THE ONTOGENY OF CYTOCHROME P450 (CYP450) ACTIVITY, EXPRESSION AND VOLATILE ORGANIC COMPOUND (VOC) METABOLISM IN SPRAGUE-DAWLEY (S-D) RATS

by

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(Under the Direction of James V. Bruckner)

ABSTRACT

CYP450 levels are altered by development and minimum metabolism occurs in the young due to an under developed hepatic system. A minimum amount of data is available for developmental toxicological studies. Rodents are often used as surrogates for toxicological and pharmaceutical studies. In the current study, Sprague-Dawley (S-D) rats were used to assess the enzymatic activity, protein expression and metabolic capabilities of developing post-natal day (PND) rodents. Cytochrome P450 (CYP450) enzymes are members of Phase I metabolism which are involved in the oxidation, reduction and hydrolysis of compounds. CYP2E1, CYP1A1/2 and CYP2B1/2 are the predominate isozymes responsible for the metabolism of xenobiotics. PNDs 1, 5, 10, 15, 21, 30, 40, 50, 60 and 90 hepatic microsomes were used to measure the ontogeny of each isozyme enzymatic activity and protein expression. CYP2E1 activity and expression showed a decrease with an increase in age, with peak levels occurring at PND 21; CYP1A1/2 showed slightly lower activity and expression in younger animals with peak levels occurring between PND 21 through 40; CYP2B1/2 activity and expression was also slightly lower in younger animals with peak levels occurring at PND 21. After the enzymatic activity and protein

expression was established, the metabolic capacity of PND 10, 15, 21, 60 and 90 were determined using the Michaelis-Menten parameters K_m and V_{max} in hepatic microsomal samples. The volatile organic compounds (VOCs) dichloromethane (DCM), trichloroethylene (TCE) and perchloroethylene (PERC) were used to measure the hepatic microsomal metabolism. Each VOC is commonly used in industrial settings and are metabolized by Phase I CYP450s. DCM, TCE and PERC showed an increase in metabolism with an increase in time and protein concentration. Although TCE and PERC are high and low metabolizing compounds, respectively, which form similar metabolites, DCM and TCE metabolism showed a similar pattern throughout development while PERC remained relatively constant. In spite of these differences, each compound showed peak Vmax levels at PND 21. The current study has shown that rodents have the highest enzymatic activity, protein expression and metabolic ability prior to adulthood; therefore, this data could prove useful in the risk assessment of VOCs for developing humans.

INDEX WORDS: Cytochrome P450 (CYP450), CYP2E1, CYP1A1/2, CYP2B1/2, Dichloromethane (DCM), Trichloroethylene (TCE), Perchloroethylene (PERC), Michaelis-Menten, K_m, V_{max}

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DEDICATION

This accomplishment is dedicated to the African kings, queens and tribes; the slaves, maids and sharecroppers; the factory workers, preachers and teachers, whose blood run through my veins.

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CHAPTER 1

INTRODUCTION

There have been few developmental toxicological studies addressing the metabolism of xenobiotics in maturing subjects. The metabolism of a compound typically involves the conversion of the compound to a more active or inactive form. Many factors affect the rate and extent of metabolism including age, gender, race and lifestyle (Bruckner 1999; Ma and Lu 2003). Metabolism is characterized as Phase I and Phase II reactions. Cytochrome P450 (CYP450) isozymes participate in Phase I metabolism and are usually involved in the biotransformation of a compound to an oxidized form, which is then either excreted or subjected to phase II metabolism.

Rats are often used as surrogates for humans in toxicological studies. The majority of toxicological data available for most chemicals do not differentiate between human developmental stages as described by the National Academy of Science (NAS) in healthy subjects (Alcorn and McNamara 2002). Thus it is necessary to characterize the maturation of xenobiotic metabolism in rodents and humans throughout all maturational stages, in order to assess the rodent's suitability as a model for maturing humans.

Although the field of toxicology is heavily dependent upon rodent data for human extrapolation, some data are available on the developmental enzymatic expression of CYP450 enzymes in rodents. Characterizing the maturation of hepatic CYP450 expression would be beneficial to biological sciences as a whole, as it would not only provide more comprehensive knowledge of the rate of oxidative metabolism at different life stages, but also provide caution as too which classes of chemicals may be of particular concern.

Background

Many factors may cause variations in xenobiotic metabolism including gender, age, disease state, body weight, therapeutic treatments and ethnicity.

Among these factors, potential age- and gender-dependent differences in drug efficacy and toxicity are presently receiving significant public and scientific attention. In 1988, the US Congress requested the National Academy of Sciences (NAS) to establish a committee within the National Research Council (NRC) to study scientific and policy issues concerning pesticides in the diets of infants and children (Goldman and Koduru 2000). The NRC report in 1993 entitled *Pesticides in the Diets of Infants and Children* prompted the passage of the Food Quality Protection Act of 1996 (FQPA), which required the US Environmental Protection Agency (EPA) to make more realistic assessments of the health risks posed by pesticides by assessing aggregate and cumulative risks. Following the FQPA in 1997, former President Clinton signed a historic executive order requiring for the first time that all federal agencies ensure that their policies and rules address disproportionate environmental health and safety risks to infants and children (Goldman and Koduru 2000).

Previous studies of pesticides and other chemicals demonstrated that toxicity was frequently age- and compound-dependent (Bruckner and Weil 1999). There is relatively little basis in the scientific and medical literature for prediction of benefits or risk posed to children by most drugs and other chemicals (Blanck *et al.* 1986; Sexton *et al.* 2005). Evidence suggests there may be 'windows of vulnerability' or short periods of development when chemical exposure can permanently alter organ structure or function (Selevan *et al.* 2000; Scheuplein *et al.* 2002). Therefore, studies are sorely needed to address potential pharmacodynamic and pharmacokinetic changes during maturation.

Human Metabolic Development

Children and adults differ both physiologically and behaviorally; therefore, the effects of exogenous and endogenous compounds may differ in children when compared to adults (Morford *et al.* 2004). Pharmacokinetic differences between children and adults with respect to the clearance of therapeutic agents have been recognized for years (Ginsberg *et al.* 2004; Makri *et al.* 2004). It has been shown that children are not small adults. Children have smaller body size; different ratios of fat, muscle and water; higher breathing and metabolism rates per body weight; and immaturity of clearance systems and enzymatic reactions (Ginsberg *et al.* 2004; Makri *et al.* 2004)). As the young mature, 'windows of vulnerability' may occur that could result in an increase in toxic effects (Bruckner and Weil 1999; Scheuplein *et al.* 2002).

Postnatal developmental groups in humans are classified as full-term newborns, neonates (birth – 4 weeks), infants (4 weeks – 1 year), young children (1 – 5 years), older children (6 – 12 years) and adolescents (13 – 18 years). The major periods of rapid growth include infancy and puberty. Although adolescence is marked by the second most rapid period of physiological development, xenobiotic metabolism appears to peak during infancy and early childhood (Bruckner 1999). Some investigators have concluded that activities of some enzymes reach adult levels by age one, but it was emphasized by NAS (1993) that maturation of some organ systems, which can influence kinetics, does not occur until adolescence (Imaoka *et al.* 1991; Vieira *et al.* 1996; Cresteil 1998; Goldman and Koduru 2000; Hines and McCarver 2002; Johnsrud *et al.* 2003).

The liver is able to convert toxic parent compounds to less toxic metabolites, and hasten the removal of endogenous and exogenous compounds from the body by excreting them into the

bile and urine. CYP450s metabolize endogenous as well as exogenous compounds that enter the body. Although CYP450s are present in a number of organs including the lungs, brain and kidney, they are largely found in the liver and expressed mainly in hepatocytes, with the highest concentrations in the centrilobular zone inside the acinus (Pineiro-Carrero and Pineiro 2004; Gonzalez 2006).

The CYP450 enzymes are broken into the family (more than 40% identity of amino acid sequence), subfamily (more than 55% identity of amino acid sequence) and the individual gene (Cederbaum 2006). Each isozyme is different. There are several factors that have been shown to affect CYP450 activity (Bebia *et al.* 2004). The genetic makeup of an individual can cause alterations in metabolic activity. Japanese have been shown to express less CYP2E1 in the liver than Caucasians (Chen *et al.* 2002). The second cause of variation is the inducibility of CYP450 activity, as a result of exposure to drugs, endogenous compounds and environmental agents, i.e., acetylsalicylic acid (aspirin), ethanol, tobacco and phenobarbitol (Gebremichael *et al.* 1995; Eke *et al.* 1997; Czekaj *et al.* 2000; Pustylnyak *et al.* 2005). The third cause of variability is inhibition of CYP450 activity by drugs, endogenous agents and environmental compounds (Aimova and Stivorova 2005). Finally, host factors can cause interindividual variation in CYP450 metabolism. These include disease state, diet and hormonal influences. Liver cirrhosis and diabetes have both been show to alter the metabolic activity of CYP450s, along with growth and sex hormones (Bandiera *et al.* 1992; Borlakoglu *et al.* 1993; Clewell *et al.* 2002; Makri *et al.* 2004).

Although information is available for the ontogeny of some CYP450 human isozymes, the data are usually gained from measuring enzymatic activity in post-mortem liver samples from children who died from various causes, including disease (Vieira *et al.* 1996; Cresteil 1998;

Bjorkman 2006). During development, changes occur in the liver which may affect both rates and metabolic pathways. Prolonged action of some drugs can result from the neonatal liver's decreased capacity to metabolize, detoxify and excrete the xenobiotics.

As the body grows, the size of the liver changes, leading to changes in total hepatic metabolic capacity (Clewell *et al.* 2002; Anderson and Palmer 2006). Enzymatic content is more variable than hepatic blood flow in neonates, while age-dependent changes in blood flow appear to be more important in other groups. There is greater intersubject variability in neonates than in older children (Bjorkman 2004; Nong *et al.* 2006). Although CYP450 activity has been reported to be approximately 50% higher in adults when compared with the fetus and neonates (Clewell *et al.* 2002 and Diehl-Jones and Askin 2002), it is believed that most isozymes reach adult levels by year one (Alcorn and McNamara 2002).

The liver is the largest organ in neonates (Diehl-Jones and Askin 2002). Neonates are generally exposed to exogenous compounds by inhalation or ingestion of breast milk or formula. Most intermediary and xenobiotic metabolic functions are immature in newborns, leading to reduced metabolic rates and prolonged elimination of substances that are dependent on these pathways (Makri *et al.* 2004). Drinking water, formula and food consumption increase in infancy (Fiorotto *et al.* 1991; Gentry *et al.* 2003). At one year of age, phase I/II metabolism are still immature, thereby producing certain metabolites that differ from those in adults (Clewell *et al.* 2002; Makri *et al.* 2004). Infants often exhibit an increase in metabolic clearance, which has been interpreted as an enhanced capacity to metabolize drugs due to an enhanced liver size or hepatic blood flow (Anderson an Palmer 2006). Phase I reactions reach maturity by the end of the young child stage (2-6 yrs) and older children Phase I/II metabolism are fully functional (Clewell *et al.* 2002; Makri *et al.* 2004).

Due to growth spurts, hormonal fluctuation, lifestyle choices, dietary habits, risk-taking behavior and occupation, adolescence is designated as the second period of highest biological change in humans (Bruckner 1999; Scheuplein *et al.* 2002; Makri *et al.* 2004) Absorption, distribution and elimination of xenobiotics are mature by adolescence, although hormone-induced changes possibly alter CYP450 metabolism during puberty (Makri *et al.* 2004).

Development is characterized by rapid and relatively continuous changes in the state of differentiation of the organism. Development toxicity is defined as the occurrence of adverse effects on the developing organism that may result from exposure prior to conception (either parent), during prenatal development, or postnatally to the time of sexual maturation (Selevan *et al.* 2000). Exposure to toxicants during development can lead to irreversible effects (Corley *et al.* 2003). The long-term effects caused by developmental toxicity include death of the developing organism, structural abnormality, altered growth and functional deficiency (Selevan *et al.* 2000).

CYP2E1, CYP1A1/2 and CYP2B1/2 are Phase I CYP450 isozymes that are involved in the metabolism of xenobiotics. CYP2E1 oxidizes small hydrophobic exogenous compounds and is induced by ethanol and acetone (Cederbaum 2006; Gonzalez 2006). Johnsrud *et al.* 2003 found that the largest increase of protein content of the isozymes occurred within the first postnatal months. Adult levels were reached by 90 days. They also showed that CYP2E1 protein content significantly increased from the third trimester through the postnatal period and through adulthood, although the in the older age group were taken from individuals 94 samples ranging in age from day 91 – 18 years.

The CYP1A family has been shown to bioactivate several procarcinogens (Aimova and Stiborova 2005; Ma and Lu 2003). CYP1A2 is the last isozyme to form in humans (Cresteil

1998; Sonnier 1998). It is absent during the fetal and neonatal period of development, but increases between 1 and 3 months of age (Cresteil 1998; Sonnier and Cresteil 1998; Hines and McCarver 2002). In a study by Hakkola *et al.* 1996, several xenobiotic metabolizing isozymes were determined at the mRNA level by reverse transcriptase-polymerase chain reaction (RT-PCR) in human placenta. CYP1A1 was not only expressed, but also induced in placenta of smoking mothers. CYP1A1 is not present in adult liver (Sonnier and Cresteil 1998) due to the decline in mRNA expression with age (Hines and McCarver 2002).

Theophylline is a medication metabolized by CYP1A1 and CYP2E1. It is used to treat respiratory ailments such as asthma, chronic bronchitis and emphysema. In a study by Bjorkman *et al.* (2004), theophylline was used to measure pharmacokinetic parameters in neonates, 6-month, 1-year, 2-year, 5-year and 10-year-olds. The demethylation of imipramine was used as a probe to measure the activity of CYP1A2. It was estimated that neonates possess 5%, 6-month-olds possess 51%, 1-year-olds possess 63%, 2-year-olds possess 81% and 5-year-olds possessing 107% of CYP1A2 activity of adults. The hydroxylation of chlorzoxazone was used as a probe to measure the activity of CYP2E1. Neonates possessed 2%, 6-month-olds possessed 43%, 1-year-olds possessed 48%, 2-year-olds possessed 57% and 5-year-olds possessed 75% of CYP2E1 activity of adults. The-10-year-olds were assumed to have normal adult levels of CYP1A1 and CYP2E1 activity.

CYP2B6 is a human isozyme found in the liver that is similar to the rodent enzyme CYP2B1/2. It is believed to play an important role in the activation of cyclophosphamide, an alkylating anticancer prodrug (Code *et al.* 1997). The expression of CYP2B6 is very low when compared to other CYP450s, accounting for less than 1% of total CYP450. CYP2B6 shows large interindividual variability and racial differences, with undetectable enzymes levels in 70%

of Japanese and 15% of Causasians. It has been shown to be a high affinity / low capacity enzyme that is capable of metabolizing compounds such as ketamine at lower concentrations (Hijazi *et al.* 2002).

Human versus Rodent

The most fundamental assumption in hazard identification is that if a chemical is hazardous to animal, it is hazardous to humans (Goldman and Koduru 2000). Age differences may be less pronounced in human than in rodents, as humans are more mature at birth than rodents in terms of liver metabolism. Although the animal research may reveal mechanisms of toxicity extrapolation of temporal trends across species is difficult, because organs and their associated functions mature at different rates in different species. Rodent studies provide valuable information on mechanism and specific immaturities that may be broadly applicable to infants and children (Ginsberg *et al.* 2004; Rodriguez *et al.* 2007). Humans reach adulthood in 18-25 years, with bursts of development during both early childhood and puberty. In contrast, rats attain adult status very quickly (Morford *et al.* 2004). Rodents have been very useful in delination of the toxicokinetic basis of age-dependent differences in heavy-metal toxicity, but subhuman primates are generally the animal of choice to examine toxicodynamic factors (Ginsberg *et al.* 2004; Makri *et al.* 2004).

Very limited work has been done to assess the toxicity of xenobiotics in children. The majority of such studies have involved determination of maximally-tolerated doses of chemotherapeutic agents. In the current study, Sprague-Dawley rat hepatic microsomal samples

will be collected on days 5, 10, 15, 21, 30, 40, 50, 60 and 90 of life, in order to assess the maturation of total CYP450, CYP2E1, CYP1A1/2 and CYP2B1/2 expression and activity.

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CHAPTER 2

ONTOGENY OF HEPATIC CYTOCHROME P450 (CYP450) ENYMATIC ACTIVITY AND PROTEIN EXPRESSION IN SPRAGUE-DAWLEY (S-D) RATS [1]

[1] B. McPhail, C. White, S. Murildhara, J. Wilson, J. Bruckner. To be submitted to *Drug Metabolism and Disposition*.

ABSTRACT

Widespread concerns about safety and efficacy of pediatric drugs, and potential susceptibility of infants and children to chemicals, have led to the use of immature rats as test surrogates. Our objective was to test the following hypothesis: The ontogeny of hepatic microsomal cytochrome P450s 2E1, 1A1/2 and 2B1/2 in Sprague-Dawley (S-D) rats resembles maturation in humans closely enough to reliably extrapolate results of metabolic studies of these isozymes' substrates in developing rats to humans. Microsomes were prepared from livers from unsexed postnatal day (PND) 1 – 90 S-D rats, and total CYP450s, as well as CYP2E1, CYP1A1/2 and CYP2B1/2 activities and protein were quantified. Isozyme protein levels were determined to be gender-independent in another experiment. Total CYP450s rose rapidly from PND 1 – 15 and gradually increased thereafter to adult levels. CYP2E1 and CYP1A1/2 developmental profiles in rats exhibited a very different pattern (i.e., rapid rise after birth, elevated levels from PND 21 – 40/50, and substantial decline to relatively low adult values). These isozymes' activity and protein levels were well correlated. CYP2B1/2 activity also peaked on PND 21, but declined irregularly to adult values. High metabolic capacity was evident in the weanlings and postweanlings when the three isozymes' activities were calculated for the entire liver. The metabolic activation of chemical substrates for the three isozymes may be greater, and the resulting adverse effects expect to be more severe in immature rats than in children. Immature S-D rats may be better surrogates for substrates of other isozymes, as total hepatic CYP450s progressively increase during maturation of each species.

INTRODUCTION

Mammalian species modify the structure of a variety of endogenous and exogenous compounds by enzyme-catalyzed processes. Cytochrome P450 (CYP450) isozymes catalyze Phase I reactions, notably oxidation and dealkylation. Metabolites may be more or less biologically active than the parent compound. Metabolites are usually more water soluble, so they are excreted or serve as substrates for Phase II reactions. The expression and action of CYP450s are critical in the systemic clearance of many drugs and other chemicals in children (Bjorkman, 2006; Gow *et al.*, 2001; Pineiro-Carrero *et al.*., 2004) and adults. Orthologous CYP450s in rats and humans oxidize many chemicals similarly. Nevertheless, there are significant interspecies differences in the expression and activity of some forms (Nedelcheva and Gut, 1994). Such differences in CYP450s of interest in the current study will be described below.

The public has major concerns about the sensitivity of children to pharmaceuticals, pesticides and other chemicals. In 1988, the U.S. Congress requested that the National Academy of Sciences appoint a committee to assess uncertainties associated with exposure and susceptibility to pesticides in infants' and children's diets. As a result of this committee's report in 1993, the Food Quality Protection Act (FQPA) of 1996 was passed. The FQPA required that the U.S. EPA apply an additional 10X safety factor when assessing risks of pre- and postnatal toxicity. In 1997, it was reported that 78.2% of high-production-volume chemicals in the U.S. had not been tested for developmental toxicity (Goldman, 2000). Former President Clinton signed an executive order in 1997, requiring all federal agencies to consider how their activities impacted the health of children. The Pediatric Research Act, of 2003, required pharmaceutical

companies to assess safety and efficacy of new drugs used by children. Due to ethical questions involved in pediatric pharmacokinetic, toxicology and efficacy investigations, data attained from animal studies often must be relied upon (Selevan *et al.*., 2000).

CYP1A1/2, CYP2E1 and CYP2B1/2 mediate the metabolism of a variety of drugs and other organic compounds present in home, occupational and environmental settings. Metabolic activation of polyaromatic hydrocarbons (PAHs) to proximate carcinogens is of interest to our research group. CYP1A1/2 isozymes metabolize PAHs, aflatoxin B1, aromatic amines, estradiol and several common medicinals (Hines and McCarver, 2002; Omiecinski *et al.*, 1999). The CYP1A family consists of two subfamilies: 1A1 and 1A2. Metabolic activation of PAHs by 1A1 involves the aryl hydrocarbon receptor (AhR), which is regulated by the nuclear pregnane X receptor (PXR), that in turn is activated upon PAH binding (Ma and Lu, 2003).

CYP2E1 is the predominant constitutive human and rat isozyme responsible for metabolism of: ethanol; drugs such as acetaminophen, caffeine, chlorzoxazone, isoniazid and certain anesthetics; and low levels of many volatile organic compounds (VOCs), (Guengerich *et al.*., 1991; Hines and McCarver, 2002). CYP2E1 is induced by many of its substrates, including acetaminophen, aspirin, ethanol and acetone (Morel *et al.*. 1999; Johnsrud *et al.*, 2003, Gonzalez, 2005). Oxidation of some VOCs (e.g., heptane, toluene, xylenes) results in their detoxification and hastened elimination, while oxidation of others (e.g., benzene, tetrachloroethylene, vinyl chloride, ethylene dibromide) produces reactive cytotoxic/mutagenic metabolites.

CYP2B1/2 and its apparent human equivalent, CYP2B6, catalyze metabolism of moderate to high doses of VOCs, barbiturates and several other important pharmaceuticals (Omiecinski *et al.*, 1999). 2B1 and 2B2 have overlapping substrate specificities and 97% gene homology in their sequence (Omiecinski *et al.*, 1990). The 2B6 gene is activated by two ligand-

activated transcription factors, PXR and the constitutive androstane receptor (CAR). Activated CAR and PXR regulate overlapping genes, including CYP3A and CYP2B6 (Maglich *et al.*, 2002).

Limited human and even fewer rodent data are available on the ontogeny of hepatic CYP450s. Therefore, there is little metabolic basis for judging the relevance of much immature rodent data to infants and children. Thus, the primary objective of the current study was to contrast the maturation of total hepatic CYP450s, as well as expression of CYP2B1/2, CYP2E1 and CYP1A1/2 activities and protein, in Sprague-Dawley (S-D) rats with published human ontogeny data. Male and female pups were examined to determine whether expression of the selected isozymes was sex-dependent. A wide range of ages was studied to ensure that expression during key developmental periods (e.g., neonatal, preweaning, prepubescent, adolescent) was thoroughly characterized. The findings described here may prove useful in assessing immature S-D rats suitability to serve as surrogates for prediction of infants', children's and adolescents' abilities to metabolize and respond to a variety of drugs, VOCs and PAHs.

MATERIALS AND METHODS

Animals Housing: Timed pregnant female S-D rats were obtained from Charles River Laboratories, Raleigh, NC. Dams were housed in rat shoebox polystyrene cages, acclimated to a 12-h light/dark cycle in a temperature (25 ± 2°C)- and humidity- (40% ± 10) controlled room, and allowed to deliver their pups over a period of 14 days. Tap water and Purina Rat Chow 5001 rat food were provided *ad libitum* to the dams and the pups as they matured.

