestation and are not expected to increase until several weeks after birth (Morselli, 1976).

As mentioned above, DZ and NDZ accumulate in fetal tissues after a single dose is administered. A post-mortem analysis of a 31-week-old fetus whose mother had been administered diazepam chronically since 27 weeks revealed an accumulation of DZ and NDZ in amounts ranging from 8-284 ng/gm in the brain, heart, lungs, kidneys, liver and placenta. Highest levels of drug and metabolite were found in the heart, lungs and brain. A 10:1 ratio of NDZ:DZ occurred in the lung and placenta, while other tissues demonstrated a ratio of 3:2. (Mandelli et al, 1975). The extensive uptake in cardiac tissue may be significant in light of the loss of normal beat-to-beat fluctuations observed in fetuses of mothers administered diazepam (Scher et al, 1972).

The increased free fraction of diazepam in maternal blood

combined with the lipophilic nature of the drug favor its transfer from the plasma into the breast milk. The maternal plasma:breast milk ratios for DZ as well as NDZ average about 2:1. Lethargy and sedation were evident in breast-fed infants whose mothers received 10 mg doses of diazepam 3x daily (Patrick et al, 1972). Moreover, measurable amounts of benzodiazepines persisted in the breast milk 6 days after a single post-partum dose (Cole and Hailey, 1975). Continuous treatment in a lactating mother may lead to an increased accumulation in the infant's tissues.

4. The Materno-placento-fetal Unit

Although the pharmacology of diazepam in pregnancy has hitherto been discussed in terms of the mother, the placenta and the fetus, it is perhaps more realistic to describe the pharmacokinetics of the drug as a unit, the materno-placento-fetal unit. This unit may be divided into compartments based on tissue properties that govern distribution and the physicochemical features of the drug which will determine a drug's displacement at each tissue interface. Multicompartmental models have been proposed to explain diazepam disposition in the unit. A simple model describes the mother as 2 compartments, a central one (the plasma) and a peripheral or 'deep' one (the peripheral tissues). The entire fetus may be thought of as an additional deep compartment (Levy, 1981; Levy and Hayton, 1973). This model predicts that when a drug enters the deep fetal compartment, elimination continues from both maternal compartments and eventually a steady-state will be reached where the drug concentration in

the fetus will exceed that in the mother (Krauer et al, 1980; Moore and McBride, 1978).

The importance of the pharmacokinetic models lies in their ability to predict information about the disposition of a particular drug that is not revealed by routine blood or urine analysis. From this, a more accurate picture of 'true' exposure of the fetus can be given. This may provide insight into the etiology of the hypothermia, apnea, limpness, etc. seen in newborns of women treated with diazepam. It has not yet been established what effect, if any, prenatal exposure to and consequent accumulation of diazepam will have on the long-term physiological and neurological development of these infants.

Inducing Agents and Drug Metabolism

In 1954, Brown and Miller demonstrated that hepatic oxidative activity in animals could be enhanced by treatment with carcinogenic dyes. Evidence soon mounted showing that the oxidative capacity of humans could be similarly induced (Chen et al, 1962; Douglas et al, 1963; Yaffe et al, 1966). Moreover, studies of the effects of inducing agents revealed the existence of multiple forms of the enzyme responsible for oxidative drug metabolism. The administration of ethanol to volunteer subjects enhanced the oxidation of pentobarbitone but not benzo(a)pyrene (Rubin and Lieber, 1968). In patients receiving rifampicin, phenytoin or phenobarbitone, ethylmorphine demethylation was increased to a greater extent

than benzo(a)pyrene hydroxylation (Farrel et al, 1979).

Within six years of Alvares' discovery of cytochrome P450 in human liver (1969), efforts were underway to isolate and purify this protein, with the help of inducing agents. It was found that phenobarbital generally induced the synthesis of cytochrome P450 as well as P450 reductase and caused proliferation of hepatic smooth endoplasmic reticulum (Thaler et al, 1972). Polycyclic aromatic hydrocarbons (PAH), including benzo(a)pyrene (present in cigarette smoke), β-naphthoflavone (β-NF) and 3-methylcholanthrene appeared to induce a P448 form of the enzyme (Boobis and Davies, 1984; Gillette and Stripp, 1974).

In 1973, the Boston Collaborative Drug Surveillance Program examined the effects of cigarette smoke exposure on the pharmacological response to a number of drugs. Smoking significantly attenuated the sedative effects of diazepam. It is possible that cigarette smoke may be inducing the isozyme of P450 responsible for diazepam oxidation.

An examination of enzyme inducibility in pregnancy is of special concern. Induction of maternal hepatic monooxygenases may alter the concentration of active drug or metabolite that reaches the fetal compartment. The elevated hydroxylase activity in the placentae of women who smoke has been well documented (Nebert et al, 1969; Pelkonen et al, 1972; Vaught et al, 1979; Welch et al, 1968). Investigating the responsiveness of fetal enzyme activity toward inducing agents may shed light on the process of development of these enzymes and on the ability of the fetus to confront potentially toxic substrates.

Unfortunately, little data exists to describe human fetal liver inducibility. Pelkonen found that maternal cigarette smoking had no effect on the oxidations of either benzo(a)pyrene or N-methylaniline in human fetal liver at mid-gestation, while benzo(a)pyrene hydroxylase activity in the placenta was highly induced (Pelkonen et al, 1972).

Studies in the rat revealed increases in fetal liver activity following exposure to PAH during the last few days of gestation (Schlede et al, 1972). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) administered to pregnant rabbits induced the LM6, a P448 form not present in the adult animal, suggesting qualitative, in addition to quantitative differences between fetal and adult hepatic oxidative activities (Norman et al, 1978). It appears that PAH inducers, such as 3-methylcholanthrene and TCDD are more potent inducers in the perinate than phenobarbital (Cresteil et al, 1979; Guenthner and Mannering, 1977). The effect of inducing agents on diazepam metabolism in the materno-placento-fetal unit has not been studied.

An Animal Model

An animal model chosen for studying the metabolism of diazepam and the effects of induction in the materno-fetal unit should meet several criteria. Firstly, it must possess gestational properties similar to the human. This infers comparable patterns and degrees of fetal development and

maturation, as well as anatomical considerations such as litter size and placental structure. Because the emphasis is on drug-metabolizing abilities, a successful animal model will display a metabolic pathway and pharmacokinetic profile of diazepam that can be compared to the human. In addition, those enzymes responsible for biotransformation should exhibit sufficient responsiveness to enable meaningful studies of induction.

The guinea pig has demonstrated itself to be superior to other commonly available laboratory animals such as the mouse and rat in fulfilling most of the above requirements. Its period of gestation is reasonably long, 67 days, and is divided into trimesters with steroidal changes paralleling those observed in the human. Both the mouse and the rat have short periods of gestation and give birth to large litters of pups with incomplete physical development. Guinea pig litters average 3 pups possessing precocious physical features.

The newborn guinea pig possesses a high body fat content, similar to humans (Thomas and Lowy, 1983). This may be related to the placental transfer of essential fatty acids observed in the guinea pig, rabbit and human (Thomas and Lowy, 1984).

The guinea pig is the only rodent species to possess a placenta that is both discoidal and hemomonochorial, features distinctive of the human placenta. The mouse, rat and hamster possess a trichorial placenta and the rabbit a dichorial one (Ecobichon, 1984).

Hepatic drug-metabolizing activity is low in the fetal guinea pig, but sharply increases soon after birth, resembling

human fetal enzymatic maturation. The maturation of oxidative abilities occurs more rapidly in the guinea pig than in the rat, possibly due to the longer gestational period (Mitchell, 1983) and higher level of maturity at birth. More specifically, differences in fetal activities among species have been attributed to differences in the development or quantity of hepatic smooth endoplasmic reticulum (Gillette and Stripp, 1975). Its precociousness has also been the explanation for the fetal guinea pig's greater responsiveness to transplacental exposure to inducing agents such as phenobarbital or 3-methylcholanthrene compared to the rat (Schlede et al, 1973; Waddel, 1972).

With respect to diazepam metabolism, neonatal rats preferentially metabolize diazepam *in vitro* by C3 hydroxylation to N-methyl oxazepam. Only at high doses is N-desmethyldiazepam found. The guinea pig displays the opposite profile; NDZ is the preferred metabolite while methyl oxazepam is found only when DZ doses are very high. In this respect, guinea pigs resemble human neonatal *in vitro* diazepam metabolism (Ackermann and Richter, 1977; Marcucci et al, 1973).

Thus the guinea pig appears to be an animal model well-suited to the study of diazepam disposition and metabolism in the materno-fetal unit.

Formulation of the Thesis

Although data on the plasma levels of DZ and NDZ in the human materno-placento-fetal unit is plentiful, little attention has been directed specifically toward the influence of drug-metabolizing systems on these levels. This may be partly due to the lack of a good animal model.

Firstly, this investigation will attempt to establish the usefulness of the pregnant guinea pig as a model for studying diazepam metabolism by answering the following questions. What is the profile of metabolism and distribution of diazepam in the guinea pig materno-placento-fetal unit? Does tissue accumulation of drug and/or metabolite occur?

An accumulation of the active metabolite NDZ in fetal tissues may be a direct consequence of drug biotransformation. Secondly, this research will determine the rates of diazepam N-demethylase activity in the guinea pig materno-placento-fetal unit. Are the fetal liver and placenta capable of oxidizing diazepam, and if so, what is their contribution to overall biotransformation by the unit?

Thirdly, biotransformation can be further characterized by studying the effects of known enzyme-inducing agents and this study will examine how metabolism of diazepam in the guinea pig materno-placento-fetal unit is affected by administration of β-NF, a prototype inducer of cytochrome P448.

Finally, diazepam and cigarette smoke is one of the most

common drug combinations known, and while clinical data has revealed an interaction between the pharmacological effects of diazepam and cigarette smoking, it has never been studied in a pregnant subject or animal model. Since this may be of toxicological significance to the developing fetus, this study will assess the influence of cigarette smoke exposure on diazepam metabolism in the materno-placento-fetal unit of the guinea pig.

MATERIALS AND METHODS

Chemicals and Reagents

Chemical	*Manufacturer*
Acetic acid (glacial)	Allied
Acetone	Fisher
Acetonitrile	Fisher
Acetylacetone	Sigma
Aminopyrine	K&K
Ammonium acetate	Baker
Benzo(a)pyrene	Sigma
Bovine serum albumin	Sigma
Brilliant blue	Sigma
n-Butyl acetate	Baker
Chloroform	Fisher
N-Desmethyldiazepam	Hoffman-LaRoche
Diazepam	Hoffman-LaRoche
Dipalmitoyl phosphatidylcholine	Sigma
Ethanol	Consolidated
Ether	Fisher
Formaldehyde	Fisher
Glucose-6-phosphate dehydrogenase	Sigma
Glucose-6-phosphate monosodium salt	Sigma
Heparin sodium	Fisher
Hexane	Fisher
Hydrochloric acid	BDH
Magnesium chloride	AnalaR

Methane 5%/Argon	Medigas
Methanol	Fisher
α-Naphthoflavone	Eastman
β-Naphthoflavone	Aldrich
Nicotinamide (Niacinamide)	Sigma
Nicotinamide adenine dinucleotide phosphate	Sigma
Nicotinamide adenine dinucleotide phosphate, reduced	Sigma
Phosphoric acid	Fisher
Potassium chloride	Fisher
Potassium dihydrogen phosphate	Fisher
Propane-1,2-diol (Propylene glycol)	BDH
SKF 525-A (proadifen)	Smith Kline & French
Sodium chloride	Fisher
di-Sodium hydrogen orthophosphate	AnalaR
Sodium hydroxide	Fisher
THAM Tris(hydroxymethyl)aminomethane	Fisher
Tobacco	Imperial Tobacco
Trichloroacetic acid	Fisher

Animals

Hartley albino guinea pigs, 400-500 gm, were obtained from Charles River Canada Inc. (St. Constant, Quebec) and were housed in one room in the McIntyre Animal Centre and acclimatized for at least 1 week prior to breeding. Guinea pigs

were placed in 60 cm x 88 cm cages bedded with soft wood shavings and were fed Purina guinea pig chow and water *ad libitum*. A room temperature of 22°C was maintained with a controlled 12 hours light/12 hours dark cycle. Animals were weighed daily.

Breeding

Upon commencement of breeding, 3 nulliparous female guinea pigs were placed together in a cage with one male. A female guinea pig's estrous cycle lasts 15-17 days with an estrus period of 9-11 hours (Sisk, 1976). Shortly preceding estrus, the vaginal epithelial membrane opens and remains open for approximately 2 days. All female guinea pigs were checked daily for opening of this membrane. If open, a vaginal wash was performed, using 0.1 ml sterile saline applied with a 1 ml syringe. A slide was prepared and examined under light microscopy for the presence of spermatozoa. A positive slide was counted as day 0. The gestation period lasts approximately 67 days.

Pregnant guinea pigs used in the studies of cigarette smoke exposure were transported to the Pathology Department Animal Centre on days 45-50 of gestation. The animals were placed 1 or 2 to a 45 cm x 50 cm cage containing beta-chip hardwood bedding. Guinea pigs were fed guinea pig chow and water *ad libitum* and were weighed daily.

Toward the end of pregnancy, the symphysis pubis relaxes, so that 3-4 days prior to term, there is a palpable separation of the 2 halves of the pelvis (Hisaw et al, 1944). At one day prior to term, when the pelvic separation was 10-15 mm, animals

were anaesthetized with chloroform and fetuses removed by Caesarian section. Litters averaged 3 pups. Animals were killed by cardiac puncture.

Drug Treatment

Diazepam was dissolved in propylene glycol to produce a stock solution of 10 mg/ml. Guinea pigs were administered oral doses of diazepam using a 5 ml syringe attached to a 38 mm 20 gauge curved feeding needle.

Kinetic Studies

1. Plasma Elimination

At 0.5, 1, 2, 3, 4 and 5 hours after dosing with 20 mg/kg diazepam, a group of 8 guinea pigs were lightly anaesthetized with diethyl ether and 1.0 ml blood samples were collected by cardiac puncture using 20 gauge 1.5 inch hypodermic needles attached to heparinized syringes. Between sampling, hypothermia was prevented by warming guinea pigs with a heat lamp. Blood samples were spun on a Damon/IEC clinical centrifuge for 20 minutes and the plasma was removed and stored at -20°C until analysis.

2. Placental Transfer and Tissue Distribution

Five pregnant guinea pigs at 65-67 days gestation were administered oral doses of 10 mg/kg diazepam as described

above. After 60 minutes animals were euthanized. Blood was removed from the dam by cardiac puncture and exsanguination and the liver, brain and abdominal adipose tissue were excised. From all pups, blood samples were drawn and livers, brains, adipose and placentae were excised. Plasma and tissues were stored at -20°C until quantitation.

Quantitative Analysis

1. Extraction

DZ and NDZ were extracted from plasma by a rapid method developed by Kuhnz and Nau (1983). Butyl acetate (0.2 ml) was added to 0.1 ml of plasma and shaken for 30 seconds on a vortex mixer. After centrifuging for 2 minutes, 1-4 µl of the organic layer were injected directly into the gas chromatograph.

For tissue quantitation, 1.0 gm samples of liver, brain and adipose tissue were homogenized with 5.0 ml of 0.06 M phosphate buffer, pH 7.4, to produce 20% w/v homogenates. Two grams of placental tissue were homogenized in 4 ml buffer to form a 50% w/v homogenate. A volume of 0.5 ml of each tissue homogenate was combined with 0.5 ml butyl acetate, mixed for 30 seconds and centrifuged for 5 minutes, followed by removal of an aliquot of the organic layer.

2. Gas-liquid Chromatography

DZ and NDZ residues in plasma and tissues were quantitated using a Hewlett Packard model 5710A gas chromatograph equipped with a 63-Ni electron capture detector and a 0.2 cm x 1.2 m

coiled Pyrex column packed with 3% OV-1 on 80/100 mesh. The column was deactivated with repeated 3.0 μl injections of dipalmitoyl phosphatidylcholine, 5.0 mg/ml in ethanol, every 3 hours during use. This treatment improved peak sharpness and shortened the retention time of NDZ without affecting the DZ peak (Rutherford, 1977). The carrier gas was 5% methane/argon. Temperatures for oven, detector and injector were 245°, 310° and 260°C respectively. Gas flow rates averaged 48.4 ml/min.

Calibration curves for DZ and NDZ were constructed after extracting drug and metabolite from plasma and tissue homogenates of untreated animals spiked with known concentrations of DZ and NDZ. Peak heights of standards of DZ and NDZ in butyl acetate were measured and compared with calibration curve values to assess the efficiency of extraction for each tissue.

For plasma and tissue homogenates, peak heights of DZ and NDZ of each sample were recorded. Levels were quantitated by calculation from a standard curve constructed for each new period of chromatographic analysis. Limits of sensitivity were 20-30 ng DZ or NDZ per ml plasma and 30-40 ng DZ or NDZ per gm tissue.

Exposure to Inducing Agents

1. β-Naphthoflavone

β-NF was dissolved in peanut oil to 25 mg/ml. Six pregnant guinea pigs near term (63-65 days of gestation) received daily

oral doses of 40 mg/kg for 3 days. Animals were sacrificed within 24 hours of the final dose and dam and fetal livers and placentae were removed and weighed. A group of 5 control guinea pigs received equivalent volumes of peanut oil.

2. Cigarette Smoke

Pregnant guinea pigs (55-57 days of gestation) were exposed to cigarette smoke using the Mason inhalation system (Bilimoria and Ecobichon, 1980). Animals were placed in cylindrical restraining cages and subjected to a nose-only inhalation of smoke that filled a central chamber. Unfiltered cigarettes prepared from a blend of flue-cured tobacco were loaded onto a rotating dial. Every 5 seconds, a 35 ml volume of smoke was drawn off a cigarette, diluted 1:5 with air and pumped into the central inhalation chamber. The chamber contained a smoke concentration of 11.43 mg particulate matter per ml (Bilimoria and Ecobichon, 1980). During each dosing period of 200 seconds, pregnant guinea pigs were exposed to 40 puffs. Animals were dosed thrice daily, at 9:00 am, 12:00 pm and 3:00 pm. Between dosing periods animals were returned to their cages and provided food and water *ad libitum*. Dosing was maintained daily for the last 10 days of gestation. Animals were sacrificed within 24 hours of the final dose and tissues were removed.

Microsomal Preparation

To prepare microsomes suitable for *in vitro* assays, all tissues and buffer solutions were maintained on ice at 0°-4°C to prevent loss of enzymatic activity.

Under chloroform anaesthesia, an incision was made across the abdomen of the guinea pig at term (65-67 days of gestation). Pups were removed from their amniotic sacs, initiating respiration. After umbilical cords were clamped, the dam was killed by cardiac puncture. Umbilical cords were then cut and pups were separated from the dam. The placentae were dissected from the amniotic tissue, blotted dry, weighed and placed together in saline on ice. The dam liver was then excised and the gall bladder carefully removed. The liver was weighed and a 5.0 gm sample placed on ice. Each pup was weighed, anaesthetized with chloroform and killed by cardiac puncture. Individual fetal livers were weighed and then pooled.

All tissues were placed in separate glass vials, capped and stored at -80°C until used. Thawing was performed gradually to preserve enzyme activity. Frozen vials were partially thawed by maintaining them at 4°C for 60 minutes. Vials were subsequently placed on ice, opened, filled with cold saline and left standing until tissue pliancy returned, after 45 minutes. Liver tissues were finely minced and washed 3-5 times in cold saline to remove excess blood. Placentae were minced well and washed 5-10 times in saline to avert heme contamination.

Crude microsomal preparations were made from these tissues by a method of differential centrifugation. After draining

tissues, samples were homogenized in 0.06 M phosphate buffer, pH 7.4, in a glass homogenizing tube with a motor-driven Teflon pestle to produce a 20% w/v homogenate. Whereas each dam liver was represented by an individual tissue sample, fetal liver and placental homogenates represented the pooled tissues of an entire litter.

Homogenates were poured into 30 ml tubes and centrifuged at 4°C on a Damon/IEC centrifuge model B-20A at 9,000 g for 15 minutes using a 8/76 rotor. The supernatant was removed, care being taken to avoid displacing the surface lipid layer or the pellet, containing nuclei, mitochondria and cell debris. Supernatant fractions were placed in 10 ml polycarbonate tubes and centrifuged at 4°C on a Beckman ultracentrifuge model L3-40 at 100,000 g for 60 minutes using a type 40 rotor. The supernatants from this centrifugation were aspirated, leaving only the microsome-containing pellets. The centrifuge tubes were washed with 1.15% KCl, 0.02 M tris buffer, pH 7.4, to remove the pellets which were homogenized and resuspended in 1.15% KCl buffer. Dam liver microsomal preparations contained 500 mg tissue/ml, fetal liver, 1 gm/ml and placenta, 2 gm/ml. Aliquots of 1-2 ml of each microsomal preparation were frozen at -20°C for later determination of protein concentration. The remainder was maintained at 0°-4°C and used in enzymatic assays within 60 minutes.

Protein Determination

Protein concentrations of individual microsomal preparations were determined in duplicate by the colorimetric method of Bradford (1976).

The reagent consisted of 100 mg G-250 Coomassie Brilliant Blue dye combined with 50 ml absolute ethanol and 100 ml phosphoric acid diluted to 1 L with distilled water. After filtering the reagent with suction through a 60 ml Pyrex Buchner funnel, the solution was kept at room temperature and remained stable for several weeks. Samples of microsomes to be analysed for protein content were diluted 1:10 in distilled water. A volume of 5.0 ml of reagent was added to 0.1 ml of each sample duplicate and to a blank containing 0.1 ml of distilled water. The samples were shaken on a vortex mixer and after 5 minutes, absorbances were read at 595 nm on a Pye Unicam spectrophotometer model SP6-500. Protein concentrations were calculated by comparing absorbance values to those of a standard curve prepared with known concentrations of bovine serum albumin.

Microsomal Enzyme Assays

The N-demethylation of diazepam and aminopyrine and the hydroxylation of benzo(a)pyrene were assayed *in vitro* by incubation of substrate with microsomes at 37°C in the presence of oxygen and a NADP-NADPH generating system (Kato and

Gillette, 1965; LaDu et al, 1955).

1. N-demethylation

Upon *in vitro* N-demethylation of a substrate such as diazepam or aminopyrine by monooxygenases present in microsomal preparations, the methyl group removed can be trapped in the form of formaldehyde. Formaldehyde production was measured colorimetrically based on the Hantzsch reaction (Cochin and Axelrod, 1959; Nash, 1953) where formaldehyde formed combines with a beta-diketone (acetylacetone) and an amine (ammonium acetate) to form a diacetyl product of yellow-green color. This product was quantitated directly by measuring its absorption spectrophotometrically.

Stock solutions of diazepam and aminopyrine were prepared prior to each assay. Diazepam, 10 mg, was mixed with 10 ml of methanol to achieve a concentration of 3.51 mM and 115.65 mg aminopyrine were dissolved in 50 ml 0.05 M Tris-HCl buffer, pH 7.4, to produce a concentration of 10 mM.

To 25 ml Erlenmeyer flasks were added a)25 μmol magnesium chloride, b)50 μmol glucose-6-phosphate, c)0.5 units glucose-6-phosphate dehydrogenase, d)100μmol nicotinamide, e)1μmol NADP, f)the appropriate volume of prepared microsomes (dam liver: 0.5 ml containing 250 mg tissue; fetal liver: 1.0 ml containing 1 gm tissue; placenta: 1.0 ml containing 2 gm tissue), g)substrate solution: 0.35 μmol diazepam or 15 μmol aminopyrine and h)0.05 M Tris-HCl buffer, pH 7.4, in sufficient volume to yield a total final volume of 5.0 ml in each flask. Blanks contained buffer in lieu of NADP.

When inhibition of activity was studied, SKF-525A was

dissolved in Tris-HCl buffer and incubations of dam liver contained 0.1, 1.0 or 10.0 μmol. α-Naphthoflavone (α-NF) was dissolved in methanol and 1 μmol was added to each mixture. Controls received an equivalent volume of methanol.

Flasks were placed in a Dubnoff metabolic shaking incubator at 37°C for 5 minutes of preincubation. At time zero the reaction was initiated by addition of diazepam or NADP (for the aminopyrine assay). The reaction was stopped at 10 minutes by addition of 2.5 ml 20% trichloroacetic acid. Samples were transferred to 15 ml test tubes and centrifuged on a clinical centrifuge for 15 minutes. Two ml of each supernatant were mixed with 2.0 ml of Nash reagent B (15.0 gm ammonium acetate, 0.30 ml acetic acid and 0.20 ml acetylacetone adjusted to 100 ml with distilled water) and incubated standing in a Fisher Versa water-bath heated to 58°C to allow development of color. After 8 minutes samples were removed and absorbances read at 412 nm on a spectrophotometer. After subtracting absorbances of blank samples, values were compared to those obtained from a standard curve prepared with known concentrations of formaldehyde incubated with Nash reagent B.

2. Aromatic Hydrocarbon Hydroxylation

The hydroxylation of benzo(a)pyrene by microsomal enzymes was assayed in duplicate by measuring the formation of 3-OH benzo(a)pyrene fluorometrically (Nebert and Gelboin, 1968; Wattenberg et al, 1962).

Prior to each experiment, fresh solutions of benzo(a)pyrene, 0.2 mg/ml methanol, and NADPH, 0.6 mg/ml 0.1 M Tris-HCl/6 mM magnesium chloride buffer, pH 7.5, were prepared.

Erlenmeyer flasks, (25 ml), contained a)0.35 ml distilled water, b)0.4 μmol NADPH, c)microsomes: 0.1 ml of a 1:5 dilution of dam liver microsomes (to reduce the concentration of endogenous proteases) containing 10 mg tissue; 0.1 ml fetal liver containing 0.1 gm tissue; 0.1 ml placenta containing 0.2 gm tissue and d)39.6 nmol benzo(a)pyrene for a total volume of 1.0 ml. Blanks contained Tris-HCl buffer instead of NADPH.

For inhibition studies, 0.5 μmol α-NF was added to the incubation mixtures. Controls received equal volumes of methanol.

Mixtures were preincubated for 5 minutes in a Dubnoff incubator at 37°C. The reaction commenced at time zero with the addition of benzo(a)pyrene substrate. After 15 minutes the reaction was stopped by the addition of 1.0 ml acetone. A volume of 3.0 ml of n-Hexane was then added to each flask and agitation continued for another 15 minutes to promote extraction. Mixtures were subsequently transferred to 15 ml test tubes and kept at 4°C for 24 hours. An aliquot of 2 ml from the organic layer of each tube was extracted with 5 ml 1N NaOH by agitating on a vortex mixer for 1 minute. After this step the organic layer was discarded, and after 10 minutes, the remaining aqueous layer was read on a Turner fluorometer model 111 with fluoresecence and excitation set at 525 and 405 nm respectively. Concentrations of hydroxylated product were estimated by referring to a standard curve prepared with known concentrations of 3-OH benzo(a)pyrene.

Statistical Methods

The Student's t test was used to assess statistically significant differences betweens means of unrelated samples. For related samples, the paired t test was used (Colton, 1974). Statistical significance was established at $p<0.05$.

RESULTS

Establishment of Experimental Conditions

1. Tissue Extraction and Chromatographic Analysis

Because of its high lipophilicity, diazepam is a compound that is readily extracted into organic solvents. In order to select a solvent that would provide the best extraction, as well as chromatographs free of interfering peaks, several established analytical techniques were tested. Extraction of DZ and NDZ from guinea pig plasma into ether, with subsequent evaporation of the solvent and redissolution of residues into acetonitrile (Marcucci et al, 1970), proved lengthy and cumbersome. Plasma extraction of drug and metabolites using benzene as a solvent (Berlin et al, 1972) would on occasion elicit interfering chromatographic peaks of unknown origin. When n-butyl acetate was substituted as the organic solvent for extracting drug from plasma (Kuhnz and Nau, 1983), clean peaks were obtained, as shown in Figure 2. Extraction of DZ and NDZ from the liver, brain, adipose tissue and placenta was similarly devoid of extraneous peaks. The retention times (Tr) were 4.0 min for DZ and 5.3 for NDZ.

Known concentrations of DZ and NDZ were added to samples of plasma and tissues from untreated animals for the purpose of constructing calibration curves to assess the efficiency of extraction from each tissue. The percent recovery of drug and metabolite from each biological tissue is recorded in Table 1. Aliquots of 1 ml of dam plasma were spiked with 0, 10, 25, 50,

Figure 2

A gas-liquid chromatogram of diazepam and N-desmethyldiazepam. Sixty minutes after dosing a pregnant guinea pig at term with 10 mg/kg diazepam, plasma was removed and extracted with n-butyl acetate to remove drug and metabolites. DZ and NDZ were separated on a coiled Pyrex column (0.2 cm x 1.2 m) packed with 3% OV-1 on 80-100 mesh contained by a Hewlett Packard model 5710A gas chromatograph equipped with a 63-Ni electron capture detector.