Microsomal Preparation: Male and female pups of different ages were sacrificed by cervical dislocation and their liver collected. Due to their small size, livers from multiple unsexed rats were pooled as follows: twenty postnatal day (PND) 1 pups per sample; twelve PND 5 pups per sample; five PND 10 pups per sample; five PND 15 pups per sample; and three PND 21 pups per sample. The entire liver was removed from male and female PND 30 rats. A 5-g portion of liver was taken from male and female PND 40, 50, 60 and 90 animals. The homogenized liver(s) was (were) centrifuged at 12,000g for 30 min at 4°C. The supernatant was collected and centrifuged 105,000g using a Beckman Coulter Optima XL-100K ultracentrifuge (Beckman Coulter, Fullerton, CA) at 4°C for 60 min. Microsomes were resuspended in Tris-KC1 at pH 7.4 and stored at -80°C prior to use.

Microsomal Protein and CYP450s: Hepatic microsomal protein concentrations and total CYP450 levels were measured by conventional spectrophotometric procedures with a Gilford Response Spectrophotometer (GRS) at 660 nm.

CYP2E1 Activity: Assays were conducted in sample vials containing 0.25 ml of ascorbic acid, 0.2 ml of a NADPH regenerating system and 0.1 ml of 1:4 dilution of hepatic microsomal suspension. Perchloric acid (0.5 ml) was added to each control sample. The vials were placed into a 37°C water bath for 2 min before 50 µl of 0.1 mM *p*-nitrophenol (PNP) solution was added. The samples were incubated for an additional 5 min, before 0.5 ml of perchloric acid was added to the non-control samples. Samples were then centrifuged at 12,000 g for 10 min, the supernatant removed, and 0.1 ml of 10N NaOH added. The hydroxylation of the substrate PNP

to a 4-nitrocatechol (4-NC) was determined with a GRS according to Koop *et al.* (1989). PNP has been considered to be one of the most specific substrates for monitoring 2E1 activity, though Zerilli *et al.* (1998) reported that CYP3A may also play a minor role in PNP hydroxylation.

CYP1A1/2 and CYP2B1/2 Activities: The fluorimetric technique of Lubet *et al.*. (1985) was used to measure formation of resorufin by CYP1A1/2 [i.e. ethoxyresorufin *O*-deethylase (EROD)] and by CYP2B1/2 [i.e. pentoxyresorufin *O*-dealkylase (PROD)] activities. EROD activity in rat liver was reported to be supported primarily by CYP1A1, while PROD dealkylation was found to be largely catalyzed by CYP2B1/2, with minor contributions by CYPs 2C11, 2C6 and 3A2 (Burke *et al.*., 1994). A 3% bovine serum albumin (BSA) solution was prepared using 0.1 M PO_4 buffer at pH 6.5. Twenty-five µl of undiluted microsomal sample and 6 µl of substrate (ethoxyresorfin or pentoxyresorufin) were added to 2.3 ml of BSA. The samples were shaken for 4 min in a 37°C water bath, after which 100 µl of NADPH were added. The samples were then placed into a Shimadzu RF 5301PC spectrofluorimeter, and measurements of resorufin formation recorded and calculated at 0.5 and 2.5 min.

Western Blot Analyses of Isozyme Proteins: Microsomal proteins samples were prepared using 10 mM TRIS HC1 buffer (pH 7.4) containing 10 mM EDTA and 20% (v/v) glycerol. Microsomal protein samples were loaded into a NuPAGE 10% Bis-Tris Gel, and electrophoresis carried out at a concentration of 10 ug/ml. The protein was then transferred to a nitrocellulose transfer membrane. For detection of CYP1A1/2, CYP2E1 and CYP2B1/2, nitrocellulose membranes were treated with 3% blocking buffer for 1 h at room temperature. The membranes were then incubated with rabbit anti-rat CYP1A1/2 (1:3000 dilution), mouse anti-rat CYP2E1

(1:2000 dilution) (Bio-Rad, USA) or mouse anti-rat CYP2B1/2 (1:1000 dilution) polyclonal primary antibody (Bio-Rad, USA). The secondary antibody for CYP2E1 and CYP2B1/2 was rabbit anti-mouse IgG conjugated with alkaline phosphatase (Bio-Rad, USA) in 1:4000 and 1:2400 dilutions, respectively. The secondary antibody for CYP1A1/2 was goat anti-rabbit IgG conjugated with alkaline phosphatase in a 1:3000 dilution. Chemiluminescent substrate and enhancer were used, along with a FOTO/Analyst camera and software to visualize substrate activity in the immunoblots. A Kodak 1D Imaging System was used to record the results.

Estimations of Enzymatic Content in Intact Liver: Total CYP2E1, CYP2B1/2 and CYP1A1/2 activities and microsomal protein levels were calculated in the liver of different-aged rats. These estimations take into account the contribution of liver size/weight of the different age-groups. The calculation are straight-forward. Total activity of each isozyme in the liver is determined by multiplying activity *(*in nmol or pmol product/mg microsomal protein) X mg microsomal protein/g liver X g liver. Microsomal protein content of the entire liver is estimated by multiplying mg microsomal protein/g liver X g liver. These values are also normalized for body weight, so the CYP450 oxidation and N-dealkylation capacities of the liver of different age-groups can be compared.

Statistics and Correlation: The statistical significance of apparent differences in group means was assessed using ANOVA and Duncan's Multiple Range Test (DMRT) for each study [(Knodt's Statistical Analysis Software Package (Bellingham, WA)]. Linear regression was used to determine the degree of correlation between enzymatic activity and protein of each isozyme.

RESULTS

The ontogeny of CYP450s in the liver was thoroughly characterized in maturing male and female S-D rats. CYP450 levels on days 1, 5 and 10 increased with age (Fig. 2.1). Levels remained relatively constant from PND 15 – 60. Amounts were uncharacteristically low in 30-day-old pups. The mature, young (PND 90) adults exhibited the highest CYP450 levels. The maturational pattern of total CYP450s, of course, is not necessarily indicative of the maturation of individual isoforms. These developmental differences were clearly evident when CYP2E1, CYP1A1/2 and CYP2B1/2 activities were measured in the developing S-D rats (Table 2.1).

The developmental profiles of hepatic microsomal CYP2E1 activity/protein and total CYP450 levels were the opposites of one another (Figs. 2.1 and 2.2). Although 2E1 activity was extremely low on PND 1, it dramatically increased by day 5 (Fig. 2.2A) and slowly rose to a peak on day 21. It then progressively diminished with increasing age, reaching its lowest levels in the young adults. Western blot analyses were performed to learn whether developmental changes in hepatic 2E1 activity paralleled changes in 2E1 protein. 2E1 protein developmental profiles in Fig. 2.2B mirrored the activity profiles in Fig. 2.2A. The protein levels were relatively high by PND 5, peaked at PND 15 – 21, and steadily decreased through PND 60. Good correlation was found when 2E1 activity and protein content were plotted against one another (i.e., $r^2 = 0.88$) (Fig. 2.3). The 2E1 protein content of livers from male and female rats was quantified separately, in order to determine whether there were gender differences in the maturational pattern or quantity of the isozyme. It can be seen in Fig. 2.4 that protein expression in maturing males paralleled that in females. CYP2E1 protein content consistently appeared to be slightly higher in males until PND 60, though the apparent differences were not sufficient to

be statistically significant. A representative Western blot of a male and a female from the PND 5 – 90 groups was included in Fig. 2.4.

The ontogeny of hepatic microsomal CYP1A1/2 resembled that of CYP2E1 (Figs. 2.2A and 2.5A). The 1A1/2 profile did differ somewhat in that it: a) was very low on days 5 and 10, modestly increased on day 15, highest from day 21 – 40; and b) fell to the lowest level measured throughout the monitoring period in the 90-day-old adults (Fig. 2.5A). It can be seen in Figs. 2.5A and B that the maturational pattern for 1A1/2 protein expression was similar to that for 1A1/2 activity. The magnitude of change in enzymatic protein, however, was less pronounced than that in enzymatic activity over the 90-day evaluation period. Nevertheless, there was a relatively high degree of correlation (i.e., $r^2 = 0.94$), when activity of the isozyme was plotted against its protein level (Fig 2.6). A representative Western blot from a male and a female in each age-group was also shown in Fig. 2.7. Assessment of microsomal 1A1/2 protein content in the liver of male and female S-D rats revealed no sex differences from PND 1 – 90 (Fig. 5).

The maturation of CYP2B1/2, CYP2E1 and CYP1A1/2 shows the same general profiles, though 2B1/2 differs in several respects. 2B1/2 activity is similar to the others in that it is relatively low at birth, maximal at weaning (PND 21), somewhat diminished during the postweaning and adolescent periods, and low again in young adult (PND 90) animals (Fig. 2.8A). As only 2B2 is expressed in naïve rats (Wrighton and Stevens, 1992), our values likely reflect this particular isozyme. 2B2 activity is unique in that it exhibits a very high, distinct peak on PND 21. Considerable fluctuation is then manifest from PND 30 – 60. 2B1/2 protein (Fig. 2.8B) shows the same general profile as activity, but the amplitude of the age-dependent changes in the former are much less pronounced. The precipitous drop in 2B2 activity after PND 21 is not evident in protein levels (Figs. 2.8A and B). Accordingly, the degree of correlation ($r^2 =$

0.52) between 2B1/2 protein and activity (Fig. 2.9) is much lower than for the other two isozymes. There are no pronounced sex differences in the time-course of expression of hepatic 2B1/2 protein (Fig. 2.10).

Body and liver weights were measured to define their relative growth in S-D rats during development. The increases in liver and body weight roughly paralleled one another from birth to adulthood (Figs. 2.11A and B). Body and liver weights rose relatively slowly from PND 1 – 21, to be followed by rapid increases after weaning. Male and female weights were comparable from birth through PND 50. Thereafter, both liver and body weights increased more rapidly in males. There was also more intersubject variability within each gender at PND 60 and 90. This was verified with the sexually mature males being higher and female being lower than the average weight when compare to both sexes. A plot of liver/body weight ratio diminished slightly from birth until PND 10 due to a somewhat more rapid rise in body weight (Fig. 2.11C). Increase in the ratio from PND 15 – 30 was attributable to a relatively rapid liver weight gain. The final drop in the ratio from PND 50 – 90 reflected marked body weight gain.

The ontogeny of levels of hepatic CYP450s, as well as CYP2E1, 1A1/2 and 2B1/2 activities and protein levels in unsexed S-D rats, are expressed in several ways in Tables 2.1– 3. The numerical values for each parameter plotted in Figs. 2.1, 2.2, 2.5 and 2.8 are included in Table 2.1. As described previously, total CYP450 levels and isozyme activities and protein content are negligible at birth, rise rapidly to maxima at/after weaning, and diminish substantially until adult levels are reached at PND 90. When the total amounts of microsomal protein and CYP450s in the entire liver are calculated, there are progressive increases through PND 50 (Table 2.2). The PND 60 values appear to be higher than those at PND 50, but intersubject variance precludes statistically significant differences. The total activity of each

isozyme in the animals' liver steadily rises for the initial 40 days of life, then falls to quite modest values in young (PND 90) adults. The early increase and pubescent decline in hepatic microsomal protein are not so pronounced when the entire liver levels are expressed per kg body weight (Table 2.3). The total amount of CYP450 in the liver progressively increases over the 90 days. The same age-dependent trends, that are evident for the isozyme activities in the entire liver, are manifest when the values are normalized to body weight. The metabolic activities in livers of preweanlings and weanlings, however, appear higher relative to maximal activities in the latter case.

DISCUSSION

The developmental profile of CYP2E1 in the liver of S-D rats differed from that of total CYP450s, which rose from birth to adulthood. Borlakoglu *et al.* (1993) detected CYP2E1 mRNA, but no 2E1 protein in the liver of newborn S-D rats. 2E1 activity was present on PND 4, but was not measured again until PND 60. Imaoka *et al.* (1991) quantified hepatic 2E1 protein in just 3 ages of immature Wistar rats. These researchers did observe a substantial increase between PND 7 and 21, followed by a pronounced decline at PND 49. A similar pattern was manifest in S-D rats in the current, more comprehensive investigation, which included nine immature age-groups. 2E1 activity and protein paralleled one another during maturation. Each rose dramatically during the first 5 days of life, peaked at weaning (PND 21) and progressively diminished until PND 60 – 90. Morel *et al.* (1999) saw a progressive reduction in PNP hydroxylation by liver microsomes of 35-, 49- and 63-day-old S-D rats. Nakajima *et al.*. (1992) measured a slower rate of trichloroethylene (TCE) metabolism and lower CYP2E1 content in

126- than in 21-day-old Wistar rats. The oxidation of TCE, in the concentration studied, was catalyzed primarily by CYP2E1.

The pattern of maturation of human hepatic microsomal CYP2E1 differs in some respects from that seen in S-D rats. Vieira *et al.* (1996) report that 2E1 protein and activity could not be detected in human fetal liver, but that they "surge" within the first hours after birth. Transcriptional activation of the CYP2E1 gene is believed to be linked to its demethylation. We did not find 2E1 activity or protein in PND 1 rats, but quite high levels are attained by PND 5. Both activity and protein peak around PND 21, and then steadily decrease until adulthood. In contrast, 2E1 in human infants slowly rises during their first year, and according to Vieira *et al.*. (1996) gradually increases thereafter until adult values are essentially reached by age 10. Treluyer *et al.* (1996) describes a similar developmental profile in liver of victims of sudden infant death syndrome. Unfortunately, each research group had access to the liver from just 4 or 5 donors between the ages of 1 and 10. Johnsrud *et al.* (2003), however, recently published the results of an extensive assessment of 2E1 protein levels in the liver of 73 human fetuses 8 – 37 weeks old and 165 immature donors 1 day – 18 years old. Immunodetectable 2E1 was found in most third trimester samples. 2E1 increased for the first 90 days after birth. There was no further increase thereafter, though 4-fold intersubject variation was manifest in all age-groups. This developmental pattern is clearly different from that exhibited by S-D rats.

The ontogeny of CYP1A1/2 in S-D rat liver resembled that of CYP2E1. The preweaning increase and subsequent adolescent decrease in 1A1/2, however, were more accentuated. 1A1/2 activity, when calculated as a percentage of its maximum, was substantially lower at the beginning and ending of the 90-day monitoring period than was 1A1/2 protein. Gebremichael *et al.* (1995) monitored hepatic microsomal EROD activity in male PND 7, 14, 21, 50 and 100 male

S-D rats. Their maturational profile resembled ours, with one notable exception. They reported a marked increase between PND 50 and 100, whereas we observed a marked decline. Imaoka *et al.* (1991) saw a modest rise and fall in 1A2 protein in male Wistar rats between 7 and 98 days, but negligible 1A1 protein levels at any of these times.

Studies of the ontogenesis of CYP1A1 and 1A2 have revealed 1A1 in human fetal liver, but 1A2 as the constitutive form in adult liver (Shimada *et al.*., 1996). 1A1 is inducible in adults by PAHs and other compounds that bind to the Ah receptor (Ma and Lu, 2003). 1A2 protein and activity were found to be very low during the first 3 months of life, but to attain 50% of adult values at 1 year (Sonnier and Cresteil, 1998). 1A1-mediated EROD activity in liver microsomes was found to be lower in 16 children < 13 years old than in 16 persons 13 – 40 years old (Sy *et al.,* 2001). 1A2-catalyzed EROD activity was not different in these age-groups. Blanco *et al.* (2000) reported no difference in maximal 1A2 activity between two broad age groups (< 10 and 10 – 60 years). In summary, liver microsomal 1A2 and 1A1/2 activities were very low in human and S-D rat neonates, respectively. 1A1/2 activity increased markedly in rat weanlings, but fell to very low levels in young adults. CYP1A2 apparently rises slowly during infancy and childhood to low, variable levels comparable to those in human adults.

The developmental expression of CYP2B1/2 did not follow the patterns characteristic of 2E1 and 1A1/2 in S-D rats. In the current study, 2B1/2 activity was initially detected in S-D neonates at PND 1. It remained quite low through PND 15, before increasing more than 6-fold in the PND 21 pups. Imaoka *et al.* (1991) found little 2B1 protein at any time during the development of Wistar rats, but did see a modest increase in 2B2 protein between PND 7 and 21. Despite low activity, 2B1/2 protein in the present investigation was quite high during the first 15 days of life. Omiecinski *et al.* (1990) could not detect 2B1 or 2B2 mRNA in liver microsomes of

unsexed PND 15 or 20 S-D rats, but measured high levels in PND 22 and 46 animals. 2B1/2 protein levels remained high from PND 21 – 90 in the current study. 2B1/2 (PROD) activity, in stark contrast, was low and quite variable from PND 30 – 90. Gebremichael *et al.*. (1995) similarly observed vacillating PROD activity in PND 21, 50 and 100 male S-D rats. Not surprisingly, the inconsistent 2B1/2 activity from PND 21 – 90 and relatively consistent 2B1/2 protein in our study resulted in a low degree of correlation ($r^2 = 0.52$) between the two indices.

There are very few data available on the ontogeny of CYP2B6, the apparent human equivalent of CYP2B1/2. The results of a single study suggest that infants at 1 year attain ~ 10% of adult 2B6 protein levels (Tateishi *et al.*., 1997). The isozyme is involved in the biotransformation of several important drugs and toxicants, including cyclophosphamide, testosterone, methadone, diazepam and high levels of VOCs (Omiecinski *et al.*, 1999).

Sex seems to play little role in the ontogeny of hepatic CYP1A1/2, 2B1/2 and 2E1 protein in S-D rats. CYP1B1/2 levels in female pups appear to be slightly higher around weaning, although at no time are values for these or other isozymes significantly different from those of males. Imaoka *et al.* (1991) describe no gender differences in hepatic 2B1 protein content of maturing Wistar rats, though 2B2 content is marginally elevated in males at PND 21. 2E1 protein levels appear to be modestly elevated in males throughout the first 50 days in the current assessment, however the levels are not significantly different in the two sexes. Imaoka *et al.* (1991) report somewhat higher CYP2E1 content in male PND 7 and 21 Wistar rats. Evaluation of the rates of hydroxylation of two 2E1 substrates reveal that 7-week-old male S-Ds oxidize chlorzoxazone somewhat more rapidly than females, while the converse is true for PNP (Morel *et al.*., 1999). Oxidation of TCE, another 2E1 substrate, is no different in PND 21 male and female Wistar pups (Nakajima *et al.*, 1992). These researchers see no sex difference in total

CYP450 levels in the immature animals, but find significantly higher levels in male adults. Imaoka *et al.*. (1991) find that CYP450 increases 3-fold during development and peaks at 98 days in male Wistars, but attains its maximum (i.e., 2-fold increase) by day 21 and remains constant thereafter in females. MacLeod *et al.* (1972) report similar age-dependent gender variance in hepatic CYP450 content in Long Evans rats.

Very little information is available on the role of gender in the ontogeny of hepatic CYP450s in humans. This can be attributed primarily to the small number of liver donors in most studies. Johnsrud *et al.* (2003) did measure CYP2E1 protein in liver microsomes from 165 individuals 1 day – 18 years old. The investigators find $\geq$ 4-fold intersubject variation in 2E1 content, but no significant sex difference. Sex-related differences in most CYP450s in adults are usually minor. Shimada *et al.* (1994), for example, report only one such difference in 7 isozymes in hepatic microsomes from 30 Japanese and 30 Caucasian men and women. CYP1A2 activity was somewhat higher in the Caucasian men. Bebia *et al.* (2004) describe no gender difference in CYP1A2 activity in 161 subjects assessed *in vivo*, but slightly higher CYP2E1-mediated metabolism of chlorzoxazone by middle-aged men. It appears from findings in these and other relatively large-scale studies that sex disparity in human adults is quite modest in contrast to that commonly observed in adult rats.

The developmental profile for total CYP450s in rat liver was fundamentally different from profiles for CYP2E1, CYP1A1/2 and CYP2B1/2. These three isozymes obviously account for a limited portion of total CYP450s. The current assessment of the ontogeny of these isozymes is the most comprehensive to date in S-D rats. Nakajima *et al.* (1992) compared only 21- with 126-day-old Wistar rats, while Borlakoglu *et al.* (1993) reported progressive increases in CYP450s in 3 ages of unsexed, postnatal S-D rats. Imaoka *et al.* (1991) conducted a

comprehensive lifetime assessment of Wistars, but measured CYP450 levels in just 3 ages of immature animals. Watanabe *et al.* (1993) used a semiquantitative microphotometric technique to carry out the most complete developmental study of total CYP450s found in the literature. They saw a rapid rise in CYP450 content of liver cells of male S-D rats from PND 1 – 10. Thereafter, CYP450 content in centrilobular hepatocytes progressively increased until PND 60, through the increase in periportal hepatocytes was relatively modest. We measured similar age-dependent rises in total CYP450s in unsexed S-D rats, with some additional increase between PND 60 and 90.

The ontogeny of total CYP450s in human liver differs from that of rats during early stages of development. Treluyer *et al.* (1996) found that CYP450 levels in newborns' liver were ~ ⅓ of adult levels. The levels gradually rose during infancy and early childhood. Shimada *et al.* (1996) observed that the content of CYP450s in fetal liver was 30 – 60% of that in adults, and that the cytochromes gradually approached adult levels during the first 10 years of life. These findings indicate that humans are born with substantially more hepatic CYP450s than S-D rats. Levels in rats rise rapidly during the first 2 – 3 weeks and then slowly increase to adult levels, much like they do in children.

Liver volume, as well as enzyme activity, is a major determinant of the metabolic clearance of drugs and other chemicals. Liver and body weights of S-D rats in the present project steadily increased after weaning until adulthood. Liver weight normalized to body weight, however, rose significantly from PND 15 – 30, remained elevated from PND 30 – 50, and declined somewhat thereafter. Mirfazaelian *et al.* (2007) recently described a similar liver/body weight time-course in maturing male S-D rats. Human liver/body weight declined

gradually from child to adult values (Johnson *et al.*, 2005; Murry *et al.*, 2000; Urata *et al.*., 1995).

There are many drugs that exhibit higher systemic clearance in children than in adults (Blanco *et al.*., 2000; Alcorn and McNamara *et al.*, 2002). Murry *et al.* (1995) found that antipyrine clearance *in vivo*, especially when normalized to body weight, decreased significantly during human maturation. Blanco and co-workers saw no difference between persons < 10 and 10 – 60 years old in hepatic microsomal EROD, ethoxycoumarin deethylase (CYP2E1) or other isozyme maximal activities. Higher metabolic clearance in children is likely due to their larger liver volume and more rapid hepatic blood flow. There are few published data on hepatic blood flow in immature rats or humans. Wynne *et al.* (1989), however, did report high correlation between liver volume and apparent liver blood flow in human adults. Postweanling/prepubescent S-D rats exhibit relatively high liver/body weight ratios.

Findings in the current investigation suggest that the use of immature rats as surrogates for children may overestimate the metabolism and risks of toxicity from CYP2E1, 2B1/2 and 1A1/2 substrates. Activities and protein levels of these hepatic isozymes in weanling/post-weanling rats are substantially higher than in adults. In contrast, the rates of biotransformation of similar substrates *in vitro* are comparable in older children, adolescents and adult humans. High metabolic capacity in our immature rats is apparent when the isozyme activities are calculated for the entire liver and for liver normalized to body weight. Although age-dependent liver blood flow rates are unavailable, it is very likely that rates are considerably higher in immature subjects of each species, and in immature rats than in children. This factor is, of course, important for blood flow-limited substrates. The net effect of age-dependent differences in liver size, blood flow and metabolic constants can be taken into account by use of

physiologically-based pharmacokinetic (PBPK) models. An objective of our work is to use the age-dependent metabolic constants for construction of PBPK models for VOCs. In light of the foregoing, it seems reasonable to anticipate that VOCs, PAHs and other classes of chemicals bioactivated by CYP2E1, 2B1/2 and 1A1/2 will present greater toxicity and/or carcinogenicity risks to immature rats than to immature humans. Nevertheless, as total hepatic CYP450 levels progressively increase during development in each species, immature rats may be better surrogates for children for substrates of other CYP450 isozymes.