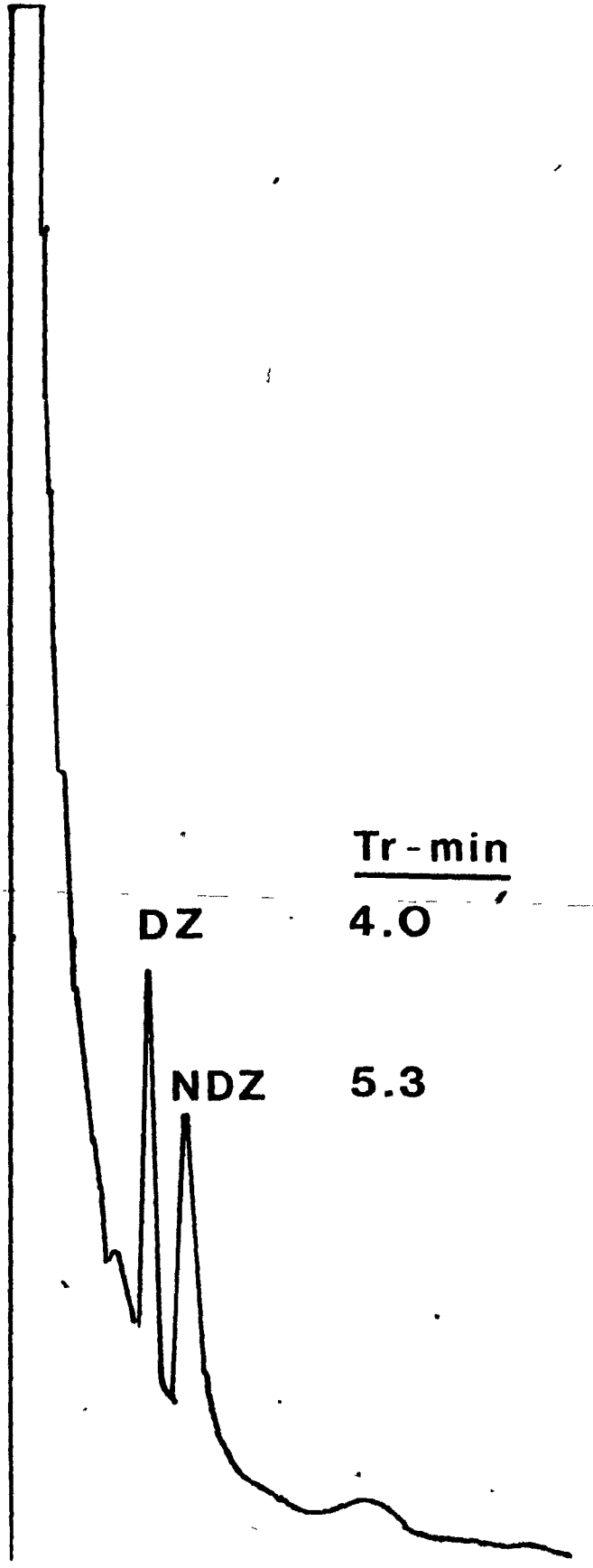

Tr - min
DZ 4.0
NDZ 5.3

Table 1
Percent recovery of diazepam and N-desmethyl-diazepam from plasma and tissues

	Range (ng/ml)		% Recovery	
Tissue	DZ	NDZ	DZ	NDZ
Dam				
plasma	10-500	50-1000	101	81.6
liver	10-200	50-1000	67.8	47.2
adipose	10-200	50-1000	70.0	46.2
brain	10-200	50-1000	75.8	47.6
fetus				
liver	10-200	50-1000	72.3	64.0
adipose	10-200	50-1000	70.0	46.2
brain	10-200	50-1000	72.0	50.0
placenta	10-200	50-1000	74.0	62.3

Percent recovery was assessed in 20% w/v tissue homogenates of liver, adipose and brain and 50% w/v tissue homogenates of placenta.

100, 200 and 500 ng DZ and 0, 50, 100, 250, 500 and 1000 ng NDZ. One ml samples of homogenates of dam liver, adipose tissue and brain as well as fetal liver, adipose tissue, brain and placenta received 0, 10, 20, 50, 100 and 200 ng DZ and 0, 50, 100, 250, 500 and 1000 ng NDZ. The values for percent recovery for each tissue exhibited little fluctuation over the range of concentrations of DZ and NDZ assayed, therefore average values were calculated. Diazepam was more efficiently extracted from plasma and tissues than NDZ. NDZ was more easily extracted from fetal liver than dam liver.

2. Dosage Regimens

To study the plasma elimination of diazepam, guinea pigs were administered single doses of 20 mg/kg orally. Although this is a high dose relative to body weight, diazepam has a wide margin of safety and toxic side effects were never observed after drug administration. Animals were treated with a high dose to enhance sedation, facilitating cardiac puncture by requiring less ether to achieve anaesthesia. Employing this dose also insured detection of drug and metabolite in the plasma for up to 6 hours after administration.

To study the effects of β-NF, reports in the literature describe a 2 or 3-day regimen for dosing rats, employing a daily dose of 80 mg/kg (Anderson and Priest, 1980; Le Provost et al, 1982; Saito and Strobel, 1981). When this dose was administered to guinea pigs, a high mortality rate was encountered. Consequently, the dose used was reduced to 40 mg/kg.

3. Diazepam N-Demethylase Assay

The in vitro N-demethylation of diazepam was measured by modification of the assay for the N-demethylation of aminopyrine (LaDu et al, 1955). Figure 3 depicts diazepam N-demethylation, expressed as nmoles substrate N-demethylated per minute per mg microsomal protein, for a period of 30 minutes at diazepam concentrations ranging from 5 to 40 μg DZ per ml incubation mixture. The activity of N-demethylase after 10 minutes of incubation with 20 μg/ml DZ was chosen for routine assay purposes except where indicated.

When 20 μg/ml DZ was incubated with increasing concentrations of microsomal protein, the quantity of nmoles N-demethylated increased correspondingly. Upon addition of 1 μmol SKF-525A, a P450 inhibitor, activity was inhibited by 17%, while 10 μmol SKF-525A inhibited N-demethylation by 95%.

A curvilinear dose-response relationship existed for 5-40 μg/ml DZ.

Pharmacokinetics of Diazepam

1. Drug Metabolism

The *in vivo* metabolism of diazepam was examined after orally dosing pregnant guinea pigs with 10 mg DZ/kg body weight. Standards containing known concentrations of diazepam metabolites including N-desmethydiazepam, temazepam and oxazepam were analysed by gas chromatography (Figure 4) and compared to a chromatogram prepared from guinea pig plasma (Figure 2). In all experiments with nonpregnant or pregnant animals, NDZ was the only metabolite detected in the plasma.

Figure 3

The *in vitro* N-demethylation of diazepam in hepatic microsomes of a guinea pig dam at term. Diazepam N-demethylase activity, expressed as nmol diazepam N-demethylated per mg microsomal protein, is plotted against minutes of incubation. Rates of N-demethylation are recorded for concentrations of 5.0, 10, 20 and 40 μg/ml diazepam present in incubation mixtures.

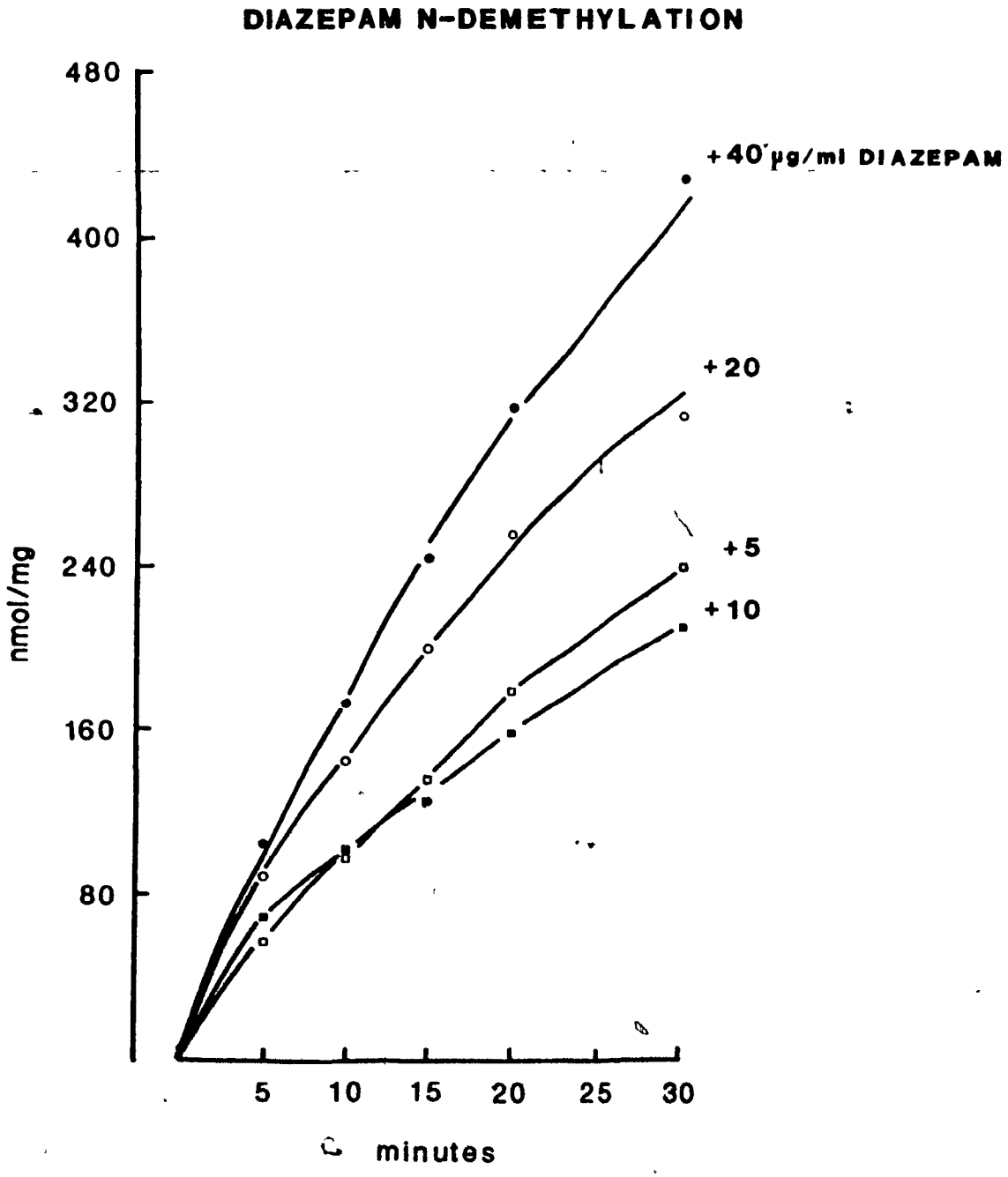
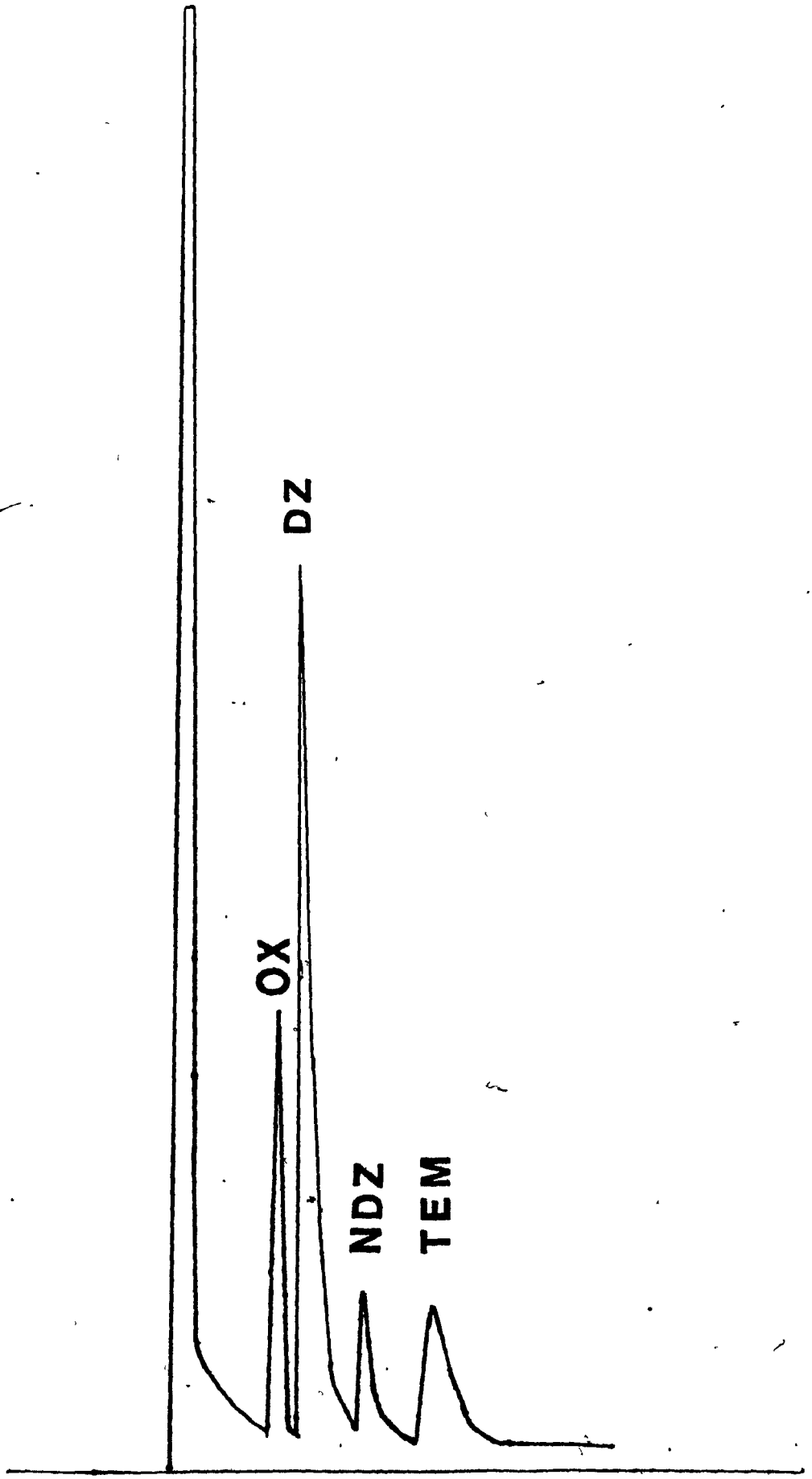

DIAZEPAM N-DEMETHYLATION
480
400
320
240
160
80
nmol/mg
+40 µg/ml DIAZEPAM
+20
+5
+10
5
10
15
20
25
30
minutes

Figure 4

A gas-liquid chromatogram of diazepam and metabolites. Standards of benzodiazepines were prepared from stock solutions of individual compounds dissolved in acetonitrile that included diazepam (DZ) 1.0 μg/ml, N-desmethyldiazepam (NDZ) 1.0 μg/ml, oxazepam (OX) 5.0 μg/ml, and temazepam (TEM) 1.0 μg/ml. Standards were chromatographed on a coiled Pyrex column (0.2 cm x 1.2 m) packed with 3% OV-1 on 80-100 mesh contained by a Hewlett Packard model 5710A gas chromatograph equipped with a 63-Ni electron capture detector. Peak heights represent 5 ng OX, 6 ng DZ, 2 ng NDZ and 8 ng TEM.


DZ
OX
NDZ
TEM

Therefore, N-demethylation appears to be the dominant route of oxidation of diazepam in this species.

2. Plasma Elimination

Some pharmacokinetic properties of diazepam distribution and metabolism in the guinea pig were determined after serial removal of blood samples from 8 guinea pigs dosed with 20 mg DZ/kg. When drug levels were fitted to a log plasma concentration vs. time curve, as is shown in Figure 5, the disappearance of both DZ and the major metabolite NDZ conformed to an open 2 compartment model with α (distributive) and β (elimination) phases. The peak plasma concentration of DZ had already been reached by the time of the first sampling, at 30 minutes. The α phase continued until 60 minutes, after which a linear rate of disappearance was observed. Active biotransformation of DZ with the formation of NDZ occurred during the first hour post-treatment, attaining a peak NDZ plasma concentration of 1.34 μg/ml. By 2 hours after treatment, the plasma disappearance of NDZ had attained a linear rate, though it was slower than that of DZ.

Table 2 summarizes the pharmacokinetic parameters of DZ and NDZ that were calculated from Figure 5. DZ was rapidly eliminated with a βt1/2 of 1.4 hours while NDZ was more slowly removed. The βt1/2 for NDZ was estimated from values obtained from analyses carried out to 6 hours post-treatment. The rapid rate of clearance of DZ from the plasma, 18.0 L/hr, was reflected in the large volume of distribution (Vd=36.4 L), suggesting active sequestration of the drug at peripheral sites.

Figure 5

The semi-logarithmic plot of guinea pig plasma levels of diazepam and N-desmethyldiazepam versus time. After oral administration of 20 mg/kg diazepam to 8 guinea pigs, blood samples were removed at 0.5, 1, 2, 3, 4 and 5 hours by cardiac puncture. Levels of DZ (●) and NDZ (o) in the plasma were quantitated following solvent extraction and gas-liquid chromatography.

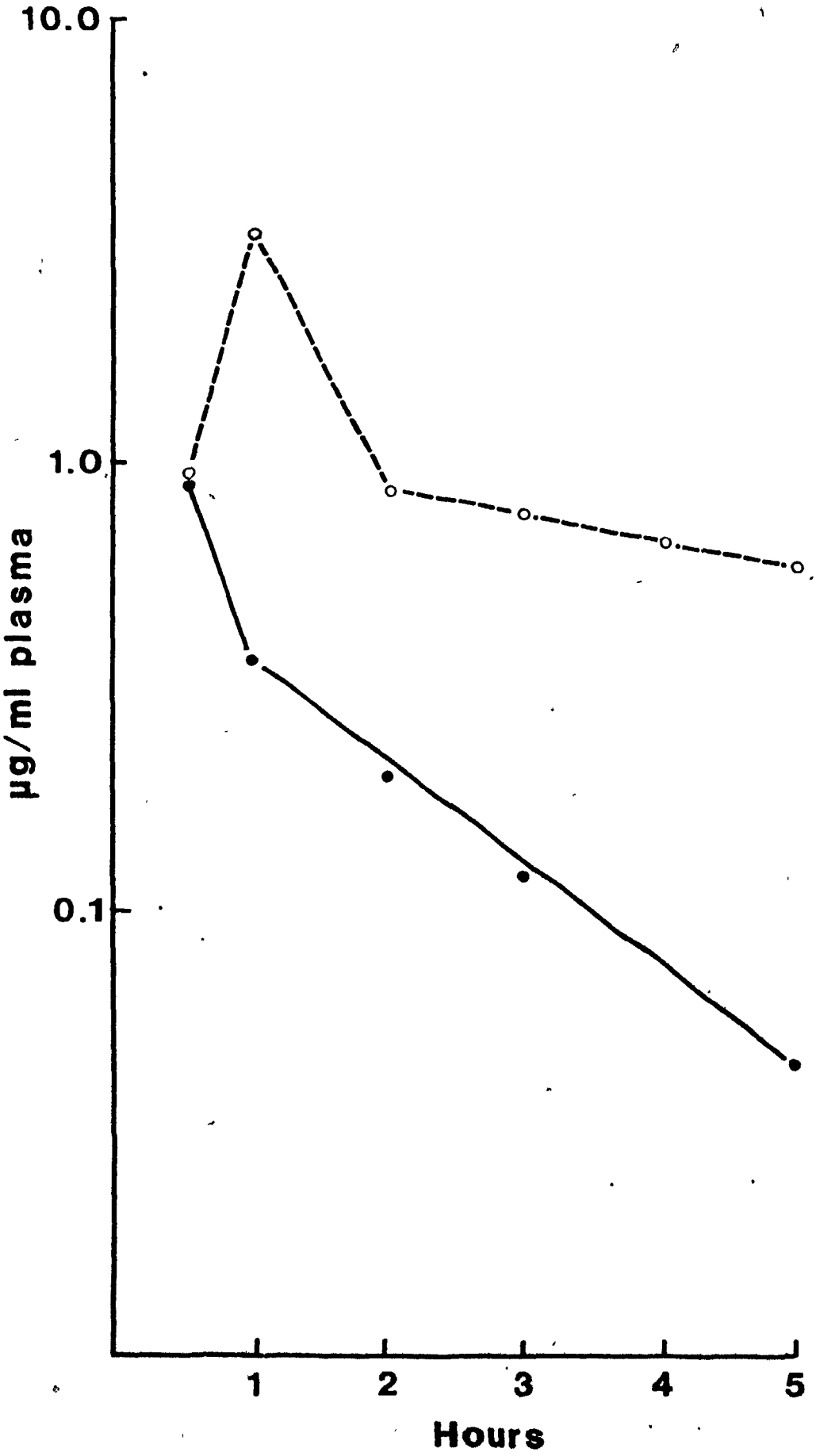

10.0
1.0
0.1
µg/ml plasma
1
2
3
4
5
Hours

Table 2
Pharmacokinetic parameters of diazepam metabolism in the adult guinea pig

Parameter	DZ	NDZ
β t1/2 (hr)	1.4	5.3
Vd (L)	36.4	-
Cl (L/hr)	18.0	-

βt1/2: elimination half-life; Vd: volume of distribution; Cl: plasma clearance.

3. Plasma and Tissue Distribution

Five pregnant guinea pigs of 65-67 days gestation were dosed orally with 10 mg DZ/kg and after 60 minutes, the animals were killed and DZ and NDZ levels were quantitated in maternal plasma, liver, adipose tissue and brain, and plasma, liver, adipose tissue, brain and placenta of individual fetuses. As can be seen in Figure 6, the placental transfer of diazepam was considerable. The parent drug and metabolite were highly distributed to all tissues investigated, with livers and brains exhibiting the greatest accumulations. In all biological tissues, NDZ levels exceeded DZ levels. The DZ and NDZ levels in dam plasma were not significantly different from those measured in fetal plasma. Hepatic NDZ concentrations in the dam were significantly different than fetal levels ($p<0.05$). The difference in brain DZ levels between dam and fetus was also statistically significant ($p<0.05$).

The sequestration of drug and metabolite intimated by the large Vd value reported above was confirmed. Table 3 depicts the ratios of tissue to total (bound + free) plasma DZ and NDZ concentrations in dam and pups. Ratios ranged from 0.8 in fetal brain/plasma DZ to 65.4 in maternal liver/plasma NDZ. In general, tissue levels greatly exceeded plasma levels and the pattern of tissue distribution was similar in dam and pups. In maternal tissues, the highest ratios were seen in the liver with values of 30.5 for DZ and 65.4 for NDZ. Among fetal tissues, the highest DZ and NDZ ratios were also seen in the liver, although these values, 4.0 and 15.3, were considerably lower than the corresponding dam ratios. This difference may

Figure 6

Plasma and tissue levels of diazepam and N-desmethyldiazepam in the materno-placento-fetal unit of the term guinea pig. Pregnant guinea pigs at 65-67 days gestation were dosed orally with 10 mg/kg diazepam, and after 60 minutes, animals were killed and plasma and tissues were removed. DZ and NDZ residues in maternal plasma, liver, adipose tissue and brain, and fetal plasma, liver, adipose tissue, brain and placenta were quantitated by gas-liquid chromatography following solvent extraction. Levels of DZ (open bar) and NDZ (hatched bar) are expressed as μg per gm tissue and μg per ml plasma. For dam tissues, values represent the mean of 5 animals ± S.E. For fetal tissues, values represent the mean ± S.E. of average values of 5 litters.

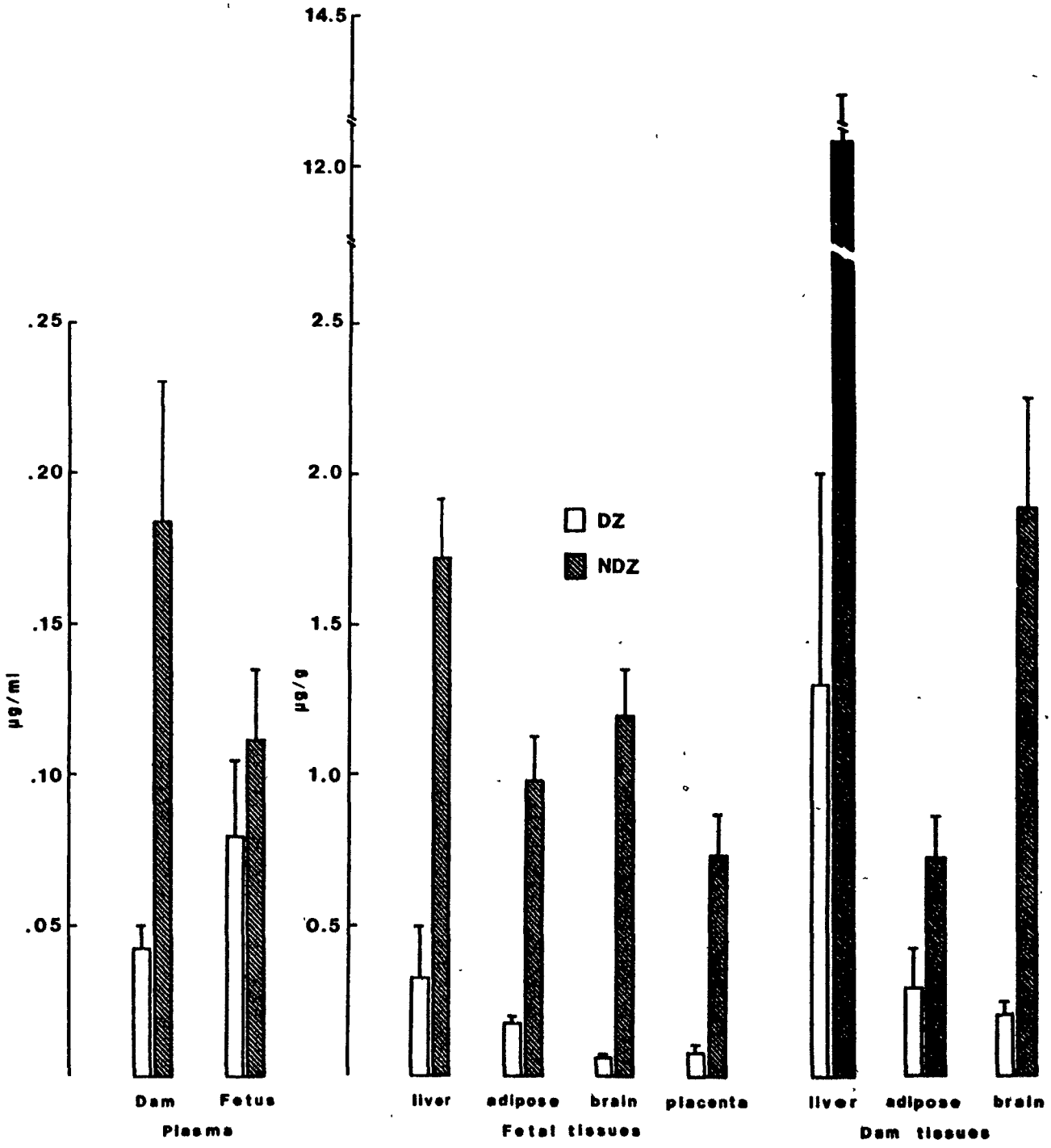

14.5
12.0
2.5
2.0
1.5
1.0
0.5
.25
.20
.15
.10
.05
µg/ml
µg/g
DZ
NDZ
Dam
Fetus
Plasma
liver
adipose
brain
placenta
Fetal tissues
liver
adipose
brain
Dam tissues

Table 3
Tissue/plasma ratios of diazepam and N-desmethyl-diazepam in guinea pig dam and pups at term[1]

	Dam		Pups	
	DZ	NDZ	DZ	NDZ
Liver/Plasma	30.5	65.4	4.0	15.3
Adipose/Plasma	7.1	3.9	2.2	8.7
Brain/Plasma	4.9	10.2	0.8	10.7
Placenta/Plasma	-	-	0.9	6.6

[1] Guinea pigs were at 65-67 days gestation. Ratios are derived from the means of tissue levels of 5 dams and the means of averages of tissue levels of 5 pup litters.

signify deficient N-demethylase activity in the fetal liver.