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FIGURES AND TABLES:

Fig. 2.1. Ontogeny of total CYP450s in the liver of unsexed S-D rats from postnatal day (PND) 1 – 90. The livers from PND 1 – 21 animals were pooled as described in the Methods. A 5-g sample from 3 male and 3 female PND 30 – 90 rats was analyzed. Total CYP450 levels are expressed as nmol/mg microsomal protein. Bar heights and bracket represent mean + S.E. Group values with different letters are significantly different from one another at $p \leq 0.05$. ND – Not detected.

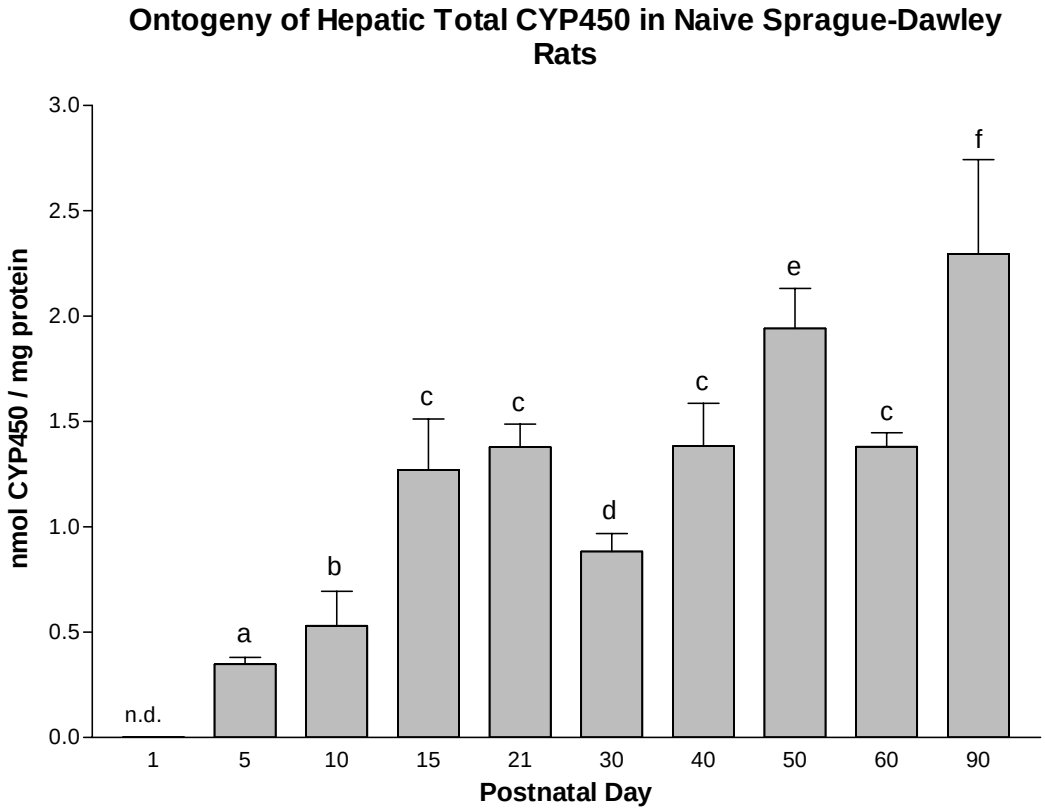
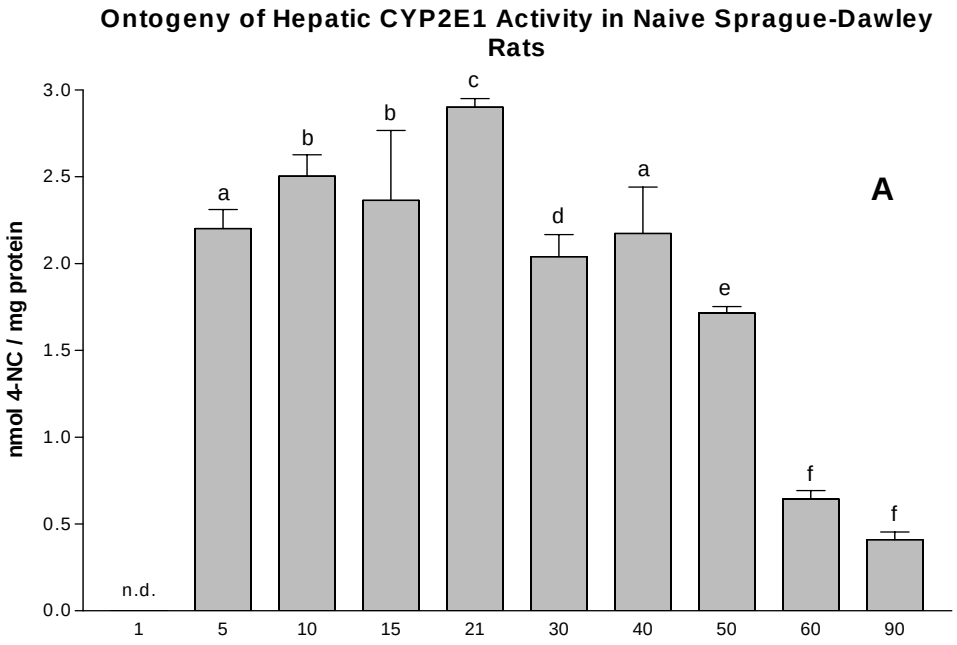

Ontogeny of Hepatic Total CYP450 in Naive Sprague-Dawley Rats
nmol CYP450 / mg protein
3.0
2.5
2.0
1.5
1.0
0.5
0.0
n.d.
a
b
c
c
d
c
e
c
f
1
5
10
15
21
30
40
50
60
90
Postnatal Day

Fig. 2.2. Age dependence of hepatic microsomal CYP2E1 activity (A) and protein levels (B) in unsexed S-D rats. The livers from postnatal day (PND) 1 – 21 animals were pooled as described in the Methods. A 5-g sample was analyzed from 3 male and 3 female PND 30 – 90 rats. CYP2E1 activity is expressed as nmol 4-nitrocatechol (4-NC) formed/mg microsomal protein. CYP2E1 protein is represented as density in arbitrary units. Bar heights and bracket represent mean + S.E. Group values with different letters are significantly different from one another at $p \leq 0.05$. ND – Not detected.


Ontogeny of Hepatic CYP2E1 Activity in Naive Sprague-Dawley Rats
nmol 4-NC / mg protein
3.0
2.5
2.0
1.5
1.0
0.5
0.0
n.d.
a
b
b
c
d
a
e
f
f
A
1
5
10
15
21
30
40
50
60
90

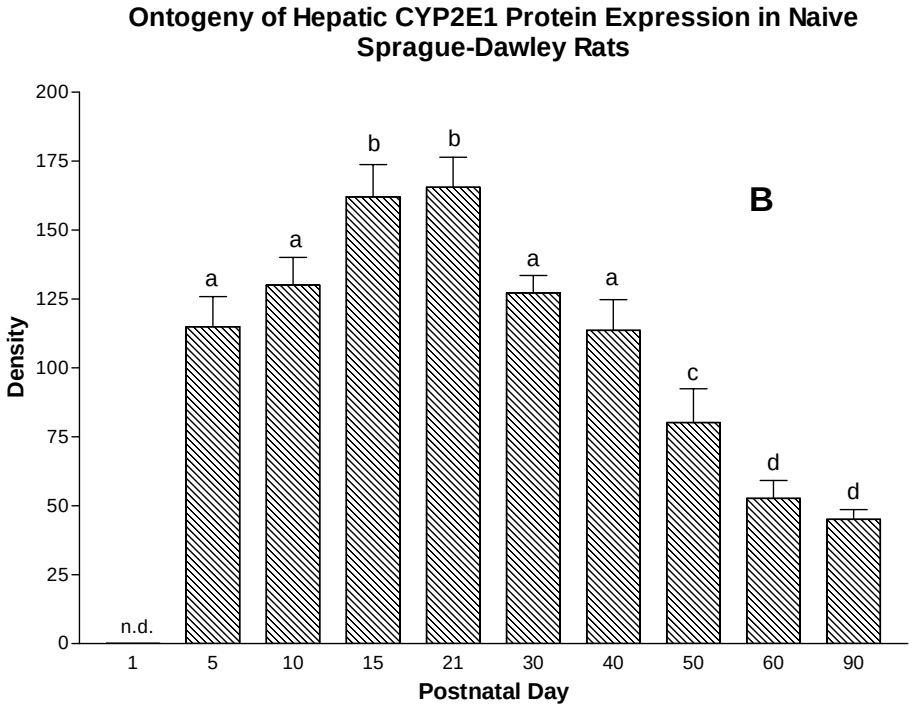

Ontogeny of Hepatic CYP2E1 Protein Expression in Naive Sprague-Dawley Rats
Density
200
175
150
125
100
75
50
25
0
n.d.
a
a
b
b
a
a
c
d
d
B
1
5
10
15
21
30
40
50
60
90
Postnatal Day

Fig. 2.3. Correlation of CYP2E1 activity [nmol 4-nitrocatechol (4-NC) formed/mg microsomal protein] and CYP2E1 protein (density in arbitrary units). A different symbol represents the mean value for each age-group (in days). A correlation coefficient (r^2) of 0.88 was calculated by nonlinear regression.

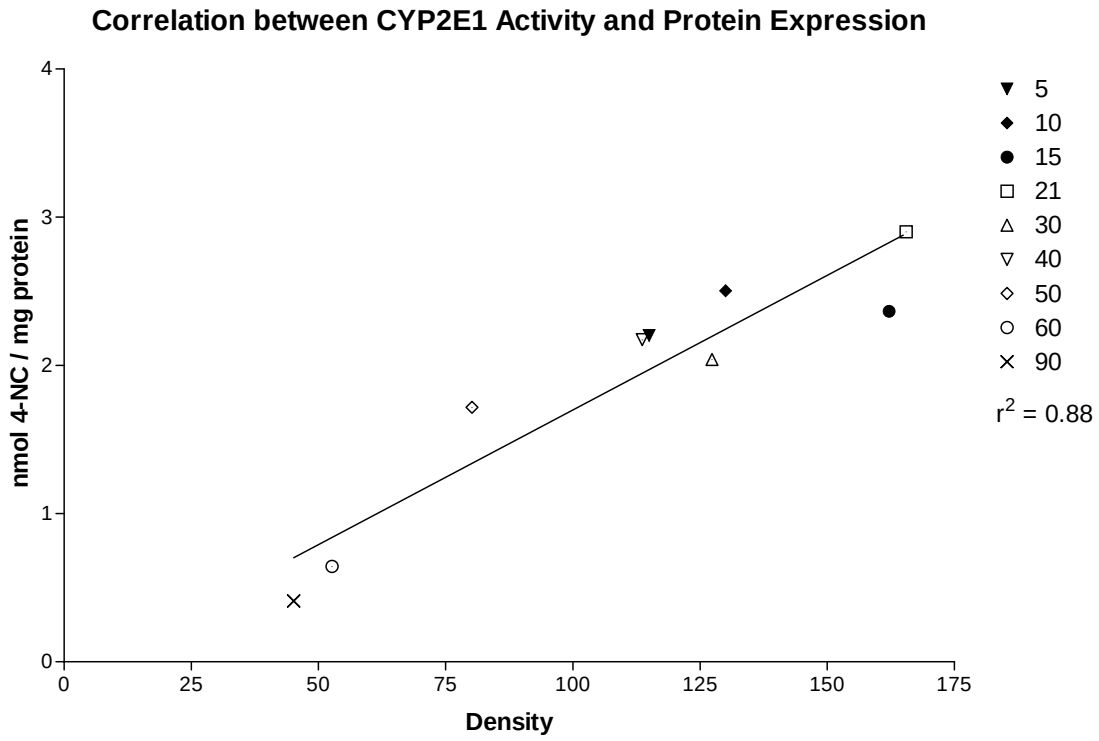
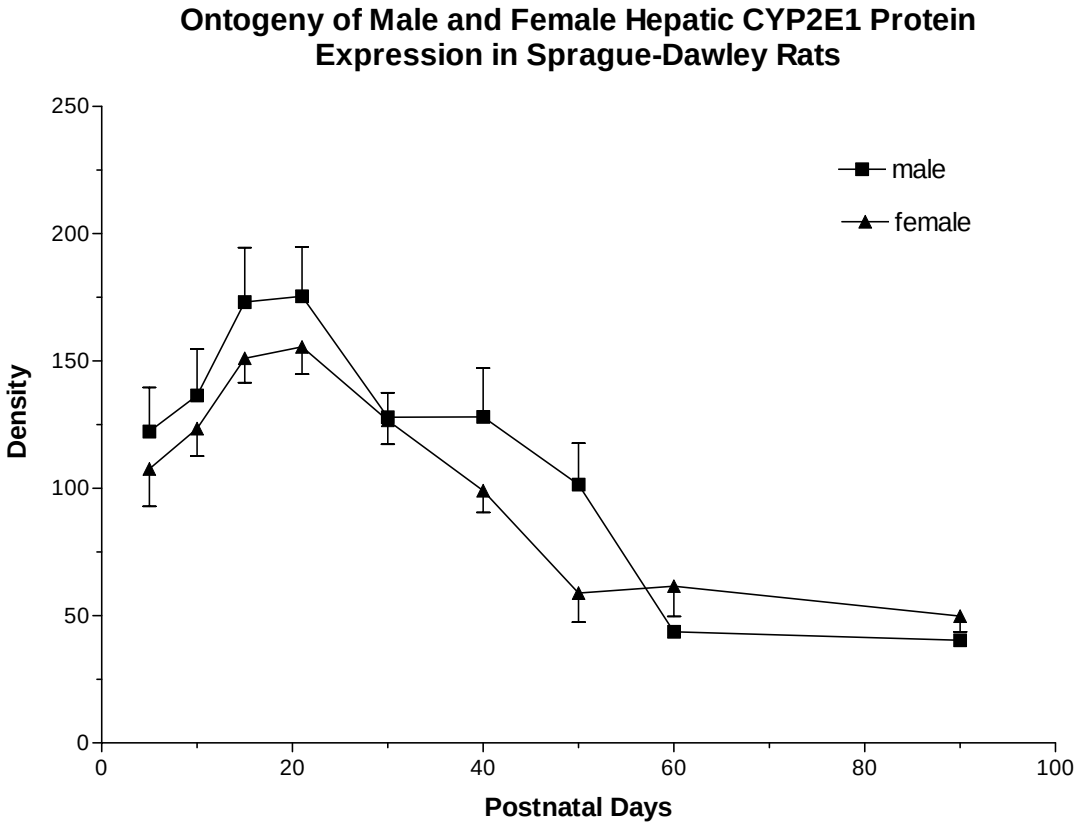

Correlation between CYP2E1 Activity and Protein Expression
nmol 4-NC / mg protein
Density
0
1
2
3
4
0
25
50
75
100
125
150
175
5
10
15
21
30
40
50
60
90
r2 = 0.88

Fig. 2.4. Sex-dependency of liver microsomal CYP2E1 protein expression in S-D rats. The livers from sexed postnatal day (PND) 1 – 21 animals were pooled as described in the Methods. A 5-g sample from PND 30 – 90 rats was analyzed. CYP2E1 protein is represented as density in arbitrary units. Each point represents the mean ± S.E. for a group of 6 rats. There were no statistically-significant gender differences. Representative Western blots from a male and a female from each age-group are shown.


Ontogeny of Male and Female Hepatic CYP2E1 Protein Expression in Sprague-Dawley Rats
Density
Postnatal Days
male
female
0
50
100
150
200
250
0
20
40
60
80
100

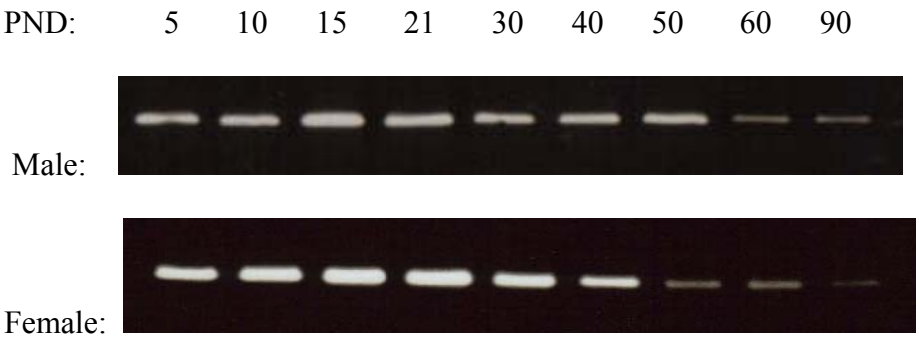
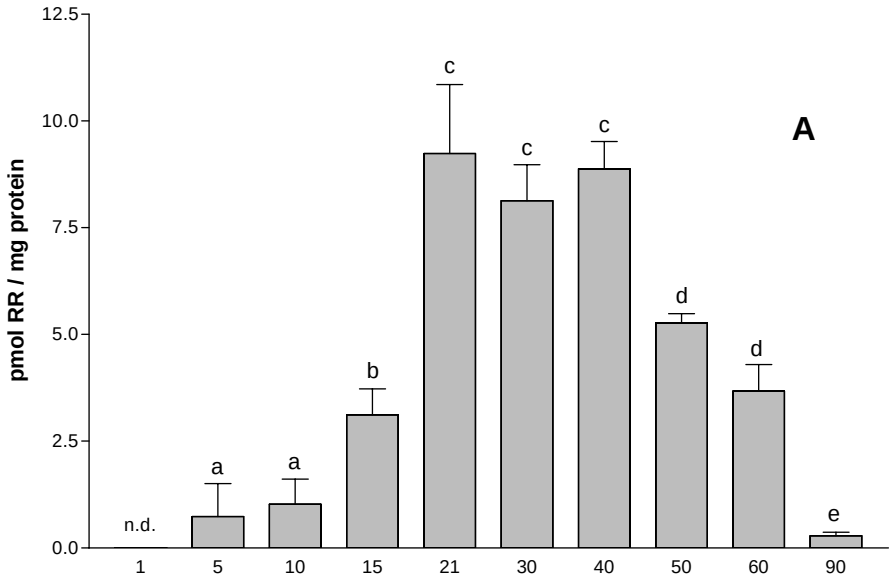

PND: 5 10 15 21 30 40 50 60 90
Male:
Female:

Fig. 2.5. Age-dependence of hepatic microsomal CYP1A1/2 activity (A) and protein expression (B) in unsexed S-D rats. The livers from postnatal day (PND) 1 – 21 animals were pooled as described in the Methods. A 5-g liver sample was analyzed from 3 male and 3 female PND 30 – 90 rats. CYP1A1/2 activity is expressed as pmol resorufin (RR) formed/mg microsomal protein. CYP1A1/2 protein is represented as density in arbitrary units. Bar heights and bracket represent mean + S.E. Group values with different letters are significantly different from one another at $p \leq 0.05$. ND – not detected.

Ontogeny of Hepatic CYP1A1/2 Activity in Naive Sprague-Dawley Rats



Ontogeny of Hepatic CYP1A1/2 Protein Expression in Naive Sprague-Dawley Rats

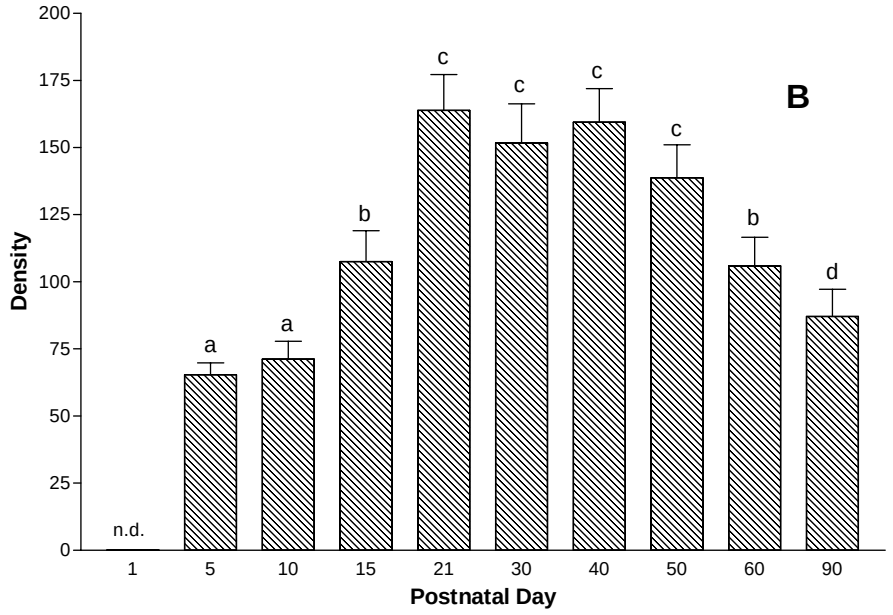


Fig. 2.6. Correlation of CYP1A1/2 activity [pmol resorufin (RR) formed/mg microsomal protein] and CYP1A1/2 protein (density in arbitrary units). A different symbol represents the mean value for different age-groups (in days). A correlation coefficient (r^2) of 0.94 was calculated by nonlinear regression.