Microsomal Enzyme Activity

Drug metabolizing ability in the materno-placento-fetal unit of the guinea pig was assessed *in vitro* by means of specific assays that measured N-demethylase and hydroxylase functions. In addition to diazepam, the N-demethylation of aminopyrine was investigated. This assay was initially performed as a basis for developing the diazepam assay and then maintained for comparison purposes, aminopyrine being a substrate for the same oxidative pathway. The hydroxylation of benzo(a)pyrene was also examined because of its known responsiveness to PAH-type inducers.

The tissues from which microsomes were prepared included the livers of the dam and fetuses, these tissues being the major drug-metabolizing organs of the body. To evaluate the fetal contribution to overall diazepam metabolism in the materno-placento-fetal unit, placental activity was also studied. The dam liver contained 6-12 mg protein/gm tissue, the fetal liver 2-5 mg/gm and the placenta 2.5-3.5 mg/gm.

1. The Effect of Pregnancy

An initial investigation examined these three oxidative pathways in non-pregnant control guinea pigs and pregnant animals at 65-67 days gestation (Table 4). No differences were observed between the two groups for any of the three assays. The two N-demethylase substrates, diazepam and aminopyrine,

Table 4
Effect of pregnancy on hepatic microsomal enzyme activity[1]

Activity[2]	Control	Pregnant
DZ (nmol/min/mg)	14.75 ± 1.48 (5)	14.59 ± 2.06 (9)
AP (nmol/min/mg)	8.40 ± 0.72 (5)	10.64 ± 1.26 (10)
BP (ng/min/mg)	123.40 ± 7.44 (5)	121.00 ± 15.3 (10)

[1] Pregnant guinea pigs are at 65-67 days gestation.

[2] DZ = diazepam N-demethylase, AP = aminopyrine N-demethylase, BP = benzo(a)pyrene hydroxylase.

Data represent mean values ± S.E.; number of animals shown in parentheses.

appeared to be oxidized at comparable rates.

Control activities for the three assays in dam liver, fetal liver and placenta appear in Table 5. Not surprisingly, fetal oxidation was deficient compared to maternal microsomal activity for all three pathways. The placental microsomes exhibited low but measurable N-demethylase ability toward both diazepam and aminopyrine substrates, the rates being nearly one half of those seen in the fetal liver. Fetal liver hydroxylase activity was even more deficient than maternal activity compared to N-demethylation. In the placenta, hydroxylation of benzo(a)pyrene was either undetectable or extremely low.

While N-demethylase activity toward aminopyrine or diazepam was not significantly different in the dam liver, the fetal liver and the placenta demonstrated a significantly better ability to N-demethylate the diazepam substrate ($p<0.05$).

2. Treatment with Naphthoflavones

Following the daily treatment of 63-65 day pregnant guinea pigs with either β-NF 40mg/kg or vehicle (peanut oil), microsomal oxidative abilities were examined (Table 6). The only significant induction ($p<0.05$) was observed in diazepam N-demethylation in dam liver microsomes. It is possible that the high degree of variability consistently observed among tissue samples, reflected in the large S.E. values, may have prevented the detection of other significant results.

As a further test of P448 responsiveness, the effect of adding α-NF, a selective P448 inhibitor, to incubation flasks was measured. As seen in Figure 7, the presence of 0.2 mM α-NF

Table 5
Microsomal enzyme activities in the pregnant guinea pig[1]

	Dam liver	Fetal liver	Placenta
DZ	14.59 ± 2.06(9)	1.28 ± 0.22(10)	0.48 ± 0.09(7)
AP	10.64 ± 1.26(10)	0.60 ± 0.14(10)	0.24 ± 0.05(9)
BP	121.0 ± 15.3(10)	1.08 ± 0.29(10)	0.06 ± 0.02(10)

[1] Guinea pigs were at 65-67 days gestation.

DZ = nmoles diazepam N-demethylated per min per mg microsomal protein; AP = nmoles aminopyrine N-demethylated per min per mg microsomal protein; BP = ng 3-OH benzo(a)pyrene formed per min per mg microsomal protein.

Data represent mean values ± S.E.; number in parentheses indicates the number of animals (dam liver) or the number of pooled litter samples (fetal liver, placenta).

Table 6
Effect of β-NF on microsomal enzyme activities in pregnant guinea pigs[1]

Activity[2]		Dam liver	Fetal liver	Placenta
DZ	Control	16.6 ± 2.4(5)	2.20 ± 0.75(5)	0.962 ± 0.582(5)
	Treated	32.9 ± 4.7(6)*	2.67 ± 0.78(6)	0.586 ± 0.200(6)
AP	Control	13.1 ± 2.0(4)	0.53 ± 0.53(4)	0.164 ± 0.080(4)
	Treated	16.5 ± 2.7(5)	1.86 ± 0.64(5)	0.466 ± 0.186(2)
BP	Control	96.1 ± 17.7(5)	1.08 ± 0.17(4)	0.310 ± 0.050(5)
	Treated	125.4 ± 23.0(6)	1.52 ± 0.26(6)	0.475 ± 0.138(5)

[1] Guinea pigs were at 63-65 days gestation. Treated animals received oral β-naphthoflavone (β-NF) 40 mg/kg daily for 3 days. Control animals received the appropriate volume of peanut oil. Animals were sacrificed within 24 hours of final dose.

[2] DZ = nmoles diazepam N-demethylated per min per mg microsomal protein; AP = nmoles aminopyrine N-demethylated per min per mg microsomal protein; BP = ng 3-OH-benzo(a)pyrene formed per min per mg microsomal protein.

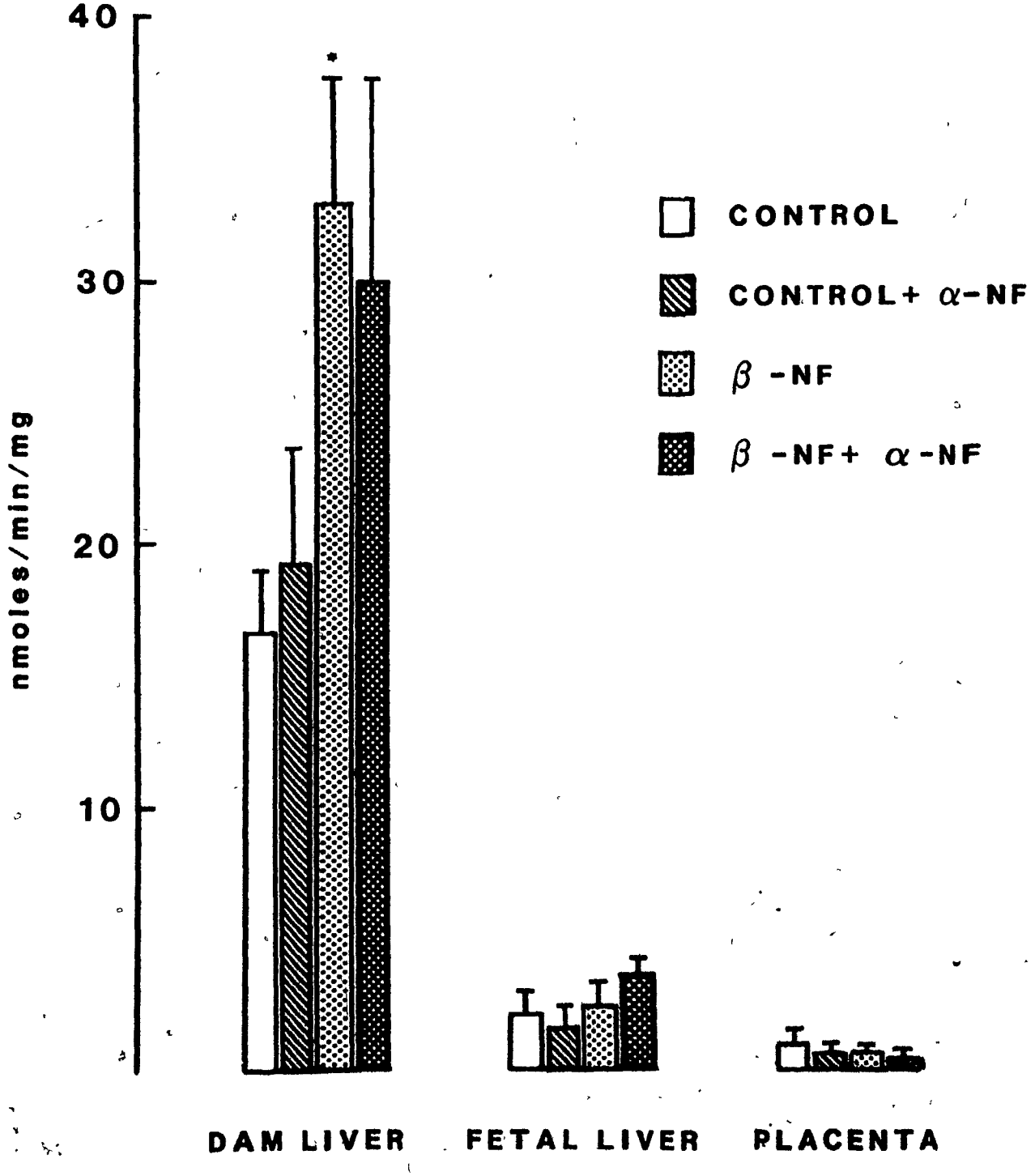
Data represent mean values ± S.E.; number in parentheses indicates the number of animals (dam liver) or the number of pooled litter samples (fetal liver, placenta).

* Value significantly different from control ($p<0.05$).

Figure 7

The effect of α-naphthoflavone on *in vitro* diazepam N-demethylation by control and β-naphthoflavone-treated pregnant guinea pigs. Six pregnant guinea pigs at 63-65 days gestation were administered daily oral doses of 40 mg/kg β-NF for 3 days. Five control animals received peanut oil. N-Demethylation was measured in microsomal preparations of dam liver, fetal liver and placenta in the presence and absence of 0.2 mM α-NF. Activity is expressed as nmol diazepam N-demethylated per min per mg microsomal protein in controls (open bar), controls + α-NF (hatched bar), β-NF-treated (stippled bar) and β-NF-treated + α-NF (cross-hatched bar). For dam liver, values represent the mean ± S.E. of individual liver samples; for fetal liver and placenta, values represent the mean ± S.E. of pooled litter samples. The asterisk (*) indicates values significantly different from control ($p<0.05$).

DIAZEPAM N-DEMETHYLATION



in incubations of dam liver microsomes was not able to reverse the significant induction of diazepam N-demethylation observed after treatment of guinea pigs with β-NF. The inhibitor had no effect on microsomal activity of untreated controls. In both fetal liver and placenta, α-NF did not significantly affect diazepam N-demethylase activity in either control or β-NF-treated animals. In a similar fashion, aminopyrine N-demethylation was unaffected by the addition of α-NF to microsomes of dam liver, fetal liver and placenta from either control or β-NF-treated animals (Figure 8).

In contrast, when 0.5 mM α-NF was present in microsomal incubations of dam liver and placenta, significant reductions ($p<0.05$) in the hydroxylation of benzo(a)pyrene were observed (Figure 9). In dam liver, hydroxylase activity was inhibited to similar extents in microsomes from both control and β-NF-treated animals. In the placenta, only α-NF-treated microsomes from control tissues exhibited a statistically significant reduction in activity. Hydroxylation in the fetal liver was not inhibited by addition of α-NF. In the control microsomes there appeared to be a paradoxical activation of hydroxylase activity, but this was not statistically significant.

3. Smoke Exposure

After exposing pregnant guinea pigs thrice daily for 10 consecutive days to either cigarette smoke or air, microsomal activity was assessed *in vitro*. As shown in Figure 10, diazepam and aminopyrine N-demethylation exhibited parallel responses. No induction was observed in either the dam liver or

Figure 8

The effect of α-naphthoflavone on *in vitro* aminopyrine N-demethylation by control and β-naphthoflavone-treated pregnant guinea pigs. Five pregnant guinea pigs at 63-65 days gestation were administered daily oral doses of 40 mg/kg β-NF for 3 days. Four control animals received peanut oil. N-Demethylation was measured in microsomal preparations of dam liver, fetal liver and placenta in the presence and absence of 0.2 mM α-NF. Activity is expressed as nmol aminopyrine N-demethylated per min per mg microsomal protein in controls (open bar), controls + α-NF (hatched bar), β-NF-treated (stippled bar) and β-NF-treated + α-NF (cross-hatched bar). For dam liver, values represent the mean ± S.E. of individual liver samples; for fetal liver and placenta, values represent the mean ± S.E. of pooled litter samples. The asterisk (*) indicates values significantly different from control ($p<0.05$).