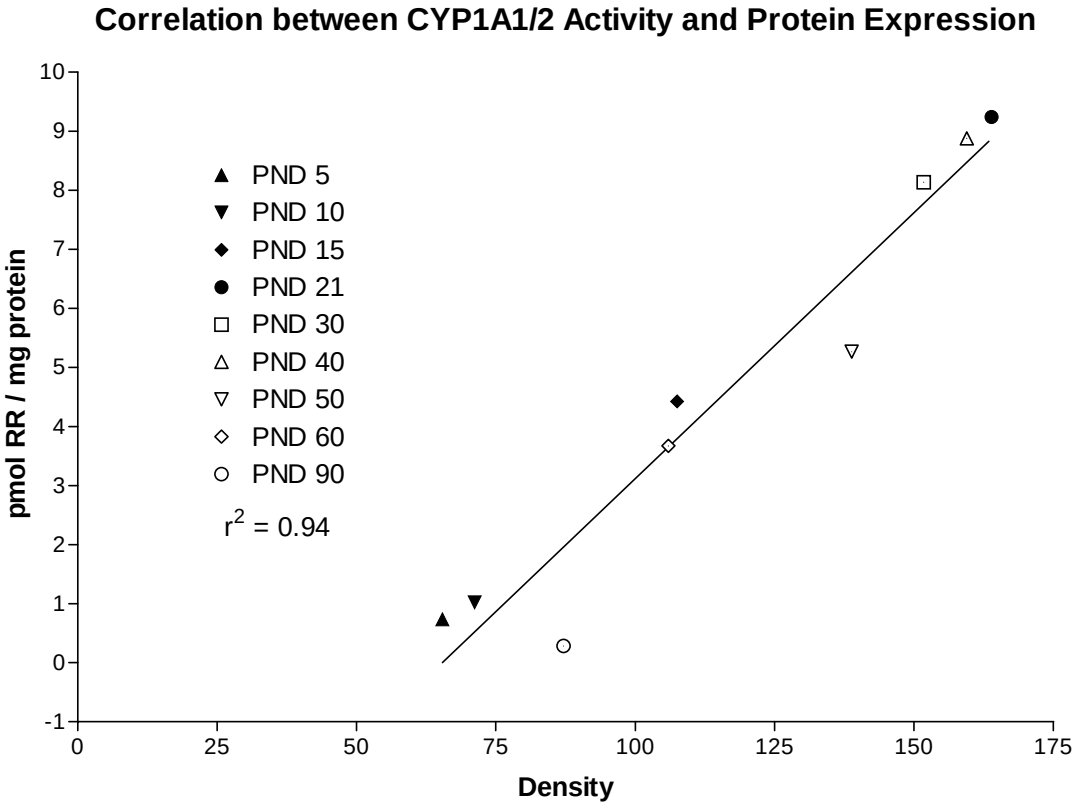
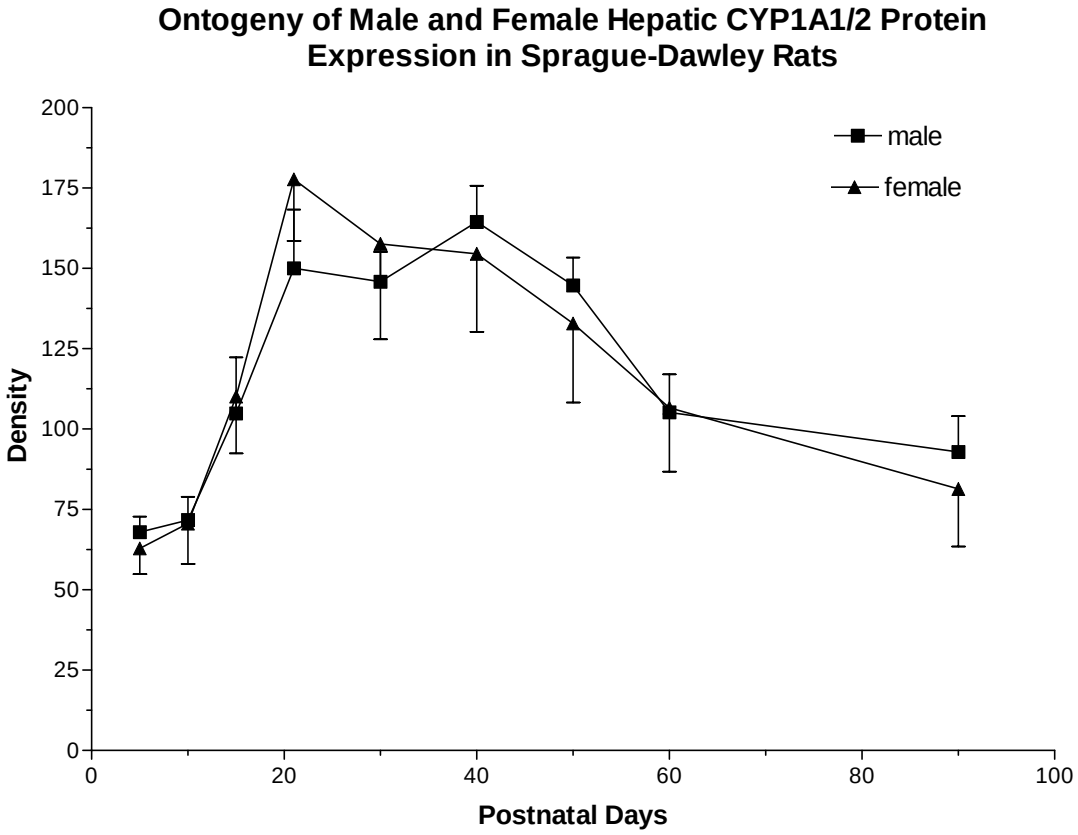

Correlation between CYP1A1/2 Activity and Protein Expression
pmol RR / mg protein
Density
PND 5
PND 10
PND 15
PND 21
PND 30
PND 40
PND 50
PND 60
PND 90
r2 = 0.94
10
9
8
7
6
5
4
3
2
1
0
-1
0
25
50
75
100
125
150
175

Fig. 2.7. Sex-dependency of liver microsomal CYP1A1/2 protein expression in S-D rats. The livers from sexed postnatal day (PND) 1 – 21 animals were pooled as described in the Methods. A 5-g sample from PND 30 – 90 rats was analyzed. CYP1A1/2 protein is represented as density in arbitrary units. Each point represents mean ± S.E. for a group of 6 rats. There were no statistically-significant gender differences. Representative Western blots from a male and a female from each age-group are shown.


Ontogeny of Male and Female Hepatic CYP1A1/2 Protein Expression in Sprague-Dawley Rats
male
female
Density
Postnatal Days
0
25
50
75
100
125
150
175
200
0
20
40
60
80
100

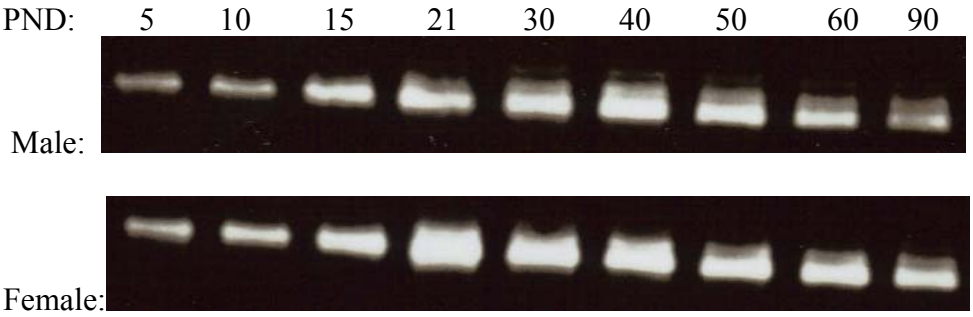
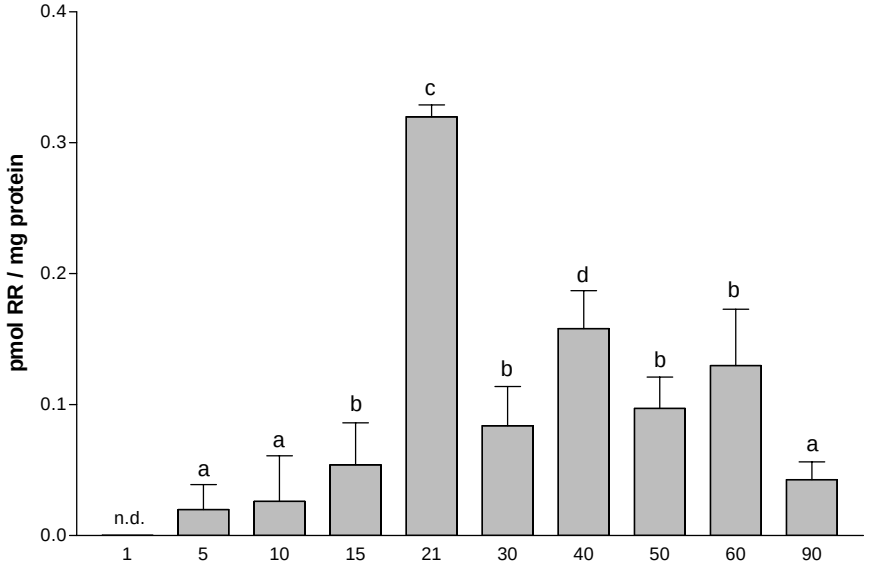

PND: 5 10 15 21 30 40 50 60 90
Male:
Female:

Fig. 2.8. Age dependence of hepatic microsomal CYP2B1/2 activity (A) and protein levels (B) in unsexed S-D rats. The livers from postnatal day (PND) 1 – 21 animals were pooled as described in the Methods. A 5-g sample was analyzed from 3 male and 3 female PND 30 – 90 rats. CYP2B1/2 activity is expressed as pmol resorufin (RR) formed/mg microsomal protein. CYP2B1/2 protein is represented as density in arbitrary units. Bar heights and bracket represent mean + S.E. Group values with different letters are significantly different from one another at $p \leq 0.05$. ND – not detected.

Ontogeny of Hepatic CYP2B1/2 Activity in Naive Sprague-Dawley Rats



Ontogeny of Hepatic CYP2B1/2 Protein Expression in Naive Sprague-Dawley Rats

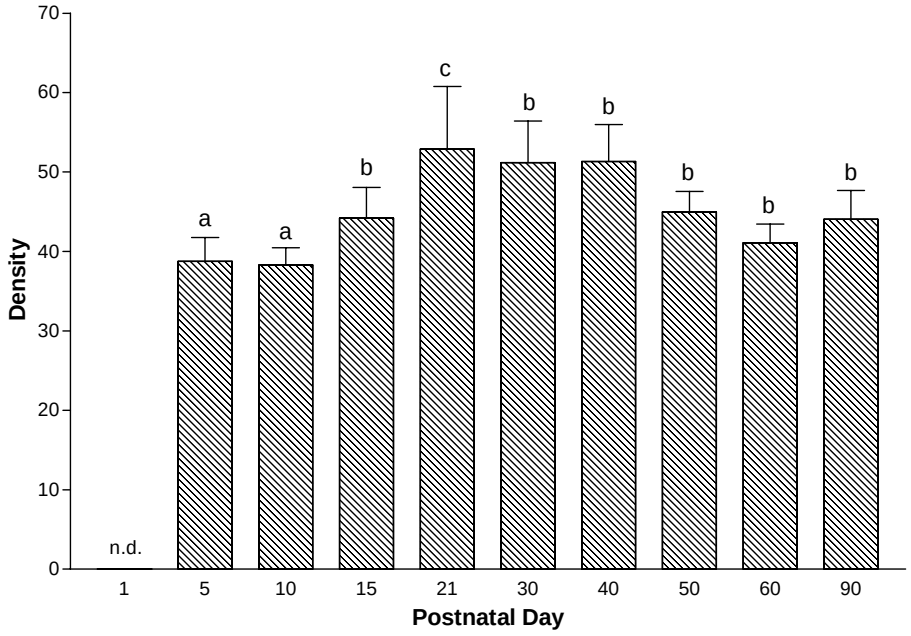


Fig. 2.9. Correlation of CYP2B1/2 activity [pmol resorufin (RR) formed/mg micromal protein] and CYP2B1/2 protein (density in arbitrary units). A different symbol represents the mean value for each age-group (in days). A correlation coefficient (r^2) of 0.52 was calculated by nonlinear regression.

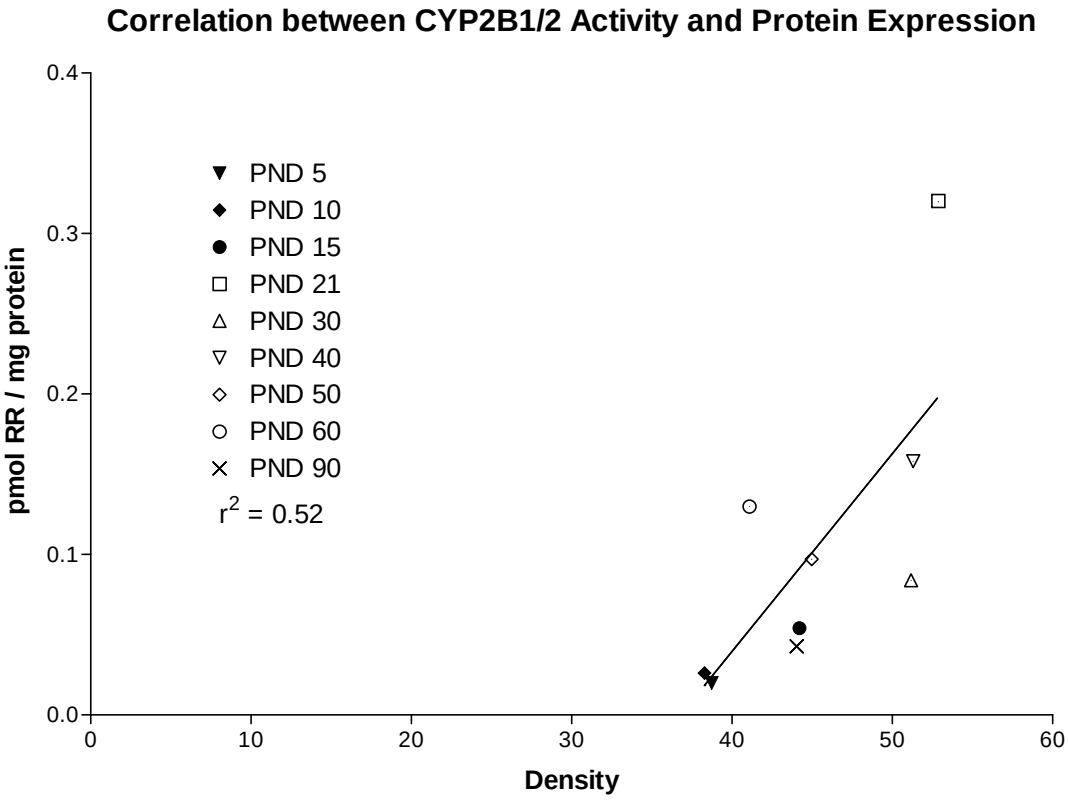
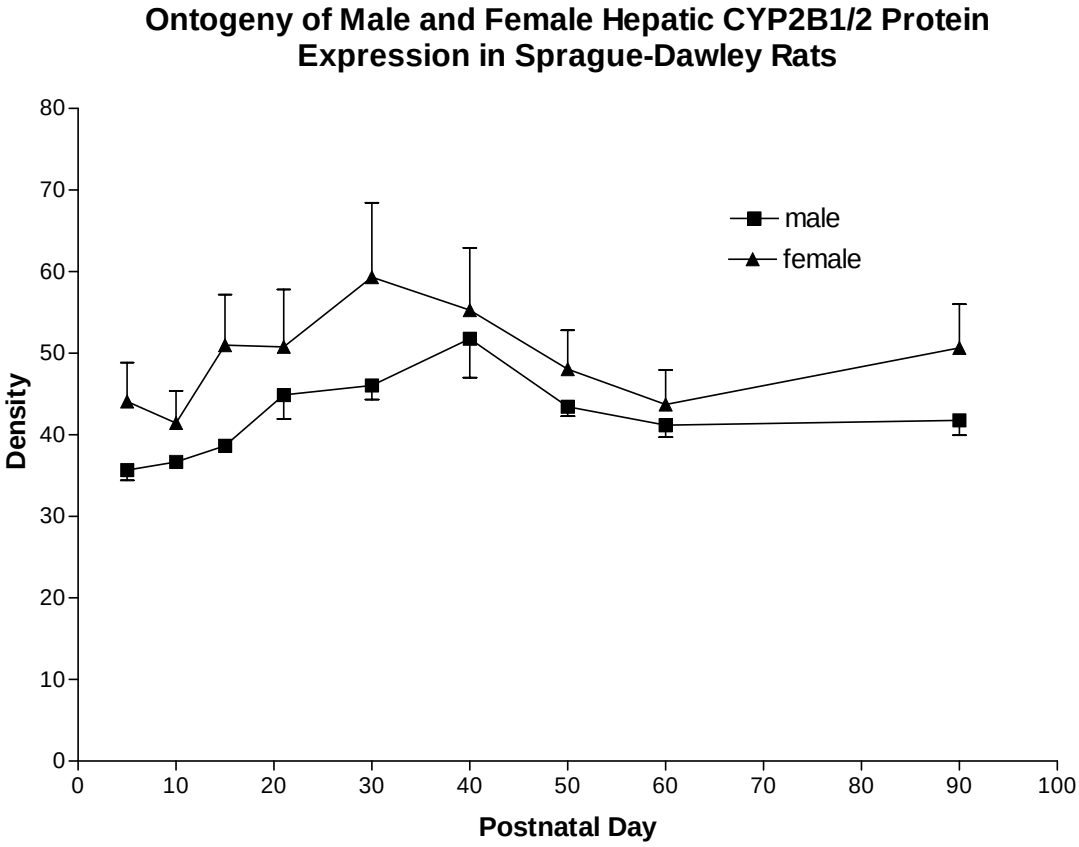

Correlation between CYP2B1/2 Activity and Protein Expression
pmol RR / mg protein
0.4
0.3
0.2
0.1
0.0
PND 5
PND 10
PND 15
PND 21
PND 30
PND 40
PND 50
PND 60
PND 90
r2 = 0.52
0
10
20
30
40
50
60
Density

Fig. 2.10. Sex-dependency of liver microsomal CYP2B1/2 protein expression in S-D rats. The livers from sexed postnatal day (PND) 1 – 21 rats were pooled as described in the Methods. A 5-g sample from PND 30 – 90 rats was analyzed. CYP2B1/2 protein is represented as density in arbitrary units. Each point represents the mean ± S.E for a group of 6 rats. There were no statistically significant gender differences. Representative Western blots from a male and a female from each age group are shown.


Ontogeny of Male and Female Hepatic CYP2B1/2 Protein Expression in Sprague-Dawley Rats
Density
Postnatal Day
male
female
0
10
20
30
40
50
60
70
80
90
100

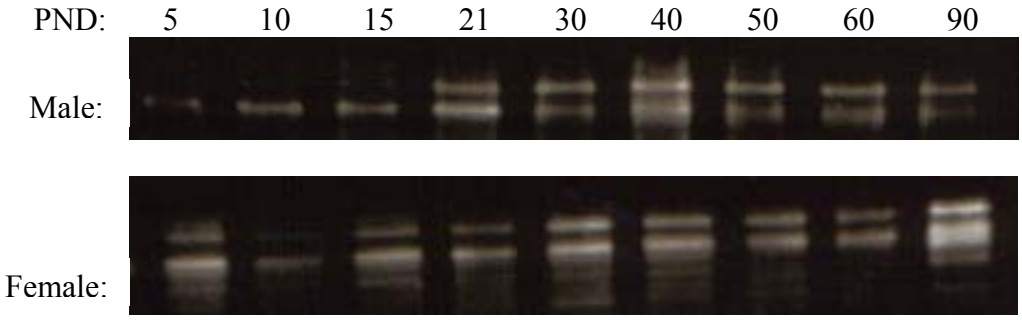
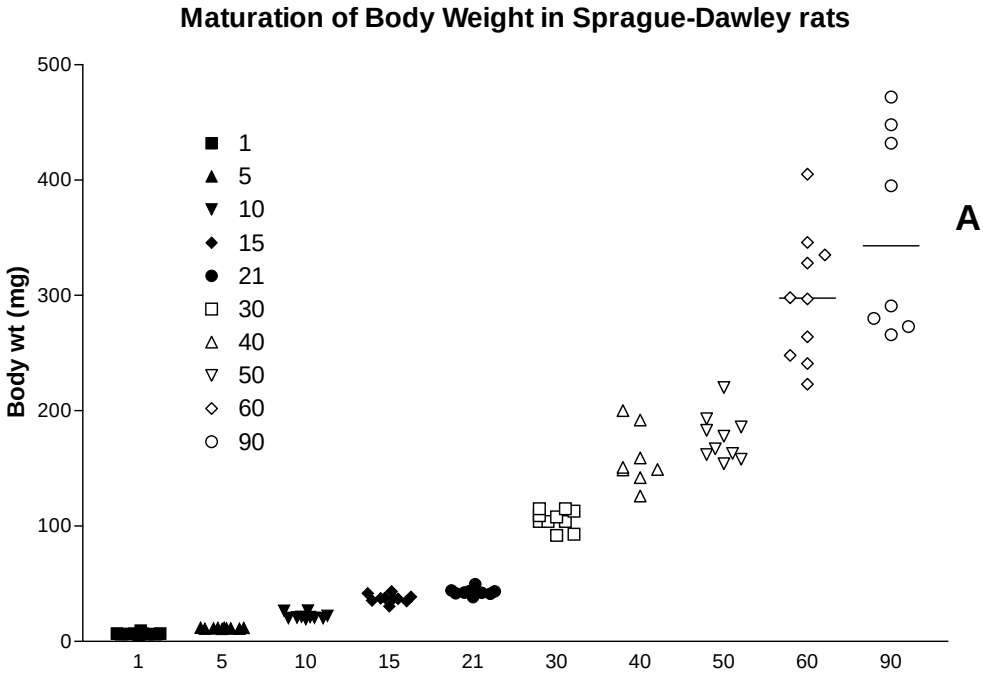
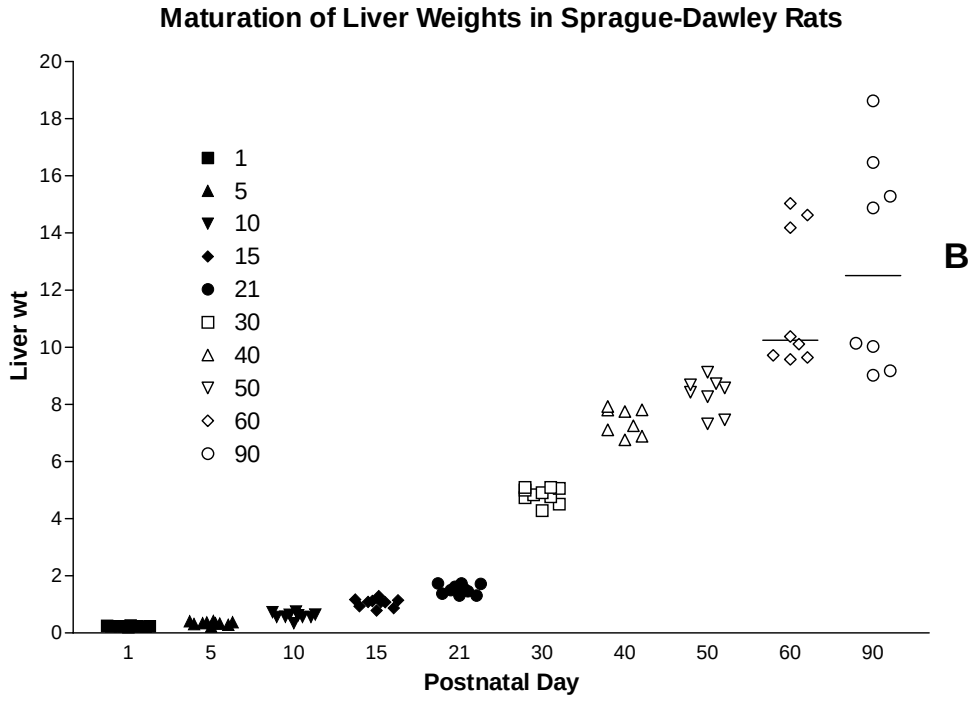

PND:
5
10
15
21
30
40
50
60
90
Male:
Female:

Fig. 2.11. Growth of the body (A), liver (B) and normalized liver (C) during maturation of unsexed S-D rats. Symbols for each age-group are included in A and B. Each datapoint in A and B represents the value for an individual animal. Normalized liver weight values (liver/body weight ratios) in C are mean ± SE (n = 6). The horizontal line indicates a weight change between each gender.


Maturation of Body Weight in Sprague-Dawley rats
Body wt (mg)
500
400
300
200
100
0
1
5
10
15
21
30
40
50
60
90
A


Maturation of Liver Weights in Sprague-Dawley Rats
Liver wt
20
18
16
14
12
10
8
6
4
2
0
1
5
10
15
21
30
40
50
60
90
Postnatal Day
B

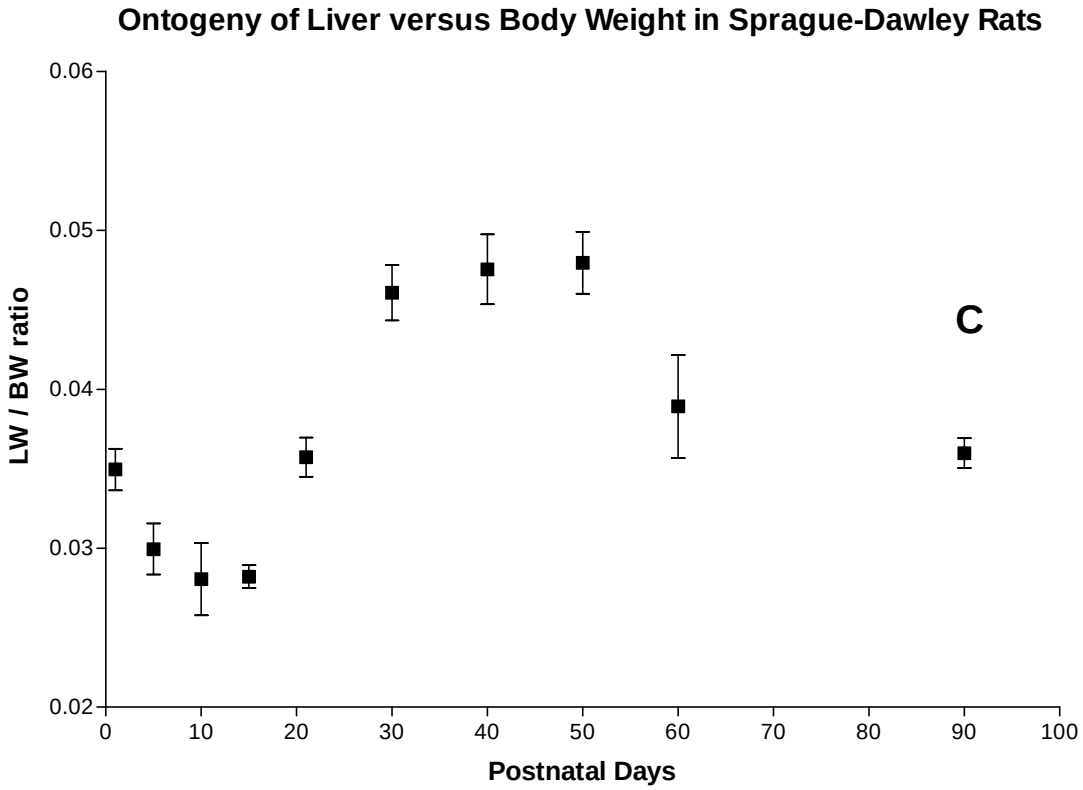

Ontogeny of Liver versus Body Weight in Sprague-Dawley Rats
LW / BW ratio
0.06
0.05
0.04
0.03
0.02
0
10
20
30
40
50
60
70
80
90
100
Postnatal Days
C

TABLE 2.1. *Ontogeny of hepatic microsomal CYP450 and CYP2E1, 1A1/2 and 2B1/2 activities in unsexed S-D rats*

Results are expressed as mean ± S.E. (n in Methods). Levels of total CYP450s are expressed as nmol/mg microsomal protein. CYP2E1, CYP1A1/2 and 2B1/2 activities are expressed as nmol 4-NC, pmol resorufin and pmol resorufin formed per mg microsomal protein, respectively. Group values with different letters are significantly different from one another at $p \leq 0.05$.

Postnatal day	Total CYP450	CYP2E1	CYP1A1/2	CYP2B1/2
1	0	0	0	0.01 ± 0.02 [a]
5	0.35 ± 0.03 [a]	2.20 ± 0.11 [a]	0.74 ± 0.02 [a]	0.02 ± 0.02 [a]
10	0.53 ± 0.16 [b]	2.50 ± 0.12 [b]	1.02 ± 0.59 [a]	0.03 ± 0.04 [a]
15	1.27 ± 0.24 [c]	2.46 ± 0.34 [b]	4.42 ± 2.09 [b]	0.05 ± 0.03 [b]
21	1.38 ± 0.10 [c]	2.90 ± 0.05 [c]	9.24 ± 1.61 [c]	0.32 ± 0.01 [c]
30	0.88 ± 0.08 [d]	2.04 ± 0.13 [d]	8.14 ± 0.84 [c]	0.08 ± 0.03 [b]
40	1.38 ± 0.20 [c]	2.17 ± 0.27 [a]	8.88 ± 0.64 [c]	0.16 ± 0.05 [d]
50	1.94 ± 0.19 [e]	1.72 ± 0.04 [e]	5.27 ± 0.22 [d]	0.10 ± 0.02 [b]
60	1.38 ± 0.07[c]	0.64 ± 0.05 [f]	3.67 ± 0.62 [d]	0.13 ± 0.04 [d]
90	2.30 ± 0.45 [h]	0.41 ± 0.04 [f]	0.28 ± 0.08 [e]	0.04 ± 0.01 [a]

TABLE 2.2. *Estimated amounts of microsomal protein, CYP450s and CYP2E1, 1A1/2 and 2B1/2 activities in the entire livers of unsexed S-D rats.*

Results are expressed as mean ± S.E. (n in Methods). Protein is expressed in mg/entire liver. Levels of total CYP450s are expressed as nmol/entire liver. CYP2E1, CYP1A1/2 and 2B1/2 activities are expressed as nmol 4-NC, pmol resorufin and pmol resorufin formed / entire livers, respectively. Group values with different letters are significantly different from one another at $p \leq 0.05$.

Postnatal day	Microsomal protein	Total CYP450	CYP2E1	CYP1A1/2	CYP2B1/2
1	5.5 ± 0.6 [a]	0	0	0	0.002 ± 0.0 [a]
5	3.7 ± 0.4 [a]	0.12 ± 0.02 [a]	0.7 ± 0.1 [a]	0.2 ± 0.03 [a]	0.01 ± 0.01 [a]
10	1.0 ± 0.2 [a]	0.32 ± 0.08 [a]	1.5 ± 0.1 [a]	0.6 ± 0.1 [a]	0.02 ± 0.02 [a]
15	17.3 ± 3.3 [a]	1.35 ± 0.37 [a]	2.3 ± 1.01 [a]	4.8 ± 1.8 [a]	0.06 ± 0.04 [a]
21	28.0 ± 1.2 [a]	2.19 ± 0.19 [a]	4.5 ± 0.2 [a]	14.8 ± 2.1 [a]	0.51 ± 0.03 [a]
30	94.6 ± 6.2 [b]	4.11 ± 0.40 [a]	9.8 ± 0.6 [b]	38.9 ± 3.6 [b]	0.49 ± 0.13 [a]
40	134.8 ± 4.4 [c]	10.0 ± 1.4 [b]	20.1 ± 2.2 [c]	81.9 ± 7.1 [c]	1.98 ± 0.21 [b]
50	173.7 ± 7.7 [d]	16.2 ± 2.1 [b]	20.4 ± 0.9 [c]	43.3 ± 1.8 [d]	0.79 ± 0.20 [c]
60	193.9 ± 13.4 [d]	14.9 ± 1.3 [b]	7.9 ± 0.9 [d]	48.1 ±14.1 [d]	2.20 ± 0.36 [b]
90	175.2 ± 23.9 [d]	25.3 ± 6.8 [c]	4.5 ± 0.9 [a]	3.1 ± 0.9 [f]	0.34 ± 0.14 [a]

TABLE 2.3. *Estimated amounts of microsomal protein, CYP450s and CYP2E1, 1A1/2 and 2B1/2 activities in the normalized liver of unsexed S-D rats*

Results are expressed as mean ± S.E. (n in Methods). Protein is expressed in mg/ liver/kg b.w. Levels of total CYP450s are expressed as nmol/ liver / kg b.w. CYP2E1, CYP1A1/2 and 2B1/2 activities are expressed as nmol 4-NC, pmol resorufin and pmol resorufin formed / livers / kg b.w., respectively. Group values with different letters are significantly different from one another at $p \leq 0.05$.

Postnatal day	Microsomal protein	Total CYP450	CYP2E1	CYP1A1/2	CYP2B1/2
1	877.9 ± 85.9 [a]	0	0	0	0.3 ± 0.03 [a]
5	322.0 ± 73.4 [b]	10.4 ± 1.9 [a]	56.7 ± 6.4 [a]	18.6 ± 2.73 [a]	0.98 ± 0.7 [a]
10	486.0 ± 22.1 [c]	15.4 ± 3.7 [a]	75.8 ± 7.9 [a]	30.8 ± 2.38 [a]	1.66 ± 1.1 [a]
15	469.5 ± 75.4 [c]	35.5 ± 7.6 [a]	94.1 ± 10.7[b]	123.1 ± 38.5 [b]	1.47 ± 0.9 [a]
21	642.3 ± 34.7 [d]	51.4 ± 3.6 [a]	103.7 ± 4.7 [b]	346.0 ± 40.8 [c]	11.9 ± 0.4 [b]
30	890.1 ± 67.9 [a]	24.8 ± 8.2 [a]	92.3 ± 6.5 [b]	369.5 ± 42.5 [c]	4.79 ± 1.4 [c]
40	892.7 ± 71.7 [a]	63.9 ± 8.0 [a]	103.1 ± 11.1 [c]	535.7 ± 49.8 [d]	13.1 ± 1.7 [b]
50	969.8 ± 42.5 [a]	90.5 ± 12.2[a]	115.0 ± 5.4 [c]	242.0 ± 10.9 [e]	4.60 ± 1.1 [a]
60	718.0 ± 97.8 [d]	46.7 ± 6.5 [b]	29.8 ± 4.7 [d]	187.1 ± 60.5 [e]	8.44 ± 1.6 [d]
90	572.6 ± 38.4 [d]	42.3 ± 9.1 [b]	14.6 ± 1.7 [d]	10.0 ± 2.80 [a]	1.49 ± 0.5 [a]

CHAPTER 3

HEPATIC METABOLISM OF DICHLOROMETHANE (DCM), TRICHLOROETHYLENE (TCE) AND PERCHLOROETHYLENE (PERC) IN DEVELOPING SPRAGUE-DAWLEY (S-D) RATS [2]

[2] B. McPhail, B. Cummings, S. Murildhara, J. Wilson and J. Bruckner. To be submitted to *Toxicological Sciences*.

ABSTRACT

Cytochrome P450 (CYP) enzymes play an important role in the metabolism and disposition of a wide range of xenobiotics. Published experimental findings indicate that CYP450 enzymes mature at different rates in the liver of human infants. Relatively little information is available on the maturation of hepatic CYP450s in rats, a common animal model. Commonly found volatile organic compounds (VOCs), dichloromethane (DCM), trichloroethylene (TCE) and perchloroethylene (PERC) are predominately metabolized by hepatic CYP450s. In the current study, postnatal days 10, 15, 21, 60 and 90 hepatic microsomal samples from Sprague-Dawley (S-D) rats were used to measure the Michaelis-Menten parameters, K_m and V_{max}, for DCM, TCE and PERC. Time and protein concentration profiles were initially performed to determine the appropriate conditions for hepatic metabolism in each PND. Each compound showed an increase in metabolism with an increase in time and hepatic protein. It was evident through the time and protein concentration profiles that DCM and TCE were better substrates for CYP450 hepatic metabolism than PERC prior to the execution of the saturation kinetic studies. The hepatic saturation kinetic study showed that peak V_{max} levels occurred at PND 21 for DCM (292 nmol/min/mg protein), TCE (7.48 nmol/min/mg protein) and PERC (0.36 nmol/min/mg protein). K_m did not show a significant difference in maturation for DCM, but peak at PND 21 for TCE and PERC. Characterizing the maturation of hepatic metabolic capacity in rats may prove useful in assessing their applicability as models for children for other xenobiotics which undergo metabolic activation.

INTRODUCTION

There is increasing public concern and scientific interest in infants and children as potentially sensitive subpopulations exposed to pesticides, volatile organic compounds (VOCs), endocrine disrupters and other xenobiotics. The Food Quality Protection Act, of 1996, provided for an additional uncertainty factor of 10 for hazard assessments of pesticides and certain other chemicals for which data pertinent to children are lacking. This is the case for almost all chemicals (Goldman, 1998). The additional 10X "safety" factor is considered to be comprised of two factors of 3 or 3.16 (Renwick, 1998; Dourson *et al.*, 2002). One provides for uncertainty about a compound's toxicodynamics (TD), while the other accounts for uncertainty about toxicokinetics (TK) in children. Physiological and biochemical changes during maturation alter the TK of chemicals, and hence the amount(s) of the bioactive form(s) that reach and affect selected cells and target organs (Scheuplein *et al.*, 2002). Toxic actions are dynamic phenomenoa with concurrent absorption, distribution, metabolic activation and/or inactivation, and elimination (ADME) determining the target tissue dose, and in turn the magnitude and duration of adverse effect(s) (Andersen *et al.*, 1987). There have been kinetics and metabolism studies of only a limited number of drugs in children (Ginsberg *et al.*, 2002, 2004). This paucity of pediatric data led to passage of the Pediatric Research Act, of 2003, which requires pharmaceutical companies to assess safety and efficacy of new drugs used by children. Empirically-based information about ADME and potential risks other classes of chemicals pose to infants and children are almost non-existent.

Immature rodents have been proposed as models for investigation of exposure conditions under which a chemical may be toxic to immature humans. It should be recognized that mice, rats and other small animals are much more immature at birth than humans (NAS, 1993). Anatomical, physiological and biochemical maturation, however, occur very rapidly in mice and rats. Hepatic microsomal cytochrome P450 2E1 (CYP2E1) activity and protein expression, for example, rise from negligible amounts at birth of rats to maximal levels within 21 days (Imaoka *et al.*, 1991). Organ systems and their associated functions develop at different rates in different species (Dourson *et al.*, 2002). This makes it difficult to extrapolate from immature rats to humans, unless cross-species temporal relationships are understood. Quite a lot, for example, is known about the relative time-courses of regional development of the rodent and primate brains (Rice and Barone, 2000). There are species similarities and differences in the ontogeny of different brain areas that extend well into the postnatal period. McPhail *et al.* (2008) have recently characterized the time-courses of expression of hepatic CYP450s (total), CYP2E1, CYP2B1/2, and CYP1A1/2 in Sprague-Dawley (S-D) rats. Johnsrud *et al.* (2003) conducted a comprehensive delineation of the expression of hepatic CYP2E1 in persons 1 day – 18 years old. CYP2E1, the isozyme primarily responsible for metabolic activation of low doses of many VOCs (Guengerich *et al.*, 1991), is a constitutive component of rat and human CYP450s. Knowledge of the temporal expression of CYP2E1 in each species provides a scientific basis for extrapolating from results of TK or toxicity studies of halocarbons in a given age-group of rats to children of a particular age.

The concurrent physiological and biochemical (P&B) processes, that govern the systemic disposition of toxicants and their metabolites, add a layer of complexity to the dissimilar development of these processes in different species. An age-related change in one parameter can

be augmented or offset by simultaneous changes in other processes. As newborns have relatively low levels of plasma proteins, protein-bound drugs such as lidocaine would be expected to be cleared rapidly from the neonates' blood. The neonate's high body water content, however, results in a larger volume of distribution for the water-soluble compound, so its systemic clearance is comparable in neonates and adults. Metabolic activation of cyclophosphamide, an anticancer drug, is highest in children, but enhanced detoxification pathways and urinary excretion give it a shorter half-life and make it less toxic in children than adults (Crom *et al.*, 1987). In light of such complexities, the net effect of immaturity on chemical ADME and toxicity in different immature species is difficult to predict (Bruckner, 2000).

Physiologically-based toxicokinetic (PBTK) modeling is a logical, scientifically-based means of dealing with the aforementioned complexity in children (Clewell *et al.*, 2002, 2004). These mathematical models are based on a realistic description of the anatomy and physiology of the experimental subject. The power of such modeling lies in its ability to estimate, with reasonable accuracy, the amount and time-course of the bioactive form of a chemical in its target tissue(s). PBTK models for toxic/carcinogenic chemicals are typically developed using rodents, for which P&B input parameters are known and used in model construction. Separate TK datasets from rodent experiments are utilized to recalibrate the models and then to assess the accuracy of their internal dosimetry simulations. Validated rodent models can be scaled up to humans allometrically and/or by input of measured human P&B indices. A common approach is to use a validated animal model to predict target organ dosimetry for exposure conditions found to be toxic to the animal. The human model is then run to estimate what level of exposure is required to produce a comparable tissue dose in humans. The concept of dose equivalence

reasonably assumes that a given tissue dose of toxicant produces equivalent adverse effects in other species (Andersen, 1987). The concept does not take possible PD differences between species into account.

The overall goal of an ongoing project in our laboratories is to establish PBTK models for prevalent VOCs in immature rats, for subsequent use in the risk assessments of the chemicals in children. National monitoring surveys in the U.S. have demonstrated the common occurrence of a number of VOCs in the blood of nonoccupationally-exposed individuals (Churchill *et al.*, 2001; Blount *et al.*, 2006). 1,1,2-Trichloroethylene (TCE), 1,1,2,2-tetrachloroethylene (perchloroethylene) (PERC) and 1,2-dichloromethane (methylene chloride) (MC) were among the most frequently-detected VOCs. TCE and PERC were found more often detected in the U.S. groundwater than MC (Moran *et al.*, 2007). A variety of VOCs, including TCE and PERC, have been found in the blood of elementary school-age children (Sexton *et al.*, 2005) and even younger children from inner-city neighborhoods (Sexton *et al.*, 2006). PERC levels were substantially higher than TCE or MC levels, due to PERC's longer half-life. Clewell *et al.* (2004) developed a life-style PBTK model to predict the kinetics of PCE, MC and their key metabolite in children's blood. The most important age-dependent factor was enzymatic immaturity in neonates. Age-specific VOC metabolism rates were calculated by the researchers from adult rates, age-dependent liver volume and linearly-interpolated fractional enzymatic activity. More recently Rodriguez *et al.* (2007) used PBTK modeling to simulate blood levels, total amounts metabolized and brain concentrations of 6 VOCs, including TCE, PERC and MC, in 10-day-old and adult rats. Metabolic rate constants had to be scaled from adult values. In order to reduce model uncertainty, the objective of current project was to accurately define

metabolic rate constants for TCE, PERC and MC at frequent intervals from birth to sexual maturity of S-D rats.

MATERIALS AND METHODS

Animal Housing Timed pregnant female S-D rats were obtained from Charles River Laboratories, (Raleigh, NC). Dams were housed in rat shoebox polystyrene cages and acclimated to a 12-h light/dark cycle in a temperature (25° C ± 2)- and humidity- (40% ± 10) controlled room. Tap water and Purina Rat Chow 5001 commercial rat food were provided *ad libitum* to the dams and the pups as they matured.

Microsomal preparation Male and female pups of different ages were sacrificed by cervical dislocation. The liver was perfused with saline. Due to their small size, it was necessary to pool the pups' livers on: postnatal day (PND) 10, using 5 pups per sample; PND 15, using 5 pups per sample; and PND 21, using 3 pups per sample. A 5-g portion of perfused liver was taken from PND 60 and 90 animals. The homogenized liver was centrifuged using a Beckman Avante (JE25) centrifuge (Palo Alto, CA) for 30 min at 4° C at 12,000 g. The supernatant was collected and centrifuged using a Beckman Coulter Optima XL-100K ultracentrifuge (Beckman Coulter, Fullerton, CA) at 4° C at 105,000 g for 60 min. Microsomal pellets were resuspended in Tris-KC1 buffer at pH 7.4 and stored at -80° C prior to use.

Protein measurement The standard method of Lowry *et al.* (1951) was used to quantify hepatic microsomal protein. A 1:20 microsomal dilution was used to measure the absorbance with a Gilford Response Spectrophotometer at 660 nm.

Incubation mixture A cofactor mixture containing 0.5 mM β-$NADP^{+}$, 10 mL glucose-6-phosphate and 5 mM $MgC1_2$ was prepared in a total volume of 0.54 ml for incubations with substrates. Glucose-6-phosphate dehydrogenase was also added to the mixture.

Measurement of VOC metabolism Sample vials were kept on ice, until incubations commenced. The 0.46-ml cofactor mixture was added to a microsomal suspension containing 8 mg protein/ml and Tris-KC1 buffer (pH 7.4), and preincubated for 2 min. Each substrate (5% as a Alkamuls® emulsion) was added to start the reaction in a 20-ml headspace vial. Controls utilized no microsomal protein. After the addition of substrate and NADPH to the microsomal mixture, each 20-ml headspace vial was capped quickly with a Teflon-coated rubber septa and a metal washer that was tightly crimped. The vials were kept in a thermoregulated water bath (37° C) shaker for varying times. Two hundred microliters of esterizier were added to the reference and sample vials to stop the reaction at the end of the incubation period. Metabolism was reflected by the disappearance of each chemical from the vial's headspace.

Trichloroethylene The hepatic microsomal metabolism of TCE was measured to determine the proper protein and TCE concentrations to use in defining metabolic constants. In order to determine the most appropriate enzymatic (protein) concentration, 0.25, 0.5, 1, 2, and 4 mg of microsomal protein from each age-group of rat were incubated for 5 min with 500 ng of TCE.

Subsequently, 4 mg of microsomal protein were incubated with 500 ng of TCE for 1, 3, 5, 7and 10 min to elucidate time-activity relationships. In order to establish substrate-activity relationships, 25, 50, 100, 300, 500, 700 and 900 ng of TCE in a 5% Alkamuls® emulsion were incubated for 3 min with 1 mg of microsomal protein from the PND 10, 15, 21, 60 and 90 animals.

Dichloromethane The most desirable protein concentration, incubation time and substrate concentration were determined by measuring the rate of disappearance of DCM from the headspace of incubation vials. Time-metabolic activity relationships for the different age-groups were established by incubating 1 mg of protein and 40 µg of substrate for 1, 3, 5, 7 and 10 min. Enzyme-activity relationships were elucidated by incubating 0.25, 0.5, 1, 2 and 4 mg microsomal protein with 40 µg of DCM for 5 min. Substrate-activity relationships were established by incubating 1 mg of protein for 7 min with 6, 10, 20, 40 and 80 µg DCM in a 5% Alkamuls® emulsion.

Perchloroethylene The disappearance of PERC from the headspace of a vial containing the incubation mixture was monitored, in order to assess the influence of time, hepatic microsomal protein concentration and substrate level on PERC metabolism. A time profile was initially characterized by incubating 2 mg of protein from members of each age-group with 150 ng of PERC for 1, 3, 5, 7 and 10min. After the appropriate incubation time was determined, 1, 2 and 4 mg of microsomal protein were used to ascertain the appropriate concentration to utilize for a 5-min incubation of 150 ng of substrate. A saturation experiment was then conducted in which the

rate of the PERC disappearance was measured for a series of substrate concentrations (150, 100, 150, 175 and 200 ng). A 3-min incubation and 2 mg of microsomal protein were employed.

Chemical Analyses Vial headspace samples were analyzed using a PerkinElmer Clause 500 Gas Chromatograph (GC) equipped with a TurboMatrix 110 Headspace Sampler. The air samples were injected onto a stainless steel column packed with 10% OV-17 Chromosorb W-AW (80-100 mesh). Operating conditions for analysis were as follows: injector temperature, 200° C; ECD detector, 360° C; column temperature was isothermal and set to 60° C for 5 min. The carrier gas was ultra pure nitrogen. Standard curves were created with each experiment and used to calculate the concentration of DCM.

Statistical Method The statistical significance of apparent differences in group means was assessed using ANOVA and Duncan's Multiple Range Test (DMRT) for each study [(Knodt's Statistical Analysis Software Package (Bellingham, WA)].

RESULTS

To our knowledge, Michealis-Menten parameters for DCM, TCE and PERC have never been examined in developing S-D rats. Prior to assessing the kinetics of each compound, time- and protein- dependencies were determined. The results from these studies were used to determine the loss of each compound through substrate disappearance.

The initial substrate concentrations used were based on a previously published data in adult rats (Lipscomb 1998). The final substrate concentrations used were based on these

published data along with the concentration at which GC saturation occurred. This was to assure that the maximum disappearance rate that occurred was not due to instrument limitations.

The rate of loss for each PND was determined at multiple hepatic microsomal protein concentrations. Initial protein concentrations were used based on previously published studies in adult rats (Reitz *et al.* 1989). Protein concentrations in the current study ranged from 0.25 – 4 mg of hepatic microsomal protein.