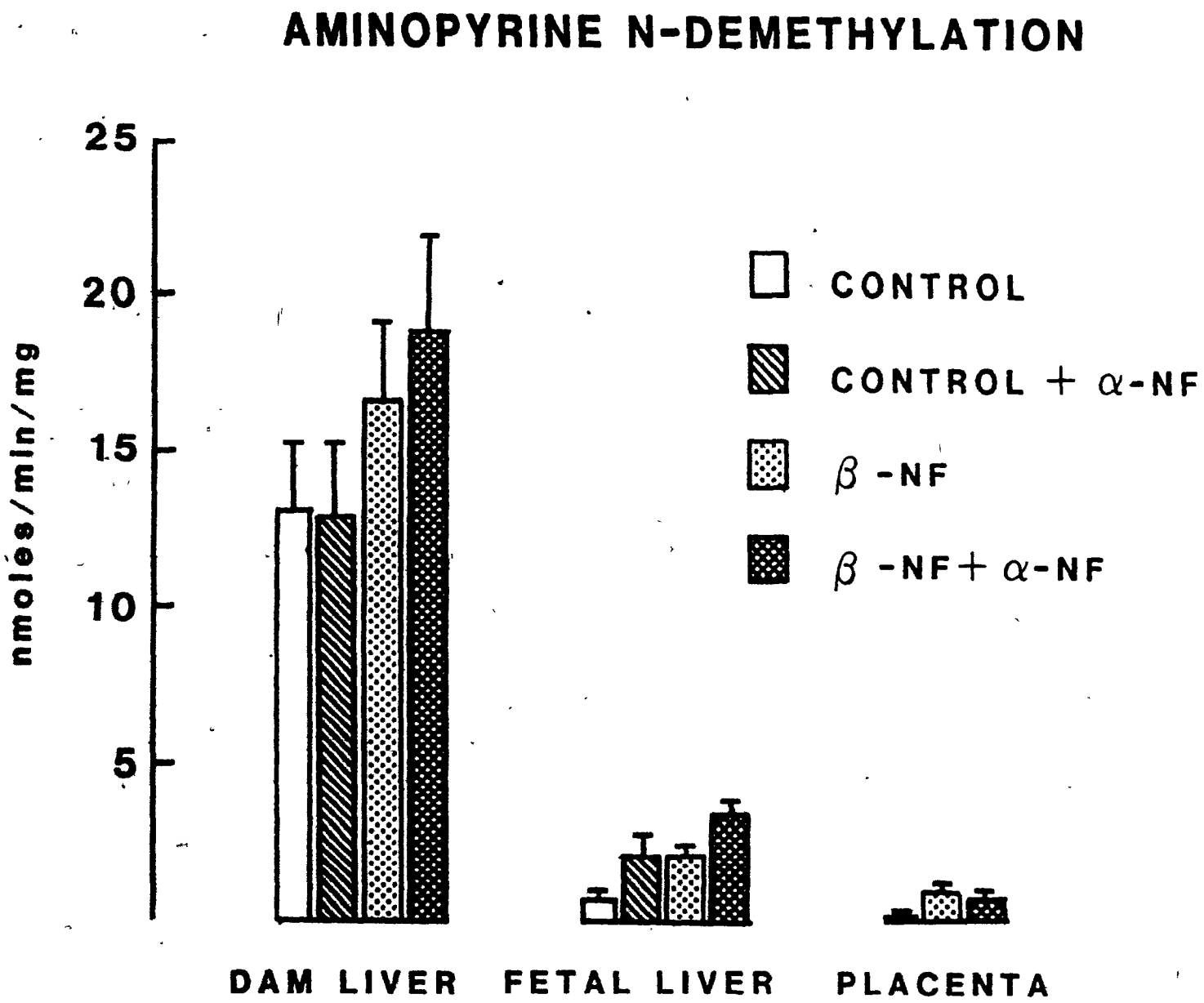

AMINOPYRINE N-DEMETHYLATION
25
20
15
10
5
nmoles/min/mg
CONTROL
CONTROL + α-NF
β -NF
β -NF+ α-NF
DAM LIVER
FETAL LIVER
PLACENTA

Figure 9

The effect of α-naphthoflavone on *in vitro* benzo(a)pyrene hydroxylation by control and β-naphthoflavone-treated pregnant guinea pigs. Six pregnant guinea pigs at 63-65 days gestation were administered daily oral doses of 40 mg/kg β-NF for 3 days. Five control animals received peanut oil. Hydroxylation was measured in microsomal preparations of dam liver, fetal liver and placenta in the presence and absence of 0.5 mM α-NF. Activity is expressed as ng 3-OH benzo(a)pyrene formed per min per mg microsomal protein in controls (open bar), controls + α-NF (hatched bar), β-NF-treated (stippled bar) and β-NF-treated + α-NF (cross-hatched bar). For dam liver, values represent the mean ± S.E. of individual liver samples; for fetal liver and placenta, values represent the mean ± S.E. of pooled litter samples. The single asterisk (*) indicates values significantly different from control animals. The double asterisk (**) indicates values significantly different from β-NF-treated animals ($p<0.05$).

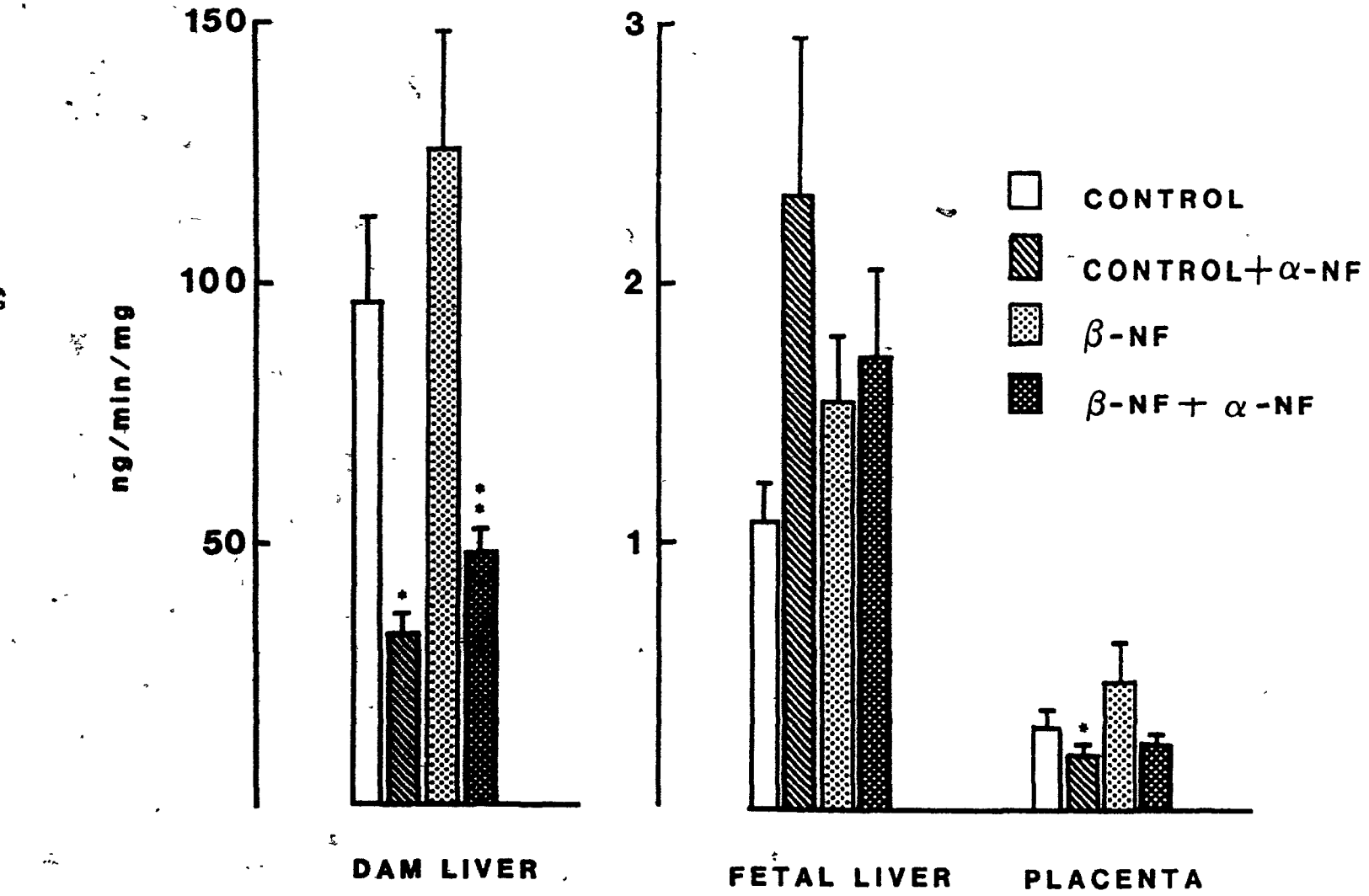
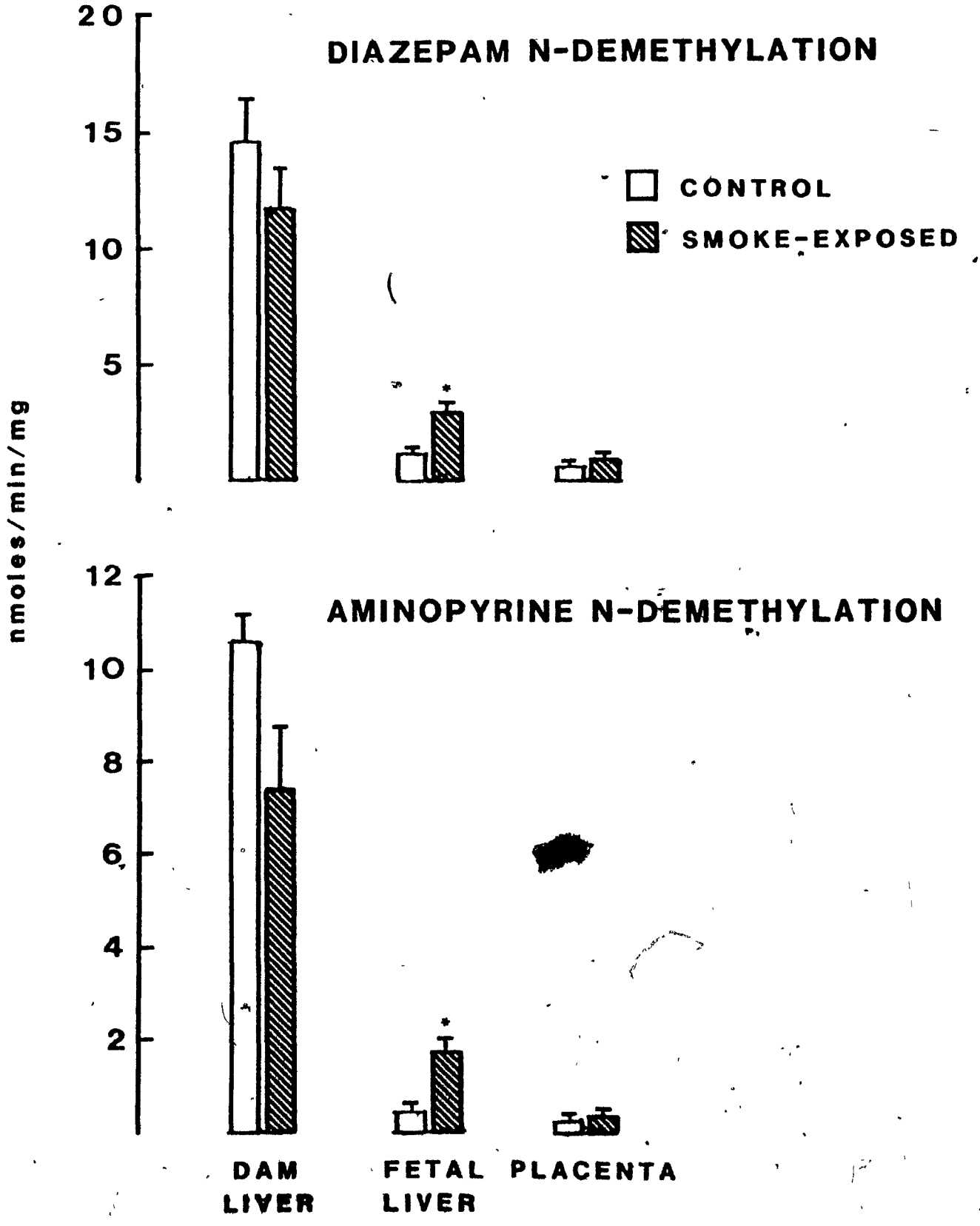

BENZO(a)PYRENE HYDROXYLATION
ng/min/mg
150
100
50
3
2
1
CONTROL
CONTROL+α-NF
β-NF
β-NF + α-NF
DAM LIVER
FETAL LIVER
PLACENTA

Figure 10

The effect of cigarette smoke exposure on the N-demethylation of diazepam and aminopyrine in the pregnant guinea pig. Pregnant guinea pigs at 55-57 days gestation were exposed thrice daily to 40 puffs of a 1:5 dilution of cigarette smoke for the last 10 days of gestation. Control animals were exposed to ambient air. N-Demethylation in dam liver, fetal liver and placental microsomes of control (open bar) and smoke-exposed (hatched bar) guinea pigs is expressed as nmol substrate N-demethylated per min per mg microsomal protein. For dam liver, values represent the mean ± S.E. of individual liver samples; for fetal liver and placenta, values represent the mean ± S.E. of pooled litter samples. The asterisk (*) indicates values significantly different from control ($p<0.05$).


DIAZEPAM N-DEMETHYLATION
CONTROL
SMOKE-EXPOSED
20
15
10
5
nmoles/min/mg
AMINOPYRINE N-DEMETHYLATION
12
10
8
6
4
2
DAM LIVER
FETAL LIVER
PLACENTA

placental microsomes, while fetal liver activity was significantly enhanced compared to controls ($p<0.05$). Exposure to cigarette smoke proved more effective at inducing the hydroxylation of benzo(a)pyrene (Figure 11). While dam liver activity was unchanged, both fetal liver and placental microsomes of smoke-exposed animals displayed marked increases in hydroxylase activity ($p<0.05$).

The efficacy of smoke exposure as an inducing agent is evaluated in Table 7. N-demethylase activity in fetal liver alone was induced 2-4 fold in exposed animals, while hydroxylase activity was induced in fetal liver and placenta by as much as 24.6 and 12.8 fold respectively.

Hepatic responsiveness to cigarette smoke was further examined by comparing the dose-response relationships of diazepam N-demethylation for microsomes prepared from control or smoke-exposed in dam and fetal livers (Figure 12). The N-demethylase activity was saturated in dam liver microsomes at 40 μg DZ/ml for both control and smoke-exposed animals. Cigarette smoke exposure had no effect on N-demethylation for concentrations of diazepam ranging from 5-200 μg/ml. In contrast, microsomes from fetal livers of smoke-exposed animals displayed significantly induced N-demethylase activity for concentrations of diazepam that ranged from 5-40 μg/ml. The microsomal fetal liver N-demethylase was saturated at 20 μg DZ/ml. The subsequent reduction in rate at higher diazepam concentrations may reflect product inhibition of the enzyme.

Figure 11

The effect of cigarette smoke exposure on the hydroxylation of benzo(a)pyrene in the pregnant guinea pig. Pregnant guinea pigs at 55-57 days gestation were exposed thrice daily to 40 puffs of a 1:5 dilution of cigarette smoke for the last 10 days of gestation. Control animals were exposed to ambient air. Hydroxylation in dam liver, fetal liver and placental microsomes of control (open bar) and smoke-exposed (hatched bar) guinea pigs is expressed as ng 3-OH benzo(a)pyrene formed per min per mg microsomal protein. For dam liver, values represent the mean ± S.E. of individual liver samples; for fetal liver and placenta, values represent the mean ± S.E. of pooled litter samples. The asterisk (*) indicates values significantly different from control ($p<0.05$).