Figure 3.1 demonstrates the effect of an increase in protein concentrations on the disappearance of DCM. Each PND showed an increase in substrate disappearance with an increase in protein concentration. The disappearance of DCM was linear between 0.25 and 2 mg of protein. Rates of disappearance did not vary greatly among each PND. However, PND 15 showed the highest level of substrate disappearance, while PND 10 showed the least amount of substrate loss.

Figure 3.2 demonstrates that the rate of TCE disappearance was also linear with regards to hepatic microsomal protein concentrations for each PND. Unlike DCM, the rates of TCE disappearance were slightly higher for PND 21. Similar to TCE, PND 21 showed an increase in PERC disappearance with an increase in protein concentration (Figure 3.3). Unlike DCM and TCE, PERC disappearance was linear from 1 to 4 mg of protein. These data demonstrated that the rates of PERC disappearance are linear within the range of protein used for each PND.

As a result of the following studies, 1 mg/ml of hepatic microsomal protein were used for DCM and TCE in subsequent studies. Due to the lack of substrate disappearance at lower protein concentrations, 2 mg/ml of hepatic microsomal protein were used for PERC.

Next, time dependencies were determined for each substrate. Microsomes for each PND were incubated for 1, 3, 5, 7 and 10 minutes. Figure 3.4 demonstrates that the rate of loss of

DCM was time-dependent from 1 to 7 minutes. The disappearance of DCM was linear for each PND at these times. Similar results were observed for TCE (Figure 3.5). In contrast, the rate of PERC disappearance did not appear to increase as greatly as DCM and TCE with an increase in time (Figure 3.6). However, there was linearity between 1 and 5 minutes for PERC disappearance for each PND. Based on these data, DCM was incubated for 7 minutes and TCE and PERC were incubated for 3 minutes in all subsequent studies.

Figure 3.7 presents the kinetics of DCM disappearance using the above time and concentrations. Figure 3.7A demonstrates the rate of DCM disappearance at various substrate concentrations for each PND. Preweanlings (PND 10 and 15) and weanlings (PND 21) had slightly higher rates of DCM disappearance at lower substrate concentrations, compared to older (PND 60 and 90) animals. The maximum rate of disappearance occurred in PND 15 and 21 pups at 100 uM of DCM. The rates of disappearance for DCM in PND 21 microsomes were significantly different from those in PND 10, 60 and 90 microsomes. Figure 3.7B depicts the Michealis-Menten saturation plot for DCM. Similar to the above data, the rate of DCM disappearance was significantly higher in the young (PND 10, 15 and 21) hepatic microsomes, compared to older (PND 60 and 90) animals.

Figure 3.8 demonstrates the disappearance of TCE in each PND. Unlike DCM, each PND showed a similar rate of loss for TCE at lower substrate concentrations. The rate of disappearance for TCE was significantly different between younger and older animals at higher concentrations. Microsomes from PND 10 rats had significantly higher rates of disappearance at 5 uM of TCE, compared to all other PND. Although each PND showed an increase in substrate disappearance at higher substrate concentrations, PND 15 and 21 had the highest rate of disappearance at the higher TCE concentrations. Figure 3.8B demonstrated the Michealis-

Menten plot for TCE disappearance. PND 10 had significantly higher rates of loss at lower concentrations compared to each PND. As the substrate concentration increased, PND 10 rates of loss decreased. Similar to Figure 3.8A, PND 15 and 21 had higher rates of disappearance at higher substrate concentrations when compared to each PND (figure 3.8B).

In contrast to TCE, the rate of disappearance for PERC was higher in PND 60 and 90 microsomes (Figure 3.9). Figure 3.9A demonstrates that the rate of PERC disappearance was significantly different in younger pups (PND 10, 15 and 21) compared to older (PND 60 and 90) animals at each substrate concentration. Peak substrate disappearance occurred at 2.3 uM of PERC for each PND, and was exceptionally high for PND 60 and 90. Michealis-Menten analysis for PERC demonstrated similar Km and Vmax values for each PND. Similar to data presented in Figure 3.9A, the rates of disappearance in microsomes from PND 60 and 90 rats had higher velocities than those from PND 10, 15 and 21 (Figure 3.9B). However, unlike, data presented in Figure 3.9A, PND 21 had significantly higher rates of PERC disappearance at higher concentrations.

Lineweaver-Burke analysis was used to further analyze the above data. Figure 2.10 demonstrates the Michealis constant and Vmax of each compound for each PND as calculated using the above data. Reciprocal plots for DCM (Figure 3.10A), TCE (Figure 3.10B) and PERC (Figure 3.10C) resulted in similar Km and Vmax as those determined experimentally.

The calculated Vmax of each compound is presented in Table 3.1. A developmental trend was evident for each PND. The Vmax for DCM increased from PND 10 to 21. Peak velocity occurred in weaning rats (PND 21). The lowest Vmax occurred during sexual maturity (PND 60), which was significantly different from PND 21. A similar developmental pattern was exhibited for TCE. Vmax peaked at PND 21, which was significantly different from all other

PND. PND 60 had the lowest Vmax, while the Vmax for PND 90 was similar to that of preweanlings (PND 15). Contrary to DCM and TCE, the Vmax for PERC remained relatively constant for PND 10, 15, 60 and 90. The Vmax for PERC peaked at PND 21, which was significantly higher than each PND.

Table 2.1 also presents the calculated Km for each compound. Similar to Vmax, a developmental trend was also shown for Km. The Michealis constant for DCM increased from PND 10 to PND 21. The Km decreased during sexual maturity. Unlike DCM, the Km for TCE was lowest at PND 10 and 60, compared to PND 15, 21 and 90. The TCE Km peaked at PND 21, which was significantly different from that calculated at PND 10 and 60. PERC did not show a similar developmental pattern for Km as that seen with DCM and TCE. Km values for PERC where higher in younger animals (PND 10, 15 and 21). Peak Km levels for PERC occurred at PND 21, which were significantly different from each PND.

In addition to the Km and Vmax, the in vitro clearance was determined for each compound at each PND (Table 3.1). In general, younger animals showed a higher clearance for DCM and TCE. Peak clearance levels were observed at PND 10, which were significantly higher from PNDs for both compounds.

Unlike DCM and TCE, PERC showed higher levels of in vitro clearance in older (PND 60 and 90) animals compared to younger animals. Clearance levels in preweanlings (PND 10 and 15) and weanlings (PND 21) remained constant. The highest rate of clearance occurred at PND 60, which were significantly different from PND 10, 15 and 21.

DISCUSSION

CYP2E1 and CYP2B1/2 are the predominant isozymes involved in the biotransformation of VOCs (i.e. perchloroethylene, trichloroethylene, dichloromethane) to their reactive metabolites (Sakai *et al.* 2002; Lash *et al.* 2000; Fisher *et al.* 2004; Forkert *et al.* 2005). Studies have shown that CYP450 enzymatic activity and protein expression changes with age in rodents and humans (Imoaka *et al.* 1991; Jonhri *et al.* 2007; Hines and McCarver 2002).

Data are often unavailable for saturation kinetic parameters for the young. CYP450 enzymatic activity and protein expression changes during development (Johri *et al.* 2006; Imoaka *et al.* 1999; Morel *et al.* 1999). Due to the lack of information for enzymatic maturation, K_m and V_{max} are often estimated when using PBPK models; therefore, data are needed to provide boundaries for their values (Andersen 2003).

Metabolic rates such as K_m and V_{max} can be determined using *in vitro* studies (Andersen 2003; Kedderis 1997). In the present study, fixed time and protein concentrations and varying substrate concentrations were used to determine the saturation kinetics for DCM, TCE and PERC. DCM and TCE are predominately metabolized by CYP2E1 at low concentrations (Jonsson *et al.* 2001; Lash *et al.* 2000; Watanabe and Guengerich 2006). PERC is a poor substrate for CYP2E1 when compare to TCE; therefore, CYP2B1/2 may be the predominate isozyme responsible for PERC metabolism (Lash *et al.* 2001; Fisher *et al.* 2004; Lash and Parker 2001).

The rate at which an enzyme is capable of catalyzing a reaction is directly proportional to the total amount of enzyme present in that organ system (Kedderis 1997; Lipscomb *et al.* 2003). A study by Kim *et al.* (2007) found that the production of COHb was directly related to the

CYP2E1 content in the livers of animals treated with DCM. The previous study from the current project indicates a peak in CYP2E1, CYP1A1/2 and CYP2B1/2 enzymatic activity and protein expression at PND 21 when compared to each PND. The peak in enzymatic content is evident in the current study by a peak in Km and Vmax for DCM, TCE and PERC prior to adulthood.

Michaelis-Menten constants were determined for DCM, TCE and PERC. The Vmax for each compound peaked at PND 21. DCM and TCE had the lowest Vmax at PND 60, which was significantly different from PND 21. TCE Vmax showed similar levels in the preweanling animals and adults. Unlike DCM and TCE, PERC Vmax remained relatively constant throughout development for PNDs 10, 15, 60 and 90, which was significantly different from PND 21.

The Km values were also determined. DCM showed no significant difference in Km values throughout development; however, DCM and TCE showed a similar pattern in development. TCE Km values increased in the young, peaked at PND 21 and decrease to adult levels. The Km for PERC also peaked at PND 21. PERC Km values were not significantly different from PNDs 10, 15, 60 and 90, but PNDs 10 and 15 were slightly higher than PNDs 60 and 90.

Various components can attribute to the variability of Vmax which includes age, gender, ethnicity and body composition (Jonsson *et al.* 2001). A study by Clewell *et al.* (2004) found that most volatiles dose metric peaked early in life and decreased with an increase in age. Although animal studies are commonly used for risk assessment, age-dependent differences in VOCs susceptibility to toxicity are poorly understood. PBPK models can be used to determine the impact of differences in key metabolic enzymes due to normal variation in enzyme activities

within the general population and also to differences in metabolism across age groups, such as children versus adults (Gentry *et al.* 2003).

Minimal data are available for kinetic parameters used in PBPK studies for juvenile animals. Currently, blood flow data are not available for rodents prior to adolescences (Delp *et al.* 1998). V_{max} values are often scaled for juvenile studies, while Km values are attained from previously published adult values (Rodriguez *et al.* 2007). Although computer simulate data is capable of showing the difference between juvenile versus adult species, the accuracy of estimations are unknown due to the lack of juvenile data. A study by Rodriguez *et al.* (2007) concluded that the lower metabolic capacity of the young was attributed to the smaller liver size when compare to adults. However, peak hepatic capacity occurred for DCM, TCE and PERC at PND 21, which is the day of weaning for S-D rats.

Human data often exclude enzymatic information after 1 year of age due to lack of samples; therefore, several assumptions are made for age-dependent PBPK models (Cresteil 1998; Hines and McCarver 2002; Rodriguez *et al.* 2007). Children have a larger proportional liver size and hepatic blood flow when compared to adults, which increase hepatic clearance (Andersen and Palmer 2006; Ginsberg *et al.* 2002). In an intact system, blood flow and liver size contributes to the bioactivation of a compound (Bjorkman 2004; Kedderis 1997). A human based study by Nong *et al.* (2006) found that enzymatic content is a more sensitive parameter in neonates than blood flow, whereas flow rates became the rate limiting step in all other age groups. This study further demonstrated how the degradation of toluene, a compound metabolized by CYP2E1, was limited by enzyme content at birth and evolved to a flow-limited condition with maturity.

Due to ethical considerations, pharmacokinetic data for environmental contaminates are not readily available for children. PBPK models are becoming increasingly important in risk assessment and in characterizing the distribution of a compound in an intact human with data attained from animal studies (Gargas and Clewell 1990; Gentry *et al.* 2003). They are capable of integrating developmental changes such as physiology, enzymatic content and tissue composition to provide information on pharmacokinetic and tissue dose of a chemical (Krishnan and Johansson 2005). Often these models lack metabolic rate constants which are experimentally obtained (Rodriguez *et al.* 2007).

Previously published data exist for each compound in adult rodents but not developing pups. Andersen *et al.* (1987) was able to successfully show the similarities of the *in vivo* metabolism of DCM in humans and rodents. This study demonstrated that rodent kinetic data could be used to predict human risk to chemicals

The maturational pattern of Km and Vmax for DCM, TCE and PERC has never been experimentally determined in rodents, a commonly used surrogate for human toxicological studies. Rodent studies commonly estimated juvenile K_m and V_{max} values based on calculated values obtained from enzymatic studies (Ginsberg *et al.* 2002) or simulated data (Rodriguez *et al.* 2007). In the current studies, the disappearance of each compound was higher in adolescent pups (PND 21) than adults. The isozymes which are involved in DCM, TCE and PERC metabolism have not shown a peak in development for human studies (Cresteil 1998; Sonnier and Cresteil 1998; Ginsberg *et al.* 2002). These data suggest that S-D rats may not be an appropriate model for developmental studies; however, additional studies are needed in order to assess their suitability for pediatric toxicological studies.

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FIGURES AND TABLE:

Fig. 3.1. Effects of microsomal protein on DCM disappearance in age-dependent rats. Various hepatic microsomal protein concentration (0.25, 0.5, 1, 2 and 4 mg/ml) were incubated at 37°C with 1024 uM of substrate for 5 min. for each PND. The livers from postnatal day (PND) 10-21 animals were pooled as described in the Methods and a 5-g liver sample was analyzed from PND 60 and 90 rats. Each point represents the mean ± S.E. for a group of 4 rats. Linearity was determined by linear regression.

Effects of microsomal protein on DCM disappearance in age-dependent rats

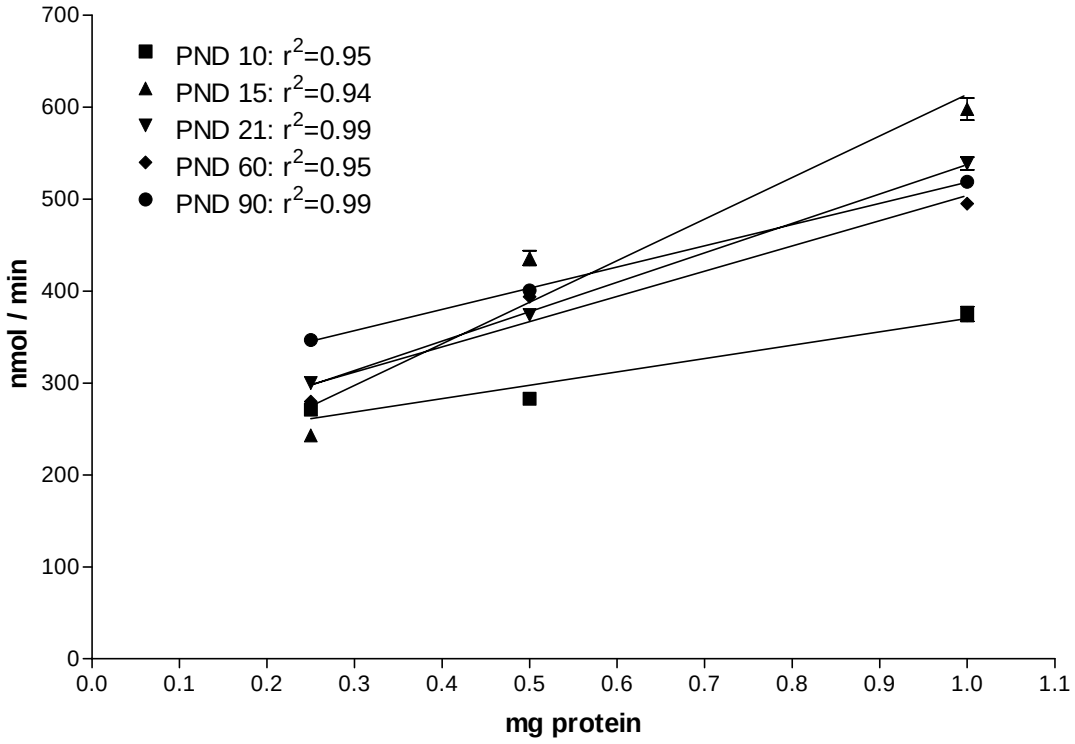

Fig. 3.2. Effects of microsomal protein on TCE disappearance in age-dependent rats. Each PND was incubated (37°C) at various hepatic microsomal protein concentration (0.25, 0.5, 1, 2 and 4 mg/ml) in 8.272 μM of substrate for 5 min. The livers from postnatal day (PND) 10-21 animals were pooled as described in the Methods and a 5-g liver sample was analyzed from PND 60 and 90 rats. Each point represents the mean ± S.E. for a group of 4 rats. Linearity was determined by linear regression.

Effects of microsomal protein on TCE disappearance in age-dependent rats

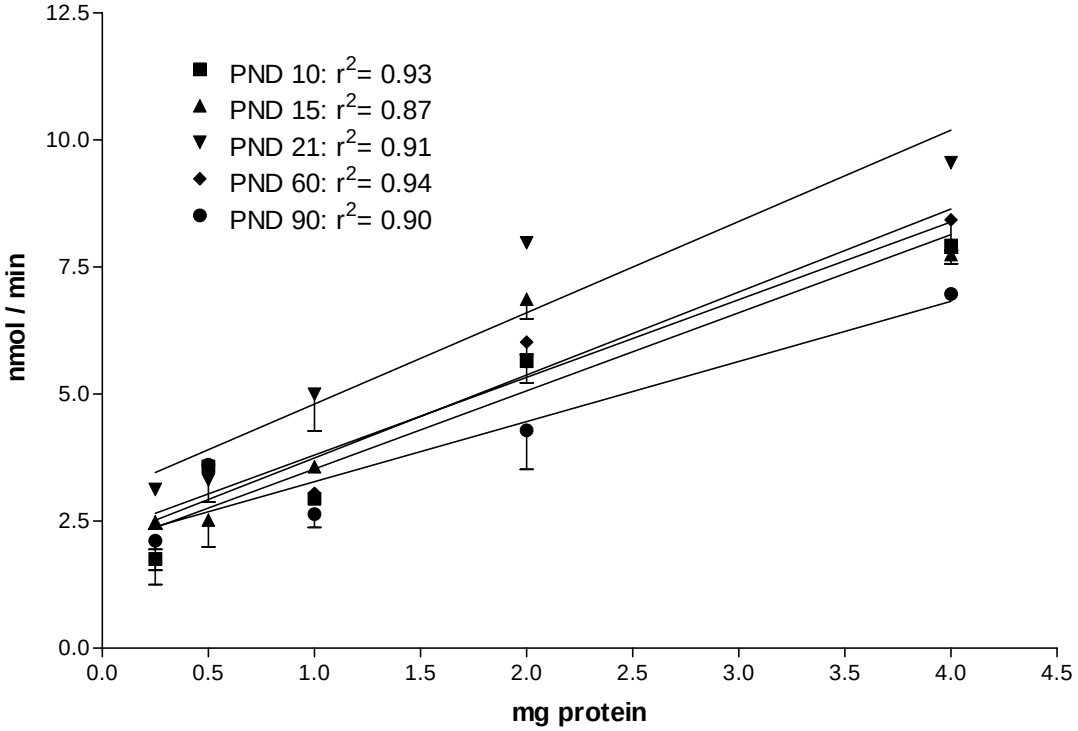

Fig. 3.3. Effects of microsomal protein on PERC disappearance in age-dependent rats. Various hepatic microsomal protein concentration (1, 2 and 4 mg/ml) were incubated for 5 min in 2.966 μM of substrate at 37°C for each PND. The livers from postnatal day (PND) 10-21 animals were pooled as described in the Methods and a 5-g liver sample was analyzed from PND 60 and 90 rats. Each point represents the mean ± S.E. for a group of 4 rats. Linearity was determined by linear regression.

Effects of microsomal protein on PERC disappearance in age-dependent rats

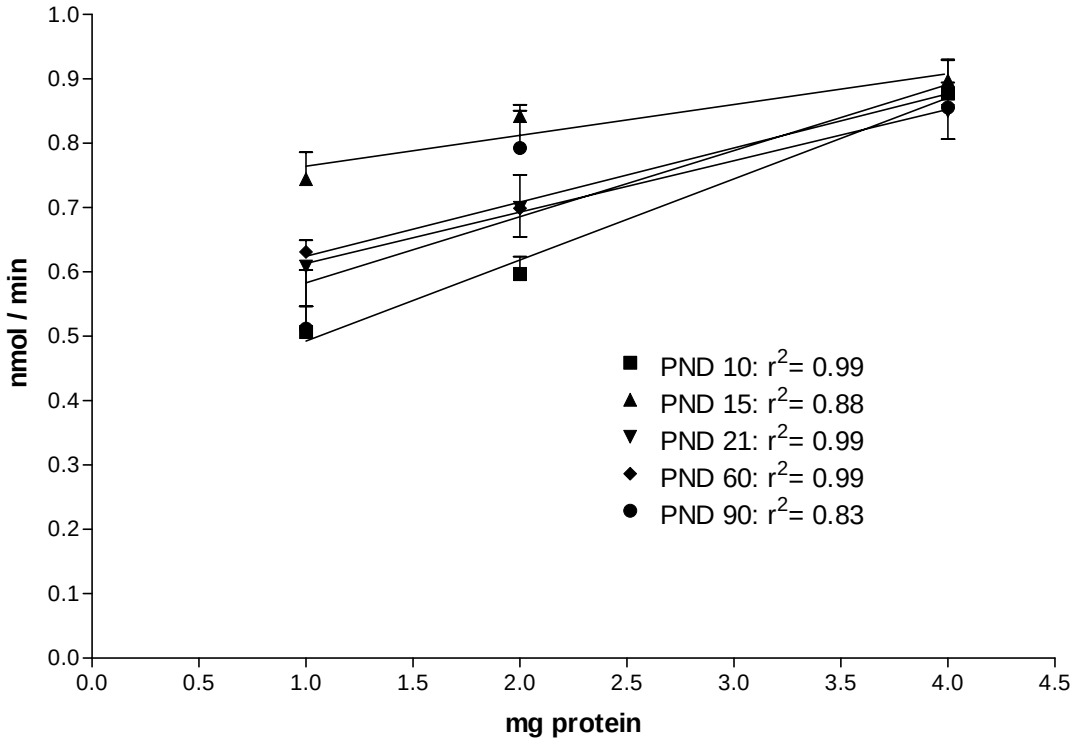

Fig. 3.4. Effects of time on DCM disappearance in age-dependent rats. One mg of hepatic microsomal protein was incubated (37°C) in 1024 uM at various times (1, 3, 5 and 7 min) for each PND. The livers from postnatal day (PND) 10-21 animals were pooled as described in the Methods and a 5-g liver sample was analyzed from PND 60 and 90 rats. Each point represents the mean ± S.E. for a group of 4 rats. Linearity was determined by linear regression.