BENZO(a)PYRENE HYDROXYLATION

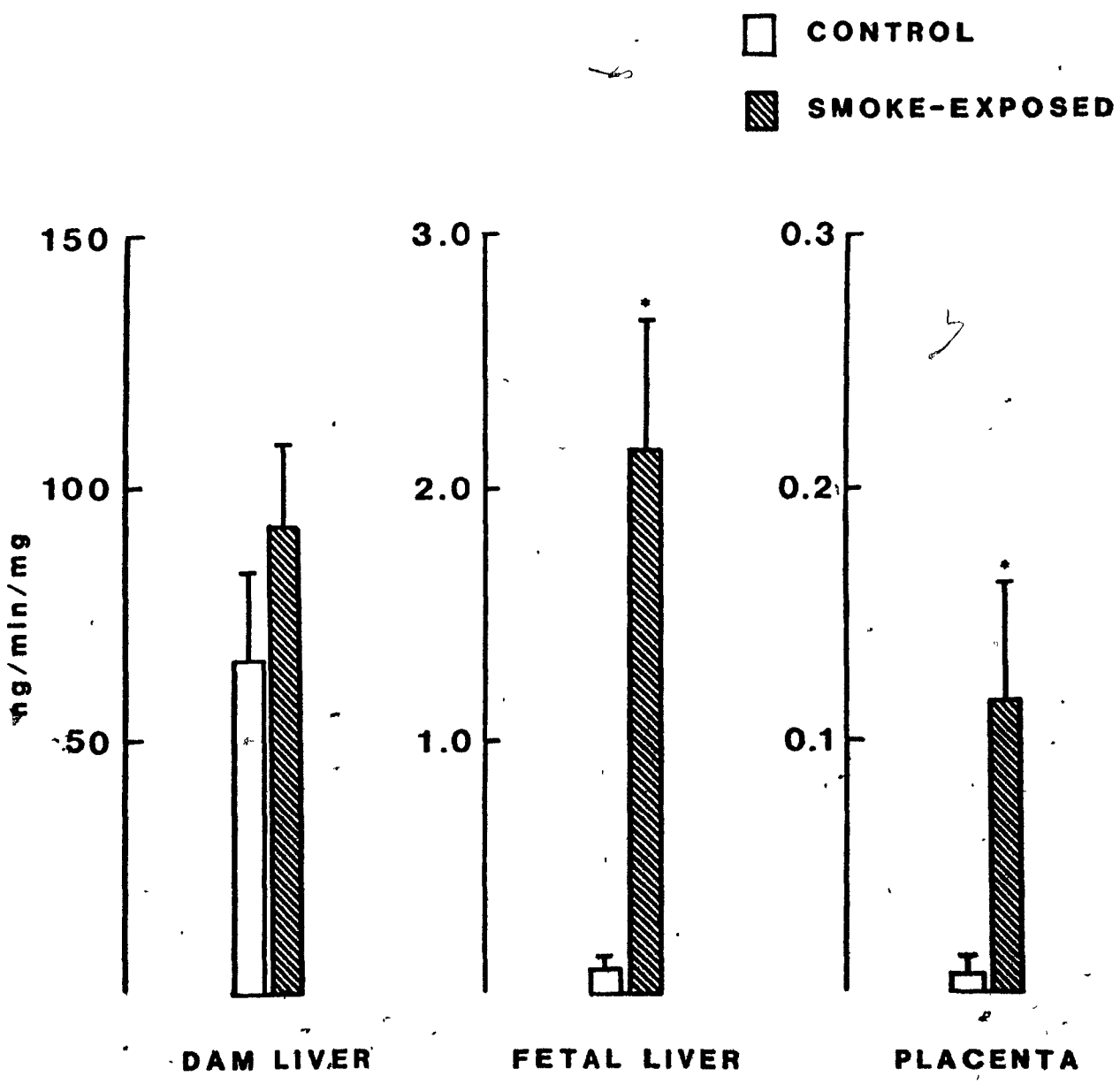


Table 7
Ratios of smoke-exposed/control microsomal enzyme activities in the term guinea pig[1]

Activity[2]	Dam liver	Fetal liver	Placenta
DZ(nmol/min/mg)	0.57	2.23	0.71
AP(nmol/min/mg)	0.51	3.92	0.93
BP(ng/min/mg)	1.39	24.6	12.8

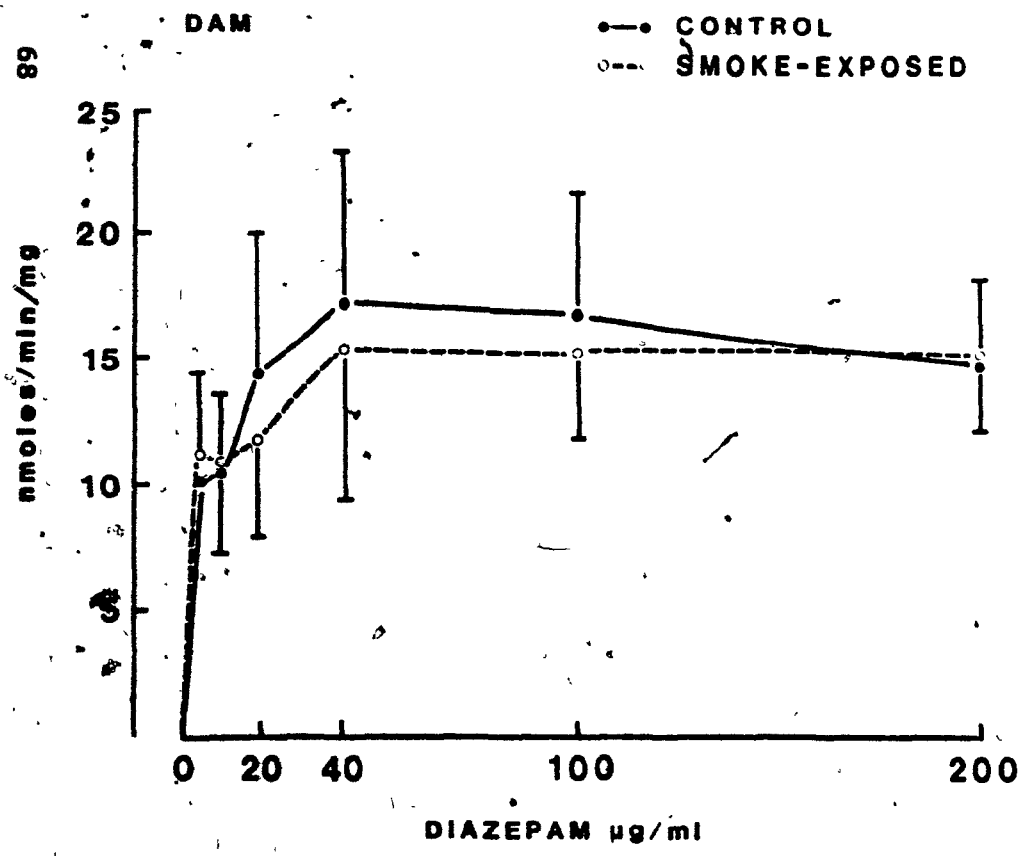
[1] Guinea pigs were at 65-67 days gestation. Animals were exposed to 40 puffs of a 1:5 dilution of cigarette smoke thrice daily for 10 days. Control animals were handled in a similar way but exposed to ambient air. Animals were sacrificed within 24 hours of last exposure.

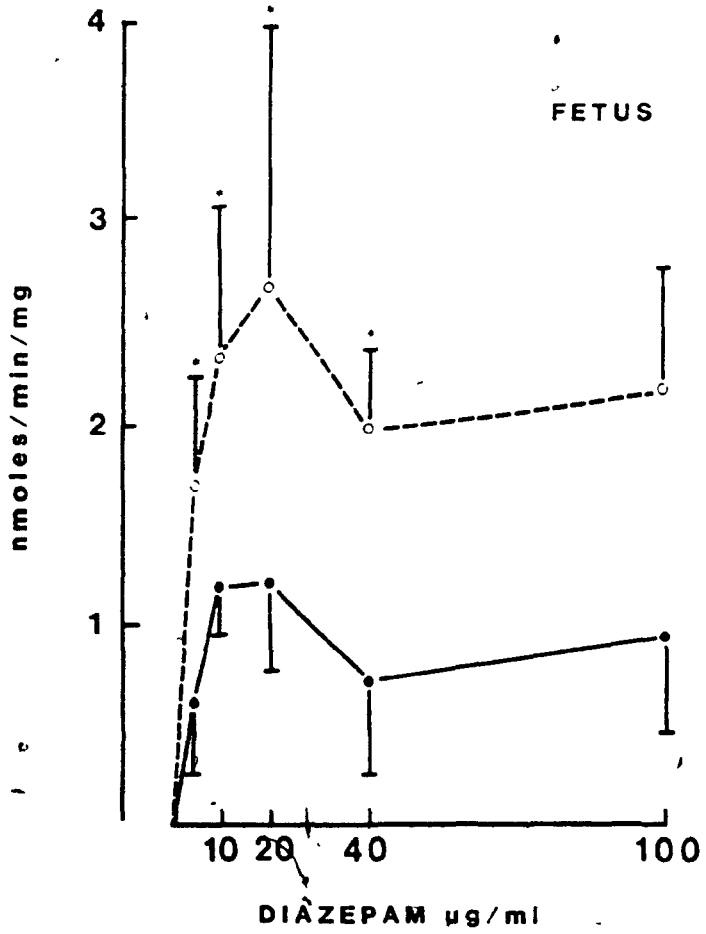
[2] DZ = diazepam N-demethylase; AP = aminopyrine N-demethylase; BP = benzo(a)pyrene hydroxylase.

Figure 12

A comparison of hepatic diazepam N-demethylase activity in the control and smoke-exposed dam and fetal guinea pig. Diazepam metabolism in microsomes from control and smoke-exposed guinea pigs, expressed as nmol diazepam N-demethylated per min per mg microsomal protein, is plotted against increasing concentrations of diazepam (μg/ml) present in incubation mixtures. Values for dam hepatic activity represent the mean ± S.D. of individual liver samples; values of fetal hepatic activity represent the mean ± S.D. of pooled litter samples. An asterisk indicates values significantly different from control ($p<0.05$).

HEPATIC N-DEMETHYLASE





DISCUSSION

The results described in the previous section attempt to shed light on the pharmacokinetic properties, placental transfer, disposition and biotransformation of diazepam in the materno-placento-fetal unit of the guinea pig.

The plasma βt1/2 of 1.4 hours for diazepam (Fig. 5, Table 2) compares favorably with the value of 2.4 hours measured by Klotz et al (1976). Klotz also reported DZ βt1/2 values of 7.6, 2.7 and 1.1 for dog, rabbit and rat respectively. These values are considerably lower than the average half-life of 33 hours reported for humans. Plasma clearance rates of total (bound + free) diazepam were much higher in the dog (18.9 ml/min/kg), rabbit (30.5 ml/min/kg), guinea pig (18.9 ml/min/kg) and rat (81.6 ml/min/kg) than in man (0.35 ml/min/kg). Klotz noted that DZ blood clearance rates exceeded hepatic blood flow rates in these species. This suggests that in laboratory animals diazepam may be cleared non-restrictively, i.e. both bound and free drug are being removed rapidly from the plasma by tissues. This is in contrast to the human, where diazepam behaves as a restrictive agent whose clearance depends on the free fraction of drug in the plasma (Nau et al, 1984). A non-restrictive behaviour and active uptake by tissues would account for the extremely high Vd (36.4 L) measured in guinea pigs (Table 2).

Studying the disposition of diazepam in the guinea pig has revealed that considerable tissue uptake occurs within 60 minutes of administration (Figure 6). The rapid and widespread distribution of diazepam has been confirmed in several species. Igari injected C14-diazepam into rats and discovered that the

drug was rapidly distributed to the liver, kidney, lungs, brain, heart and small intestine (Igari et al, 1982). Metabolites were detectable in plasma and tissues immediately after injection. Diazepam and metabolites were more slowly distributed to the skin and adipose tissue. The delayed appearance in these tissues was attributed to a large tissue volume and/or low blood flow. This may explain the relatively low levels of drug and metabolite quantitated in guinea pig adipose tissue 60 minutes after DZ administration (Figure 6). Unlike the guinea pig, where higher levels of metabolite than parent compound were seen, DZ tissue levels in the rat exceeded those of NDZ in all tissues studied except the liver and small intestine. This may reflect the different major routes of DZ oxidation in these two species. Oxazepam (OX) was also observed in all tissues in the rat, whereas this metabolite was not detected in guinea pig plasma or tissues (Figure 2). Lower metabolite levels in rat tissues most likely indicated a more rapid excretion in this species. This finding agrees with Marcucci's suggestion that diazepam is short-acting in the rat and that the metabolites do not persist (Marcucci et al, 1969). He discovered that while NDZ and OX accumulated in mouse brain, these metabolites were soon undetectable in rat brain. He suggested that the persistence of DZ metabolites in mouse brain may be the cause of the prolonged anticonvulsant action of diazepam observed in this species.

Marcucci also examined DZ, NDZ and OX levels in the blood, brain and adipose tissue of the guinea pig (Marcucci et al, 1970). Similar to the findings presented here, OX was

undetectable in these tissues and NDZ levels exceeded DZ levels after 30 minutes in the blood and brain (Figure 2, Figure 6). Peak adipose tissue concentrations of NDZ were not reached until 3 hours, after which the levels of metabolite exceeded those of the parent compound. Guinea pigs are therefore similar to mice in their ability to accumulate N-demethylated product, but differ in their inability to produce the hydroxylated products temazepam and oxazepam (Fig.1, Fig.2).

It is obvious from the results that the guinea pig placenta poses no barrier to the transplacental acquisition of DZ by the fetus, higher tissue levels being observed than that measured in the plasma of the dam (Figure 6). Idanpaan-Heikkila's group (1971) was among the first to show the accumulating properties of diazepam in fetal tissues in studies with mice, hamsters and monkeys. In mice, diazepam and metabolites crossed the placenta more rapidly in late than in early pregnancy with the highest fetal concentrations appearing from 1 hour after dosing. Maternal liver and brain concentrations were highest in the materno-placento-fetal unit and were comprised mostly of metabolite. In the hamster, diazepam crossed the placenta much more slowly with maternal plasma levels being consistently higher than fetal tissue concentrations. The highest fetal accumulation was observed in the spinal cord. In monkeys, ratios of fetal:maternal plasma diazepam levels were slightly greater than 1.0 from 30 minutes to 24 hours after the administration of 5 mg DZ/kg, similar to results measured in humans (Erkkola et al, 1973). By 30 minutes, DZ and metabolite levels were higher in fetal liver,

lung, spinal cord and brain than in maternal plasma, a pattern also observed in the guinea pig model. High concentrations of drug and metabolite were detected in the fetal cerebellum and spinal cord of the monkey and were associated with the hypotonicity observed in "floppy infant syndrome" (Shannon et al, 1972). In the pregnant rat, Simmons et al (1983) found that 50% of the dose was quickly eliminated by the dam. Approximately equal levels of drug and metabolites were found in maternal and fetal brains.

The guinea pig appears to behave more like the monkey than any other species examined by displaying high accumulations of drug and metabolite in fetal tissues, with levels exceeding those of the dam plasma. In both species, the liver and the brain showed the greatest accumulation. Differences in placental transfer and tissue disposition between the rat and the guinea pig may be due in part to anatomical and developmental considerations, as well as the pathways responsible for biotransformation. Because the rat placenta is dichorial and because oxidation proceeds along a route favoring a more water-soluble excretory product (oxazepam), significant levels of drug and/or metabolite may not accumulate in the fetal tissues. In contrast, the monochorial guinea pig placenta and oxidation of diazepam to a highly lipid-soluble, non-conjugatable product (NDZ) would facilitate accumulation in the materno-placento-fetal unit.

The usefulness of the guinea pig model can be further evaluated by comparing the biotransformational capacities of various species. The differences in metabolic pathways among

the rat, mouse and guinea pig have already been confirmed by the presence of oxazepam in rat and mouse tissues. When mouse liver microsomes were incubated with up to 100 μg diazepam, a greater proportion of NDZ was formed, while rat liver microsomes preferentially hydroxylated diazepam with only limited N-demethylation occurring (Marcucci et al, 1969). By incubating microsomes from either species with temazepam, the relative abilities of the rat and the mouse to N-demethylate were determined. Mouse liver N-demethylase activity proceeded at a rate at least 10 fold higher than in rat liver microsomes.

Although there are no reports of *in vitro* DZ metabolism in the materno-fetal unit, Marcucci's group (1973) compared oxidative capabilities of the newborn and adult rat and guinea pig. As was seen in the present study (Table 5), newborn animals were deficient in their oxidative abilities compared to adult animals. In the newborn rat, not only was hydroxylation the preferred route of metabolism, N-demethylated product was undetectable at low concentrations of diazepam. Newborn guinea pig N-demethylase activity was deficient compared to adult activity for all concentrations. A very interesting finding was the formation of temazepam upon incubation of microsomes from newborn guinea pigs with more than 200 μg diazepam. Above this quantity of substrate, N-demethylation sharply declined while the rate of hydroxylation slightly increased. Ackermann and Richter (1977) detected hydroxylation of DZ upon incubation of human fetal liver microsomes with high concentrations of diazepam. No temazepam was detected in the microsomes of adult guinea pigs incubated with 20-500 μg diazepam. Although no

products of hydroxylation were detected *in vivo* in the term guinea pig fetuses (Figure 6), it is possible that *in vitro* hydroxylation may have occurred at high concentrations of substrate; the Hantzsch reaction, a colorimetric procedure, only permits the detection of N-demethylated product. Marcucci assayed for the various products by chromatographic quantitation.

Marcucci's work revealed important differences in oxidative capacities between newborn and adult animals in both rats and guinea pigs (Marcucci et al, 1973). Unlike the adult rat, the newborn rat showed no capacity to perform N-demethylation at low DZ concentrations. Unlike the adult guinea pig, the newborn guinea pig exhibited a capacity to hydroxylate diazepam but at very high concentrations of substrate. These findings suggest that the perinate possesses qualitatively different means of oxidation compared to the adult animal. Those enzymes responsible for biotransformation may undergo changes that characterize hepatic drug-metabolizing ability at the various stages of pre- and postnatal development.

The deficiency in N-demethylase activity in fetal guinea pig liver was more pronounced for aminopyrine than diazepam (Table 5). Fetal N-demethylase activity was 2-fold higher toward DZ in both liver and placental microsomes while in adult liver, the N-demethylation of diazepam and aminopyrine were not significantly different. If both compounds are substrates for the same N-demethylating enzyme, then the significant differences between fetal N-demethylation of aminopyrine and

diazepam may reflect a development-related reduction in enzyme affinity for the aminopyrine substrate that changes as the hepatic enzymes mature. These differences in affinity for the substrates suggest that qualitative differences may exist between adult and fetal N-demethylase enzymes.

The contribution of placental microsomal enzyme activity toward overall fetal oxidative ability cannot be overlooked (Table 5). Placental N-demethylase activity was nearly half that of the term fetus, and therefore could contribute to the levels of NDZ found in the fetus. The existence of placental monooxygenase activity has been demonstrated for many substrates that belong to the category of PAH agents (Kapitulnik et al, 1976; Manchester and Jacoby, 1981; Pelkonen et al, 1979; Welch et al; 1968). The fact that the guinea pig placenta demonstrated an ability to oxidize a therapeutic agent such as diazepam may be of some significance.

The pattern of benzo(a)pyrene hydroxylase activity in dam liver, fetal liver and placental microsomes was clearly different from N-demethylation in these tissues. It is likely that benzo(a)pyrene is oxidized by an isozyme of P450 different from the form responsible for N-demethylation. Other differences between hydroxylase and N-demethylase activities were uncovered by examining their responses to the application of inducing agents. When guinea pigs were administered 40 mg/kg β-NF, a known P448 inducer, only dam liver diazepam N-demethylation was induced, and only 2-fold. However, the dose employed was half of that recommended to achieve measurable induction in order to avoid toxicity to the guinea pigs. The

lack of induction might better be explained by evidence that the guinea pig is a poor responder to PAH-type inducers. Abe and Watanabe (1982) demonstrated refractoriness of 4 different guinea pig strains to the induction of aryl hydrocarbon hydroxylation by treatment with 3-methylcholanthrene. Moreover, guinea pigs are a species known for their resistance to chemical carcinogens requiring metabolic activation, usually by enzymes responsible for PAH oxidation (Alvares et al, 1970; Weisburger and Weisburger, 1973). In contrast, guinea pig diazpam N-demethylase activity was induced approximately 4-fold by treatment with phenobarbital (Marcucci et al, 1970). In the guinea pig at 63 days gestation, acute pretreatment with phenobarbital signficantly induced benzo(a)pyrene hydroxylation and p-chloro-N-methylaniline demethylation in both dam and fetal livers (Kuenzig et al, 1974). The lack of response to PAH inducers suggests that the guinea pig possesses a form of P450 that is unique from the highly-inducible P448 of the rat or LM4 of the rabbit (Guengerich et al, 1982; Norman et al, 1978).

α-NF has been used as an *in vitro* probe to detect multiple forms of the same monooxygenase by studying the response of a particular pathway in different tissues. Though it had no effect on the N-demethylation of either diazepam or aminopyrine, α-NF achieved a reduction in the hydroxylation of benzo(a)pyrene. Interestingly, inhibition was observed only in the microsomal preparations from the dam liver and placenta, and ocurred to approximately the same extent in samples from control and β-NF-treated guinea pigs. The fetal liver appeared

to be unique in its resistance to inhibition by α-NF. Pelkonen (1976) showed that α-NF preferentially inhibited human placental benzo(a)pyrene hydroxylation without inhibiting fetal or adult hepatic activity. His study revealed a 2-fold enhancement of fetal liver benzo(a)pyrene hydroxylation by α-NF. This dual action of α-NF has been seen by others. Gelboin et al (1981) found α-NF to selectively inhibit human placental benzo(a)pyrene hydroxylase activity while enhancing adult hepatic activity. Similarly, Kapitulnik and colleagues (1977) found human hepatic oxidation of benzo(a)pyrene, zoxazolamine and antipyrine to be enhanced by α-NF. The ability of α-NF to inhibit one form of P450 while enhancing the activity of another becomes evident when the metabolism of substrates with affinities for particular forms of P450 is measured in the presence of α-NF. Biphenyl 4-hydroxylation was inhibited while biphenyl 2-hydroxylation was enhanced in the presence of α-NF in liver microsomes from control and 3-methylcholanthrene treated guinea pigs (Thorgeirsson et al, 1979). As was seen with β-NF (Figure 9), pre-treating animals with the P448 inducer 3-methylcholanthrene appeared to have no effect, as inhibition and enhancement occurred to the same extent in both groups. The results depicted in Figure 9 suggest that the form of P450 responsible for the hydroxylation of benzo(a)pyrene in the fetal liver is different from that of the dam liver and placenta. In addition, the lack of response of the N-demethylase to the presence of inhibitor infers that hydroxylation and N-demethylation may be catalysed by two distinct enzymes.

A major objective of this investigation was to study the effects of cigarette smoke exposure on diazepam metabolism in the materno-placento-fetal unit. Cigarette smoke has long been established as an inducer of drug biotransformation due mainly to its PAH constituents. Nicotine hydroxylation (Beckett and Triggs, 1967), phenacetin O-dealkylation (Pantuck et al, 1973), antipyrine hydroxylation (Hart et al, 1976), theophylline N-demethylation (Powell et al, 1977), imipramine N-demethylation (Perel et al, 1975) and pentazocine hydroxylation (Vaughan et al, 1976) are some of the pathways shown to be induced in smokers. In the pregnant subject, cigarette smoke exposure resulted in enhanced oxidation by placental microsomes of substrates including benzo(a)pyrene, 3-methyl-4-monomethylaminoazobenzene, zoxazolamine and 7-ethoxyresorufin (Kapitulnik et al, 1976; Manchester and Jacoby, 1981; Nebert et al, 1969; Pelkonen et al, 1979; Welch et al, 1968). In addition, cigarette smoke exposure has resulted in adverse effects to the fetus. Increased morbidity and low birth weight possibly due to fetal hypoxia are the most common adverse effects (Naeye, 1978; Johnston, 1981). Other changes include enlarged and lesioned placentae (Johnston, 1981), decreases in fetal heart-rate variability (Kelly and O'Conor, 1984; Lehtovirta et al, 1983), increased fetal breathing movements (Thaler et al, 1980), increased levels of cord blood immunoglobulins (Cedarqvist et al, 1984), diminished fetal reactivity (Phelan, 1980) and decreased intervillous placental blood flow (Lehtovirta and Forss, 1978; Rauramo et al, 1983). A change in placental blood flow could exert some

influence on placental transfer and disposition of drugs in the fetus.

In pregnant guinea pigs, cigarette smoke did not induce N-demethylation of either diazepam or aminopyrine in dam liver and placental microsomes (Figure 10). However, N-demethylase activity toward both substrates was induced in fetal liver microsomes. Exposure to cigarette smoke may have induced a form of P450 active in N-demethylation in the fetal liver that was either not present or not inducible in the other tissues examined. The difference in responsiveness between dam liver and fetal liver N-demethylase activities is evident from Figure 12. The observed induction of N-demethylation was not dependent on the concentration of diazepam present in the incubation, and may reflect differences in the genetic regulation of adult and fetal hepatic function.

Not unexpectedly, hydroxylation of benzo(a)pyrene proved more sensitive to induction by cigarette smoke (Figure 11). The extent of induction of fetal liver activity (24-fold) and placental activity (13-fold) was remarkable in view of the documented resistance of guinea pigs to induction by PAH compounds. The considerable responsiveness of the hydroxylase in fetal tissues compared to the dam liver confers a unique qualitative identity upon the monooxygenase responsible for benzo(a)pyrene hydroxylation in the fetus.

That quantitative differences in oxidative activity exist between the dam and term fetus has been demonstrated by lower rates of oxidation toward a range of substrates in the fetuses of many species. Indeed, perinates are not expected to achieve

an adult drug-metabolizing capacity until days or weeks after birth (Neims et al, 1976). The concept that the fetal hepatic monooxygenases responsible for particular pathways of drug metabolism may be qualitatively distinct from those in the dam is not as widespread. Ioannides and co-workers (1984) have stated that the age-dependent changes in oxidative activity in the perinate are qualitative as well as quantitative. Biphenyl 2-hydroxylation was high in newborn rats but undetectable in untreated adults (Basu et al, 1971). Ethoxyresorufin O-deethylase also decreased with age (Ioannides et al, 1984). Qualitative differences in the response to inducers have also been revealed. Treatment of pregnant rabbits with TCDD primarily induced the LM6 in the neonatal liver and the LM4 in the adult liver (Norman et al, 1978). The result of exposing pregnant guinea pigs to cigarette smoke has added one more piece of evidence to support this hypothesis.

SUMMARY AND CONCLUSIONS

Diazepam was oxidized to N-desmethyldiazepam in the guinea pig, the same major metabolite that is produced in humans. A rapid transfer of diazepam across the placenta was observed and 60 minutes after dosing, the drug had accumulated in the maternal liver, adipose tissue and brain, and fetal liver, adipose tissue, brain and placenta. Most ratios of tissue:plasma DZ and NDZ levels were greater than 1.0. The patterns of distribution of DZ and NDZ were similar in the dam and fetal compartments. NDZ levels exceeded DZ levels in all tissues with the largest accumulations observed in the brain and liver. The persistence of the active metabolite NDZ in guinea pig fetal tissues may serve as a useful mechanism for exploring the adverse symptomatology of "floppy infant syndrome" in humans.

The *in vitro* diazepam metabolism by guinea pig tissues displayed the same profile as that of humans. The fetal liver was deficient at oxidation of diazepam, aminopyrine and benzo(a)pyrene. The guinea pig placenta demonstrated an ability to N-demethylate both diazepam and aminopyrine. The placenta may play a significant role in activation or detoxification as substrates potentially toxic to the fetus cross the placenta.

Fetal and maternal hepatic microsomal monooxygenases appeared to be qualitatively different. the inducer β-NF increased hepatic DZ N-demethylation in the dam but not in the fetus. Fetal hepatic microsomal hydroxylation was not inhibited by α-NF whereas the activity in the dam was markedly decreased.

The placental hydroxylation of benzo(a)pyrene was also inhibited by α-NF. The lack of response by fetal liver suggests a development-related tissue dependence of P450 activity.

Further qualitative differences between dam and fetal liver activity were suggested by a significant induction of N-demethylase activity in fetal but not dam liver microsomes from guinea pigs exposed to cigarette smoke. Discordant responses of N-demethylase and hydroxylase activities between the dam and fetal livers may reflect characteristics of enzymatic development under genetic control.

In the human, the responsiveness of hepatic function of the term fetus who is exposed to cigarette smoke may be of importance in determining the fetal tissue levels of pharmacologically active compounds. The state of fetal liver oxidative function and its potential for modification by exposure to cigarette smoke should be considered when women are treated with with therapeutic agents during late pregnancy.

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