Effects of time on DCM disappearance in age-dependent rats

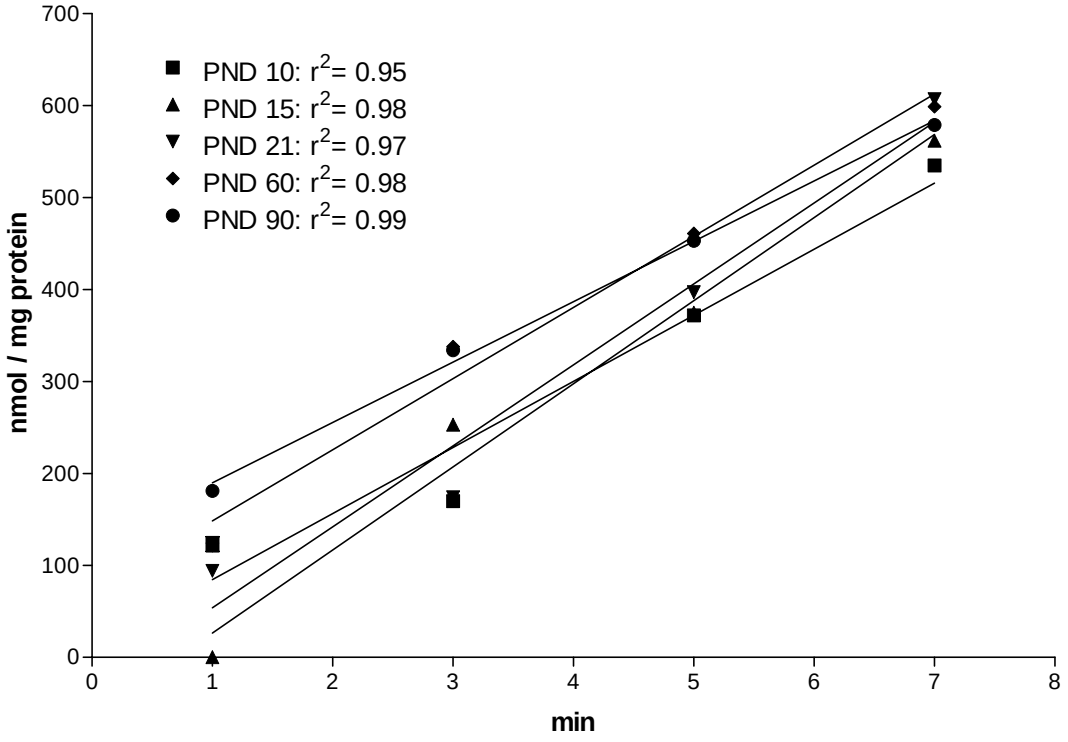

Fig. 3.5. Effects of time on TCE disappearance in age-dependent rats. Each PND was incubate at 37°C using 1 mg of hepatic microsomal protein in 4.963 μM of substrate at various times (1, 3, 5 and 7 min). The livers from postnatal day (PND) 10-21 animals were pooled as described in the Methods and a 5-g liver sample was analyzed from PND 60 and 90 rats. Each point represents the mean ± S.E. for a group of 4 rats. Linearity was determined by linear regression.

Effects of time on TCE disappearance in age-dependent rats

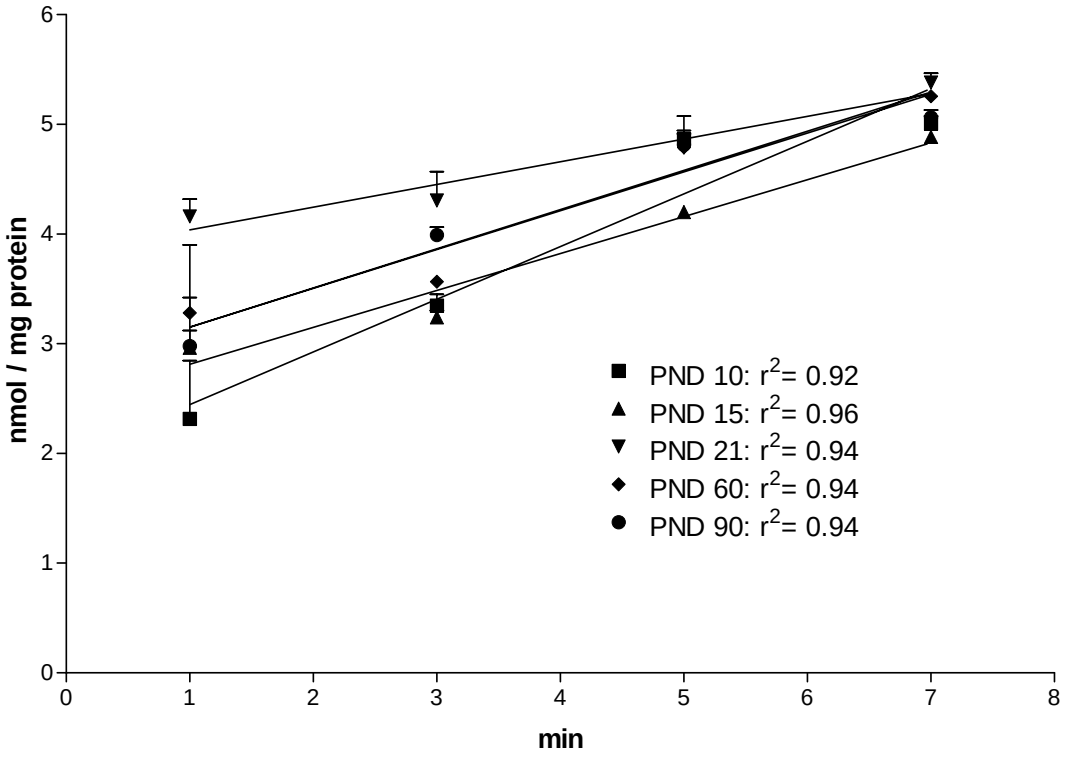

Fig. 3.6. Effects of time on PERC disappearance in age-dependent rats. Compound metabolism for each PND was incubated at 37°C for 1, 3 and 5 min using 1 mg of hepatic microsomal protein in 2.966 μM of substrate. The livers from postnatal day (PND) 10-21 animals were pooled as described in the Methods and a 5-g liver sample was analyzed from PND 60 and 90 rats. Each point represents the mean ± S.E. for a group of 4 rats. Linearity was determined by linear regression.

Effects of time on PERC disappearance in age-dependent rats

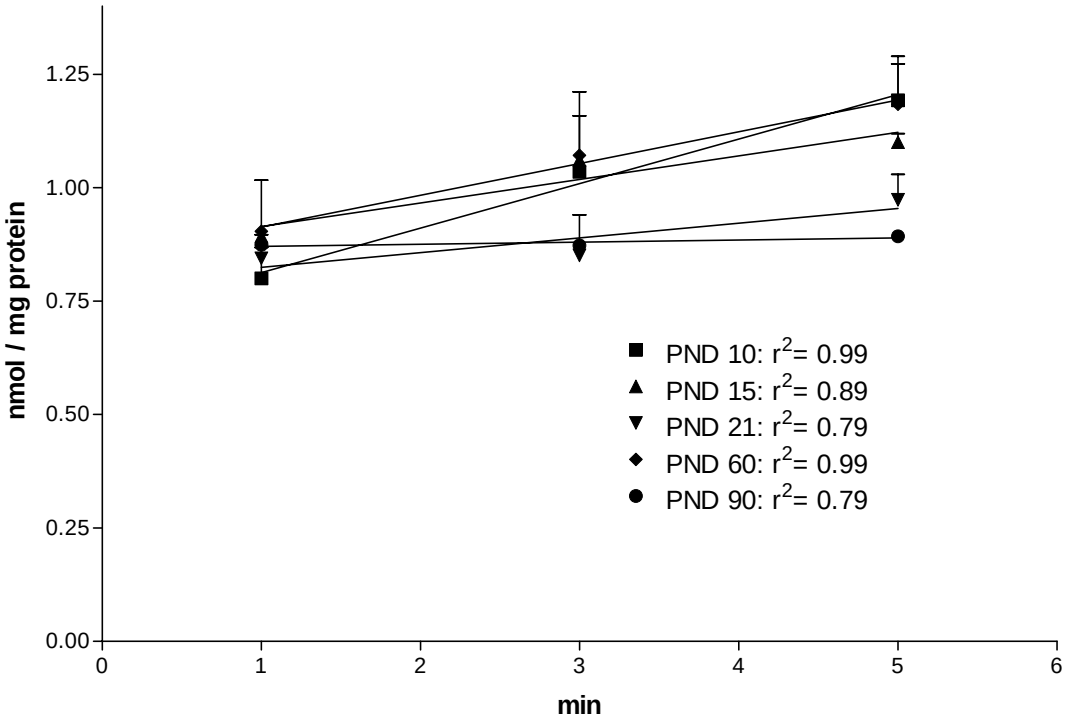

Fig. 3.7. Age-dependent metabolism of DCM in hepatic microsomal samples. (A) Each PND was incubated for 7 min at 37°C in 1 mg of hepatic microsomal protein using 4 µl of substrate at various concentrations (6, 10, 20, 40 and 80 µg). The livers from postnatal day (PND) 10-21 animals were pooled as described in the Methods and a 5-g liver sample was analyzed from PND 60 and 90 rats. Each point represents the mean ± S.E. for a group of 4 rats. (B) Michealis-Menten saturation plot $v = (Vmax*[S]) / (Km + [S])$. Michealis-Menten rate constants were calculated using SigmaPlot and are represented in table 1. Letters denote statistical differences ($p < 0.05$) among each PND for each substrate concentration.

Age-dependent metabolism of DCM in hepatic microsomal samples

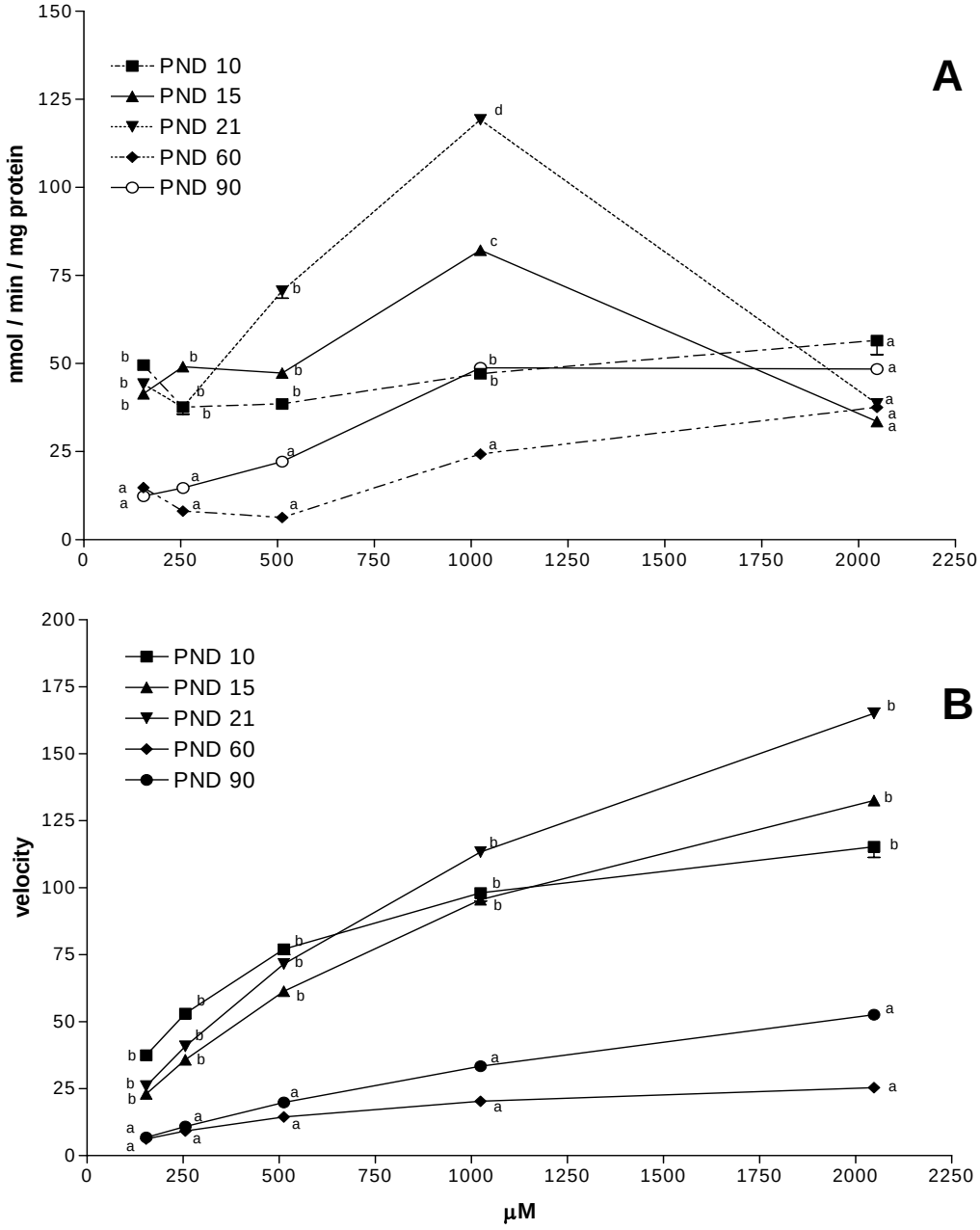

Fig. 3.8. Age-dependent metabolism of TCE in hepatic microsomal samples. (A) Ten µl of TCE at various concentrations (25, 50, 100, 300, 500, 700 and 900 ng) were incubated at 37°C for 3 min in 1 mg of hepatic microsomal protein for each PND. The livers from postnatal day (PND) 10-21 animals were pooled as described in the Methods and a 5-g liver sample was analyzed from PND 60 and 90 rats. Each point represents the mean ± S.E. for a group of 4 rats. (B) Michealis-Menten saturation plot using $v = (Vmax*[S]) / (Km + [S])$. Michealis-Menten rate constants were calculated using SigmaPlot and are represented in table 1 Statistical differences ($p < 0.05$) among each PND for each substrate concentration are denoted by different letters.

Age-dependent metabolism of TCE in hepatic microsomal samples

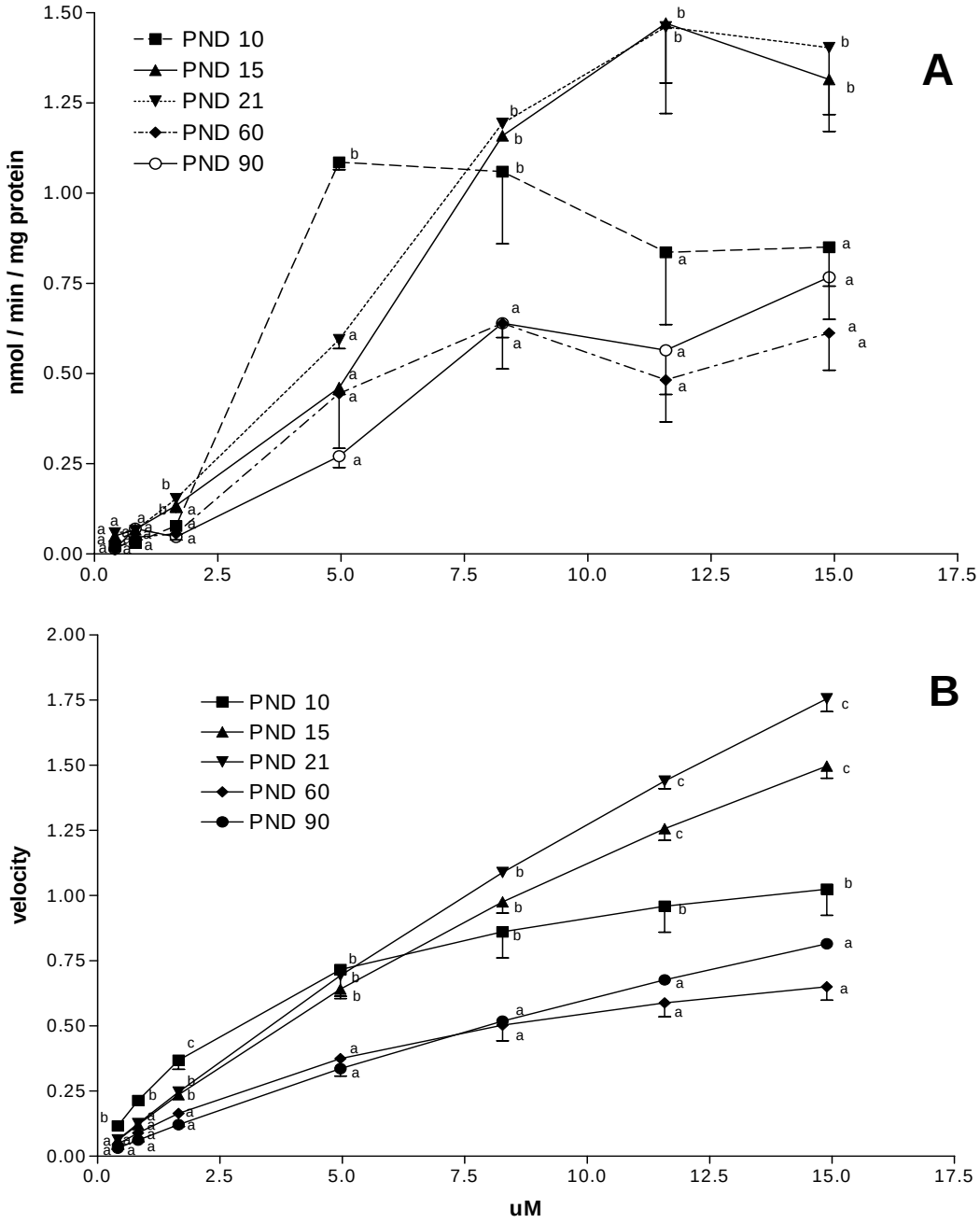

Fig. 3.9. Age-dependent metabolism of PERC in hepatic microsomal samples. (A) Each age group was incubated (37°C) in 2 mg of hepatic microsomal protein for 3 min using 10 µl of substrate at various concentrations (50, 100, 150, 175, 200 ng). The livers from postnatal day (PND) 10-21 animals were pooled as described in the Methods and a 5-g liver sample was analyzed from PND 60 and 90 rats. Each point represents the mean ± S.E. for a group of 4 rats. (B) Michealis-Menten saturation plot using $v = (Vmax*[S]) / (Km + [S])$. Michealis-Menten rate constants were calculated using SigmaPlot and are represented in table 1. Letters denote statistical differences ($p < 0.05$) among each PND for each substrate concentration.

Age-dependent metabolism of PERC in hepatic microsomal samples

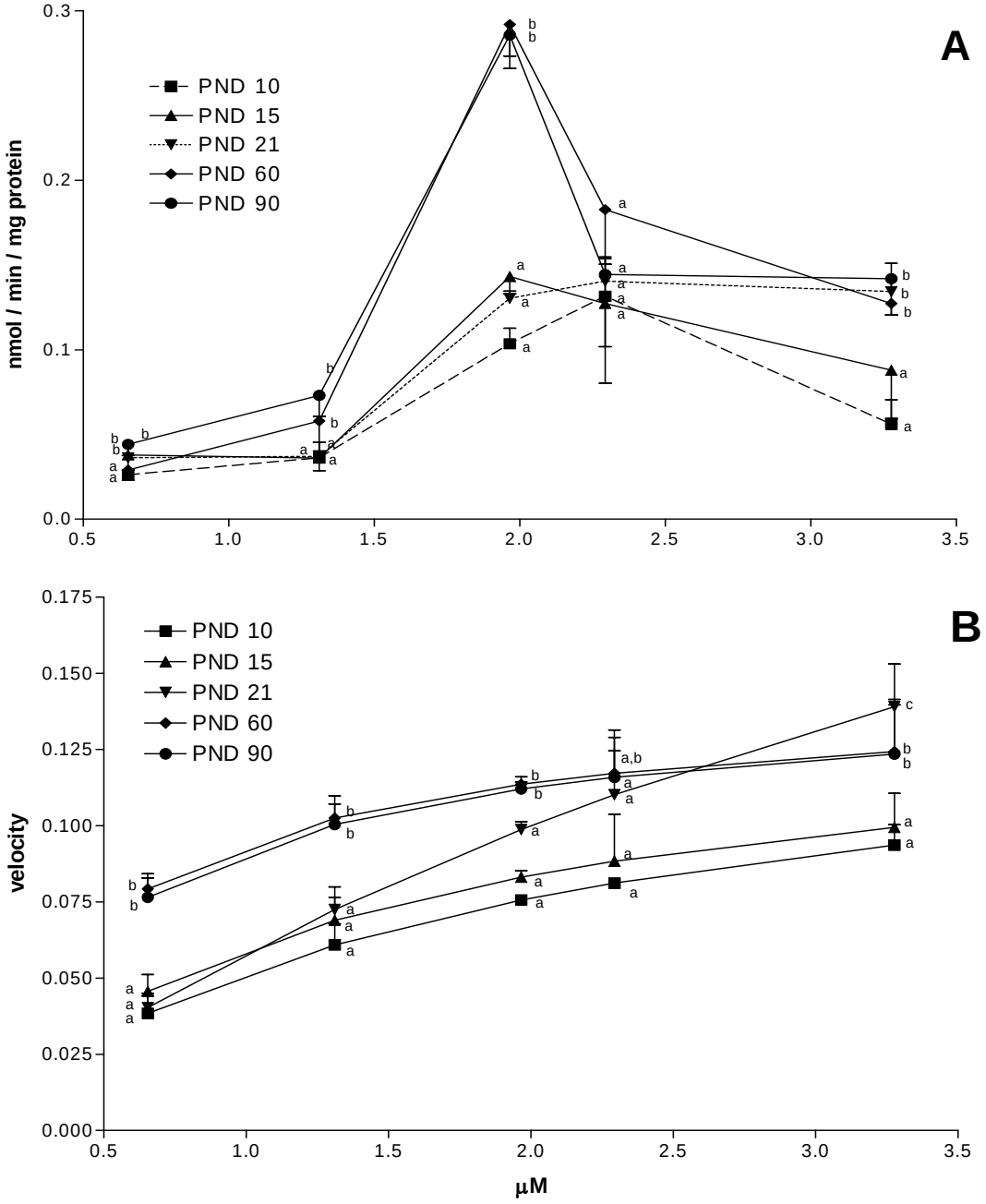

Fig. 3.10. Lineweaver-Burke plots for DCM (A), TCE (B) and PERC (C) in developing S-D rats.

Lineweaver-Burke plots for DCM (A), TCE (B) and PERC (C) in developing S-D rats

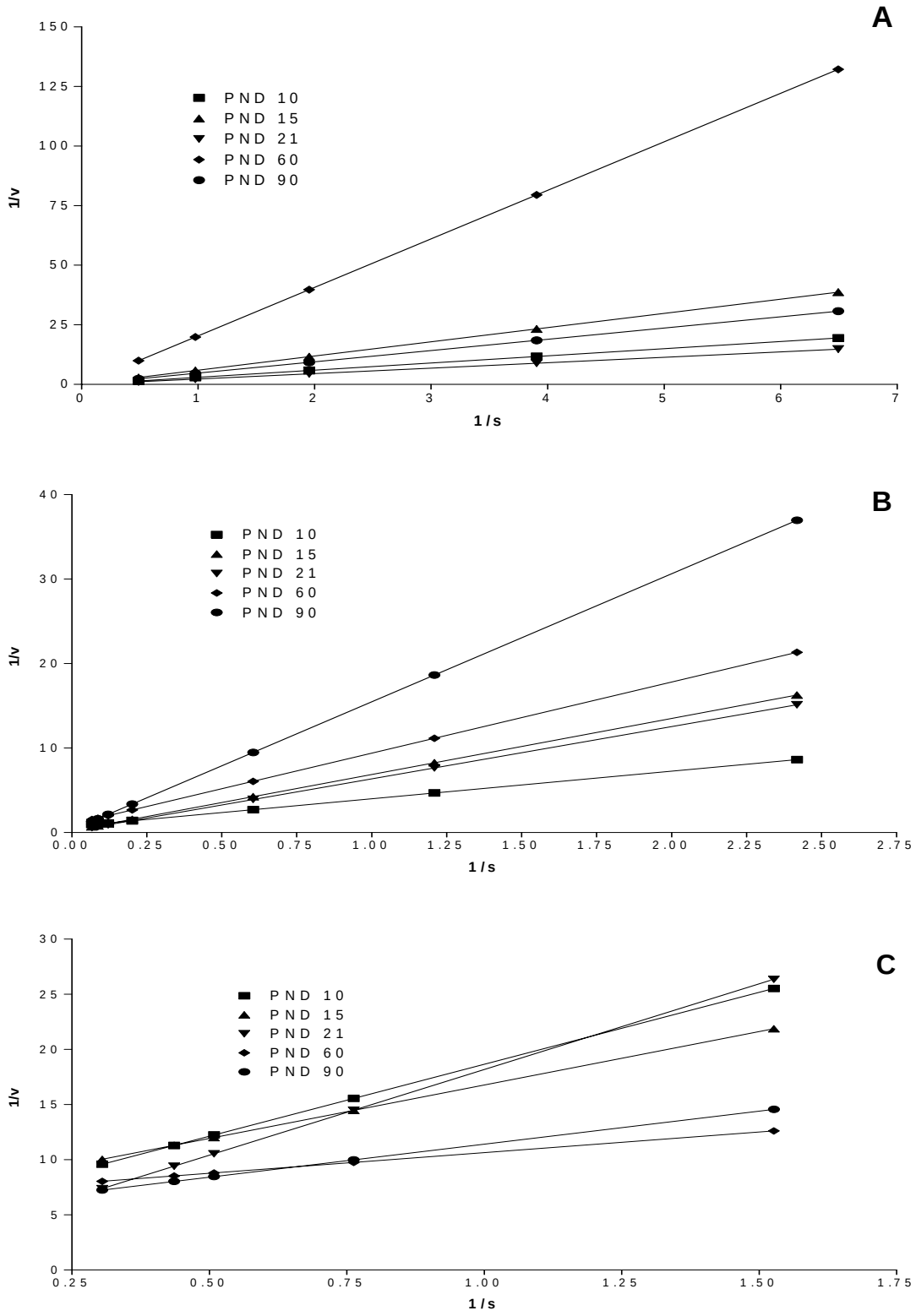

Table 3.1. Michaelis-Menten constants for DCM, TCE and PERC from age-dependent *in vitro* metabolism. Results are represented as mean ± S.E. (n=4). V_{max} is expressed in units of nmol/min/mg protein; K_m is expressed in units of μM; *in vitro* clearance is expressed in units of min^{-1} for each compound. Statistical significant ($p < 0.05$) is denoted by letters: [a] denotes the statistical difference ($p < 0.05$) from PND 10, [b] denotes the statistical difference ($p < 0.05$) from PND 15, [c] denotes the statistical difference ($p < 0.05$) from PND 21, [d] denotes the statistical difference ($p < 0.05$) from PND 60 and [e] denotes the statistical difference ($p < 0.05$) from PND 90.

	PND 10	PND 15	PND 21	PND 60	PND 90
DCM					
Vmax	140.3 ± 95	216.3 ± 97	292 ± 107	34 ± 7.5 [c]	117.7 ± 49
Km	421.3 ± 112	1290 ± 314	665.6 ± 317	692 ± 246	555 ± 188
Vmax/Km	0.0003 ± 0.08	0.0002 ± 0.04	0.0004 ± 0.06	0.00005± 0.04 [a]	0.0002 ± 0.04 [a]
TCE					
Vmax	1.36 ± 0.32 [b,c]	4.51 ± 0.42 [c]	7.48 ± 1.44	1.03 ± 0.22 [b,c]	3.136 ± 0.72 [c]
Km	4.42 ± 0.94 [b,c,e]	29.93 ± 4.57	48.61 ± 10.40	8.665 ± 2.97 [c,e]	37.14 ± 12.02
Vmax/Km	0.0003 ± 0.01	0.00015 ± 0.01 [a]	0.00016 ± 0.00 [a]	0.00016 ± 0.06 [a]	0.00008 ± 0.01 [a]
PERC					
Vmax	0.15 ± 0.03 [c]	0.14 ± 0.00 [c]	0.36 ± 0.01	0.14 ± 0.01 [c]	0.15 ± 0.03 [c]
Km	1.83 ± 0.36 [c]	1.37 ± 0.25 [c]	5.16 ± 0.41	0.54 ± 0.16 [c]	0.59 ± 0.20 [c]
Vmax/Km	0.0001 ± 0.02 [d]	0.0001 ± 0.02 [d]	0.00007 ± 0.00 [d]	0.0003 ± 0.13	0.00027 ± 0.04

Table 3.2. Lineweaver Burke values for DCM, TCE and PERC. The reciprocal plot was performed in order to validate the calculated Michaelis-Menten parameters. Vmax is the reciprocal of the y-intercept; Km is the intercept of the x-intercept; Vmax/Km is the intercept of the slope.

	PND 10	**PND 15**	**PND 21**	**PND 60**	**PND 90**
DCM					
Vmax	140.29	216.31	291.97	34.0	117.0
Km	421.05	1289.99	665.78	692.04	554.63
Vmax/Km	0.333	0.1677	0.4387	0.049	0.2115
TCE					
Vmax	1.335	4.507	5.593	1.029	3.136
Km	4.425	29.93	34.59	8.665	47.53
Vmax/Km	0.306	0.151	0.162	0.119	0.066
PERC					
Vmax	0.178	0.141	0.378	0.145	0.184
Km	2.321	1.366	5.872	0.543	1.101
Vmax/Km	0.077	0.103	0.064	0.267	0.077

CHAPTER 4

CONCLUSION

Animals are often used as surrogates for humans in toxicological studies (Andersen *et al.* 1991; Goldman *et al.* 2000; Ginsberg *et al.* 2004; Rodriguez *et al.* 2007). Human risk assessments are usually based on the results of studies of homologous animal population model. Minimal data are available from developmental studies in humans and rodents. Studies of immature humans are scarce due to ethical considerations and a lack of pediatric tissue samples. Until recently, developmental studies were not of great concern; therefore, rodent maturational studies were not commonly performed.

Many obstacles are present when performing human developmental studies. For example, samples from different age groups are often combined despite developmental changes that occur. Also, the tissue samples used are often from diseased patients (Vieira *et al.* 1996; Cresteil 1998; Sonnier and Cresteil 1998; Hines and McCarver 2002; Bjorkman 2004).

Very few studies have quantified the ontogeny of Phase I cytochrome P450 (CYP450) isozymes in developing rodents. In the current study, the ontogeny of CYP2E1, CYP1A1/2 and CYP2B1/2 enzymatic activity and protein expression were quantified in maturing Sprague-Dawley (S-D) rats. In addition, the K_m and V_{max} for the volatile organic compounds dichloromethane (DCM), trichloroethylene (TCE) and perchloroethylene (PERC) were determined in developing S-D rats. An overall objective of the current project was to determine whether the immature S-D rat would be an appropriate model for developmental toxicological studies of chemicals that serve as substrates for these isozymes. The following sections were designed to compare existing human and rodent data to the data obtain in the current project.

Ontogeny of CYP450 Isozymes

It is important to recognize that children are not miniature adults (Bebia *et al.* 2004). Windows of vulnerability may exist during development of a variety of organ systems (Bruckner and Weil 1999; Selevan *et al.* 2000; Scheuplein *et al.* 2002). Phase I cytochrome P450 (CYP450) enzymes develop at different rates during maturation. Although CYP450s are expressed to a limited extent in newborns, they generally develop at a rapid rate. Enzymatic changes that occur during childhood, sometimes produce metabolites that differ from those produced by adults (Makri *et al.* 2004).

Studies that involve children's samples generally use tissue from diseased patients postmortem. Although fetuses used in research are normally stillborn or aborted, samples are often derived from children suffering from severe infections, immature pulmonary function that result in respiratory distress, hypertension, hydro or microcephaly, non-reversible hyprotrophy or sudden death (Cresteil 1998; Bjorkman 2004). Adult human studies also often lack a sufficient amount of samples (Bjorkman 2004; Blanco *et al.* 2000). Rodent studies can be performed on a substantial number of healthy animals of selected ages. Several rodent studies have shown an increase in CYP450 activity, which sometimes exceeded adult levels, in developing rodents; however, unlike the current study, these studies did not include each period of development (Imaoka *et al.* 1991; Gerbremichael *et al.* 1995; Morel *et al.* 1999). In the current project, the activity and expression of hepatic CYP2E1, CYP1A1/2 and CYP2B1/2 peaked prior to adulthood in naïve rats.

In our study, hepatic enzymatic activity and protein expression of CYP2E1 was not present at PND 1, but dramatically increased from PND 5-15, and peaked at PND 21, followed by a steady decrease to adulthood. Previous studies have suggested the onset of CYP2E1

activity occurs within 24 hours of birth of rodents, followed by peak activity prior to adulthood (Imaoka *et al.* 1991; Borlakoglu *et al.* 1993; Morel *et al. 1999*). Humans have been shown to reach adult levels by 1 year of age (Vieira 1996; Cresteil 1998; Hines and McCarver 2002; Johnsrud *et al.* 2003; Nong *et al.* 2006). Due to the lack of samples, developmental stage (i.e., infant, younger children, older children and adolescents) were often grouped together. Rodent studies can provide sufficient numbers of subject/samples for each stage of development. In a study by Wauthier *et al.* 2006, male Wistar rats showed a decrease in hepatic CYP2E1 activity in 24-month-old animals compared to 9-month-old animals. Morel *et al.* showed peak CYP2E1 at 5 weeks of age with a subsequent decrease in adults and further decreased in geriatric animals. These data did not peak in activity during development.

CYP1A2 has been shown to be primarily responsible for the metabolism of pharmaceutical compounds, while CYP1A1 metabolizes a number of environmental pollutants and carcinogens (e.g., polycylic hydrocarbons) (Sonnier 1997). CYP1A1 is present in extremely low concentrations in human liver (Gomez-Lechon 2001; Sy 2001; Stiborova 2005; Sonnier 1998). In a study by Cresteil *et al.* (1998) CYP1A2 was found to be the last isozyme detected in the human liver at one month of age. An increase in CYP1A2 occurred between 1-3 months of age and reachd 50% of adults levels by year one. Human data also have suggested that CYP1A2 activity in 5-year-olds exceeds that in adults by 7% (Bjorkman 2004).

Exthoxyresorufin is a commonly used substrate that is metabolized by the CYP1A subfamily (Stiborova *et al.* 2005; Sy *et al.* 2001). Gebremichael *et al.* 1995 showed EROD activity peak near PND 21 in S-D rats. Similarly, our data concluded that an increase in EROD activity and protein expression occurred with age, with peak expression and activity at PND 21. Thus, weanlings and prepubescent animals have higher CYP1A1/2 levels than adults.

CYP2B1/2 is not present in humans but a similar isozyme, CYP2B6, exists (Nakajima *et al.* 1997; Cresteil 1998). Both CYP2B1/2 and CYP2B6 metabolize pentoxyresorufin. They are also both induced by phenobarbital (PB) and are regulated by the constitutive androstane receptor (CAR) (Gomez-Lechon *et al.* 2001; Muangmoonchai *et al.* 2001; Lamba *et al.* 2003; Faucette *et al.* 2004). CYP2B6 metabolizes many clinical drugs, recreational drugs and environmental contaminants (Lamba *et al.* 2003). Nevertheless, it is considered to be a minor CYP450 isozyme quantitatively and is one of the least characterized isoforms (Jinno *et al.* 2003; Faucette *et al.* 2004). Studies have shown that CYP2B is usually present in very low levels in the liver of adult humans and rodents (Alcorn and McNamara 2002). Higher levels of CYP2B1/2 have been formed in the younger animals than in adults (Imaoka *et al.* 1991; Johri *et al.* 2006). Omiecinski *et al.* 1990 showed a peak in the metabolism of PB, an inducer of CYP2B1/2, three weeks postnatal. Our current data show an increase in PROD activity in younger animals with peak activity occurring at weaning PND 21. However, expression of CYP2B1/2 protein remained relatively constant throughout development.

Saturation Kinetics of Compounds

Minimal data are available for biotransformation of environmental toxicants by children (Ginsberg *et al.* 2002). Physiologically-based pharmacokinetics (PBPK) models are becoming increasingly useful for characterizing the distribution of a compound and its metabolites in humans (Gargas and Clewell 1990; Gentry *et al.* 2003; Clewell *et al.* 2004). These models are capable of integrating developmental changes in physiology, enzymatic content and tissue composition to provide simulations of time-courses of blood and tissue doses of chemicals

(Krishnan and Johansson 2005). However, PBPK models often lack metabolic rate constants which must be obtained experimentally (Gargas and Clewell 1990; Beliveau *et al.* 2003; Rodriguez *et al.* 2007).

The Michealis-Menten parameters, K_m and V_{max}, are necessary for input into PBPK models. They are also often associated with classical kinetics. Classical kinetics assumes that one molecule of substrate is catalyzed by one enzyme and produce a single product. The V_{max} is a measure of the velocity at which the substrate exceeds the amount of enzyme present. The K_m is the rate at which V_{max} is half maximal (Harper 2003). In the current project, each VOC substrate is metabolized by more than one CYP450 isozyme. Unlike common practice, product formation was not measured in the current project. The disappearance of each parent compound, as a result of metabolism, was used to determine the K_m and V_{max}.

In the current study, the Michealis-Menten parameters, K_m and V_{max}, were determined for DCM, TCE and PERC in postnatal day (PND) 10, 15, 21, 60 and 90 S-D rats. Each compound undergoes Phase I oxidation at low substrate concentrations. The amount of enzymatic protein present in a system is a determinant of the rate of biotransformation (Kedderis 1997; Lipscomb *et al.* 2003). Forkert *et al.* (2005) showed that metabolism by P450 isozymes was dependent on enzyme concentrations and incubation times. In the current studies, each compound had an increase in substrate disappearance with an increase in protein concentration. Each reaction also exhibited an increase in substrate disappearance with an increase in time. The disappearances of DCM and TCE were greater than that of PERC for both the time and protein concentration profiles.

DCM, TCE and PERC are three VOCs that are metabolized by several isozymes. CYP2E1 is high affinity, low capacity isozyme that primarily metabolize DCM (Kim *et al.* 2007;

Sakai *et al.* 2002; Watanabe and Guengerich 2006). DCM is also metabolized to some extent by CYP2B1/2. It is a low affinity, high capacity isozyme. A study by Kim and Kim (1996), demonstrated the induction of DCM metabolism with both the CYP2E1 inducer benzene and the CYP2B1/2 inducer PB.

In 1987, Andersen and colleagues, determine the metabolic parameters, V_{max} and K_m, for DCM in mice, rats, hamsters and humans. These data were obtained by gas uptake experiments. The constants were placed into a PBPK model for DCM exposure. In 1989, Reitz *et al.*, did a similar study using liver microsomes from mice, rats, hamsters and humans. Each study confirmed that CYP450 metabolism was a saturable pathway for DCM metabolism. The current data indicate no significant difference in K_m among each immature age-group of rats. V_{max}, however, showed a decrease at sexual maturity (PND 60), which was significantly different from that for weanlings (PND 21). Therefore if previous studies indicate that there are no variations of K_m and V_{max} among species, the current study results could infer that there are not developmental changes of K_m and V_{max} among various aged children.

CYP2E1 is also a high affinity low capacity isozyme for TCE (Nakajima *et al.* 1990; Lee *et al.* 2000). Several studies have shown that other isozymes contribute to the metabolism of TCE. Among the isozymes that can metabolize TCE are CYP2B1/2 and CYP1A1/2 (Hanioka *et al.* 1997; Forkert *et al.* 2005; Nakajima *et al.* 1990; Lash *et al.* 2000). A study by Forkert *et al.* (2005) showed that CYP2E1 and CYP2B1 were the major isoforms responsible for TCE metabolism in rat liver, and that CYP2E1 is the major isoform responsible for TCE metabolism in humans.

Previous studies have shown changes in TCE metabolism with age of rodents. A study by Nakajima *et al.* (1992) revealed that TCE metabolism was lower in 18-week-old Wistar rats

than in 3-weeks-old animals. Fisher *et al.* (1990) also showed that PND 21 rats were able to metabolize TCE better that adult male and female rats. In their study, peak K_m and V_{max} values were manifest in nursing rats (PND 19-21) exposed to TCE than adults. In the current study, peak K_m and V_{max} values for TCE metabolism were found at PND 21.

Controversy exits about the dominant isozyme responsible for PERC metabolism. Some suggest CYP2B1/2 is the primary isozyme responsible for PERC metabolism (Philip *et al.* 2007; Hanioka *et al.* 1994). Others suggest CYP2E1 may be the major isozyme that metabolizes PERC (Lash *et al.* 2007).

CYP2B1/2 is a high capacity low affinity isozyme. There is limited ability of both rodents and humans to metabolize PERC (Dallas *et al.* 1995). In a study by Reitz *et al.* 1996, differences between rodents' and humans' capacity to metabolize PERC was assessed. Although PERC could be metabolized at lower, non-saturating concentrations, the extent of metabolism differed. Mice were able to metabolize PERC more extensively than rats, and rats metabolized it to a greater extent than humans (Reitz *et al.* 1996; Lash *et al.* 2000). Our current study assessed the influence of maturation on PERC metabolism. Peak K_m and V_{max} values were observed on PND 21. However, PERC metabolism in PND 10, 15, 60 and 90 rats remained relatively constant, without any significant differences occurring from day to day. These results coincide with the protein expression of CYP2B1/2, which supports the hypothesis that CYP2B1/2 is the dominant isozyme responsible for PERC metabolism rather than CYP2E1.

In a study by Rodriguez *et al.* (2007), PND 10, 60 (adult) and 22-month- old (aged) S-D rats were used to predict age differences in the kinetics of VOCs through physiologically based pharmacokinetic modeling (PBPK). Included in that study were DCM, TCE and PERC. Due to the lack of data, younger animals V_{max} and K_m had to be extrapolated from adult animal data.

Rodriguez *et al.* 2007 determined that younger animals were less capable of metabolizing VOCs. The researchers concluded that due to the smaller liver size of young animals, their metabolic capabilities were less than 5% of adult rats. CYP2E1, CYP1A1/2 and CYP2B1/2 are the isozymes primarily responsible for the metabolism of VOCs in rats. The current study indicates that the amounts of CYP2E1, CYP1A1/2, and CYP2B1/2 per entire liver and per body weight, peak prior to adulthood (PND 90). PND 90 rats had the lowest amount of microsomal protein, as well as CYP2B1/2, CYP2E1 and CYP1A1/2 activity, when compared to younger animals. Although PND 21 rats had peak activity and protein expression for each isozyme, these parameters were maximal in the PND 40 rats when determined for the entire liver. The PND 90 adults exhibited the highest levels of total CYP450s when expressed per mg microsomal protein or per entire liver.

In a similar enzymatic study by Johri *et al.* (2006), developmental changes of CYP450 isozymes were observed in Albino Wistar rats administered deltamethrin (DLM). Peak enzymatic activity and RNA expression occurred prior to adulthood for each isozyme. Michealis-Menten parameters were also determined for DLM. Anand *et al.* (2006) determined the K_m and V_{max} for DLM in PND 10, 21 and 40 Sprague-Dawley rats. The V_{max} for PND 40 rats was much higher than at PND 10 and 21, but the K_m remained constant. However, the lack of change in K_m may have been attributed to the variation of protein and time used for assays for each age group. For example, 10 mg microsomal protein/ml were incubated for 30 min for PND 10 samples, while PND 21 and 40 samples were incubated for 15 min in 4 and 2 mg protein/ml, respectively. It has been shown that various protein concentrations and various lengths of time affect the extent and rate of metabolism (Forkert *et al.* 2005). An increase in time and/or protein concentration will increase metabolism. Therefore, the increases in protein concentrations and

time that were used for younger animals in Anand *et al.* (2006) study indicate changes in enzymatic capability with age.

In the current study, CYP2E1, CYP1A1/2 and CYP2B1/2 activity and protein expression increased from birth, peaked at PND 21 and decreased thereafter to adult levels. Consequently, the K_m and V_{max} for DCM, TCE and PERC peaked at PND 21 and decreased during sexual maturity. Unlike the current study, human studies rarely account for such changes during development

Many factors must be taken into consideration when assessing potential risks of chemicals to children. These include the transfer of water-soluble and lipid-soluble compounds via breast milk; pharmacokinetic and pharmacodynamic factors; differences in hepatic blood flow and size during development (Gow *et al.* 2001; Alcorn and McNamara 2002; Gentry *et al.* 2003; Krishnan and Johanson 2005). Researchers commonly rely on PBPK models to simulate pharmacokinetic data for pharmaceutical compounds in children (Clewell *et al.* 2004; Daston *et al.* 2004). Many concerns arise from the use of such PBPK simulations, since drugs are frequently water-soluble, while many environmental contaminates are lipophilic. Lipophilic compounds cross the membrane barriers in the body more easily than water-soluble compounds. Water-soluble compounds generally are rapidly excreted via the bile and urine (Clewell *et al.* 2004). Water-soluble compounds will be more diluted when the proportion of water in the body is higher, as in infancy, while lipophilic compound will be less concentrated with an increase in fat, as in female adolescents (Makri *et al.* 2004).

The National Research Council (NRC) suggested that children are at disproportionate risk from environmental health threats, because they receive greater exposures per unit of body weight and their developing organ systems are immature (Lash *et al.* 2000). A study by Malhe *et*

al. (2007) found that age-related changes occurred for various volatile organic compounds' partition coefficients, including DCM, TCE and PERC. PND 10, PND 60 and 12-month-old animals were used to determine tissue: air and blood: air partition coefficients in maturing S-D rats. This study suggests that the decrease in fat in PND 10 pups contributes to the lower PC values for lipophilic compound when compared to adults.

Summary

The current project is the first to quantify total CYP450, as well as CYP2E1, CYP1A1/2 and CYP2B1/2 activity and protein in developing S-D rats. It is also the first project to determine V_{max} and K_m values for developing rodents for three of the most commonly found VOCs, DCM, TCE and PERC. The current studies reveal that pronounced changes in hepatic oxidative capacity occur during the maturation of S-D rats. These findings concur with and expand upon findings in some other CYP450 developmental studies that indicate peak enzymatic activity and expression occur around PND 21 (Gerbmicheal *et al.* 1995; Fisher *et a*l 1989).

VOC metabolic capacity peaked prior to adulthood in SD rats. V_{max} values for DCM, TCE and PERC were relatively low at PND 10, peaked at PND 21, and decreased significantly by PND 60. K_m values were not consistent for any VOC from one study period to another. The highest K_ms coincided with the highest V_{max} on PND 21, so intrinsic clearance values were not elevated. This phenomenon may be due to the onset of participation of enzymes in addition to CYP2E1, the isoform primarily responsible for oxidation of low doses of TCE and DCM. CYP1A1/2, 2B1/2, 2C11, and 2C12 have also been shown to participate in biotransformation of the VOCs, as have glutathione S-transferase. Thus, it is quite likely there is substantially more

VOC flux in weanling than in neonatal or adult rats. The weanlings would be anticipated to be the most susceptible age-group, since each of the VOCs must undergo bioactiviation to exert cytotoxicity or carcinogenicity.

It was rather difficult to compare previous K_m and V_{max} values from other studies to the current project, since most of these were *in vivo* studies. However, studies that were performed *in vitro* generally measured metabolite formation and not parent compound disappearance. Kim *et al.* (1996), did measure the disappearance of DCM, but their results were not reproducible. Nevertheless, Andersen *et al.* (1987), Reitz *et al.* (1996) and Elfarra *et al.* (1998) each found that adult rodents were appropriate models for human PBPK models for DCM, PERC and TCE, respectively.

Although rodent data are commonly used in human PBPK models, human studies generally do not indicate prematurational peaks for CYP2E1 or CYP1A1/2 activities (Ginsberg *et al.* 2002; Cresteil 1998). Therefore, the S-D rat may not be a suitable surrogate for developing human toxicological studies for compounds that are metabolized by CYP1A1/2 and CYP2E1. More extensive human and rodent metabolism and PK studies are needed to determine whether S-D rats are appropriate surrogates for maturational research.

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